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DOI:

[10.1021/acs.langmuir.7b02244](https://doi.org/10.1021/acs.langmuir.7b02244)

Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

Zaki, A. M., & Carbone, P. (2017). How the incorporation of Pluronic block copolymers modulates the response of lipid membranes to mechanical stress. *Langmuir*. <https://doi.org/10.1021/acs.langmuir.7b02244>

Published in:

Langmuir

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Journal:	Langmuir
Manuscript ID	la-2017-02244g.R1
Manuscript Type:	Article
Date Submitted by the Author:	08-Sep-2017
Complete List of Authors:	Zaki, Afroditi; The University of Manchester, School of Chemical Engineering and Analytical Science Carbone, Paola; The University of Manchester, School of Chemical Engineering and Analytical Science

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How the incorporation of Pluronic® block copolymers modulates the response of lipid membranes to mechanical stress

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Abstract

We employ atomistic Molecular Dynamics simulations to investigate the effect that the incorporation of the nonionic amphiphilic copolymer known as Pluronic® L64 has on the mechanical stability of a DPPC membrane. The simulations reveal that the incorporation of the polymer chains leads to membranes that can sustain increasing mechanical stresses. Analysis of mechanical, structural and dynamic properties of the membrane shows that the polymer chains interact strongly with the lipids in the vicinity, restraining their mobility and imparting better mechanical stability to the membrane. The hybrid membranes under tension remain thicker, more ordered and stiffer in comparison to their lipid analogues. Trans-bilayer lipid movements (flip-flop) are observed and appear to be triggered by the presence of the polymer chains. A careful analysis of the pore formation under high tensions reveals two distinctive mechanisms that depend on the distribution of the hydrophilic polymer blocks in the bilayer. Finally, the rate of growth of the formed membrane defects is slowed down in the presence of polymers. These findings show that Pluronic® block copolymers could be exploited

for the formation of optimized hybrid nanodevices with controlled elastic and dynamic properties.

Introduction

The mechanism by which polymers modify and interact with lipid bilayers is a crucial step for the development of new polymer-based drug nano-carriers.¹⁻⁴ Such nanocarriers can be developed entirely from polymers (polymersomes, micelles, dendrimers),⁵ or, to minimize the side effects, be self-assembled from a mixture of polymers and lipids (hybrid particles). The latter type of vesicles should have the advantage of retaining the good mechanical properties typical of the polymer vesicles and of displaying milder adverse effects.^{5,6} For example it has been shown that grafting lipid vesicles with Polyethylene glycol (PEG) increases the vesicles' stability during blood circulation^{7,8} and that polyelectrolyte-coated DOPC vesicles possess improved mechanical stability under pH shock.^{9,10} In particular, the incorporation of amphiphilic block copolymers in phospholipid vesicles have shown to enhance not only the vesicles' mechanical stability but also the loading rates of drugs and the drug cellular uptake.^{11,12} Experimental data showed that these hybrid vesicles have substantially increased shelf stability, resistance against rupture and fusion, and are characterized by decreased water permeability.^{8,13} In addition, recent *in-vivo* studies on mice showed that amphiphilic block copolymer-lipid vesicles loaded with an anticancer drug cocktail cause reduced organ toxicity and higher drug load release at the target cells.¹⁴ While the data clearly indicate that the hybrid polymer-lipid vesicles possess improved mechanical properties, it is less clear what molecular mechanism drives such changes. The polymer molecular weight, its microstructure and, more complicated to rationalize, the procedure followed for the self-assembly seem to be all variables affecting the final stability of the vesicles.^{6,11} Specifically, for amphiphilic copolymers, the hydrophobic/hydrophilic ratio and the molar mass of the polymers seem to be the main factors affecting both the cellular uptake and the change in the membrane

properties.¹⁵ Due to the difficulty in gaining an atomistically resolved picture of the vesicles' structure, molecular simulations have been extensively used to investigate the effect that polymers, surfactants or small molecules have on the structure of lipid bilayers.^{16–22}

Specifically, Srinivas *et al.*²³ have firstly shown, using coarse-grained simulations, that diblock copolymers of polyethylene glycol (PEG-PEE) translocate within preformed lipid bilayers that consequently increase their thickness. The simulations showed that the hydrophilic block tends to reside at the water/bilayer interface while the hydrophobic block moves inside the bilayer tail region. Despite the numerous computational studies on polymer/lipid membranes, most of them focus on structural and dynamic properties of stress-free systems and there is a lack in the investigation of the membranes' mechanical properties. One of the few such studies, is the work by Tomasini *et al.*²⁴ who, using a mesoscopic model, showed that the (PEG-PEE) copolymer modifies the bilayer mechanical properties and highlighted how the hydrophilic-lipophilic balance and the polymer chain molecular weight can either enhance or reduce the vesicles mechanical stability.

