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Lorena Redondo-Morata, Patricia Losada-Pérez, Marina Inés Giannotti

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# 1 Lipid bilayers: phase behavior and 2 nanomechanics

3 **LORENA REDONDO-MORATA**

4 *Center for Infection and Immunity of Lille, INSERM U1019, CNRS UMR 8204, F-59000 Lille, France.*

5 **PATRICIA LOSADA-PÉREZ**

6 *Experimental Soft Matter and Thermal Physics (EST) group, Department of Physics, Université  
7 Libre de Bruxelles, 1050 Brussels, Belgium*

8 **MARINA INÉS GIANNOTTI**

9 *Biomedical Research Networking Center on Bioengineering, Biomaterials and Nanomedicine  
10 (CIBER-BBN), Spain.*

11 *Institut de Bioenginyeria de Catalunya (IBEC), The Barcelona Institute of Science and Technology  
12 (BIST), 08028 Barcelona, Spain.*

13 *Departament de Ciència de Materials i Química Física, Universitat de Barcelona, 08028  
14 Barcelona, Spain.*

15 [miannotti@ibecbarcelona.eu](mailto:miannotti@ibecbarcelona.eu)

16

17 **Keywords:**

18 *Lipid phase behavior / phase transition / phase coexistence / nanomechanics / thermodynamics  
19 / Atomic Force Microscopy (AFM)/ Quartz crystal microbalance with dissipation monitoring  
20 (QCM-D)*

21

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## 43 1. Abstract

44 Lipid membranes are involved in many physiological processes like recognition, signaling, fusion  
 45 or remodeling of the cell membrane or some of its internal compartments. Within the cell, they  
 46 are the ultimate barrier, while maintaining the fluidity or flexibility required for a myriad of  
 47 processes, including membrane protein assembly. The physical properties of *in vitro* model  
 48 membranes as model cell membranes have been extensively studied with a variety of  
 49 techniques, from classical thermodynamics to advanced modern microscopies. Here we review  
 50 the nanomechanics of solid-supported lipid membranes with a focus in their phase behavior.  
 51 Relevant information obtained by quartz crystal microbalance with dissipation monitoring  
 52 (QCM-D) and atomic force microscopy (AFM) as complementary techniques in the  
 53 nano/mesoscale interface is presented. Membrane morphological and mechanical  
 54 characterization will be discussed in the framework of its phase behavior, phase transitions and  
 55 coexistence, in simple and complex models, and upon the presence of cholesterol.

## 2. Introduction

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Cells can be thermodynamically defined as open systems in constant exchange of mass, energy and information with the environment. The membrane is the ultimate boundary for the cell, confining it from the medium and some of its internal compartments. The cell membrane is a fundamental structure of the cell, providing a support matrix for proteins and carbohydrates. As complex systems, cell membrane performance is the result of lipids and proteins working together, with main functions like being barriers, mediating the exchange of molecules and information, promoting signaling and adhesion, and being metabolically self-renewing structures.

Cell membranes are materials with unique physical properties allowing cells to rapidly change shape, squeeze, stretch, pinch off smaller units, fuse, and reseal. This is the result of being viscous sheets with both fluid and elastic properties. Cell membranes are supramolecular structures, where the lipids and proteins interact through non-covalent bonding. Membranes are curved surfaces, vesicular in nature, whose curvature is deeply influenced by the lipid packing. The lipid bilayer, made of millions of building blocks held together by weak interactions, is in part responsible for the physical, dynamic and mechanical properties of the cell membrane. In the membrane, lipids are mainly organized in lamellar phases, where two leaflets of lipids are self-assembled exposing the polar headgroups to the aqueous interface and keeping the fatty-acyl chains aligned opposed and parallel to one another to form the hydrophobic core.

The enterprising scientist Alec Bangham was the discoverer in 1958 of the ‘multilamellar smectic mesophases’ or, as himself referred to less seriously, the ‘bangasomes’ (Bangham et al., 1958) - which were eventually named ‘liposomes’ to define microscopic lipid vesicles (Sessa & Weissmann, 1968). When the Babraham Institute (Cambridge, UK) acquired its first electron microscope in 1961, Bangham had the privilege to firstly observe the dispersions of phospholipids in water solutions of negative stains (Bangham & Horne, 1964). In the following years, Bangham and his collaborators performed the key experiments to demonstrate that lipid bilayers maintain concentration gradients of ions such as potassium and sodium. Indeed, David D. Deamer wrote in his memoir *“It was the membrane equivalent of finding the double helix structure of DNA, another Cambridge discovery in the life sciences”* (Deamer, 2010). This was reflected in the well-known lecture by Bangham in the University of Bristol in 1975, entitled “Membranes came first”, where he proposed that something similar to liposomes had been available to house the first forms of life. Indeed, all living organisms have a membrane.

88 Later in the early 1970s, Singer and Nicolson proposed the fluid-mosaic-model, depicting the  
89 cell membranes as two-dimensional liquids where all lipid and protein molecules diffuse easily  
90 (Singer & Nicolson, 1972). Concerning the composition, besides all the proteins and  
91 carbohydrates, lipids are the main components of the cell membrane in terms of molar fraction.  
92 Lipids are a broad family which covers many different chemical structure sphingolipids  
93 (ceramides, sphingomyelin, gangliosides, sphingosines), sterols (cholesterol and vitamins), and  
94 phospholipids, each of them contributing to different and crucial physicochemical properties.

95 Gradual progress was made in the knowledge of the complexity of the biological membranes.  
96 The most famous hypothesis in the field is the membrane raft proposed in 1997 by K. Simons  
97 and E. Ikonen (Simons & Ikonen, 1997). The idea was based on the fact that portions of  
98 membrane were found to be detergent resistant, that may give rise to a virtual  
99 compartmentalization of the cell. This hypothesis generated a lot of literature and became a  
100 recursive concept in the field. Nowadays it is well known that there is a variety of nanostructures  
101 in the membrane of heterogeneous sizes and functions, and the methods that allow us to  
102 observe these nanodomains *in vivo* are only beginning to emerge (Goñi, 2019b; Pinkwart et al.,  
103 2019). Nanodomains can be considered putative heterogeneous structures within the  
104 membrane which are due partly to phase separation.

105 The mechanical role of the lipid membrane in force-triggered and force-sensing mechanisms in  
106 cells is of significance and adds to the better-established role of the mechanosensitive proteins  
107 (Vogel, 2006)(Kechagia et al., 2019). Membrane conformational changes such as bending,  
108 vesiculation or tubulation are involved in cellular processes including adhesion, signaling,  
109 endocytosis or membrane resealing (van Meer & de Kroon, 2011)(van Meer et al.,  
110 2008)(Hassinger et al., 2017). For instance, in endocytosis, the endocytic system needs to  
111 generate enough force to form an endocytic vesicle by bending the membrane. These  
112 mechanisms generally require the membrane separation from the cytoskeleton as well as strong  
113 bending, for which the membrane chemical composition and physicochemical properties, often  
114 highly localized and dynamic, are key players (Sheetz, 2001)(van Meer et al., 2008). It is now  
115 clear that the lipid packing and composition are direct modulators of the membrane curvature  
116 and elasticity, at different scales, even locally at the nanometer scale (Yeagle, 1989)(Vereb et  
117 al., 2003). Understanding the lipid bilayers structural and mechanical properties and the  
118 involvement and role of each individual component turns out to be essential in order to identify  
119 their contribution to the overall membrane traits. Structural and physical properties of lipid  
120 bilayers include shape and local distribution of components (phases and domains), and related  
121 mechanical stability in response to compression, bending or stretching, their ability to fuse, etc.

122 We review the nanomechanics of solid-supported lipid membranes and how these relate to lipid  
123 membrane phase behavior. To this end, we chose two complementary techniques with  
124 nano/mesoscale sensitivity to mechanical and viscoelastic properties, namely quartz crystal  
125 microbalance with dissipation monitoring (QCM-D) and atomic force microscopy (AFM). QCM-D  
126 is a label-free surface-sensitive technique, whose working principle is based on the inverse  
127 piezoelectric effect. Real-time simultaneous measurements of resonant frequency and energy  
128 dissipation make QCM-D very suitable for gravimetric and viscoelastic characterization of solid-  
129 supported nanoscale sized films. AFM is a technique that allows the observation of surfaces  
130 under controlled liquid environment, to resolve topographical features with nanoscale  
131 resolution and to measure and apply forces in the pN-nN range. Therefore, AFM is appropriate  
132 for imaging the topography of solid-supported lipid membranes and probing the physical and  
133 mechanical properties at the nanoscale by means of force spectroscopy, providing high spatial  
134 and force resolution. QCM-D is complementary to AFM, useful for probing changes in  
135 viscoelastic properties during solid-supported film formation. The complementarity of AFM and  
136 QCM-D manifests at different levels: i) AFM measurements are performed at a local level, while  
137 QCM-D provides a global characterization of the solid-supported films; ii) both techniques  
138 enable dynamic mechanical analysis by applying a small oscillatory stress, however, the AFM tip  
139 scans and deforms supported layers from the top, whereas QCM-D measures the sensor  
140 oscillation from the bottom. The combination of AFM and QCM-D for supported lipid  
141 membranes has been employed in previous works (see, for instance (R. Richter et al., 2003,  
142 2003; Van Lehn et al., 2014). In the following sections we briefly describe the membrane models  
143 and discuss the working principles behind both approaches, highlighting the quantitative and  
144 qualitative mechanical information that can be extracted using each technique. Specific  
145 examples on the usefulness to monitor phase transitions on one-component to more complex  
146 bilayers, and resolve domain coexistence are also provided, including the key role of cholesterol.

### 147 3. Experimental approaches to study membrane mechanics

#### 148 3.1. Model systems: supported vesicles layers (SVLs) lipid bilayers (SLBs)

149 Due to the extreme complexity of biological membranes, cell membrane mimetics are excellent  
150 approaches to study membrane properties and biological processes at the cellular and  
151 subcellular level. A great deal of what we know about these processes comes from modeling  
152 lipid bilayers *in vitro*. Various membrane systems are established as biomimetic structures. They  
153 include vesicles: freely-suspended and supported liposomes, giant unilamellar vesicles (GUVs);  
154 micelles, bicelles; suspended and supported lipid films: Langmuir monolayers, phospholipid  
155 bilayers nanodiscs, black lipid membranes (BLM), supported lipid bilayers (SLB), tethered bilayer

156 lipid membranes (tBLM), polymer-cushioned membranes, protein-tethered bilayer lipid  
157 membranes (ptBLM) and hybrid bilayers (Sebaaly et al., 2019)(Dimova, 2019)(Doktorova et al.,  
158 2018)(Siontorou et al., 2017)(Rascol et al., 2016). Among these, the giant unilamellar vesicles  
159 (GUVs) have been extensively used since they offer a perfect stage to study the mechanical,  
160 thermodynamic, electrical, and rheological properties of the overall GUV and lipid bilayer as a  
161 function of membrane composition, surrounding media and temperature (Dimova, 2019)(Evan  
162 Evans et al., 2003)(Kahya et al., 2004). Being heterogeneous and dynamic at the nanoscale,  
163 nanotechnology-based techniques can further explore biomembranes locally with resolution at  
164 the nanometric level. For many of these techniques, like surface analytical techniques, solid-  
165 supported models are the most adequate, including supported lipid monolayers, supported  
166 vesicles layers (SVLs) or supported lipid bilayers (SLBs).

167 SVLs are the precursor solid-supported systems to planar SLBs formed by vesicle fusion and  
168 rupture. They result from spontaneous adsorption of small vesicles of diameter  $d \leq 200$  nm onto  
169 solid surfaces. The geometry of SVLs embodies membrane curvature, tension and osmotic stress  
170 within the supported layer. This makes SVLs useful biomimetic platforms to probe membrane  
171 deformation using surface-sensitive techniques and testing ground for adhesion, budding and  
172 lipid membrane exchange and fusion (Lipowsky & Seifert, 1991; Hurley et al., 2010; Tabaei et  
173 al., 2016; Steinkühler et al., 2019). SVLs are generally formed by small soft adsorbates which are  
174 mimics to small endosomes or exosomes. These are optically inaccessible systems and the  
175 experimental investigation of their deformation is not straightforward. Adsorbed vesicles onto  
176 inorganic surfaces also serve as model systems relevant to biocompatibility studies. Vesicle  
177 adsorption is very often accompanied by vesicle deformation upon contact with the solid  
178 support. The extent of vesicle deformation depends on several factors such as vesicle size,  
179 mechanical properties of the vesicles, adhesion strength of the surface, osmotic pressure  
180 difference over the vesicle, etc. The fate of vesicles upon adsorption on a solid support depends  
181 both on vesicle-vesicle and vesicle surface interactions. In the absence of osmotic stress and at  
182 a concentration where vesicle surface coverage enhances fusion probability, the rupture of  
183 vesicles will depend strongly on the adhesion strength. Specifically on how this imbalances the  
184 energetic competition between adhesion energy and an elastic stretching of the membrane  
185 (Lipowsky & Seifert, 1991). If the adhesion strength  $W$  is large enough and exceeds the lysis  
186 tension of the membrane, the vesicles will rupture. SVLs are typically formed on surfaces whose  
187 adhesion strength is not sufficient to induce vesicle rupture, such as Au, TiO<sub>2</sub>, Pt (Tero, 2012).

188 SLBs are very manageable platforms relatively simple to obtain and that retain two-dimensional  
189 order and lateral mobility. They are ideal to study lipid lateral interactions and membrane local

190 mechanical properties, growth of lipid domains, as well as interactions between the lipid  
191 membrane and proteins, peptides and drugs, cell signaling, etc. They also offer an excellent  
192 environment for inserting membrane proteins. There are many different methods that can be  
193 used to obtain SLBs, like the spin-coating and hydration (Mennicke & Salditt, 2002),  
194 microcontact printing (Strulson & Maurer, 2011), solvent-exchange deposition (Tabaei et al.,  
195 2016; Hohner et al., 2010), and the most widely used methods like the Langmuir–  
196 Blodgett/Schäfer deposition to prepare mono and bilayers (Kurniawan et al., 2018), and the  
197 liposome fusion and rupture method for bilayers (Hardy et al., 2013)(Mingeot-Leclercq et al.,  
198 2008). The liposome rupture method remains the most popular and simple one, based on  
199 depositing small unilamellar vesicles (SUVs) from a suspension onto a flat substrate where the  
200 adhesion strength is high, generally mica or silicon oxide, but can also be formed on bare or  
201 gold-coated glass, following the needs of the analysis technique (Mingeot-Leclercq et al., 2008;  
202 Ralf P Richter & Brisson, 2005)(Choi et al., 2016; B. Gumí-Audenis et al., 2018; Seeger et al., 2010;  
203 Winkler et al., 2020). Once in contact with the substrate the SUVs start fusing between them,  
204 deforming, flattening, and finally rupturing to form a continuous film. It is important to have in  
205 mind that the final SLB structure is affected by variables like the lipid vesicles composition,  
206 concentration, and size, the physicochemical environment pH, temperature, and ionic strength,  
207 as well as the surface roughness and charge density (Reimhult et al., 2003).

