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Regular Article

Structure and morphology of vesicular dispersions based on novel phosphatidyl glucose and phosphatidyl choline with different acyl chains

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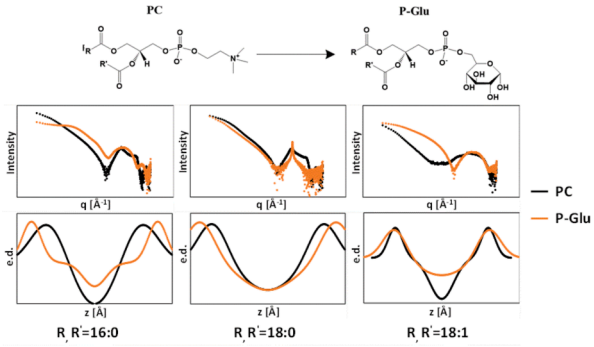
GRAPHICAL ABSTRACT



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ABSTRACT

Hypothesis: Phospholipids are widely used in food and pharmacological formulations. However, these typically suffer from limitations such as low colloidal stability. Promising stability has been observed for vesicles based on phosphatidylglucose (P-Glu), but fundamental knowledge on this lipid is missing and those observations were made using P-Glu containing mixed acyl groups. The acyl groups are expected to influence the properties of phosphatidylglucose to a large extent.

Experiments: Using an enzyme-based method, P-Glu containing either palmitic (DPP-Glu), stearic (DSP-Glu) or oleic (DOP-Glu) acid were synthesized. The morphology of the lipid dispersions was studied using small angle x-ray scattering and cryogenic transmission electron microscopy and the data was modelled to extract bilayer structural parameters. Phosphatidylcholine lipids containing the same fatty acids were studied for comparison.

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Findings: All phosphatidylcholine lipids formed mainly multilamellar vesicles. DOP-Glu formed unilamellar vesicles (ULVs), while disc like objects were observed in the case of DPP-Glu and DSP-Glu formed predominantly bilayer stacks. In the 1:1 mixture of the DOPC and DOP-Glu, ULVs were formed. The bilayer thickness increased as follows: DOP-Glu < DPP-Glu < DSP-Glu and in the PC series the same trend was seen for the lamellar spacing. DSP-Glu had similar lamellar spacing as DSPC.

1. Introduction

Phospholipids (PLs) are amphiphilic molecules that are highly abundant in nature and constitute one of the major components of cell membranes of all living organisms. The most abundant class of PLs from natural origin is phosphatidylcholine (PC), also referred to as lecithin, and the most utilized sources for PC isolation are from vegetable sources and egg yolk. PCs are zwitterionic at physiological pH and normally self-assemble into bilayer structures, forming vesicles upon dispersion in excess aqueous solution. PC based liposomal formulations are good drug delivery systems and have been extensively researched for several decades [1,2,3] with several examples of successful formulations in clinical use and even more in clinical studies [4].

Purification of PL can be achieved to some extent via the selection and optimisation of extraction, precipitation and chromatography protocols. These products are useful for a limited number of applications. Mainly because, the isolation of high purity fractions, that constitute a single, well-defined chemical structure of PC, or fractions of PLs other than PC, from natural sources is demanding and the yields are very limited [5,6]. Formulations made from solely PC often suffer from limited colloidal stability (vesicle aggregation and fusion), chemical instability (oxidation and hydrolysis), complicated release mechanisms at the target site and long-term stability issues (phase separation and leakage of cargo) [7,8]. Both stability and release properties are highly dependent on environmental conditions. These challenges are usually circumvented by more complicated excipient formulations, where PC constitutes the carrier lipid, and other components such as cholesterol [9], polyethylene glycol (PEG) [10], targeting ligands [11] and stimuli sensitive lipids or polymers [12] are introduced for better liposome stability, longer circulation time *in vivo*, and better retention and controlled release of cargo. As all of these mechanisms take place in an aqueous environment, these challenges could be circumvented by drying or lyophilisation of liposome based formulations, but these processes usually require stabilizing excipients such as carbohydrates [13]. An alternative approach could be the use of PLs other than PC, with modified head groups, that improve their properties as excipients.

For the isolation of high purity, non-natural PLs with well-defined chemical structures, a synthetic approach must be applied [5,14]. Most research regarding enzymatic modification of PLs is focused on modifications of the hydrophobic part, where typically lipases or phospholipases are utilized for transesterification reactions in order to synthesise a PC with a single well-defined fatty acid composition. The research on modification of the hydrophilic head is more limited and only one family of enzymes is available with the appropriate catalytic activity, namely phospholipase D (PLD). The enzymatic transphosphatidylation reaction is most preferably done in a biphasic reaction system where a donor phospholipid (PC or lecithin) is incubated together with a high excess of acceptor alcohol [15]. The conjugated head group is released by cleavage of the phosphodiester bond between the phosphate group and the head, and the new acceptor alcohol is introduced in its place. This approach has been used on a large scale for the production of well-defined PCs and other industrially relevant natural PLs, such as phosphatidylserine (PS) and phosphatidylglycerol (PG). Considerable attention has also been paid to the synthesis of *de novo* PLs with modified head groups for various purposes, such as prodrug functionalization of the PL by introduction of terpenes [16] or doxorubicin [17], targeted delivery [18] or improved vesicle stability [19,20]. Usually, this research remains at a lab scale, and very few *de novo* PLs have been produced in high enough quantities to do any physicochemical characterization, and therefore not much is known about their potential as formulation excipients.

In our previous study, we developed an enzyme catalysed reaction system for the synthesis of glycophospholipids with various saccharide conjugates replacing the choline head group [21]. One of the saccharides we used was glucose, which upon conjugation with the PL, converts the previously zwitterionic PC into the negatively charged phosphatidylglucose (P-Glu). The chemical structures of the commercial unmodified PC and the modified P-Glu are shown in Fig. 1. The new head group with glucose is highly hydroxylated and possesses the ability to form multiple hydrogen bonds with closely located PLs and the surrounding water. In addition, the net charge also becomes negative upon the modification. Song et al. and Wu et al. have shown that vesicles formed with glycophospholipids have higher colloidal stability and the leakage of enclosed cargo is lower compared to PC vesicles [19,20]. Due to the large fraction of hydroxyl groups, saccharides and other carbohydrates are commonly used as cryoprotective agents during freezing and dehydration [13]. This could potentially give phospholipids conjugated with saccharides beneficial properties for use in dehydrated or lyophilized liposomal formulations, and therefore make them interesting as a new class of PL formulation ingredients.

The self-assembly behaviour of PC has been extensively studied using small angle x-ray scattering (SAXS), cryogenic transmission electron microscopy (cryo-TEM) and other techniques, giving information about which structures are formed as well as fine details of these structures, such as bilayer thickness, etc. This knowledge forms the basis for existing practical use of PC in pharmaceutical and other applications. Unfortunately, the corresponding fundamental information on P-Glu and its self assembly structures is lacking. Although PLs with mixed fatty acids are used in several applications, and promising stability has been observed for mixed chain P-Glu, further possibilities to modulate the lipid properties can be obtained by proper selection of single fatty acids as building blocks of the lipids. The influence of the fatty acids on the self assembly behaviour of the lipids is thus of considerable interest for all PLs including P-Glu.