Among the various amphiphilic block copolymers that can be employed to fabricate these hybrid vesicles, Pluronic®, a series of triblock copolymers of ethylene oxide (EO) and propylene oxide (PO) arranged along the chain as a EO-PO-EO sequence, look particularly interesting. Due to their amphiphilic nature, in aqueous environments they form micelles,²⁵ which are suitable for the encapsulation of drugs, proteins and genes and can therefore be used as nanocarriers. Their properties can be controlled by tuning the hydrophilic-lipophilic balance and their molecular weight. Being already FDA approved, they are currently widely used in the cosmetic and pharmaceutical industry,¹⁵ however they demonstrate increased levels of toxicity if used in high dosage. On the other hand, Pluronic® coated nanoparticles developed for medical imaging purposes were characterized as highly biocompatible and relatively non-toxic,¹⁵ opening the possibility for Pluronic® copolymers to be employed in hybrid systems for nanomedical applications. Experimental data^{26,27} and molecular simulations^{17,21,28} on stress-free lipid bilayers containing different types of Pluronic® copolymers

have already showed that the incorporation of the chains within the bilayer alters the membrane thickness, the lipid diffusivity and packing properties and the bilayer permeability. The data have also suggested that in some cases the amphiphilic copolymers are able to heal a damaged membrane by stabilizing its structure, but in other cases, that the presence of the copolymers disturbs the lipid packing and enhances membrane permeability.²⁹

The aim of this work is to elucidate the impact that the incorporation of Pluronic® chains has on various properties of phospholipid bilayers when the membrane is subjected to mechanical stresses. This work focuses specifically on a Pluronic® labeled as L64 (consisting of a 40% EO content and a PO molecular weight of 1800 g/mol³⁰) (Figure 1a) which is one of the best experimentally characterized among the copolymer series^{31–37} and shows spontaneous adsorption into phospholipid bilayers.³⁸

The well characterized 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) lipid (found in abundance in mammalian cell membranes as one of their main constituents) was selected for the construction of the model lipid bilayer^{39,40} (Figure 1b). It is a zwitterionic phospholipid and has been used -among others- for the development of various types of hybrid nanoparticles,¹¹ including binary polymer/lipid mixtures,¹² core-shell type polymer-lipid nanoparticles^{6,11} and polymer-caged liposomes.⁴¹

Figure 1: (a) Molecular structure of the L64 Pluronic®. The blocks in brackets highlight the ethylene oxide (EO) (green) and the propylene oxide (PO) (red) segments. (b) Molecular structure of the DPPC lipid.

Methodology

Systems

Lipid Bilayers. The Berger *et al.*⁴² force field and the single point charge SPC model⁴³ were used to model the lipid and the water molecules, respectively. The lipid bilayer was built starting from a previously equilibrated DPPC lipid bilayer consisting of 128 lipids and 3655 water molecules, downloaded from <http://wcm.ucalgary.ca/tieleman/downloads/dppc.itp>. The water molecules were then removed and the box was duplicated in the *x*- and *y*- dimensions to generate a bilayer of 512 lipids which was then placed in the center of a box of dimensions 13×13×10 nm³. Approximately 33000 water molecules were added and the system was then energy minimized following the steepest descent method and equilibrated in an NVT and subsequently, in an NPT ensemble.

Polymer-Lipid Bilayers. Hybrid polymer-lipid (HPL) systems were obtained by adding L64 polymer chains to the water phase of the pure lipid membrane system and allowing the polymers to insert into the preformed bilayer. The L64 polymer was modeled using the unmodified GROMOS force field with partial charges obtained from DFT calculations. For the method followed for the development and validation of the force field parameters, we refer the reader to the original work.¹⁷ For convenience, the set of charges is reported in the supporting information (Table S1).

In order to elucidate the effect of polymer concentration, either one or twelve L64 chains were added resulting in polymer mass fractions in the polymer-lipid mixture of approximately 1.2 wt% and 12.7 wt% (in DPPC/L64 molar ratios this translates to 82:1 and 7:1, respectively). In the text, the hybrid systems will be labeled as *LowHPL* (DPPC/L64 82:1) and *HighHPL* (DPPC/L64 7:1) for brevity. The twelve L64 chains were initially randomly distributed in the entire *xy*-plane covering most of the surface of one of the leaflets and acting as coating, mimicking the preparation of polymer-coated liposomes.² Finally, the space was again filled with enough water molecules to fill a box of similar size as the pure lipid

system. Energy minimization was carried out followed by an NVT and an initial NPT equilibration with the polymer chains being position restrained. This initial equilibration step was necessary to relax the water molecules around the polymer and the lipids and to ensure that the thermodynamic properties have equilibrated before allowing the polymers to move freely. The restraints were then removed and the systems were subjected to an additional 100 ns NPT run within which the polymer chains translocate and remain incorporated in the bilayer. The final configurations were used as the starting points for the production runs where the membranes were expanded as will be described below.

