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## Modulation of gastric lipase adsorption onto mixed galactolipid-phospholipid films by addition of phytosterols

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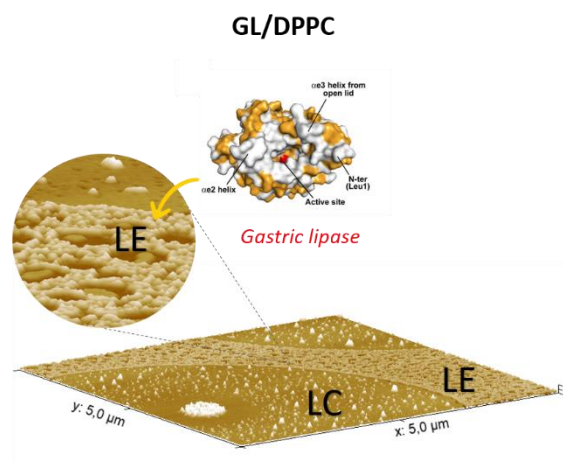
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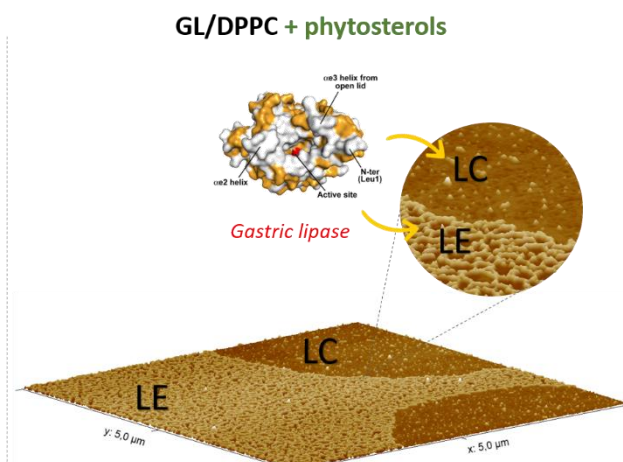
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# 1 GRAPHICAL ABSTRACT

## Interfacial studies



*Lipase adsorption onto the fluid phase (LE)  
Exclusion from the LC domains and phase boundaries*



*Lipase adsorption onto the fluid phase (LE)  
But also at the level of the defects created by the  
inclusion of pS into the LC domains and at phase  
boundaries*

2

3

## 4 HIGHLIGHTS

- 5 • rDGL binds to the fluid phase of homogeneous galactolipid monolayer.
- 6 • The orientation of galactolipid polar heads limits the adsorption capacity of rDGL.
- 7 • rDGL was excluded from condensed phase domains in mixed heterogenous galactolipid-
- 8 phospholipid monolayer.
- 9 • Defects created in the condensed phase by addition of phytosterol favor rDGL adsorption.



32 **Abbreviations**

33 AFM: atomic force microscopy

34 BMM: bovine milk membranes

35 DGDG: digalactosyldiacylglycerol

36 DPPC: dipalmitoylphosphatidylcholine

37 GL: galactolipids

38 HGL: human gastric lipase

39 HMM: human milk membranes

40 IRS: Interfacial recognition site

41 LC: liquid condensed phase

42 LE: liquid expanded phase

43 MGDG: monogalactosyldiacylglycerol

44 MPL: milk polar lipids

45 PTL: pancreatic triacylglycerol lipase

46 rDGL: recombinant dog gastric lipase

47

48

49 **ABSTRACT**

50 The rapid and preferential adsorption of a gastric lipase recombinant dog gastric lipase (rDGL) in  
51 heterogeneous films of phospholipids and triacylglycerols has previously been unveiled using  
52 Langmuir films analyzed by tensiometry, ellipsometry and Langmuir-Blodgett transfer coupled to  
53 atomic force microscopy. Here we invest the adsorption behavior of rDGL in heterogeneous  
54 galactolipid and mixed galactolipid-phospholipid or galactolipid-phospholipid-phytosterol films  
55 representative of plant membrane. Again rDGL, preferentially got adsorbed at the expanded lipid  
56 phases of the films underlining the genericity of such adsorption behavior. The addition of  
57 phytosterols to these mixtures resulted in the creation of defects, favoring the adsorption of rDGL  
58 at the fluid phases, but also improving the adsorption capacities of the lipase at the phase boundaries  
59 and towards the defects in the condensed phase. rDGL, like all gastric lipases, does not show any  
60 activity on galactolipids and phospholipids but its adsorption impacts their lateral organization and  
61 may change the adsorption and activity of other lipolytic enzymes in the course of digestion.

62 **KEYWORDS: adsorption, gastric lipase, galactolipids, heterogeneous biomimetic**  
63 **membranes, plant lipids, ellipsometry/tensiometry, atomic force microscopy**

64

## 65 **1 INTRODUCTION**

66 Lipases (EC 3.1.1.3, triacylglycerol hydrolase) play an important role in lipid metabolism, and have  
67 been found in most living organisms, from the plant kingdom to microorganisms and animals  
68 (Huang, 1987; Jaeger et al., 1994). Lipases are water-soluble enzymes that catalyze the hydrolysis  
69 of the ester bonds of insoluble triacylglycerols (TAG). Prior to the hydrolysis of their substrates,  
70 lipases must therefore get adsorbed onto the lipid-water interface (Aloulou et al., 2006). The  
71 digestion of lipids is a complex phenomenon, which depends on their chemical structure, their  
72 organization in water (monolayers, bilayers, micelles, oil/water emulsions...) and the ability of  
73 lipolytic enzymes to interact with these lipid assemblies. Indeed, the mechanisms involved in  
74 enzymatic lipolysis depends on the interfacial organization of the lipid substrates.

75 In humans, lipid digestion starts in the stomach where gastric lipase is responsible for 10%  
76 to 25% of the TAG total digestion (Carrière et al., 2001; Lengsfeld et al., 2004) and is very central  
77 during neonatal period as it is mature at birth (Bourlieu et al., 2015). It is worth nothing that gastric  
78 lipase is a true lipase, acting on TAG digestion but not on polar lipids, such as phospholipids or  
79 galactolipids. Human gastric lipase (HGL) is a globular enzyme of 50 kDa, belonging to the  $\alpha/\beta$   
80 hydrolase family, and possessing a classical Ser-His-Asp catalytic triad (Canaan et al., 1999;  
81 Roussel et al., 1999). The active site is covered by a lid, which has to be displaced to permit the  
82 TAG substrates to have access to the enzyme active site. Changes in the conformation (opening)  
83 of the lid have been observed in the homologous dog gastric lipase, co-crystallized in the presence  
84 of substrate analogs (Roussel et al., 2002). The lid opening generates a large hydrophobic ring, that  
85 is part of the interfacial recognition site (IRS) located around the active site entrance (Figure 1).

86 Gastric lipase has been found to be highly active in the acidic environment of the stomach  
87 with an optimum at pH 4-5.4, thanks to a remarkable stability and adsorption capacity at the

88 lipid/water interface at low pH, in contrast to other lipases (Bénarouche et al., 2013; Chahinian et  
89 al., 2006). This pH-dependent interfacial adsorption is driven by hydrophobic interactions between  
90 the substrates and the large hydrophobic ring, part of the IRS (Figure 1.B) (Chahinian et al., 2006)  
91 and electrostatic interactions, rDGL surface potential being mostly positive at low pH (Figure 1.C).  
92 This combination of interactions probably explains why rDGL is a highly tensioactive lipase with  
93 a high penetration capacity into phospholipid layers (Bénarouche et al., 2013).

94 Prior to hydrolyzing TAG, lipases frequently have to get adsorbed onto interfacial films composed  
95 of polar lipids and/or amphiphilic proteins (Bénarouche et al., 2017), in which a small fraction of  
96 TAG playing various metabolic part can also be found (Lerique et al., 1994). This is typically the  
97 case of milk fat globules and phospholipid-stabilized emulsions. Polar lipids are not hydrolyzed in  
98 the upper gastrointestinal tract by gastric lipase, but this enzyme has a strong ability to get inserted  
99 into phospholipid (PL) monolayers thanks to its high surfactant capacity (Bénarouche et al., 2013;  
100 de La Fournière et al., 1994) and thus, has access to the TAG substrate. In addition, gastric lipolysis  
101 favors the activity of pancreatic lipase (Gargouri et al., 1986) and has been recently reported to  
102 significantly affect the production and the degradation of intermediate TAG digestion products  
103 during the intestinal phase (Infantes-Garcia et al., 2021). Given the synergy between the gastric  
104 and intestinal digestion phases, it is crucial to obtain knowledge on the protein-lipid interactions  
105 occurring during the gastric phase, including the impact of gastric lipase adsorption onto the  
106 interfacial organization of membrane lipids, even though gastric lipase is not active on  
107 phospholipids (Carrière et al., 1991) nor on galactolipids (Wattanakul et al., 2019).