In this study we have synthesized and isolated a series of three phosphatidylglucose (P-Glu) species with well-defined fatty acid compositions: Saturated DPP-Glu (C16:0), saturated DSP-Glu (C18:0) and unsaturated DOP-Glu (C18:1). We have used cryo-TEM and SAXS to study vesicle morphology and structural features, such as bilayer thickness both in pure water and physiological PBS solutions at 25 °C and 37 °C. Special attention was dedicated to differences between the non-modified PC and the corresponding P-Glu as well as the effect of the length and degree of saturation of the hydrophobic chain on structures formed by the modified lipids. These characterisation studies are important to achieve a basic understanding of the physicochemical properties of *de novo* PLs and expand the number and functionality of available ingredients for more diverse and better formulations for future clinical applications.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-*Glycero*-3-Phosphatidylcholine (DOPC) > 98 % purity was purchased from Larodan (Solna, Sweden), 1,2-Dipalmitoyl-*sn*-*Glycero*-3-Phosphatidylcholine (DPPC) from Lipoid GmbH

(Ludwigshafen, Germany) and 1,2-Distearoyl-*sn-Glycero*-3-Phosphatidylcholine (DSPC) > 99 % purity from Avanti polar lipids (Birmingham, AL, USA). Chloroform and methanol (99 %) were purchased from VWR Chemicals. NaCl, KH_2PO_4 and Na_2HPO_4 (99.9 %) were purchased from VWR Chemicals. Milli Q purified water (18 MΩ cm) was used for all experiments.

2.2. Synthesis of modified phospholipids

The modified phospholipids were prepared as described by Barchan et al. [21]. In short, the donor phospholipid (DOPC, DPPC or DSPC) was dissolved in chloroform and exposed to the transphosphatidylation reaction by addition of acetate/phosphate buffer pH 5.6, containing the saccharide acceptor substrate and PLD enzyme. The reactions were typically run for 24 h at 30 °C, 400 rpm, with a high excess (25–50 times) of acceptor substrate to supress hydrolysis. After the reaction was complete, the modified phospholipids were isolated and excess sugar was removed by a simple extraction procedure with chloroform. After centrifugation, the organic phase was recovered, and the solvent was removed by evaporation. The lipids were then re-dissolved in $CHCl_3$:MeOH (93:7, v/v) and purified by flash chromatography on a silica column. The lipids were eluted with a gradient of $CHCl_3$:MeOH:HAc (8:3:1, v/v). Fractions containing the purified lipid were pooled and concentrated by evaporation before lyophilization.

2.3. Preparation of vesicles

A protocol adapted from Dabkowska et al. [22] was used to prepare the vesicles. In summary, stock solutions of each lipid in 2:1 (v/v) ratio of $CHCl_3$:MeOH were prepared and vortexed until the lipid was fully dissolved. From each stock solution, a lipid film was prepared by evaporation of the solvent using a gentle N_2 flow. An additional lipid film of a 1:1 (w/w) mixture of DOPC:DOP-Glu was prepared by mixing the stock solutions of the relevant lipids. The lipid films were then hydrated with Milli Q water to a final nominal concentration of 10 mg/mL (see final concentration of each lipid in Table 1) and vortexed until the lipid was fully suspended. The coarse suspensions were then sonicated with a tip sonicator (Vibra-Cell VCX 130, Sonics & Materials Inc., Newton, CT, USA) in a water bath with the following settings: 7.5 min total sonication time, divided in pulses of 10 s sonication and 10 s cooling time at 50 % amplitude with 3 mm stepped sonication tip. Samples of lipids containing palmitic acid residues (DPPC and DPP-Glu) were heated to 46 °C, and samples containing stearic acid residues (DSPC and DSP-Glu) were heated to 60 °C prior to sonication. All other lipid samples were handled at ambient temperature of 25 °C.

Effect of salt: A stock solution of 20X PBS was prepared and added to the vesicle samples in order to study the effect of salt on the vesicle morphology. The small dilution of the samples caused upon addition of the PBS stock solution (5 % v/v) was neglected. The final concentration of PBS in the samples was 155 mM NaCl, 1.06 mM KH_2PO_4, 2.97 mM Na_2HPO_4, pH 7.3.

Table 1
Final lipid concentration in vesicles.

Lipid	Concentration in SAXS experiments	Concentration in DLS experiments
DOPC	10 mg/mL	0.10 mg/mL
DPPC	10 mg/mL	0.10 mg/mL
DSPC	10 mg/mL	0.10 mg/mL
DOP-Glu	10 mg/mL	0.10 mg/mL
DPP-Glu	<20 mg/mL*	<0.20 mg/mL
DSP-Glu	5 mg/mL**	0.05 mg/mL
DOPC/DOP-Glu 1:1 (w/w)	10 mg/mL	0.10 mg/mL

*Estimated concentration. Lipid sample was not completely dry when the lipid films were made.
** Not enough lipid sample to prepare higher concentration.

2.4. Synchrotron SAXS

SAXS measurements were performed at the CoSAXS beamline, MAX IV (Lund, Sweden) using the sample autoloader into a quartz capillary (diameter = 1.5 mm) and an Eiger2 4 M SAXS detector placed 5 m from the sample. Measurements were performed at 25 °C and 37 °C, controlled by setting the sample temperature in the loading plate to 25 °C (actual = 25.5 ± 0.1 °C) and 37 °C (actual = 36.8 ± 0.3 °C) for the temperature scan. For each sample, 400 frames were collected with an exposure time of 20 ms per frame over a q range of 0.0024–0.36 $Å^{-1}$. The 2D SAXS data were reduced, frame averaged, normalised and the background subtracted using the MatFRAIA algorithm provided by MAX IV [23].

2.5. Data modelling

SAXS data were fitted using the procedure described by Pal et al. [24]. The form factor is given by the square of the Fourier transform of the bilayer (electron density profile, EDP) according to Eq. (1) and illustrated by the theoretical EDP in Fig. 2. The EDP is described by two Gaussians of width σ_H, centered at $z = \pm z_H$, that represent the head groups, which are assumed to be similar, and a third Gaussian at the bilayer center ($z = 0$), representing the terminal methyl groups.

$$\rho(z) = \rho_{ch_2} + \overline{\rho}_h \left[e^{\left(-\frac{(z-z_H)^2}{2\sigma_H^2}\right)} + e^{\left(-\frac{(z+z_H)^2}{2\sigma_H^2}\right)} \right] + \overline{\rho}_c \left(e^{-\frac{z^2}{2\sigma_c^2}} \right) \quad (1)$$

The electron densities are defined relative to the methylene electron density. The bilayer thickness $d_B = 2(z_h + \sigma_h)$ and the water layer thickness $d_w = (d - d_B)$ can be estimated from the values of the model parameters obtained from the fits. For the structure factor in the multilamellar systems we have used a modified Caillé structure factor.

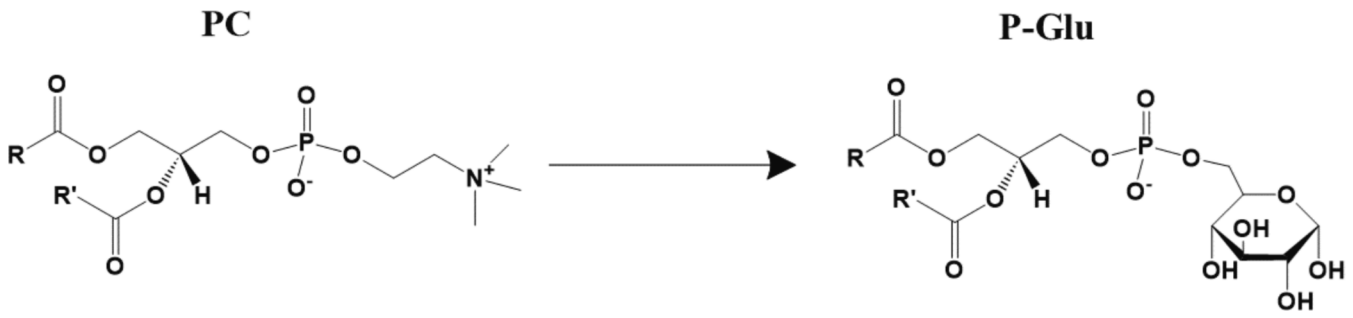

Fig. 1. Conversion of the natural lipid phosphatidylcholine (PC) to the modified one phosphatidylglucose (P-Glu). Glucose is used in excess as a second substrate and the reaction is catalysed by phospholipase D.