Simulations under mechanical stress

In order to assess the resistance of the membranes under tension, both the NP_NAT (constant surface area) and NPT (isothermal-isobaric) thermodynamic ensembles were preliminary tested on the DPPC bilayers. Specifically for the NPT ensemble, two loading rates were applied and the results (i.e., area per lipid and surface tension) obtained from the three different procedures were compared. For the high loading rate NPT simulations (NPT-HR), lateral pressures (P_L) of increasing magnitude ranging from -10 bar to P_{LC} (P_{LC} : critical rupture lateral pressure) were applied on the equilibrated tensionless membrane. On the contrary, for the low loading rate simulations (NPT-LR), the lateral pressure was applied to the final configuration of each previously equilibrated NPT simulation. The latter procedure of gradually increasing the pressure allowed the system to adjust to the expansion and produced comparable area-per-lipid and surface tension data with the NP_NAT ensemble simulations (Figure S1) and therefore was selected as the method to expand the HPL systems. The pressure coupling was semi-isotropic, with the pressure applied in the direction normal to the membrane (P_N) (set at 1 bar) being coupled separately from the lateral pressure (P_L). The temperature was set at 323.15 K, approximately 8 K above the gel-to-liquid phase transition temperature of unsupported DPPC.⁴⁴ The temperature and pressure were controlled with the Nose-Hoover thermostat⁴⁵⁻⁴⁷ and the Parrinello-Rahman barostat^{48,49}

with time constants of 0.5 ps and 5 ps, respectively. Periodic boundary conditions were applied in all three dimensions. The bonds were constrained using the LINCS algorithm.⁵⁰ The Particle-Mesh Ewald summation⁵¹ was adopted for the treatment of the long range electrostatic interactions, as it produces the most reliable results for systems with charges. A cubic interpolation was chosen with a grid spacing of 0.16 nm. The Lennard-Jones interactions and the short range Coulomb interactions were cut-off at 1 nm. The time step was 2 fs and the leap-frog algorithm was selected for the integration of the equations of motion. The total length of each production run was 500 ns, of which the final 50-200 ns were used for the calculations, depending on the type of analysis. The MD simulations were performed using the GROMACS software package (version 4.6.5).⁵² The VMD software (version 1.9)⁵³ was used for the visualisation of the systems and the MDAnalysis package^{54,55} was employed for the development of home-made analysis tools.

Results and Discussion

Effect of the L64 chains on the elastic properties of the membrane

Within few nanoseconds after removing the position restraints, the polymer chains started to interact with the headgroups of the phospholipids and translocated into the bilayer where they remained adsorbed for the total length of the simulation (Figures 2b and 2c). This observation was not surprising as the poloxamers are surface-active polymers and rapidly adsorb onto oil-water interfaces⁵⁶⁻⁶⁰ and one of their main applications is as stabilizers for oil/water emulsions.^{38,57} Most of the polymer chains remained localized in the upper leaflet and the hydrophobic nature of the PO segment of the polymers allowed, within the simulation time, only few of the chains to penetrate into the hydrophobic center of the bilayer and to move to the opposite leaflet (Figures 2c and 2f). The PEO block on the other hand, mostly lied close to the lipid/water interface, interacting closely with the hydrophilic heads of the lipids. The spatial distribution of the PEO blocks could depend on the polymer

Figure 2: *Upper Panel*: Mass density profiles of all the system components in $P_L = 1$ bar, averaged over the final 50 ns of each trajectory. *Lower Panel*: Representative snapshots of the membranes in water. (a),(d) DPPC; (b),(e) *LowHPL*; (c),(f) *HighHPL*. Color code of the snapshots: water in blue, lipids in yellow, polymer PEO blocks in green and polymer PPO blocks in red.

content, as for similar mass fractions, the PEG blocks of amphiphilic copolymers have been found to behave similarly, whereas for higher polymer content, they were forced to extend in the water phase.²³ Especially for the *HighHPL* system, the large difference in polymer concentration between the two leaflets induces a significant asymmetry in the membrane and has implications on its structural properties and fluidity, as it will be shown later (Figures 6, 7 and 8). The above equilibrated systems were then subjected to an increasingly high external lateral pressure as described in the Methodology section aiming to assess the mechanical properties of the membranes under tension. Experimentally, the mechanical strength of vesicles is investigated via the micropipette aspiration technique which probes the stress-strain curves and provides information on the membrane tension, the areal strain upon which lysis occurs and the area compressibility modulus (K_A).⁶¹ Similarly, the surface tension-area strain curve can be obtained from the simulations carried out at different lateral pressures and is reported in Figure 3a. The surface tension (γ) is calculated as:

$$\gamma = L_z(P_N - P_L) \quad (1)$$

where L_z is the box length in the z -dimension, $P_N = P_{zz}$ and $P_L = \frac{1}{2}[P_{xx} + P_{yy}]$, with P_{xx} , P_{yy} and P_{zz} being the diagonal components of the pressure tensor. The areal strain (ϵ_A) is defined as the relative increase in the membrane surface area under tension with respect to the surface area of a tensionless state:

$$\epsilon_A = \frac{A_i}{A_0} - 1 \quad (2)$$

where A_i and A_0 are the bilayer surface areas in the systems under lateral pressure P_{Li} and in the tensionless system, respectively.

As expected, the membranes responded to the applied pressure with an increase in their surface tension (γ) as they tried to minimize the exposure of their hydrophobic constituents to water. All the membranes can withstand a 70-77% expansion of their surface area prior

the formation of a pore which leads to their rupture. For the polymer-free bilayer, the area per lipid at rupture is $\sim 1.1 \text{ nm}^2$, which is in good agreement with previous studies of DPPC membranes under tension.^{16,62,63} The corresponding critical surface tension is approximately 20 mN/m lower than that found in previous computational work.¹⁶ This can be attributed to the larger size of the membrane patch simulated here, which is expected to lead to reduced bilayer cohesion and stability.¹⁶ The data show that low polymer content does not affect the response of the membrane to the applied pressure but that for high polymer content, the *HighHPL* membrane is characterized by higher surface tension compared to the other two systems, indicating stronger intermolecular attractive interactions at the interface. This increase in the surface tension upon addition of the copolymer is in disagreement with the results shown by Dissipative Particles Dynamics²⁴ where however most of the hydrophilic part of the copolymer chains (PEO-PEE) resided at the outer part of the bilayer (e.g. towards the water phase). In the present case instead the PEO blocks are totally incorporated inside the membrane. The data also shows that, even though the rupture occurs at similar ϵ_A in all cases, the *HighHPL* membrane can sustain an increased surface tension of 75.8 mN/m prior to failure compared to the pure lipid membrane that breaks at 71.8 mN/m. Moreover, Figure 3b shows that while in the case of the pure DPPC, the critical areal strain is reached for $P_{Lc} = -110$ bar, a higher lateral pressure of -130 bar is required to cause rupture in the HPL bilayers (Figure 3b). Here it is important to notice that the critical pressure (P_{Lc}) is a size-dependent property and that small deviations in the box dimensions affect its values which therefore should be interpreted with caution. Figure 3b also demonstrates the resistance of the hybrid membranes to stretching when lateral pressure is imposed and how this resistance becomes more prominent at higher pressures. A significant increase in the lysis tension of HPL vesicles has also been found experimentally from Chen *et al.*¹² who showed that the hybrid vesicles of phospholipids and PDMS-*co*-PEO copolymers are mechanically more stable than the pure lipid ones, as the critical areal strain and the critical rupture surface tension significantly increase when polymers are present and the vesicles can withstand higher expansion of their

surface area before lysis. It is worth mentioning that the expansion the hybrid membrane can sustain before rupture in an experiment is much lower (no more than 8%) than the critical strain calculated from simulations (exceeding 70%). This discrepancy between experiments and simulations has been observed previously⁶³ and is attributed to the dynamic nature of the pore formation process that depends on the loading rate. At the much higher loading rates applied in simulations, the critical rupture tension moves to higher values, as explained by the kinetic model proposed by Evans *et al.*⁶⁴

The elasticity of a membrane can be assessed computationally and experimentally from the calculation of the compressibility modulus (K_A) which is related to the resistance of a membrane to the expansion of its surface area when it is under mechanical stress.⁶⁵ For small area expansion, it can be calculated via the slope of the stress-strain curve⁶⁶ and for larger membrane expansion, it can be calculated via equation 3:

$$K_A = k_B T \frac{\langle A \rangle}{\langle \delta A^2 \rangle} \quad (3)$$

where $\langle A \rangle$ is the mean total area, $\langle \delta A^2 \rangle$ is the mean square fluctuations of the area and $k_B T$ is the thermal energy. Membranes with high K_A , respond moderately to the applied stress showing a low areal strain for a given lateral tension and can be characterized as more cohesive. On the contrary, membranes that respond to the applied tension with high area fluctuations show increased elasticity.^{67,68} For the pure DPPC bilayer at zero surface tension, K_A is found to be 247.7 ± 30.0 dyn/cm, which is in good agreement with the experimentally measured value of 231 dyn/cm.³⁹ Upon increasing tension, the compressibility modulus of both the polymer-free and hybrid membranes decreases (Figure 4), a trend also previously observed by Feller and Pastor⁶⁶ for DPPC membranes. Looking at the impact of the polymers on K_A , it is clear that the *LowHPL* membrane behaves similarly to the pure lipid membrane, but when the polymer content is high, the membrane becomes stiffer (i.e., K_A increases with respect to the pure lipid and the *LowHPL* systems), implying that higher energy would be required for its surface area to expand. This effect disappears when

