Lipid-lipid interactions of *Escherichia coli* mimetic inner membrane at human physiological temperature

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Keywords: phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, lipid-lipid interactions, bacterial mimetic membranes, physiological temperature

Abstract

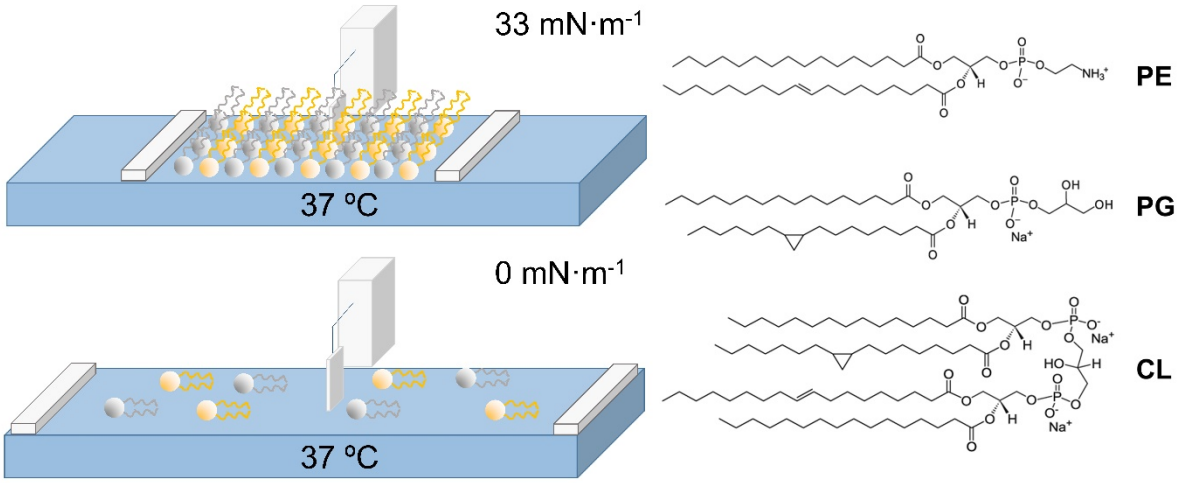
The current strategies to eradicate bacteria require that the antimicrobial agent either penetrate or disrupt the bacterial membrane. In *Escherichia coli* (*E.coli)* as a model of Gram negative strains, the antimicrobials have to cross two barriers - the outer and the inner membrane being the latter composed by ~ 77 % phosphatidylethanolamine (PE), ~ 13 % phosphatidylglycerol (PG) and ~ 10 % cardiolipin (CL) lipids. Each one of these lipid families shares the same headgroup, but contains acyl chains with varying length and degree of unsaturation. Bacteria adapt their membrane lipid composition and metabolism in response to environmental signals, such as the temperature, resulting in different interactions with exogenous molecules, e.g. antibacterial agents. Herein, bacterial model membranes are prepared to evaluate the lipid-lipid interactions in Langmuir monolayers of binary mixtures at several molar ratios of PE and PG or CL at human physiological temperature (37ºC). Both PE:PG and PE:CL monolayers were stable at 37 ºC and presented higher molecular areas (> 20 $Å^2 \cdot molecule^{-1}$) than at 23 ºC. However, these lipid mixtures presented liquid-expanded state and rigidity (inverse of the compressibility modulus ~ 90 $mN \cdot m^{-1}$) slightly lower than at 23ºC. Such athermalicity at biologically relevant temperatures may favour the preservation of the biological functions of *E.coli*.

1 Introduction

Most recent strategies to eradicate bacteria, rely on disruption of the bacterial membrane with nano-formulated antimicrobial agents that ultimately causes cell death with less probability for resistance development (Ivanova et al. 2017; Ferreres et al. 2018; Hoyo et al. 2019a). However, the majority of clinically relevant antibiotics need to cross the membrane of Gram-negative bacteria such as *Escherichia coli* (*E.coli)* in order to exert their antibacterial effect. The outer membrane of *E.coli* is mainly composed of lipopolysaccharides, while the inner membrane contains mainly lipids (~ 77 % phosphatidylethanolamine (PE), ~ 13 % phosphatidylglycerol (PG), and ~ 10 % cardiolipin (CL)) and proteins (Shokri and Larsson 2004; Yeagle 2016). Langmuir and Langmuir Blodgett (LB) films have been used to study *in vitro* the antibacterial mechanism of new agents (Fernandes et al. 2017; Ivanova et al. 2018). Simple and reproducible mammalian (Nichols-Smith et al. 2004; Domènech et al. 2006), thylakoid (Hoyo et al. 2012; Hoyo et al. 2015; Hoyo et al. 2016a; Hoyo et al. 2016b), and bacterial (Gidalevitz et al. 2003; Clausell et al. 2007; López-Montero et al. 2008; López-Montero et al. 2010; Michel et al. 2015; Michel et al. 2017) membrane models have been prepared using these techniques for studying the interactions of exogenous molecules, including antibacterial agents, with bacterial membranes. Previous works reported the application of inner bacterial membrane models composed of a single lipid, e.g. dipalmitoylphosphatidylglycerol (DPPG) (Gidalevitz et al. 2003) and palmitoyloleoylphosphatidylglycerol (POPG) (Gidalevitz et al. 2003; Clausell et al. 2007), while the outer bacterial membrane was mimicked by lipopolysaccharides (LPS) (Clausell et al. 2007). The validity of these models was compromised by the use of only one lipid and the absence of PE, the major lipid present in bacterial inner membranes (Yeagle 2016). Michel et al. (Michel et al. 2015) mimicked separately the outer and inner leaflets by the use of LPS and a ternary mixture of monounsaturated bacterial phospholipids. Furthermore, the same authors prepared a similar model with a unique structure combining the LB and Langmuir

Schaefer techniques (Michel et al. 2017). Both models lacked the use of natural lipid constituents of the bacterial membranes. López-Montero *et al.* (López-Montero et al. 2008; López-Montero et al. 2010) solved this drawback using an *E. coli* polar lipid extract (PLE) comprised of a myriad of lipid structures sharing the same headgroup. Recently, we prepared biomimetic membrane models using also a myriad of lipid structures to evaluate the lipid-lipid interactions in binary mixtures composed of PE and PG or CL at 23 ºC. The models revealed that all tested ratios of lipids were in fluid state (liquid expanded -LE-) and minor changes in terms of membrane rigidity were observed among the PE content in the mixture (Hoyo et al. 2019b).