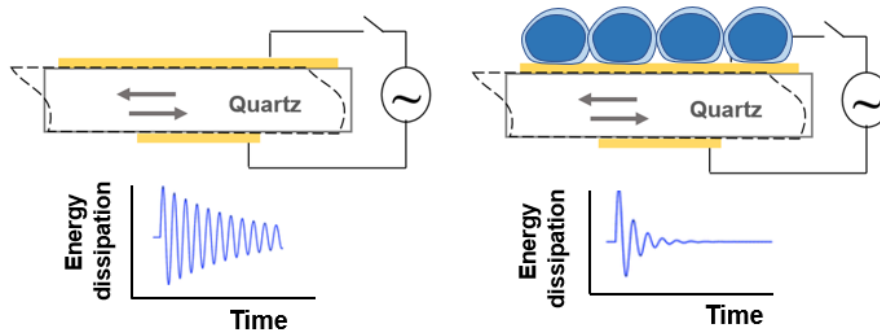
### 208 3.1 QCM-D

209 Quartz crystal microbalance with dissipation monitoring (QCM-D) is an acoustic-based surface-  
210 sensitive technique that enables label-free, real-time simultaneous measurements of wet-mass  
211 and viscoelastic properties of solid-supported nano/meso-scaled adlayers.

212 The QCM-D sensor consists of an AT-cut quartz crystal being sandwiched between two  
213 electrodes. When an AC voltage is applied across the electrodes with a frequency close to the  
214 resonant frequency of the quartz crystal, a mechanical deformation is induced, resulting in a  
215 standing shear wave. The surfaces of the electrodes coincide with the antinodes of the standing  
216 shear wave with wavelength  $\lambda = 2d/n$ , with  $d$  the quartz thickness and  $n$  the (odd) overtone  
217 number. The resonance frequency at each overtone is  $f_n = nv/2d$ , with  $v$  the speed of sound in  
218 quartz. The shear waves propagate as evanescent waves decaying across the boundary between  
219 the crystal and the fluid environment (air or liquid) with a penetration depth  $\delta = \sqrt{\eta_L / \pi f_n \rho_L}$ ,  
220 which depends on the overtone frequency  $f_n$  and on the viscosity  $\eta_L$  and density  $\rho_L$  of the fluid in  
221 contact with the sensor surface (Reviakine et al., 2011). The penetration depth of a 5 MHz shear  
222 wave in water is  $\delta \sim 250$  nm, rendering QCM-D surface-specific.  $D$  is defined as the ratio



223 between the dissipated energy during one vibration period and the total energy of the crystal at  
 224 that instant  $D = E_{lost} / 2\pi E_{stored}$ . In the so-called 'ring-down method' measurements, energy  
 225 dissipation  $D$  can be calculated when the AC voltage driving the quartz crystal oscillation is  
 226 turned off. The working principle of QCM-D is reflected in Figure 1, upon the presence of a soft,  
 227 viscoelastic layer such as a layer of lipid SVLs. The amplitude of the crystal oscillation decays  
 228 exponentially and much faster when in contact with a viscoelastic layer, characterized by a large  
 229 energy dissipation response.



230

231 **Figure 1.** Schematic view of a QCM-D sensor excited by an AC voltage. Left panel: Bare Au-coated  
 232 sensor in liquid environment, the dampening of the oscillations takes place slowly. Right panel:  
 233 The presence of an adsorbed lipid vesicle layer (SVL) induces a faster damping of the oscillations  
 234 and thus a larger energy dissipation.

235 Commercial QCM-D instruments allow for monitoring changes in frequency and dissipation for  
 236 several overtones ( $n = 3, 5, 7, \dots, 13$ ),  $\Delta f_n$  and  $\Delta D_n$ , upon the presence of a nano/mesoscale layer.  
 237 The spectroscopic data at multiple frequencies with varying detection range enables assessing  
 238 the spatial homogeneity and rigidity of the adsorbed layer. For homogeneous thin and rigid  
 239 films,  $\Delta D_n \sim 0$ ,  $\Delta f_n$  overtones overlap and a simple relationship between mass adsorbed and  
 240 change in frequency holds, the so-called Sauerbrey relation:  $\Delta m = -C\Delta f / n$ , with  $C$  being the  
 241 Sauerbrey constant which for a 5 MHz quartz crystal reads  $C = d\rho_{quartz} / f_0 = 17.7 \text{ ng/cm}^2$   
 242 (Sauerbrey, 1959). For thicker and softer films  $\Delta D_n > 0$ , overtones do not overlap and the  
 243 ensemble quartz-film-fluid can be represented by simple models combining elastic components  
 244 and viscous dashpots. These models enable to extract effective viscoelastic parameters of the  
 245 adsorbed film such as shear viscosity, thickness and shear storage modulus (Cho et al., 2007;  
 246 Voinova et al., 1999).

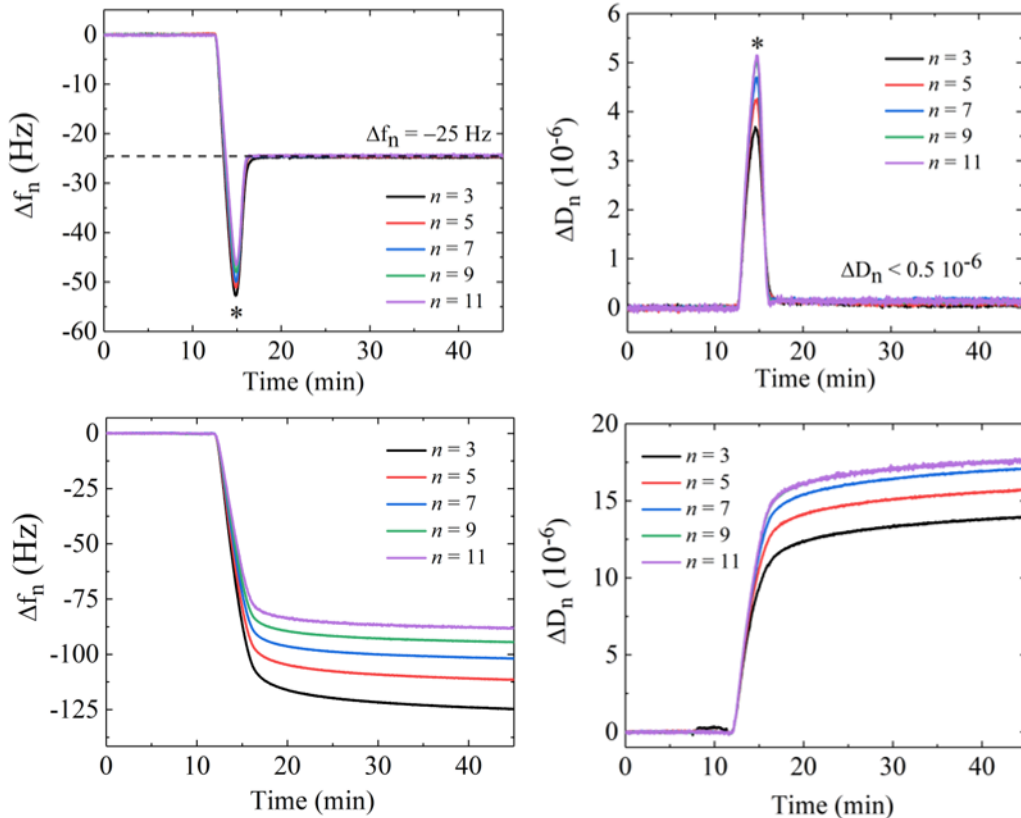
247 The capability of monitoring the viscoelastic properties during film formation is particularly  
 248 useful in the case of solid-SLB formation. QCM-D has been a valuable tool in deciphering the  
 249 kinetic pathways of SLB formation from precursor vesicles and how those depend on relevant

250 conditions such as solid surface adhesion strength, medium ionic strength, vesicle mechanics,  
251 etc. (Cho et al., 2010; Jing et al., 2013; C. A. Keller & Kasemo, 1998; Pramanik et al., 2016; R P  
252 Richter, 2006).

253 Figure 2 shows a typical QCM-D experiment where real-time monitoring of the adsorption of  
254 zwitterionic DOPC vesicles onto SiO<sub>2</sub> and oxidized Au was carried out at 37 °C. The vesicles are  
255 dispersed in TRIS-buffer (10 mM TRIS, 100 mM NaCl, pH 8) at a concentration of 0.1 mg/mL and  
256 their hydrodynamic diameter  $d = 79 \pm 20$  nm. As initially discussed by Keller and Kasemo (C. A.  
257 Keller & Kasemo, 1998), the adsorption kinetics is surface-specific. The mechanistic picture of  $\Delta f$   
258 and  $\Delta D$  changes is governed by a delicate balance between the adhesive contribution to the free  
259 energy from lipid-surface interactions and the opposing effect of bending and stretching the  
260 membrane (Lipowsky & Seifert, 1991). DOPC vesicles adsorbing on SiO<sub>2</sub> follow a two-step  
261 adsorption process consisting of i) adsorption of a critical number of vesicles and ii) fusion,  
262 rupture and formation of an SLB. This is reflected in the  $\Delta f_n$  and  $\Delta D_n$  signals depicted in the upper  
263 panels of Figure 2. After a  $\sim 10$  min baseline in buffer ( $\Delta f_n$  and  $\Delta D_n = 0$ ), DOPC vesicles added at  
264 a very small flow rate adsorb onto the SiO<sub>2</sub>-coated quartz sensor ( $\Delta f_n$  decreases and  $\Delta D_n$   
265 increases) until a maximum number (minimum in  $\Delta f_n$  and maximum in  $\Delta D_n$ ). Adsorbed vesicles  
266 then fuse and rupture as a consequence of the large adhesion strength of SiO<sub>2</sub>, thus releasing  
267 the entrapped aqueous buffer. As a consequence,  $\Delta f_n$  increases (mass loss) to a constant plateau  
268 of  $\Delta f_n \sim -25$  Hz and the  $\Delta D_n$  signal decreases back to a very small value ( $\Delta D_n < 0.5 \cdot 10^{-6}$ ). The final  
269  $\Delta f_n$  and  $\Delta D_n$  values and the fact that overtones signals overlap is consistent with the formation  
270 of a homogeneous rigid and thin DOPC SLB. The pathway of SLB formation is not unique and  
271 depends strongly on the ionic conditions (head group charges of the constituent lipids, buffer  
272 ionic strength presence of divalent cations, pH). Vesicles containing positively charged lipids like  
273 DOTAP SUVs rupture individually on SiO<sub>2</sub> as a consequence of stronger vesicle-support  
274 electrostatic interactions (R. Richter et al., 2003).

275 When DOPC vesicles adsorb onto an oxidized Au surface, a monotonic  $\Delta f_n$  decrease and  $\Delta D_n$   
276 increase can be observed reaching constant non-zero plateau values with non-overlapping  
277 overtones. Such time-dependent responses provide evidence that oxidized Au facilitates non-  
278 ruptured vesicle adsorption towards the formation of acoustically non-rigid vesicle layers with  
279 saturated coverage. As pointed out by Lind and Cárdenas (Lind & Cárdenas, 2016), it is worth  
280 mentioning that local vesicle rupture events and formation of small bilayer patches cannot be  
281 ruled out. As a matter of fact, QCM-D is very sensitive to hydrodynamic (wet) mass and the local,  
282 partial formation of SLBs might be masked by the adsorption of vesicles on top or in between  
283 the bilayer patches. In this case, complementary QCM-D and AFM measurements are

284 particularly useful to obtain a complete picture of the heterogeneous layers SLBs with co-  
 285 adsorbed vesicles formed. For example, the complementarity of AFM and QCM-D has proven  
 286 successful in experimentally confirming defect (protrusion)-driven nanoparticle interactions  
 287 with SLBs (Van Lehn et al., 2014).

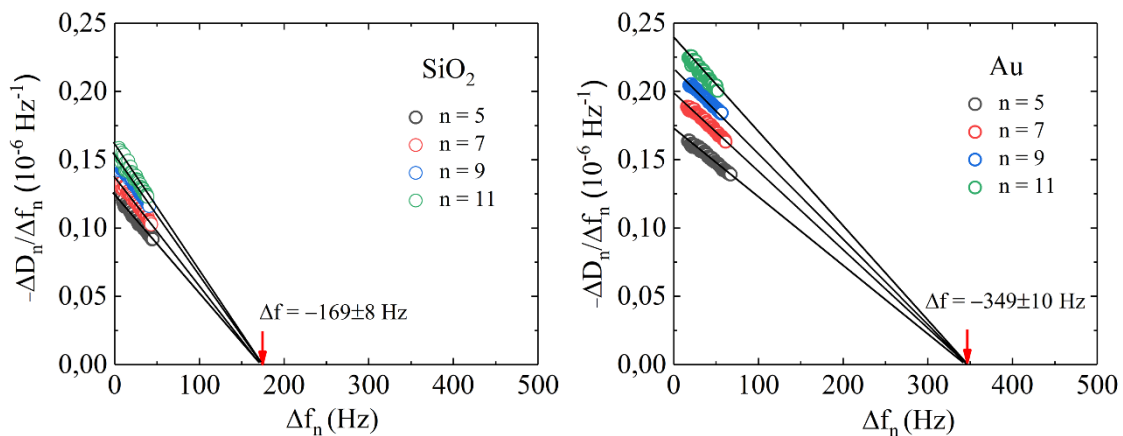


288

289 **Figure 2.** Time dependence of  $\Delta f_n$  and  $\Delta D_n$  during a QCM-D experiment of DOPC vesicle adsorption  
 290 at 37°C onto SiO<sub>2</sub> (upper panels) and Au (lower panels).

291 Apart from kinetic information, QCM-D can be also useful to estimate the extent of vesicle  
 292 deformation upon adsorption following a model-free approach introduced by Tellechea *et al.*  
 293 (Tellechea et al., 2009). This method consists in plotting  $-\Delta D/\Delta f$  ratio vs  $-\Delta f$  for all overtones  
 294 during initial adsorption, which shows a linear decrease over a large range of frequency shifts.  
 295 Extrapolation of this linear decrease to a frequency-independent intercept with the  $-\Delta f$  axis  
 296 (where overtones intersect) provides a value of the thickness of the adsorbed vesicle layer  $h$  or  
 297 Sauerbrey thickness:  $h = -\Delta f C / \rho$ , where  $C = 17.7 \text{ ng/cm}^2 \text{ Hz}$  and  $\rho = 1 \text{ g/cm}^3$  is the density of  
 298 the film. This approach assumes a complete surface coverage, where the presence of trapped  
 299 buffer has been diminished to occupy only the void spaces between densely packed vesicles (the  
 300  $-\Delta D/\Delta f$  ratio is close to zero and the  $-\Delta f$  intercept values were the same on the extrapolation of  
 301 a linear regression) (Tellechea et al., 2009; Reviakine et al., 2012; Olsson et al., 2013). Figure 3

302 displays the extrapolated Sauerbrey thickness for DOPC SUVs adsorbed onto SiO<sub>2</sub> and Au  
 303 surfaces. The respective effective frequencies read  $h(\text{SiO}_2) = 30 \pm 2$  nm and  $h(\text{Au}) = 62 \pm 5$  nm. If  
 304 one compares these values with the original vesicle diameter in bulk measured by DLS ( $d = 79 \pm$   
 305  $20$  nm), vesicles were deformed to a greater extent onto SiO<sub>2</sub> surfaces. A more exact approach  
 306 based on a hydrodynamic model was recently introduced by Cho and co-workers (Gillissen et  
 307 al., 2017) to estimate the deformation of small vesicles at low surface coverage. The model  
 308 enables to estimate adsorbed vesicle shape and bending energy by using hydrodynamic  
 309 spectroscopy (frequency shifts at several overtones). It treats vesicles as ellipsoids and relates  
 310 the so-called QCM-D force to the scaled viscous penetration depth  $\delta/a$  with  $\delta$  the viscous  
 311 penetration depth, and  $a$  the non-deformed vesicle radius, in order to probe the adsorbed  
 312 particle aspect ratio. Further details on the model can be found in (Gillissen et al., 2017).

