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DOI:
[10.1039/C9CP01293A](https://doi.org/10.1039/C9CP01293A)

Document Version
Accepted author manuscript

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

Citation for published version (APA):

Zaki, A. M., & Carbone, P. (2019). Amphiphilic copolymers change the nature of the ordered-to-disordered phase transition of lipid membranes from discontinuous to continuous. *Physical Chemistry Chemical Physics*, 21(25), 13746-13757. <https://doi.org/10.1039/C9CP01293A>

Published in:
Physical Chemistry Chemical Physics

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Cite this: DOI: 10.1039/xxxxxxxxxx

Amphiphilic copolymers change the nature of the ordered-to-disordered phase transition of lipid membranes from discontinuous to continuous[†]

Afroditi Maria Zaki*[‡] and Paola Carbone

Received Date

Accepted Date

DOI: 10.1039/xxxxxxxxxx

www.rsc.org/journalname

The phase behaviour as a function of temperature is explored for *pure* phospholipid (DPPC) and *hybrid* lipid-polymer (DPPC/Pluronic L64) bilayers with the aid of atomistic MD simulations. The range of the fixed-temperature simulations includes temperatures below and above the known melting temperature (T_m) for DPPC membranes. For the *pure* lipid bilayer, the main phase transition is discontinuous, as verified by the abrupt changes observed in the membrane structure, elasticity and the lipid diffusivity near the critical temperature T_m , which lies in the region 298.15–303.15 K. A pre-transition step is detected at 298.15 K which has been identified as the ripple phase (P'_β), where ordered and disordered lipids coexist, causing thickness fluctuations. In the ordered gel phase, the positional ordering as assessed by the lipid radial distribution functions is long-range and some degree of hexagonal packing is measured. The *hybrid* bilayers on the other hand, transform from a more ordered to a disordered phase in a continuous manner, without finite jumps in their properties. No signs of the ripple phase are identified and the ordered phase exhibits very limited hexagonal packing and some positional ordering that decays fast. The effect of the inserted polymers in the two phases is reversed; at low temperatures, they render the membrane thinner, less cohesive and less ordered compared to the *pure* one, with the lipids assuming faster diffusion rates, whereas at high temperatures, the polymer interaction with the lipids acts in reducing their diffusivity, but also they increase the lipid tail ordering and the membrane stiffness. The ability of the amphiphilic L64 copolymers to change the nature of the main phase transition of lipid membranes and their properties both in the ordered and the disordered phase is of vital importance for the prediction of both the efficacy of hybrid lipid/polymer nanoparticles as drug delivery vehicles as well as their potential adverse implications during interactions with healthy cell membranes.

1 Introduction

In the field of nanotechnology and nanomedicine, a plethora of studies has focused on the design and formulation of nanoparticles that will serve for therapeutic purposes, such as drug delivery.^{1–10} This concept is fascinating as it envisions nanocarriers that will selectively target cancer cells, interact

with their cell membranes and be triggered to release the encapsulated drug minimising the unsought side effects. One of the most promising approaches involves the construction of such nanoparticles from a combination of lipid molecules with synthetic polymers, with the aim to produce mechanically stable and non-toxic hybrid polymer-lipid (HPL) nanoparticles. Multiple combinations of the materials and the development techniques have been tested,^{6,8,11,12} but in the majority of studies the mechanical, structural and dynamic properties of the lipid L_α phase of these systems are usually investigated,⁸ as this is considered the biologically relevant phase. However, it has been found that the cell membranes contain domains of higher lipid order known as "rafts", as a result of the inhomogeneity induced by the presence of sterols.¹³ In analogy, such domains may also be formed in the synthetic hybrid polymer-lipid vesicles with yet unknown implications in their efficiency. Indeed, Nam

School of Chemical Engineering and Analytical Science, The University of Manchester, Oxford Road, Manchester M13 9PL, United Kingdom;

E-mail: afroditi.maria.zaki@manchester.ac.uk, paola.carbone@manchester.ac.uk

[†] Electronic Supplementary Information (ESI) available: [The chemical structures of the materials, partial density profiles of the *hybrid* membranes, polymer-lipid RDFs and tabulated DPPC areas per lipid, lipid diffusion coefficients and diffusion pre-exponential factors]. See DOI: 10.1039/cXCP00000x/

[‡] Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom;

E-mail: afroditi-maria.zaki@bioch.ox.ac.uk

et al. studied the temperature-induced demixing behaviour of HPL vesicles that leads to the formation of polymer-rich and lipid-rich nanodomains with impact on their drug-release properties.¹⁴ Besides the significance of the efficiency of the hybrid nanoparticles, the interaction of free polymer chains with the healthy mammalian cell membranes during a drug therapy is inevitable. The polymers that are not successively incorporated in the nanoparticles will freely diffuse in the blood stream and an interaction with the cell membranes must be predicted and well understood. For the above reasons, it is vital to further explore the behaviour of HPLs as a function of temperature.

The temperature-induced phase behaviour of lipid membranes has been studied both experimentally via NMR, fluorescence measurements and calorimetry on unilamellar and multilamellar vesicles^{15–17} and computationally^{18–24} with MC, MD and DPD studies on bilayers. At very low temperatures, the lipids are in the sub-gel phase (L_c), where they assume a solid-like behaviour, displaying high ordering and they are practically immobile. With increasing temperature, they transition into the gel phase, which possesses some different characteristics, depending on the type of lipid and is labelled as L'_β or L_β , depending on whether the hydrocarbon tails are tilted or not. At the gel phase, the lipids are positionally and orientationally ordered, but demonstrate some limited lateral mobility. When the temperature exceeds a critical value (T_m), they melt into liquid crystals (L_α phase); the positional and orientational order vanishes and the bilayers behave like fluids.¹⁹ The transition is mirrored in a change in structural properties, such as lipid tail packing, thickness and area per lipid.¹³ The sharp change in these properties and the hysteresis observed indicate that this transition is discontinuous (i.e., first order). Even though the L_α is well characterized, the gel phase and the actual transition process are less clearly understood.

