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Phospholipase D catalysed synthesis and characterization

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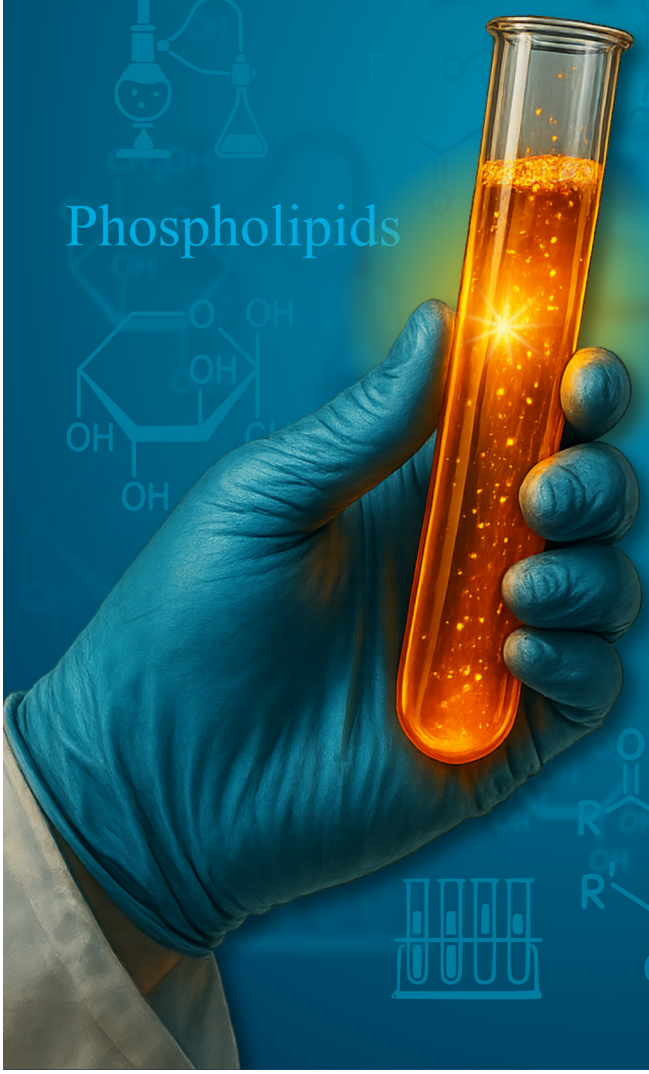

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Nikolina Barchan

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UNIVERSITY

DOCTORAL DISSERTATION

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Abstract: Phospholipids are amphiphilic molecules that self-assemble into organized structures such as bilayers and vesicles when dispersed in aqueous solutions. This property in addition to their biocompatibility, biodegradability, and natural abundance in high titres makes phospholipids highly attractive as formulation excipients for a wide range of biological and technical applications. However, isolating phospholipids other than the commonly occurring phosphatidylcholine is challenging, particularly concerning the hydrophobic tails that often come in a variety of lengths and degree of unsaturation. This can be limiting in their use in technically advanced formulations that demand precise and reproducible chemical specifications. To address this, the modification of phospholipid head groups has emerged as a valuable strategy, not only for enriching natural phospholipids but also for introducing novel functionalities with tailored properties. Such modifications has the potential to enable refined formulations with enhanced characteristics including improved drug delivery, higher encapsulation efficiency of pharmaceutical ingredients, and better colloidal stability.

In this thesis, we investigate the structural modification of phospholipid head groups through the conjugation of small carbohydrates. Using a phospholipase D (PLD)-catalysed transphosphati-dylation reaction, we successfully conjugated various carbohydrates to the phospholipid head group. The resulting glycophospholipid conjugates were characterized for their self-assembly behaviour using small-angle X-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryo-TEM). Our findings reveal that head group modifications have a profound impact on lipid self-assembly. The type of carbohydrate in the head group significantly influenced vesicle structures, resulting in variations in size and lamellarity. Small changes in the orientation of hydroxyl groups, as dictated by different carbohydrates, induced substantial differences in vesicle morphology. The use of PLD catalyzed enzymatic transphosphatidylation represents a powerful strategy for synthesis of novel glycophospholipid materials, which are otherwise difficult to achieve through traditional synthetic methods. By harnessing the complex nature and structurally rich diversity of carbohydrates, this approach enables the synthesis of an extensive variety of glycophospholipid conjugates with distinct properties. This work demonstrates an effective method for designing advanced lipid-based materials tailored for diverse applications such as drug delivery, nanotechnology, and biomedicine, where precise control of self-assembly and functionality is essential.

Key words: glycophospholipid, transphosphatidylation, phospholipase D, polar lipid, lipid self-assembly, carbohydrate

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Till alla mina nära och kära

Table of Contents

Abstract

Phospholipids are amphiphilic molecules that self-assemble into organized structures such as bilayers, which gives lamellar phases at low water content and vesicles when dispersed in excess aqueous solutions. This property in addition to their biocompatibility, biodegradability, and natural abundance in high titres makes phospholipids highly attractive as formulation excipients for a wide range of biological and technical applications. However, isolating phospholipids other than the commonly occurring phosphatidylcholine is challenging, particularly concerning the hydrophobic tails that often come in a variety of lengths and degree of unsaturation. This can be limiting in their use in technically advanced formulations that demand precise and reproducible chemical specifications.

To address this, the modification of phospholipid head groups has emerged as a valuable strategy, not only for enriching natural phospholipids but also for introducing novel functionalities with tailored properties. Such modifications have the potential to enable refined formulations with enhanced characteristics including improved drug delivery, higher encapsulation efficiency of pharmaceutical ingredients, and better colloidal stability.

In this thesis, we investigate the structural modification of phospholipid head groups through the conjugation of small carbohydrates. Using a phospholipase D (PLD)-catalysed transphosphatidylation reaction, we successfully conjugated various carbohydrates to the phospholipid head group. The resulting glycophospholipid conjugates were characterized for their self-assembly behaviour using small-angle X-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryo-TEM). Our findings reveal that head group modifications have a profound impact on lipid self-assembly. The type of carbohydrate in the head group significantly influenced vesicle structures, resulting in variations in size and lamellarity. Small changes in the orientation of hydroxyl groups, as dictated by different carbohydrates, induced substantial differences in vesicle morphology.

The use of PLD catalyzed enzymatic transphosphatidylation represents a powerful strategy for synthesis of novel glycophospholipid materials, which are otherwise difficult to achieve through traditional synthetic methods. By harnessing the complex nature and structurally rich diversity of carbohydrates, this approach enables the synthesis of an extensive variety of glycophospholipid conjugates with distinct properties. This work demonstrates an effective method for designing advanced lipid-based materials tailored for diverse applications such as drug delivery, nanotechnology, and biomedicine, where precise control of self-assembly and functionality is essential.

Popular Science Summary

You have probably heard the saying "like dissolves like" meaning that oil and water cannot be mixed into a homogeneous solution. Even with vigorous shaking, the oil droplets will just disperse temporarily, resulting in a cloudy mixture. After a few minutes, the oil droplets have merged into larger droplets and eventually formed a separate oil phase that floats on top of the water. But, as with so much else in this world, there is a grey area between those extreme opposites of hydrophilic substances, such as water, and hydrophobic substances, such as oil (or lipids, as the scientific name is, and includes all substances that are insoluble in water, but can be dissolved in organic solvents). Amphiphilic molecules fall into this grey area; they possess properties from both sides. They have a water-loving hydrophilic part and a water-repellent hydrophobic part. This unique duality allows them to interact with both hydrophilic and hydrophobic substances, facilitating the combination of oil and water into reasonably stable colloidal systems. Amphiphiles achieve this by orienting their hydrophilic parts towards the water and their hydrophobic parts towards the oil. By this arrangement, they create structures that can be useful for various applications.

A classic example of this is mayonnaise, which is an emulsion between oil and water that is stabilized by the amphiphilic phospholipids naturally found in egg yolk. This gives the mayonnaise a smooth, lovely texture and keeps it stable for months before it starts to separate.

Phospholipids also make up majority of the cell membrane, the protective barrier surrounding all cells. They create a bilayer with their hydrophilic parts facing outwards and the hydrophobic parts hidden inside the bilayer. Essentially, the phospholipids form small capsules that enclose all the cell components. This concept can be extended to the creation of artificial cell membranes, that instead of enclosing cell material, can be loaded with other interesting components, such as drugs. This type of spherical assemblies is called liposomes and constitute a very attractive method for drug formulation and delivery, not least in cancer treatment [1-4]. They protect the sensitive active ingredients of drugs from the body’s aggressive immune system. Additionally, because phospholipids are well-known to the body, they reduce the risk of triggering an immune response.

Isolation of the desired type of phospholipids from natural sources such as egg yolks or seed oils can be challenging, especially when high purity is required. Additionally, liposomes made from these natural phospholipids sometimes suffer from stability problems. To address these challenges, selected parts of the phospholipids can be modified, and small adjustments can be made in their chemical structure. These modifications can introduce new properties, such as enhanced colloidal stability, improved formulation characteristics, or increased purity.

In this thesis, I investigate how enzymes can be used to modify the hydrophilic, water-loving part of the phospholipid to create a new and relatively unexplored type of phospholipids called glycophospholipids. For those familiar with the terminology, the word "glyco" indicates modifications that involve carbohydrates (sugars). These modifications confer a negative charge to the phospholipid, which contributes to increase colloidal stability of the liposomes. Additionally, these modified phospholipids interact differently with each other compared to their natural counterparts, resulting in different liposome structures and characteristics. The objective of this work was to synthesize and purify these new glycophospholipids and investigate their chemical structures in detail. By examining how they self-assemble into different liposomal structures and evaluate their characteristics, we contribute to the fundamental understanding of these molecules. This knowledge is essential to evaluate their potential role as future drug formulating excipients.

