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A combined FTIR and DSC study on the bilayer-stabilising effect of electrostatic interactions in ion paired lipids

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ABSTRACT

Investigating lipid ion pair formation is important for understanding the mechanisms of lipid-mediated drug resistance in bacteria. In this study we have used the charged amphiphiles dipalmitoylphosphatidylglycerol (DPPG) and dihexadecyldimethylammonium bromide (DHDAB), as a model to evaluate the formation of ion pairs by a combined Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) analysis. FTIR was employed to study the environment of the DPPC headgroup phosphate and lipid/surfactant alkane chains, in vesicles formed by the two amphiphiles mixed in various molar ratios. An increase of the absorbance ratio of 1221–1201 cm^{-1} in the asymmetric phosphate stretching mode was found to follow a sigmoidal relationship with the proportion of DHDAB, increasing to a plateau above a DPPG/DHDAB 1:1 molar ratio of, providing evidence that the PG headgroup phosphate is involved in ion pairing. A consistent red shift was measured for the position of the symmetric CH_2 stretch band for the lipid/surfactant 1:1 molar ratio mixture, which is indicative of an increased ordering of the hydrophobic chains. The DSC experiments yielded information about the thermotropic and the mixing behaviour of the lipid/surfactant systems. DPPG and DHDAB seem to form an ion pair with cluster compound characteristics at the equimolar ratio. Most interestingly, the DPPG/DHDAB 2:1 molar ratio mixture is characterized by strong intermolecular interactions, which result in a pronounced stabilization of the gel phase, possibly through the formation of a closely-associated ion triplet configuration in which the charges are delocalised across the headgroups.

1. Introduction

The vast diversity of lipid species present in biological membranes and their compositional plasticity are considered to be important for maintaining the functional homeostasis of membrane proteins in response to environmental fluctuations in temperature, pH and solute content [1]. Alterations in the lipid composition of some biomembranes may also induce changes in membrane function, facilitated solely by lipid–lipid interactions. Such lipid-mediated functional plasticity plays an important role in the survival mechanisms of bacteria exposed to environmental stress [2]. In the case of the Gram positive opportunistic pathogen *Staphylococcus aureus*, increased synthesis of the aminoacyl lipid lysyl-phosphatidylglycerol (L-PG), has been shown to protect the bacteria from defensive cationic membrane-active antimicrobial peptides (AMPs) and thus allow them colonise human epithelia and even establish, maintain and spread infections [3]. The formation of ion pairs between the phosphate of the phosphatidylcholine (PG) headgroup and the amines of the L-PG headgroup, has been shown to fine tune both the charge and ordering of lipids in reconstituted *S. aureus* lipid membranes, thus attenuating interaction with AMPs [4]. The ability of L-PG and other aminoacyl lipids to protect bacteria from host innate immunity has usually been studied in simple model systems, designed to examine the effect of lipid composition on AMP binding, rather than focusing on how L-PG influences the physical properties of lipid membranes [5]. The use of synthetic L-PG for examining its behaviour and influence in model membranes is complicated by its alkali-lability [6] and multiple headgroup pK_a [4], which also exist in synthetic L-PG analogues [7,8]. In this study we have employed a very simple model system with which to explore the physicochemical effects of lipid ion pairing on membrane properties, using a range of different techniques.

We chose a structurally simple binary lipid system to evaluate different methods used for the investigation of ion pair formation, using various mixtures of the anionic phospholipid dipalmitoylphosphatidylglycerol (DPPG) and the quaternary ammonium surfactant

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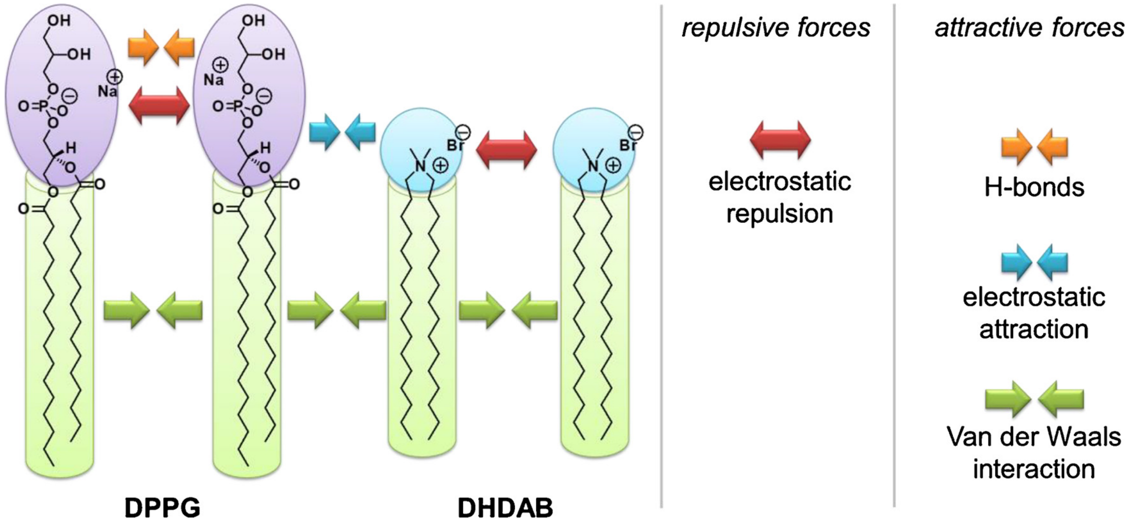