Even though for three-dimensional systems the melting is a first-order transition, in two-dimensional systems it can be more complicated. It has been claimed that 2D melting involves an intermediate step with the system transitioning first from gel to a hexatic phase and then from a hexatic to a liquid crystalline phase.²⁵ Other studies have detected an intermediate ripple phase (P'_β) for some lipid types, which constitutes a coexistence phase of ordered and disordered lipids that leads to an anomalous swelling.^{19,26–28} Marrink et al.,²⁴ attempted a more detailed understanding of the gel formation mechanism on DPPC lipids, modelled with a coarse-grained representation, and described it as a four-step process, but at the same time rejected the hypothesis of a hexatic phase. More recently, Katira et al.²⁰ using the same model provided evidence of that the gel phase is in fact hexatic, as it displays short-range positional and quasi-long range orientational order, and they claimed that the melting in lipid membranes is a first-order hexatic-to-liquid transformation.

However, the lipid-containing membranes that are of interest in nanomedicine are rarely pure. The cell membranes contain numerous other molecules, such as proteins and sterols. Also, mixed systems are being tested for the development of drug delivery carriers, which are very often a combination of polymers

and lipids. These inserted molecules are expected to affect the phases and the phase transition mechanisms of the membranes. For example, it has been found that at temperatures above the T_m , sterols can induce a third phase, labelled as liquid-ordered phase (L_o) by forcing the surrounding lipid tails to become more ordered,^{13,23} whereas they perturb the acyl tail packing at low temperatures.²³ Interestingly, in concentrations higher than 60% of cholesterol, the phase transition vanishes, as no finite jump at the structural properties is observed.²³ Studies on the effect of polymers on the lipid phases are scarce. Thakkar et al. performing DPD simulations of polymer-grafted lipid bilayers observed a weakening of the transition and a shift toward lower temperatures with increasing polymer content.²⁹

The focus of this study is to investigate and comprehend the mechanisms of response to temperature of bilayers of different compositions; on the one hand, a *pure* lipid bilayer composed only by DPPC phospholipids (see Fig. S1(b)) and on the other hand a *hybrid* polymer-lipid bilayer, constructed by incorporating the amphiphilic L64 Pluronic copolymer (see Fig. S1(a)) into the DPPC membrane. Amphiphilic copolymers have been tested experimentally in conjunction with surfactants for drug delivery applications and have shown to improve the mechanical stability of the mixed vesicles.⁸ The L64 Pluronic in particular has demonstrated the ability to improve the drug entrapment efficiency and optimise the drug release rate in niosomes.³⁰ Moreover, due to the polymer's thermal sensitivity, the hybrid vesicles have shown the highest release rates in mild-hyperthermia temperatures (42 °C) and the lowest in storage temperatures (25 °C),³¹ a property that is desired for drug delivery systems. Here, by analysing structural, elastic and dynamic properties at both the low temperature and the high temperature region, we will attempt to clarify the phase transformation process of a DPPC membrane and then compare it to the transformation that the DPPC/L64 bilayer undergoes. In addition, we will discuss the different impact that is observed on the different membrane phases. The investigation of these systems under mechanical stress in the liquid crystalline phase, as explored in our previous work³² revealed that the L64 Pluronic copolymers make the L_α membranes stiffer, more ordered and less fluid but to our knowledge, no evidence exists on their effect on the lipid membrane gel phase.

2 Methods

2.1 System Set-up

Two systems were the subject of this work; a *pure* DPPC bilayer and a *hybrid* DPPC/L64 bilayer of approximately 12.7 wt% polymer mass fraction (the lipid/polymer ratio is calculated as DPPC/L64 7:1). For the representation of the lipid, the Berger et al.³³ force field was adopted. Even though this set of parameters has been developed for DPPC lipids in the liquid-crystalline phase, it has been shown to successively reproduce the properties of the gel phase as well and has been adopted in previous studies that focused on the investigation of the phase behaviour of DPPC bilayers.^{21,34} For the water, the single point charge SPC model³⁵

was employed, as the Berger lipid force field was developed in combination with this water model. For the L64 polymer, the unmodified GROMOS force field was used and DFT calculations were performed for the derivation of the partial charges. Further details on the model development and validation can be found in the original work by Nawaz et al.³⁶

First, a previously equilibrated DPPC lipid bilayer consisting of 128 lipids and 3655 water molecules was downloaded from <http://wcm.ualgary.ca/tieleman/downloads/dppc.itp>. The water molecules were eliminated and subsequently, the box was duplicated in the x - and y - dimensions in order to generate a bilayer of 512 lipids which was then placed in the center of a box of dimensions $13 \times 13 \times 10 \text{ nm}^3$. For the construction of the *hybrid* membrane, 12 L64 polymer chains were placed in the empty space above one of the leaflets. The chains were randomly distributed in order to occupy most of the xy - plane and effectively act as a coating for the lipid bilayer, in analogy to the preparation process of polymer-coated liposomes.³

