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Contribution of headgroup and chain length of glycerophospholipids to thermal stability and permeability of liposomes loaded with calcein

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ABSTRACT

Biological membranes are complex systems that are composed of lipids, proteins and carbohydrates. They are difficult to study, so it is established practice to use lipid vesicles that consist of closed 'shells' of phospholipid bilayers as model systems to study various functional and structural aspects of lipid organisation. To define the effects of the structural properties of lipid vesicles on their phase behaviour, we investigated their headgroup and chain length, and the chemical bonds by which their acyl chains are attached to the glycerol moiety of glycerophospholipid species, in terms of phase transition temperature, enthalpy change and calcein permeability. We used differential scanning calorimetry to measure the temperature and enthalpy changes of phase transition, and fluorescence to follow calcein release through the bilayer structure. Our data show that longer acyl chains increase the stability of the lipid bilayers, whereas higher salt concentrations decrease the thermal stability and widen the phase transitions of these lipid bilayers. We discuss the possible reasons for the observed phase transition behaviour.

1. Introduction

Lipids are one of the major constituents of cell membranes. As well as their structural role, lipid membranes have biological roles, in terms of modulation of the other constituents of the membranes (*e.g.*, proteins) (Lee, 2004; Marsh, 2008; Yeagle, 2014) and the physicochemical properties of the membranes themselves (Galvagnion et al., 2016; Haque et al., 2001). In general, the membrane lipids consist of two regions: the polar headgroup, onto which the one or two hydrocarbon (acyl) chains are attached. The headgroup can carry a net charge, and the acyl chains can be either saturated or unsaturated, and they give the lipid molecule its hydrophobic character.

This dual nature of lipid molecules enables the spontaneous formation of bilayers in aqueous solutions, which can be either planar or vesicular, whereby the hydrophobic carbon chains are hidden on the inside and are thus 'protected' by the polar headgroups that face the aqueous solution (Lamond, 2002). The structure of the lipids determines their physicochemical properties (*e.g.*, phase transition temperature, membrane curvature), and consequently influences their interactions with the other constituents in the system.

One approach in understanding the structural effects of lipids on the properties of a lipid bilayer is to study the phase transitions of the lipids from the gel phase (L_β) – an ordered state – to the liquid crystalline phase (L_α) – a disordered state. In addition to the main transition, there is an interconversion between two different gel phases. This interconversion is called the 'pretransition' and it involves transformation of the L_β phase to the rippled gel phase (P_β) (Koynova and Caffrey, 1998). In the gel phase, the lipid molecules do not show much lateral motion as all of the hydrocarbon chains are in the *trans* conformation, which leads to a very closely packed structure. As the temperature rises, the lipids move into the liquid crystalline phase, where some carbons on the hydrocarbon chains switch to the *cis* conformation. This can lead to 'kinks' in the acyl chain structure, which can cause the bilayer to lose its tightly packed structure, and thus to become more liquid (Akabori and Nagle, 2015; Huang and Li, 1999; Leekumjorn and Sum, 2007; Suurkuusk et al., 1976).

As biological membranes are a mixture of different lipids, it is difficult to study the structural effects of isolated membrane lipids on the membrane properties, as the biological membranes have a cumulative effect due to all of their constituents (Baoukina et al., 2017; Haque et al., 2001; Nickels et al., 2017). Model membranes offer much more controlled settings, especially as lipid vesicles can be made from only one lipid species. Therefore, the selected parameters to be investigated can be evaluated in a much more straightforward way, and in comparison with other varieties of lipids determined in separate experiments (Eeman and Deleu, 2010).

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Such model membranes can also be used to study extremophiles – microorganisms that can survive under extreme conditions in habitants such as hot springs and salt lakes (Rothschild and Mancinelli, 2001). Understanding the contributions of individual structural factors to the stability of extremophiles is important, because these microorganisms have the potential to enter into large-scale use in the field of biotechnology (Coker, 2016).

Synthetic glycerophospholipids can be used as model membranes to study the bilayer stability, water permeability (Mathai et al., 2008) and salt tolerance of biological membranes. Comparisons of ether linkages *versus* ester linkages is also of special interest, because this choice represents a well-localised chemical perturbation that affects the physical properties of the membranes (Guler et al., 2009). It has been reported that ether-linked lipids are more stable than ester-linked lipids (Baba et al., 1999; Balleza et al., 2014; Gmajner and Ulrih, 2011). Indeed, the glycerol ether lipids are one of the most remarkable features that distinguish members of Archaea from those of Bacteria and Eucarya (Phillips et al., 1970). In Bacteria and Eucarya, the membrane phospholipids feature a *sn*-glycerol-3-phosphate scaffold, which is ester linked to the fatty acid (acyl) chains. In contrast, archaeal lipids feature the 'opposite' glycerol stereoisometry, as *sn*-glycerol 1-phosphate, with isoprenoid groups connected *via*ether linkages (Pakchung et al., 2006; Pereto et al., 2004; Ulrih et al., 2009).

With the aim to better understand the molecular mechanisms of adaptation of Archaea to extreme environmental conditions, we investigated the effects of their structural properties on their phase behaviour (*e.g.*, phase transition temperature), in terms of headgroup and chain length, and chemical bonds by which of the acyl chains are attached to the glycerol moiety of the glycerophospholipid species. Subsequently, the stability of the lipid vesicles and the enthalpy of their transitions were determined using differential scanning calorimetry (DSC) (Ali et al., 2000; Gmajner and Ulrih, 2011; Lewis et al., 1996). Additionally, we followed the release of the fluorescent probe calcein over a selected temperature range using emission fluorescence spectrometry (Gmajner et al., 2011a, 2011b; Maherani et al., 2013; Patel et al., 2009). The effects of ionic strength on the transition temperatures and enthalpy of the transition and on the release of calcein from vesicles composed from different lipids was also investigated. The contributions of these individual structural factors on the thermal stability and permeability of the lipid membranes are discussed.

2. Materials and methods

2.1. Materials

The following lipids were purchased in powder form from Avanti Polar Lipids Inc. (Alabama, USA): 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC); 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE); 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-*myo*-inositol) ammonium salt (DPPI); and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt (DPPG). The structures of these lipids are shown in Scheme 1. NaCl, NaOH, chloroform, ethanol and methanol were from Merck KGaA (Damstadt, Germany), and 4-(2-hydroxyethyl)-1-piperasinethanesulphonic acid (HEPES) and calcein were from Sigma Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Preparation of liposomes