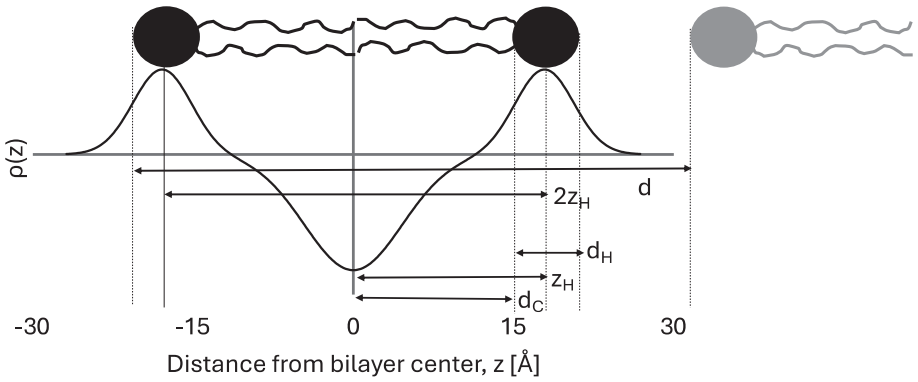

Fig. 2. Representation of a typical electron density profile, EDP, or ρ versus distance from the bilayer centre, z, of a symmetric lipid bilayer. The zero value of z is set to the midplane of the bilayer. The EDP is described by two Gaussians with full width at half maximum (FWHM) $d_H = 2\sigma_H$, centered at $z = \pm z_H$, that represent the head groups, which are assumed to be similar, and a third Gaussian at the bilayer center (z = 0), representing the terminal methyl groups. The bilayer is described by the hydrocarbon chain length d_C, the head group thickness d_H, the bilayer thickness d_B, the bilayer repeat distance d in multilayer systems/MLVs and the distance between the minimum and maxima of the electron density profile z_H. Particularly well defined is the peak to peak distance, $2z_H$ as discussed in the text. The bilayer thickness is defined as $d_B = 2(z_H + \sigma_H)$.

$$S(q) = N + 2\sum_{k=1}^{N-1}(N-k)\cos(kqd) \times e^{-(d/2\pi)^2 q^2 \eta\gamma}(\pi k)^{-(d/2\pi)^2 q^2 \eta} \quad (2)$$

Here N is the mean number of bilayers in the stack, while γ is the Euler's constant and d is the lamellar periodicity. The Caillé parameter is given by

$$\eta = \frac{q^2 k_B T}{8\pi\sqrt{KB}} \quad (3)$$

where K (= κ/d) and B are the bending and bulk moduli of the bilayer stack respectively.

2.6. In house WAXS measurements

Vesicle samples dispersed in Milli Q, used for synchrotron SAXS measurements, were additionally measured using wide angle x-ray scattering (WAXS) in order to study the fluidity of the bilayer. Measurements were performed on a SAXSLab Ganesha 300XL (SAXSLAB ApS, Skovlunde, Denmark) with a 2D 300 K Pilatus detector (Dectris) and Genix 3D x-ray source ($\lambda_{x-ray} = 1.54$ Å). The vesicle samples were sealed in reusable quartz capillaries and mounted in a temperature controlled sample holder, where the temperature is regulated by an external water bath. WAXS data were collected for 20 min at 25 °C over a q range of 0.6–2.8 $Å^{-1}$. The 2D data was reduced to the 1D radially averaged scattering pattern using the SAXSGui software and the solvent background was manually subtracted using MATLAB R2020b.

For data with sharp peaks (DPP-Glu, DPPC and DSPC), the position of the peak was determined using SasView (version 5.05, https://www.sasview.org/) by fitting with a Lorentzian peak (model name: peak_lorentz) in a limited q range around the peak. For each sample, all parameters were manually estimated, then allowed to fit simultaneously.

2.7. Cryo-TEM

The morphology of the vesicles was studied with a JEM-2200FS transmission electron microscope (nCHREM at Lund University), operated at 200 kV. The images were recorded with a TemCam-F416 camera (TVIPS) using SerialEM software. Cu grids coated with a lacey-carbon film (Ted Pella) were plasma cleaned and hydrophilized (GloCube Quorum), before 4 μl sample was applied and the sample was blotted for three seconds on the non-coated side to remove excess liquid. The grids were then plunged into liquid ethane (−184 °C) using an automatic plunge freeze system (Leica EM GP) for rapid vitrification. The plunged grids were stored in liquid nitrogen (−196 °C) until transferred by a cryotransfer tomography holder (Fischione Model 2550) for imaging under the microscope.

3. Results and discussion

3.1. Single component lipid vesicle dispersions in water

The phospholipid derivative DOP-Glu, which has not previously been fully characterised, as well as the novel analogues with palmitic acid or stearic acid acyl chains were for the first time synthesized and isolated in high enough quantities to prepare and physicochemically characterize aqueous vesicle dispersions. These lipids were compared with the three corresponding commercially available unmodified phosphatidylcholines. All lipid preparations, as well as the 1:1 (w/w) mixture of DOP-Glu/DOPC, were dispersed in water and sonicated to prepare vesicles. The vesicle sizes and morphologies were studied by dynamic light scattering (DLS, presented in SI), cryo-TEM and SAXS. The SAXS data were modelled to extract more detailed information regarding the type of structures formed and the effect of lipid modification on the bilayer parameters.

3.2. Vesicles prepared from PC lipids: morphology and bilayer properties

Lipid vesicles prepared from PC lipids were studied by SAXS and cryo-TEM and the data are shown in Fig. 3 and Table 2. Fitting of the SAXS data at 25 °C revealed that all lipids in the PC series formed primarily multilamellar vesicular (MLV) structures in excess aqueous solvent (Fig. 3A–F), as expected from previous studies [25–27]. The vesicle morphologies were studied by cryo-TEM imaging, as well, and the observations were in good agreement with the SAXS data. From cryo-TEM, DOPC vesicles were mostly present as spherical MLVs with a low number of correlated bilayers and a sub-population of smaller unilamellar vesicles (ULVs) (Fig. 3G). This agrees well with the broad peak visible in the SAXS data, corresponding to the bilayer form factor, which has a small but sharp peak visible at $q \approx 0.1$ $Å^{-1}$, corresponding to correlated bilayers (Fig. 3A).

In the cryo-TEM images for DPPC vesicles, MLVs were also observed, but with a higher number of bilayers in the MLV. In addition, the MLVs appear faceted (Fig. 3H), due to the crystalline state of the lipid chains at temperatures below their chain melting temperature ($T_m = 41$ °C[28]). The crystalline state of the lipid chains was further observed in WAXS data for this sample (Fig. 4A), in which a sharp peak at $q = 1.50 \pm 0.01$ $Å^{-1}$ (Table 3) was observed, corresponding to a repeat distance of 4.19 ± 0.03 Å as previously observed in the literature [29].