Populärvetenskaplig sammanfattning

Du har säkert hört att ”lika löser lika” vilket innebär att olja och vatten inte kan blandas till en homogen lösning. Trots kraftig omskakning kommer oljedropparna endast finfördelas tillfälligt, och resultera i en grumlig blandning. Efter några minuter har oljedropparna gått samman till större droppar och slutligen bildat en separat oljefas som flyter ovanpå vattnet. Men, som för så mycket annat här i världen, finns det en gråskala mellan dessa extrema motsatser av hydrofila ämnen, såsom vatten, och hydrofoba ämnen, som olja (eller lipider, som det vetenskapliga namnet är, och innefattar alla ämnen som är olösliga i vatten, men går att lösa i organiskt lösningsmedel). I denna gråskala ingår amfifila molekyler; det vill säga molekyler som besitter egenskaper från båda sidor. De har en vattenälskande hydrofil del och en vattenskyende hydrofob del. Detta gör att de kan interagera med både hydrofila och hydrofoba substanser och möjliggör att olja och vatten kan kombineras till någorlunda stabila kolloidala system. Detta sker genom att amfililerna riktar sina vattenälskande delar mot vattnet och de vattenskyende delarna mot oljan. På så vis bildas strukturer som kan vara väldigt användbara för olika applikationer.

Ett klassiskt exempel på detta är majonnäs, vilket är en emulsion mellan olja och vatten som stabiliseras av de amfilila fosfolipiderna som finns naturligt i äggula. Detta ger majonnäsen en len, härlig konsistens och gör att den håller sig stabil i flera månader innan den börjar separera.

Fosfolipider utgör även merparten av cellmembranet som är den skyddande barriär som finns runt alla celler. Där bildar de ett dubbellager med sina hydrofila delar utåt och de hydrofoba delarna gömda inuti dubbellagret. Man skulle kunna uttrycka det som att fosfolipiderna utgör små kapslar som innesluter cellens alla komponenter. Detta koncept kan vidare användas för att skapa artificiella cellmembran, som istället för att innesluta cellmaterial, kan fyllas med andra intressanta komponenter, t ex läkemedel. Denna typ av kapslar kallas för liposomer och utgör en mycket attraktiv metod för att formulera och administrera läkemedel, inte minst inom cancerbehandling [1-4]. Liposomerna innesluter de känsliga aktiva ingredienserna i läkemedlet och skyddar dem mot de aggressiva försvarsmekanismer som vårt immunförsvar utgör. Dessutom är fosfolipider välkända för kroppen, vilket minskar risken för att de ska trigga en immunrespons.

Det kan vara svårt att isolera den önskade typen av fosfolipider från naturliga källor såsom äggulor eller fröoljor, i synnerhet när det ställs höga krav på renheten av dessa ämnen. Ibland lider även liposomer från dessa naturliga fosfolipider av stabilitetsproblem. För att komma till bukt med detta kan utvalda delar av fosfolipiderna modifieras med små justeringar i deras kemiska uppbyggnad. På så vis kan nya egenskaper introduceras, vilket bland annat kan ge ökad kolloidal stabilitet, förbättrade formuleringsegenskaper eller ökad renhet.

I den här avhandlingen undersöker jag hur man med hjälp av enzymer kan modifiera den hydrofila, vattenälskande delen av fosfolipiden för att generera en ny och nästan helt outforskad typ av fosfolipider, nämligen glykofosfolipider. För den insatte avslöjar ordet ”glyko” att det rör sig om modifieringar som innefattar kolhydrater (socker). Dessa modifieringar resulterar i att fosfolipiderna får en negativ laddning, vilket bidrar till att öka den kolloidala stabiliteten. Detta gör även att fosfolipiderna interagerar annorlunda med varandra jämfört med de naturliga fosfolipiderna, vilket leder till att deras liposomstrukturer ser ut, och beter sig annorlunda jämfört med deras naturliga motsvarigheter. Arbetet i den här avhandlingen syftar till att syntetisera och rena upp dessa nya glykofosfolipider, samt att undersöka deras kemiska uppbyggnad i detalj. Detta för att se hur den nya kemiska strukturen påverkar deras liposomstrukturer när de blandas i vatten. Detta är en del av den fundamentala grundforskning som är nödvändig för att förstå vilka egenskaper dessa nya molekyler besitter och är avgörande för att utvärdera vilken roll de kan spela i utformningen av framtidens läkemedelsexcipienter.

List of Publications

I. **Synthesis of glycophospholipid conjugates with mono- and disaccharides by enzymatic transphosphatidylation**
Nikolina Barchan, Patrick Adlercreutz
European Journal of Lipid Science and Technology, vol 126, Issue 3, 2024

II. **Chemical structure determination of novel glycophospholipids**
Nikolina Barchan, Zoltan Tacaks, Patrick Adlercreutz, 2025
(Manuscript)

III. **Structure and morphology of vesicular dispersions based on novel phosphatidyl glucose and phosphatidyl choline with different acyl chains**
Nikolina Barchan, Jennifer Gilbert, Antara Pal, Tommy Nylander, Patrick Adlercreutz
Journal of Colloid and Interface Science, vol 682, pp. 94-103, 2025

IV. **Structure and morphology of vesicular dispersions based on novel glycophospholipid conjugates with different monosaccharide head groups**
Nikolina Barchan, Jennifer Gilbert, Antara Pal, Tommy Nylander, Patrick Adlercreutz, 2025
(Manuscript)

Publications not included in this thesis:

V. **Redefining Poly in Alkylpolyglucosides**
Stefan Ulvenlund, Maria Andersson, Patrick Adlercreutz, Maria Viloria Cols, Nikolina Barchan, Tobias Halthur, Catharina Salomonsson *Household and Personal Care Today, vol 11 (2) p79-82*, 2016

VI. **Surface properties of carboxylated alkyl glycosides**
Ngoc T. N. Ngo, Nikolina Barchan, Carl Grey, Patrick Adlercreutz, 2020
(Manuscript).

Author Contributions

I. NB conceptualized and designed the study together with PA. NB performed all laboratory work, performed the data interpretation and visualization, wrote the original draft and submitted the manuscript as corresponding author.

II. NB conceptualized and designed the study together with PA and ZT. NB provided the lipid material, did all sample preparation and performed MS analysis. NMR experiments were conducted by ZT and data analysis was done by PA and ZT. NB wrote the original draft with contributions from the co-authors.

III. NB conceptualized and designed the study together with PA and TN. NB provided the lipid material, arranged for the beam time and conducted all experiments at MaxIV together with JG. NB performed or took part in all data curation, wrote the original draft with contributions from the co-authors and is the main author of the manuscript.

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Abbreviations

LMSD	LIPID MAPS® Structure Database
PL	Phospholipid
PC	Phosphatidylcholine
PLD	Phospholipase D
TLC	Thin layer chromatography
HPLC	High performance liquid chromatography
CAD	Charged aerosol detector
MS	Mass spectrometry
NMR	Magnetic nuclear resonance spectroscopy
SAXS	Small angle X-ray scattering
WAXS	Wide angle X-ray scattering
Cryo-TEM	Cryogenic electron transmission microscopy
DOPC	dioleoylphosphatidylcholine
HILIC	Hydrophilic interaction chromatography
m/z	Mass over charge
DOPA	dioleoylphosphatidic acid
PA	Phosphatidic acid
MLV	Multilamellar vesicle
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
CPP	Critical packing parameter
PEG	Polyethylene glycol
ULV	Unilamellar vesicle
MVV	Multivesicular vesicle
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLC	Phospholipase C
EC	Enzyme commission number
DOP-Glu	dioleoylphosphatidyl-glucose
DOP-Gal	dioleoylphosphatidyl-galactose
DOP-Fru	dioleoylphosphatidyl-fructose
DOP-Xyl	dioleoylphosphatidyl-xylose
DPPC	dipalmitylphosphatidylcholine
DSPC	distearoylphosphatidylcholine
DPP-Glu	dipalmitylphosphatidyl-glucose
DSP-Glu	distearoylphosphatidyl-glucose

Introduction

The exact definition of a lipid can be debated, but generally from a chemical point of view, lipids are classified as a heterogeneous group of "hydrophobic substances that are soluble in organic solvents but insoluble in water" [5]. There is a great diversity of different lipid structures, and the LIPID MAPS® Structure Database (LMSD) is a classification system for biologically relevant lipids. Since its introduction in 2007, it has grown to include almost 50 000 lipid structures [6]. According to their classification system, there are eight categories of lipids as displayed in Figure 1.

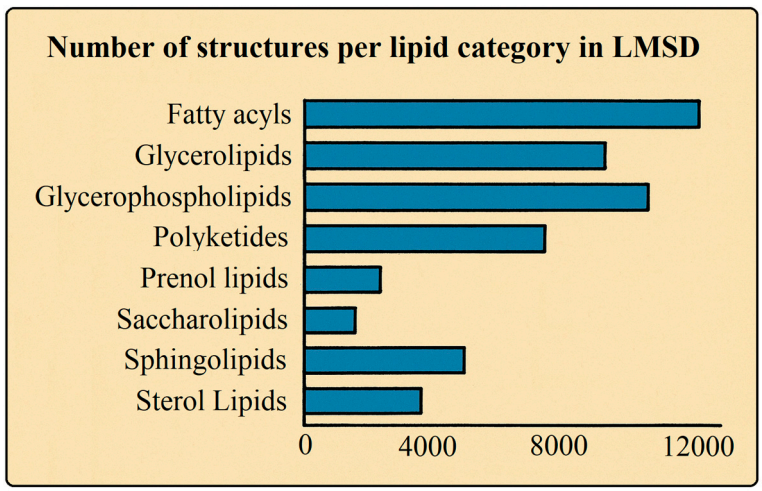

Figure 1. Natural lipids by category as documented in LMSD [6].

A major lipid category is the glycerophospholipids (PLs), which are the protagonists of this thesis. To date, 10188 different chemical structures of naturally occurring PLs have been documented in the LMSD. Even though the work performed within the scope of this thesis does not qualify for curation in the database, since it involves synthetically modified PLs, it contributes to further diversify the realm of polar lipids and constitutes an interesting new type of phospholipid; glycero-glycophospholipids. This thesis will summarize my efforts in finding an applicable reaction- and purification system for their synthesis and isolation, the application of

various analytical techniques for quantification and characterisation of their chemical structures, as well as some initial physico-chemical characterisation of their self-assembly behaviour in aqueous solution. But first, a brief introduction of the field of phospholipids, to put the research in some larger perspective.

Since the early discovery of the supramolecular nanostructures that are formed when polar lipids are dispersed in aqueous environment, the research and number of industrial applications have increased tremendously, and it is a constantly growing field. Not least in the pharmaceutical industry where these nanostructures are used as carriers in cleverly designed delivery systems for drugs, nucleic acids and viral material in vaccines.