Multilamellar liposomes were prepared with DPPC, DSPC, DBPC, DHPC, DPPE, DPPI and DPPG by the thin-layer method (Lasic, 1993). Briefly, 10 mg lipids were dissolved as stock solutions in either 1 mL chloroform (DPPC, DSPC, DBPC, DHPC) or 1 mL chloroform: methanol (7:3; v/v; DPPE, DPPI, DPPG). The liposome aqueous suspensions were prepared by transferring 1 mL phospholipids stock solution into rotary flasks. First, the organic solvents were completely evaporated off under reduced pressure (17 mbar), to provide a thin phospholipid film on the wall of the flasks. These lipid films were hydrated by adding 2 mL 20 mM HEPES or 2 mL 50 mM calcein without or with NaCl (0–500 mM), with the mixtures shaken for 2 h above the phase transition temperature of the phospholipids (Šentjurc et al., 1999). The final lipid concentration was 0.5 mg/mL (c (DPPC) = 0.68 mM, c (DSPC) = 0.63 mM, c (DBPC) = 0.55 mM, c (DHPC) = 0.71 mM, c (DPPE) = 0.72 mM, c (DPPI) = 0.60 mM, c (DPPG) = 0.67 mM). The pH of all solutions was 7.0, except the pH of DPPE, which was adjusted to 8.0. These liposomes were in the form of multilamellar vesicles (MLVs) as proved before (Gmajner et al., 2011a, 2011b), and they were used for the DSC measurements. For the fluorescence emission spectrometry, the MLVs were converted into large unilamellar vesicles (LUVs) using the extrusion technique. Here, after six freeze–thaw cycles in liquid nitrogen, the MLVs were extruded 21 times through a 100-nm polycarbonate membrane using a mini extruder (Avanti Polar Lipids, Alabaster, Alabama, USA).

2.3. Determination of phospholipid concentration

Phospholipid C assays were used for quantitative determination of phospholipid concentrations *in-vitro* (Wako Pure Chemical Industries Ltd., Japan). This assay is an enzymatic method that uses choline oxidase in a reaction that can be measured spectrophotometrically.

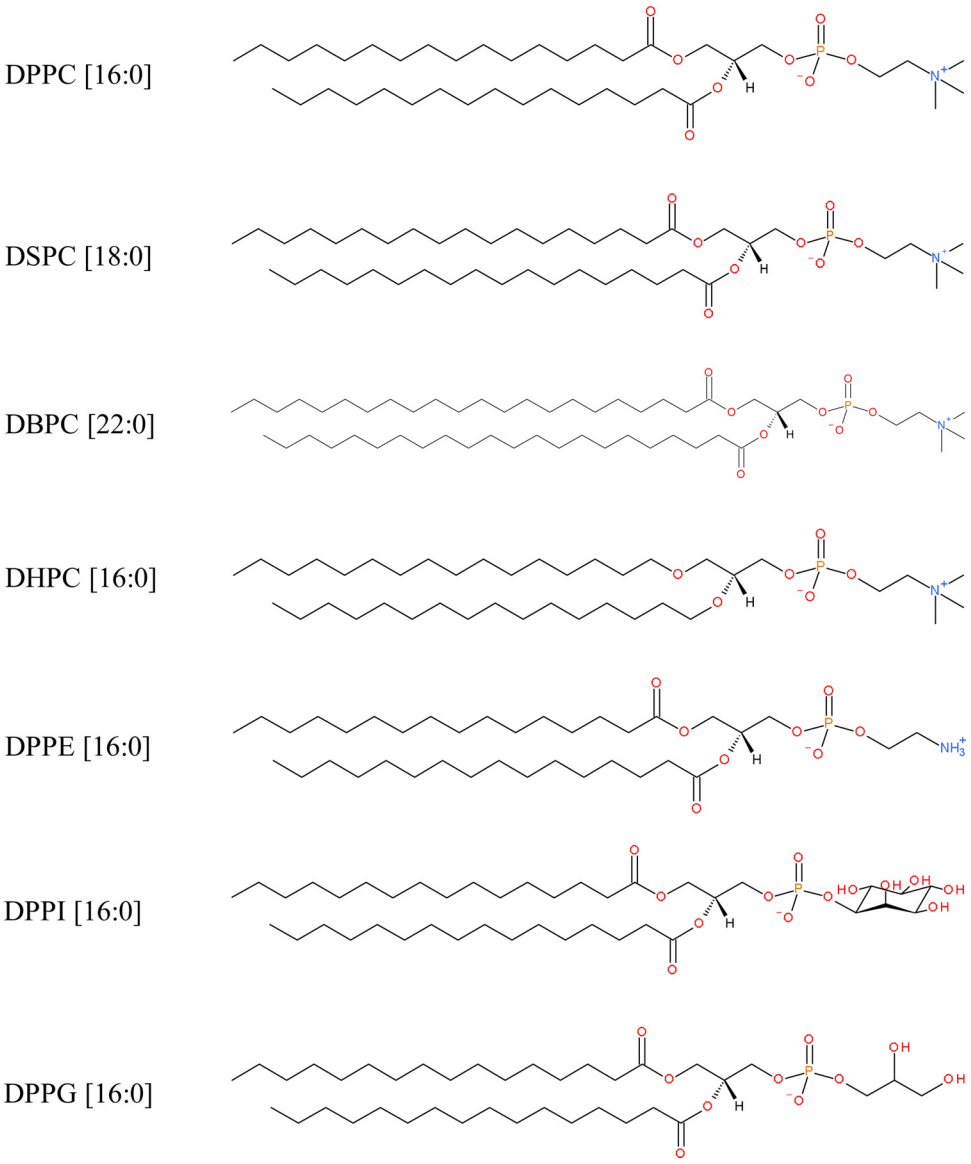
2.4. Differential scanning calorimetry

Differential scanning calorimetry is a very useful technique to investigate heat-induced changes in lipid structures. The gel-to-liquid crystalline phase transitions of the phospholipids were monitored using a NANO DSC series III system (Calorimetry Science, Provo, USA). The concentration of all lipids was 0.5 mg/ml (c (DPPC) = 0.68 mM, c (DSPC) = 0.63 mM, c (DBPC) = 0.55 mM, c (DHPC) = 0.71 mM, c (DPPE) = 0.72 mM, c (DPPI) = 0.60 mM, c (DPPG) = 0.67 mM). The phase transitions of the MLVs prepared from DHPC, DPPC, DSPC, DBPC, DPPI, DPPG and DPPE were monitored at pH 7.0 and 8.0, in 20 mM HEPES with 0, 50, 100 and 500 mM NaCl. The reference cell was filled with 20 mM HEPES buffer at pH 7.0 or pH 8.0 without or with NaCl. Before measurements, the samples were degassed under vacuum and then loaded into the calorimetric cell. Each sample was heated/cooled repeatedly in the temperature range from 10 °C to 70 °C and to 90 °C for DBPC and DPPE, at a heating rate of 1 °C/min. The first DSC scan was used to determine the temperature of the gel-to-liquid-crystalline phase transition (T_m) of the lipids, and the model-independent calorimetric enthalpy (ΔH_{cal}). The subsequent scans were used to determine the reversibility of the lipid-phase transitions. The NanoAnalyse software (TA Instruments, USA) was used to evaluate the enthalpies and temperatures of the transitions from the DSC thermograms.

2.5. Fluorescence emission intensity of released calcein

The calcein-loaded LUVs prepared from different lipids were used to determine the vesicle permeability as a function of temperature and NaCl concentration, as changes in the fluorescence intensity followed using a fluorescence spectrophotometer (Cary Eclipse; Varian, Mulgrave, Australia). Calcein is a small fluorophore that can be released from vesicles due to lipid bilayer relaxation (Butterfield and Lashuel, 2010).