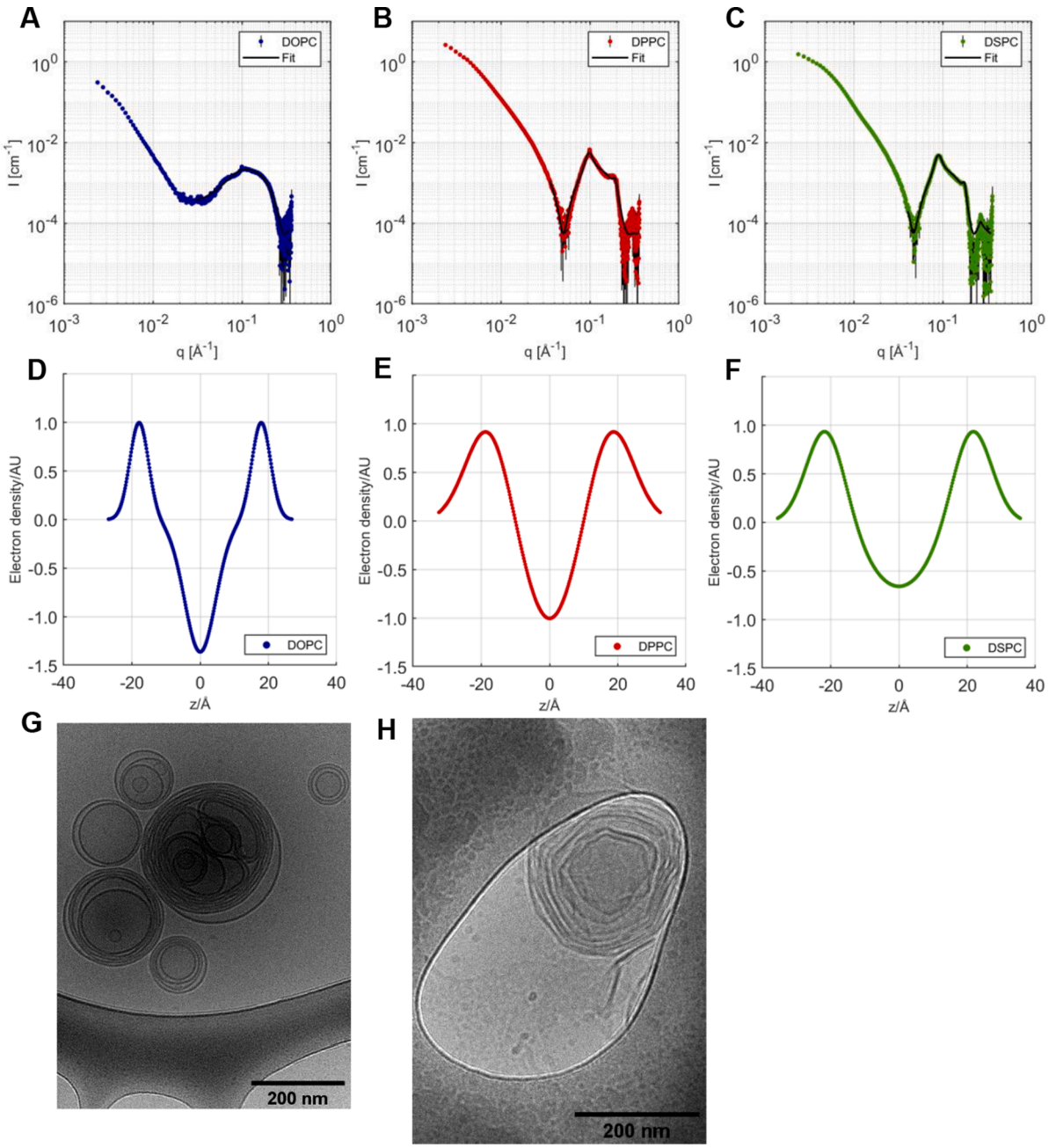

Fig. 3. SAXS and cryo-TEM data of unmodified PC lipids, DOPC (left), DPPC (middle) and DSPC (right). Top: SAXS scattering profiles (A-C), Middle: Electron density profiles (EDP, D-F)), Bottom: Cryo-TEM images (G-H).

Cryo-TEM imaging of stearic acid containing lipids (DSPC and DSP-Glu) was challenging due to the high viscosity of those samples, preventing proper blotting during sample preparation. For those lipids the structural analysis is based on SAXS data only. The SAXS data for DSPC and DPPC are very similar, indicating that MLVs with a high number of coordinated bilayers are also present for DSPC (Fig. 3B,C). In the WAXS data for DSPC (Fig. 4B), a sharp peak was observed (Table 3), indicating the expected crystalline state of the lipid chains for DSPC at this temperature (T_m = 55 °C) [28].

Due to the high contrast between phosphate groups in the lipid head group and the acyl chains, the bilayer thickness in terms of $2z_H$ can be calculated with high accuracy from the electron density profiles (EDP) based on the method described by Pabst et al. [30]. The value of $2z_H$ was directly extracted from the EDP as indicated in Fig. 2. The head-head spacing of PC esterified with different fatty acids increased in the order of oleic acid (18:1) < palmitic acid (16:0) < stearic acid (18:0), which is in agreement with their increasing effective chain-length and correlates well with results presented by Nagle and Tristan-Nagle [31].

In addition to the head-head spacing, the EDP can in principle be used to extract other bilayer parameters such as the thickness of the head group and acyl chain regions. The challenge is that for some systems the contrast between heads and tails is not high, as is the case here, and the modelling is not accurate enough to give such detailed insights into the bilayer structures [31].

In the case of multilamellar vesicles (MLVs), the lamellar repeat distance, d, can also be estimated from the SAXS data. In the PC series, d increased in the same manner as the bilayer thickness; oleic acid (18:1) < palmitic acid (16:0) < stearic acid (18:1) (Table 2).

Table 2
Properties of vesicles formed from PC and P-Glu lipids based on the SAXS and Cryo-TEM data.

Lipid sample	Dominant Morphology	2 z_H [Å]	d [Å]
Unmodified PC Lipids			
DOPC (18:1)	MLVs	35.8 ± 1.3	53.7 ± 0.5
DPPC (16:0)	MLVs	38.3 ± 1.1	65.1 ± 0.1
DSPC (18:0)	MLVs	42.8 ± 1.2	71.1 ± 0.1
Modified Lipids			
DOP-Glu (18:1)	ULVs	36.2 ± 1.3	
DPP-Glu (16:0)	Discs	48.8 ± 0.1	
DSP-Glu (18:0)	Stacks	52.6 ± 0.7	67.3 ± 0.1
Lipid Mixture 1:1 (w/w)			
DOP-Glu/DOPC	ULVs	33.8 ± 2.3	

3.3. *Vesicles prepared from P-Glu lipids: morphology and bilayer properties*

The structure of vesicles formed from the modified lipids in the P-Glu series varied depending on the acyl chain, and also had different morphologies compared to their unmodified analogues as shown in Table 2 and Fig. 5. Fig. 5 shows the SAXS curves for the P-Glu series (Fig. 5A–C) and the corresponding electron density profiles for the P-Glu series compared with the analogous PC lipids (Fig. 5D–F).

The extracted electron density profiles show differences in the bilayer structure as well as in the thickness of the lipid bilayer. The head-head spacing increased in the order of oleic acid (18:1) < palmitic acid (16:0) < stearic acid (18:0), which is similar to the observations for the unmodified lipids (Table 2). Comparison of the head-head spacing between the modified glucophospholipids and their corresponding PC analogues showed that the P-Glu lipids had a larger distance between the head groups in the lipids with the saturated chains, i.e. DPPC/DPP-Glu and DSPC/DSP-Glu (Table 2), resulting in thicker bilayers compared to their PC analogues. In the case of DOPC/DOP-Glu, there was no significant difference.

When comparing the morphology of the dispersions it is interesting to note that a dominant MLV structure was not observed for any of the glucophospholipids in contrast to their choline analogues, where MLVs were dominant for all lipids.