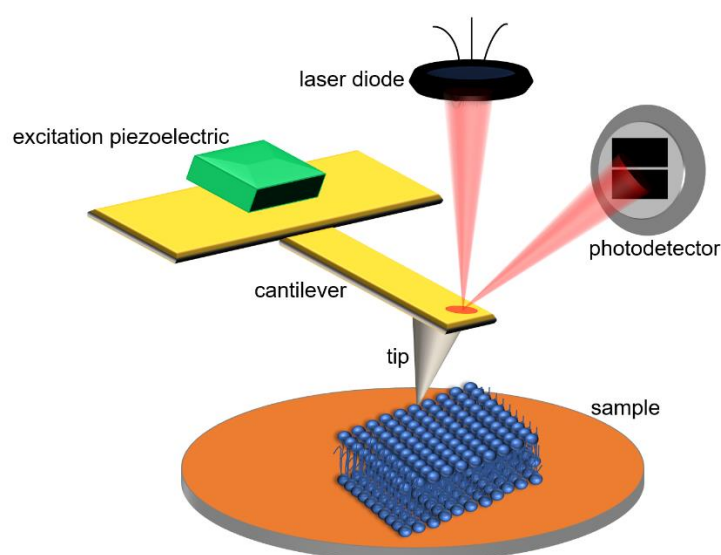


313  
 314 **Figure 3.**  $\Delta D_n / -\Delta f_n$  ratio as a function of frequency at different overtones for DOPC vesicles  
 315 adsorbed onto SiO<sub>2</sub> and Au.

### 316 3.2. Atomic Force Microscopy (AFM)-based methodology

317 Classical structural techniques such as X-ray, NMR or electron microscopy rely on ensemble  
 318 averaging of molecular properties. Yet, by these means, the underlying molecular dynamics can  
 319 be hidden, since the signal comes from the average of many unsynchronized molecules in the  
 320 bulk. The picture changes when using single-molecule techniques. The measured signal of one -  
 321 or a few- molecules reflects the stochasticity of thermally induced processes when randomly  
 322 crossing free-energy barriers. Single-molecule optical microscopy techniques are particularly  
 323 well suited for the study of the dynamics of molecules. However, these latter methods rely on  
 324 the detection of fluorescent markers attached to proteins or lipids, which means that the  
 325 resolution is limited to  $\sim 200$  nm due to diffraction of light and to a few tens of nm thanks to  
 326 super-resolution techniques. The atomic force microscopy (AFM) was since its invention in 1986  
 327 (Binnig et al., 1986) quickly positioned among the single-molecule, high-resolution structural

328 analysis techniques. It provides information concerning structure, function-related  
329 conformational changes and supramolecular assemblies, crucial for a complete understanding  
330 of biological processes. Nowadays, AFM is in its thirties and has become an invaluable tool for  
331 studies at the micro- and nanoscale. As a stand-alone, high-resolution imaging technique and  
332 force transducer, it is today an established methodology among the biophysical community. The  
333 AFM uses a nanometer-sharp tip -resembling a record player needle- attached to a  
334 (micro)cantilever to sense the sample surface while scanning it using piezoelectric elements. An  
335 optical system is used to detect the cantilever deflection (or force), thanks to a laser diode beam  
336 reflected on the back of the cantilever and detected by a photodiode. Changes in deflection due  
337 to sample protrusions while the tip scans the sample are translated into electrical signals thanks  
338 to a feedback mechanism. This is schematically represented in Figure 4.



339

340 **Figure 4.** Schematic representation of an AFM instrument. The deflection of the cantilever in the  
341 normal direction is monitored by a photodetector which collects the reflection of a laser beam  
342 focused at the back of the AFM cantilever. In the tapping mode, thanks to a piezoactuator, the  
343 AFM cantilever is excited near its resonance frequency.

344 One of the main strengths of AFM relies on the possibility of working in a controlled environment  
345 (medium composition and temperature) and with great spatial resolution. AFM-imaging mode  
346 provides submolecular resolution on a large variety of biological samples: from single molecules,  
347 that is, DNA or proteins, to supramolecular assemblies such as SLBs, or even entire cells (Giles  
348 et al., 1993)(Dufrêne et al., 2017; Parot et al., 2007). Therefore, AFM has become a well-  
349 established technique for imaging the topography of lipid membranes that show homogeneous  
350 or phase-separated morphology, permitting then to increase the bilayer complexity, from

351 bilayers of one component to multicomponent ones (El Kirat et al., 2010; B. Gumí-Audenis et al.,  
352 2016a; Morandat et al., 2013; L Redondo-Morata et al., 2014).

353 For the study of biological samples, it is common to use AFM-imaging dynamic modes that have  
354 an intermittent tip-sample contact. Among them, the most often used is oscillation or tapping  
355 mode, in which the AFM cantilever is excited near its resonance frequency. The resulting  
356 oscillating tip is intermittently contacting -tapping- the surface, giving rise to a damped  
357 oscillation amplitude. Additionally, the resonance frequency is also shifted due to the probe-  
358 surface interaction. The topography of the surface is reconstructed by monitoring the amplitude  
359 of the oscillating cantilever, which is kept constant by adjusting the z-piezo position through a  
360 feedback loop. While scanning in the X-Y direction, the amplitude of the cantilever oscillation is  
361 kept constant thanks to a feedback control. However, biomolecules are dynamic in essence;  
362 hence, to understand how biomolecules work it was only natural to think about increasing the  
363 temporal resolution of conventional AFMs. The first time that the concept of High-Speed (HS)  
364 AFM was mentioned -to our knowledge- was in 1991 by Barrett and Quate (Barrett, 1991), who  
365 did a fairly attempt with the technology at that time. Other research groups, as Paul Hansma's,  
366 would challenge the practical speed limitations. It was only in 2010 when the laboratory leaded  
367 by Toshio Ando in Kanazawa University (Japan), filmed individual myosin molecules walking on  
368 an actin filament (T Ando et al., 2008), operating their AFM instrument at a speed about 1000  
369 times faster than the conventional instruments of that time. Besides the visual impact and  
370 scientific insight of those movies, these experiments illustrated that HS-AFM could obtain  
371 concomitantly structural and dynamic data, providing insights inaccessible by any other method  
372 (Toshio Ando, 2017; Chiaruttini et al., 2015; Kodera et al., 2010; Mierzwa et al., 2017). The basic  
373 principles, advantages and limitations of the most common AFM bioimaging mode are nicely  
374 detailed in a recent review from Dufrêne *et al.* (Dufrêne et al., 2017).

375 Following the capability of the AFM for mechanical manipulation, to sense and apply small forces  
376 accurately (pN-nN range), it rapidly developed into an excellent technique to study interactions  
377 -inter and intramolecular forces- at the molecular level, the so-called AFM-based force  
378 spectroscopy (AFM-FS). The manipulation of single molecules (single molecule force  
379 spectroscopy) has led to fascinating new insights in the mechanics of proteins, polysaccharides,  
380 synthetic polymers, and DNA at the molecular level (Florin et al., 1994)(Lee et al.,  
381 1994)(Radmacher, 1997)(Noy et al., 1997)(Clausen-Schaumann et al., 2000)(Rief et al.,  
382 1997)(Hugel & Seitz, 2001)(P E Marszalek et al., 1999; Piotr E Marszalek et al., 2001)(Giannotti  
383 & Vancso, 2007).

384 In the case of lipid bilayers, AFM offers the unique opportunity to probe local physical and  
385 mechanical properties, determining the interaction forces with nanoscale lateral resolution,  
386 thereby providing new insights into membrane molecular mechanisms. For the nanomechanical  
387 characterization with AFM, an SLB is generally first imaged and then a series of force-distance  
388 curves is recorded over the same area. In each force-distance curve, the AFM tip away from the  
389 surface is approached and retracted at constant velocity while the cantilever deflection is  
390 recorded as a function of the Z piezo position. Each portion of the force-distance curve provides  
391 information about the physical and mechanical properties of the bilayer, schematized in Figure  
392 5. Upon mechanical contact during approach, the cantilever deflection increases and the SLB is  
393 elastically compressed by the AFM probe until the tip suddenly breaks through the SLB, jumping  
394 into a contact with the substrate (Figure 5, b). The first part of the curve, corresponding to small  
395 elastic deformation upon compression, can be used to estimate elastic descriptors of the  
396 membrane, like the Young modulus ( $E$ ), the area compression-expansion modulus ( $K_A$ ) and  
397 bending modulus ( $k_c$ ) (Dufrêne & Lee, 2000; Picas et al., 2012; L Redondo-Morata et al., 2016).  
398 When performing very small elastic deformations (far from the bilayer rupture) it is also often  
399 used for this calculation the retraction part of the curve. To estimate the elasticity of the lipid  
400 film, the most common model used is the Hertz model, where the surface is approximate to an  
401 infinitely half-space of an isotropic elastic solid and the indenter as non-deformable. The  
402 parameter describing the sample is the Poisson's ratio ( $\nu$ ), which depends on the nature of the  
403 material. For soft biological samples, the Poisson's ratio is generally set to 0.5 (as for  
404 incompressible materials). The geometry of the indenter determines the contact area. The  
405 original Hertz model considers the shallow contact between two spherical bodies, but several  
406 extensions were made for different indenter geometries (D. C. Lin et al., 2007). Again, when  
407 performing experiments with small deformations far from the onset of the lipid bilayer rupture,  
408 the hysteresis found between the approach and the retract curves is considered as dissipation  
409 work and can be used to obtain intrinsic viscoelastic parameters of the viscoelastic material, by  
410 the adaptation of common viscoelastic models as power-law rheology or the Kelvin-Voigt model  
411 (Garcia et al., 2020).

412 Moreover, different types of short-range interactions can be measured -DLVO, hydration or  
413 steric forces-, for further information review here (Lorena Redondo-Morata et al., 2012).







437 Dufrêne et al. (Dufrene et al., 1997, 1998)(Dufrêne & Lee, 2000; S Garcia-Manyes & Sanz, 2010;  
 438 B. Gumí-Audenis et al., 2016a; L. Redondo-Morata et al., 2012; Schneider et al., 2000).  $F_b$  is  
 439 generally of several nanonewtons (nN) and it is considered as a direct measurement of the  
 440 lateral interactions within the lipid bilayer, at a specified loading rate. From the step in the  
 441 separation before and after the breakthrough, the SLB thickness at different compression forces  
 442 (and at zero force) can be extracted.

443 The mechanical rupture of lipid bilayers is of thermal fluctuation nature, whose destructive  
 444 action is facilitated and directed by the application of the external force. The penetration of the  
 445 AFM tip through the SLB has been modeled and conceived as a two-state process with an  
 446 associated energy barrier (Butt & Franz, 2002; Franz et al., 2002; Simona Loi et al., 2002).  
 447 Specifically, two models describe the activation process. The first is the continuum nucleation  
 448 model, which considers the bilayer as a molecular thin homogeneous film -a two-dimensional  
 449 fluid layer- between the solid substrate and the solid surface of the AFM tip. The second model  
 450 ponders the molecular nature of the lipid bilayer and proposes that each molecule in the SLB  
 451 has specific binding sites corresponding to energetically favorable positions. These sites are  
 452 energetically equivalent, and as the SLB is pressed by the AFM tip, the energy of the molecules  
 453 significantly increases, leading them to jump apart and create a hole under the tip. After a critical  
 454 number of molecules have jumped out of the contact area, the tip indents the SLB due to the  
 455 high pressure of the remaining molecules, breaking the bilayer. Determining the energy barriers,  
 456 represented by the Arrhenius law, governing the lipid membranes rupture process contributes  
 457 to the understanding of the extent of the lateral interactions in the bilayer, and this is generally  
 458 achieved by following the variation of the  $F_b$  with the loading rate ( $r$ ) by varying the tip-sample  
 459 approaching velocity ( $v$ ) in what is called the dynamic force spectroscopy (DFS). Out of  
 460 equilibrium, the  $F_b$  increases linearly with the logarithm of  $r$  (and of  $v$  as well) (Butt & Franz,  
 461 2002; S Loi et al., 2002). The loading rate dependence with the thermomechanically activated  
 462 nature of the bilayer rupture kinetics leads to the evaluation of the activation energy of the  
 463 bilayer failure in the absence of an external force ( $\Delta E_0$ ). The activation energy  $\Delta E$  for the  
 464 formation of a hole in the bilayer that is large enough to initiate rupture and lead the tip  
 465 breakthrough can then be calculated following the expression proposed by Butt et al.:

$$\Delta E(F_b) = -k_B \cdot T \cdot \ln \left[ \left( \frac{0.693 \cdot k_s}{A} \right) \cdot \frac{dv}{dF_b} \right] \quad (\text{Eq. 1})$$

466 where  $k_B$  is the Boltzmann factor,  $T$  is the absolute temperature,  $k_s$  is the cantilever spring  
 467 constant,  $A$  is the frequency at which the AFM tip attempts to penetrate the bilayer, generally

468 approximated to the resonance frequency of the cantilever,  $v$  is the tip velocity, and  $F_b$  the  
469 breakthrough force.

470 When retracting the AFM tip away from the SLB, it sometimes remains connected to the bilayer  
471 through a lipid tube (Figure 5, d). While the tip moves further away, the membrane tube grows  
472 longer until it breaks at a certain distance, and the cantilever returns to the equilibrium position.  
473 This tube pulling process occurs at constant force, the tube growing force ( $F_{\text{tube}}$ ) (Armond et al.,  
474 2011; Berta Gumí-Audenis et al., 2018; Maeda et al., 2002; Ralf P Richter & Brisson, 2003), and  
475 it is observed as a force plateau in the retract part of the force-separation curve (Figure 5), at  
476 several tens of pN from which  $F_{\text{tube}}$  and the tube growing distance ( $d$ ) can be recorded. Pulling  
477 lipid tubes with an AFM tip out of SLBs is a simplified but analogous situation to some biological  
478 processes involving mechanical and conformational adjustments -bending, vesiculation,  
479 tubulation-like formation of membrane tethers, endocytic vesicles, etc. (Schmidtke & Diamond,  
480 2000; Shao et al., 1998; Sheetz, 2001; van Meer et al., 2008). Separation of a membrane segment  
481 from the cytoskeleton as well as strong membrane bending are both involved, for which the  
482 membrane chemical composition and physicochemical properties are key players. Pulling  
483 tethers from cells with an AFM probe is a widely explored approach to evaluate the membrane  
484 mechanics on entire cells (Brochard-Wyart et al., 2006; Marcus & Hochmuth, 2002; Nawaz et  
485 al., 2015; Sun et al., 2005). Recently, it has also been established as a method to locally probe  
486 SLB mechanics and the contribution of the underlying substrate to the measured properties on  
487 SLBs (Berta Gumí-Audenis et al., 2018).