The LUVs filled with calcein were prepared by hydrating the dried lipids with 50 mM calcein (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 20 mM HEPES, pH 7.0 (8.0 for DPPE) containing 0, 50, 100 or 500 mM NaCl. Gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) columns was used to remove the extra-vesicular calcein. The calcein released from the LUVs was assayed



Scheme 1. Chemical structures of the glycerophospholipids used in the present study.

using a fluorescence spectrophotometer. The temperature-dependent release of calcein from DHPC, DPPC, DSPC, DBPC, DPPI and DPPG LUVs was studied for all lipids in the temperature range of 20 °C to 90 °C at pH 7.0, except for DPPE at pH 8.0. The heating rate was 1 °C/min. The concentration of lipids used for the calcein release was 0.023 mg/mL (c (DPPC) = 31 μM, c (DSPC) = 29 μM, c (DBPC) = 25 μM, c (DHPC) = 33 μM, c (DPPE) = 33 μM, c (DPPI) = 28 μM, c (DPPG) = 31 μM). The intrinsic emission fluorescence of the calcein was measured at 515 nm, with an excitation wavelength of 495 nm; a 5 nm excitation and emission slit width was used. The calcein levels released from the LUVs were calculated according to Eq. (1):

$$\text{Calcein release (\%)} = (F - F_{min})/ (F_{min} - F_{min}) \times 100 \quad (1)$$

where F is the fluorescence emission intensity (measured at the different temperatures), F_{min} (base line) is the minimal fluorescence of free calcein giving the same emission intensity as the LUVs filled with calcein at the start of each experiment, and F_{max} is the maximal fluorescence of calcein obtained after addition of 10 μL 16 mM Triton X-100 (Sigma-Aldric Chemie GmbH, Steinheim, Germany) to the phospholipid solution, either at the beginning or at the end of the experiment.

3. Results and discussion

3.1. Differential scanning calorimetry measurements

The temperature-induced disruption of MLVs composed of the different phospholipids was investigated using DSC. Fig. 1 shows the DSC melting profiles of MLVs in the absence and presence of the selected NaCl concentrations (0, 50, 100, 500 mM). DSC thermograms were used to calculate the thermodynamic parameters of enthalpy and temperature of the phase transitions of the pure phospholipid MLVs. The pretransition peak in DSC thermogram is energetically small and it is difficult to measure it accuratelly. It is influened by NaCl and other compounds which interacts with polar head groups of lipids. Due to the difficulty to measure it in the presence of NaCl, it is not shown it in DSC thermograms.

The results of thermodynamic analysis are summarised in Table 1.

For DSC measurements MLVs have been used. The determination of enthalpy of the transition of lipids for MLVs is more precise than for the LUV or SUV. As reported in literature, DSC results obtained with SUV made from DPPC are rather difficult and complicated for interpretation. The main reason is that gel-phase SUVs have tendency to fuse into

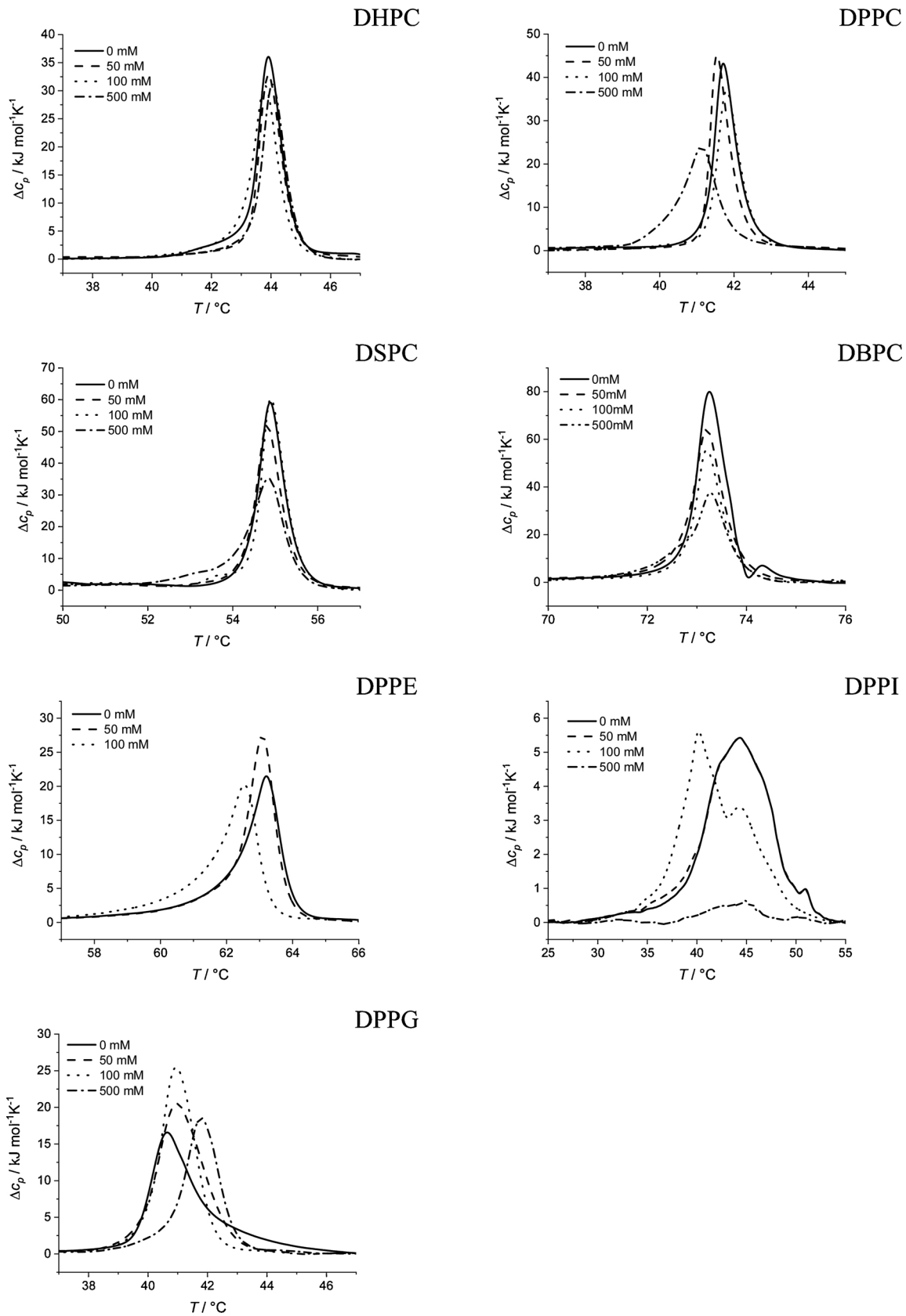


Fig. 1. Differential scanning calorimetry profiles for NaCl effects on the phase transitions of the different multilamellar vesicles (as indicated).

larger vesicles (Biltonen and Lichtenberg, 1993). In fact, according to literature, upon heating SUVs (T_m = 37 °C) are in coexistence with large unilamellar vesicles, LUVs (T_m = 41 °C). When the SUVs were incubated for sufficiently long periods of time below T_m, essentially all of them fused into LUVs which exhibited a melting temperature of about 41 °C and an enthalpy change that is close to that observed for the

Table 1
Thermodynamic profiles of the phase transitions in terms of the NaCl effects on the different multilamellar vesicles.