DOP-Glu vesicles are well-described by only the bilayer form factor, indicating that the dominant morphology is that of ULVs. Although there is possibly a minor contribution from MLVs, as the broad peak shape is not entirely symmetric, the sharp peak at q ≈ 0.1 $Å^{-1}$ visible for DOPC is no longer present. This agrees well with the cryo-TEM data, where smaller and more uniformly sized ULVs are visible (Fig. 5G). This change from MLVs to ULVs can be attributed to the structurally larger and more bulky hydrophilic part of the P-Glu, due to both the introduction of glucose and the net negative charge in the P-Glu lipids, in contrast to the choline head group and zwitterionic net neutral of the PC lipids.

The broad peak visible in the SAXS data for DPP-Glu can also be described well by the bilayer form factor, indicating there are no or only a low number of correlated bilayers present in the sample, unlike those observed for DPPC. However, the bilayers are not present as ULVs in this sample. In the cryo-TEM images, disc-like structures with a diameter of approx. 15–20 nm can be observed (Fig. 5H) and the lower q (q < 0.045 $Å^{-1}$) region of the data is well-described by fitting with an ellipsoidal model with polar radius of 61.14 ± 0.04 Å and equatorial radius of 175.89 ± 0.06 Å (Figure S1, Table S1). Such disc-like structures have been reported previously for ganglioside containing lipid samples [32]. These types of nanosized bilayer disc have been observed in mixed systems where the PLs form a bilayer and the other components accumulate around the high curvature edges [33]. Interestingly, similar types of non-vesicular structures was also observed for cryo-TEM of DPPC vesicles containing 5 mol % DOPE-PEG2000 together with highly

Table 3
Peak position and calculated d spacing for the gel phase vesicle samples. The error in peak position is from the instrument resolution (as the fit error was lower) and in d spacing was from propagation of the peak position error.

Sample	Peak position/$Å^{-1}$	d spacing/Å
DPP-Glu	1.51 ± 0.01	4.16 ± 0.03
DPPC	1.50 ± 0.01	4.19 ± 0.03
DSP-Glu	N/A	
DSPC	1.51 ± 0.02	4.16 ± 0.06

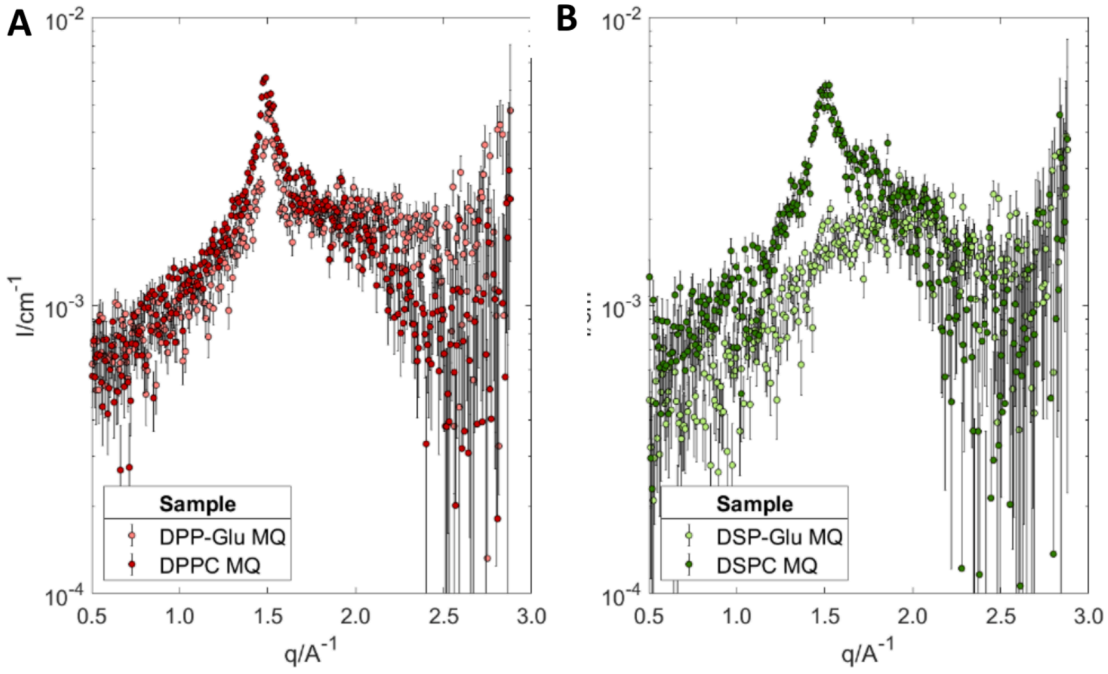

Fig. 4. WAXS data measured for vesicle samples at 25 °C. (A) DPPX and (B) DSPX where X =C or Glu.

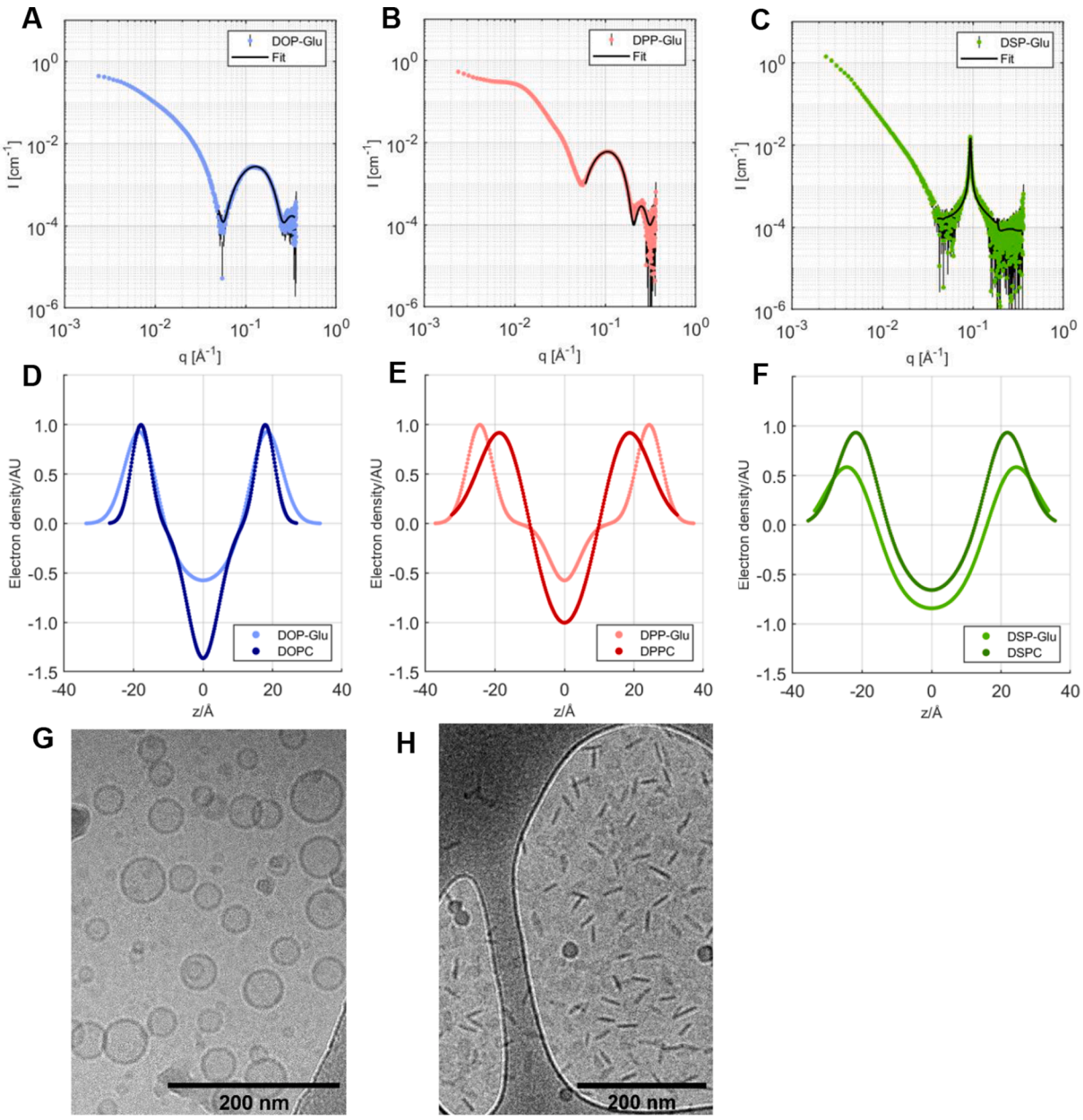

Fig. 5. SAXS data and cryo-TEM profile of vesicles formed from glucose modified lipids: DOP-Glu, DPP-Glu, DSP-Glu. Top: SAXS scattering profiles (A-C); Middle: Electron density profiles (EDPs, D-F)) comparing the glucose modified lipids with the corresponding phosphatidylcholine lipids; Bottom: Cryo-TEM image of DOP-Glu (G) and DPP-Glu (H) dispersions.