108 Polar lipid films are useful tools to study the interfacial properties of lipases, irrespective  
109 of their lipolytic activity. Langmuir films used as model interfaces allows determining the impact  
110 of various physicochemical parameters, including the lipid packing and composition at the

111 air/water interface, on the adsorption and insertion capacity of a lipase. The variations of surface  
112 pressure are related to interactions between molecules, which are directly connected to the  
113 variations of molecular areas. The coupling of surface pressure measurements and ellipsometry  
114 provides insights on the thickness and refractive index of the surface layer formed, and thus on the  
115 phenomena occurring in one micrometer range below the interface.

116 Previous studies have been done on the adsorption behavior of gastric lipase onto model  
117 heterogenous films mimicking the outer layer of the membrane of human and bovine milk fat  
118 globule (Bourlieu et al., 2016, 2020). These studies have shown that the coexistence of expanded  
119 and condensed phases influenced sternly lipase adsorption: lipase got rapidly inserted onto the fluid  
120 phase and at the phase boundaries. In addition, different levels of insertion were observed  
121 suggesting a molecular cooperation and besides hydrophobic interactions local negative charges  
122 reinforced adsorption.

123 However, to the best of our knowledge, no study has yet tackled the question of adsorption  
124 and penetration mechanisms of gastric lipase onto plant cell membranes, especially the nature  
125 widespread photosynthetic membranes. On the contrary to animal cell membranes, plant  
126 photosynthetic membranes to save up phosphorus do not contain phospholipids but  
127 galactoglycerophospholipids (or galactolipids), which accounts for more than 70% of polar lipids  
128 (Dörmann & Benning, 2002). In addition to their biological and structural functions in membranes,  
129 galactolipids are of nutritional importance since they are rich in polyunsaturated fatty acids  
130 (PUFA), essentials to cell functions in the human body, and especially in the  $\alpha$ -linolenic acid  
131 (C18:3 n-3, ALA) (Sahaka et al., 2020). Phytosterols and carotenes are also major constituents of  
132 plant cell membranes (Grosjean et al., 2015; Silvius, 2005). They play a key role in maintaining  
133 biologic properties of membranes by controlling their structural properties such as their



134 permeability, fluidity and rigidity (Dufourc, 2008; Silva et al., 2007). The chemical structures of  
135 phytosterols are similar to those of animal cholesterol, nevertheless the absorption of the majority  
136 of phytosterols in the intestinal tract of mammals is up to ten times lower than that of cholesterol  
137 (von Bergmann et al., 2005). In humans, phytosterol consumption has been shown to induce a  
138 lowering of plasma cholesterol levels, thus preventing cardiovascular diseases and atherosclerosis  
139 (Moghadasian & Frohlich, 1999). Sterol molecules are known to promote hydrophobic interactions  
140 between membrane lipid acyl chains, which introduce a higher order and a subsequent rigidification  
141 of membranes (Silva et al., 2011). At the air/water interface, the incorporation of phytosterols  
142 molecules in a phospholipid film has been shown to reorganize the monolayer, by modifying the  
143 packing and orientation of the polar heads of phospholipids (Hąc-Wydro et al., 2007).

144         In this context, the present work aimed at determining the interaction of gastric lipase with  
145 mixed films of galactolipids, galactolipids/phospholipids, and  
146 galactolipids/phospholipids/phytosterols, mimicking plant photosynthetic membranes.  
147 Biophysical tools such as the combination of ellipsometry and tensiometry were used to monitored  
148 the lipid-protein interactions at the air/water interface. Atomic force microscopy was further used  
149 to study the distribution of the enzyme into the heterogeneous lipid systems. Recombinant dog  
150 gastric lipase (Figure 1) was used as a representative gastric lipase available in a purified and well  
151 characterized form (Roussel et al., 1999) and with close homology with human gastric lipase.  
152 Vegetal membrane composition was approached using mixed monolayers of purified  
153 phospholipids (PL), galactolipids (GL) and phytosterols (pS) presenting liquid expanded/liquid  
154 condensed (LE/LC) phase coexistence at 20 mN/m and 20°C.

155

156 **2 EXPERIMENTAL SECTION**

157 Chloroform and methanol were purchased from Sigma Aldrich Ltd. (St. Quentin Fallavier, France).

158 If not stated otherwise, all biophysical characterizations were conducted at least in triplicate.

159 **2.1 Preparation of lipid mixtures**

160 Glycerophospholipids (dipalmitoylphosphatidylcholine, DPPC) and galactolipids  
161 (monogalactosyldiacylglycerol, MGDG, and digalactosyldiacylglycerol, DGDG) were purchased  
162 from Avanti Polar Lipids (see supplementary data for their fatty acid composition). Canola  
163 phytosterols (pS) from the deodorization distillates of canola oil were obtained as a gift from  
164 Cognis France (Estarac, France). The typical molar composition (estimated from usual normalized  
165 GC procedures) was:  $\beta$ -sitosterol (50.3 mol%), campesterol (38.9 mol%) and brassicasterol (10.5  
166 mol%).  $\beta$ -sitosterol and campesterol are the most commonly found in the human diet due to their  
167 high composition in nuts and oilseed species (Cañabate-Díaz et al., 2007; Phillips et al., 2005).  
168 Brassicasterol is a phytosterol found in some unicellular algae and terrestrial plants such as  
169 rapeseed (Ikekawa et al., 1968; Itoh et al., 1973).

170 Simple binary mixture of MGDG and DGDG (60:40, mol/mol) was prepared, namely GL. Ternary  
171 and quaternary mixtures of GL, DPPC and pS, namely GL/DPPC (50:50, mol/mol) and  
172 GL/DPPC/pS (45:45:10 mol/mol/mol), respectively, were also prepared to mimic the composition  
173 of natural vegetal membrane (see Table 1 for relative molar composition). Lipid-lipid interactions  
174 and molecular organization at the air/water interface of the three monolayers studied are reported  
175 in Kergomard et al. (2022).

176

177

## 178 **2.2 Enzyme purifications and preparation of aliquots**

179 Recombinant dog gastric lipase (rDGL) (86% amino acid sequence identity with HGL) was  
180 produced in transgenic maize by Meristem Therapeutics (Clermont-Ferrand, France) and purified  
181 as described previously (Roussel et al., 1999). rDGL stock solution was prepared at a concentration  
182 of 1.8 mg/mL in 10 mM sodium acetate buffer, 100 mM NaCl, 20 mM CaCl<sub>2</sub>, pH 5, and stored at  
183 -20°C. Diluted aliquots at a final concentration of 1.92 mg/mL (40 nM) were prepared in the same  
184 buffer before being used in monolayer experiments.

## 185 **2.3 Ellipsometry and surface pressure measurements at the air/water interface**

186 Kinetic measurements over 5 hours were performed using circular Teflon trough of 8 mL (surface  
187 area of 27 cm<sup>2</sup>). Kinetic measurements over 2 hours before Langmuir Blodgett transfer of the  
188 interfacial film were performed using Langmuir trough of 50 mL and a surface area of 35 cm<sup>2</sup>.

189 Before each experiment, the Teflon trough have been carefully cleaned with UP water and ethanol  
190 to get rid of surface-active residual impurities. Control ellipsometric and tensiometric  
191 measurements were performed during half an hour on 10 mM acetate buffer, 100 mM NaCl, 20  
192 mM CaCl<sub>2</sub>, pH 5 before experiments to check the cleaned surface before experiments. The surface  
193 pressure ( $\pi$ ) and the ellipsometric angle ( $\Delta$ ) were recorded at the same time.  $\pi$  was measured  
194 according to the Wilhelmy-plate method using a filter paper connected to a microelectronic  
195 feedback system to measure the surface pressure (Nima Technology, UK). Values of  $\pi$  were  
196 recorded every 4 s with a precision of  $\pm 0.2$  mN/m. Ellipsometric measurements were carried out  
197 using a home-made automated ellipsometer in a “null ellipsometer” configuration (Berge &  
198 Renault, 1993; Bourlieu et al., 2020). The laser beam probed a surface of 1 mm<sup>2</sup> and a depth in the

199 order of 1  $\mu\text{m}$  and provided insight on the thickness of the interfacial film formed at the interface.  
200 Values of the ellipsometric angle ( $\Delta$ ,  $^\circ$ ) were recorded every 4 s with a precision of  $\pm 0.5$   $^\circ$ .