The easiest and most cost-effective approach to isolate the phospholipid material is from natural sources with as little purification as possible. This result in a complex mixture called lecithin, which primarily consists of phosphatidylcholine (PC) but also includes other lipid categories and PL classes. The final composition varies depending on several factors, such as the type of source, isolation procedure, and time for harvest. Elaborated isolation protocols including several extraction and chromatographic steps can be employed to achieve a phospholipid product with relatively small batch-to-batch variation, but the resulting product will never be a pure lipid material with a homogenous chemical composition, especially not in regards to the acyl chain composition [7]. For some applications, a lower grade purity lipid mixture is acceptable, but as the complexity and regulatory requirements for a formulation increases, these ingredients may no longer be sufficient to reach the high demands on batch reproducibility and product quality. Due to the low titres of PLs other than PC in lecithin, their isolation from natural sources is not always economically feasible, especially not in high purity. Instead, their analogues can be produced in a semi-synthetic reaction starting from PC. In addition, such an approach can be used to produce entirely synthetic PLs, with new intrinsic chemical structures.

Semi-synthetic routes for purification of complex phospholipid material provide simple and highly used methods to achieve lipid material with a well-defined chemical composition. It can be done both chemically and in enzyme catalysed reactions utilizing lipases or phospholipases. Modification of the acyl chains is readily researched and industrially employed for the synthesis of PLs with a desired fatty acid composition [8-11]. Less attention has been paid to PLs with modified head groups [12], but there are patents for such industrial applications e.g. for the production of phosphatidylserine, although most of the research is done in laboratory scale. Whereas the modification of acyl chains and enrichment of PL classes from natural sources can be done either chemically or enzymatically, the alteration of head group is almost exclusively done enzymatically in a transphosphatidylation reaction between a donor PL and an acceptor alcohol using phospholipase D (PLD) as catalyst [13].

As the availability of well-defined lipid material grow, and new properties can be tailored into the intrinsic chemical structure of the lipids, the pharmacological possibilities expand. This can lead to improved formulations with more efficient administration and less side effects, better treatment efficacy, less troublesome administration for patients and easier transportation and storage of formulated materials [14].

Aim and scope of thesis

The aim of this thesis was to produce and characterize novel phospholipid-saccharide conjugates as a step in the collective effort of expanding the fundamental knowledge of these potential formulation ingredients. The work involved development of a reaction system that yielded the desired products in a simultaneous investigation of reaction conditions such as solvent, enzyme, reactant concentrations and reaction duration as presented in **Paper I**. This resulted in a process that could be applied for the synthesis of several different glycophospholipid conjugates**.**

Due to the complex nature of carbohydrates and their tautomeric equilibrium in water, **Paper II** delves deeper into the chemical characterization of glycophospholipids conjugates to elucidate the lipid-carbohydrate linkage and the spatial configuration of the conjugated saccharides. This kind of characterisation is important to understand fine details in their chemical structure, where even small structural variations, such as the orientation of the hydroxyl groups of different saccharide isomers, can have large effects on the lipid self-assembly behaviour, which was investigated in **Paper III** and **Paper IV**. In **Paper III** we compared the vesicle morphology in dispersions of commercial phosphatidylcholine to that of our newly synthesized phosphatidylglucose. This investigation aimed to evaluate the impact of the new head group on vesicle morphology and bilayer characteristics across a series of phosphatidyl conjugates with different acyl composition. We also investigated differences that arise from variation in acyl chain composition. In **Paper IV** we focused on the head group variations and investigated how conjugation with different saccharides resulted in aggregation structures with widely diverse morphologies. The wealth of successful industrial applications of PL liposome formulations arises from numerous studies of fundamental vesicle properties such as the ones we used in the papers presented in this thesis [15]. Our ambition for this work is to give a small contribution to the field, by initial characterisation of a few novel and unexplored glycophospholipids.

Analytical techniques

To follow the transphosphatidylation progress and evaluate the reaction outcome, a combination of different analytical techniques were required. For the work presented in this thesis, the analysis of transphosphatidylation reaction and the phosphatidyl-saccharide conjugates chemical structures were based on four techniques; Thin Layer chromatography (TLC), High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS) and Magnetic Nuclear Resonance (NMR). For later characterization of vesicle morphology and bilayer characteristics a combination of Small Angle X-ray Scattering (SAXS), Wide Angle X-ray Scattering (WAXS) and Cryogenic Electron Transmission Microscopy (Cryo-TEM) were conducted. In the following sections, very brief information about the techniques and their relevance for the work conducted for this thesis is presented.

Thin Layer Chromatography (TLC)

Thin layer chromatography is a simple chromatographic method that separates the analytes based on their interaction with a stationary phase (silica) when migrating in an organic solvent matrix. It is easy to operate and requires very little method optimization. It served as a rapid, qualitative screening method to detect transphosphatidylation and hydrolysis products, as well as simultaneous consumption of donor substrate during the transphosphatidylation reaction between dioleoylphosphocholine (DOPC) and various saccharides, as outlined in **Paper I**. It was also instrumental to follow the elution pattern of isolated product fractions during flash chromatography for isolation of pure lipid materials. However, accurate quantification is not possible, the technique requires extensive manual operation, and the number of samples that can be analysed simultaneously is limited, and therefore complementary quantitative techniques are required.

High Performance Liquid Chromatography (HPLC)

For quantitative analysis of transphosphatidylation reaction progress, High Performance Liquid Chromatography is a good option. In similarity with TLC it separates the analytes based on their interaction with a stationary phase. It has a high sample capacity and very little manual work during operation, and is a good method for high throughput analysis. However, it requires method optimization of analytical conditions and calibration with standards for proper quantification. It does not give any information on chemical structures, and for new PLs that are not commercially available for purchasing, their chemical identity need to be verified with other techniques. Moreover, each substance needs calibration for correct quantification.

A HILIC separation mode coupled with Charged Aerosol Detector (CAD) gave quantitative information to enable calculations on reaction yields in **Paper I** based on relative peak areas. It was also useful for sample purity determination after isolation of the phosphatidyl saccharide conjugates that were studied in **Paper II**, **Paper III** and **Paper IV**. Due to the lack of DOP-saccharide reference materials, the purity could not be determined quantitatively, but based on relative peak areas for the detected compounds.

Mass Spectrometry (MS)

Mass spectrometry is a powerful technique to evaluate chemical structures. By exposing the molecules for a high electric voltage, they ionize and disintegrate into smaller fragments. The fragments are then separated based on their mass over charge (m/z). Subsequent fragmentation of the previous fragments break down the molecule into smaller fragments and separate them according to their individual m/z value. This fragmentation pattern can then be elucidated to give information of the chemical structure of the original molecule.

Even though MS is a good method for identification of chemical structures, it can be challenging to obtain the right amount of fragmentation to unravel some intrinsic molecular structures. Such an example is elucidation of saccharide orientation in the phosphatidyl conjugates. Typically, the fragmentation occurs at the phosphodiester bond between the saccharide and phospholipid, resulting in free monosaccharide and phosphatidic acid (PA). From such fragmentation, the chemical structure can be deduced based on molecular weight of each fragment, but it is not possible to know the position of the chemical bond on the carbohydrate that conjugated it to the lipid.

MS analysis was used to confirm the correct identity of the detected peaks in the HPLC separation, based on their m/z. It was particularly useful to confirm

conjugation of sugars to PLs of the synthesised phosphatidyl-saccharide products in **Papers I-IV**, but also to evaluate the identity of by-product formation that originated from alcohol stabilizers present in the reaction solvent as detected in **Paper I**.

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance spectroscopy (NMR) take stance in the differences in nuclear spin of all nuclei in a sample, to give information on atom arrangements and chemical bonds within the molecule. Since the saccharides used for transphosphatidylation reaction evaluated in this thesis each exist as an equilibrium of different tautomers when dissolved in water, in combination with a few potential hydroxyl groups available for conjugation, there are many possibilities for how the saccharide on the resulting glycophospholipid conjugate could be oriented. NMR was used to unravel the location of the covalent bond between saccharide and PL and the spatial configuration of the conjugated saccharides in **Paper II**.

Small/Wide Angle X-ray Scattering (SAXS/WAXS)

Small Angle X-ray Scattering (SAXS), and Wide Angle X-ray Scattering (WAXS), are ideal techniques to study lipid self-assembly structures in aqueous environment with minimal sample intervention. The obtained scattering pattern originates from structures within the sample and reflects differences in electron densities throughout the sample. A lipid bilayer has a region of high electron density (the phosphate groups) and low electron density (the hydrocarbon tails) compared to the surrounding medium (water), which results in high scattering contrasts. For sample with order, this generates a distinct pattern. Fitting of different models to the scattering data, and subsequent extraction of the electron density profile can give valuable insights in the lipid membrane at different length scales [16]. This includes information about lipid arrangement and spacing between lipid bilayers in multi lamellar vesicles (MLVs). SAXS and WAXS were used in **Paper III** and **Paper IV** for characterisation of different vesicle morphologies and bilayer parameters due to differences in self-assembly behaviour after modification with different saccharide head groups.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryogenic Transmission Electron Microscopy (cryo-TEM), is a high resolution imaging technique that can resolve very fine details of a sample. This makes it incredibly useful to study particle morphology and distinguish between different self-assembly structures. In contrast to SAXS/WAXS, which generates a sample average, cryo-TEM will present every individual structure that is studied under the microscope, which makes it a good starting point for characterization of lipid self-assembly and selection of a fitting model for SAXS/WAXS data. However, it shows the structures of a very isolated part of a much larger sample, and could thus be misleading, if the studied part is not representative for the whole population. Cryo-TEM was used in **Paper III** and **Paper IV** for identification of different vesicle morphologies of modified phosphatidyl-saccharide conjugates.

Phospholipids

The following chapters of this thesis present the theoretical foundation, discussing the fundamental aspects of phospholipids, including their chemical compositions and characteristic properties, which are essential for understanding the scope of this work. These sections provide a rationale for modification of phospholipid structures and describe the enzyme-catalysed method employed in synthesizing the glycophospholipids that form the basis of this research. Subsequently the focus shifts to our learnings on selection of optimal parameters for the transphosphatidylation reaction, followed by the findings from our structural and self-assembly characterisation of the novel glycophospholipids conjugates.