Bacteria regulate their metabolism, membrane lipid composition and the degree of unsaturation of their hydrocarbon chains as a response to environmental signals (Larsson and Törnkvist 1996; Shokri and Larsson 2004). Several biomimetic membrane studies have shown relevant differences in the lipid-lipid (Suárez-Germà et al. 2011) or lipid-polymer (Krajewska et al. 2013a; Krajewska et al. 2013b) interactions upon increasing the temperature. Therefore, studying the role of temperature in bacterial mimetic inner membranes could anticipate the interactions between novel antibacterial agents and the membrane. Herein, our previous study of PE:PG and PE:CL monolayers at 23 ºC (Hoyo et al. 2019b) is reproduced at human physiological temperature of 37 ºC. The surface pressure-area isotherms of Langmuir films describe the physical states, rigidity and thermodynamic properties of the monolayers at the air/water interface (Scheme 1). The use of natural *E. coli* lipid extracts of the myriad of structures corresponding to each lipid, and the study of several binary mixtures of lipids, including the biologically relevant one, allows for a reliable evaluation of the lipid-lipid interactions in *E.coli* mimetic membranes to serve potentially as a model for validation of novel antibacterial agents.



Scheme 1. Langmuir monolayer formation and representative chemical structure of the myriad of lipid structures that shares the same headgroup: phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) in lipid extracts from *E. coli* (see section 2.1).

2 Materials and methods

2.1 Materials

Avanti Polar Lipids provided PE (♯ 840027), PG (♯ 841188) and CL (♯ 841199) extracted from *E.coli*. Each lipid represents a myriad of structures that share the same headgroup, but differ in their degree and position of unsaturation. $CHCl_3$ and phosphate buffer solution (PBS) tablets were provided by Sigma-Aldrich (Spain). Ultrapure MilliQ water with a resistivity of 18.2 MΩ · cm^{-1} was used in cleaning procedures and for PBS at pH 7.4 preparation.

2.2 Surface pressure – area isotherms

PE, PG, and CL solutions in $CHCl_3$ (0.5 mg · mL^{-1}) and the corresponding mixtures at different molar ratios were prepared by mixing each stock solution and were stored at - 20 ºC until used. Surface pressure – area (π-A) isotherms were performed in a Langmuir trough equipped with

two mobile barriers (KSV NIMA, model KN2002, Finland) with a total area of 273 cm^2 mounted on an antivibration table and housed in an insulation box at 37 ± 1 ºC. The temperature was maintained by connecting a thermostatic water bath to the inner circuit of the Langmuir trough and placing a temperature probe in the subphase. The Langmuir trough was cleaned with $CHCl_3$ and water. After subphase addition, the surface was further cleaned by suctioning. Immediately, 25 µL of the lipid or lipid mixture solution was added dropwise into the trough, and after 10 minutes evaporation of $CHCl_3$, the barriers were compressed at 25 $cm^2 \cdot min^{-1}$. π – A isotherms were performed by triplicate.

2.3 Data analysis

Physical states

The inverse of the compressibility modulus (C_s^{-1}) was obtained from the π-A isotherms calculated according to Equation 1, where A is the mean area per molecule ($Å^2 \cdot molecule^{-1}$), π the surface pressure ($mN \cdot m^{-1}$) and T the absolute temperature (K).

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)_T \qquad \text{Equation 1}$$

Thermodynamic study

The mixing energy (ΔG_{mix}) of a mixture is obtained from the following equation in which A^E represents the excess area, A_1 and A_2 the area per molecule for the individual components, A_{12} the mean area per molecule for the mixture, x_1 and x_2 the molar fraction for each component, G^E the excess free energy of mixing, N_A the Avogadro's number, R the gas constant and T the absolute temperature.

$$A^E = A_{12} - (x_1 A_1 + x_2 A_2) \quad \text{Equation 2}$$

$$G^E = N_A \int_0^{\pi} A^E \, d\pi \quad \text{Equation 3}$$

$$\Delta G_{mix} = \Delta G_{id} + G^E \quad \text{Equation 4}$$

$$\Delta G_{id} = RT\,(x_1 \ln x_1 + x_2 \ln x_2) \quad \text{Equation 5}$$

3. Results

3.1 π-A isotherms, physical states and mixing behaviour of PE:PG system

The lift-off area for PE and PG at 37 ºC was observed at 135 and 185 $Å^2 \cdot molecule^{-1}$ and the collapse at π = 46 and 45 $mN \cdot m^{-1}$, respectively (fig. 1). The isotherms were continuous and the C_s^{-1} curves reached their maximum ($C_s^{-1}{}_{max}$) at ≈ 94 and 80 $mN \cdot m^{-1}$ (inset of fig. 1) for PE and PG respectively, confirming the LE (Vitovič et al. 2006) physical state of the system. The studied PE:PG mixtures show similar isotherms and C_s^{-1} curves, mainly differing in the lift-off area that was higher upon increasing the amount of PG in the mixture. PE and PG showed similar size and shape. However, the PG headgroup is slightly larger than the headgroup of PE, explaining the differences observed in the lift-off area. The average molecular area (fig. 2A) showed slight random deviations from the additivity rule (dashed line), confirming the ideal behaviour of the PE:PG Langmuir films regardless the PG content. The ΔG_{mix} curves (fig. 2B) presented negative values for all tested mixtures, confirming their stability (Roche et al. 2006).