#### 201 **2.4 Adsorption of rDGL onto multicomponent lipid monolayers and at the air/water interface**

202 A homogenous monolayer was formed by spreading a few microliters of 1 mM solution of lipids  
203 in  $\text{CHCl}_3/\text{MeOH}$  (2:1, v/v) over the surface of the buffer solution until an initial pressure of  $20 \pm 1$   
204  $\text{mN/m}$  was reached (Bénarouche et al., 2013). After stabilization of the film over 5 minutes, rDGL  
205 was further injected in the sub-phase at a final concentration of 40 nM. The increase of the surface  
206 pressure and ellipsometric angle due to protein adsorption onto the lipid monolayer was  
207 continuously monitored until reaching the equilibrium.

#### 208 **2.5 Visualization of phase separations and lipase distribution in heterogeneous film by atomic** 209 **force microscopy**

210 For AFM imaging, interfacial films were transferred onto a freshly-cleaved mica plate using the  
211 Langmuir-Blodgett method. The transfer was processed after 2 hours kinetics at a constant surface  
212 pressure and at a very low speed ( $0.5 \text{ mm}\cdot\text{min}^{-1}$ ). Imaging was carried out with an AFM (Multimode  
213 Nanoscope 5, Bruker, France) in contact mode QNM in air ( $20^\circ\text{C}$ ), using a standard silicon  
214 cantilever ( $0.06 \text{ N/m}$ , SNL-10, Bruker, France), and at a scan rate of 1 Hz. The force was minimized  
215 during all scans and the scanner size was  $100 \times 100 \mu\text{m}^2$ . The processed images analyzed by the  
216 open-source platform Gwyddion are representative of at least duplicated experiments. ImageJ  
217 software was used to perform the analysis of AFM images. The functions *Plot Profile*, *Threshold*,  
218 *Particle Analysis*, *Make Binary* and *Dilate* were applied to study the different height levels as well  
219 as the shape of the protein networks. ImageJ software was used to perform different height analyses

220 (based on the analyses of five plot profiles taken randomly in the AFM image) and thresholds on  
221 the raw AFM images (see supplementary data), in line with the study of Bourlieu et al. (2016)

222

## 223 **3 RESULTS**

### 224 **3.1. Ellipsometric and tensiometric adsorption kinetics of rDGL onto galactolipids** 225 **(homogenous phase) and mixed galactolipid-phospholipids (heterogenous phase) monolayers**

226 The adsorption kinetics of rDGL onto the GL monolayer was studied by tensiometry and  
227 ellipsometry, at 20 mN/m. Under these conditions, the GL monolayer forms a liquid expanded (LE)  
228 phase. The results obtained on this homogenous system were then compared with the rDGL  
229 adsorption kinetics onto heterogenous film based on mixture of galactolipids and phospholipids  
230 (GL/DPPC monolayer). Under these conditions, some phase separation occurs with the coexistence  
231 of liquid condensed (LC) and LE phases at 20 mN/m.

232 The gastric lipase was injected into the subphase after 5 minutes equilibrium of the GL and  
233 GL/DPPC lipid films, respectively. The adsorption kinetics were monitored over five hours. The  
234 variations with time in the ellipsometric angle and surface pressure are shown Figure 2.

235 In Figure 2.A, the surface pressure increased quickly after the injection of the enzyme in the  
236 subphase below the GL monolayer, while the ellipsometric angle remained stable over the first few  
237 minutes of kinetics, reflecting the accumulation of the gastric lipase below the air/water interface.  
238 After one hour kinetic, the surface pressure started to slowly decrease, together with a drastic  
239 increase in the ellipsometric angle from 6.9° to a maximum value of 12.9°, reflecting a  
240 reorganization at the interface and the subsequent adsorption of the enzyme onto the lipid  
241 monolayer. The evolution of the surface pressure and ellipsometric angle after injection of gastric  
242 lipase in the subphase of the GL/DPPC system (fig. 2.B) followed a similar evolution. The  
243 maximum increase in the surface pressure ( $\Delta\pi_{\max}$ ) was similar for both systems ( $\Delta\pi_{\max}=1.2$  mN/m  
244 vs 1.4 mN/m, for GL and GL/DPPC films, respectively). Nevertheless, for the GL<sub>50</sub>/DPPC<sub>50</sub>

245 monolayer, the ellipsometric angle started to increase right after the injection of the lipase into the  
246 subphase, reflecting a faster adsorption of the rDGL onto the heterogeneous film, probably due to  
247 the different orientation of the GL polar heads in the presence of DPPC. Indeed, the lag phase  
248 before the start of rDGL adsorption observed in the case of the GL monolayer could be due to the  
249 packing and orientation of the GL polar heads, which could hinder the access of the enzyme active  
250 site to the interface. In addition, it is important to point that addition of DPPC triggers phase  
251 separation which has been demonstrated as a structural feature favoring rDGL adsorption. For both  
252 systems, an equilibrium plateau was reached from 3h and until the end of the kinetics, with similar  
253 values for both GL and GL/DPPC systems ( $\delta\Delta=10.5 \pm 0.5^\circ$ ).

254 The  $\Delta\pi_{\max}$  values obtained upon rDGL adsorption on GL and GL/DPPC films are lower than those  
255 previously recorded upon rDGL adsorption onto pure phospholipid (PC) films (Bénarouche et al.,  
256 2017) or films mimicking bovine milk fat membranes and presenting phases coexistence (Bourlieu  
257 et al., 2016). This may result from weaker electrostatic interactions of rDGL with the neutral GL  
258 (compared to milk polar lipids (MPL)) which in general have charged polar heads. In addition to  
259 weaker electrostatic interactions, the galactose residues of GL polar heads may also generate some  
260 steric hindrance for lipase penetration into the film. Difference in fatty acyl moieties (chain length  
261 and unsaturation degree) may also influence lipid packing and rDGL adsorption.

262

263 **3.2. Visualization of lipase organization into galactolipid monolayers by atomic force**  
264 **microscopy**

265 AFM images of monomolecular films in the presence of rDGL were made after sampling the  
266 interface using the Langmuir Blodgett method after 2h of kinetics. The use of AFM allowed the  
267 topographic visualization of the interface at the nanoscopic scale. The AFM images of the interface  
268 were compared before and after the rDGL injection to highlight the distribution of the enzyme in  
269 the GL films and to determine the reorganization of the monolayers induced by the lipid-protein  
270 interactions. Details on AFM study on GL mixed films in the absence of rDGL are reported in  
271 Kergomard et al. (2022).

272 The injection of rDGL in the subphase (40 nM) clearly modified the morphology of the GL film  
273 (Figure 3) and triggered the formation of homogeneous patches onto the fluid phase after 2h of  
274 kinetics. These patches, assumed to reflect protein adsorption, had a mean width of 300 nm, which  
275 could correspond to 60 rDGL molecules, taking a width of about 5 nm for a rDGL molecule  
276 (Bénarouche et al., 2013). The roughness of the GL film before the enzyme injection was no longer  
277 visible on the AFM images in the presence of rDGL, suggesting a modification of the galactolipid-  
278 galactolipid interactions as well as a reorganization of the galactolipid polar heads induced by the  
279 adsorption of the rDGL onto the lipid film. Based on the analysis of five sections of the AFM  
280 image, the height variations of the fluid lipid background ranged from 0 to 0.5 nm (average  
281 height= $0.24 \pm 0.12$  nm), representing about 57% of the interfacial film. After rDGL adsorption, the  
282 cross-section profiles of the AFM images displayed several peaks of varying heights and were  
283 divided into two categories depending on their height levels and occurrences. A first height level  
284 was identified, with peaks ranging from 0.5 to 2.0 nm (average height= $0.98 \pm 0.4$  nm), accounting  
285 for 17% of the interfacial film. A second level was also detected, with heights ranging from 2.0 nm



286 to 5.5 nm (average height= $4.0 \pm 0.8$  nm), representing about 26% of the interface on the AFM  
287 image. On this second height population, 55% of the heights were included between 3.9 and 4.5  
288 nm, which could correspond to the entire cross-section of rDGL molecules (Roussel et al., 2002)  
289 and thus of protein patches covering an average area of 336 nm<sup>2</sup> (15 pixels per AFM image), which  
290 would correspond to a consistent value of 47 rDGL molecules, taking a cross-sectional area of 7.2  
291 nm<sup>2</sup> at 20 mN/m, as reported by (Bénarouche et al., 2013).