488 When pulling tethers from cells, the  $F_{\text{tube}}$  depends on the membrane properties like bending  
489 stiffness ( $k_c$ ) and in-plane membrane tension ( $\sigma$ ), and also on the adhesion between the  
490 membrane and the cytoskeleton (Sheetz, 2001). In regions where the membrane has separated  
491 from the cytoskeleton -bleb or free membrane-, as there is no direct interaction with the  
492 cytoskeleton,  $F_{\text{tube}}$  strictly relies on  $k_c$  and  $\sigma$  (Dai & Sheetz, 1999). However, the cytoskeleton  
493 adhesion and  $\sigma$  are difficult terms to separate, and therefore the concept of apparent membrane  
494 tension,  $\sigma_{\text{app}}$ , has been proposed to include the adhesion energy parameter  $\gamma$  (Sheetz, 2001),  
495  $\sigma_{\text{app}} = \sigma + \gamma$ . When the lipid tube grows under thermodynamic equilibrium, at the limit of zero  
496 velocity (static thermodynamic analysis), the following mathematical expression relates the  
497 membrane parameters mentioned above with the  $F_{\text{tube}}$  (Armond et al., 2011; Canham, 1970;  
498 Dailant et al., 2005; E Evans & Yeung, 1994; Berta Gumí-Audenis et al., 2018; Hochmuth et al.,  
499 1996; Marcus & Hochmuth, 2002; Roux, 2013):  $\sigma_{\text{app}} = \frac{F_{\text{tube}}^2}{8k_c\pi^2}$ .

500 The plus of this approach, AFM-based pulling lipid tubes from SLBs, is that it combines the  
501 advantage of the AFM local probing with nanoscale lateral and force resolution, with the  
502 simplicity of the SLB model preparation. Besides, the local nanomechanical properties of SLBs  
503 can be explored through the combination of the tubing force spectroscopy approach and the  $F_b$   
504 analysis exposed before, within the same experimental procedure (Berta Gumí-Audenis et al.,  
505 2018).

506 Although it will not be extensively discussed here, we find important to mention that the  
507 constant force approach can also be used to evaluate the membrane rupture kinetics in SLBs  
508 and supported lipid multibilayers (SLMs). The AFM tip is approached at constant velocity, and  
509 then compresses the membrane at a constant force (force clamp, FC). Through measuring the  
510 time it takes to penetrate the bilayer -time to breakthrough ( $t_b$ )- distribution at different  
511 compression forces, the AFM-FC mode allows to estimate the rate of bilayer failure and calculate  
512 the location of the energy barrier maximum -distance to the transition state- along the reaction  
513 coordinate ( $\Delta x$ ) and the activation energy ( $E_0$ ) that characterize the energy barrier (L Redondo-  
514 Morata et al., 2012a; Relat-Goberna et al., 2017). Both  $E_0$  as well as  $\Delta x$  were found to be higher  
515 for SLBs than for supported lipid multibilayers, endorsing previous suspicion that the mechanical  
516 properties of lipid bilayers obtained from nano-indentation are strongly influenced by the  
517 underlying hard substrate.

## 518 4. Phase behavior and nanomechanics. From one component 519 membranes to higher complexity

### 520 4.1. One-component membranes

#### 521 4.1.1. The gel and the fluid phase

522 To start with the simplest system and increase the complexity in this revision -which does not  
523 intend to be exhaustive- consider a one-component phospholipid bilayer, which is thermalized  
524 at a temperature far from its  $T_m$ , the temperature of the main phase transition, at which a  
525 disordering of the hydrophobic tails happens followed by a sudden increase of the lateral  
526 mobility. We are then able to distinct two different main phases, gel-like or solid ordered phase  
527 ( $s_o$ ) below  $T_m$ , and fluid-like phase or liquid disordered ( $l_d$ ) above  $T_m$ .

528 A mechanical descriptor of lipid bilayers that shows significant differences between both phases  
529 is the bending modulus  $k_c$ . At  $T < T_m$ ,  $k_c$  is an order of magnitude larger than at  $T > T_m$ , rendering  
530 lipid vesicles quite stiff, while softer and more deformable above  $T_m$ . In the case of SVLs, where  
531 probing deformation is not straightforward, QCM-D has proved to be a useful tool to estimate  
532 the extent of vesicle deformation upon adsorption onto surfaces. Reviakine and co-workers

533 (Reviakine et al., 2012) reported deformation measurements of small DMPC vesicles on TiO<sub>2</sub>  
534 above and below T<sub>m</sub>, and experimentally found out that adsorbed vesicle deformation correlates  
535 with the bilayer bending modulus. Liposomes in the fluid phase bear a smaller bending modulus  
536 and deform to a greater extent.

537 When imaged with the AFM, the SLBs show typically 4-6 nm of height. The measured height is  
538 also influenced by both the elasticity of each bilayer and the particular AFM methodology, *i.e.*  
539 acoustic-modulation “tapping” mode, contact mode, etc, where the representation of the  
540 interface of the lipid bilayer changes. In terms of its mechanical properties, whether we are  
541 measuring the elastic deformation or the yield threshold force, a one-component SLB yields a  
542 monomodal distribution. While the distribution of deformations or breakthrough forces of the  
543 bilayer, being a stochastic process, have been often approached to Gaussian distributions, other  
544 distributions based on different theoretical frameworks can also be found in the literature, like  
545 the models proposed by Franz *et al.* (Butt & Franz, 2002) for the two-state activation process of  
546 the bilayer rupture, mentioned in section 3.2.

547 In the first place, it could be said that, in a general way, the bilayer thickness depends on the  
548 nature of the lipids -solid-like bilayers are usually higher than liquid-like counterparts of similar  
549 number of carbons in the hydrocarbon chains. In an s<sub>0</sub> phase, the hydrocarbon chains are  
550 stretched, so the apparent height is greater, and this is observed when imaging SLBs by AFM.  
551 Consequently, stretched acyl chains lead to stronger van der Waals interactions between  
552 neighboring molecules, which translates into a shorter intermolecular distance between  
553 phospholipids, and a greater mechanical resilience, both elastically and upon AFM tip  
554 penetration.

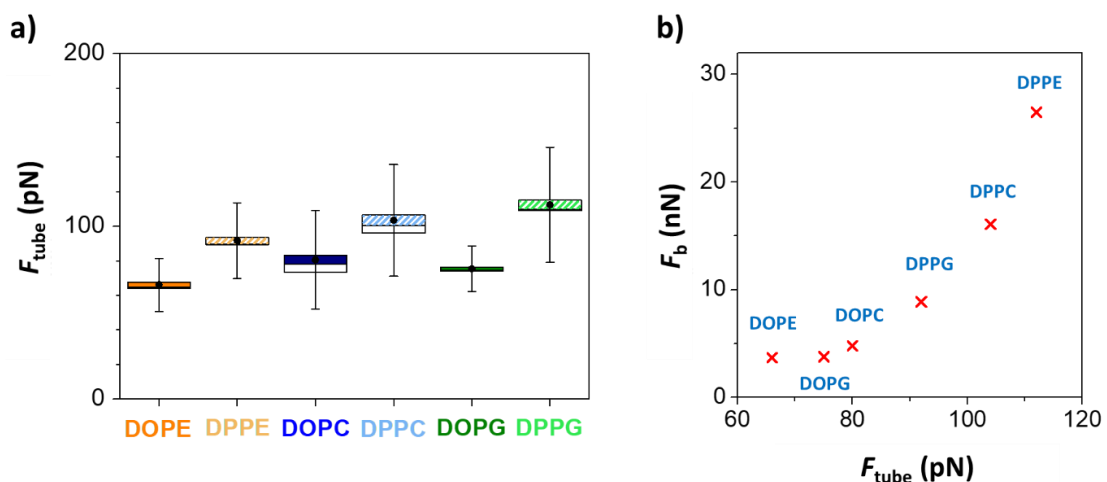
555 For example, let's briefly consider the elastic deformation of DOPC and DPPC, as models of l<sub>d</sub>  
556 and s<sub>0</sub> SLBs, respectively. The slope of the curve in the contact zone (elastic regime), as we have  
557 commented in section 3.2, is related to the elastic modulus of the material. At room  
558 temperature, the slope for the DOPC SLB, which is in the fluid phase, is lower than that for the  
559 solid DPPC SLB. This indicates that DPPC bears a larger Young modulus than DOPC and thus the  
560 membranes in the s<sub>0</sub> phase are therefore stiffer than in the l<sub>d</sub> phase. Both slopes are smaller  
561 than the one that can be measured on the bare substrate (often assumed as an infinitely stiff  
562 material, such as mica).

563 Besides the evidenced difference in elasticity for the fluid and gel-like membranes, l<sub>d</sub> SLBs show  
564 in general lower F<sub>b</sub> values than s<sub>0</sub> SLBs. A good example are homologous series phospholipids;  
565 while at room temperature DLPC (T<sub>m</sub> ~ -2 °C) SLBs break at force values between 2 and 13 nN,

566 according to the ionic environment, DPPC ( $T_m \sim 41.5$  °C) SLBs are pierced by the AFM probe, in  
567 similar conditions, in the force range that goes from 7 to 30 nN (Lorena Redondo-Morata et al.,  
568 2012). It is also noticeable that the  $F_b$  increase with the logarithm of the loading rate is lower for  
569 the fluid than for the gel membranes (Lorena Redondo-Morata et al., 2012). This can be  
570 expected, if we consider the Bell-Evans or assimilable model, the most likely breaking force  
571 depends linearly on the logarithm of the loading rate  $r$ ,  $F(r) = \left(\frac{k_B T}{x}\right) \ln \frac{rx}{K_0 k_B T}$ , where  $k_B$  is the  
572 Boltzmann constant,  $x$  is the distance to the transition state and  $K_0$  is the off-rate constant at 0  
573 force (E Evans & Ritchie, 1997). In this case,  $x$  is directly related to the distance between  
574 neighboring phospholipid molecules. This intermolecular distance, therefore  $x$ , is smaller in  $s_o$   
575 than in  $l_d$ .

576 Early work showed how AFM-FS is able to explore quantitatively the molecular determinants  
577 that provide mechanical stability to the supported membrane (S Garcia-Manyes, Oncins, et al.,  
578 2005a; Sergi Garcia-Manyes et al., 2010). By systematically and independently changing the  
579 chemical composition of the lipid headgroup, the length of the hydrophobic tail, and the number  
580 of chain unsaturations, {Citation} it was possible to describe the mechanical stability of each  
581 bilayer as a direct signature of membrane lateral organization (S Garcia-Manyes et al., 2010).  
582 The distribution of  $F_b$  values as a function of the length of the hydrophobic chain showed that  
583 on average, increasing each hydrocarbon tail by 2 extra  $-\text{CH}_2-$  groups results in  $\sim 7$  nN increase  
584 of the  $F_b$ , equivalent to an enthalpy increase by roughly 2 kJ/mol (Berg et al., 2002). In the same  
585 study, the mechanical properties of the linear DPPC were compared with its analogous branched  
586 phospholipid, DPhPC, to investigate the effect of acyl chain branching. The distribution of  $F_b$   
587 showed a decrease from  $\sim 20$  nN to  $\sim 12$  nN, revealing an important contribution of the lateral -  
588  $\text{CH}_3$  groups on the molecular packing of the bilayer. This observation can be rationalized in terms  
589 of the larger area per lipid for the branched DPhPC ( $76.8 \text{ \AA}^2$ ) compared to that for the linear  
590 DPPC ( $62.0 \text{ \AA}^2$ ), as revealed by molecular dynamics simulations conducted in the fluid phase  
591 (Shinoda et al., 2007). The nanomechanical effect of the  $-\text{cis}$  double bonds present in the acyl  
592 chains was also investigated. The  $F_b$  values distributions for each particular bilayer showed an  
593 inversely proportional trend between mechanical stability and number of unsaturations. It was  
594 estimated that upon introduction of one additional unsaturation in each acyl chain,  $F_b$  values  
595 decrease by  $\sim 5$  nN. Interestingly, introducing an asymmetric unsaturation resulted in an even  
596 further mechanical destabilization. The resulting molecular tilt reduces the effective packing  
597 between molecules, giving rise to a lower  $T_m$  (Berg et al., 2002). The findings regarding the  
598 mechanical implications of the headgroup chemistry were, however, far less straightforward  
599 and highly unanticipated. Comparing phospholipids with the same hydrocarbon tails but

600 different headgroups, a huge range of forces required to indent the bilayer in the same  
 601 experimental conditions, spanning from  $\sim 3$  nN (DPPA) all the way up to  $\sim 66$  nN (DPPG).  
 602 Furthermore, the lateral packing differences observed between fluid and gel-like bilayers is also  
 603 reflected in the tube growing force, pulled as the AFM probe moves away from the SLBs, as this  
 604 approach has demonstrated to be sensible to the membrane chemical composition in one-  
 605 component SLBs (Maeda et al., 2002)(Berta Gumí-Audenis et al., 2018). Higher  $F_{\text{tube}}$  values for  
 606 DPPC with respect to DMPC have already been observed by Maeda *et al.* (Maeda et al., 2002)  
 607 and, as reported in Gumí-Audenis *et al.* (Berta Gumí-Audenis et al., 2018),  $F_{\text{tube}}$  is lower for  $l_d$   
 608 mica-SLBs (in the range of 65–80 pN) than for  $s_o$  bilayers of the same headgroup (in the range of  
 609 90–110 pN) (Figure 6a). Additionally, while in the  $l_d$  state the  $F_{\text{tube}}$  slightly augments from PE to  
 610 PC ( $66 \pm 15$  pN for DOPE,  $80 \pm 25$  pN for DOPC and  $75 \pm 13$  pN for DOPG), it clearly increases  
 611 when changing the headgroup for  $s_o$  membranes from PE to PC and then PG ( $92 \pm 20$  pN for  
 612 DPPE,  $104 \pm 30$  pN for DPPC and  $112 \pm 30$  pN for DPPG). Hence,  $F_{\text{tube}}$  is generally higher for  $s_o$   
 613 than for  $l_d$  and goes along with the lateral packing associated to different phospholipid  
 614 headgroups, in a way that is comparable to the trend observed on the  $F_b$  approach, as can be  
 615 seen in Figure 6b.