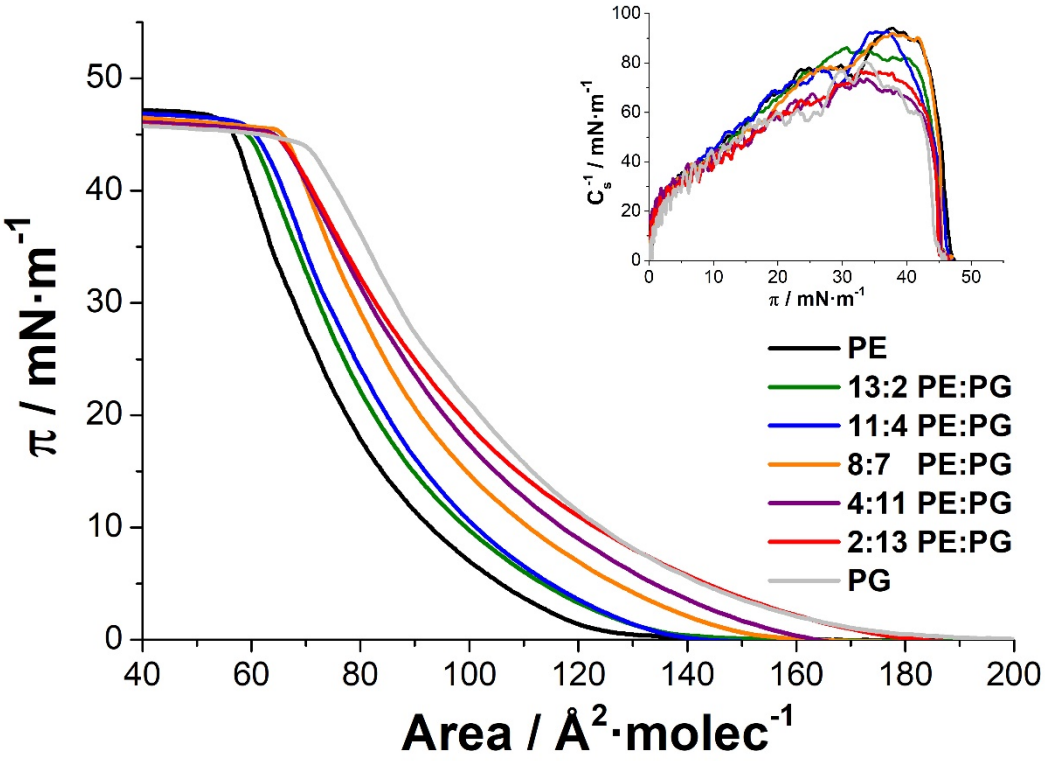


Fig. 1 π-A isotherms for PE, PG and PE:PG mixtures on PBS subphase at 37 ºC. Inset: Inverse of the compressibility modulus *vs*. surface pressure corresponding to the described π-A isotherms.

3.2 π-A isotherms, physical states and mixing behaviour of PE:CL system

The CL π-A isotherm at 37 ºC (fig. 3) showed the lift-off area at 240 Å^2·molecule^{-1} and the collapse of the film at π = 47 mN·m^{-1}. The $C_s^{-1}{}_{max} \approx 94$ mN·m^{-1} (inset of fig. 3) indicates the LE physical state of the monolayer (Vitovič et al. 2006). The comparable hydrocarbon chains of PE and CL lead to similar isotherms and C_s^{-1} curves for their mixed monolayers. The increased molecular area observed upon increasing the CL content is correlated with the larger volume that occupy the four hydrocarbon chains in CL compared to the volume of two hydrocarbon chains in PE (Baumgärtner et al. 2007; Boyd et al. 2017).