Finally, the boxes were filled with approximately 33000 water molecules, which amounts to ~ 64 water molecules per lipid. This value is large enough¹⁷ to minimise the effects from of the periodic boundaries in the z -dimension that would otherwise lead the systems to behave as multilamellar, which are known to display different phase behaviours from their unilamellar analogues.^{21,37} This number of water molecules is higher than the equilibrium hydration level of the L'_β phase (11 molecules/lipid), but is not expected to affect the phase behaviour.^{34,38} An energy minimisation step was carried out with the steepest descent method and the systems were then equilibrated in an NVT and subsequently, in an NPT ensemble. During the equilibration, the polymers were positionally restrained and hence remained in the water phase. The water and the lipid molecules were instead allowed to reorganise and the target temperature and pressure were stabilised. The *hybrid* system was subjected to an additional NPT run of 100 ns, but this time the restraints were removed and the polymers rapidly translocated in the bilayer interior. A production run of 500 ns was performed for both membranes an $T=323.15 \text{ K}$ and $P=1 \text{ bar}$, to ensure adequate equilibration and to verify that the properties of at least the *pure* bilayer match the previously obtained values from experiments and simulations.

2.2 Simulations at fixed temperature

In order to explore the phase behaviour of the membranes, equilibrium MD simulations were carried out at fixed temperatures ranging from 278.15 K to 328.15 K with an increment of 5 K. This range includes temperatures above and below the phase transition of DPPC bilayers, as it has been experimentally observed that the main phase transition occurs at $\sim 315 \text{ K}$.³⁹ The starting points of these simulations were the equilibrated *pure* and *hybrid* membranes obtained as described in the previous paragraph. To erase any thermal history, the systems were annealed to 450.15 K with a rate of 2.5 K ns^{-1} and immediately cooled to the desired temperature each time with the same rate.

It should be noted that in following this procedure, the slow

disordered-to-ordered transition rates should be considered and the limitations of the short time and length scale of atomistic simulations may get in the way of accurately reproducing the phase changes. Coppock and Kindt⁴⁰ have addressed this problem by developing a stripe growth strategy in order to study the growth rates of the ordered lipid regions, therefore in this work we will interpret the observed ordered phase and the observed transition temperature with caution.

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 and pressure were controlled with the Nosé–Hoover thermostat^{41–43} and the Parrinello–Rahman barostat^{44,45} with time constants of 0.5 and 5 ps, respectively. Periodic boundary conditions (PBC) were applied in all three dimensions. The bonds were constrained using the P-LINCS algorithm.⁴⁶ The Particle-Mesh Ewald summation⁴⁷ was adopted for the treatment of the long range electrostatic interactions, as it produces the most reliable results for systems with charges.⁴⁸ 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. 1.5- μs long equilibrium MD runs were subsequently performed and were repeated twice to verify the reproducibility of the results. For the analysis, the final 100 ns–400 ns were used depending on the type of analysis. The MD simulations were performed using the GROMACS software package (version 4.6.5).⁴⁹ The VMD software (version 1.9)⁵⁰ was used for the visualisation of the systems and the MDAnalysis package^{51,52} was employed for the development of tailor-made analysis tools.

Table 1 Details of the fixed-temperature simulations

System	N_{DPPC}	N_{L64}	N_{Water}	$T \text{ [K]}$	Time [μs]
DPPC <i>pure</i>	512	0	32963	278.15, 283.15, 288.15, 293.15, 298.15, 303.15, 308.15, 313.15, 318.15, 323.15, 328.15	1.5
DPPC/L64 <i>hybrid</i>	512	12	32963	278.15, 283.15, 288.15, 293.15, 298.15, 303.15, 308.15, 313.15, 318.15, 323.15, 328.15	1.5

3 Results and Discussion

3.1 Polymer-lipid interactions

During the initial unrestrained NPT run at 323.15 K, the L64 polymer was inserted into the upper leaflet of the lipid membrane and a few of the chains overcame the energetic barrier and moved across to the lower leaflet. For the entire duration of all the simulations performed at the fixed temperatures, the polymer remained incorporated in the membrane and showed limited motion. The polymer chains' root mean square deviation (RMSD) (Fig. S2) captures the very slow polymer diffusion, especially at

the lower temperatures, and shows that the chain almost ceases to move after the first 100 ns of the simulations. The partial density profiles of the system components shown in Fig. S3 capture the location of the L64 chains and do not reveal significant differences among the various temperatures. In all cases, the two leaflets are characterized by an inhomogeneity caused by the unequal partition of the twelve L64 chains. The PEO blocks show a preference for the lipid headgroup region and are absent from the center of the bilayer, whereas the hydrophobic PPO segments penetrate deeper into the lipid tail region. The radial distribution functions of the lipid phosphorus atoms around the PEO and the PPO polymer moieties are illustrated in Fig. S4 and do not reveal a dependence of the polymer-lipid interaction on the temperature. They confirm however the more favourable PEO-lipid headgroup against the PPO-lipid headgroup interactions. Two main peaks are observed at distances 0.45 nm and 0.83 nm for the PEO centre of mass (CoM) and 0.45 and 0.85 for the PPO CoM, but there is higher probability for the phosphorus to be found at the distance that corresponds to the first peak from the PEO rather than the PPO group.