616  
 617 **Figure 6.** AFM-pulling lipid tubes sensible to the membrane composition in one-component SLBs.  
 618 a)  $F_{\text{tube}}$  values for single component SLBs of different composition (DOPE, DPPE, DOPC, DPPC,  
 619 DOPG and DPPG). Boxchart for  $F_{\text{tube}}$  values show the mean ( $\bullet$ ), SE (box) and SD (bars). b)  
 620 Correlation between  $F_b$  and  $F_{\text{tube}}$  values for single component SLBs. The individual phospholipids  
 621 contain a constant tail length of 18 C with 1 unsaturation (DOPE, DOPG and DOPC) or 16 C and  
 622 fully saturated (DPPE, DPPC and DPPG), in fluid and gel state at room temperature (RT),  
 623 respectively. All the measurements were performed in 150 mM NaCl, 20 mM  $\text{MgCl}_2$ , 20 mM

624 HEPES (pH 7.4) buffer solution and at RT. Adapted from Ref. (Berta Gumí-Audenis et al., 2018)  
625 with permission from the Royal Society of Chemistry.

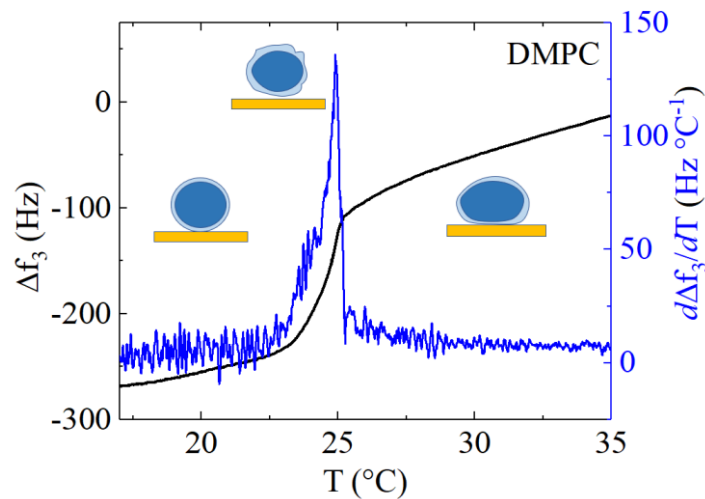
626 Taken together, force spectroscopy experiments on SLBs are in qualitative agreement with the  
627 literature that correlates the physicochemical properties of membranes with the chemical  
628 composition of their phospholipid hydrophobic chains (Rawicz et al., 2000). Noteworthy,  
629 thermodynamic quantities such as the melting temperature or the order parameter can be  
630 translated into the realm of mechanical stability. The order parameter is usually calculated from  
631 NMR-based measurements and provides information about the overall membrane order and  
632 more particularly the conformations that the atoms within the lipid tails adopt (Piggot et al.,  
633 2017).

#### 634 4.1.2. The thermal transition

##### 635 4.1.2.1. Main transition

636 A useful application of QCM-D is the detection of thermotropic phase transitions within SLBs  
637 and SVLs, with the asset that it is label-free, it does not require long temperature equilibration  
638 times nor a large amount of sample. When temperature is varied in the absence of any  
639 transition,  $\Delta f$  and  $\Delta D$  typically show a regular, linear temperature dependence, as a reflection of  
640 regular, temperature-dependent changes of the viscosity and density of the surrounding liquid  
641 medium. When approaching the main phase transition temperature, both frequency and the  
642 dissipation responses will differ from a regular temperature dependence, displaying anomalies  
643 that reveal that structural changes are taking place (Losada-Pérez et al., 2014; Neupane et al.,  
644 2018). Figure 7 displays changes in the frequency and its derivative of an SVL of DMPC of vesicles  
645 of around 130 nm, with main transition temperature  $T_m \sim 24$  °C in a rather narrow temperature  
646 range. The shape of frequency shift curves and its respective temperature derivative are  
647 reminiscent of the enthalpy jump and its heat capacity derivative typically obtained from  
648 calorimetric measurements (Heimburg, 1998; Losada-Pérez et al., 2014). Dissipation typically  
649 shows jumps and minima in the same temperature range. Therefore, extrema in the first-order  
650 derivative of frequency and dissipation curves stand as a token of the layer thermotropic phase  
651 transitions. Calorimetry is a state-of-the art technique for the detection of phase transitions  
652 based on changes in thermal properties when lipids undergo the main (first-order) transition  
653 (heat absorption or release), while QCM-D is based on changes in viscoelastic properties of the  
654 adsorbed vesicles as a result of changes in bilayer thickness, rigidity and vesicle shape. Although  
655 also detectable in SLBs, the size of the jumps in  $\Delta f$  and  $\Delta D$  is several orders of magnitude smaller  
656 than the size of the jumps observed for SVLs (Wargenau & Tufenkji, 2014; Hasan & Mechler,  
657 2015).





658

659 **Figure 7.** Temperature dependence of the frequency shift  $d\Delta f_3/dT$  (black solid line) and its first-  
 660 order derivative (blue solid line) of a DMPC SVL upon heating at 0.2 °C/min.

661 Reviakine and coworkers provided a comprehensive interpretation of the mechanisms by which  
 662 QCM-D detects phase transitions in lipid membranes and the related relative contribution of  
 663 thickness, stiffness and hydrodynamic channels to the  $\Delta f$  and  $\Delta D$  fingerprints (Peschel et al.,  
 664 2016). Upon heating, lipid bilayers in both SVLs and SLBs change from a thicker and stiffer  $s_o$   
 665 phase to a thinner and less stiff  $l_d$  one. Both stiffness and thickness changes in the lipid layer  
 666 properties across the transition encompass changes in the  $\Delta f$  signal (decrease upon heating). In  
 667 the case of the  $\Delta D$ , the stiffness and the thickness contributions oppose each other since stiffer  
 668 layers dissipate less. In the SLBs, this leads to an overtone dependence of the transition peak  
 669 (Peschel et al., 2016). In thicker and softer SVLs, changes in the shape of the adsorbed vesicles  
 670 might induce further hydrodynamic channels that contribute to  $\Delta D$ . As a consequence, the size  
 671 of the maximum in SVLs is several orders of magnitude larger than the one observed in SLBs.

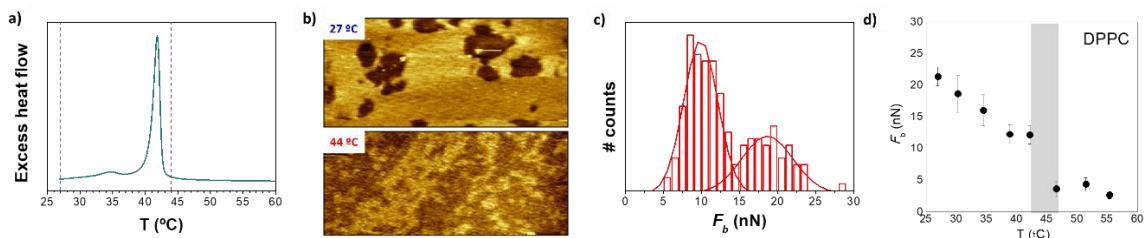
672 The thermotropic phase behavior of pure SLBs has been widely related with the  
 673 (nano)mechanical properties of the bilayer. Combining the previously exposed approaches, the  
 674 AFM-FS sensitivity to the lipid phase of SLBs is evidenced, positioning it as an appropriate tool  
 675 to determine the phase thermal transitions. Temperature controlled-AFM has been established  
 676 as a suitable tool to analyze both the topographical and mechanical evolution of biologically  
 677 relevant issues that are temperature dependent at the nanometer scale.

678 Seminal work of Leonenko *et al.* demonstrated the existence of several phase transitions in DPPC  
 679 and DOPC mica-supported bilayers (Z. V. Leonenko et al., 2004). Data of both topographical  
 680 imaging and force measurements provide invaluable insight into membrane behavior during  
 681 phase transitions. For DPPC SLBs,  $F_b$  values decrease as temperature increases, and beyond the



682 main transition, DPPC shows similar force plots as the fluid-phase DOPC bilayers. This  
 683 dependence of the  $F_b$  on the phase state of the SLB below and above  $T_m$  has been further  
 684 reported for different lipids, like DMPC ( $T_m \sim 24 \text{ }^\circ\text{C}$ ) that changes  $F_b$  from 13-14 nN at 20  $^\circ\text{C}$   
 685 below  $T_m$  to about 6 nN at or 40  $^\circ\text{C}$  DPPC ( $T_m \sim 42 \text{ }^\circ\text{C}$ ) that shows a switch of  $F_b$  from around 20-  
 686 25 nN at 27  $^\circ\text{C}$  to around 3-5 nN at 55  $^\circ\text{C}$  (S Garcia-Manyes, Guell, et al., 2005; L Redondo-Morata  
 687 et al., 2012b). When the DPPC SLB is maintained a temperature slightly above  $T_m$ , coexistence  
 688 of the gel and fluid phase occurs and a bimodal  $F_b$  distribution is obtained (Figure 8), as reported  
 689 in Gumí-Audenis *et al.* (B. Gumí-Audenis et al., 2016a). This is because the thermal transitions in  
 690 SLBs generally happen at temperatures slightly higher and in a broader range than in vesicle  
 691 suspensions as a consequence of the underlying substrate affecting lateral order and interleaflet  
 692 coupling (S Garcia-Manyes, Guell, et al., 2005; Z. V Leonenko et al., 2004; L Redondo-Morata et  
 693 al., 2012b; Seeger et al., 2010). In Garcia-Manyes *et al.* it was shown that the main phase  
 694 transition occurs in a range of temperatures of *ca.* 10-13  $^\circ\text{C}$  (S Garcia-Manyes, Oncins, et al.,  
 695 2005b).

696 There is an effect of temperature on the mechanical stability for the SLBs because the increase  
 697 of temperature directly affects the molecules fluctuations provoking an increment in the area  
 698 per molecule -lower packing density-, especially noticeable in the gel phase, with the  
 699 consequent decrease of the  $F_b$  values. At temperatures around the  $T_m$  of the lipid, and for a  
 700 temperature range of a few  $^\circ\text{C}$ , a pronounced drop in  $F_b$  takes place, as the SLB gradually  
 701 transitions to the fluid phase, thereby softening. Several degrees over the  $T_m$ , the  $F_b$  values are  
 702 on the order of the ones typically observed for  $l_d$  SLBs at room temperature (Alessandrini & Facci,  
 703 2012; S Garcia-Manyes, Oncins, et al., 2005a; L Redondo-Morata et al., 2012b). This can be seen  
 704 in the example shown in Figure 8d, where the DPPC mica-SLB shows an  $F_b$  value around of 21  
 705 nN at 27  $^\circ\text{C}$  and slight decreases with temperature as the 42–45  $^\circ\text{C}$  range is reached. At higher  
 706 temperatures, the  $F_b$  remains approximately constant at *ca.* 3.5 nN. The  $F_b$ -temperature trend  
 707 displays a break around the  $T_m$  value of DPPC.



708

709 **Figure 8.** Phase transition of DPPC bilayers: a) DSC thermogram of DPPC vesicle suspension; b) 6  
 710  $\mu\text{m} \times 2.5 \mu\text{m}$  AFM images of DPPC silicon-SLB at 27  $^\circ\text{C}$  and 44  $^\circ\text{C}$ ; c)  $F_b$  histogram at 44  $^\circ\text{C}$  and

711 gaussian fits to the bimodal distribution. All experiments performed in 150 mM NaCl, 20 mM  
712 MgCl<sub>2</sub>, 20 mM HEPES (pH 7.4) buffer solution. Adapted from Ref. (B. Gumí-Audenis et al., 2016a).  
713 d) Mean  $F_b$  value of DPPC mica-SLB as a function of temperature, in 10 mM HEPES, 20 mM MgCl<sub>2</sub>  
714 and 150 mM NaCl (pH 7.4). The shadowed vertical line marks the observed transition  
715 temperature range. Adapted from Ref. (L Redondo-Morata et al., 2012b)

716 Additionally, Mouritsen and coworkers proposed that AFM topography at a varying temperature  
717 could decouple the phase transition of the two different leaflets of the mica-supported bilayer  
718 (D. Keller et al., 2005). The low temperature transition was proposed to be related to the melting  
719 of the leaflet far from the substrate surface, and the second transition to the phase transition of  
720 the leaflet in contact with the mica. Such decoupling would respond to the stronger interaction  
721 between the lipid headgroups of the proximal monolayer and the mica support.

#### 722 4.1.2.2. Pretransition

723 Some lipids present a pretransition before the main first-order transition which gives rise to an  
724 intermediate order in the lipid bilayers referred to as ripple phase. Ripple phases are curved  
725 structures with undulating surface topography. AFM imaging revealed the structure of the ripple  
726 phase in hydrated conditions at the nanometer scale (Kaasgaard et al., 2003), and such particular  
727 structure has been recently explored nanomechanically (Majewska et al., 2020). It can be found  
728 when solid phase lipids experience an increase in temperature and go into the fluid phase, and  
729 alternatively it occurs when the fluid phase is cooled down. The observation of this phase  
730 transition process is characterized by a melting point temperature of the ripple phase to fluid  
731 phase, nucleation point and growth directionality for the formation of the ripple phase. HS-AFM  
732 coupled to a temperature-controlled system can report directly ripple to fluid phase transitions  
733 (reversibly) in real time and at high resolution (Takahashi et al., 2016). Transition processes from  
734 ripple phase to fluid phase and from ripple phase to metastable ripple phase to fluid phase,  
735 could be reversibly, phenomenologically and quantitatively studied. In Takahashi *et al.*, results  
736 showed phase transition hysteresis in fast cooling and heating processes, while both melting  
737 and condensation occurred at 24.15 °C in quasi-steady state situation. A second metastable  
738 ripple phase with larger periodicity was formed at the ripple phase to fluid phase transition when  
739 the buffer contained Ca<sup>2+</sup>. Thermodynamically, a lipid system containing small domains does not  
740 necessarily correspond to a phase coexistence regime, but they may constitute a one phase  
741 regime with micro- or nanoscale heterogeneities<sup>1</sup> (Heimburg, 2000). Altogether, these findings

---

<sup>1</sup> We will further visit this topic in Section 5).

742 may have relevance in more complex cellular systems where lipid nanodomains form and  
743 dissociate reversibly.

#### 744 4.2 Phase coexistence

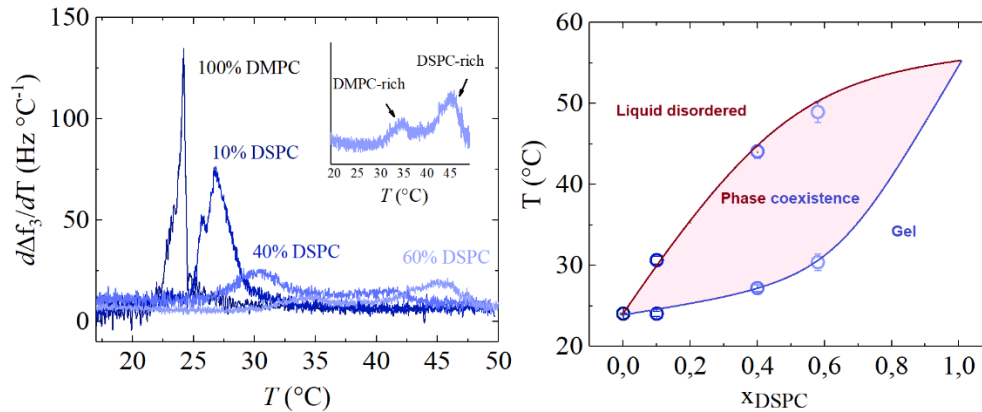
745 Phase segregation and coexistence in lipid bilayers generally happens in the simplest models  
746 when mixing a fluid and a gel-like phospholipids, and in the more complex mixtures, like those  
747 containing phospholipids, sphingomyelins, ceramides, and cholesterol. At room temperature  
748 the bilayers composed of a mixture of a gel and a fluid phospholipid generally segregate into  
749 different domains, showing a continuous fluid phase enriched with  $l_d$  lipid and thicker dispersed  
750 domains associated to a phase enriched in the  $s_o$  lipid. When the  $T_m$  of the gel-phase lipid is  
751 overcome, the binary phospholipid system becomes homogeneous and fluid. Among the many  
752 that can be found in the literature, we here mention some examples of these binary systems.