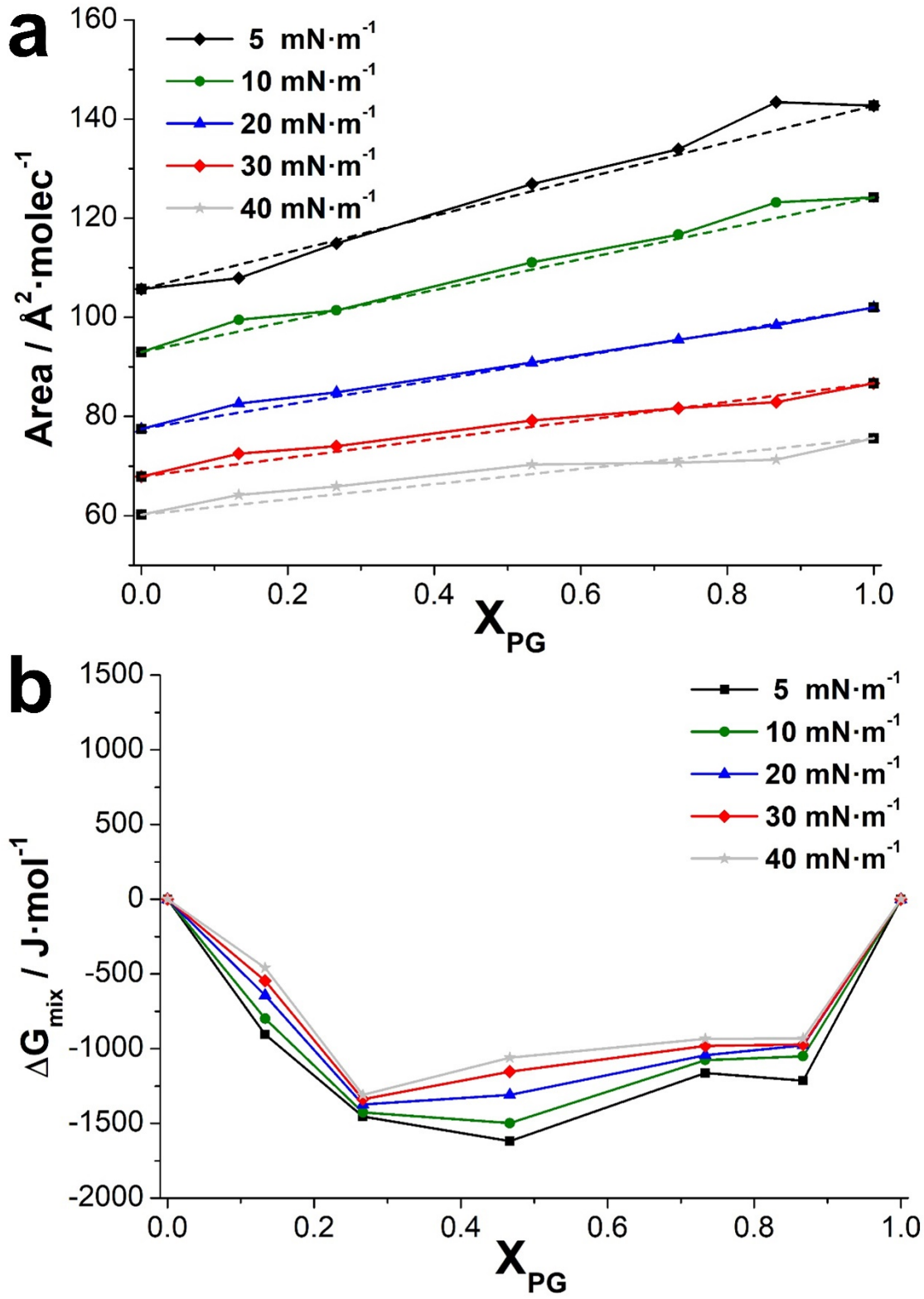


Fig. 2 a) Mean area per molecule *vs.* molar fraction and b) mixing energy *vs.* molar fraction at several surface pressures for the PE:PG system at 37 ºC. The discontinuous straight line represents the ideal behaviour for each surface pressure.

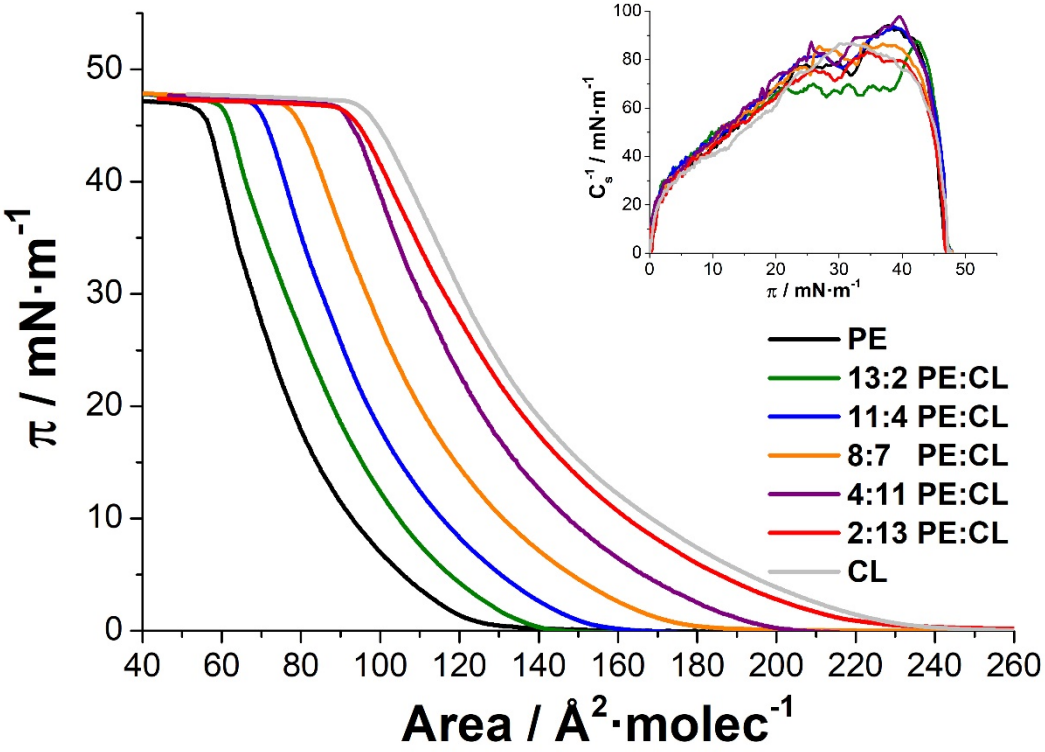
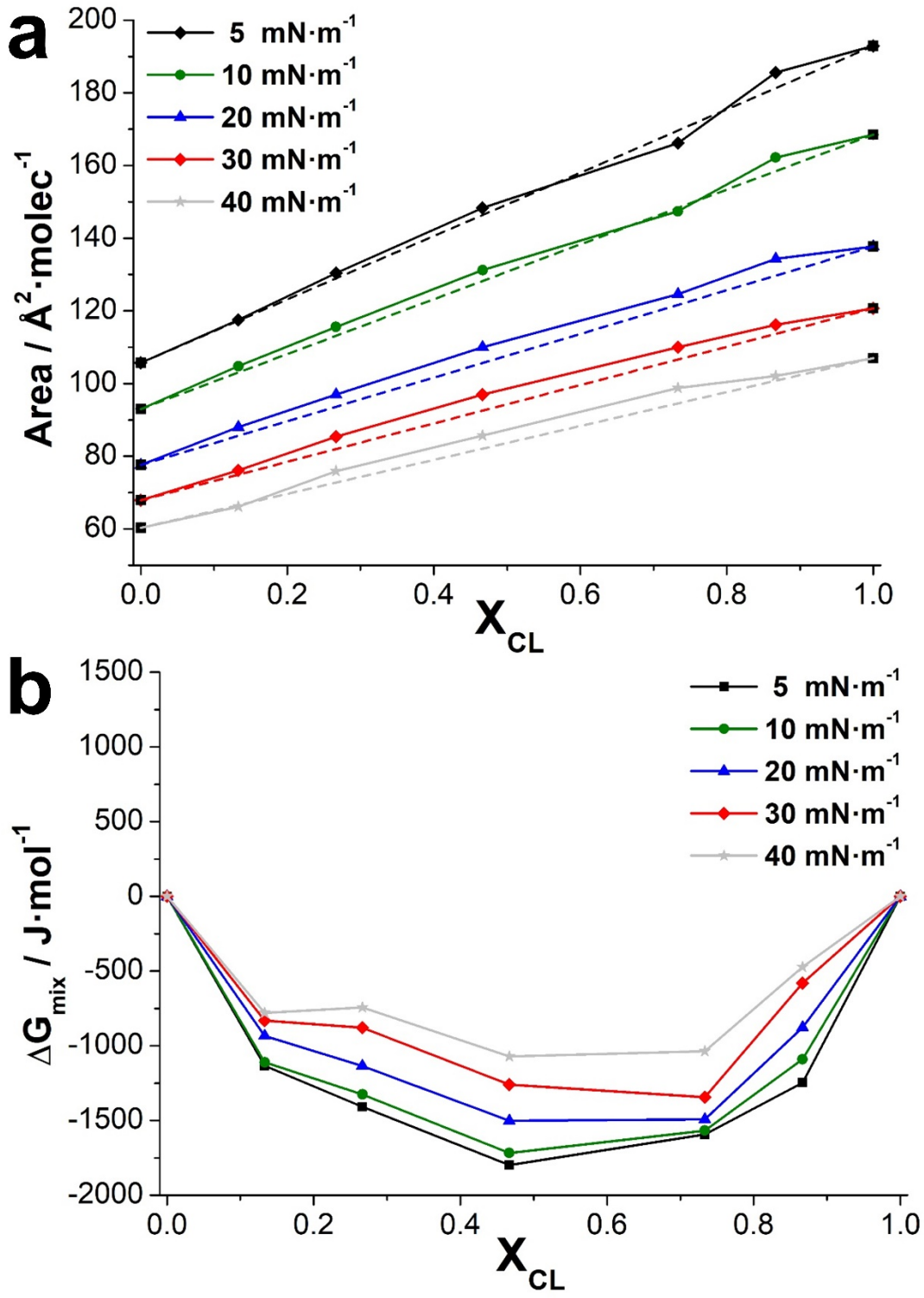