3.2 Pure and hybrid membrane phase behaviour

The response of the membranes to the applied temperature is mirrored in the changes that are observed in their structural properties. The area per lipid (A_{lip}) can be obtained easily only for the *pure* membranes and these values are included in Table S1 and compared against the experimental ones, as a measure of the ability of the force field to reproduce both the L_α and the L'_β phase of DPPC. The A_{lip} for the L_α phase at 323.15 K is in very good agreement with experiment,¹⁷ but slightly deviates for the L'_β phase at 293.15 K (experiment¹⁷: 0.48 nm², simulations: 0.53 nm²), probably due to the limitations of the Berger force field which does not allow for a perfectly-formed gel phase. To compare the *pure* and the *hybrid* system, the membrane areal strain ϵ_A , calculated as the relative change in the surface area with respect to the reference surface area A_0 at 278.15 K, is plotted against temperature in Fig. 1(a). In the *pure* bilayer and at low temperatures, no effect of the temperature is observed and the average surface area is constant. When the temperature exceeds 298.15 K, the increase in area rises to 12% and at 308.15 K, an increase of 17% is reached. In the high temperature region, the rate of areal change drops, but it continues to gradually increase as the membrane is heated. The *hybrid* bilayer expands instead in a continuous manner and a critical temperature cannot be specified. At high T , the adjustment of the area to the increasing temperature is more pronounced for the *pure* membrane, whereas the polymers hinder the expansion of the *hybrid* membrane, which remains below 15% even at the highest T .

The lipid hydrocarbon tails undergo conformational changes when the temperature increases, described by the fraction of *trans* dihedrals and the average order parameter ($\langle S_{CD} \rangle$) (Fig. 1(b) and 1(c)). In general, in the L'_β phase of saturated lipids, the *trans* conformation is preferable from an energetic point of view and very high percentages of the tails are expected to assume *trans* dihedral angles.²² In the L_α phase, the number of *gauche*

conformers grows as the tails regain their flexibility which gives space for the appearance of defects. A first-order transition between the L'_β and the L_α phase is accompanied by a sharp change in the fraction of *trans*.²¹ As seen in Fig. 1(b), in the *pure* lipid ordered phase, more than 90% of the dihedrals are *trans*, while this percentage drops to 82% at 303.15 K and continues to decrease at higher T . These values compare very well with previous computational studies.^{21,34} The acyl tails of the *hybrid* membranes do not assume *trans* conformations of equally high percentages in the low T region and the fraction slowly drops upon increasing the temperature. In the high T region, the two systems reach a similar fraction of *trans* conformers.

A comparison among the structural properties shown in Fig. 1, reveals that the conformational isomerism of the tail carbon atoms is the last property to respond to the phase transition, dropping abruptly by 6.3% at 303.15 K, whereas the other properties already demonstrate a slight change at 298.15 K.

Fig. 1 Membrane structural properties for the DPPC and the DPPC/L64 systems with respect to temperature. (a) Areal strain (ϵ_A), (b) fraction of *trans* dihedrals, (c) average deuterium order parameter ($\langle S_{CD} \rangle$) and (d) average phosphorus-phosphorus distance. The values correspond to the time averages of the final 100 ns of the trajectories. In plots (a) and (b) the standard errors of the mean are included but are too small to be visible, no errors are included in plot (c) and finally, the error bars in plot (d) correspond to the standard error of the mean.

Additionally to the fraction of *trans* conformers, the packing of the lipid acyl chains in a bilayer structure is also a well-studied property of the phase of the bilayer. It can be measured with the use of the deuterium order parameter (S_{CD}), which can be quantified via ²H NMR measurements⁵³ and also obtained from simulations. The details of the calculation of the order parameter can be found in our previous work.³² A mean value for S_{CD} is computed for the *sn*-1 tails of 512 lipids and the final 100 ns of the simulations.

At the ordered phase of the DPPC bilayer, the increased S_{CD} denotes that the lipid tails are highly aligned in parallel to the bilayer normal. The value of S_{CD} is maintained at a plateau and a sharp jump occurs first at 298.15 K and then at 303.15 K, where the tails become more disordered. On the contrary, this

change is more gradual for the DPPC/L64 bilayer and the lipid tails are less ordered in the low T region and more ordered in the high T region than the lipids of the DPPC membrane. This observation shows that the polymers hinder the alignment of the lipids in the gel-like phase, but in the liquid-crystalline phase they instead enable the lipid tails to self-organise in closely packed arrangements. This is the same effect that cholesterol induces in lipid membranes in the L_α phase, leading to the emergence of a new phase known as liquid-ordered phase (L_o). In this phase, the membrane is still fluid, but the lipids show increased ordering.²³ It is also interesting to look at the S_{CD} along the lipid tail and how it changes when moving towards the bilayer center. Fig. 2(a) clearly highlights the main phase transition for the *pure* membrane, as a gap is observed between the curves of S_{CD} in the gel-like and in the liquid-crystalline phase. At 298.15 K, the lipid tails seem to undergo a pre-transition step, during which they slightly lose their alignment, before it massively drops at 303.15 K. The distinction between the L'_β and the L_α phase is also apparent from the position of the maximum in the S_{CD} along the tail. In the L'_β phase, the middle part of the tail is highly ordered, in contrast to the L_α phase, where the fatty acid tails are more ordered near the headgroup region and the S_{CD} drops when moving towards the terminal carbon atoms. In the case of the *hybrid* system, no gap distinguishing between two phases is present and the tails slowly become more disoriented with increasing T . At all temperatures, the part of the tails close to the headgroup remains more aligned and this could be attributed to the polymer chains, and especially the presence of hydrophilic PEO segments, which are mostly located in that region (see Fig. S3). Their effect is two-fold; on one hand they disrupt the tight lipid packing at low temperatures reducing the S_{CD} and on the other hand, at high temperatures they favourably interact with the lipid headgroups, forcing them to remain ordered and affecting mainly the part of the tail that is in their vicinity.