faceted vesicular structures [34]. The sharp peak observed in the WAXS data for DPPC is also present for this sample at the same position (Fig. 4A, Table 3), indicating that the lipid chains are crystalline.

Unlike the other glucose-based lipids, DSP-Glu is the only modified lipid with correlated bilayers, present mostly as bilayer stacks with a minor sub-population of MLVs. Interestingly the d spacing for DSP-Glu was similar to that of DSPC, although the bilayer is thicker. This could possibly be a result of the difference in curvature between the different morphologies, DSPC formed MLVs, whereas DSP-Glu formed more bilayer stacks. No peak was visible in the WAXS of the DSP-Glu sample (Fig. 4B). This was most likely due to insufficient concentration of lipid, as the sample data matched the buffer background. For this sample, there were also considerable issues with precipitation and sedimentation leading to loss of sample.

3.4. *Mixed DOPC/DOP-Glu lipid vesicle dispersion in water*

From the studies of single component lipid vesicles, it is clear that the exchange of choline with glucose in the phospholipid head group changes the lipid bilayer. One interesting question is to determine how the degree of glycosylation affects the bilayer properties. For this purpose, we investigated vesicles prepared from a 1:1 (w/w) mixture of DOP-Glu and DOPC, which corresponds to a molar ratio of 1.1:1 DOPC/DOP-Glu and the results are shown in Fig. 6.

Although there is no clear sharp peak indicating the presence of correlated bilayers, there is a plateau on the top of the bilayer form factor peak, indicating the contribution of another structure (Fig. 6A). This shows that the addition of DOP-Glu to DOPC changed the vesicle morphology from mostly MLVs to mostly ULVs, although a small number of MLVs were still observed by cryo-TEM imaging. The ULVs were similar in appearance, and size compared to the vesicles observed for the

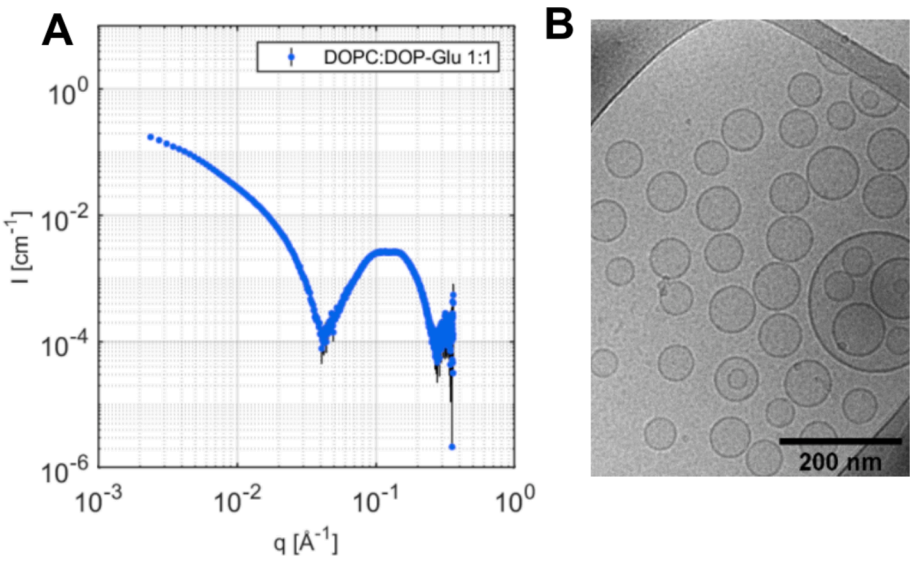

Fig. 6. SAXS (A) and cryo-TEM (B) data of a mixture of DOP-Glu/DOPC (1:1 w/w) at 25 °C in water.

pure DOP-Glu sample (Fig. 5G). This shows that also a smaller fraction of glycosylated/negatively charged lipid alters the vesicle morphology towards ULVs. This can be compared to a similar change from MLV to ULV observed due to the presence of another negatively charged lipid, phosphatidyl serine (PS), to PC [35]. A transition from MLVs towards ULVs can be beneficial, since MLVs usually have a limited loading capacity and have a shorter circulation time *in vivo* compared to ULVs due to their rapid clearance from circulation.

3.5. Effect of ionic strength on vesicle structure

As discussed above, glycosylation introduces a negative net charge to the lipid. In order to investigate the effect of the charge screening, the vesicle samples were also studied in PBS.

For all PC lipids, a minor increase in definition of the correlation peak, indicating more ordered bilayers, was observed for the samples in PBS compared to Milli Q. This effect was more pronounced for DOPC and DPPC than DSPC (Figure S5). We also note that for DPPC two sharp peaks are now clearly visible in the presence of PBS. The bilayer thicknesses did not change significantly within the limits of errors as judged from the peak position in the scattering curves with the increased ionic strength, indicating that the changes are due to interbilayer interactions.

A shift to higher q of the bilayer correlation peak from 0.0995 ± 0.0003 Å^{-1} in MQ to 0.113 ± 0.0003 Å^{-1} in PBS can be observed for DOPC, corresponding to a decrease in inter-bilayer distance.

The effect of ionic strength on bilayers from zwitterionic phosphatidylcholine lipids of varying chain length has previously been investigated by Petrache et al. [36]. Their x-ray diffraction data showed that there were no changes with added salt for the membrane structure, as we also observed, or bending rigidity. However, their data on lamellar stacks and multilamellar vesicles showed that they swell in 100 mM KBr and KCl, primarily due to weakening of the van der Waals attraction between neighbouring bilayers.

However for DOPC vesicles, del Mel et al. observed an increase in bending rigidity with increasing salt concentration from 0 to 450 mM NaCl based on data from neutron spin echo (NSE) spectroscopy, accompanied by an increase in bilayer thickness at intermediate salt concentrations as observed by SAXS [37].

For DOP-Glu (Figure S5A), for which there were no correlated bilayers in the sample, there was no significant difference in the data between the samples in PBS or Milli Q. For DSP-Glu, however, there was a minor broadening of the bilayer correlation peak in PBS indicating a decrease in order of the bilayer stack (Figure S5C).

The limited salt effects show that the major effect on bilayer properties is due to the glucose modification rather than the net negative charge of the modified lipids.

3.6. Effect of temperature

The SAXS experiments were carried out at 25 °C and 37 °C, and the results are shown in Fig. S6. Based on the scattering profiles, no clear difference could be detected for either the PC or the modified glucose conjugated lipids at the different temperatures.