Fig. 3 π-A isotherms for PE, CL and PE:CL mixtures on PBS subphase at 37 ºC. Inset: Inverse of the compressibility modulus *vs*. surface pressure corresponding to the described π-A isotherms.

The mean area per molecule *vs*. molar fraction curves (fig. 4A) exhibited slightly positive deviations from the additivity rule (dashed line), corroborating that PE:CL system constitutes slightly non-ideal films at $\pi > 5$ mN·m^{-1}. The negative ΔG_{mix} values (fig. 4B) observed for all the mixtures indicate the stability (Roche et al. 2006) of the studied PE:CL mixtures.



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172 **Fig. 4** a) Mean area per molecule *vs.* molar fraction and b) mixing energy *vs.* molar fraction at
173 several surface pressures for the PE:CL system at 37ºC. The discontinuous straight line
174 represents the ideal behaviour for each surface pressure.

4. Discussion

The current work used a myriad of natural lipid structures that share the same headgroup, but contain acyl chains with different length and degree of unsaturation, for preparing model bacterial membranes. PE:PG and PE:CL mixtures at 37 ºC showed higher lift-off areas (fig. 1 and fig. 3) than those observed at 23 ºC (Hoyo et al. 2019b). On the contrary, the collapse surface pressure was lowered upon the increase of temperature. Higher temperature favours the lipid vibrations and the dissociation of ionisable groups (Krajewska et al. 2013b) -PG and CL headgroups - inducing higher molecular areas. The PE:PG system yielded a higher lift-off area difference ~30 $Å^2$·molecule^{-1} than PE:CL ~20 $Å^2$·molecule^{-1} upon the temperature increase, attributed to the stronger interactions observed in the PE:PG system compared to PE:CL at 23 ºC (Hoyo et al. 2019b). Similarly, Suárez-Germà et al. (Suárez-Germà et al. 2011) observed that monolayers of lipids with PE headgroup presented higher lift-off area as the number of unsaturations of the hydrocarbon chains or the temperature was increased, according to observations for DPPG (Krajewska et al. 2013b) and dipalmitoylphosphatidylcholine (Krajewska et al. 2013a). The characteristics of the acyl chains (unsaturation and chains length) contributed to the steric hindrances between individual lipids resulting in a lower packing.

The $C_s^{-1}{}_{max}$ for the single lipids and their corresponding mixtures decreased below 100 mN·m^{-1}, indicating that all studied systems present LE physical state in accordance to the results observed at 23ºC (Hoyo et al. 2019b), where only PE lipid monolayer reached the liquid condensed (LC) state. The increase of the temperature favours the dissociation of ionisable groups (Krajewska et al. 2013b) and lowers the packing, thus yielding fluid phases. The rigidity of the studied systems at 23 ºC was slightly higher than at 37º C. The characteristics of the isotherms and the LE state observed for the binary PE:PG and PE:CL systems (fig. 1 and 3) are in line with previous studies using a natural ternary mixture (Clausell et al. 2004; López-

199 Montero et al. 2008). The presence of unsaturations promoted the formation of fluid phases in
200 our PE:PG and PE:CL systems (inset of fig. 1 and 3). Such phenomenon was also described
201 (Suárez-Germà et al. 2011) for palmitoyloleoylphosphatidylethanolamine (POPE) and POPG
202 that became less rigid upon increasing the number of unsaturations whereas the saturated
203 dipalmitoylphosphatidylethanolamine reached solid state (S) (Hernández-Borrell and
204 Domènech 2017). The PE:PG and PE:CL systems formed almost ideal monolayers at 37 ºC
205 (fig. 2A and 4A). In contrast, the PE:CL system showed non-ideal films at 23 ºC, inducing non-
206 favoured mixtures when the amount of PE or CL was above 20 % and $\pi \geq 30$ mN·m^{-1} (Hoyo et
207 al. 2019b). This observation is explained by the higher repulsion and vibration between lipids
208 upon increasing the temperature.