Figure 3: (a) Surface tension-areal strain curve and (b) Lateral pressure-areal strain curve for the two lipid/polymer ratios investigated here. The open symbols denote the rupture points. The standard errors of the mean are included, but are too small to be visible.

P_L becomes higher than -70 bar and above this pressure value all the membranes show a significant softening irrespective of their polymer content.

Figure 4: Area compressibility modulus (K_A) of membranes with different lipid/polymer ratios versus the applied lateral pressures (P_L). The error bars correspond to the standard errors of the mean, calculated from block averaging⁶⁹ of four independent blocks of 100 ns each.

An initial understanding of the molecular mechanism behind the improved mechanical properties of the HPL membranes is obtained looking at the area (A_{occ}) occupied by both the lipid and polymer molecules across the bilayer. To calculate A_{occ} we followed the method described in the work of Falck *et al.*⁶⁸ and later also used by Nawaz *et al.*,¹⁷ where the system is sliced across the z -axis (i.e., perpendicular to the membrane plane) and in each slice the atoms either belonging to the phospholipid molecules or to the polymer chain are mapped onto a two-dimensional grid. If a grid point lies within the Van der Waals radius of the atom, it is considered lipid-occupied or polymer-occupied, respectively. The total area profiles (Figure 5) reveal a thinning of the membrane as the expansion increases. For

the polymer-free bilayer, the two leaflets present, as expected, a symmetric profile and the only effect that applying a lateral pressure has on the membrane is to increase -significantly- its surface area. This increased surface area forces the lipids to spread out resulting in an increased lipid-occupied area, A_{occ} .¹ For the system containing one polymer chain, a small difference in the area per lipid between the two leaflets is observed for low pressures, but at higher pressures this asymmetry vanishes. Comparing the profiles calculated for the HPL membranes to that for the lipid system, it can be seen how the presence of the polymer makes the bilayer more resistant to the expansion, as in the former the increase in the lipid-occupied area is more limited, especially as the pressure becomes high. Finally, the *HighHPL* system presents highly asymmetric profiles indicating that the applied tension has a much more pronounced effect on the structure of the polymer-free leaflet. The polymer chains occupy approximately 30 nm^2 and are mainly located close to the headgroup region of one leaflet (the one from which they passively translocated inside the membrane), but some chains extend to the other leaflet and span the bilayer across the z -axis indicating that the polymer translocation is not inhibited by high polymer concentration.

Figure 5: Lipid-occupied and polymer-occupied area profiles averaged over the final 10 frames of the trajectories, for all the studied lateral pressures P_L below the critical rupture pressure P_{Lc} . (a) Pure DPPC bilayer, (b) *LowHPL* bilayer and (c) *HighHPL* bilayer. Given that the models used for the lipids and the polymers are united-atom representations, the half of the force field σ value was used as the Van der Waals radius.

Effect of the L64 chains on the structural properties of the membrane

The ordering of the lipid acyl chains of the membrane was determined by calculating the carbon-deuterium order parameter S_{CD} for the *sn-1* acyl chains (see Figure 1) using equation 4:

$$S_z = \frac{3}{2} \langle \cos^2 \theta_z \rangle - \frac{1}{2} \quad (4)$$

where θ_z is the angle between the bilayer normal and the vector from C_{n-1} to C_{n+1} (C is the carbon atom). Since a united-atom model was used for the lipids, the C-D vector is replaced by a vector joining two successive carbon atoms on the same side of the acyl chain. The brackets indicate average over time and lipids. The order parameter S_z is related to the deuterium order parameter as $S_{CD} = -\frac{1}{2}S_z$. The absolute value of S_{CD} lies between 0 (isotropic orientation) and 0.5 (orientation along the bilayer normal).⁷⁰

Irrespectively to the pressure applied and the presence of the polymer chains in the bilayer, the overall tendency is that the lipid ordering decreases with increasing distance from the lipid heads, approaching the value of zero at the terminal methyl groups, following the typical trend that has been previously observed both experimentally⁷⁰ and computationally^{40,44} for tensionless membranes (Figure 6a). The orientation of the acyl chains is however clearly reduced when the membrane is expanded, a result that is rather expected, since the membrane is characterized by a higher free volume allowing the lipids to assume a large number of configurations and also a direct consequence of the membrane thinning (see above). However, a more interesting finding is that the polymers enhance the lipid tails' ordering, especially in the proximity of the headgroup region where the polymer chains are mostly located (see Figure 2). The effect is negligible for low lateral pressure, but becomes evident for P_L exceeding the value of -30 bar. The polymer concentration also seems to play a role in the lipid tail packing as the values of the S_{CD} are higher when more polymers are incorporated in the bilayer. Similar behavior is observed for the *sn-2* fatty acid tails