Kucerka et al. [38] have studied the effect of temperature on bilayer thickness of many of the most common PCs and found that the bilayer thickness decreases linearly with increased temperature (20-60 °C). This effect was most noticeable for saturated lipids (up to −0.083 Å/°C) and a weaker, but still noticeable correlation (up to −0.056 Å/°C) was seen for monounsaturated lipids. Szekely et al, 2011 [39] observed a similar trend when they studied bilayer thickness of charged PL (PS) as function of temperature (10–80 °C) with −0.049 Å/°C for saturated DLPS and −0.038 Å/°C for monounsaturated DOPS. Due to the limited temperature range in the present study, no clear temperature effects were to be expected and this is also what the results showed.

4. Conclusions

Phospholipids are used for a variety of applications, including being key components of pharmaceutical formulations, often having the function to form vesicles (liposomes). In the vast majority of the applications, the phospholipid used is PC and, in a few cases, other common and commercially available phospholipids, such as phosphatidylethanolamine or phosphatidylglycerol [14]. Relatively poor stability to storage and various types of handling constitutes a severe limitation [7]. The methodology used to stabilize the formulations is to use additives such as cholesterol or polyethylene glycol modified lipids [9,10], but the use of a more well-defined phospholipid with more favorable intrinsic properties would be highly attractive. We here note that polyethylene glycol lipids can be rather polydisperse and cannot be synthesized with mild enzymatic methods. The promising observation has been made that vesicles prepared from P-Glu have better storage stability than the corresponding PC based vesicles [19]. Furthermore, they had excellent stability towards dehydration/rehydration cycles. However, the P-Glu product used in that study was synthesized from technical grade soybean PC and contained a complex mixture of fatty acids (originating from soybean) and thus the influence of the nature of the acyl groups could

not be deduced. Furthermore, the vesicles prepared were just superficially characterized. The wealth of applications of PC is made possible by the numerous studies of the fundamental properties of vesicles prepared from PC using methods such as SAXS and cryo-TEM [26,27,37]. For most other phospholipids, information from such studies is scarce and in the case of P-Glu, it is totally lacking.

In the present study, for the first time, P-Glu with three different acyl chains was synthesized and purified in sufficient amounts to properly characterize them and the vesicles prepared thereof. The corresponding PCs containing the same acyl groups were studied for comparison. For the first time, vesicles prepared of P-Glu were studied using SAXS, WAXS, and cryo-TEM. The introduction of glucose in the phospholipid head group to replace choline had a large effect on vesicle morphology. The vesicles got smaller in size and the typical MLVs of DOPC transformed to smaller ULVs with DOP-Glu. The same trend was observed when mixing DOPC with DOP-Glu in a 1:1 (w/w) mixture. The transition from MLV to ULV can be regarded as a consequence of the swelling limit of bilayer microstructures as discussed in the review of Dubois and Zemb [40], where they discuss the transformation between different microstructures formed by bilayers. In MLVs, as in lamellar liquid crystalline phases, the bilayers of phosphatidylcholine are stacked together at a certain separation that can be regarded as a balance between attractive van der Waals forces and the repulsion through the entropy gain from fluctuation of the membrane [41]. The shift from MLVs to ULVs has been observed previously by addition of anionic phospholipids to PC [34], which suggests that electrostatic repulsion can also contribute. In the present study, the ULV forming lipid P-Glu carries a net charge and hence electrostatics contribute to repulsive forces that decouple the bilayers in the MLV. However, we saw a limited effect of increasing the ionic strength that would screen the electrostatic repulsion. Instead, it is likely that the introduction of the more hydrated sugar group might increase the flexibility of the bilayer, which in turn increases the undulation forces preventing the formation of multilayer structures. It is also possible that the attractive van der Waals forces are weaker between DOP-Glu bilayers. The acyl groups had dramatic effects on vesicle morphology and bilayer thickness of vesicles prepared from the glucose conjugated lipids. DOP-Glu formed ULVs while DPP-Glu formed thin disc-like structures and DSP-Glu formed an increased portion of bilayer stacks rather than MLVs. This dramatic change in the morphology of the lipid assemblies can most probably be attributed to a balance of interactions from the increased size of the head group that follows from the conjugation with glucose as well as the change in head-head interactions with the increased hydrogen bonding capacity of the glucose head group. Effects of the fatty acid component of P-Glu on the self assembly behaviour has not been studied before, and it is interesting to note that the fatty acids have a much larger impact on the morphology of structures formed by P-Glu than in the case of PC. Only minute effects of ionic strength and temperature were observed for P-Glu vesicles, which indicates robustness and can make them valuable in future applications.

Further detailed studies of phospholipids with various carbohydrates in their polar head group are warranted since even slight changes in hydrogen bonding patterns can influence the self-assembly behaviour. Already variation of the acyl groups produced a variety of structures and variation of the carbohydrate part is expected to induce even larger structural diversity. It is also highly motivated to study the colloidal stability of the vesicles based on various carbohydrate-containing phospholipids with well-defined fatty acid parts, as well as their applicability in the formulation of pharmacologically relevant cargos.

CRediT authorship contribution statement

Nikolina Barchan: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jennifer Gilbert:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Antara Pal:** Writing – review & editing, Formal analysis, Data curation. **Tommy Nylander:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Patrick Adlercreutz:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2024.11.153.

Data availability

Data will be made available on request.

References

[1] A.G. Kohli, P.H. Kierstead, V.J. Venditto, C.L. Walsh, F.C. Szoka, Designer lipids for drug delivery: From heads to tails, Journal of Controlled Release 190 (2014) 274–287, https://doi.org/10.1016/j.jconrel.2014.04.047.
[2] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. 4 (2) (2005) 145–160.
[3] A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: classification, preparation, and applications, Nanoscale Res. Lett. 8 (2013) 1–9.
[4] P. van Hoogevest, H. Tiemessen, J.M. Metselaar, S. Drescher, A. Fahr, The use of phospholipids to make pharmaceutical form line extensions, Eur. J. Lipid Sci. Technol. 123 (4) (2021) 2000297.
[5] P. Van Hoogevest, A. Wendel, The use of natural and synthetic phospholipids as pharmaceutical excipients, Eur. J. Lipid Sci. Technol. 116 (9) (2014) 1088–1107.
[6] O. Bogojevic, J.V. Nygaard, L. Wiking, C. Arevång, Z. Guo, Designer phospholipids–structural retrieval, chemo-/bio-synthesis and isotopic labeling, Biotechnol. Adv. 60 (2022) 108025.
[7] T.M. Allen, P.R. Cullis, Liposomal drug delivery systems: from concept to clinical applications, Adv. Drug Deliv. Rev. 65 (1) (2013) 36–48.
[8] M. Grit, D.J. Crommelin, Chemical stability of liposomes: implications for their physical stability, Chem. Phys. Lipids 64 (1–3) (1993) 3–18.
[9] J. De Gier, J.G. Mandersloot, L.L.M. Van Deenen, Lipid composition and permeability of liposomes, Biochim. Biophys. Acta (BBA) – Biomembr. 150 (4) (1968) 666–675, https://doi.org/10.1016/0005-2736(68)90056-4.
[10] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, FEBS Lett. 268 (1) (1990) 235–237.
[11] L. Nobs, F. Buchegger, R. Gurny, E. Allémann, Current methods for attaching targeting ligands to liposomes and nanoparticles, J. Pharm. Sci. 93 (8) (2004) 1980–1992.
[12] Y. Lee, D. Thompson, Stimuli-responsive liposomes for drug delivery, Wiley Interdiscipl. Rev.: Nanomed. Nanobiotechnol. 9 (5) (2017) e1450.
[13] P.T. Ingvarsson, M. Yang, H.M. Nielsen, J. Rantanen, C. Foged, Stabilization of liposomes during drying, Exp. Opin. Drug Deliv. 8 (3) (2011) 375–388.
[14] J. Li, X. Wang, T. Zhang, C. Wang, Z. Huang, X. Luo, Y. Deng, A review on phospholipids and their main applications in drug delivery systems, Asian J. Pharmaceut. Sci. 10 (2) (2015) 81–98.