Characteristics and functions

Natural occurrence and biological relevance

Phospholipids are the primary structural components of cell membranes across all living organisms. They build the bilayers, which are the barriers between the intracellular cytoplasm and the extracellular environment and the envelope that contains the cell organelles. These bilayers do not only enclose and protect cellular compartments but also provide the structural framework for various cellular functions. It hosts an array of signalling molecules, receptors, and integral proteins responsible for processes like nutrient transport, waste removal, cell signalling, and communication with the extracellular environment.

The composition of phospholipids and the distribution of fatty acids within the membrane vary widely depending on the type of cell and organism. This diversity is essential for achieving the specific physical and functional properties required by different membranes. Phospholipid head group composition contribute to chemical characteristics of the membrane such as ion transport, signalling and protein interactions. The fatty acid chains play a pivotal role for key membrane properties such as membrane fluidity and permeability.

The membrane lipids are inherently dynamic, undergoing constant rearrangements and compositional changes to maintain optimal structural and functional properties. This adaptability allows the membrane to remain rigid enough to hold cellular structures in place while preserving sufficient flexibility and fluidity to respond to

external conditions such as variations in temperature or electrolyte concentrations. This finely tuned balance of lipid diversity ensures that membranes are dynamic, adaptable structures capable of maintaining cellular integrity, responding to environmental changes, and supporting complex biological processes [17].

Chemical structure

Phospholipids can be categorized into two groups based on their backbone structure: phosphosphingolipids and glycerophospholipids. Throughout this thesis, the term 'phospholipid' will exclusively refer to glycerophospholipids. They are composed of a glycerol backbone, which is esterified with acyl chains in the sn1 and sn2 positions and a phosphate group at the sn3 position. The phosphate group can further be conjugated with an alcohol, giving rise to different classes of phospholipids. The chemical structure of a common phospholipid, dioeloylphosphatidylcholine (DOPC), which is the starting point for the work performed in this thesis, is displayed in Figure 2. Phosphatidyl Choline (PC), -Ethanolamine (PE), -Serine (PS), -Glycerol (PG), -Inositol (PI) and non-conjugated phosphatidic acid (PA) are the main PL head group classes and the most abundant naturally occurring PLs. All lipids within each class share the same head group but varies in the acyl chain composition. At physiological pH, PC and PE are zwitterionic possessing both a negative charge on the phosphate group and a positive charge on the choline and ethanolamine respectively. PS, PG, PI and PA on the other hand are anionic, with only the negative charge on the phosphate group. The typical acyl composition of natural PLs almost exclusively contains even numbered carbon fatty acids that vary between 14 and 22 carbon atoms in length with various degrees of unsaturation. This vast array of acyl chain combinations, varying in length, degree of unsaturation, and their positioning on the glycerol backbone, combined with the diversity of available head groups, generates an extensive range of possible chemical PL structures.

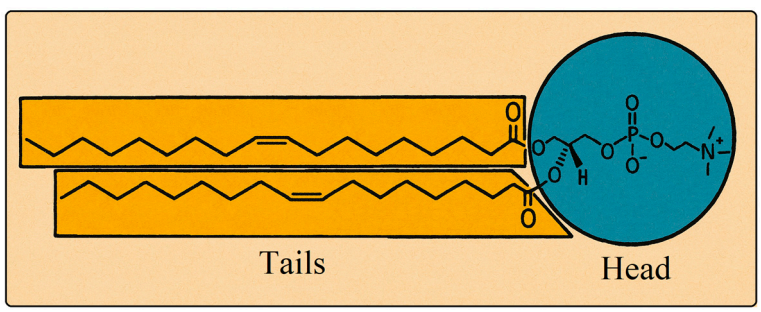

Figure 2. Chemical structure of dioleoylphosphatidylcholine (DOPC). Phospholipids comprise of two distinct regions, contributing to their amphiphilic nature. The hydrophilic ”head,” highlighted in blue, includes the glycerol backbone with a phosphate group at the sn3 position, conjugated with choline. The hydrophobic ”tails”, highlighted in yellow, consist of two oleoyl fatty acid chains. Phospholipids differ in their fatty acid composition and type of conjugation to the phosphate group.

Sources

The primary source of phospholipids is vegetable oils, including soybean, rapeseed, sunflower and flax seed. They are isolated from the oils as a by-product in the degumming process [18]. The purification proceeds via validated purification procedures including solvent precipitation, extraction and chromatography steps and result in fractions with different degrees of purity [7]. The PL content in crude vegetable oils is typically around 2-3 % of total lipid content [19]. PLs are also commonly derived from animal tissue such as egg yolk, milk and krill, which are naturally abundant in PLs. In a hen egg the PL content can constitute around 30 % of the total lipid fraction [20]. Processes for isolating PLs from animal sources are typically designed to provide PLs of high purity as their primary product. As a result, PLs derived from animal sources are generally more expensive than the vegetable derived counterparts. Few attempts have also been focused on the microbial production of PLs in fermentation processes, but the titres are very low, less than 1 % of the dry matter, and those processes have limited economical value [21].

Regardless of the source, the isolated PL product constitutes a mixture of different classes of PLs varying in acyl chain length and degree of unsaturation. This mixture is often referred to as lecithin and the dominating component is always PC, as this is the most abundant type of PL across all organisms. The composition of lecithin can drastically vary depending on the source and protocol used for isolation. This makes isolation of high purity PL with a single, well-defined PL composition in high yields challenging with such methods. For applications where a very high purity is required, synthetically produced PLs are necessary [7]. More information on synthetic PLs, and how we used this approach for the synthesis of glycophospholipids with new saccharide head groups, will follow in later chapters of this thesis and in **Paper I**.

Properties

Phospholipids are amphiphilic molecules, which means that they possess properties of both hydrophilic and hydrophobic character. The phosphate group and its conjugated alcohol constitute the hydrophilic part of the molecule and the fatty acids on the glycerol backbone defines the hydrophobic part. When dispersed in aqueous media, they spontaneously self-assemble. This behaviour is driven by the hydrophobic effect, where the hydrophobic tails minimize contact with water, while the hydrophilic head groups interact with each other and the surrounding aqueous environment. The type and morphology of the formed structures depend on several interacting parameters characterised by the intrinsic properties of the phospholipid and on the surrounding medium. A useful way to predict the preferred aggregation structure of a lipid is the critical packing parameter (CPP) which is defined as:

$$CPP = \frac{v}{a_0 \cdot l_c} \quad (1)$$

Where v=volume of hydrocarbon chain(s), a_0=effective head group area and l_c=hydrocarbon chain length [22-24]. Single chained surfactants with a large polar head group typically exhibit a CPP of less than 1/3. These molecules are water soluble and tend to self-assemble into spherical micelles in aqueous environments, with the hydrophobic tail hidden inside the structure, and the hydrophilic head facing outwards. As the balance between the hydrophilic and hydrophobic parts of the molecule shifts towards higher hydrophobicity, the CPP increases. This shift alters the geometry of the aggregates formed.

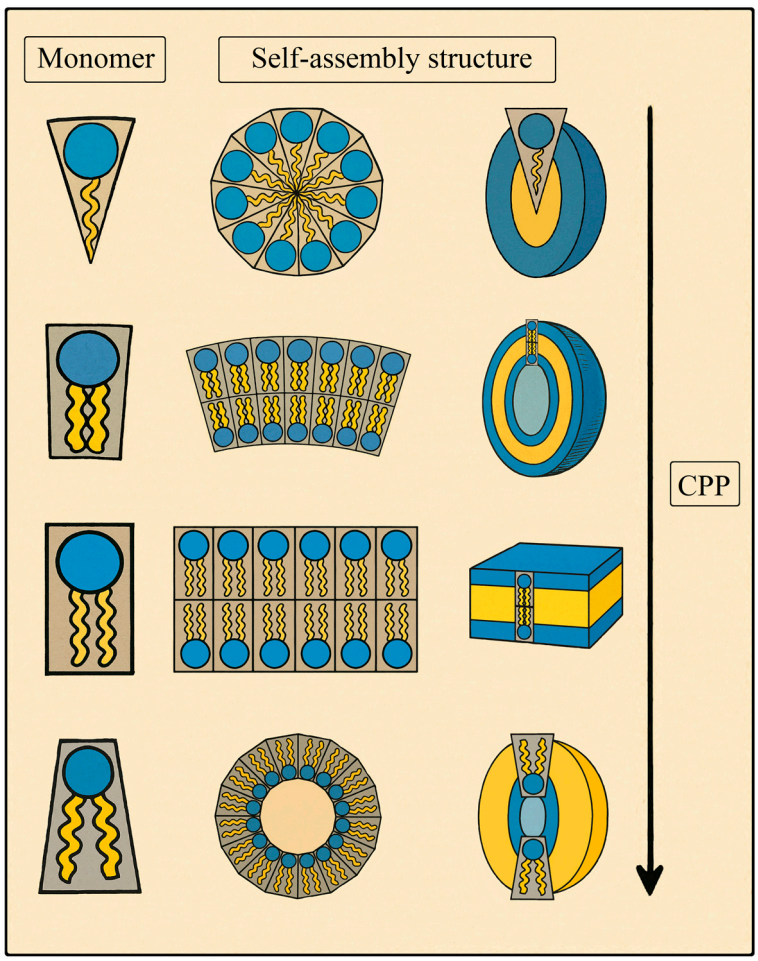

Figure 3. Monomer geometry (left) and corresponding self-assembly structures (middle and right) of polar lipids with different CPPs. As the balance between the hydrophobic and hydrophilic regions shifts towards higher CPPs, the structures evolve from water-soluble small micelles, via bilayer structures like vesicles, eventually forming inverted structures, such as reverse micelles that are soluble in organic solvent.