Figure 6: (a) Deuterium order parameter (S_{CD}) profiles for the *sn*-1 lipid acyl tail. The carbon atom number increases towards the center of the bilayer. Solid line, filled circles polymer-free DPPC; dashed line, filled squares DPPC/L64 82:1; pointed line, filled diamonds DPPC/L64 7:1. (b) The average deuterium order parameter (S_{CD}) for the *HighHPL* system, calculated for all the lipids (circles), for the leaflet without polymers (squares) and for the leaflet with polymers (diamonds). The calculations are averaged over the final 100 ns of the trajectory.

(Figure S2). As the packing of the lipid tails is directly related to the total membrane area, the evident increase of the order parameter at high pressures and high lipid/polymer ratio could be linked to the lower areal strain that was observed for this system. Figure 6b reports the average deuterium order parameters ($\langle -S_{CD} \rangle$) (averaged over all the acyl carbon atoms) for the *HighHPL* as a function of the applied pressure and calculated separately for the polymer-rich and the polymer-free leaflet. It can be clearly seen that the polymers force the lipids to remain ordered and (as we will see later) prevent their lateral diffusion. The difference in lipid packing properties between the polymer-rich and the polymer-free leaflet, has implications on the corresponding leaflet thickness. The thickness of each leaflet is calculated as the distance between the maximum and the minimum of the DPPC density profiles (Figure S3) and is shown in Figure 7. Increasing external lateral pressure induces a significant thinning on the polymer-free leaflet, whereas the polymer-rich leaflet maintains an almost constant thickness irrespectively of the area expansion.

Effect of the L64 chains on the fluidity of the membrane

A major implication of the inclusion of the polymer in the lipid bilayer is the alteration of the membrane fluidity. The membrane fluidity is related to the lipid lateral diffusion (i.e., in the membrane plane) (D_{lip}) which can be quantified by the mean square displacement (MSD) of the lipid phosphate groups averaged over all the lipids and the final 200 ns of each trajectory (Figure S4). The (D_{lip}) can be calculated by employing the Einstein's relation:

$$D_{lip} = \lim_{t \rightarrow \infty} \frac{1}{2Dt} \langle \|\vec{r}(t) - \vec{r}(0)\|^2 \rangle \quad (5)$$

where D equals 2, as the calculation is restricted in two dimensions and $\vec{r}(t)$ is the position of the phosphorus atom after a time interval t . The diffusion coefficient is calculated from the slope of a least squares straight line fitted to the MSD curve in the region 20-180 ns. The D_{lip} in the pure DPPC and tension-free system is $8.1 \pm 0.4 \cdot 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$, values

Figure 7: Membrane leaflet thickness (H_L) versus the applied lateral pressures (P_L), for the *HighHPL* system, calculated separately for the leaflet with (diamonds) and without (squares) polymers. The thickness was calculated as the distance between the maximum (headgroup region) and the minimum (bilayer center) in the phosphate density profiles.

that compare well with the corresponding experimental data⁷¹ that lie within $1.5\text{--}6\cdot 10^{-12}\text{ m}^2\cdot\text{s}^{-1}$ and is in excellent agreement with previous computational values.⁴⁴ The lipid lateral diffusivity increases when the bilayer is expanded as can be seen in Figure 8a. The increased free area around the lipid molecules facilitates their translational movement since, according to the free-area theory,⁷² the lipids can translate laterally when there is adjacent free space that exceeds a critical value. However, the mobility of the DPPC molecules when tension is applied increases less abruptly in the case of the hybrid membranes. Especially, for the high polymer concentration, the lipid displacement seems to be highly restricted and increases only moderately with the lateral pressure. A similar behaviour has been observed computationally^{67,68} for lipid membranes adsorbing relatively small molecules such as cholesterol or fullerenes and experimentally for supported membranes with adsorbed macromolecules.^{73,74} In particular, Granick and co-workers^{73,74} using spatially resolved measurements noticed a bimodal distribution of the lipid diffusion coefficient corresponding to slow and rapid diffusion. The slow movement was attributed to polymer-rich domains, where the lipids were bound to the polymer chains which dictate the lipid mobility. The small system size of the simulations does not allow the observation of only polymer-rich domains, but since the polymers are localized mainly in one of the leaflets, the diffusivity can be calculated separately for the polymer-rich and polymer-free monolayers in the high lipid/polymer ratio system. Interestingly, Figure 8b clearly shows that the lipids within the hybrid membrane diffuse with different rates; the polymer-bound lipids remain "trapped" by the chains and diffuse slowly, even for large area expansions, whereas the polymer-free lipids move faster with diffusion coefficients that approach those of the pure bilayer. The second interesting finding from Figure 8b is that even when the bilayers are expanded, the lipid diffusion remains limited, despite the increase in the free area around them.