209 The thermodynamic study showed negative ΔG_{mix} values for all studied systems (fig. 2B and
210 4B), thus indicating the stability of the mixtures and the absence of phase separation (Gzyl-
211 Malcher et al. 2008) domains for both PE:PG and PE:CL systems, regardless the PE:lipid ratio,
212 contrarily to the PE:CL system unstable at 23 ºC. The formation of domains was observed for
213 the POPE:CL (Domènech et al. 2006) and POPE:POPG 3:1 (Seeger et al. 2009; Picas et al.
214 2010; Suárez-Germà et al. 2011) systems both in LB and supported planar bilayers - widely
215 used for biomimetic membranes construction (Domènech et al. 2006; Hoyo et al. 2013) - below
216 and above the melting temperature. These observations were attributed to the tendency of POPE
217 to form LC phase (Suárez-Germà et al. 2011) and the presence of Ca^{2+} ions that resulted in
218 stronger interaction with POPG than with POPE (Picas et al. 2010), thus inducing phase
219 separation. Oppositely to the POPE:POPG and POPE:CL systems, the thermodynamic studies
220 of biomimetic membranes with a natural myriad of bacterial lipids indicated the absence of
221 phase separation domains at 37 ºC (López-Montero et al. 2008). Nevertheless, the same authors
222 (López-Montero et al. 2008) observed their presence by epifluorescence microscopy, whereas
223 Domènech et al. (Doménech et al. 2006) did not detect such domains by atomic force

microscopy (AFM), in line with our thermodynamic results (fig. 2B and fig. 4B). Most probably, the use of selective fluorescent dyes favors the visualization of such domains in biomimetic membranes (López-Montero et al. 2008), as previously observed in natural bacterial membranes (Mileykovskaya et al. 2001). These apparently contradictory results with some previous studies may be explained by the limitations of the thermodynamic study applied to a multicomponent system. Despite the fact that we have studied binary PE:PG and PE:CL systems at several molar ratios, each lipid named by its headgroup contains several structures, corresponding to different chain length and unsaturation, building a multicomponent system. Therefore the emergence of nanoscale lipid domains could be possible as observed in biological membranes (Jacobson et al. 2007).

The temperature of the bacterial membrane environment is an important parameter, since some of the lipid constituents of these membranes present phase transition close to the physiological human temperature (Silvius 1982). Therefore, the physical state influences both the permeability of the membrane and the interactions of the lipids with other membrane components, altering consequently their function (Barrera et al. 2012). Several mechanisms have been reported by which antibacterial agents target bacteria and alter their membrane properties (Epand et al. 2016). Therefore, the physical state and the fluidity of the bacterial membrane is of high relevance for the development of bactericidal agents. Several bacterial model membranes with different degree of similarity to the real membranes are described in the literature, but most of them are composed by only one or two lipid structures. Despite that some of these studies used unsaturated lipids to enhance the fluidity of the resulting membranes, the effect of the biological myriad of lipid structures was not evaluated, thus the reduction of fluidity was not considered neither the different interaction of the lipid structures with the antibacterial agent. In our work, the fluidity of the model membrane formed at 37 ºC was compared to the fluidity of a membrane formed at 23 ºC, concluding the athermalicity of the

249 rigidity and physical state for PE:PG and PE:CL systems at biologically relevant temperatures.
250 These results correlate with previous reports for other lipid systems at LE state (López-Montero
251 et al. 2008) and may explain the ability of *E.coli* membranes to maintain their functionality
252 across a broad temperature range (Remaut and Fronzes 2014) due to intermolecular hydrogen
253 bonding between lipids and transmembrane proteins allowing the correct protein insertion and
254 function in the membrane (Domènech et al. 2006). The observed athermalicity suggested that
255 the effect of antibacterial agents on model lipid membranes would be similar, regardless room
256 or human physiological temperature.
257
258

5. Conclusions

E.coli model membranes were prepared using bacterium's natural lipid structures. These biomimetic membranes present almost athermal physical state and rigidity, which may be related to the ability of bacteria to maintain their functions at human physiological temperatures. These observations are of high relevance for the design of novel antimicrobial agents targeting the bacterial membranes. Similar behaviour of the bacterial membrane in the range of 23 to 37 ^{o}C was detected, despite that the phase transition temperature of some lipid structures present in *E. coli* membranes is close to human physiological temperature. Independently from the different strategies that bacteria adopt to adapt their lipid membrane composition to the temperature of the environment, their bulk membrane behaviour in the studied temperature range was not significantly altered. Novel bactericidal agents targeting the bacterial membrane may be engineered considering the athermalicity of the membrane bulk properties. The simple and reliable bacterial membrane model described in this work may be further used for mechanistic studies in vitro of the interactions between bactericidal agents and bacteria.

Acknowledgements

The research leading to these results has received funding from European Community's Horizon 2020 Framework Program H2020 (H2020-720851 project PROTECT - Pre-commercial lines for production of surface nanostructured antimicrobial and anti-biofilm textiles, medical devices and water treatment membranes)

Notes

The authors declare no competing financial interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Abbreviations

Cardiolipin from *E. coli* (CL), dipalmitoylphosphatidylglycerol (DPPG), *Escherichia coli (E.coli)*, *E. coli* polar lipid extract (PLE), Langmuir-Blodgett (LB), liquid condensed (LC), liquid expanded (LE), palmitoyloleoylphosphatidylethanolamine (POPE), palmitoyloleoylphosphatidylglycerol (POPG), phosphatidylethanolamine from *E. coli* (PE), phosphatidylglycerol from *E. coli* (PG)

References

Barrera FN, Fendos J, Engelman DM (2012) Membrane physical properties influence transmembrane helix formation. Proc Natl Acad Sci U S A 109:14422–14427 . doi: 10.1073/pnas.1212665109

Baumgärtner P, Geiger M, Zieseniss S, Malleier J, Huntington JA, Hochrainer K, Bielek E, Stoeckelhuber M, Lauber K, Scherfeld D, Schwille P, Wäldele K, Beyer K, Engelmann B (2007) Phosphatidylethanolamine critically supports internalization of cell-penetrating protein C inhibitor. J Cell Biol 179:793–804 . doi: 10.1083/jcb.200707165