Fig. 1. Structures of the investigated lipids and schematic overview of different forces acting between the amphiphiles. DPPG = dipalmitoylglycero-3-phosphoglycerol; DHDAB = dihexadecyldimethylammonium bromide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dihexadecyldimethylammonium bromide (DHDAB) (Fig. 1). The combination of techniques we have used has allowed us to differentiate between simple charge neutralization between the anionic DPPG and cationic DHDAB and the formation of real ion pairs, which act as a complex compound. Although our model is structurally very different from the natural PG/L-PG mixture, it possesses various desired characteristics needed to prove the utility of the investigation methods. With respect to the design of our Fourier transform infrared spectroscopy (FTIR) experiments, it was necessary to avoid the presence of phosphate and carbonyl functions in both lipids, because the IR bands of both groups are very sensitive to lipid packing and hydration characteristics. Using DHDAB has several advantages, its lack of both phosphate and ester groups prevents masking of the PO_2^- stretch bands for the DPPG, and its two C16 alkyl chains reduce the risk of packing inhomogeneity arising from hydrophobic mismatch. The various possible intermolecular interactions within DPPG/DHDAB mixtures, are summarised in Fig. 1.

The effect of ion pairing on the surface charge properties and dispersion stability of DPPG/DHDAB vesicles in the gel phase was determined using a combination of zeta potential and dynamic light scattering (DLS) measurements. FTIR spectra provided a useful tool for quantifying DPPG/DHDAB ion pairs through analysis of the phosphate stretch bands [9], and lipid ordering effects were determined by analysing the CH_2 stretching modes [10]. Differential scanning calorimetry (DSC) experiments were performed to get information about the thermotropic behaviour of the lipid mixtures, and therefore allowed an assessment of their mixing behaviour, and the effect of ion pairing on gel phase stability. This combination of techniques and the results obtained in the DPPG/DHDAB system, sheds light on the complexity of the mixing behaviour of the two lipids and demonstrates that neutralization of the lipid dispersions does not yield the most stable bilayer structures. This has a number of implications for both the stability of bacterial membranes containing ion paired lipids and their interactions with components of the innate immune system.

2. Materials and methods

2.1. Materials

High Purity Water with a specific resistivity of 18.2 MΩ cm and a pH between 5 and 6, was used throughout these experiments (ElgaPURELAB®Ultra, Elga Veolia, High Wycombe UK). 1,2-Dipalmitoyl-*sn-glycero*-3-phospho-(*rac*-1-glycerol) sodium salt (DPPG) was supplied by Avanti Polar Lipids (Alabaster, AL, USA) and dihexadecyldimethylammonium bromide with a purity of 97% was purchased from Sigma Aldrich (Poole, Dorset, UK). Analytical grade chloroform and Methanol were purchased from either Fisher Scientific (Loughborough, UK), or Merck (Darmstadt, Germany).

2.2. Zeta-potential and particle size measurements

Lipid/surfactant dispersions were prepared by the film hydration procedure to obtain multilamellar vesicles [12], from DPPG/DHDAB mixtures dissolved in chloroform/methanol (9/1, v/v). Lipid films of different mixtures (xDHDAB = 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8) and of the pure lipids were obtained by evaporating the solvent at 40 °C for 1 h at 200 mbar and for a further 3 h at 10 mbar. After formation of dry lipid films, sterile filtered dispersion medium (1 mM NaCl to get a suitable conductivity for the ζ-potential measurement), was added to give a final total lipid/surfactant concentration of 0.025 mg mL^{-1}. The lipid dispersions were then incubated at 60 °C (at least 5 °C above the gel–fluid phase transition of the phospholipids) while shaking (1400 rpm) for 30 min (Eppendorf Thermomixer 5436). Finally, the lipid dispersions were sonicated at 37 kHz (100% power output, sonication bath FB11201, Fisherbrand, FisherScientific, Germany) for 6 min.

The electrophoretic measurements were performed using the laser-Doppler-electrophoresis technique on a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Malvern, UK) at 25 °C. Three measurements consisting of 30 runs with a voltage of 50 V were performed. For the calculations, a viscosity of $\eta = 0.8872$ mPa s, a dielectric constant of $\varepsilon = 78.5$ F m^{-1}, and a refractive index of 1.33 were assumed. The analysis was performed using the Zetasizer software (version 6.34). The mobility μ of the diffusing aggregates was converted into the ζ-potential using the Smoluchowski relationship $\zeta = \mu\eta/\varepsilon$. DLS measurements were also performed with the Zetasizer Nano ZS ZEN3600. The scattering angle was 173°. Three measurements consisting of 15 runs with a duration time of 20 s for each run were performed at 25 °C. For the calculations, a viscosity of $\eta = 0.8872$ mPa·s and a refractive index of 1.33 were assumed. The Zetasizer software (version 6.34) and the ALV-Correlation software version 3.0 calculated the z-average (diameter) and PDI using the cumulants method, from the intensity distribution autocorrelation function.