[15] J. Damnjanovic, Y. Iwasaki, Phospholipase D as a catalyst: application in phospholipid synthesis, molecular structure and protein engineering, J. Biosci. Bioeng. 116 (3) (2013) 271–280, https://doi.org/10.1016/j.jbiosc.2013.03.008.
[16] Y. Yamamoto, M. Hosokawa, H. Kurihara, K. Miyashita, Preparation of phosphatidylated terpenes via phospholipase d-mediated transphosphatidylation, J. Am. Oil Chem. Soc. 85 (4) (2008) 313.
[17] X. Tao, N. Jia, N. Cheng, Y. Ren, X. Cao, M. Liu, D. Wei, F.-Q. Wang, Design and evaluation of a phospholipase D based drug delivery strategy of novel phosphatidyl-prodrug, Biomaterials 131 (2017) 1–14, https://doi.org/10.1016/j.biomaterials.2017.03.045.
[18] S. Kawamura, Y. Ito, T. Hirokawa, E. Hikiyama, S. Yamada, S. Shuto, Ligand–phospholipid conjugation: a versatile strategy for developing long-acting ligands that bind to membrane proteins by restricting the subcellular localization of the ligand, J. Med. Chem. 61 (9) (2018) 4020–4029, https://doi.org/10.1021/acs.jmedchem.8b00041.
[19] S. Song, L.Z. Cheong, M. Falkeborg, L. Liu, M. Dong, et al., Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability, PLOS ONE 8 (9) (2013) e73891, https://doi.org/10.1371/journal.pone.0073891.
[20] H. Wu, H. Zhang, X. Li, F. Secundo, X. Mao, Preparation and characterization of phosphatidyl-agar oligosaccharide liposomes for astaxanthin encapsulation, Food Chemistry 404 (2023) 134601, https://doi.org/10.1016/j.foodchem.2022.134601.
[21] N. Barchan, P. Adlercreutz, Synthesis of glycophospholipid conjugates with mono- and disaccharides by enzymatic transphosphatidylation, Eur. J. Lipid Sci. Technol. (2024).
[22] A.P. Dabkowska, C.S. Niman, G. Piret, H. Persson, H.P. Wacklin, H. Linke, C. N. Prinz, T. Nylander, Fluid and highly curved model membranes on vertical nanowire arrays, Nano Lett. 14 (8) (2014) 4286–4292.
[23] A.B. Jensen, T.E.K. Christensen, C. Weninger, H. Birkedal, Very large-scale diffraction investigations enabled by a matrix-multiplication facilitated radial and azimuthal integration algorithm: MatFRAIA, J. Synchrotron Radiat. 29 (2022) 1420–1428, https://doi.org/10.1107/S1600577522008232.
[24] A. Pal, P. Bharath, S.G. Dastidar, V.A. Raghunathan, Thermal unbinding and ordering of amphiphile bilayers in the presence of salt, J. Colloid Interface Sci. 402 (2013) 151–156, https://doi.org/10.1016/j.jcis.2013.03.063.
[25] G. Pabst, R. Koschuch, B. Pozo-Navas, M. Rappolt, K. Lohner, P. Laggner, Structural analysis of weakly ordered membrane stacks, Journal of Applied Crystallography 36 (6) (2003) 1378–1388, https://doi.org/10.1107/S0021889803017527.
[26] J.F. Nagle, R. Zhang, S. Tristram-Nagle, W. Sun, H.I. Petrache, R.M. Suter, X-ray structure determination of fully hydrated L alpha phase dipalmitoylphosphatidylcholine bilayers, Biophys. J . 70 (3) (1996) 1419–1431.
[27] S. Tristram-Nagle, H.I. Petrache, J.F. Nagle, Structure and interactions of fully hydrated dioleoylphosphatidylcholine bilayers, Biophys. J . 75 (2) (1998) 917–925.
[28] M. Caffrey, J. Hogan, Lipidat - a database of lipid phase-transition temperatures and enthalpy changes - DMPC data subset analysis, Chem. Phys. Lipids 61 (1) (1992) 1–109, https://doi.org/10.1016/0009-3084(92)90002-7.
[29] T.J. McIntosh, Effect of cholesterol on structure of phosphatidylcholine bilayers, Biochim. Biophys. Acta 513 (1) (1978) 43–58, https://doi.org/10.1016/0005-2736(78)90110-4.
[30] G. Pabst, M. Rappolt, H. Amenitsch, P. Laggner, Structural information from multilamellar liposomes at full hydration: Full q-range fitting with high quality x-ray data, Physical Review E 62 (3) (2000) 4000–4009, https://doi.org/10.1103/PhysRevE.62.4000.
[31] J.F. Nagle, S. Tristram-Nagle, Structure of lipid bilayers, Biochim. Biophys. Acta (BBA)-Rev. Biomembr. 1469 (3) (2000) 159–195.
[32] E.H. Mojumdar, C. Grey, E. Sparr, Self-assembly in ganglioside–phospholipid systems: the co-existence of vesicles, micelles, and discs, Int. J. Mol. Sci. 21 (1) (2019) 56.
[33] E. Johansson, A. Lundquist, S.S. Zuo, K. Edwards, Nanosized bilayer disks: attractive model membranes for drug partition studies, Biochim. Et Biophys. Acta-Biomembr. 1768 (6) (2007) 1518–1525, https://doi.org/10.1016/j.bbamem.2007.03.006.
[34] V. Nele, M.N. Holme, U. Kauscher, M.R. Thomas, J.J. Doutch, M.M. Stevens, Effect of formulation method, lipid composition, and PEGylation on vesicle lamellarity: a small-angle neutron scattering study, Langmuir 35 (18) (2019) 6064–6074, https://doi.org/10.1021/acs.langmuir.8b04256.
[35] N. Kučerka, J. Pencer, J.N. Sachs, J.F. Nagle, J. Katsaras, Curvature Effect on the Structure of Phospholipid Bilayers, Langmuir 23 (3) (2007) 1292–1299, https://doi.org/10.1021/la062455t.
[36] H.I. Petrache, S. Tristram-Nagle, D. Harries, N. Kucerka, J.F. Nagle, V.A. Parsegian, Swelling of phospholipids by monovalent salt, J. Lipid Res. 47 (2) (2006) 302–309.
[37] J.U. De Mel, S. Gupta, R.M. Perera, L. Ngo, P. Zolnierczuk, M. Bleuel, S.V. Pingali, G.J. Schneider, Influence of external NaCl salt on membrane rigidity of neutral DOPC vesicles, Langmuir 36 (32) (2020) 9356–9367, https://doi.org/10.1021/acs.langmuir.0c01004.
[38] N. Kučerka, M.-P. Nieh, J. Katsaras, Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidylcholines as a function of temperature. Biochimica et Biophysica Acta (BBA) -, Biomembranes 1808 (11) (2011) 2761–2771, https://doi.org/10.1016/j.bbamem.2011.07.022.
[39] P. Szekely, T. Dvir, R. Asor, R. Resh, A. Steiner, O. Szekely, A. Ginsburg, J. Mosenkis, V. Guralnick, Y. Dan, Effect of temperature on the structure of charged membranes, J. Phys. Chem. B 115 (49) (2011) 14501–14506.
[40] M. Dubois, T. Zemb, Swelling limits for bilayer microstructures: the implosion of lamellar structure versus disordered lamellae, Curr. Opin. Colloid Interface Sci. 5 (1–2) (2000) 27–37, https://doi.org/10.1016/S1359-0294(00)00032-7.
[41] H. Wennerstrom, The unbinding transition and lamellar phase lamellar phase coexistence, Langmuir 6 (4) (1990) 834–838, https://doi.org/10.1021/la00094a020.