292 **3.3. Visualization of lipase organization into galactolipid-phospholipids monolayers by**  
293 **atomic force microscopy**

294 A saturated phospholipid (DPPC) was added at a molar ratio of 50% in the GL mixture, in  
295 order to induce phase heterogeneity and determine the impact of such heterogeneity on the rDGL  
296 adsorption by introduction of a liquid condensed phase (LC) in the monolayer. The AFM images  
297 ( $5 \times 5 \mu\text{m}^2$  and  $2.5 \times 2.5 \mu\text{m}^2$ ) of the interfacial film before and after the injection of the rDGL in the  
298 subphase of the GL/DPPC monolayer are presented Figure 4.A.

299 After injection of the enzyme, the LC domains have merged, adopting a more circular  
300 shape, with heights of 1.7 to 2.3 nm (average height =  $1.8 \pm 0.1$  nm), covering an average surface of  
301 36% of the interfacial film. At the very center and on the sides of the LC domains, bright peaks of  
302 2.0 to 8.0 nm in height were also visible, circularly distributed, pointing the heterogeneity of the  
303 gel phase domains. Onto the fluid phase (LE), an irregular protein network was formed, much  
304 denser than the one observed in the case of the GL film (fig. 3), and made of small grains forming  
305 interconnected units of  $50.0 \pm 20.0$  nm width. A sharp demarcation was also visible between the  
306 protein network in the fluid phase and the condensed domains, indicating rDGL exclusion from the  
307 LC-LE phase boundary and LC domains.

308 Size analyses and thresholds on AFM images led to the identification of height levels  
309 induced by the interactions of rDGL with the heterogeneous membrane, which are summarized  
310 Table 2. As for the GL monolayer, two different height profiles were identified at the fluid phase  
311 level, with similar profiles, indicating the preferential adsorption of the enzyme at the level of the  
312 fluid phase. A new higher height profile was identified at the level of the condensed domains,  
313 where the lipase probably has more difficulty to adsorb due to the high packing.

314  
315 **3.4. Impact of phytosterol addition in galactolipid-phospholipids mixture on the adsorption**  
316 **behavior of the gastric lipase**

317 The inclusion of phytosterols in the GL/DPPC mixture has been shown to generate defects at the  
318 level of the condensed phase, due to the preferential interactions between the phytosterols and the  
319 saturated fatty acids (Kergomard et al. (2022)). The AFM images Figure 4.B revealed that 2h after  
320 the injection of rDGL into the subphase, a regular protein network has formed onto the fluid phase.  
321 This network is composed of interconnected units of the same width as the protein network formed  
322 at the interface of the GL film ( $30.0 \pm 0.0$  nm), suggesting a similar organization of the proteins.  
323 Additionally, the delineation between the fluid phase protein network and the condensed phase  
324 domains was no longer visible on the AFM images, in contrast to the GL<sub>50</sub>/DPPC<sub>50</sub> film, indicating  
325 a modification of the protein-lipid interactions and probably a better miscibility of the components  
326 at the interface when pS were added in the system. As for the two previous samples, image and  
327 size analyses have revealed different levels of rDGL adsorption on the lipid film, which are  
328 reported Table 2.

## 329 **4 DISCUSSION**

330 rDGL has been shown to exhibit a high affinity for the air/water or lipid/water interface covered  
331 by polar lipids and to form monolayers, which distinguishes it from other globular proteins that  
332 tend to form multilayers (Bolanos-Garcia et al., 2008; Bourlieu et al., 2016). The formation of a  
333 monolayer was also deduced from rDGL adsorption onto a siliconized hydrophobic surface,  
334 monitored using a quartz crystal microbalance (Chahinian et al., 2006). This is probably due to its  
335 structural adaptation that avoids interfacial denaturation and involves a localized conformation  
336 change (lid opening) with the formation of an IRS on one side of the molecule (Figure 1) (Mateos-  
337 Diaz et al., 2017). The binding capacity of the rDGL at the air/water interface was monitored as  
338 the increase in the surface pressure  $\pi_{\max}$ , correlated to the variations of the lipid packing and the  
339 adsorption of enzyme molecules onto the monolayer. Here, the combination of data obtained with  
340 ellipsometry, tensiometry, and atomic force microscopy analyses provided insights on the  
341 physicochemical behavior and the distribution of rDGL in mixed GL films at the air/water  
342 interface.

### 343 **4.1. The particular orientation of the galactolipid polar heads could limit the enzyme** 344 **adsorption at the interface of the monolayer despite the high surface activity of rDGL**

345 The adsorption of rDGL onto mixed GL film (initial  $\pi=20$  mN/m) triggered a slight increase in the  
346 surface pressure ( $\Delta\pi_{\max}=1.2$  mN/m). Although this result reflects lipid-protein interactions at the  
347 air-water interface, this increase in the surface pressure was more discrete than those reported by  
348 Bottier (2006) upon the adsorption of pin-a isoform of puroindoline, a small globular protein (13  
349 kDa), onto homogenous monolayers of pure MGDG or DGDG isolated from wheat. Indeed, a  
350 pressure increase of 4.4 and 2.5 mN/m at 20 mN/m was obtained upon adsorption of pin-a onto

351 pure MGDG and DGDG films, respectively. The insertion of pin-a onto the GL films could be  
352 explained by the fluidity of GL as well as by the establishment of hydrophobic and Van der Waals  
353 interactions between the galactosyl head groups and the numerous tryptophan moieties of  
354 puroindolines, favoring their penetration into GL films. Furthermore, the overlap of the MGDG  
355 heads from 20 mN/m could have induced the formation of local instabilities, favoring the insertion  
356 of the protein in the film, explaining the greater increase of pressure. Conversely, the larger polar  
357 heads of DGDG could have limited the insertion of pin-a, thus explaining the lower pressure  
358 increase. Indeed, despite the larger polar head of DGDG than MGDG, digalactosyl residues possess  
359 the ability to adopt a tilted orientation, at 40° to the interface normal, in contrast to the polar head  
360 of pure MGDG, which was oriented parallel to the interface (Bottier, 2006). The particular  
361 orientation of the digalactosyl residue of DGDG is consistent with the results obtained by Chu et  
362 al. (2010) on the digestibility of an olive oil stabilized by either DPPC or DGDG, showing that the  
363 air/water interface occupied by DGDG was more resistant to the adsorption of bile salts, pancreatic  
364 lipase and colipase compared to the interface occupied by DPPC.

365 The small increase in surface pressure and the latency phase observed before the increase of the  
366 ellipsometric angle in the case of rDGL is therefore more consistent with a discrete adsorption  
367 behavior onto the mixed GL monolayers at 20 mN/m. The more limited insertion of rDGL could  
368 have been due to the larger size of the protein (50 kDa versus 13 kDa for pin-a puroindoline), but  
369 also to the fact that the monolayer was composed of a mixture of GL, and not of pure MGDG or  
370 DGDG. Indeed, in the equimolar mixture, a reorientation of the DGDG polar heads was observed,  
371 with the digalactosyl moiety orienting parallel to the interface, suggesting strong interactions  
372 between MGDG and DGDG and allowing tighter packing of mixed galactolipid molecules at the  
373 interface that could inhibit the lipase adsorption (Bottier, 2006). Moreover, the adsorption kinetics

374 properties of gastric lipase could also result from the interactions between the substrates and the  
375 active site of the enzyme. The IRS of rDGL being composed of several basic residues (Figure 1.C),  
376 it is less likely to develop electrostatic interactions with an uncharged galactolipid monolayer than  
377 with a charged phospholipid monolayer. This feature probably explains the lower adsorption  
378 capacity of rDGL to GL monolayer. The absence of charge at an interface decreases the  
379 establishment of electrostatic interactions with the enzyme, which could slow down or even hinder  
380 its adsorption.