The membrane thickness (Fig. 1(d)) is measured from the average distance of the phosphorus atoms of the two leaflets and it mirrors the change in lipid packing, as it follows the same behaviour as the S_{CD} . The two systems differ in the low T region, with the *pure* membrane being significantly thicker, but in the disordered phase, the thickness is similar for both systems. The increased tail orientation that is observed for the DPPC bilayer in the ordered phase in comparison to the DPPC/L64 bilayer explains the discrepancy in the thickness of the two systems. Similarly, in the disordered phase, the DPPC/L64 lipid tails are only slightly more ordered, without apparent effect on the thickness.

These finite jumps in the structural properties of the DPPC system signal a discontinuous transition from the disordered to the ordered phase. The melting temperature (T_m) lies in the region between 298.15 and 303.15 K, which is an underestimation of the experimentally obtained T_m for DPPC which is at ~ 315 K, but is in very good agreement with previous computational studies employing the same force field.^{21,34} It should be noted that the Berger force field was parameterised for lipids in the liquid-crystalline phase³³ and, despite the underestimation of the T_m , it has been proved that it can closely reproduce the

Fig. 2 Deuterium order parameter (S_{CD}) profiles for the *sn-1* lipid acyl tail at different temperatures. The carbon atom number increases toward the center of the bilayer. (a) DPPC and (b) DPPC/L64.

properties of the gel phase.²² In addition, the starting points of the simulations were fluid bilayers and, despite the long simulations performed that ensure proper equilibration, the disordered-to-ordered transition is slower and exhibits hysteresis. It has also been verified that it can be dependent on the cooling rate.^{34,40} Interestingly, the nature of the transition changes when the polymer chains are incorporated in the lipid membranes, as the membrane structure changes gradually with increasing T . This implies that the transformation is no longer discontinuous, but instead the membrane transitions between phases continuously. These two different behaviours are also mirrored in the system enthalpy curve (H) as a function of temperature, shown in Fig. 3(a). A small discontinuous jump is observed when the *pure* bilayer's temperature changes from 298.15 K to 303.15 K. This jump corresponds to the transition enthalpy and is absent in the respective curve for the *hybrid* bilayer. The discontinuity in the enthalpy of the *pure* system is reflected on the system heat capacity (C_p), which is dependent on the enthalpy fluctuations. In the *hybrid* membrane, the C_p is roughly maintained constant (within the errors), whereas a maximum in the *pure* membrane's C_p is detected near the transition temperature.

Similar thermodynamic calculations were performed by Waheed et al.²³ on cholesterol-containing lipid membranes subjected to simulated annealing. They found that the enthalpy discontinuity is prominent in cholesterol-free systems and is weakened with increasing cholesterol content until it vanishes for concentrations of 60%. Analogously, a maximum in C_p appears around the melting temperature, which also disappears for high cholesterol concentrations. Their interpretation of the results was similar; high amounts of cholesterol gradually change the lipid ordering and a clear first-order transition no longer takes place. Notably, our data indicate however that this effect on the nature of the main phase transition of the lipids occurs for much lower polymer concentrations.

Fig. 3 (a) The total enthalpy (H) and (b) the heat capacity (C_p) for the DPPC and the DPPC/L64 systems at all temperatures. The values are calculated via block averaging⁵⁴ from five independent blocks for the final 300 ns of the trajectories. The standard errors of the mean are included in the total enthalpy plot, but are too small to be visible.

3.3 Membrane positional and orientational ordering

For a more detailed analysis of the lipid phases, we looked at the radial distributions functions (RDFs) of the terminal carbon atoms of the $sn-1$ and the $sn-2$ lipid tails on the xy -plane (see Fig. S1(b)). The lateral RDFs are a means to investigate the degree of positional ordering of the lipids and therefore to characterize the phase of each membrane at varying temperatures. In Fig. 4, the RDFs for the lowest and the highest temperatures are presented. At the highest investigated temperature (328.15 K), the order decays at very short distances with only three clear peaks observed, an indication of the disordered fluid phase. The RDFs of the two bilayers are almost identical and the polymers seem to have zero effect on the structure of terminal carbon atoms in such high temperatures. At the lowest investigated temperature (278.15 K), the order is long-range extending up to ~ 6 nm for the DPPC and ~ 3.5 nm for the DPPC/L64 bilayer, but the amplitude of the peaks decreases with distance, as is expected for a gel phase.^{23,55} In this case however, the effect of the incorporated polymer chains is evident; the RDF of the DPPC/L64 system decays faster than the respective RDF of the DPPC system, demonstrating a disturbance of the lipid ordering due to the presence of the polymer. The peak heights of the RDFs of the two systems ($g_{max}(r)$) in the ordered phase can be fitted fairly well to a power law equation (Fig. 5):

$$g_{max}(r) = g_0 + \frac{1}{r^n} \quad (1)$$

where n corresponds to the rate of decay and is found to be 1.25 and 1.43 for the DPPC and the DPPC/L64 membrane, respectively, verifying the faster positional order decay in the presence of polymers. These values compare well to previous studies which reported n in the range 1–2, both with and without the addition of cholesterol.^{23,55}

The packing of the lipid tails can be visually assessed by snapshots of bilayer slices perpendicular to the z -axis and close to the centre of the membrane. Fig. 6 captures the packing of tail particles at four different temperatures and for both systems. At 278.15 and 293.15 K, a large portion of the xy -plane is occupied

by hexagonally packed particles in the *pure* lipid bilayer. Any perturbation from the hexagonal ordering can be attributed to the buckling of the membrane as can be seen from Fig. 9.