753 The capability of QCM-D to detect lateral phase coexistence and map phase diagrams for binary  
754 lipid mixtures of saturated phospholipids will be provided for binary mixtures of homologous  
755 phosphatidylcholines DMPC and DSPC, whose chemical structures differ in four methylene  
756 groups. This system has been thoroughly investigated experimentally and theoretically in free  
757 standing lipid systems (Ehrig et al., 2011; Fidorra et al., 2009; Mabrey & Sturtevant, 1976).  
758 DMPC: DSPC mixing behavior is driven by the large hydrophobic mismatch between the two  
759 components and departs from ideality as a consequence of the large difference in  $T_m$  of the pure  
760 constituents.

761 Figure 9 (left panel) shows representative QCM-D signature of phase transitions we now revisit  
762 taking place upon heating pure SVLs of pure DMPC and three DMPC: DSPC mixtures (Losada-  
763 Pérez, Mertens, et al., 2015). The diameter of the precursor vesicles forming the SVLs was  
764 around 130 nm. For pure DMPC, the main transition takes place in a rather narrow temperature  
765 range around  $T_m \sim 24$  °C. The first-order derivatives of  $\Delta f$  curves capture the typical behaviour  
766 of two-component lipid mixtures displaying  $s_o$  and  $l_d$  phase coexistence. As very low DSPC  
767 concentration (DSPC mole fraction = 0.1), the transition is shifted towards higher temperatures  
768 and takes place in a broader temperature range indicating a loss of intermolecular cooperation  
769 between DMPC molecules to undergo the main transition due to the presence of DSPC. For the  
770 mixtures close to the equimolar concentration two small peaks can be observed indicating the  
771 coexistence of two phases,  $s_o$  and  $l_d$ . The peaks are not simple additions of the peaks of the  
772 individual constituents, the size of each peak scales with the concentration of each lipid in each  
773 coexisting phase. For instance, the inset of Figure 9 displays the peaks of the mixture  
774 DMPC: DSPC (40:60). The low-temperature peak corresponds to the DMPC-rich phase, while the

775 high-temperature peak to the DSPC-rich phase. As expected, the size of the latter is higher, since  
 776 the concentration of DSPC in the mixture is larger.

777 The analysis of the peaks using the tangent method (Ehrig et al., 2011) to determine the onset  
 778 and completion temperatures enables to map the phase diagram of this mixture. The solid lines  
 779 are added as a guide to the eye, delimiting the phase coexistence region, dark red (liquidus line),  
 780 light blue (solidus line) and follow experimental heat capacity values by Maubrey *et al.* (Mabrey  
 781 & Sturtevant, 1976) obtained for multilamellar vesicles of the same concentration.



782

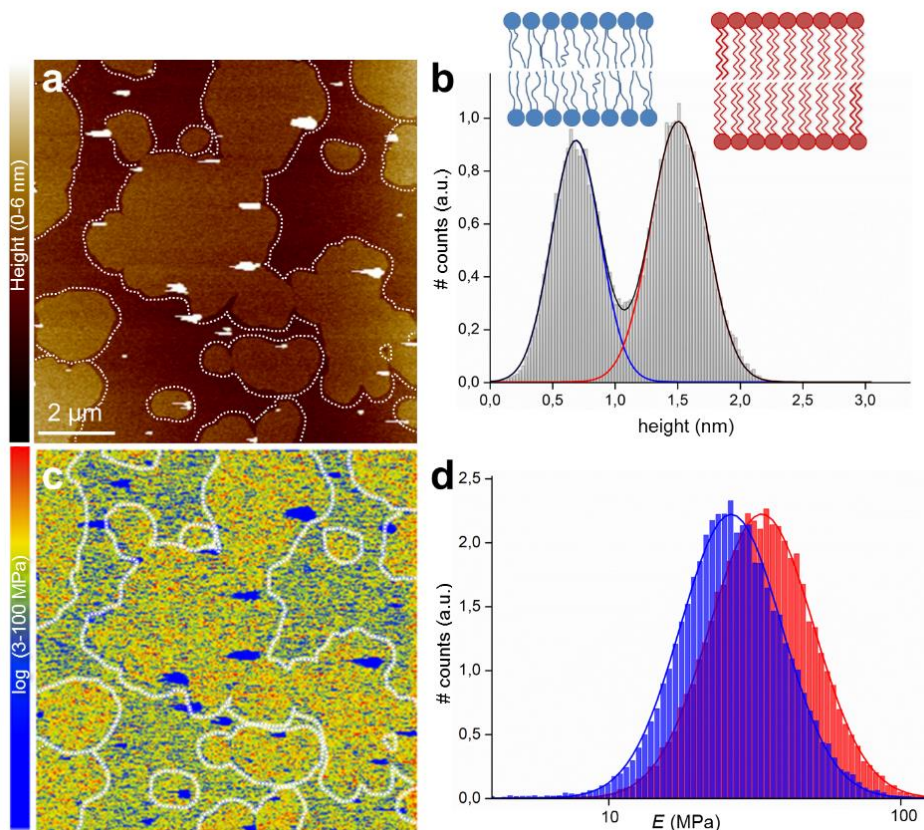
783 **Figure 9.** Left panel: temperature profiles of the first-order temperature derivative of the  
 784 frequency shifts for pure DMPC and DMPC:DPSC mixtures 10%, 40% and 60% mole fraction (in  
 785 percentage) of DSPC. Right panel: phase diagram obtained from extracting onset and completion  
 786 temperatures from the peaks (Losada-Pérez, Mertens, et al., 2015).

787 As discussed in the previous sections, the nanomechanical properties determined by AFM-FS on  
 788 SLBs are distinctive of the chemical structure and molecular organization of a bilayer at specific  
 789 environmental conditions. This allows for a direct correlation of the elastic modulus,  $F_b$ , or  $F_{\text{tube}}$   
 790 values with the composition in phase-segregated membranes. In most cases, the AFM  
 791 topographical characterization easily identifies the segregation of an SLB in different domains,  
 792 due to their difference in thickness and compressibility. However, it is possible to reveal the  
 793 presence of different domains in an SLB on the basis of their different mechanical properties  
 794 even when the domains have a height difference which is too small to be detected by  
 795 conventional imaging.

796 Bernchou *et al.* studied, using combined fluorescence microscopy and AFM, the nucleation and  
 797 growth of domains in equimolar binary SLBs composed by DOPC and DPPC (Bernchou et al.,  
 798 2009), a mixture that shows coexistence of  $l_d$  and  $s_o$  domains at room temperature (Z. V.  
 799 Leonenko et al., 2004). These authors could observe that during cooling the shape of the  
 800 domains evolved from compact to a branched morphology, which they discussed as the domain

801 growth being controlled by the diffusion of DPPC from the liquid phase toward the solid domain  
 802 interface. In the late stages of the growth, the size and overall shape of the domains depend on  
 803 the position of the nucleation points relative to the surrounding nucleation point positions.

804 A mechanical contribution concerning the elastic deformation of the membrane before the  
 805 breakthrough point should be considered to fully comprehend the force-distance curve. A  
 806 method to study the elastic properties consists of quantitative nanomechanical mapping (Picas  
 807 et al., 2012). In (L Redondo-Morata et al., 2016), the equimolar binary lipid mixture DPPC:DOPC  
 808 was studied, where  $l_d$  and  $s_o$  coexisting domains in SLBs are large and directly visually discernable  
 809 by AFM imaging (Figure 10a) with clearly distinguishable heights ( $\sim 0.8$  nm height difference,  
 810 Figure 10b). Elastic modulus nano-mechanical mapping (Figure 10c) shows good agreement  
 811 with the  $E$  values in the literature, characterized mechanically by AFM (Picas et al., 2012; Rawicz  
 812 et al., 2000) and micropipette aspiration (Dieluweit et al., 2010). The mean  $E$  values are  
 813  $26_{-12}^{+58}$  MPa for the  $l_d$  domain and  $33_{-15}^{+75}$  MPa for the  $s_o$  domain, respectively (Figure 10d).



814

815 **Figure 10.** PF-QNM AFM Topography and Elasticity mapping of DOPC:DPPC (1:1) SLBs. a)  
 816 Topography image (nm) and b) height histogram analysis of the topography (a). The dashed lines  
 817 in a) represent the domain edges defined by edge detection. The solid lines in b) are Gaussian fits  
 818 to the height distribution, indicating an average height difference between the DOPC  $l_d$  (blue line)



819 and DPPC  $s_o$  (red line) domains of 0.82 nm. c) The corresponding stiffness map (log scale, MPa)  
 820 and d) Young's modulus values histograms (log scale, MPa) of the regions outlined in the  
 821 topography (a) and stiffness (c) maps, corresponding to the DOPC (blue) and DPPC (red) domains.  
 822 The solid lines are Gaussian fits to the distributions peaking at  $26^{+58}_{-12}$  Mpa (DOPC) and  $33^{+75}_{-15}$  Mpa  
 823 (DPPC) (from reference (L Redondo-Morata et al., 2016) with permission).  
 824 Using the Young modulus for each domain of the DOPC:DPPC mixture together with the  
 825 experimental bilayer thickness ( $h$ ), the thin shell theory can be used to calculate the area  
 826 compression-expansion modulus ( $K_A$ ) and bending modulus ( $k_c$ ) as (Rawicz et al., 2000):

$$827 \quad K_A = \frac{Eh}{(1-\nu^2)} \quad \text{and} \quad k_c = \frac{Eh^3}{24(1-\nu^2)}$$

828 When lipid domains separate in lipid mixtures, or when lipids surround membrane embedded  
 829 proteins there is, in general, a hydrophobic mismatch at the domain interfaces (Wallace et al.,  
 830 2006) or protein/bilayer interfaces (Mouritsen & Bloom, 1984), with an ensuing compression or  
 831 stretching of the hydrocarbon chains in the vicinity of the interface. This hydrocarbon tail  
 832 stretching or compression may influence the distribution of domain sizes and the kinetics of  
 833 domain separation, as well as membrane protein function. The compression/stretching of the  
 834 acyl chains of the  $s_o$  and  $l_d$  domains can be determined by minimizing the elastic energy of the  
 835 system,  $G_s(x)$ , expressed assuming volume conservation (Wallace et al., 2006) as

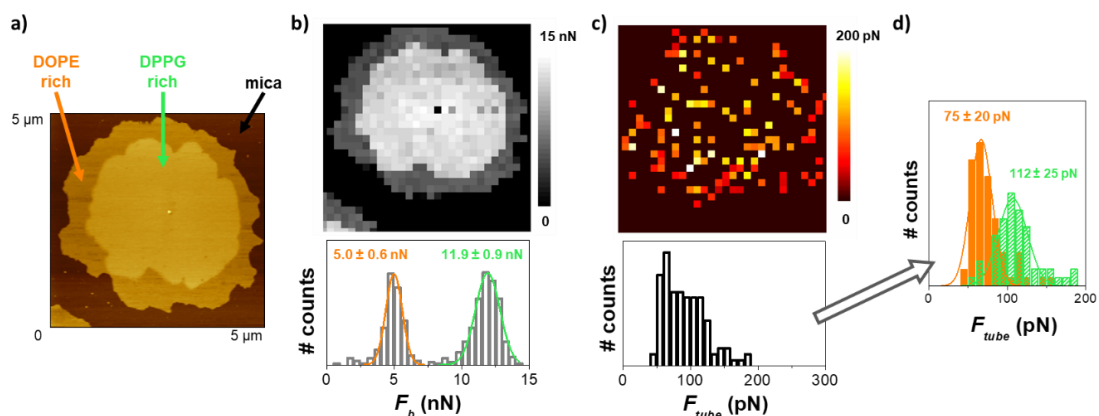
$$836 \quad G_s = \frac{K_o A_o}{2h_o^2} (x - h_o)^2 + \frac{K_d A_d}{2h_d^2} (x - h_d)^2 \quad (2)$$

837 where  $K_o$  and  $K_d$  are the stretch moduli for  $s_o$  and  $l_d$ , respectively.  $A_o$  and  $A_d$  are the lipid area  
 838 per molecule for  $s_o$  and  $l_d$ , respectively.  $A$  values are well-documented in literature (notably  
 839 through X-ray diffraction and Langmuir methods), being 0.72 nm<sup>2</sup> for  $l_d$  DOPC and 0.49 nm<sup>2</sup> for  
 840  $s_o$  DPPC at room temperature (Nagle & Tristram-Nagle, 2000).  $h_o$  and  $h_d$  are the measured  
 841 bilayer thickness of  $s_o$  and  $l_d$ , respectively. The estimated  $G_s$  values for the DOPC  $l_d$  and the DPPC  
 842  $s_o$  domains are 0.27  $k_B T$ . Hence, the experimental determination of  $E$  allows to estimate the  
 843 stretch modulus and the bending stiffness of the lipid membrane, as well as the energetic cost  
 844 of the lipid domains to accommodate to different thickness.

845 For the SLB system composed of a POPE:POPG mixture (Picas et al., 2009), AFM images revealed  
 846 the existence of two separated phases, the higher showing a region with protruding  
 847 subdomains. AFM-FS was then used to clarify the nature of each phase. The values of  $F_b$ ,  
 848 adhesion force and height extracted from the force curves were assigned to the corresponding  
 849  $s_o$  (the mechanically stable domains) and  $l_d$  phase (the mechanically labile phase). In Crespo-

850 Villanueva *et al.*, while the SLB composed of equimolar DSPC:DOPC was shown to phase  
 851 segregate, clearly demonstrated in the AFM topographical images and the correlative  $F_b$  maps  
 852 and bimodal distributions around  $\sim 40$  nN and  $\sim 5$  nN, the equimolar mixture of the two  $l_d$  lipids  
 853 DOPC:DOPS led to a homogeneous topography and narrow  $F_b$  distribution around 3.5 nN  
 854 (Crespo-Villanueva *et al.*, 2018). In Domenech *et al.*, they studied ternary mixtures of  
 855 POPC:POPE:CL, a minimum system that mimics the mitochondrial inner membrane (Domenech  
 856 *et al.*, 2007). They could observe laterally segregated domains whose height decreased with  
 857 increasing temperatures.