Phospholipids generally have a CPP of ½-1, which results in flexible, curved bilayer structures that spontaneously organize into vesicles (liposomes) when dispersed in excess aqueous solutions. At even larger CPP values, greater than 1, the hydrophobic part of the molecules dominates, reducing their solubility in water. Under these conditions inverted structures such as reverse micelles are formed, where the hydrophilic head groups are facing inwards and the hydrophobic tails outwards towards the hydrophobic environment. Additionally, the characteristics of the self-assembled structures are not solely determined by the CPP but are also influenced by properties of the surrounding medium. Factors such as temperature, ionic strength, pH, and the presence of divalent cations (e.g., Ca^{2+} or Mg^{2+}) significantly impact the size, shape, and stability of the aggregates. These environmental factors can shift the equilibrium of self-assembly, facilitating transitions between different structural forms. A schematic representation of the monomer molecular geometry and the corresponding self-assembled structures is displayed in Figure 3, illustrating how the variations in CPP explains the aggregate morphology. In this thesis, most of the studied lipids self-assemble into bilayer type of structures, like vesicles. **Paper III** and **Paper IV**, focus on exploring how the morphologies of these vesicles among glycophospholipids that are conjugates to different carbohydrate head groups vary, as a result of differences in their chemical structures.

Applications

The amphiphilic character of phospholipids makes them excellent emulsifiers and wetting agents. They are also highly versatile ingredients for a wide range of applications because of their high abundance, low toxicity, exceptional biocompatibility and biodegradability.

Due to the tendency of phospholipids to form vesicles, they are particularly attractive as excipients in pharmaceutical formulations. The vesicles are used to encapsulate therapeutic cargo and act as a protective barrier against enzymes, pH variations, bile salts, free radicals and other components of the mononuclear phagocytic system [25]. The use of vesicles as drug delivery systems offers several advantages, such as improved drug stability, reduced drug toxicity, controlled release, targeted delivery and the ability to encapsulate both hydrophilic and hydrophobic substances [26].

Since the discovery by Bangham in the 1960s [27], vesicles have attracted high attention as pharmaceutical delivery systems leading to the development of increasingly advanced formulations. In the early days of liposome-based therapeutics, formulations were primarily intended for various types of cancer therapy. Doxil, launched 1995 for treatment of ovarian cancer, was the first nanomedicine on the market that utilized these type of nanoscale structures [1]. Since then the available therapeutic liposomal formulations have grown to include

antibiotics, antioxidants, vitamins, non-steroidal anti-inflammatory drugs (NSAIDs) and genetic materials [28].

To modify bilayer properties and control vesicle stability, PLs are commonly mixed with cholesterol during preparation of liposomes. Cholesterol modulates the fluidity of the lipid bilayer and hampers leakage of encapsulated cargo. A widely used modification to increase colloidal stability is the coating of the liposome surface with polyethylene glycol polymer (PEG). PEG constitutes a steric barrier that acts as a protective layer, shielding liposomes from the mononuclear phagocytic system and extend their circulation time in vivo. Numerous other modifications have been developed to optimize liposome functionality, including the addition of targeting ligands, monitoring labels, antibody anchors, and stimuli sensitive lipids and polymers. These advancements have significantly expanded the potential applications of liposomes across various fields. For more in-depth reading, readers are encouraged to consult three excellent papers in the field by Torchilin, 2005 [2], Allen & Cullis, 2013 [29] and Lee and Thompson, 2017 [30].

Liposomes, or lipid vesicles are categorized based on size and number of correlated bilayers. Unilamellar vesicles (ULVs) are the simplest form of vesicle and contain only a single bilayer. This type of vesicles can be further subdivided into small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) and giant unilamellar vesicles (GUVs) with increasing diameter. In contrast, multilamellar vesicles (MLVs) consist of several bilayers arranged in an onion-like structure, and Multivesicular vesicles (MVVs) encapsulate smaller vesicles within a larger one. These various vesicle structures are illustrated in Figure 4.

The size and lamellarity of vesicles significantly influence their physical and functional properties, such as cargo encapsulation ability and liposome circulation half-life in vivo, which in turn affect their potential applications [25]. Smaller SUVs are particularly suitable for delivery of small hydrophilic drugs. LUVs or GUVs are more suitable for formulations involving larger biomolecules such as enzymes or nucleic acids, but their increased size makes them more susceptible to recognition and clearance by the mononuclear phagocytic system. MLVs are composed of multiple bilayers, which enhance their stability and make them more useful for application requiring prolonged circulation time and protection of sensitive cargo. MVVs are explored in advanced therapeutic formulations for combination therapy and co-encapsulation of synergistic drug combinations. Liposome structure and morphologies resulting from our physico-chemical characterisation work of various glycophospholipids conjugates are discussed in depth in **Paper III** and **Paper IV**.

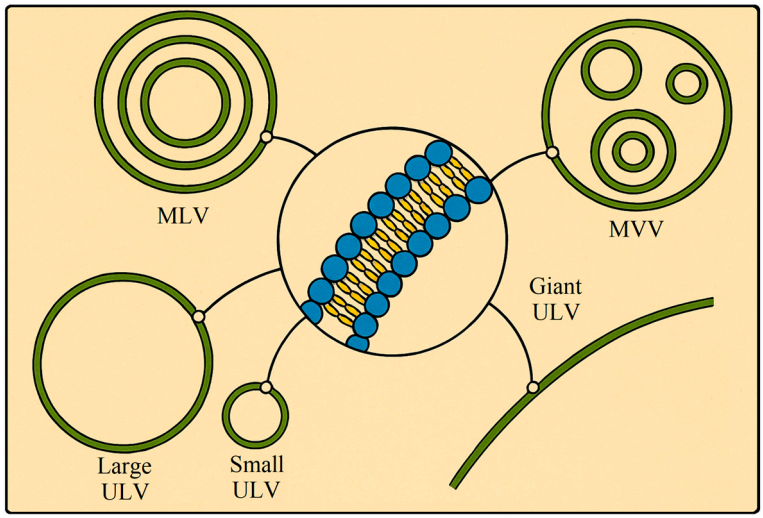

Figure 4. Different types pf phospholipid vesicle structures. MLV: Multilamellar vesicle, MVV: Multivesicular vesicle, ULV: Unilamellar vesicle.

Structural modifications of phospholipids

Why do we care about modifying them?

As previously discussed, phospholipids derived from natural sources consist of a complex mixture of lipids from a variety of PL classes, and a diverse composition of acyl chain lengths and degree of unsaturation. Isolation of PLs with high purity and a well-defined fatty acid composition is challenging. Additionally, extracting substantial quantities of PLs beyond phosphatidylcholine, which is typically the most abundant class, is particularly difficult due to their limited natural abundance.

These limitations highlight the potential of synthetic PLs, which can be manufactured with high purity and reproducibility. Synthetic PLs offer control over lipid composition and allows precise design of fatty acids to achieve desired structural and functional properties. It also provides the possibility to alter PL class by introduction of novel entities into the lipid head group. This approach does not only facilitate enrichment of less abundant PL classes, but also allows for the synthesis of entirely new phospholipids with engineered intrinsic properties. These properties could be enhanced stability, targeted functionality, or specific interactions with biomolecules, which significantly enhance their properties as formulation excipients and widens the potential for PL based formulations [31, 32].

Enzymatic modifications with phospholipases

Enzymes are highly attractive as catalysts for the modification of phospholipids because of their high specificity. They typically operate at milder conditions, and cause less pollution than traditional chemical methods, making them more environmentally friendly. Their ability to precisely target specific reactions also reduces unwanted by-products, enhance efficiency and promote greener manufacturing practices [33, 34]. Different enzymes with specificity towards the ester bonds and phosphoester bonds of the phospholipid are readily available for industrial use. For the modification of the acyl chains in the sn1 and sn2 positions on the glycerol backbone, phospholipase A1 and A2 (PLA_1 and PLA_2 respectively) can be utilized in transesterification reactions [35]. Phospholipase C (PLC) can hydrolyse the phosphate ester bond between the phosphate group and the glycerol backbone, whereas phospholipase D (PLD) catalyse both transphosphatidylation reaction and hydrolysis reaction on the terminal phosphate diester bond [36, 37]. The target sites for the different phospholipases are illustrated in Figure 5.

Phospholipases have been extensively used in food industry with several different applications such as oil refinery and degumming of crude oils, processing of egg yolk emulsifiers, treatment of dairy products and improved flour properties for baking [11]. Another important field for phospholipases is in the production and refinery of excipients for high value products such as pharmaceutical formulations [7], which is where our primary interest is focused in this thesis. The PLD enzyme and its applicability for synthesis of PLs with altered head groups is the foundation of this work and will be readily discussed in following chapters of this thesis and has a special focus in **Paper I**.

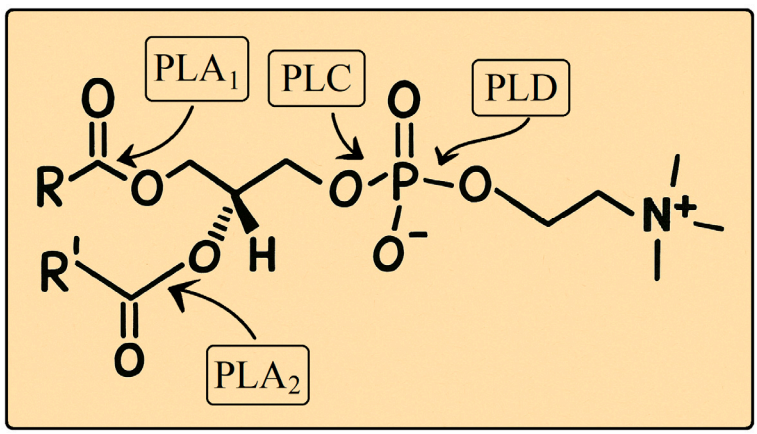

Figure 5. Activities of the different phospholipase enzymes. PLA1 and PLA2 express hydrolytic and transesterification activity towards the acyl chains in sn1 and sn2 positions respectively. PLC hydrolyses the phosphate ester bond between the phosphate group and the glycerol backbone, and PLD can express both hydrolytic, and transphosphatidylation activity towards the phosphodiester bond on the terminal side of the phosphate group.

Phospholipase D catalysed transphosphatidylation

Phospholipase D enzymes (PLDs) are hydrolytic enzymes [EC 3.1.4.4] that, in presence of water, catalyse the hydrolysis of the phosphodiester bond within the PL head group. This reaction result in the formation of phosphatidic acid (PA) and a free alcohol. In addition to their hydrolytic activity, PLDs can catalyse transphosphatidylation reaction, where an alcohol acts as the nucleophile instead of water, enabling modification of the lipid head group [31, 38-41]. The reaction scheme for hydrolysis and transphosphatidylation with PLD is illustrated in Figure 6.