This curvature explains the less densely packed regions observed in Fig. 6. A degree of similar ordering is present in the *hybrid* systems at the same temperatures. However, the presence of polymer and in particular of the PPO segments, disrupts the local packing. It should be noted that apparently disordered regions of the slices that appear polymer-free in Fig. 6 are most likely affected by the polymer presence at different depths along the bilayer normal (not shown here). At 298.15 K, the *pure* bilayer demonstrates a mixture of hexagonally packed tails, observed in the middle of the xy -plane, surrounded by disordered lipid tails. The coexistence of two regions with different degrees of ordering can be explained by Fig. 9 and will be discussed later. The *hybrid* membrane at the same temperature seems to have lost its ordering and resembles a fluid structure. No signs of hexagonal packing are apparent at 303.15 K for both membranes, which have transitioned to the fluid phase.

The orientational ordering of the tail-end carbon atoms can be quantified by Halperin and Nelson's^{56,57} local rotational invariant (ϕ_l):

$$\phi_l = \left| \frac{1}{6} \sum_{j \in nn(l)} e^{6i\theta_{lj}} \right|^2 \quad (2)$$

where the angle θ_{lj} is computed between the vector connecting the atoms l and j and an arbitrary fixed axis and for each atom l only the six nearest neighbours ($nn(l)$) are considered. The calculation was performed for the tail end carbon atoms of one of the leaflets for the final 50 ns of the trajectories and the average ϕ_l is plotted against the temperature, as shown in Fig. 7.

For temperatures exceeding 303.15 K, both membranes show very limited hexagonal packing, with values of $\langle \phi_l \rangle$ of approximately 0.17, a value which is in excellent agreement with calculations from coarse-grained simulations of DPPC by Katira et al. ($\langle \phi_l \rangle \simeq 0.18$).²⁰ In the low temperature region, the degree of hexagonal packing is slightly elevated for the *hybrid* membrane, but the change is more abrupt and significant for the *pure* system,

Fig. 4 Radial distribution functions of the terminal carbon atoms of the *sn*-1 and *sn*-2 lipid tails for (a) the lowest (278.15 K) and (b) the highest (328.15 K) temperatures investigated.

Fig. 5 Peak heights of the RDFs of the DPPC and the DPPC/L64 membranes as a function of r , for the lowest investigated temperature (278.15 K), obtained from Fig. 4. The data points are fitted to curves of the form $1 + \frac{g_0}{r^n}$ (dashed lines).

which demonstrates a maximum of orientational ordering at 298.15 K, just prior to the phase transition. The values obtained here for the gel phase are quite lower in comparison to the data presented from Katira et al. who report a value of ~ 0.4 and this is due to the undulations formed in the bilayer at low temperatures in our work, which are not reproduced by the coarse-grained model. However, the overall behaviour of the rotational invariant is qualitatively similar for the DPPC bilayer and portrays the differences in hexagonal ordering between the lipid phases of the *pure* and the *hybrid* membranes, at the low T region.

The sideviews of the bilayers for selected temperatures shown in Fig. 9 allow for a more detailed description of the tail packing and the fluctuations in thickness. As a first observation, the hybrid membranes demonstrate reduced buckling in comparison

to the DPPC membranes. The buckling of the *pure* DPPC bilayers is the result of a mixture of ordered and disordered lipids or of the inter-penetration of the lipid tails of the two leaflets (see for example at 298.15 K). In DPPC bilayers, this has been observed as a pre-transition step and is known as the ripple phase (P'_β).^{19,28} It is more prominent at temperatures close to T_m , but at 278.15 K, the lipids are mainly cross-tilted, a structure characteristic of the DPPC gel phase.^{21,22} The ripple phase is observed for short phospholipids and it is hypothesized to be the result of a competition between the packing of the headgroups and the acyl tails.¹⁹ The phase transition can be quantified calculating the distribution of the average lipid acyl chain tilt angles (Fig. 8). Our simulations predict an angle between 23° to 26° for temperatures below 298.15 K, deviating from the experimentally value of $32^\circ \pm 0.5^\circ$.⁵⁸ At 298.15 K, the distribution is shifted towards increased angles, suggesting the co-existence of more ordered and less ordered lipid regions, also supported by the membrane sideviews of Fig. 9. Abruptly, at 303.15 K, the tilt angle distribution becomes wider around an angle of 37° , until the membrane has fully transitioned into the liquid-crystalline phase (angles higher than 40°).

In the *hybrid* systems, as the polymers are located in the interior of the bilayers and occupy the entire bilayer surface, they hinder the lipid inter-penetration and retain a constant thickness across the xy -plane, reducing the membrane curvature. At low temperatures, the lipid tails exhibit some local ordering, which however does not expand across the whole membrane, but is disrupted in the vicinity of polymer chains. At the highest temperature shown (303.15 K), the *pure* and the *hybrid* bilayers present similar degrees of tail ordering, with most tails having lost their orientation and small regions of some remaining well-packed lipids. The tilt angle distribution peak (Fig. 8) is calculated to be approximately 32° , for the lowest temperature; the average angle then gradually increases to up to 44° as the lipids become more disordered. If compared to the *pure* lipid

Fig. 6 Effect of the polymer on the hexagonal lipid tail packing in 278.15 K, 293.15 K, 298.15 K and 303.15 K. The snapshots show slices of the bilayer centres of width equal to the diameter of the unified carbon atoms of the acyl tails (0.4 nm). *Upper Panel:* Pure lipid membranes; *Lower panel:* Hybrid polymer-lipid membranes. Only the tails of the lipids are shown, for clarity. Colour code: lipid tails in blue; polymer PEO blocks in green and polymer PPO blocks in red.