858 Pulling lipid tubes with the AFM probe also allows to characterize phase segregated bilayers and  
 859 laterally resolve the different domains in multicomponent SLBs (Berta Gumí-Audenis *et al.*,  
 860 2018). This is exemplified in Figure 11, a bilayer composed of the  $l_d$  lipid DOPE and the  $s_o$  lipid  
 861 DPPG in ratio DOPE:DPPG 25:75, that at room temperature forms SLB patches with coexisting  
 862 domains: gel-phase rich in DPPG, dispersed in fluid-phase rich in DOPE. When indented by the  
 863 AFM tip at a constant velocity of  $1 \mu\text{m s}^{-1}$ , a bimodal  $F_b$  distribution is obtained, with  
 864 differentiated mean  $F_b$  values associated with the fluid ( $\sim 5$  nN) and gel ( $\sim 12$  nN) domains.  
 865 Analyzing the retract force-separation curves that showed the tube pulling event allowed to  
 866 represent the  $F_{\text{tube}}$  distribution and map, where the lower force values (red pixels) are  
 867 distributed in the peripheral part of the SLB patch and higher forces (yellow pixels) in the center,  
 868 and the  $F_{\text{tube}}$  distribution is resolved when sorting according to the corresponding  $F_b$ , giving a  
 869 mean value of  $75 \pm 20$  pN for the DOPE-rich phase, and  $112 \pm 25$  pN for the DPPG-rich phase.  
 870 The fact that  $F_b$  and  $F_{\text{tube}}$  for the domains slightly differ from the values for pure DOPE and DPPG  
 871 SLBs allows to infer partial miscibility among them.



872  
 873 **Figure 11.** DOPE:DPPG (25:75). a) AC-mode AFM topographical image; b)  $F_b$  map and distribution;  
 874 c)  $F_{\text{tube}}$  map and distribution; d)  $F_{\text{tube}}$  distributions resolved by filtering according to the  $F_b$

875 distribution. Reproduced from Ref. (Berta Gumí-Audenis et al., 2018) with permission from the  
876 Royal Society of Chemistry.

877 Higher complexity models, like the ternary lipid mixtures containing an unsaturated  
878 phosphatidylcholine (PC), a saturated PC or sphingomyelin (SM), and Cholesterol (Chol) have  
879 been probably the most studied due to the physiological abundance of these lipids. Depending  
880 on the ratio of the components, these mixtures have shown to exhibit two coexisting fluid  
881 phases: an  $l_o$  phase that is enriched in Chol and SM or saturated PC, and an  $l_d$  phase that is  
882 predominantly composed of unsaturated PC (Johnston, 2007), and the possibility of a coexisting  
883  $s_o$  phase. The group of Schwille has extensively analyzed the domain organization in  
884 DOPC/SM/Chol model membranes by means of AFM in combination with fluorescence  
885 correlation spectroscopy (FSC) (S Chiantia, Kahya, et al., 2006; S Chiantia, Ries, et al., 2006;  
886 Salvatore Chiantia et al., 2007, 2008) and by performing AFM-FS experiments on the different  
887 phases they have shown that the average force needed to pierce the  $l_o$  phase of the  
888 DOPC/SM/Chol bilayer is about 4 nN higher than the one needed to pierce the  $l_d$  phase. In a  
889 parallel study, Zou and coworkers explored the influence of different cholesterol contents on  
890 the morphology and (nano)mechanical stability of phase-segregated DOPC/SM/Chol lipid  
891 bilayers by means of AFM imaging and force mapping (R M A Sullan et al., 2010). They observed  
892 consistently higher  $F_b$  values in the SM/Chol-enriched  $l_o$  domains than in the DOPC-enriched  $l_d$   
893 phase, and that the (nano)mechanical stability of both coexisting phases counterintuitively  
894 decreased with increasing Chol content. By following the dependence of the  $F_b$  value with the  
895 loading rate in both phases, they calculated the activation energies ( $\Delta E_0$ ) of bilayer rupture at  
896 zero applied force.  $\Delta E_0$  values obtained were in the range 75-125 kJ/mol for all tested Chol  
897 contents and did not show a dependence on the different lipid phases.

898 Besides sphingolipids, ceramides (Cer) have also gained attention in physicochemical studies by  
899 means of AFM, since they are known to be predominant in physiologically relevant nanodomains  
900 -which have been controversially called for many years lipid rafts (Goñi, 2019a). Longo and  
901 coworkers observed that DLPC/GalCer/Chol ternary mixtures only display  $l_d/s_o$  coexistence and  
902 no  $l_o$  phase even at high Chol content (Blanchette et al., 2006). By measuring the area/perimeter  
903 ratios of the segregated domains, the authors concluded that Chol was able to decrease the line  
904 tension between the two phases. In another study (W.-C. Lin et al., 2007), they could also  
905 observe a phase coexistence attributed to  $l_d/l_o$  phases with POPC and DOPC at 10 mol% Chol, an  
906 indication that fatty acyl chain unsaturation, cholesterol ratio and lipid hydrophobic mismatch  
907 in the organization of multi-component lipid systems.



908 Ceramide incorporation on coexisting fluid phase and ordered domains in phase-separated  
909 binary and ternary lipid mixtures was shown to affect the lipid spatial organization in domains,  
910 with the appearance of a Cer-enriched gel-like phase and the displacement of cholesterol from  
911 rafts (S Chiantia, Kahya, et al., 2006; Salvatore Chiantia et al., 2007; Ira et al., 2009). In order to  
912 directly probe and quantify the (nano)mechanical stability and rigidity of the Cer-enriched  
913 platforms, Zou *et al.* performed AFM topographical images and force mapping that allowed the  
914 determination of the  $F_b$  value and elastic modulus of the different phases (Ruby May A Sullan et  
915 al., 2009a, 2009b; Zou & Johnston, 2010). They confirmed the expulsion of cholesterol from  
916 sphingolipid/Chol-enriched domains as a result of Cer incorporation, and observed an increased  
917 mechanical stability attributed to the influence of Cer in the lipid organization and packing  
918 behavior, also reported later on GalCer rich domains on model membranes with PC and Chol  
919 (Berta Gumí-Audenis et al., 2015).

920 The group of Goñi has systematically explored the effect of the acyl compositions of  
921 sphingomyelins and the corresponding ceramide counterparts in ternary mixtures with PC and  
922 Chol (García-Arribas et al., 2016; González-Ramírez et al., 2019; Jiménez-Rojo et al., 2014)).  
923 Combining confocal microscopy, DSC, AFM imaging and FS, they compared the effects of C24:1  
924 Cer (nervonoyl ceramide, nCer) with those of C16:0 Cer (palmitoyl ceramide, pCer) in bilayers  
925 composed basically of DOPC, SM (either C24:1, nSM or C16:0, pSM) and Chol (García-Arribas et  
926 al., 2017). AFM-FS showed that nCer has a lower stiffening effect than pCer, while the presence  
927 of nSM reduces the stiffness. When the proportion of phospholipid increases beyond 60 mol %,  
928 a lateral phase separation occurs at the micrometer scale (González-Ramírez et al., 2019). The  
929 authors interpreted this data as a pCer:Chol interaction, that would predominate at the lower  
930 phospholipid concentrations. The putative pCer:Chol complexes (or nanodomains) would mix  
931 well with the phospholipid. At higher SM concentrations pSM:pCer and pSM:Chol interactions  
932 would become more important, giving rise to the coexisting  $s_o$  and  $l_o$  phases, respectively.  
933 Heterogeneity, or lateral phase separation, occurs more easily with pSM than with DPPC,  
934 indicating a higher affinity of SM over DPPC for Chol or Cer. Altogether, the findings of Goñi and  
935 coworkers show the sharp increase in complexity when membranes exhibit different  
936 sphingolipids of varying N-acyl chains, which should be a common issue in an actual cell  
937 membrane environment.

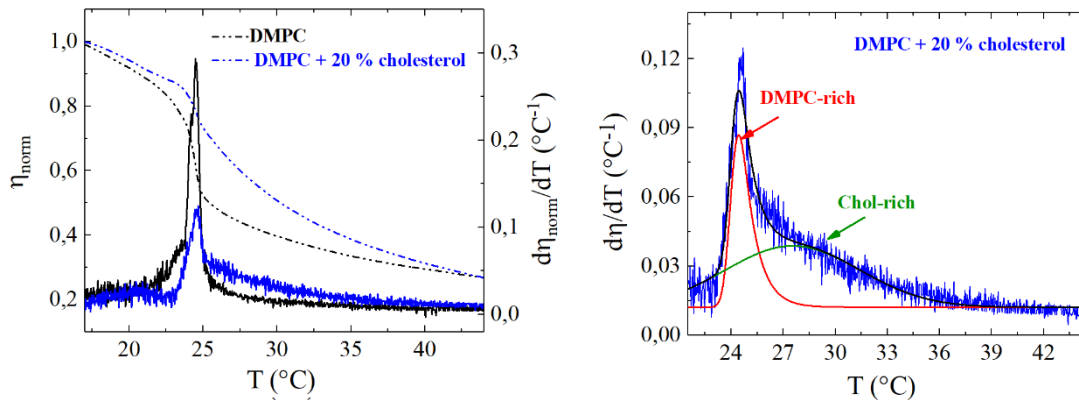
## 938 1.2. The role of cholesterol

939 Cholesterol is a fundamental constituent of the eukaryotic cell plasma membranes. Eukaryotic  
940 cell membranes are not entirely in the conventional  $l_d$  phase. Instead, they are, at least partially,  
941 in the Chol-rich liquid-ordered ( $l_o$ ) phase or a phase with similar properties.  $l_o$  phase is

942 characterized by a high degree of acyl chain order and is favored by high- $T_m$  lipids with saturated  
943 acyl chains such as sphingolipids, when they are mixed with Chol, as exposed in the previous  
944 section. Chol plays the essential function of regulating the physical properties of the cell  
945 membrane as it controls the lipid organization and phase behavior, regulates the membrane  
946 fluidity and its mechanical strength, influencing the passive permeability of water and other  
947 small molecules (Pan et al., 2008; Pinkwart et al., 2019). From the molecular perspective, Chol  
948 is considered to produce a condensing effect and induce ordering to fluid phase lipids forming  
949 the membrane and to have the opposite effect on phospholipids present in the gel phase (Hung  
950 et al., 2007; Rog et al., 2009). However, several experiments pointed out the complex effect of  
951 Chol on lipid bilayers, which depends on the molecular structure of the neighboring lipids: the  
952 degree of chain unsaturation, the length of the hydrophobic tail and the chemistry of the  
953 headgroup (Pan et al., 2008; Mills et al., 2009; Garcia-Manyes et al., 2010). In view of  
954 acknowledging the particular role of cholesterol, let us briefly outline results on the phase  
955 behavior of SVLs and SLBs consisting of binary mixtures of saturated phospholipids and  
956 cholesterol using mechanical descriptors such as shear viscosity of SVLs and nanomechanics of  
957 SLBs.

958 We first describe the phase behavior of SVLs containing binary mixtures of DMPC:Chol using  
959 QCM-D. Upon the addition of Chol to phospholipid bilayers, the sharp main phase transition in  
960 pure DMPC gradually diminishes and eventually disappears over a given Chol concentration. In  
961 the case of DMPC, SVLs showed no transition from 30% cholesterol. Figure 12 shows normalized  
962 shear viscosity  $\eta_{\text{norm}} = [\eta(t)/\eta(t=0)]$ , with  $t$  the time] for pure DMPC and DMPC:Chol (20 mol %  
963 Chol) calculated using a Voigt-based viscoelastic model (Voinova et al., 1999). At intermediate  
964 Chol concentrations, asymmetric peaks can be observed. These peaks encompass two main  
965 contributions, a sharp, narrow peak and a broader one that overlaps with the former (see right  
966 panel in Figure 12 for a more detailed view including Gaussian fitting). Two domains can be  
967 identified, namely, the sharp contribution can be ascribed to the pure DMPC-rich domains  
968 undergoing the main transition and the latter to the Chol-rich ones. The broader peak appears  
969 as a result of the reduced mobility of the hydrophobic chains of DMPC in Chol-rich domains. The  
970 presence and the size of these domains depend strongly on the concentration of cholesterol and  
971 the temperature. On increasing the amount of cholesterol, the temperature range of the overall  
972 transition increases as a result of the extension of the second broad contribution, while the size  
973 of the maximum corresponding to the melting of the single lipid decreases. No phase transition  
974 is detected at Chol concentration over 30 mol % indicating the formation of a homogeneous  
975 liquid ordered  $l_o$  phase at a macroscopic level. Such a large cholesterol concentration inhibits

976 the formation of extended domains. A similar pattern of behavior was observed for DPPC:Chol  
 977 SVLs, however, for this later system, the completion temperature of the overall transition at  
 978 intermediate cholesterol concentrations was not attained, due to the (upper) limited  
 979 temperature range of commercial QCM-D systems (Losada-Pérez, Khorshid, et al., 2015). These  
 980 results are in agreement with recent calorimetric studies of free-standing membranes of the  
 981 same systems (Mannock et al., 2010).

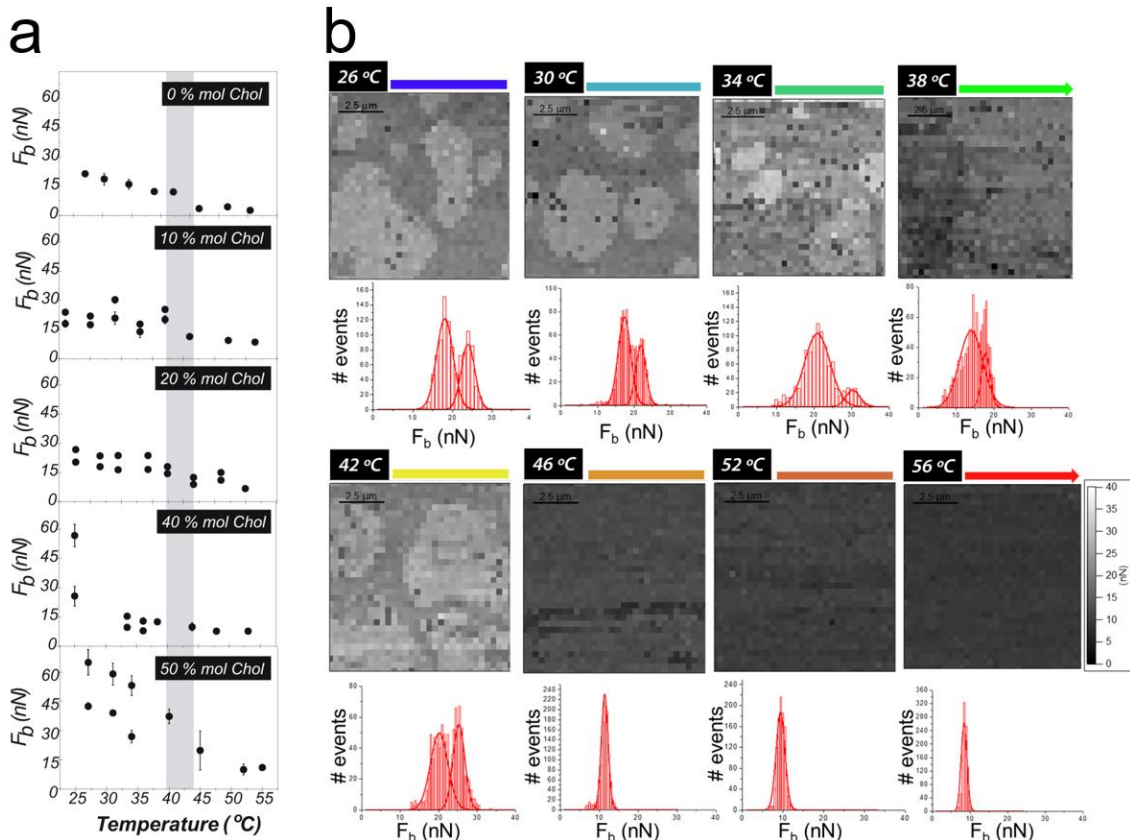


982  
 983 **Figure 12.** Left panel Temperature dependence of the normalized effective shear viscosity (dash  
 984 dotted lines) and its first-order derivative (solid line) of DMPC and DMPC + 20% cholesterol SVLs  
 985 on a supported vesicle layer on a gold-coated QCM-D quartz sensor upon heating. Right panel  
 986 closer view of the normalized shear viscosity first-order derivative for DMPC + 20% cholesterol  
 987 SVLs. Solid lines denote multiple peak gaussian fitting.