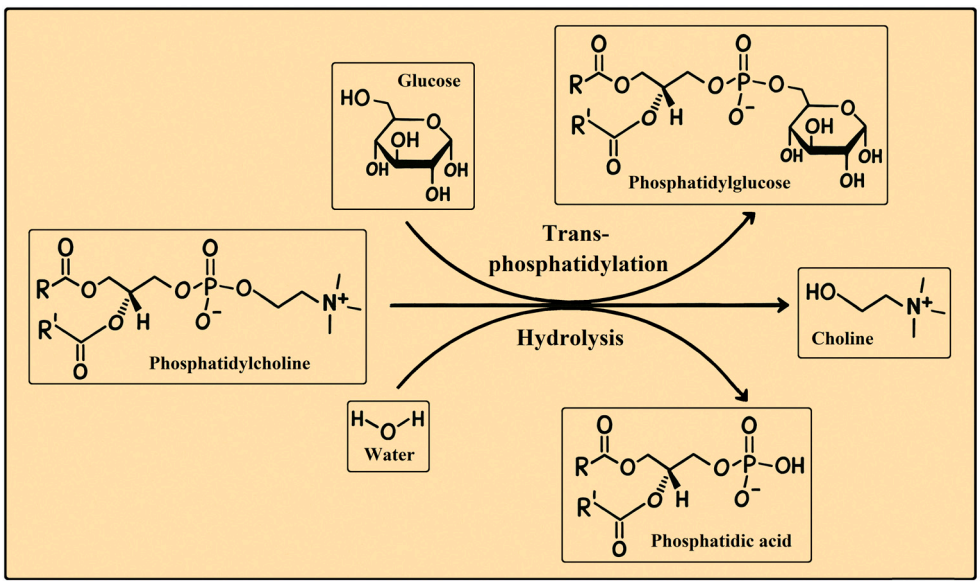

Figure 6. Reaction scheme for PC in transphosphatidylation and hydrolysis reaction catalysed by PLD. The top reaction with glucose as acceptor substrate generates the transphosphatidylation product, phosphatidylglucose (P-Glu), whereas the bottom reaction with water generates the hydrolysis product, phosphatidic acid (PA). In both reactions, choline is released as a by-product. R and R′ represent alkyl groups.

Phospholipase D enzyme

Phospholipases are abundant in microorganisms, plants and other eukaryotes where they act as hydrolases for the breakdown of phospholipids into phosphatidic acid. The composition of phospholipids that are displayed on the cell surface is important for cell regulation and signalling and PLDs play an important role in the complex membrane remodelling machinery [38, 42].

Acceptor substrates

PLDs have a broad acceptance range of natural phospholipids with varying preference for length and degree of unsaturation of the acyl chains. Most PLDs favour PC over other classes of PLs. The hydrolysis and transphosphatidylation ratio depends on the enzyme origin [36]. Regarding the acceptor substrate, PLDs accept a wide range of different types of aliphatic alcohols, cyclic alcohols, alcohols with functional groups, nucleosides and carbohydrates [37]. Generally, PLDs have a preference towards small primary alcohols, and there seem to be a clear discrimination against large and bulky substrates [39]. The first PLDs that were discovered and characterised were of plant origin such as cabbage leaves, castor beans and peanuts [38]. Generally, these enzymes have a strong dependence for Ca^{2+}, exhibit a narrow substrate acceptance for small primary alcohols, and are significantly affected by the competing hydrolysis reaction [12]. Since the discovery of bacterial PLDs from e.g. *Streptomyces* species that can accept secondary alcohols and bulkier substrates, and often show a strong preference for transphosphatidylation over hydrolysis, the potential for PLDs as catalysts in modified phospholipid synthesis has expanded greatly [31, 43, 44]. However, this is just a rough generalization, and not a strict rule that applies to all PLDs. Enzyme source, acceptor alcohol and reaction conditions such as solvent have proved to be strongly interdependent and consideration for each reaction system has to be undertaken for a high yield of transphosphatidylation product [45].

PLD catalysed transphosphatidylation is a powerful strategy for preparation of PLs with altered head groups. It enables the incorporation of entirely new properties such as different charge, size and polarity, as well as new functionality such as antioxidant properties, antibacterial properties, and various bioactive properties [39, 46]. Extensive studies on PLD catalysed modifications with diverse head groups is thoroughly discussed in these references [37, 47].

Reaction mechanism

Most PLD enzymes belong to the PLD superfamily, which catalyse transphosphatidylation reaction following a ping-pong mechanism [48, 49]. The reaction proceeds via a phosphatidyl-enzyme intermediate in a double displacement reaction, which keeps the original PL chirality [48, 50, 51]. PLDs have dual domain structures with a duplicate set of the characteristic HKD motif in their primary

structure, one motif located in each half domain of the enzyme [39, 52]. The HKD motif contain conserved His, Lys, Asp, Gly and/or Ser residues that are located in the active site and take part in its catalytic activity. The reaction mechanism of PLD from *Streptomyces* PMF is illustrated in Figure 7. The Histidine from one motif acts as a nucleophile on the PL phosphorous to form a phosphatidyl-enzyme intermediate, which is the rate limiting step [50, 53]. The other His donates a proton to the leaving alcohol (choline in PC). In a second step, the previously deprotonated His take up a proton from an incoming acceptor substrate, either water or alcohol, which then acts as a nucleophile on the phosphorous that protonates the first His, resulting in release of the phosphatidyl-enzyme intermediate and completion of the cycle [36]. The other amino acids of the HKD motifs are not directly involved in the catalysis, but the lysine residues are believed to stabilize the phosphatidyl-enzyme transition state [39]. Aspartic acid forms hydrogen bond with the nucleophilic His and stabilizes it in the right position to guide the substrate into the active site and/or guide the product out from active site after completed catalysis [50].

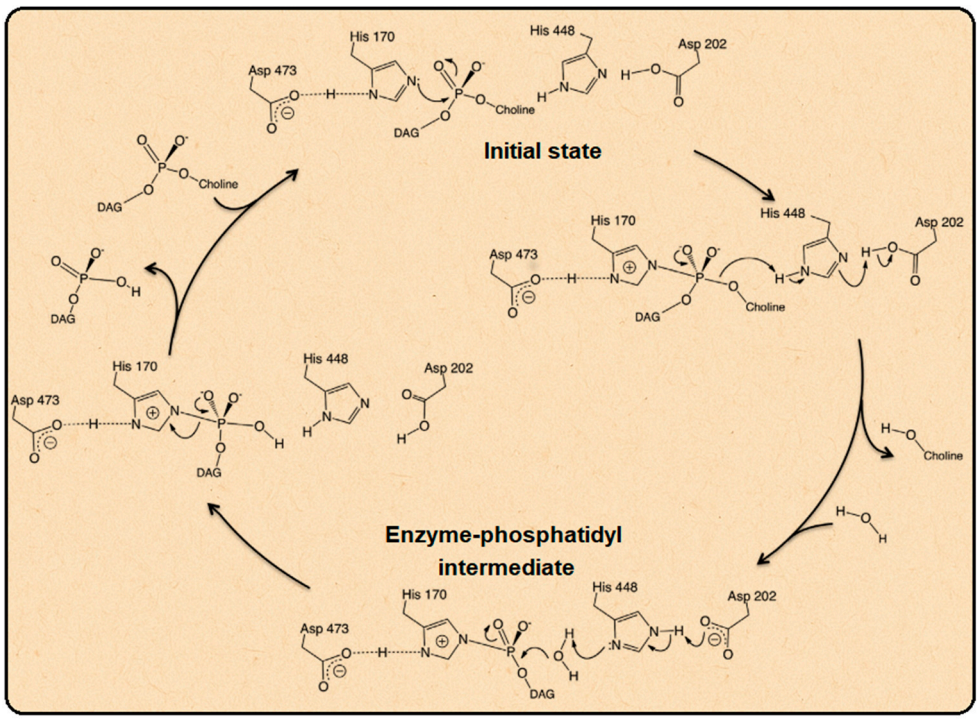

Figure 7. Reaction mechanism for PLD. The reaction procedes via a ping-pong mechanism. His 170 acts a nucleouphile on the PL phosphorous in the formation of a phosphatidyl-enzyme intermediate and His 448 donates a proton to the leaving alcohol (choline). Next, His 448 deprotonates an incoming acceptor substrate (In this case water), which performs a nucleophilic attac on the phosphatidyl-enzyme intermediate and release the hydrolysis product, PA. If the incoming acceptor substrate instead is an alcohol it will result in transphosphatidylation reaction and release of the transphosphatidylation product.
The figure is adapted from Damnjanović et al 2013 [39].

Glycophospholipids –Phospholipids modified with carbohydrates

The term “glyco” in glycophospholipid indicates that the lipid contains a carbohydrate. Surfactants and amphiphiles containing carbohydrates have attracted growing interest due to their environmentally friendly nature and low toxicity [54]. Unlike traditional surfactants, which can be harsh on the environment and poorly biodegradable, carbohydrate derived amphiphiles significantly reduce the environmental impact. Additionally, their high biocompatibilty makes them excellent candidates for systemic administration. Glucophospholipids represents a rare and unique class of phospholipids, combining the properties of glycerolipids (where carbohydrates are directly linked to the glycerol backbone) and those of conventional phospholipids [55]. Moreover, carbohydrate are important for cell recognition, and modification of liposomes with carbohydrates on the surface or incorporation of glycolipids during preparation is an interesting approach for targeted drug delivery [56].

Synthesis of modified glycophospholipids

The natural quantity of glycophospholipids is very low making it challenging to isolate more than trace amounts of pure lipid material. To overcome this limitation, a synthetic approach using PLD as catalyst is highly effective [57, 58]. In **Paper I**, the PLD catalysed reaction between dioleoylphosphatidylcholine (DOPC) and glucose was evaluated for the synthesis of dioleylphosphatidyl glucose (DOP-Glu). Due to the difference in solubility of the donor PL and the alcohol acceptor (glucose), transphosphatidylation reactions like this, are typically performed in biphasic reaction system. The donor phospholipid, with desired fatty acid composition, is dissolved in organic solvent and the carbohydrate acceptor is dissolved in the aqueous phase. The interplay between solvent, substrate and enzyme in such two-phase systems have significant impact on reaction rates and product yields [45, 59, 60]. Selecting an appropriate solvent is essential; it should effectively dissolve the phospholipid substrate, and preferably be immiscible with water to avoid stripping of essential water that is necessary for the enzyme’s

catalytic activity. Further, it should not contain any primary alcohol group that could act as a competing substrate in the transphosphatidylation reaction.