LUV lipid	NaCl (mM)	Gel-to-liquid-crystalline phase transition (T_m; °C)	Calorimetric enthalpy (ΔH; kJ/mol)
DHPC	0	43.9 ± 0.5	47.1 ± 2.4
	50	43.9 ± 0.5	38.5 ± 1.9
	100	43.8 ± 0.5	38.4 ± 1.9
	500	44.0 ± 0.5	35.6 ± 1.8
DPPC	0	41.7 ± 0.5	37.6 ± 1.9
	50	41.5 ± 0.5	34.3 ± 1.7
	100	41.8 ± 0.5	30.7 ± 1.5
	500	41.2 ± 0.5	32.8 ± 1.6
DSPC	0	54.9 ± 0.5	51.8 ± 2.6
	50	54.8 ± 0.5	46.1 ± 2.3
	100	54.9 ± 0.5	48.3 ± 2.4
	500	54.9 ± 0.5	42.9 ± 2.1
DBPC	0	73.2 ± 0.5	61.5 ± 3.1
	50	73.2 ± 0.5	57.5 ± 2.9
	100	73.2 ± 0.5	44.2 ± 2.2
	500	73.2 ± 0.5	43.8 ± 2.2
DPPE	0	63.2 ± 0.5	33.7 ± 1.7
	50	63.1 ± 0.5	34.1 ± 1.7
	100	62.5 ± 0.5	34.0 ± 1.7
	500	/ *	/ *
DPPI	0	44.5 ± 0.5	44.7 ± 2.2
	50	44.5 ± 0.5	45.8 ± 2.3
	100	44.3 ± 0.5	41.9 ± 2.1
	500	44.9 ± 0.5	4.0 ± 0.2
DPPG	0	40.6 ± 0.5	37.1 ± 1.9
	50	41.0 ± 0.5	38.3 ± 1.9
	100	40.9 ± 0.5	37.7 ± 1.9
	500	41.8 ± 0.5	30.9 ± 1.5

Experimental errors are shown in parentheses: T_m (± 0.5 °C), ΔH (± 5%).
* could not be determined, because of aggregation of MLVs.

MLVs (Biltonen and Lichtenberg, 1993).

3.1.1. Effects of chain length on T_m and ΔH

The data in Fig. 1 and Table 1 show that in comparison to DPPC (16:0), an increase in the length of the acyl group by two methylene groups (*i.e.*, $-CH_2-CH_2-$) in DSPC (18:0) and six in DBPC (22:0) thermally stabilised the MLVs. The phase transition took place at higher temperatures and was accompanied by higher ΔH. Addition of two methylene groups to DPPC without added NaCl increased T_m by approximately 13 °C and ΔH by 13 kJ/mol. Addition of six methylene groups to DPPC without added NaCl increased T_m by ˜32 °C and ΔH by 24 kJ/mol. As the hydrocarbon chain length was increased, the van der Waals interactions between the lipid chains will have become stronger and thus have required more energy to disrupt the ordered packing, and T_m increased (Mohammed et al., 2004).

3.1.2. Effects of ester versusether bonds on T_m and ΔH

1,2-Dihexanoyl-sn-glycero-3-phosphocholine is obtained by replacing the ester bond in DPPC with an ether bond. Fig. 1 and Table 1 show that this replacement increased the thermal stability of the phospholipid vesicles. The main phase transition took place at higher temperatures and was accompanied by higher ΔH. For example, without added NaCl, going from ester (DPPC) to ether (DHPC) bond, T_m was increased by 2.2 °C and ΔH by 10 kJ/mol. The reason for this increase in the thermal stability will be due to the conformational and structural differences between these ester and ether phospholipids. The sp^2 hybridised carbon atom of the carbonyl group in DPPC was replaced with the sp^3 hybridised carbon atom in DHPC. It is speculated that the carbonyl in the ester bond is responsible for reducing the overall water order, which means that the water molecules near the DHPC headgroups will be less mobile and more ordered than those near the DPPC headgroups (Kruczek et al., 2017). In addition, the removal of the carbonyl groups will reduce the hydrogen bonding between the DHPC molecules themselves, which will allow tighter packaging of the hydrocarbon chains, thus increasing T_m and ΔH (Mckeone et al., 1986; Mukherjee and Chattopadhyay, 2005).

3.1.3. Effects of a charged polar headgroup on T_m and ΔH

To follow the effects of the headgroups on the thermal stability of the lipids in the MLVs, C16 lipids were used (*i.e.*, zwitterionic DPPC, DPPE; negatively charged DPPG, DPPI). The DPPE MLVs could not be dissolved in the HEPES buffer at pH 7.0, so the pH was adjusted to 8.0. DPPE has a primary amine in its headgroup, and can thus form both intramolecular and intermolecular hydrogen bonds. There are more H-donors for amine groups (*i.e.*, $-NH_3$) than there are available H-acceptors for lipid oxygen atoms, which thus results in formation of hydrogen bonds with the water molecules. Intramolecular hydrogen bonds in DPPE are formed mainly between hydrogens from amine groups and either phosphate or carbonyl oxygens. Hydrogen bonds between H-donors from amine groups and water molecules account for 80% of the total hydrogen bonds that are made with amine groups (Leekumjorn and Sum, 2006). If the concentration of DPPE is too high, then there will not be enough water molecules surrounding the MLVs, and the DPPE molecules will be arranged in hexagonal structures, to decrease the areas of interaction. We see this process as aggregation. Increasing the pH will deprotonate the amine groups, which will thus reduce the number of hydrogen bonds that need to be formed with water molecules, and will allow the DPPE molecules to form soluble MLVs.

When comparing the data in Fig. 1 and Table 1, it can be seen that DPPE MLVs underwent phase transition at higher temperature than DPPC MLVs (ΔT = 21.5 °C), although ΔH is approximately the same. DPPE can form both intramolecular and intermolecular hydrogen bonds, which will lead to a considerably more densely packed lipid–water interface, and thus higher thermal stability of DPPE MLVs when compared to DPPC MLVs (Gurtovenko and Vattulainen, 2008; Leekumjorn and Sum, 2006). As the phase transitions of DPPC and DPPE were accompanied by the same positive ΔH at the different temperatures, it appears that they were accompanied by different positive entropy changes. Due to the higher temperature, the phase transition of the DPPE MLVs should be accompanied by a smaller change in entropy. We believe that the DPPC polar headgroup cannot form hydrogen bonds and that the methyl groups face outwards towards the water phase, which will shield the positive charge on the nitrogen atom from the water molecules. This will give the surface of the MLVs a slightly hydrophobic character, and will force the water molecules into ice-like structural cages. As these cage structures are more ordered than the surrounding water molecules, their formation decreases the entropy. When DPPC MLVs undergo phase transition, the lipid headgroups will become more loosely packed, thus losing some of their hydrophobic character, which will weaken the cage structure of the water molecules and increase the entropy. Also, the phase transition of the DPPC MLVs will be accompanied by increases in the lateral mobility of the lipid acyl chain tails, which will also contribute to an increase in entropy. On the other hand, the DPPE polar group can form hydrogen bonds with the surrounding water molecules, so when the phase transition takes place, it will be accompanied by a positive change in entropy, primarily because of increased lateral mobility of the lipid acyl chain tails. Hence, we speculate that the phase transition of the DPPC MLVs is entropically more favourable than the phase transition of the DPPE MLVs.