Figure 8: (a) Lipid lateral diffusion coefficients (D_{lip}) versus the applied lateral pressures (P_L), for all the DPPC/L64 ratios. (b) The lipid lateral diffusion coefficients (D_{lip}) for the HighHPL system, calculated for all the lipids (circles), for the leaflet without polymers (squares) and for the leaflet with polymers (diamonds).

L64 chains induce lipid flip-flops

The motion of lipids within bilayer structures is usually restricted in-plane for short time scales. The lipids can either move around their equilibrium position or diffuse laterally on the plane of the membrane when a void appears nearby due to thermal fluctuations.⁴⁴ A more rare event however, is the transverse movement of a lipid that manages to overcome the energy barrier imposed to the headgroup by the hydrophobic tail region and diffuse to the opposite leaflet. This type of movement is known as flip-flop and cannot be typically observed within the short time frame of atomistic simulations. However, experimental studies have revealed the ability of Pluronic® polymers, to disturb the internal structure of bilayers and facilitate this translational movement of lipids.²⁶ Our simulations reveal that the incorporation of Pluronic® chains within the membrane can indeed facilitate and accelerate lipid flip-flop. In the high polymer content system, at $P_L = -10$ bar, we observed two lipids from the polymer-rich bilayer to flip-flop to the polymer-free leaflet. Careful visualization of the trajectory revealed that the lipids are initially dragged to the tails region by a neighboring PEO block that also translocates deeper in the membrane.

The whole process begins after 150 ns and lasts until the end of the simulation at 500 ns (Figure 9) with the lipids occupying the space between the two leaflets most of the time and having lost their orientation along to the bilayer normal. The local bilayer structure is temporarily perturbed during the flip-flop movement of the lipids, but no water channel is generated. Flip-flops are also observed for the systems under high P_L , right before pore formation, when already the lipids have lost their ordering and the free volume is increased allowing them to move more freely. The flip-flop as investigated through experiments has shown to be correlated to the chemical structure of the incorporated polymers, with more hydrophobic Pluronic® having a significantly stronger effect.²⁶ The simulations though show that the flip-flop is feasible when the PEO enters the membrane hydrophobic region and pulls the lipids in its vicinity by interacting favourably with their headgroups, suggesting that the polar block of the copolymers determines the flip-flop process.

Figure 9: Time evolution of the distance on the z -axis of two lipids that flip-flop from the center of mass of the bilayer at $z=0$.

Pore Formation and Bilayer Rupture

When the applied pressure exceeds a threshold value, the bilayer surface area becomes too large and the membranes lose their cohesive structure and break. The critical values of the rupture tensions and the rupture areal strain for the studied systems were determined by the tension-areal strain curves (Figure 3a). The DPPC bilayer breaks when γ becomes 71.8 mN/m and the A_{lip} reaches $\sim 1.1 \text{ nm}^2$, which corresponds to $\epsilon_A=0.73$. The critical area per lipid is comparable to the results of Leontiadou *et al.*,⁶³ who find the A_{lip} of the rupture equal to 1.2 nm^2 . For the polymer-containing membranes, the critical γ increases to 73.4 mN/m and 75.8 mN/m for the *LowHPL* and the *HighHPL*, respectively, and the critical ϵ_A is between 0.70-0.77 without showing a trend relative to the polymer concentration.

To gain an insight into the pore formation mechanism and test the reproducibility of the stages that lead to the membrane rupture, the simulations at the P_{Lc} were repeated three times and the same observations were made. In all systems, prior to rupture, a pore is formed and water penetrates from both leaflets creating a channel. Immediately after the first water pore is formed, more pores appear in the bilayer and they expand rapidly leading to complete distortion of the membrane structure. Even though the stress-strain curves seem to indicate that the polymer content does not affect significantly the critical rupture point (i.e., γ is 2.4 mN/m higher for the *HighHPL* than for the *LowHPL*), a more careful look into the trajectories reveals two entirely different lysis mechanisms. For the *LowHPL* membrane, the pore appears in a region far from the location where the polymer chain is adsorbed (Figure 10a) and is caused by the increased free volume that enhances water permeability and allows the water molecules to enter the hydrophobic region of the bilayer from both leaflets, disrupting the membrane structure. The same mechanism is observed in the polymer-free membrane but in the case of the *LowHPL* membrane, the polymer chain plays the role of reducing the local lipid diffusivity and thus the local area fluctuations hindering the pore formation in its vicinity. For the *HighHPL* membrane, the pore is located close to the polymer and induced by it. At first, the PEO block of one of the chains manages to overcome the energy barrier