For development of greener processes and limiting the amount of toxic solvents, alternative reaction systems have been investigated [61]. These include purely aqueous systems, where PLs substrates are absorbed on solid support [62-64], or dispersed as vesicles [65, 66] or mixed micelles [67], or reactions in green [68] or novel solvents such as ionic liquids [69] or deep eutectic solvents [70, 71]. Despite attractive novel methods for transphosphatidylation reaction, these routes are outside the scope of this investigation, where we limit the study to the biphasic reaction system.

To optimize DOP-Glu yield, multiple reaction parameters were simultaneously investigated, including choice of solvent, pH, enzyme variety and concentration, glucose molar excess and reaction time. The optimized reaction conditions; a two-phase system consisting of chloroform and buffered aqueous solution at pH 5 with 50 times excess of glucose, achieved a transphosphatidylation product yield of approximately 75 % DOP-Glu. No hydrolysis product (PA) was detected and the product accumulated only in the organic phase.

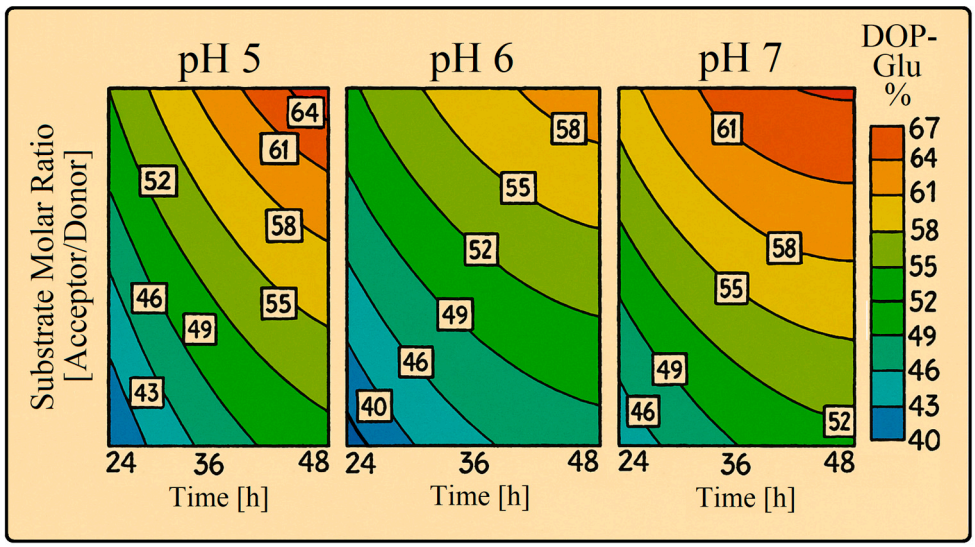

Figure 8. Simultaneous investigation of glucose excess, pH and reaction time for optimisation of DOP-Glu yield in PLD catalysed transphosphatidylation reaction between DOPC and glucose in two phase system with chloroform and buffered aqueous phase.

Figure 8 illustrates the results from simultaneous optimization of glucose excess, pH and reaction time in a biphasic reaction system with chloroform and buffered aqueous solution. The most important factor for optimal transphosphatidylation

yield was the addition of glucose in high excess, as high as solubility allowed, in order to push the reaction equilibrium towards the product side. In addition, it was important to limit the reaction time and enzyme concentration to avoid product degradation and formation of hydrolysis product in the later stages of the reaction [40]. The choice of pH was of less importance and similar yields were obtained across the evaluated pH interval of 5-7.

The optimized reaction system was subsequently used to synthesise phosphatidyl conjugates with six different mono- and disaccharides (glucose, galactose, fructose, xylose, maltose, sucrose). The resulting yields spanned between 30-80 % of transphosphatidylation product after 48-72 hours of reaction as displayed in Figure 9. Glucose, galactose and fructose, which are all isomers with the chemical formula $C_6H_{12}O_6$, resulted in around 75 % transphosphatidylation yield. Xylose, $C_5H_{10}O_5$, resulted in the fastest reaction rate and highest yield of approximately 80 % transphosphatidylation product. The disaccharides, maltose and sucrose, $C_{12}H_{22}O_{11}$, are bigger and bulkier than the monosaccharides and hence have a lower molar solubility in water, which restricted the possible molar excess to 25 times compared to the donor lipid. As a result, the disaccharides reacted slower and yielded only around 35 % transphosphatidylation product. The obtained transphosphatidylation yields correlate well with the size of the acceptor molecule, as previously reported [72], but further investigations are required to draw any definite conclusions on the reason behind the differences in reaction rates and product yields.

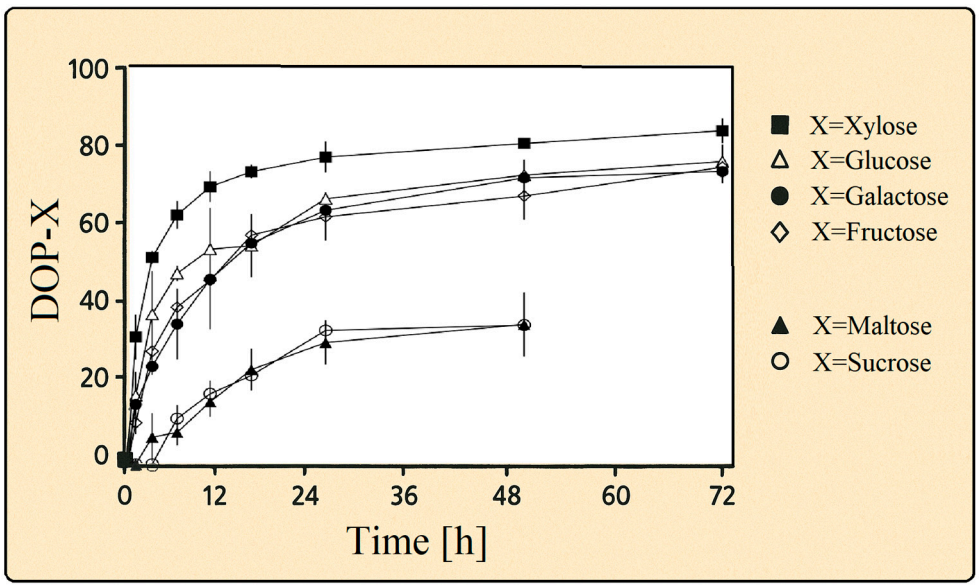

Figure 9. Reaction progress between DOPC and various carbohydrate acceptor substrates at optimized conditions. Monosaccharides (glucose, galactose, fructose and xylose) yielded 70-80 % transphosphatidylation products while disaccharides (maltose, sucrose) resulted in approximately 35 % product yields.

The resulting products from the optimized reactions were isolated for further characterisation studies on chemical structure, **Paper II,** and self-assembly behaviour, **Paper III** and **Paper IV**. The excess saccharide solution was removed by extraction and the PL saccharide product was isolated via flash chromatography on a silica column. Eluting solvent was evaporated from the pure product fractions before the product was lyophilized to a white, waxy powder.

During the solvent screening, almost nothing of the desired phosphatidylglucose product was detected after reaction in chloroform. Instead, a large, unidentified peak dominated the product profile. This peak was identified by MSMS as dioleoylphosphatidylethanol and is believed to originate from ethanol used as a stabilizer in the solvent. Small aliphatic alcohols such as methanol and ethanol are excellent acceptors for the PLD enzyme. After shifting to chloroform solvent that was stabilized with amylene instead of alcohol (that lacks a reactive hydroxyl group) this contaminating product was no longer detected.

It was surprising to achieve such fast reaction rates and high transphosphatidylation yields with xylose, given the PLD enzyme's strong preference for primary alcohols and the low abundance of linear xylose and xylofuranose (the xylose tautomer possessing a primary alcohol functionality) in aqueous solution. However, certain PLD enzymes derived from *Streptomyces* sp have demonstrated catalytic activity also with secondary alcohol acceptor substrates and have been shown efficient for their transphosphatidylation, as reported by Ferra et al, 1994 [43]. The high transphosphatidylation rate experienced towards xylose could thus be explained by a high PLD acceptance rate towards a secondary hydroxyl group of xylopyranose, resulting in lipid conjugation via C1-C4. However, this would contradict previous reports that PLDs exclusively catalyse transphosphatidylation with primary alcohols in substrates that possess both functionalities, such as carbohydrates [39, 47].

Alternatively, transphosphatidylation towards the primary hydroxyl group on xylofuranose or the linear open form of xylose dominates, despite these forms being present only in small amounts. However, this alternative seems less likely considering the high transphosphatidylation rate observed with xylose as acceptor substrate.

To further investigate this intriguing observation, it is essential to identify the chemical structures of the glycophospholipids conjugates in detail. This will enable the precise determination of the position of the lipid-carbohydrate linkage and the spatial orientation of the saccharide in the lipid conjugates, ultimately revealing the underlying mechanism responsible for these results. This topic will be discussed in more detail in **Paper II** and in the next chapter of this thesis**.**

Characterization of chemical structures

Structures and composition of phosphatidyl saccharide products

Saccharides are small carbohydrates with multiple hydroxyl groups and either an aldehyde group or a keto group. Monosaccharides can be classified according to the number of carbon atoms they contain. The saccharides relevant for this thesis are pentoses that contain five carbons, and hexoses containing six carbons. They can exist as a linear open chain (keto- or aldehyde form) or in closed ring forms. When dissolved in water, they enter an equilibrium state of all their different tautomers. The equilibrium distribution is dependent on external factors such as temperature and solvent. Different methods give somewhat deviating results, but a representative tautomeric composition for the four most relevant monosaccharides for this thesis; glucose [73], galactose [74], fructose [75] and xylose [76], in aqueous solution at 25 °C is illustrated in Figure 10. Due to the fast ring closing in water, the monosaccharides are almost exclusively present in their closed ring forms and the open structure is only present in trace amounts. This closed structure can constitute a six-membered ring consisting of five carbons and one oxygen, called pyranose, or a five-membered ring with four carbons and one oxygen called furanose. The closed ring form can further differ in orientation of the hydroxyl group on its anomeric carbon, resulting in either alpha- or beta configuration.