1,2-Dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) has a similar phase transition temperature and enthalpy to DPPC, which means that the strength of the headgroup interactions is similar. DPPI underwent phase transition at slightly higher temperatures than DPPC (ΔT = 3 °C), but over a wider temperature interval. The phase transition of DPPI started at around 31 °C and ended at 51 °C, whereas that of DPPC started at 41 °C and ended at 43 °C. This might suggest that even though the inositol groups of DPPI can form intermolecular bonds with each

other, due to steric repulsion of the inositol groups and the dynamic nature of the lipid molecules, these bonds can be easily broken and then re-formed (Zaraiskaya and Jeffrey, 2005).

3.1.4. Effects of NaCl on T_m and ΔH

Increasing the NaCl concentration from 0 to 100 mM did not significantly change the temperatures of the heat capacity maxima during the phase transitions of any of these phospholipids. Addition of NaCl to the lipid solutions did however decrease ΔH of the phase transitions of almost all of these phospholipids, with the exception of DPPE. Also, the increased ionic strength changed the shapes of the profiles. The added Na^+ ions can penetrate into the headgroups, which will induce changes in the lipid–lipid interactions, which are highly directional. The more Na^+ ions that bind to the lipids, the more their phosphate headgroups will be shifted out of the plane of the membrane. In their simulation of a DPPC bilayer in the fluid phase, Wohlert and Edholm (Wohlert and Edholm, 2004) showed that dipole components perpendicular to the bilayer plane repel, and parallel ones attract each other. Addition of NaCl will induce repelling forces of the MLV polar headgroups, and will increase the distance between the phosphate groups (Bockmann et al., 2003; Ferber et al., 2011); this might lead to small decreases in the intermolecular forces, which will thus reduce the phase transition enthalpy. NaCl did not change the phase transition enthalpy of DPPE. This will be a direct consequence of the different natures of the primary amines in the DPPE headgroup, which will result in a more densely packed lipid–water interface compared to the other phospholipids here. A more densely packed lipid–water interface will hinder the binding of Na^+ ions, which will reduce the salt effects overall (Gurtovenko and Vattulainen, 2008).

With the increased Na^+ ion concentration of 500 mM, a saturation effect was observed. The DPPE MLVs aggregated, so it was not possible to measure the enthalpy of the phase transition. The phase transition enthalpy of the DPPI MLVs dropped to almost zero, and the DSC profiles of the DSPC, DBPC and DPPG MLVs changed noticeably. This might be due to anion–lipid interactions, which will become significant only at the high Na^+ ion concentrations, as these anions are believed to only weakly interact with lipid bilayers (Ferber et al., 2011).

3.2. Fluorescence measurements

3.2.1. Detection of membrane permeabilisation by calcein release

For fluorescence measurements the LUVs instead of MLVs were used. A high turbidity of MLV is associated with a loss of intensity for two reasons: (i) in the scattering of the exciting light before it passes all of the cuvette width; and (ii) in the scattering of the fluorescent emission, part of which is scattered before it reaches the photomultiplier (Borenstain and Barenholz, 1993). The best for fluorescence measurements would be SUV, but unfortunately the comparison with MLV is not possible. In fact, according to literature, the large unilamellar vesicles, LUVs exhibited some physical properties that is close to that observed for the MLVs (Biltonen and Lichtenberg, 1993).

The disruption of the model-lipid bilayer structural integrity of LUVs with temperature was investigated without or with NaCl using the calcein dye-leakage assay. The LUVs were loaded with the fluorescent dye calcein and then incubated without or with NaCl. Any weakening of the lipid packing of the LUVs as a result of increased temperature without or with NaCl allows the calcein to leak from the LUV interior into the external buffer medium, where its fluorescence emission intensity increases (Butterfield and Lashuel, 2010). A dye-leakage data as a function of time at 25 °C for DPPC and DPPG LUV and SUV (in gel phase) were recorded in two hours. The calceine-leakage was observed for SUVs and it was negligible for LUVs (data not shown).

Fig. 2 shows the effects of temperature and NaCl on calcein release from the LUVs formed from the different lipids, and Table 2 shows the melting temperatures determined from the fluorescence curves. At T_m and in the liquid-crystalline phase, some calcein was released from the LUVs because of the increased phospholipid mobility. Eq. (1) was used to calculate the released calcein, with these data shown in Table 2 .

Almost all of the melting temperatures obtained from the fluorescence analysis (see Table 2) were in agreement with those obtained from the DSC analysis (see Table 1). For DPPI, there was a discrepancy of 10 °C, which can be attributed to the very wide T_m interval with the DSC analysis, and the subsequent uncertainty of the melting temperature evaluation.

3.2.2. Effects of chain length on calcein release

The data in Fig. 2 and Table 2 show that in comparison to DPPC (16:0), an increase in the lengths of both alkane chains by two methylene groups in DSPC (18:0) and six in DBPC (22:0) stabilised the liquid phase of the phospholipids, with less calcein released.

3.2.3. Effects of ester versusether bond on calcein release

The release of calcein from the DHPC LUVs in the liquid-crystaline phase was lower than that from the DPPC LUVs. It appears that the ether bond not only contributed to higher thermal stability of these LUVs, but also induced modest differences in the fluid phase structures of the bilayers of the DHPC *versus* DPPC LUVs. Replacement of the strongly dipolar C═O group with a methylene group can lower the membrane dipole potential in the fluid phase by 50% (Guler et al., 2009), which will lead to more ordered water molecules with DHPC than DPPC (Gawrisch et al., 1992). As a consequence of these changes, the water permeability for the fluid phase DHPC LUVs was a little smaller than that of the DPPC LUVs, which might explain the lower calcein release.

3.2.4. Effects of charged polar headgroups on calcein release

To follow the effects of the headgroups on the permeability of the liquid phase of the lipid LUVs, C16 lipids were used again (zwitterionic DPPC, DPPE; negatively charged DPPG, DPPI). The DPPE LUVs underwent phase transition at higher temperatures than for the DPPC LUVs, which means that they were thermally more stable. Fluorescence measurements show that the liquid phase of the DPPE LUVs was less permeable to calcein, which might be attributed to a higher packaging density due to a less bulky headgroup. The DPPG LUVs showed very similar calcein release to the DPPC LUVs, which suggested that these lipids form similar LUV bilayers in the fluid phase. The DPPI LUVs released less calcein in the fluid phase than the DPPC LUVs. We believe that this will be because of the vertical arrangement of the inositol groups during the phase transition, which will interact with each other and with the surrounding water molecules, to produce steric barriers that will hinder the transport of calcein across these bilayers.