988 The role of Chol in the structure and nanomechanics of SLBs has been extensively studied by  
 989 AFM (L Redondo-Morata et al., 2012b; R M A Sullan et al., 2010; Al-Rekabi & Contera, 2018; S  
 990 Garcia-Manyes et al., 2010; Adhyapak et al., 2018), as well as its interplay with sphingolipids or  
 991 other lipids (García-Arribas et al., 2016; 2006; Ahyayauch et al., 2002; Guyomarc'h et al., 2014)  
 992 and further reviewed in (B. Gumí-Audenis et al., 2016b).

993 By means of temperature-controlled AFM imaging and AFM-FS to assess the influence of Chol  
 994 on the membrane ordering and stability (Redondo-Morata et al., 2012), this work analyzed a  
 995 DPPC:Chol in representative range of compositions up to 50 mol % Chol, studying the phase  
 996 evolution upon heating (from room temperature to temperatures high above the  $T_m$  of DPPC)  
 997 and the corresponding (nano)mechanical stability. This provided a correlation between the  
 998 mechanical behavior and composition with the lateral order of each phase present in the  
 999 bilayers. It proved that low Chol contents lead to a phase segregated system, while high contents  
 1000 of Chol can give a homogeneous topography, although a bimodal  $F_b$  distribution was observed,  
 1001 possibly due to the presence of small heterogeneities that could not be resolved in the images.  
 1002 In all cases, it was demonstrated that Chol enhances the mechanical stability of the binary

1003 membrane, and an extraordinarily stable system was observed for equimolar fractions (50 mol  
1004 % Chol). In addition, even when no thermal transition was detected by the traditional bulk  
1005 analysis techniques for vesicles with high Chol content (40 and 50 mol %), it was shown that  
1006 temperature-controlled AFM-FS is capable of identifying a thermal transition for the SLBs. As  
1007 detailed in Figure 13, for high-Chol contents -40 and 50 mol %- the DPPC:Chol system shows a  
1008 double  $F_b$  distribution at room temperature, probably due to the presence of (space or time)  
1009 non-resolved heterogeneities for these compositions. Although no thermal transition was  
1010 observed in DSC thermograms of free-standing systems, when AFM-FS experiments were  
1011 performed at different temperatures for these systems, a gradual decrease of the mean  $F_b$  value  
1012 was observed until temperature reached ca. 40 °C. The continuous decrease in the  $F_b$  value with  
1013 increasing temperature may be ascribed to an increase in fluidity of the system, and it is evident  
1014 that, although not detected in DSC of DPPC:Chol vesicles, a transition occurs for DPPC:Chol SLBs  
1015 with 40 and 50 mol % Chol around 42-45 °C. Below this temperature, the system in the  $l_o$  phase  
1016 presents extraordinary lateral order and may have properties that resemble a gel-like bilayer,  
1017 and as temperature increases, it gradually becomes a system with higher lateral mobility (but  
1018 still with higher order than the DPPC  $l_d$  phase). This behavior indicates that for DPPC:Chol SLBs,  
1019 a thermal transition occurs even for high-Chol compositions, in a temperature range between  
1020 ca. 42-47 °C independently of the membrane composition, and that it may probably be of  
1021 second-order for the high-Chol DPPC:Chol bilayers (40-50 mol %), which generally appear as a  
1022 very broad transition or it is even not detected in DSC experiments of free standing vesicles or  
1023 QCM-D of SVLs.



1024

1025 **Figure 13.** a) Mean  $F_b$  value of DPPC:Chol SLBs in 10 mM HEPES, 20 mM  $\text{MgCl}_2$  and 150 mM NaCl,  
 1026 pH 7.4, with various Chol contents, as a function of temperature. The shadowed vertical line  
 1027 marks the temperature range where the main transition in pure DPPC occurs. For DPPC:Chol SLBs  
 1028 with 40 and 50 mol % Chol, although not detected in DSC of DPPC:Chol vesicles, a transition occurs  
 1029 around 42–45  $^{\circ}\text{C}$ . b)  $F_b$  maps and distributions for DPPC:Chol SPB with 10 mol % Chol, in 10 mM  
 1030 HEPES, 20 mM  $\text{MgCl}_2$  and 150 mM NaCl, pH 7.4, with increasing temperature. Reprinted from (L  
 1031 Redondo-Morata et al., 2012b).

1032 Strikingly, information about the lateral in-plane structure of such systems can be instead  
 1033 obtained by grazing incidence XR diffraction (GIXD). In (Gumi-Audenis et al., 2018), a silicon-SLB  
 1034 system was designed in order to deal with the requirement of the wetting preservation to  
 1035 guarantee the membrane stability while minimizing the background from the liquid  
 1036 environment. Using this methodology, it was confirmed that DPPC:Chol with low contents of  
 1037 Chol up to 30 mol % lead to separation into two coexisting phases at room temperature and  
 1038 concentrations higher than 30 mol % Chol appear to be a unique  $l_o$  phase. This condition was  
 1039 consistent with the earlier work (Redondo-Morata et al., 2012b) demonstrating that such  
 1040 increase of nanomechanical resistance is associated to a strong lateral interaction mediated by  
 1041 Chol molecules placed between the DPPC ones, due to a highly stable structure with most  
 1042 probably an equimolar DPPC:Chol ratio.

1043 To date, efforts continue to understand the complex role of cholesterol in biomembranes. Still,  
1044 these discoveries cannot always be directly translated to the behavior of living cell membranes.  
1045 By means of advanced fluorescence imaging and spectroscopy, Sezgin and colleagues (Pinkwart  
1046 et al., 2019) have shown that cholesterol diffuses faster than phospholipids in live membranes,  
1047 but not in model membranes. Interestingly, a detailed statistical diffusion analysis suggested  
1048 two-component diffusion for cholesterol in the plasma membrane of live cells.

1049 Recent work based on a systematic AFM-FS study on DOPC:Chol SLBs at varying ratios suggested,  
1050 however, that cholesterol may not just be responsible of increasing the mechanical stability but  
1051 also introduce irregularities across the leaflets (Adhyapak et al., 2018). At Chol ratios above 20%,  
1052 they could observe two sudden jumps in the force-distance curve, the second jump always  
1053 displaying shorten separation. They interpreted that cholesterol induced asymmetry across the  
1054 (in the inner and outer leaflets) bilayer, related to the phenomena of interleaflet coupling and  
1055 depending on Chol concentration.

1056 Recently, Contera and coworkers took a different approach to study the effect of Chol in the  
1057 mechanical properties of lipid bilayers (Al-Rekabi & Contera, 2018). Applying different  
1058 frequencies simultaneously in an AFM topography mode, namely amplitude modulation–  
1059 frequency modulation (AM–FM), they investigated the effect of Chol on DPPC bilayers in  
1060 concentrations from 0 to 60 %. The lipid bilayers probed with this methodology displayed both  
1061 elastic and viscous behavior. Cholesterol showed to modulates both, increasing elastic and  
1062 viscous components as a function of Chol concentrations. The authors interpreted that their  
1063 results may have a physiological relevance, where such viscoelastic properties may be used by  
1064 cells to modulate the propagation (elastic) or attenuation (viscous) of mechanical signals across  
1065 the cell membrane.

## 1066 5. Connection between nanoscale measurements and 1067 thermodynamic descriptors of membranes

1068

1069 When studying phase coexistence in lipid membranes a distinction between thermodynamically  
1070 stable phases and transient local domains in lipid bilayer at equilibrium should be made. Let us  
1071 briefly discuss this in line with the above-exposed results on phospholipid-cholesterol binary  
1072 mixtures. PC-Chol binary mixtures have been studied for many years using a plethora of  
1073 experimental techniques. Yet, despite their apparent simplicity being two-component systems,  
1074 their phase behavior remains controversial and, in many cases, departs from the canonical phase  
1075 diagram proposed by Vist and Davis (Vist & Davis, 1990). In fact, results depend strongly on the



1076 spatio-temporal resolution of the different experimental techniques. For instance, unlike  
1077 predicted by most phase diagrams,  $l_d$ - $l_o$  phase coexistence was not observed for micron-sized  
1078 GUVs of DPPC-cholesterol mixtures using fluorescence microscopy (Veatch & Keller, 2003),  
1079 while some other techniques captured this phenomenon for free standing MLVs vesicles (DSC  
1080 (Mannock et al., 2010), volumetric (Miyoshi et al., 2014), NMR (Davis et al., 2009), SLBs (AFM)(L  
1081 Redondo-Morata et al., 2012b), and SVLs (QCM-D)(Losada-Pérez, Khorshid, et al., 2015). These  
1082 findings suggest that, instead of macroscopic phase separation, these systems are presumably  
1083 filled with mesoscopic and nanoscopic domains, whose size and lifetime depends on  
1084 temperature and mixture composition (Winkler et al., 2017).

1085 Classically, when dealing with phase separation the Gibbs' phase rule is customarily invoked,  
1086 assuming macroscopic phase coexistence and neglecting the role of phase boundaries, which  
1087 comparably, hardly contribute to the free energy. If domains are small, the interfaces between  
1088 domains constitute an important part of the system and its contribution to the free energy is  
1089 not negligible. In this respect, using the term phase in the classical thermodynamic sense might  
1090 not be appropriate (Heimburg, 1998; Almeida et al., 2005). Following Hill (Hill, 2013) a system  
1091 can no longer be called a phase when it ceases to behave like a macroscopic system and starts  
1092 behaving like a small system.

1093 In this respect, both AFM and QCM-D have demonstrated to be valuable tools to detect domain  
1094 (nano/mesoscopic sized domains) coexistence within supported membranes of binary lipid  
1095 mixtures.

## 1096 6. Conclusions and future perspectives

1097 As mentioned in the previous sections, AFM-FS can interestingly resolve coexistence of domains  
1098 at concentrations where height differences at domain boundaries are not detectable.  
1099 Breakthrough force thus stands as a useful mechanical descriptor to resolve domain coexistence.  
1100 Even though domains cannot be considered as phases in the purely thermodynamic standpoint,  
1101 this does not mean that these systems cannot be treated in a rigorous thermodynamic way. As  
1102 a matter of fact, the breakthrough force is able to detect a thermal transition at high cholesterol  
1103 concentrations in binary PC:Chol SLBs, whose origin deserves to be revisited and further  
1104 explored in the framework of either fluctuations arising in the vicinity of a critical point, the  
1105 possibility of glassy-cholesterol-rich nanodomains or eventual cholesterol crystallization. In this  
1106 sense, the development of HS-AFM will likely provide new insights due to its unprecedented  
1107 spatio-temporal resolution, thanks to a direct correlation between structure and diffusion  
1108 (Munguira et al., 2016). Similarly, HS Force Spectroscopy has the capacity to cover a wider force

1109 spectrum, which can help to validate theoretical frameworks of the field, as well as provide a  
1110 direct correlation with Molecular Dynamics simulations (Rico et al., 2013).

1111 There is indeed plenty of room in fundamental lipid biophysics and phase behavior in particular  
1112 to use both purely mechanical and viscoelastic properties as descriptors to provide a more  
1113 complete picture of the origins of domain formation and coexistence. The ultimate lipid phase  
1114 coexistence to be fully understood are transient nanodomains, often (confusively) referred to  
1115 as lipid rafts. There is to date no agreement about the size and lifetime of rafts, and the term is  
1116 used to referred to a collection of different structures (Goñi, 2019). Based on the current  
1117 knowledge, microdomains in equilibrium are no longer considered suitable models for the  
1118 biological structure that rafts represent. Fortunately, advanced super-resolution microscopies  
1119 and fast techniques are becoming available and will shed some light in one of the most discussed  
1120 topics in biomembrane studies.

1121 In light of this, multiscale spatio-temporal measurements of mechanical properties of supported  
1122 membranes can help to experimentally address different scenarios where membrane micro- and  
1123 nano-domain formation finds theoretical support. Starting from equilibrium ternary lipid  
1124 mixtures in the vicinity of a critical point, it might be worth exploring how the presence of critical  
1125 fluctuations reflects in the membrane mechanical properties, and if these properties can be  
1126 used as physical descriptors to test critical-point universality laws. Secondly, tuning membrane  
1127 (local and global) environment by actively controlling curvature, leaflet asymmetry, solid  
1128 support topography and addition of external components would help complete the manifold  
1129 where domain formation occurs. Finally, the growing spatiotemporal capabilities of AFM are  
1130 promising tools to explore membrane organization in the realm of non-equilibrium phenomena.

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## 1138 8. Glossary of lipid acronyms

1139

Acronym	Long name
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<i>C16:0 palmitoyl Cer (aka palmitoyl Cer, pCer)</i>	N-palmitoyl-D-erythro-sphingosine
<i>C16:0 palmitoyl SM (aka palmitoyl SM)</i>	N-palmitoyl-D-erythro-sphingosylphosphorylcholine
<i>C24:1 nervonoyl Cer (aka nervonoyl Cer, nCer)</i>	N-nervonoyl-D-erythro-sphingosine
<i>C24:1 nervonoyl SM (aka nervonoyl SM)</i>	N-Nervonoyl-D-erythro-sphingosylphosphorylcholine-d9
<i>Cer</i>	ceramide
<i>Chol</i>	cholesterol
<i>CL</i>	cardiolipin
<i>DMPC</i>	1,2-dimyristoyl-sn-glycero-3-phosphocholine
<i>DOPC</i>	1,2-dioleoyl-sn-glycero-3-phosphocholine
<i>DOPE</i>	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
<i>DOPG</i>	1,2-dioleoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))]
<i>DOTAP</i>	1,2-dioleoyl-3-trimethylammonium-propane
<i>DPPA</i>	1,2-dipalmitoyl-sn-glycero-3-phosphate
<i>DPPC</i>	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
<i>DPPE</i>	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
<i>DPPG</i>	1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
<i>DSPC</i>	1,2-distearoyl-sn-glycero-3-phosphocholine
<i>GalCer</i>	Galactosylceramides
<i>PC</i>	phosphocholine
<i>PE</i>	phosphoethanolamine
<i>POPC</i>	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
<i>POPE</i>	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
<i>POPG</i>	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
<i>SM</i>	sphingomyelin

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