Fig. 7 Average local rotational invariant ($\langle\langle\phi_i\rangle\rangle$) as a function of temperature for the *pure* and the *hybrid* bilayers. The calculation has been performed on the tail-end carbon atoms of the *sn*-1 and the *sn*-2 tails. The error bars show the standard deviation of the mean computed for the final 50 ns of the trajectories.

membranes, it can be seen that even at the lowest temperatures, the lipids of the *hybrid* membranes do not assume similar tilt angles as those in the DPPC bilayer, demonstrating a resistance to the formation of a similar gel-like phase. Instead, the increase in the average chain tilt angles suggests a gradual transition into a more disordered lipid phase upon heating.

3.4 Membrane elasticity and fluidity

Besides the structure of a membrane that undergoes a phase transition, its elastic and dynamic properties are also affected. The membrane elasticity can be quantified using the area compressibility modulus (K_A) which is shown in Fig. 10 and can be obtained from the area fluctuations:

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

The compressibility modulus of the *pure* membrane spans two orders of magnitude after dropping significantly near the phase transition temperature. At 303.15 K, a minimum in K_A appears, indicative of the massive decrease of the membrane area and of high area fluctuations. Qualitatively, this behaviour agrees with the K_A curve obtained by Waheed et al.²³ from annealing simulations, but the minimum they observed was more pronounced. However, our values of K_A in the L_α phase are in better agreement with the experimental value obtained by Nagle et al.¹⁷ In the DPPC gel phase, the large values of K_A indicate a very stiff bilayer, which becomes more flexible once it transforms into the fluid phase. The elasticity of the *hybrid* membrane instead increases gradually with temperature, presenting no signs of a clear phase transition, demonstrating a behaviour equivalent to that of cholesterol-containing bilayers of high concentration.²³ The presence of polymers renders the *hybrid* membrane less stiff than the *pure* one in the low T region and stiffer in the high T region, showing that the polymer effect on the elasticity of the ordered and the disordered phase is reversed.

Fig. 8 Distribution of the lipid tail tilt angles calculated for both the *sn*-1 and the *sn*-2 tails for the final 100 ns of the trajectories. The angles are computed between the *z*-axis and the vectors formed by every other carbon atom on each tail. (a) DPPC bilayer, (b) DPPC/L64 bilayer.

A lipid bilayer is a fluid object and even though the lipids rarely manage to display any transverse motion towards the opposite leaflet, they are able to move laterally. This lateral mobility is observed even when the lipids are in the gel phase, where the rate of motion is reduced by up to two orders of magnitude,²⁴ but not eliminated. The fluidity of the lipid membranes can be measured from the lipid diffusion coefficient (D_{lip}), which according to Einstein's relation can be linked to the lipid mean square displacement (MSD) for long time scales, as shown in eqn (4):

$$D_{lip} = \lim_{t \rightarrow \infty} \frac{1}{2\mathcal{D}t} \langle \|\vec{r}(t) - \vec{r}(0)\|^2 \rangle \quad (4)$$

where $\mathcal{D}$ is the dimensionality of the diffusion which takes the value of 2 in the case of lateral (two-dimensional) movement and $\vec{r}(t)$ is the atom position at time t . At large t , the lipid motion becomes Brownian and the MSD curve is linear. The D_{lip} can then be obtained from the slope of the MSD. The D_{lip} values should be interpreted with caution, as it has been predicted that the PBC affect the computationally obtained diffusion coefficients, which are normally smaller than the experimental ones by approximately a factor of 3.⁵⁹ This fact however still allows for a comparison between the polymer-free and the polymer-rich systems. The MSD was calculated for the *sn*-1 and the *sn*-2 tail terminal carbon atoms and averaged over all the 512 system particles and the final 100 ns of the trajectories. The selection of the terminal atoms of the lipid tails was based on the fact that in the gel phase, the headgroups remain quite fluid whereas the tails are packed more tightly and their fluidity is highly affected. The lipid diffusivity follows an Arrhenius-like behaviour (Fig. 11), described by eqn (5):

$$D_{lip} = D_{lip0} e^{\left(-\frac{E_D}{RT}\right)} \quad (5)$$

where D_{lip0} is a pre-exponential factor, E_D is the activation energy for the lipid lateral diffusion and R is the gas constant. In the *pure* membrane, three distinct regions of lipid diffusivity can be identified. The lipids display limited mobility in the low T region (278.15-298.15 K), that ranges from $\sim 0.14 \cdot 10^{-12}$

to $0.19 \cdot 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$. In the coexistence phase from 298.15 K to 303.15 K, the D_{lip} abruptly increases by approximately 18 times and finally, in the high T region (303.15-328.15 K) the lipids are in the liquid-crystalline phase and exhibit higher rates of mobility, with diffusion coefficients that range from $2.5 \cdot 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$ to $12.4 \cdot 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$ and compare very well with previous experimental and computational measurements of DPPC membranes in the L_a phase.^{39,60-62} The diffusivity of the lipids of the *hybrid* system is significantly affected by the presence of the polymer. In this case, the data points can be fitted to only one Arrhenius curve, whereas a coexistence phase is missing and the D_{lip} changes gradually with increasing T . This is another indication of the different natures of the phase transformation of the two systems, as the absence of evidence for phase coexistence in the *hybrid* bilayer reasonably leads to the assumption of a continuous phase transition.⁶³ The D_{lip} ranges from $0.38 \cdot 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$ at the lowest temperature to $4.61 \cdot 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$ at the highest temperature. The values of D_{lip} can be found in Table S2 in the Supplementary Information.