3.2.5. Effects of NaCl on calcein release

All of the LUVs were resistant to the addition of NaCl, and they did not leak calcein at 25 °C when the LUV lipids were in the gel phase. In general, the T_m was lowered and the phase transition interval was widened with the increased ionic strength, especially at 500 mM NaCl. At higher salt concentrations, the anion–lipid interactions will be significantly weaker, which might lead to the disruption of the LUV membranes in the liquid–crystalline state, and thus increased calcein release. The DHPC, DSPC and DBPC LUVs appeared to be little influenced by the increased salt concentration up to 100 mM. They all showed small decreases in calcein release (100 mM NaCl: 1%, 2%, 1%, respectively), whereas increased salt with the DPPC and DPPG LUVs led to a greater decrease in the calcein release (100 m NaCl: 10%, 12%, respectively). Structural changes in the LUV membranes (*e.g.*, decreased membrane area, increased thickness) due to the increased electrolytes might have been responsible for these lowered permeabilities. On the other hand, the increased salt concentration to 100 mM NaCl for the DPPE and DPPI LUVs increased the calcein release (14%, 6%, respectively). It is possible that the NaCl will compete with the water molecules for binding to the lipid headgroups, which will increase the

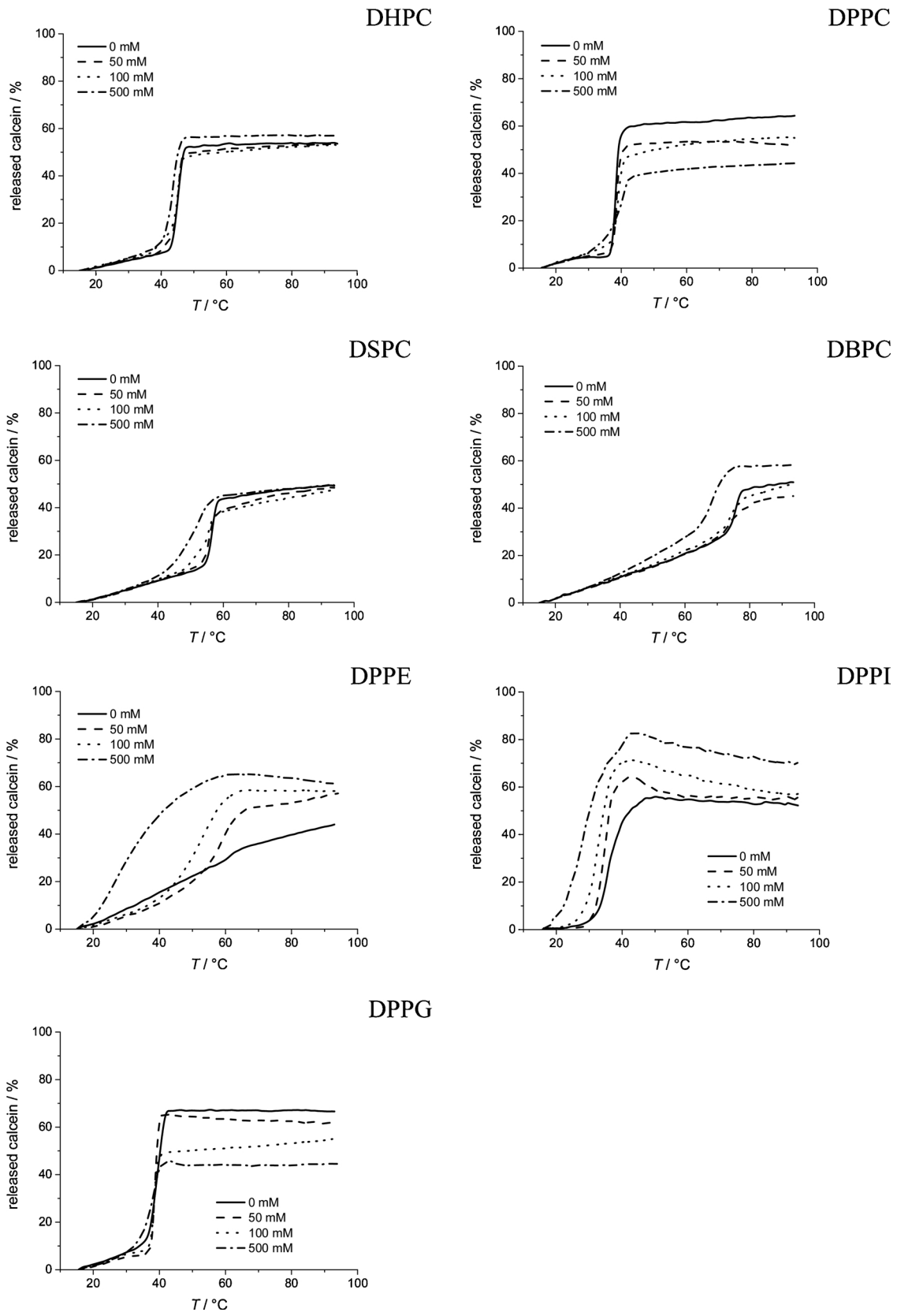


Fig. 2. Calcein release from the LUVs formed from the different lipids as a function of temperature and NaCl concentration, as defined by changes in the fluorescence intensity (normalised data).

Table 2
Phase transition temperatures of the different lipid in the large unilamellar vesicles, as determined from the calcein release fluorescence curves. By averaging y-values of last four points on fluorescence curve (at T = 91, 92, 93 and 94 °C) we estimated final calcein release percentage.

LUV lipid	NaCl (mM)	Gel-to-liquid-crystalline phase transition (T_m; °C)	Calcein release (%)
DHPC	0	45.2 ± 0.5	54
	50	45.1 ± 0.5	53
	100	44.9 ± 0.5	53
	500	43.4 ± 0.5	57
DPPC	0	41.0 ± 0.5	64
	50	41.0 ± 0.5	52
	100	41.0 ± 0.5	55
	500	40.0 ± 0.5	44
DSPC	0	55.0 ± 0.5	49
	50	55.0 ± 0.5	48
	100	53.0 ± 0.5	47
	500	50.0 ± 0.5	49
DBPC	0	75.0 ± 0.5	51
	50	74.0 ± 0.5	45
	100	74.0 ± 0.5	50
	500	69.5 ± 0.5	58
DPPE	0	64.0 ± 0.5	44
	50	60.0 ± 0.5	57
	100	55.0 ± 0.5	58
	500	/*	/*
DPPI	0	35.0 ± 0.5	51
	50	35.0 ± 0.5	52
	100	33.0 ± 0.5	57
	500	28.0 ± 0.5	68
DPPG	0	41.0 ± 0.5	67
	50	41.0 ± 0.5	62
	100	40.0 ± 0.5	55
	500	40.0 ± 0.5	45

* could not be determined, because of aggregation of MLVs.

calcein release, and for the DPPE LUVs, eventually lead to aggregation.

4. Conclusions

The lipid structure of membranes determines their physicochemical properties (*e.g.*, T_m, membrane curvature, permeability), and consequently this influences their interactions with other constituents in the system. Lipid-only model membranes offer unique insight into these interactions in well-defined systems. We used liposomes made from different phospholipids (*i.e.*, DHPC, DPPC, DSPC, DBPC, DPPE, DPPI, DPPG) to study the effects of the lipid structural properties, in terms of their headgroup and chain length, and the effects of the chemical bonds by which their acyl chains are attached to the glycerol moiety of the glycerophospholipid species on their phase behaviour (*i.e.*, T_m, ΔH, calcein permeability).