Boyd KJ, Alder NN, May ER (2017) Buckling under Pressure: Curvature-Based Lipid Segregation and Stability Modulation in Cardiolipin-Containing Bilayers. Langmuir 33:6937–6946 . doi: 10.1021/acs.langmuir.7b01185

Clausell A, Busquets MA, Pujol M, Alsina A, Cajal Y (2004) Polymyxin B-lipid interactions in Langmuir-Blodgett monolayers of Escherichia coli lipids: A thermodynamic and atomic force microscopy study. Biopolymers 75:480–490 . doi: 10.1002/bip.20165

Clausell A, Garcia-Subirats M, Pujol M, Busquets MA, Rabanal F, Cajal Y (2007) Gram-negative outer and inner membrane models: Insertion of cyclic cationic lipopeptides. J Phys Chem B 111:551–563 . doi: 10.1021/jp064757

Doménech Ò, Merino-Montero S, Montero MT, Hernández-Borrell J (2006) Surface planar bilayers of phospholipids used in protein membrane reconstitution: An atomic force microscopy study. Colloids Surfaces B Biointerfaces 47:102–106 . doi: 10.1016/j.colsurfb.2005.11.025

Domènech Ò, Sanz F, Montero MT, Hernández-Borrell J (2006) Thermodynamic and structural study of the main phospholipid components comprising the mitochondrial

316 inner membrane. Biochim Biophys Acta - Biomembr 1758:213–221 . doi:
317 10.1016/j.bbamem.2006.02.008

318 Epand RM, Walker C, Epand RF, Magarvey NA (2016) Molecular mechanisms of membrane
319 targeting antibiotics. Biochim Biophys Acta - Biomembr 1858:980–987 . doi:
320 10.1016/j.bbamem.2015.10.018

321 Fernandes MM, Ivanova K, Hoyo J, Pérez-Rafael S, Francesko A, Tzanov T (2017)
322 Nanotransformation of vancomycin overcomes the intrinsic resistance of Gram-negative
323 bacteria. ACS Appl Mater Interfaces 9:15022–15030 . doi: 10.1021/acsami.7b00217

324 Ferreres G, Bassegoda A, Hoyo J, Torrent-Burgués J, Tzanov T (2018) Metal−Enzyme
325 Nanoaggregates Eradicate Both Gram-Positive and.pdf. ACS Appl Mater Interfaces
326 10:40434–40442

327 Gidalevitz D, Ishitsuka Y, Muresan AS, Konovalov O, Waring AJ, Lehrer RI, Lee KYC
328 (2003) Interaction of antimicrobial peptide protegrin with biomembranes. Proc Natl
329 Acad Sci U S A 100:6302–6307 . doi: 10.1073/pnas.0934731100

330 Gzyl-Malcher B, Filek M, Makyła K, Paluch M (2008) Differences in surface behaviour of
331 galactolipoids originating from different kind of wheat tissue cultivated in vitro. Chem
332 Phys Lipids 155:24–30 . doi: 10.1016/j.chemphyslip.2008.06.004

333 Hernández-Borrell J, Domènech Ò (2017) Critical Temperature of 1-Palmitoyl-2-oleoyl-sn-
334 glycero-3-phosphoethanolamine Monolayers and Its Possible Biological Relevance. J
335 Phys Chem B 121:6882–6889 . doi: https://doi.org/10.1021/acs.jpcb.7b04021

336 Hoyo J, Guaus E, Oncins G, Torrent-Burgués J, Sanz F (2013) Incorporation of Ubiquinone
337 in supported lipid bilayers on ITO. J Phys Chem B 117:7498–7506 . doi:
338 10.1021/jp4004517

Hoyo J, Guaus E, Torrent-Burgués J (2016a) Monogalactosyldiacylglycerol and digalactosyldiacylglycerol role, physical states, applications and biomimetic monolayer films. Eur Phys J E 39:1–11 . doi: 10.1140/epje/i2016-16039-0

Hoyo J, Guaus E, Torrent-Burgués J (2016b) Influence of membrane galactolipids and surface pressure on plastoquinone behaviour. Bioelectrochemistry 111:123–130 . doi: 10.1016/j.bioelechem.2016.06.002

Hoyo J, Guaus E, Torrent-Burgués J, Sanz F (2015) Biomimetic monolayer films of digalactosyldiacylglycerol incorporating plastoquinone. Biochim Biophys Acta - Biomembr 1848:1341–1351 . doi: 10.1016/j.bbamem.2015.03.003

Hoyo J, Guaus E, Torrent-Burgués J, Sanz F (2012) Electrochemical behaviour of mixed LB films of ubiquinone - DPPC. J Electroanal Chem 669:6–13 . doi: 10.1016/j.jelechem.2012.01.020

Hoyo J, Ivanova K, Guaus E, Tzanov T (2019a) Multifunctional ZnO NPs-chitosan-gallic acid hybrid nanocoating to overcome contact lenses associated conditions and discomfort. J Colloid Interface Sci 543:114–121 . doi: 10.1016/j.jcis.2019.02.043

Hoyo J, Torrent-Burgués J, Tzanov T (2019b) Physical states and thermodynamic properties of model gram-negative bacterial inner membranes. Chem Phys Lipids 218:57–64 . doi: 10.1016/j.chemphyslip.2018.12.003

Ivanova A, Ivanova K, Hoyo J, Heinze T, Sanchez-Gomez S, Tzanov T (2018) Layer-By-Layer Decorated Nanoparticles with Tunable Antibacterial and Antibiofilm Properties against Both Gram-Positive and Gram-Negative Bacteria. ACS Appl Mater Interfaces 10:3314–3323 . doi: 10.1021/acsami.7b16508

Ivanova K, Ramon E, Hoyo J, Tzanov T (2017) Innovative Approaches for Controlling

Clinically Relevant Biofilms: Current Trends and Future Prospects. Curr Top Med Chem 17:1889–1914 . doi: 10.2174/1568026617666170105143315