381 Overall, the extend of rDGL adsorption onto the lipid monolayer seems to be highly dependent on  
382 the lipid packing and acyl chain composition. Indeed, a higher increase of  $\pi$  ( $\Delta\pi_{\max}=8$  mN/m) was  
383 reported for the rDGL adsorption onto 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC)  
384 monolayer in the same conditions (Bénarouche et al., 2013). Moreover, rDGL shows a higher  
385 penetration capacity into medium chain DLPC than into long chain egg PC monolayers, with  
386 critical surface pressures of insertion ( $\pi_c$ ) of 30.3 mN/m and 21.5 mN/m, respectively. The surface  
387 pressure chosen here with GL film ( $\pi=20$  mN/m) therefore represents harsh conditions for rDGL  
388 adsorption and penetration. An higher increase of the surface pressure was also reported by  
389 Bourlieu et al. (2016) on milk polar lipids with or without residual triacylglycerols MPL and  
390  $MPL_{TC}$  ( $\Delta\pi_{\max}=2.4$  mN/m and  $\Delta\pi_{\max}=6.1$  mN/m, respectively).

391 In our study, the ellipsometric data suggested that a significant amount of rDGL was adsorbed onto  
392 the GL monolayer after 1h latency phase, with a high ellipsometric angle variation of  $\delta\Delta=4.2^\circ$   
393 obtained after 2h of kinetics. This value was higher than that obtained on MPL ( $\delta\Delta=3.1^\circ$ ) but much  
394 lower ( $\delta\Delta=18.3^\circ$ ) than that obtained on  $MPL_{TC}$  after 5 hours of kinetics. Overall, the results of  
395 tensiometry and ellipsometry analysis confirmed the adsorption of rDGL onto the GL monolayer,

396 although to a lesser extent than for bovine milk membranes (MPL and MPL<sub>TC</sub>) due to the particular  
397 orientation of the polar head of galactolipids and the uncharged surface of the monolayer.

398  
399 **4.2. rDGL undergoes conformational changes upon adsorption, explaining the different level**  
400 **of adsorption/insertion onto the GL films**

401 The AFM images of the GL film evidenced the formation of protein patches at two different height  
402 levels in the fluid phase 2h after the injection of rDGL in the subphase. Different levels of rDGL  
403 adsorption have already been reported (Bénarouche et al., 2013, 2017; Bourlieu et al., 2016, 2020)  
404 as being dependent on the monolayer composition but also on the conformation of the adsorbed  
405 enzyme, which reflect its level of interaction and activity with the substrate. This may result from  
406 structural changes occurring upon protein adsorption at the air/water (or lipid/water) interface. The  
407 unfolding and denaturation of proteins at the interfaces are well-known behaviors, especially when  
408 interfacial tension is high. For example, lipases are rapidly inactivated at the oil-water interface  
409 when using pure triacylglycerols (Aloulou et al., 2006). Nevertheless, denaturation can be avoided  
410 using surfactants decreasing the interfacial tension or by the enzyme itself. Lipases like gastric  
411 lipase are also known to undergo a localized conformational change with a lid opening that controls  
412 the interfacial recognition site and activity (Mateos-Diaz et al., 2017). Coagulation/aggregation are  
413 additional phenomena that may also occur with proteins at interfaces (James & Augenstein, 1966).  
414 All these phenomena were likely to explain the different levels of insertion of the rDGL in the  
415 mixed GL film. Indeed, the lowest height level could correspond to some denatured rDGL,  
416 unfolded upon adsorption and resulting in an increase in the surface pressure, more favorable to  
417 the adsorption of the enzyme in its active conformation. The second height level corresponding to  
418 homogeneous peak sizes between 2.0 and 5.5 nm is likely to correspond to the adsorption of rDGL

419 in its active conformation, at a depth of 1 to 2 nm in the GL monolayer onto the GL monolayer at  
420 the air/water interface. This level of rDGL would fit with the adsorption model previously proposed  
421 for rDGL (Bénarouche et al., 2013). A model for the rDGL distribution in the GL monolayer under  
422 gastric conditions is proposed Figure 5.

### 423 **4.3. rDGL is excluded from condensed phase domains in mixed heterogeneous GL-DPPC** 424 **systems and exhibits similar adsorption behavior to other small globular proteins**

425 Numerous studies have been conducted in AFM to investigate the adsorption of various lipases and  
426 phospholipases onto heterogenous supported monolayers and bilayers at the air/water interface  
427 (Balashev et al., 2007; Rangl et al., 2017). Overall, results have indicated the preferential  
428 adsorption of these enzymes at the edges of low molecular packing domains, where the increase of  
429 the curvature favor the amphiphile adsorption (Nielsen et al., 1999; Prim et al., 2006).

430 The lateral distribution of the gastric lipase in monolayers of milk fat globules with phase  
431 heterogeneity has been studied in both human and bovine systems (Bourlieu et al., 2016, 2020).

432 The coexistence of liquid condensed/liquid expanded (LC/LE) phases in milk fat globule  
433 monolayers has been shown to impact the adsorption of gastric lipase. Indeed, the rDGL gets really  
434 rapidly adsorbed in the LE phase or at the phase boundaries in the bovine milk membrane (BMM)  
435 and human milk membrane (HMM) extracts, showing that the coexistence of LC/LE phase was an  
436 important driver of the rDGL adsorption.

437 Here, we introduced some phase heterogeneity in the GL monolayer by adding DPPC. As expected,  
438 the AFM images (fig. 4.A) revealed that the rDGL was excluded from the LC phase, and  
439 preferentially adsorb onto the LE phase of GL-DPPC monolayer, which present a lower molecular  
440 packing, probably due to the high composition in PUFA of GL. Indeed, the condensed domains



441 were likely to include DPPC, with tight packing of its saturated acyl chains preventing lipase  
442 penetration. Nevertheless, the bright peaks visible in the center of the condensed domains seemed  
443 to indicate an adsorption of rDGL at this level, probably due to some compositional heterogeneity  
444 between the center and the edge of the LC domains, favoring a less dense packing or the appearance  
445 of defects at the central level. On the other hand, the adsorption of the rDGL onto the LE phase  
446 triggered the formation of a dense protein network, composed by small interconnected  
447 nanodomains of irregular shape and width, in contrast to the discontinuous homogeneous network  
448 previously formed in the GL film. Several levels of rDGL insertions were identified, in line with  
449 the results of Bourlieu et al. (2016) on bovine milk fat model membranes (MPL and MPL<sub>TC</sub>), with  
450 slightly different height values in those different heterogenous systems, which could be explain by  
451 the differences of composition and lipid packing between the monolayers.

452 Additionally, the condensed domains composing the LC phase merged and their circularity was  
453 increased, indicating the existence of a line tension at the interface of the LC/LE phases. Indeed,  
454 García-Sáez et al. (2007) have shown that the size and the shape of LC domains composed by  
455 sphingomyelin and cholesterol depend on the balance between the line tension, which tends to  
456 increase the size of the domains in order to reduce the total length of the boundaries, and the  
457 entropic and electrostatic repulsions, which prevent the fusion of the LC domains. Thus, the  
458 increase of the LC domain size in our study is consistent with a higher line tension at their edges.  
459 This change in line tension could be triggered by the greater height difference between the two  
460 phases LC and LE, induced by the adsorption of rDGL onto the fluid phase. Indeed, this height  
461 difference has an unfavorable energy cut per unit length, and as a result, the domain boundary  
462 deforms to counteract this phenomenon, inducing line tension, which may be unfavorable for rDGL  
463 adsorption. Indeed, the sharp domain delineation visible on the AFM image between the fluid-

464 phase protein network and the LC domains seems to indicate that the rDGL poorly adsorbs at the  
465 phase boundaries.

466 The adsorption behavior of rDGL on heterogeneous lipid films from animal or plant sources can  
467 be compared to other globular proteins. Weise et al. (2010) studied the adsorption behavior of  
468 several lipid membrane proteins, including N-Ras, a small globular protein (21 kDa), onto  
469 heterogeneous model membranes with coexisting fluid/gel phases. The results of this study  
470 provided direct evidence of the preferential distribution of the N-Ras protein in the fluid phase, but  
471 also at the edges of the LC phase domains, leading to a favorable decrease of the energy between  
472 the phases. This adsorption of proteins at boundary phases was characteristic of proteins inserted  
473 into multiphase lipid systems but which do not show specific preferences for a given phase, so that  
474 they are expelled toward the boundaries. This localization and accumulation of proteins at the phase  
475 interface to decrease the line tension could explain the adsorption behavior of rDGL in the presence  
476 of pS.