The selection of lipids for this study was based on the concept that phosphatidylcholines are the major zwitterionic class of lipids in most eukaryotic cell membranes, and the acyl chains selected are the most common ones in biological lipids. The DSC data show that longer acyl chains increase the stability of the liposome lipid bilayers, which will be due to the stronger van der Waals interactions between the lipid chains. On the other hand, increased salt concentrations does not have a huge impact on the thermal stability, but widen the phase transitions of all liposome lipid bilayers. It is likely that the NaCl induce repelling forces for the liposome polar headgroups, which will increase the distance between the phosphate groups. The charge and the geometry of the polar headgroup is likely to be the important for the stabilisation (*e.g.* H-bonds, counter ions binging to phosphate, shielding the electrostatic repulsion by Na^+ or Cl^- ions). It is difficult to conclude from our results the effect of different concentration of the NaCl on the structure of liposomes, since the results are not so straightforward. In the case of zwitterionic phospholipids (DHPC, DPPC, DSPC and DBPC) from DSC we can see no effect on thermal stability of liposomes judged from T_m, but slight effect on ΔH (destabilisation) can be traced by increasing the concentration of NaCl. Higher concentration of NaCl *e.g.* 500 mM cause aggregation of the lipids. The measured temperatures and enthalpy changes of phase transition (*i.e.*, T_m, ΔH) also showed that replacing the ester bond with the ether bond that is common for Archaea will increase the thermal stability of the phospholipid bilayers. The properties of the bilayer phase transitions were also determined by fluorescence measurements of thermally induced calcein release. Here, the temperatures of phase transition agree well with the DSC measurements, with the calcein release from these phospholipid liposomes varying from 40% to 70%, depending on the lipid used for liposome formation and on the salt concentration.

Declaration of Competing Interest

The authors have no conflict of interest.

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References

Akabori, K., Nagle, J.F., 2015. Structure of the DMPC lipid bilayer ripple phase. Soft Matter 11, 918–926. https://doi.org/10.1039/c4sm02335h.
Ali, S., Minchey, S., Janoff, A., Mayhew, E., 2000. A differential scanning calorimetry study of phosphocholines mixed with paclitaxel and its bromoacylated taxanes. Biophys. J. 78, 246–256. https://doi.org/10.1016/S0006-3495(00)76588-X.
Baba, T., Toshima, Y., Minamikawa, H., Hato, M., Suzuki, K., Kamo, N., 1999. Formation and characterization of planar lipid bilayer membranes from synthetic phytanyl-chained glycolipids. BbaBiomembranes 1421, 91–102. https://doi.org/10.1016/S0005-2736(99)00114-5.
Balleza, D., Garcia-Arribas, A.B., Sot, J., Ruiz-Mirazo, K., Goni, F.M., 2014. Ether- versus Ester-Linked Phospholipid Bilayers Containing either Linear or Branched Apolar Chains. Biophys. J. 107, 1364–1374. https://doi.org/10.1016/j.bpj.2014.07.036.
Baoukina, S., Rozmanov, D., Tieleman, D.P., 2017. Composition fluctuations in lipid bilayers. Biophys. J. 113, 2750–2761. https://doi.org/10.1016/j.bpj.2017.10.009.
Biltonen, R.L., Lichtenberg, D., 1993. The use of differential scanning calorimetry as a tool to characterize liposome preparations. Chem. Phys. Lipids 64, 129–142. https://doi.org/10.1016/0009-3084(93)90062-8.
Bockmann, R.A., Hac, A., Heimburg, T., Grubmuller, H., 2003. Effect of sodium chloride on a lipid bilayer. Biophys. J. 85, 1647–1655. https://doi.org/10.1016/S0006-3495(03)74594-9.
Borenstain, V., Barenholz, Y., 1993. Characterization of liposomes and other lipid assemblies by multiprobe fluorescence polarization. Chem. Phys. Lipids 64, 117–127. https://doi.org/10.1016/0009-3084(93)90061-7.
Butterfield, S.M., Lashuel, H.A., 2010. Amyloidogenic protein-membrane interactions: mechanistic insight from model systems. Angew. Chemie 49, 5628–5654. https://doi.org/10.1002/anie.200906670.
Coker, J.A., 2016. Extremophiles and biotechnology: current uses and prospects. F1000Res 5. https://doi.org/10.12688/f1000research.7432.1.
Eeman, M., Deleu, M., 2010. From biological membranes to biomimetic model membranes. Biotechnol Agron. Soc. 14, 719–736. https://doi.org/10.1016/B978-0-12-409547-2.12236-X.
Ferber, U.M., Kaggwa, G., Jarvis, S.P., 2011. Direct imaging of salt effects on lipid bilayer ordering at sub-molecular resolution. Eur. Biophys. J.: EBJ 40, 329–338. https://doi.org/10.1007/s00249-010-0650-7.
Galvagnion, C., Brown, J.W.P., Ouberai, M.M., Flagmeier, P., Vendruscolo, M., Buell, A.K., Sparr, E., Dobson, C.M., 2016. Chemical properties of lipids strongly affect the kinetics of the membrane-induced aggregation of alpha-synuclein. P. Natl. Acad. Sci. USA 113, 7065–7070. https://doi.org/10.1073/pnas.1601899113.
Gawrisch, K., Ruston, D., Zimmerberg, J., Parsegian, V.A., Rand, R.P., Fuller, N., 1992. Membrane dipole potentials, hydration forces, and the ordering of water at membrane surfaces. Biophys. J. 61, 1213–1223. https://doi.org/10.1016/S0006-3495(92)81931-8.
Gmajner, D., Ahlin Grabnar, P., Tušek-Žnidarič, M., Štrus, J., Šentjurc, M., Poklar Ulrih, N., 2011a. Structural characterization of liposomes made of diether archaeal lipids and dipalmitoyl-L-[alpha]-phosphatidylcholine. Biophys. Chem. 158, 150–156. https://doi.org/10.1016/j.bpc.2011.06.014.
Gmajner, D., Ota, A., Sentjurc, M., Ulrih, N.P., 2011b. Stability of diether C-25,C-25 liposomes from the hyperthermophilic archaeon Aeropyrum pernix K1. Chem. Phys. Lipids 164, 236–245. https://doi.org/10.1016/j.chemphyslip.2011.01.005.

Gmajner, D., Ulrih, N.P., 2011. Thermotropic phase behaviour of mixed liposomes of archaeal diether and conventional diester lipids. J. Therm. Anal. Calorim. 106, 255–260. https://doi.org/10.1007/s10973-011-1596-4.

Guler, S.D., Ghosh, D.D., Pan, J., Mathai, J.C., Zeidel, M.L., Nagle, J.F., Tristram-Nagle, S., 2009. Effects of ether vs. Ester linkage on lipid bilayer structure and water permeability. Chem. Phys. Lipids 160, 33–44. https://doi.org/10.1016/j.chemphyslip.2009.04.003.