Jacobson K, Mouritsen OG, Anderson RGW (2007) Lipid rafts: At a crossroad between cell biology and physics. Nat Cell Biol 9:7–14 . doi: 10.1038/ncb0107-7

Krajewska B, Wydro P, Kyzioł A (2013a) Chitosan as a subphase disturbant of membrane lipid monolayers. The effect of temperature at varying pH: II. DPPC and cholesterol. Colloids Surfaces A Physicochem Eng Asp 434:359–364 . doi: 10.1016/j.colsurfa.2013.03.015

Krajewska B, Wydro P, Kyzioł A (2013b) Chitosan as a subphase disturbant of membrane lipid monolayers. The effect of temperature at varying pH: I. DPPG. Colloids Surfaces A Physicochem Eng Asp 434:349–358 . doi: 10.1016/j.colsurfa.2013.03.015

Larsson G, Törnkvist M (1996) Rapid sampling, cell inactivation and evaluation of low extracellular glucose concentrations during fed-batch cultivation. J Biotechnol 49:69–82 . doi: 10.1016/0168-1656(96)01534-9

López-Montero I, Arriaga LR, Monroy F, Rivas G, Tarazona P, Vélez M (2008) High fluidity and soft elasticity of the inner membrane of escherichia coli revealed by the surface rheology of model langmuir monolayers. Langmuir 24:4065–4076 . doi: 10.1021/la703350s

López-Montero I, Arriaga LR, Rivas G, Vélez M, Monroy F (2010) Lipid domains and mechanical plasticity of Escherichia coli lipid monolayers. Chem Phys Lipids 163:56–63 . doi: 10.1016/j.chemphyslip.2009.10.002

Michel JP, Wang Y, Dé E, Fontaine P, Goldmann M, Rosilio V (2015) Charge and aggregation pattern govern the interaction of plasticins with LPS monolayers mimicking

385 the external leaflet of the outer membrane of Gram-negative bacteria. Biochim Biophys
386 Acta - Biomembr 1848:2967–2979 . doi: 10.1016/j.bbamem.2015.09.005

387 Michel JP, Wang Y, Kiesel I, Gerelli Y, Rosilio V (2017) Disruption of Asymmetric Lipid
388 Bilayer Models Mimicking the Outer Membrane of Gram-Negative Bacteria by an
389 Active Plasticin. Langmuir 33:11028–11039 . doi: 10.1021/acs.langmuir.7b02864

390 Mileykovskaya E, Dowhan W, Birke RL, Zheng D, Lutterodt L, Haines TH (2001)
391 Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic
392 domains on bilayer surfaces. FEBS Lett 507:187–190 . doi: 10.1016/S0014-
393 5793(01)02948-9

394 Nichols-Smith S, Teh S-YY, Kuhl TL (2004) Thermodynamic and mechanical properties of
395 model mitochondrial membranes. Biochim Biophys Acta - Biomembr 1663:82–88 . doi:
396 10.1016/j.bbamem.2004.02.002

397 Picas L, Suárez-Germà C, Teresa Montero M, Hernández-Borrell J (2010) Force spectroscopy
398 study of langmuir-blodgett asymmetric bilayers of phosphatidylethanolamine and
399 phosphatidylglycerol. J Phys Chem B 114:3543–3549 . doi: 10.1021/jp910882e

400 Remaut H, Fronzes R (2014) Bacterial Membranes, Structural and Molecular Biology. Caister
401 Academic Press

402 Roche Y, Peretti P, Bernard S (2006) Influence of the chain length of ubiquinones on their
403 interaction with DPPC in mixed monolayers. Biochim Biophys Acta - Biomembr
404 1758:468–478 . doi: 10.1016/j.bbamem.2006.03.015

405 Seeger H, Marino G, Alessandrini A, Facci P (2009) Effect of Physical Parameters on the
406 Main Phase Transition of Supported Lipid Bilayers. Biophys J 97:1067–1076 . doi:
407 10.1016/j.bpj.2009.03.068

Shokri A, Larsson G (2004) Characterisation of the Escherichia coli membrane structure and function during fedbatch cultivation. Microb Cell Fact 3:9 . doi: 10.1186/1475-2859-3-9

Silvius JR (1982) Thermotropic Phase Transitions of Pure Lipids in Model Membranes and Their Modifications by Membrane Proteins. John Wiley Sons, Inc

Suárez-Germà C, Montero MT, Ignés-Mullol J, Hernández-Borrell J, Domènech Ò (2011) Acyl chain differences in phosphatidylethanolamine determine domain formation and LacY distribution in biomimetic model membranes. J Phys Chem B 115:12778–12784 . doi: 10.1021/jp206369k

Vitovič P, Nikolelis DP, Hianik T (2006) Study of calix[4]resorcinarene-dopamine complexation in mixed phospholipid monolayers formed at the air-water interface. Biochim Biophys Acta - Biomembr 1758:1852–1861 . doi: 10.1016/j.bbamem.2006.08.011

Yeagle PL (2016) The Membranes of Cells. Academic Press-Elsevier, San Diego, California

Figure captions

Scheme 1. Langmuir monolayer formation and representative chemical structure of the myriad of lipid structures that shares the same headgroup: phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) in lipid extracts from *E. coli* (see section 2.1).

Fig. 1 π-A isotherms for PE, PG and PE:PG mixtures on PBS subphase at 37ºC. Inset: Inverse of the compressibility modulus *vs.* surface pressure corresponding to the described π-A isotherms.

Fig. 2 a) Mean area per molecule *vs.* molar fraction and b) mixing energy *vs.* molar fraction at several surface pressures for the PE:PG system at 37ºC. Discontinuous straight line represents the ideal behaviour for each surface pressure.

Fig. 3 π-A isotherms for PE, CL and PE:CL mixtures on PBS subphase at 37ºC. Inset: Inverse of the compressibility modulus *vs.* surface pressure corresponding to the described π-A isotherms.

Fig. 4 a) Mean area per molecule *vs.* molar fraction and b) mixing energy *vs.* molar fraction at several surface pressures for the PE:CL system at 37ºC. Discontinuous straight line represents the ideal behaviour for each surface pressure.