#### 477 **4.4. The addition of pS modulated the rDGL adsorption onto mixed GL-DPPC monolayers** 478 **at the air/water interface**

479 In our previous study, the inclusion of phytosterols (pS) in the GL/DPPC film induced a drastic  
480 modification of the interfacial organization, with a dilution of the domains as well as an increase  
481 in the thickness of the condensed phases. This was attributed to interactions between GL and PL  
482 and a condensation effect of pS (Li et al., 2003; Su et al., 2007). Moreover, the addition of pS to  
483 mixed DPPC and GL monolayers was found to trigger the appearance of defects into the condensed  
484 phase domains. Previous studies on various other lipases found that defects in lipid packing could  
485 constituted preferential sites for the enzyme anchoring (Balashev et al., 2001, 2007; Brezesinski &  
486 Möhwald, 2003; Nielsen et al., 2002). The presence of local defects in the LC phase GL/DPPC/pS

487 film reflects a less dense molecular packing, which could favor rDGL adsorption. Indeed, AFM  
488 images recorded after rDGL injection in the subphase of the films revealed significant differences  
489 in the organization at the interface between GL/DPPC and GL/DPPC/pS films. In the presence of  
490 pS, a regular protein network was visible on the AFM images, not only at the fluid phase but also  
491 at the phase domain boundaries and at the defects present on the condensed phase domains. The  
492 absence of a marked delineation of the protein network level between the LE and LC phases  
493 contrasts with the system without pS. It points to an enhancement of the rDGL adsorption onto the  
494 LC domains and at the boundary phase, leading to a decrease of the height difference between the  
495 LC/LE phases and thus to a decrease of the line tension (fig. 4.B), favoring a more homogenous  
496 adsorption of rDGL at the interface. This modulation of rDGL adsorption in the presence of pS  
497 could also be explained by a modification of the lipid-lipid and protein-lipid interactions. Indeed,  
498 hydrogen bridges could be established with the hydroxyl groups of pS, as well as stacking between  
499 rDGL aromatic residues and the sterol backbone, leading to a different interfacial organization and  
500 favoring the rDGL adsorption.

501 Moreover, the two different levels of rDGL insertion with homogeneous peak sizes onto the GL-  
502 DPPC-pS film (h2, h3) were comparable to those identified in the case of rDGL adsorption onto  
503 models of HMM by Bourlieu et al. (2020). In contrast, rDGL insertion onto BMM was less  
504 homogeneous, with 3 levels of heights described, similar to the ones evidenced for the GL-DPPC  
505 film. It was speculated by the authors that the particular composition of HMM (high PUFA content,  
506 presence of DHA, presence of anionic PL, coexistence of LC/LE phase) was more favorable to the  
507 action of rDGL than the composition of BMM. Indeed, HMM contained more anionic  
508 phospholipids, which have been shown to enhance the adsorption of rDGL at pH 5 thanks to a  
509 better reorientation of the charged active site.

510 **Interest of the study for the adsorption of gastric lipase onto natural plant cell membranes**

511 Plant cell membranes are complex systems containing a large variety of lipid and protein  
512 molecules. The high proportion of DPPC in our model systems (50%) was explained by the need  
513 to create a heterogeneous system, presenting a coexistence of LC/LE phase observable in AFM,  
514 but was actually far from the palmitic acid compositions (C16 :0) found in natural plant membrane  
515 systems (plasma membrane, chloroplast thylakoids, oleosomes) (Kergomard et al., 2021). Indeed,  
516 the proportion of palmitoyl chains in natural plant systems is on average between 5-20% wt. of  
517 total lipids (Lee et al., 1998; Venkatachalam & Sathe, 2006). Nevertheless, the raft theory in plant  
518 membrane systems predicts the existence of lipid assemblies locally enriched in phytosterol and  
519 sphingolipid molecules (Mongrand et al., 2010), which are known to contain liquid-ordered  
520 domains in a fluid environment (Bhat & Panstruga, 2005). These plant membrane rafts are  
521 biochemical homologues of mammalian cell membrane rafts rich in sphingomyelin and cholesterol,  
522 known for their transient dynamic structure in the nanometer range and involved in signaling and  
523 protein transport processes and other cellular processes (Hancock, 2006; Jacobson et al., 2007;  
524 Munro, 2003).

525 Thus, although our biomimetic membranes constituted a simplified model somewhat far from the  
526 natural plant membrane composition, the general physicochemical trends and adsorption behavior  
527 of gastric lipase at these heterogeneous model membrane systems remain extendable to the protein-  
528 lipid interactions occurring at the so-called raft zones in plant cells. In particular, it appeared that  
529 the presence of phytosterols in condensed domains and, by extension, in plant rafts, may enhance  
530 the rDGL adsorption at Lo or gel phase (LC) domains.

531

532

533 **CONCLUSION**

534 We have shown that Langmuir films and their analysis by tensiometry, ellipsometry and Langmuir-  
535 Blodgett transfer coupled to atomic force microscopy provide a suitable tool for studying the  
536 adsorption and interactions of gastric lipase with biomimetic vegetal membranes. Gastric lipase  
537 binds to these heterogenous membranes with a preference for phase boundaries and defects. It  
538 induces changes in the interfacial organization of lipids although it does not display any activity  
539 on polar acylglycerols from plant membranes (Wattanakul et al., 2019). Since gastric lipase is  
540 known to promote the action of pancreatic lipase through the release of fatty acids and changes in  
541 interfacial properties (Gargouri et al., 1986), the changes observed with plant polar lipids, without  
542 lipolysis, may also trigger the adsorption and activity of other pancreatic enzymes. This will be the  
543 objective of further studies, but the present one is a step forward in the comprehension of  
544 gastrointestinal lipase interactions with plant membranes, an overlooked aspect of lipid digestion  
545 (Sahaka et al., 2020).

546 **Declaration of Competing Interest**

547 The authors declare that they have no known competing financial interests or personal relationships  
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549

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555 Kergomard wrote the manuscript and all the co-authors participated in the experimental design, the  
556 collection, the interpretation of data and the correction and implementation of the manuscript. All  
557 co-authors have approved the final article.

558

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770



771 **FIGURES**

772 Figure captions.

773 **Figure 1** 3D structure of recombinant dog gastric lipase showing the interfacial recognition site  
774 (IRS). Panel A: ribbon model showing the secondary structure elements of rDGL with the lid in  
775 the open conformation and putative orientation at lipid-water interface based on the localization of  
776 the IRS. Panel B: Top view and molecular surface representation (white=hydrophobic;  
777 yellow=polar) of rDGL showing the hydrophobic ring surrounding the active site entrance. Panel  
778 C: Top view and molecular surface representation showing its surface potential at pH 5 Surface  
779 potential scale is given below the rDGL structure, with red and blue colors corresponding to  
780 negative and positive potential, respectively. Views were prepared using PyMol software  
781 (Schrodinger, L. (2010) The PyMOL Molecular Graphics System, Version 1.3r1.) and the crystal  
782 structure of rDGL (PDB: 1K8Q). Surface potential was estimated using the TITRA software  
783 program (Petersen et al., 2001) and the protein surfaces were generated using GRASP (Nicholls,  
784 1993).

785 **Figure 2** (A) Kinetic evolution of the surface pressure (circle, red) and ellipsometric angle  
786 (triangle, blue) upon rDGL adsorption at the air/water interface onto GL lipid film and, (B) Kinetic  
787 evolution of the surface pressure (circle, red) and ellipsometric angle (triangle, blue) upon rDGL  
788 adsorption onto GL<sub>50</sub>/DPPC<sub>50</sub> monolayers performed at an initial surface pressure of 20 mN/m.  
789 The rDGL was injected in the subphase after 5 minutes stabilization of the monolayers at the  
790 air/water interface.