The impact of the polymers on the lipid mobility is different in the ordered phases in the low T region and the disordered phases in the high T region. In the ordered phase, the *hybrid* bilayer lipids diffuse faster, a result that most likely stems from the defects that arise due to the disruption that the polymers cause to the lipid tail packing properties, as has been discussed previously, and create adequate adjacent free volume to allow for lateral diffusion.⁶⁴ On the other hand, the lipids of the *pure* lipid ordered phase are closely packed and aligned, displaying limited movement. Interestingly, the reversed behaviour is observed in the disordered phase, where the polymers hinder the fast lipid motion and hence reduce the fluidity of the membrane.

From the slopes of the Arrhenius curves of each region for both the DPPC and the DPPC/L64 systems, the diffusion activation energies (E_D) can be computed (eqn (5)) and are given in Table 2. Comparing the two phases of the *pure* membrane, it is clear that the energy barrier for diffusion is higher in the L_α phase ($44.8 \text{ kJ} \cdot \text{mol}^{-1}$ vs $4.0 \text{ kJ} \cdot \text{mol}^{-1}$ in the L'_β phase), which is rather unexpected and could be attributed to the increased dependence

Fig. 9 Sideviews of the *pure* (upper panels) and the *hybrid* (lower panels) membranes at (a) 278.15 K, (b) 293.15 K, (c) 298.15 K and (d) 303.15 K, for both the *xz*– and the *yz*–planes. Colour code: lipid tails in blue; polymer PEO blocks in green and polymer PPO blocks in red.

Fig. 10 The area compressibility modulus (K_A) versus the temperature for the DPPC and the DPPC/L64 membranes. The values were calculated via block averaging of four independent blocks of 100 ns each, for the final 400 ns of the simulations. The errors bars correspond to the standard errors of the mean.

of the diffusion on temperature in the disordered phase as opposed to the ordered phase, where the temperature-dependence of D_{lip} is limited.⁶⁵ Also, the pre-exponential factors D_{lip0} (shown in Table S3) are much larger for the disordered phases showing higher frequency of attempts for diffusion,⁶⁵ which is reasonable and correlates with the larger diffusion coefficients of the L_a phase.

Fig. 11 Arrhenius plot of the logarithm of the lateral diffusion coefficients $\ln(D_{lip})$ versus the inverse temperature T^{-1} for both the DPPC and the DPPC/L64 systems. The average D_{lip} values were calculated from the mean square displacements (*MSD*) of the lipid tails terminal carbon atoms.

Table 2 Diffusion activation energies (E_D) of the lipids in the DPPC and the DPPC/L64 membranes in the regions of low temperature and high temperature

	DPPC Low T Region	DPPC High T Region	DPPC/L64
E_D [kJ·mol ⁻¹]	4.0	44.8	40.3

4 Conclusions

In this work, we explored the underlying phase transition mechanisms for lipid and mixed polymer-lipid bilayers as a

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function of temperature. The significance of the understanding of the systems' behaviours is related to their potential applications in nanomedicine and dictated by the requirements of proper nanoparticle preparation and storage protocols, efficiency and reduced side effects. The computational study of phase transitions entails large time and size scales to ensure the proper equilibration but also large enough membrane patches that allow for the phase description. The simulations that we performed are a good compromise between the large scales required and the highest possible molecular detail. The *pure* DPPC membrane, unsurprisingly, exhibited a behaviour that implies a first-order phase transition, since it was associated with sharp changes in the structural, elastic and dynamic properties. The phase transition temperature was identified in the region between 298.15 K and 303.15 K, which is a known underestimation of the T_m , as revealed from experiments.¹⁷ The data point to a two-step transition, which involves a pre-melting step at 298.15 K with the formation of the ripple phase (P'_β), where the lipid tails start losing their orientation and the bilayer is composed by a mixture of well-ordered and disordered lipids, leading to fluctuations in thickness, an increase to the elasticity, but no reduction in the all-*trans* conformation of the tail carbon atoms and no significant change in the dynamics.

On the other hand, the results obtained for the *hybrid* DPPC/L64 membrane showed a different behaviour. The bilayer properties in this case undergo a gradual change, rather than a sudden drastic jump, an indication of a phase transition of higher order. There is no clear transformation from the ordered to the disordered phase at a specific temperature and no indications of formation of a ripple phase. The *hybrid* membranes in the low T region are thinner and less stiff, show reduced positional and orientational ordering, the tails are less oriented and exhibit faster dynamics in comparison to the *pure* membranes. The impact of the polymers is reversed in the high T region, where the L64 chains slow down the lipid dynamics, induce higher tail ordering and render the membrane stiffer. Notably, the effect of the polymers is more pronounced in the ordered phase. We conclude that the L64 Pluronic copolymers, even at as low mass fraction as 12.7 %wt, have the ability to change the nature of the main transition from an ordered to a disordered phase. A similar effect has been documented for cholesterol-containing membranes, with the cholesterol weakening the first-order transition until it is no longer observed for concentrations exceeding 60%.^{23,55} This continuous temperature-dependent behaviour seen in the presence of the polymer may be related to a combination of kinetic and thermodynamic effects.

The varying effects of the incorporation of Pluronic chains into DPPC membranes at the different phases might prove to be of great significance for the preparation, storage and activity of polymer-lipid vesicles, where the temperatures may vary, but also for the interaction of the polymers with healthy cells. We have shown in our previous work³² that the Pluronic copolymers can improve the mechanical properties of the lipid membranes under stress in the fluid phase, but the present work predicts that the hybrid vesicles' properties are also temperature-dependent for a physiologically-relevant range of temperatures and this factor

should be accounted for during the preparation, storage and blood circulation steps of the drug delivery process.

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