Gurtovenko, A.A., Vattulainen, I., 2008. Effect of NaCl and KCl on phosphatidylcholine and phosphatidylethanolamine lipid membranes: insight from atomic-scale simulations for understanding salt-induced effects in the plasma membrane. J. Phys. Chem. B 112, 1953–1962. https://doi.org/10.1021/jp0750708.

Haque, M.E., McIntosh, T.J., Lentz, B.R., 2001. Influence of lipid composition on physical properties and PEG-mediated fusion of curved and uncurved model membrane vesicles: “Nature’s own” fusogenic lipid bilayer. BiochemistryUs 40, 4340–4348. https://doi.org/10.1021/bi002030k.

Huang, C.H., Li, S.S., 1999. Calorimetric and molecular mechanics studies of the thermotropic phase behavior of membrane phospholipids. Bba-Rev. Biomembranes 1422, 273–307. https://doi.org/10.1016/S0005-2736(99)00099-1.

Koynova, R., Caffrey, M., 1998. Phases and phase transitions of the phosphatidylcholines. Bba-Rev. Biomembranes 1376, 91–145. https://doi.org/10.1016/S0304-4157(98)00006-9.

Kruczek, J., Saunders, M., Khosla, M., Tu, Y.C., Pandit, S.A., 2017. Molecular dynamics simulations of ether- and ester-linked phospholipids. BbaBiomembranes 1859, 2297–2307. https://doi.org/10.1016/j.bbamem.2017.08.023.

Lamond, A.I., 2002. Molecular biology of the cell, 4th ed. Nature 417, 383. https://doi.org/10.1038/417383a.

Lasic, D.D., 1993. Liposomes: From Physics to Applications. Elsevier, Amsterdam; New York.

Lee, A.G., 2004. How lipids affect the activities of integral membrane proteins. BbaBiomembranes 1666, 62–87. https://doi.org/10.1016/j.bbamem.2004.05.012.

Leekumjorn, S., Sum, A.K., 2006. Molecular simulation study of structural and dynamic properties of mixed DPPC/DPPE bilayers. Biophys. J. 90, 3951–3965. https://doi.org/10.1529/biophysj.105.076596.

Leekumjorn, S., Sum, A.K., 2007. Molecular studies of the gel to liquid-crystalline phase transition for fully hydrated DPPC and DPPE bilayers. BbaBiomembranes 1768, 354–365. https://doi.org/10.1016/j.bbamem.2006.11.003.

Lewis, R.N.A.H., Pohle, W., McElhaney, R.N., 1996. The interfacial structure of phospholipid bilayers: Differential scanning calorimetry and Fourier transform infrared spectroscopic studies of 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine and its dialkyl and acyl-alkyl analogs. Biophys. J. 70, 2736–2746. https://doi.org/10.1016/S0006-3495(96)79843-0.

Maherani, B., Arab-Tehrany, E., Kheirolomoom, A., Geny, D., Linder, M., 2013. Calcein release behavior from liposomal bilayer; influence of physicochemical/mechanical/structural properties of lipids. Biochimie 95, 2018–2033. https://doi.org/10.1016/j.biochi.2013.07.006.

Marsh, D., 2008. Protein modulation of lipids, and vice-versa, in membranes. BbaBiomembranes 1778, 1545–1575. https://doi.org/10.1016/j.bbamem.2008.01.015.

Mathai, J.C., Tristram-Nagle, S., Nagle, J.F., Zeidel, M.L., 2008. Structural determinants of water permeability through the lipid membrane. J. Gen. Physiol. 131, 69–76. https://doi.org/10.1085/jgp.200709848.

Mckeone, B.J., Pownall, H.J., Massey, J.B., 1986. Ether phosphatidylcholines - comparison of Miscibility with Ester Phosphatidylcholines and Sphingomyelin, vesicle fusion, and association with Apolipoprotein-a-I. BiochemistryUs 25, 7711–7716. https://doi.org/10.1021/Bi00371a064.

Mohammed, A.R., Weston, N., Coombes, A.G.A., Fitzgerald, M., Perrie, Y., 2004. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. Int. J. Pharm. 285, 23–34. https://doi.org/10.1016/j.ijpharm.2004.07.010.

Mukherjee, S., Chattopadhyay, A., 2005. Influence of ester and ether linkage in phospholipids on the environment and dynamics of the membrane interface: a wavelength-selective fluorescence approach. Langmuir 21, 287–293. https://doi.org/10.1021/la048027+.

Nickels, J.D., Chatterjee, S., Stanley, C.B., Qian, S., Cheng, X.L., Myles, D.A.A., Standaert, R.F., Elkins, J.G., Katsaras, J., 2017. The in vivo structure of biological membranes and evidence for lipid domains. PLoS Biol. 15. https://doi.org/10.1371/journal.pbio.2002214.

Pakchung, A.A.H., Simpson, P.J.L., Codd, R., 2006. Life on earth. Extremophiles continue to move the goal posts. Environ. Chem. 3, 77–93. https://doi.org/10.1071/En05093.

Patel, H., Tscheka, C., Heerklotz, H., 2009. Characterizing vesicle leakage by fluorescence lifetime measurements. Soft Matter 5, 2849–2851. https://doi.org/10.1039/b908524f.

Pereto, J., Lopez-Garcia, P., Moreira, D., 2004. Ancestral lipid biosynthesis and early membrane evolution. Trends Biochem. Sci. 29, 469–477. https://doi.org/10.1016/j.tibs.2004.07.002.

Phillips, M.C., Ladbrook, B., Chapman, D., 1970. Molecular interactions in mixed lecithin systems. Biochim. Biophys. Acta 196https://doi.org/10.1016/0005-2736(70)90163-X. 35-&.

Rothschild, L.J., Mancinelli, R.L., 2001. Life in extreme environments. Nature 409, 1092–1101. https://doi.org/10.1038/35059215.

Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L., Thompson, T.E., 1976. A calorimetric and fluorescent probe study of the gel-liquid crystalline phase transition in small, single-lamellar dipalmitoylphosphatidylcholine vesicles. BiochemistryUs 15, 1393–1401. https://doi.org/10.1007/978-3-642-66564-6_4.

Šentjurc, M., Vrhovnik, K., Kristl, J., 1999. Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method. J. Control. Release 59, 87–97. https://doi.org/10.1016/S0168-3659(98)00181-3.

Ulrih, N.P., Gmajner, D., Raspor, P., 2009. Structural and physicochemical properties of polar lipids from thermophilic archaea. Appl. Microbiol. Biot. 84, 249–260. https://doi.org/10.1007/s00253-009-2102-9.

Wohlert, J., Edholm, O., 2004. The range and shielding of dipole-dipole interactions in phospholipid bilayers. Biophys. J. 87, 2433–2445. https://doi.org/10.1529/biophysj.104.044222.

Yeagle, P.L., 2014. Non-covalent binding of membrane lipids to membrane proteins. Biochim. Biophys. Acta 1838, 1548–1559. https://doi.org/10.1016/j.bbamem.2013.11.009.

Zaraiskaya, T., Jeffrey, K.R., 2005. Molecular dynamics simulations and 2H NMR study of the GalCer/DPPG lipid bilayer. Biophys. J. 88, 4017–4031. https://doi.org/10.1529/biophysj.104.054601.