Figure 10: Side-view of the membranes after the water channels have been formed. (a) *LowHPL* bilayer and (b) *HighHPL* bilayer. For the color code refer to Fig. 2. For clarity, only the Phosphorus atoms of the lipids are shown.

and moves towards the bilayer center. This PEO translocation seems to perturb the local lipid bilayer conformation, as a few nearby lipids lose their orientation and move towards the opposite leaflet. During the PEO rearrangement and the lipid flip-flop, water is dragged to the hydrophobic region of the membrane resulting in the formation of a water channel (Figure 10b). The pore again expands quickly and the bilayer breaks, similarly to the other systems.

Figure 11: Water-occupied volume of the pores formed (V_{water}) as a function of time, for 5 ns before and after the pore formation. The water-occupied volume prior to pore formation corresponds to the water molecules in the headgroup region.

The dynamics of the pore growth are also affected by the polymer chains. The volume of water in the bilayer region (V_{water}) as a function of time is illustrated in Figure 11. The V_{water} was calculated by counting the number of water molecules in the region between the phosphate groups of the two leaflets and multiplying by the volume of each molecule, calcu-

lated considering the water atoms as spheres with radii equal to the Van der Waals radii. It can be seen that the rate of increase of the V_{water} differs for the three investigated systems. Immediately after the appearance of the first pore, the growth of the water channel follows a linear trend with the slope corresponding to the rate of growth (dV_{water}/dt). In the lipid membrane, the V_{water} increases by 161 nm³/ns, whereas for the *HighHPL* system, it is reduced to 89.5 nm³/ns. In the *LowHPL* bilayer, initially the rate is calculated at 93.2 nm³/ns, but less than 3 ns after the first pore, a second one is created, increasing the dV_{water}/dt to 119.6 nm³/ns. The slower diffusion of the lipids that are bound to the polymer chains is most likely the cause of the obstruction to the pore growth in the hybrid membranes and it reveals a potential capability of Pluronic® copolymers in stabilizing defects of lipid membranes, which will be the subject of a future study focused on the pore kinetics.

Conclusions

Our simulations have revealed that the incorporation of Pluronic® L64 chains in preformed lipid membranes can alter the membrane elastic properties under mechanical stress. The hybrid membranes resist the expansion of their surface area and the critical rupture tension increases from 71.8 mN/m (lipid bilayer) to 75.8 mN/m (*HighHPL* bilayer). The presence of the polymer chains hinder the lipids diffusion in their proximity and as a result, the lipids remain closely packed, even when the applied stress forces the membrane to expand and to increase its surface area. In the HPL membranes, the lipid tails show higher orientational order than the lipids in standard lipid membranes. This higher order of the fatty acid tails limits the thinning effect of the polymer-rich leaflets under stress, whereas the polymer-free leaflets are characterized by a decreased order and thickness. The increase in the compressibility modulus of the hybrid membranes suggests that they become stiffer and therefore mechanically stronger. The reduced lipid mobility and the increased stiffness are both pa-

rameters that imply reduced permeability of small molecules,⁶⁸ which is a desired property for the design of nanocarriers with low drug leakage during blood circulation. The presence of the L64 chains is also found to facilitate the trans-bilayer lipid motions (flip-flop), even at low lateral pressures, an event that is normally very rare (one flip-flop/24 h⁷⁵), verifying previous experimental observation.²⁶ Regarding the formation of water pores, two different mechanisms were identified for the two polymer concentrations, that were driven by the distributions of the chains within the bilayer. The rate of growth of these pores is found to be reduced by the presence of polymer chains.

Our results suggest that the incorporation of amphiphilic block copolymers such as Pluronic® increases the mechanical stability of lipid membranes even at small concentration and indicate that the passive adsorption of polymers in preformed liposomes might be enough to construct hybrid vesicles with improved mechanical properties. Although our simulations have been performed on a specific Pluronic® copolymer, the details of the molecular mechanism driving the change in the mechanical and structural properties of the membrane are valid for any other copolymer of the Pluronic® family and in general for any copolymer containing PEO blocks that can translocate within the membrane.

Acknowledgement

The authors thank the N8 HPC Centre of Excellence, coordinated by the Universities of Leeds and Manchester for the computing time. This work has been sponsored by the Leverhulme grant number RPG-2013-246.

Supporting Information Available

The following files are available free of charge.

- supportinginfo.pdf: Supplementary figures.

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