791 **Figure 3** AFM images (5×5 μm<sup>2</sup> and 1×1 μm<sup>2</sup>) of GL monolayers at an initial surface pressure of  
792 20 mN/m before (-rDGL, red) and after (+rDGL, green) rDGL injection (40 nM final concentration,  
793 5h kinetics). A cross-section of height profile is representative for 1×1 μm<sup>2</sup> image after rDGL

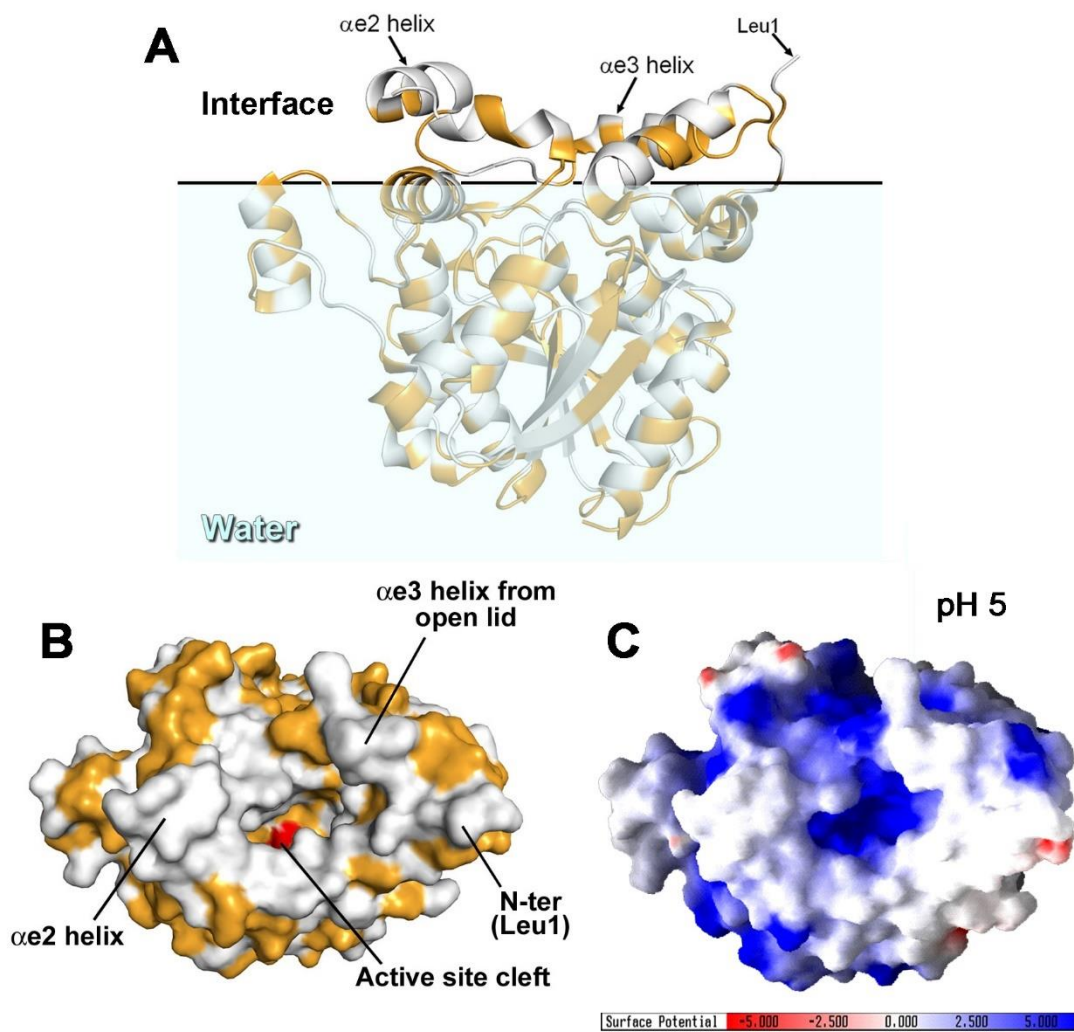
794 injection. ImageJ analysis was performed on a  $0.56 \mu\text{m}^2$  crop to determine the shape of the protein  
795 network (see material and methods). The vertical color scale from dark brown to white corresponds  
796 to an overall height of 15 nm.

797 **Figure 4** AFM images ( $5 \times 5 \mu\text{m}^2$  and  $2.5 \times 2.5 \mu\text{m}^2$ ) at an initial surface pressure of 20 mN/m before  
798 (-rDGL, red) and after (+rDGL, green) rDGL injection (40 nM final concentration, 5h kinetics) of  
799 A) GL/DPPC monolayer (50:50 mol/mol) and B) GL/DPPC/pS monolayer (45:45:10  
800 mol/mol/mol). Cross-sections of height profiles are representative for  $2.5 \times 2.5 \mu\text{m}^2$  image after  
801 rDGL injection. (LE – Liquid Expanded, fluid phase; LC – Liquid Condensed, gel phase). The  
802 vertical color scale from dark brown to white corresponds to an overall height of 15 nm.

803 **Figure 5** Proposed model of rDGL distribution in homogenous galactolipid monolayer under  
804 gastric conditions

805

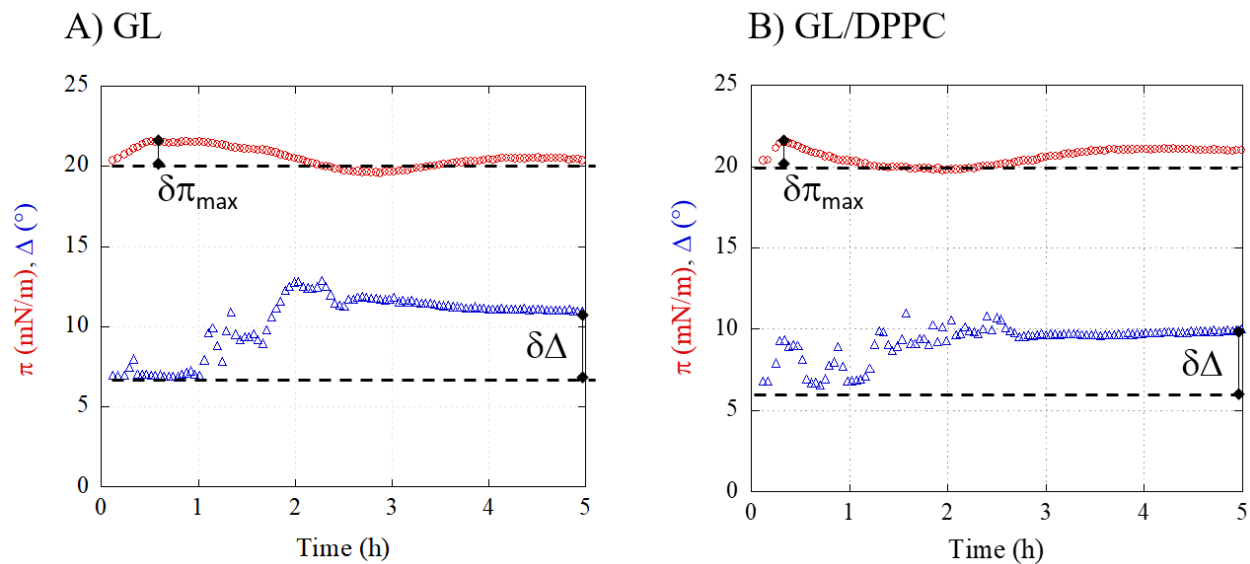
806 **Figure 1**



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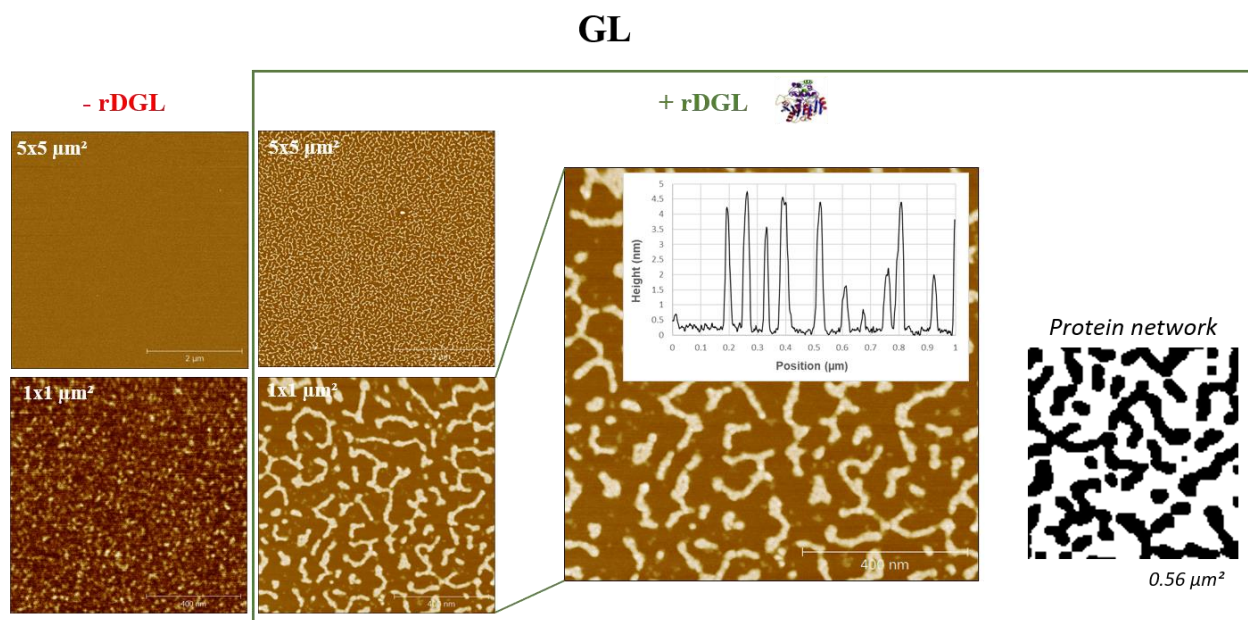
809 **Figure 2**



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812 **Figure 3**

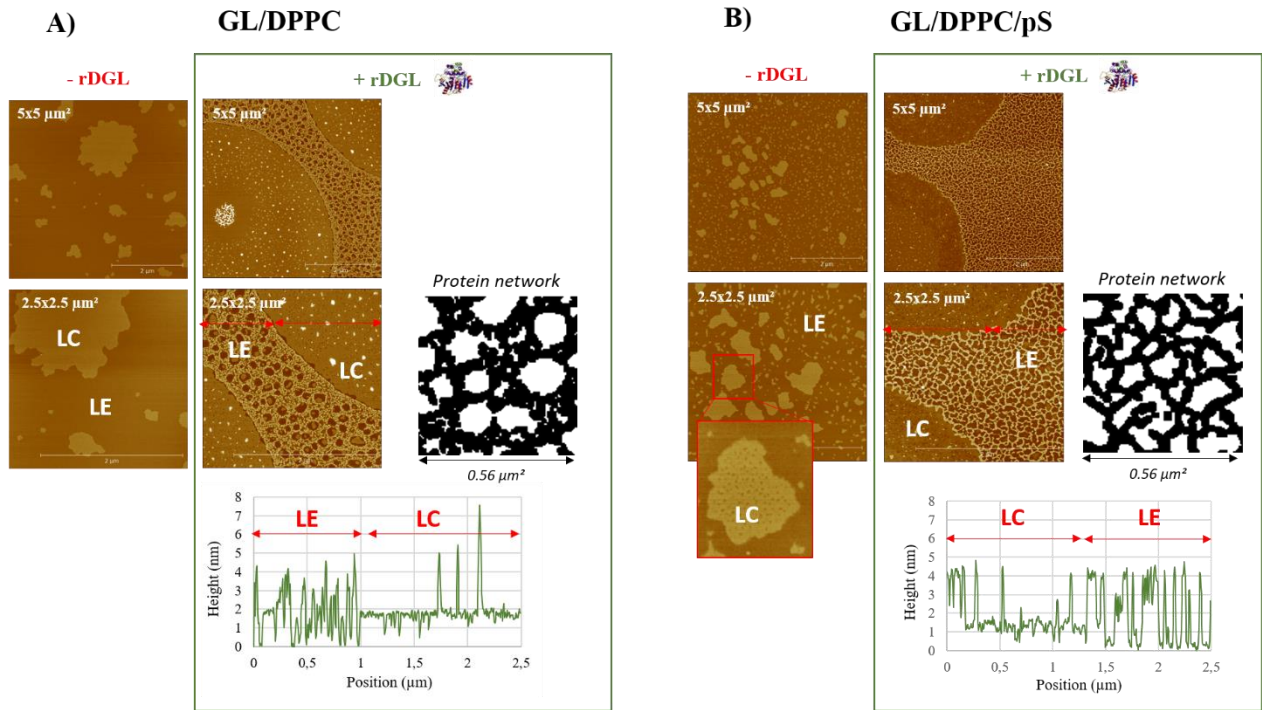


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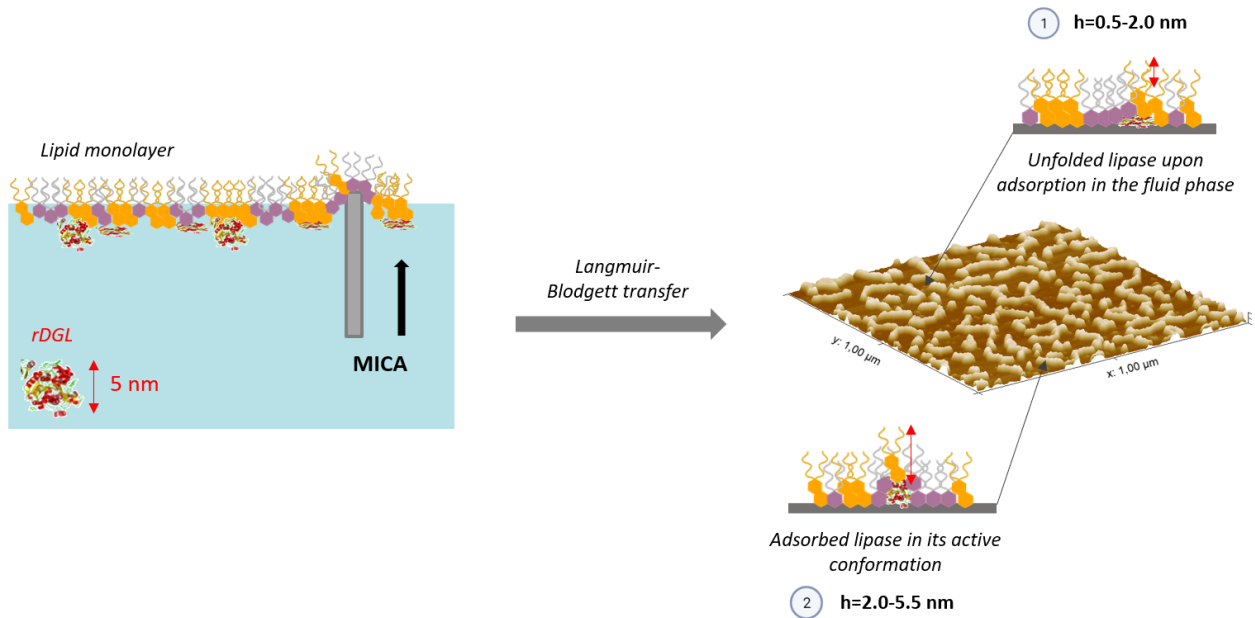
816 **Figure 4**



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819 **Figure 5**



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822 **TABLES**823 **Table 1** - Model system compositions

	Monolayer composition
(1) GL	MGDG/DGDG 60:40 mol/mol
(2) GL/DPPC	GL/DPPC 50:50 mol/mol
(3) GL/DPPC/pS	GL/DPPC/pS* 45:45:10 mol/mol/mol

\* *$\beta$ -sitosterol, campesterol, brassicasterol 50:40:10 mol/mol/mol*

824

825 **Table 2** - Summary of height levels identified in AFM images and related to rDGL interactions  
 826 with model membranes. Height values are given in nm. The percentages given are the percentage  
 827 of pixels covered by the indicated height range (-). Values obtained were based on the analysis of  
 828 five plot profiles taken randomly in the AFM image.

Monolayer	Protein adsorption (nm)		
<b>GL</b>	h1 = $0.98 \pm 0.4$ (0.5 – 2) 17%	h2 = $2.0 \pm 5.5$ (2.0 – 5.5) 26%	-
<b>GL<sub>50</sub>/DPPC<sub>50</sub></b>	h1 = $0.4 \pm 0.1$ (0.2 – 1.7) 32%	h2 = $2.6 \pm 0.2$ (2.3 – 4.8) 26%	h3 = $5.5 \pm 0.7$ (>5.5) 2%
<b>GL<sub>45</sub>/DPPC<sub>45</sub>/pS<sub>10</sub></b>	h1 = $1.2 \pm 0.2$ 0.6 – 1.5 34%	h2 = $3.6 \pm 0.6$ (2.3 – 5.0) 24%	-

829