Different sugar isomers exhibit remarkably distinct properties, such as melting point and water solubility, despite having identical chemical formulas. These differences arise from how the isomers interact with water in solution, and is influenced by the number, position, and orientation of the hydroxyl groups [77]. This arrangement determines the ability of the carbohydrate to from hydrogen bonds, and ultimately affects its chemical and physical properties. For this reason, we believe that also the self-assembly properties on a macroscale can be affected by these variations as investigated in **Paper III** and **Paper IV**, which motivates a deeper investigation of chemical structure of our phosphatidyl saccharide conjugates, **Paper II**.

From a catalytic perspective, the PLD enzyme favours acceptor substrates with primary alcohols over secondary in the transphosphatidylation reaction. This results in conjugation via a phosphodiester bond between the PL phosphorous and a primary hydroxyl group on the carbohydrate. Most of the hydroxyl groups on saccharides are secondary, which make them less accessible for transphosphatidylation reaction, and limits the number of possible conjugation sites on the carbohydrate. However, the tautomeric equilibrium of carbohydrates in water involves multiple combinations of cyclic and anomeric forms, that each possess zero-two primary hydroxyl groups. This equilibrium gives rise to several possible conjugated structures.

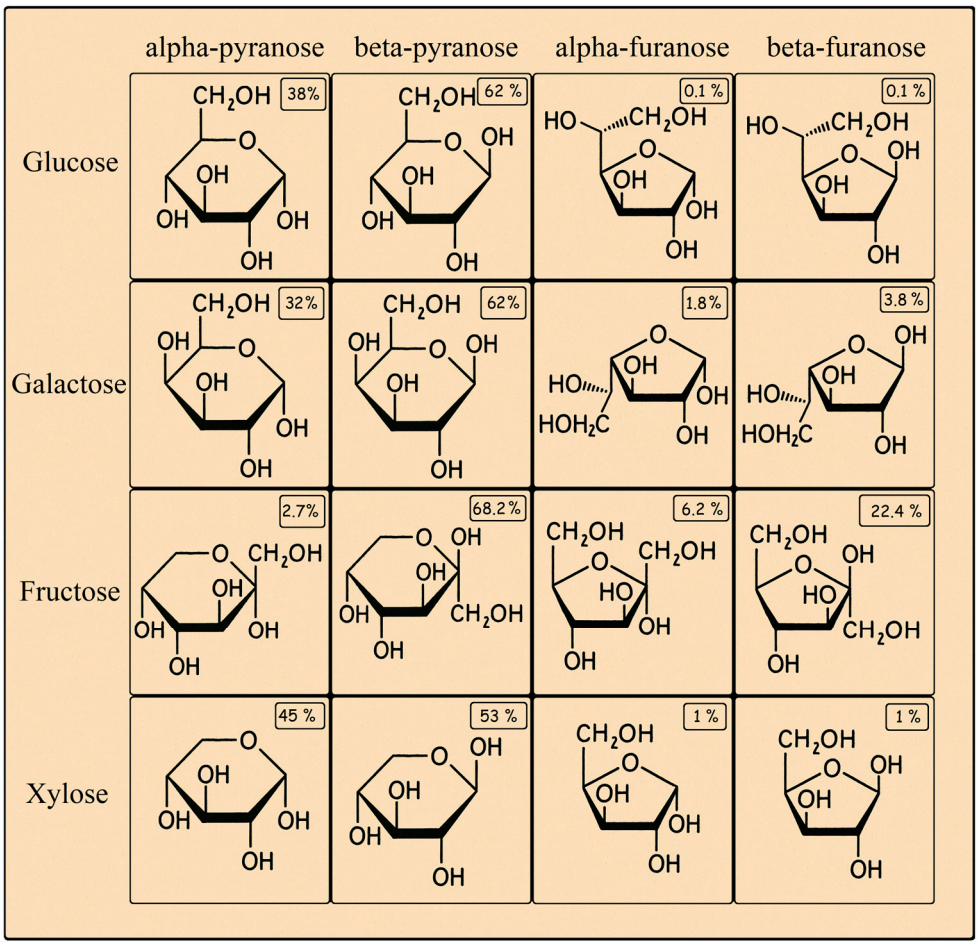

Figure 10. The different tautomers of glucose, galactose, fructose and xylose in water. The number in the upper right corner of each saccharide shows the tautomeric abundance at equilibrium in water at 25 ºC, except for fructose which is at 20 ºC.

To determine the chemical structures of the phosphatidyl-saccharide conjugates, a combination of mass spectrometry (MS) and extensive nuclear magnetic resonance (NMR) studies was employed, **Paper II**. MS provided a robust foundation by accurately determining the molecular formulas of the compounds. Using hydrophilic interaction liquid chromatography (HILIC) coupled with MS and data-dependent acquisition fragmentation, we validated the mass over charge (m/z) values and fragmentation patterns for all phosphatidyl-saccharide conjugates and confirmed conjugation between lipid and carbohydrate.

However, MS was unable to identify the exact position of the phosphodiester bond on the carbohydrate, necessitating further characterization work with NMR spectroscopy. This part of the structural analysis aimed to identify how the different carbohydrates were conjugated to the phospholipid.

Glucose and galactose, regardless of tautomeric configuration, each possess C6 as their only primary hydroxyl group, making it the most likely site for lipid conjugation. NMR analysis revealed that conjugation indeed occurs at C6 for both phosphatidylglucose and phosphatidylgalactose, resulting in single product species: 6-phosphatidylglucose and 6-phosphatidylgalactose respectively. The proportions of the different ring structures and anomeric configurations of these products remain unsolved. Since the anomeric carbon does not participate in the lipid conjugation, the carbohydrates may undergo tautomerization after conjugation, entering equilibrium for ring- and anomeric configurations when dissolved in solution. These equilibrium dynamics are influenced by the properties of the solvation matrix.

In contrast, fructose presents a more complex scenario. The furanose form of fructose provides two primary hydroxyl groups, located at C1 and C6, as potential sites for conjugation. Consequently, the transphosphatidylation reaction yielded a mixture of two distinct product species: 1-phosphatidylfructose and 6-phosphatidylfructose. The relative abundance of these two products remains uncertain and warrants further investigation as it could give valuable insights in the PLD enzyme's catalytic behaviour. In similarity with the glucose and galactose conjugates, these products may undergo rearrangement between cyclic forms and anomeric configurations post-conjugation, resulting in a complex product profile.

Unlike the other monosaccharides, which are hexoses, xylose is a pentose. In aqueous solution, xylose predominantly adopts the pyranose configuration, where primary hydroxyl groups are absent. Although its furanose form contains a primary hydroxyl group, xylofuranose is present in only minor proportions during the transphosphatidylation reaction. As discussed in previous chapters, the transphosphatidylation with xylose was surprisingly fast considering the fact that it has no- or only very low titres of primary hydroxyl groups readily available for conjugation. This led to the hypothesis that conjugation occurs via one of its secondary hydroxyls, C1-C4 instead. The NMR analysis confirmed this hypothesis, and gave strong evidence of conjugation solely via C4.

The majority of detected product was in the pyranose forms and a little amount in furanose forms.

The central objective of the remaining work on our structural analysis aims to resolve the cyclic and anomeric configurations of the resulting phosphatidyl-saccharide conjugates and, if possible, unravel the relative tautomeric composition for each product. This could give valuable insight in the PLD catalytic behaviour especially in case of fructose and xylose products.

Characterization of physico-chemical properties

As previously discussed, phospholipids self-assemble into bilayer structures that form vesicles when dispersed in excess water. Fundamental characterisation of the physico-chemical properties of these vesicles is important for their use as excipients and for basic understanding of their function *in vivo* and *in vitro* [78]. The size and shape of the vesicles plays an important role in their ability to encapsulate cargo and affect the circulation half-life of liposomes. The fluidity of the membrane influences its permeability, while lamellarity affects encapsulation efficiency, and the kinetics of drug release [79, 80].

Effect of replacing the choline head group with glucose

DOPC, DPPC and DSPC are all common lipid excipients that are known to form vesicles when dispersed in excess aqueous solution. In **Paper III**, we investigate how conjugation with glucose instead of choline affects their vesicle morphology. All lipids were studied at 25 ºC by a combination of SAXS/WAXS and cryo-TEM. The choline conjugated lipids served as reference points for the characterization of the newly synthesized glycophospholipids. The SAXS scattering curves, extracted electron density profiles and cryo-TEM images for the reference lipids are displayed in Figure 11. These lipids all formed MLVs with several correlated bilayers when dispersed in water. The saturated DPPC and DSPS (16:0 and 18:0 in regards to their fatty acid composition) were studied below their phase transition temperatures (41 ºC and 55 ºC respectively) [81], meaning that their acyl chains were in crystalline state, which resulted in faceted structures (Fig 11B and 11C). They also contained a higher number of stacked bilayers compared to the monounsaturated DOPC (18:1) (Fig. 11A). This higher degree of correlated bilayers can be explained by the denser packing and thereby stronger attractive van der Waals forces between their saturated hydrocarbon tails compared to DOPC. Due to the high viscosity of DSPC, it was not possible to do proper blotting during cryo-TEM sample preparation, and hence these vesicles could not be studied by imaging.

Modification of the lipid head groups with glucose resulted in significant changes of vesicle morphology. While the reference analogues formed MLVs, DOP-Glu formed ULVs (Fig 12A), DPP-Glu formed discs (Fig 12B) and DSP-Glu formed bilayer stacks with a small sub-population of MLVs (Fig 12C). When mixed in a 1:1 ratio of DOP-Glu:DOPC (w/w), ULVs with a similar appearance as the vesicle formed from pure DOP-Glu were formed, with only a minor sub-population of MLVs. The transition from MLVs to ULVs after modification of the head group is a consequence of the swelling limit for lamellar phases [82]. Bilayers stacked together in multi-layered structures are separated with a certain repeat distance, d, that is determined by the balance between attractive forces such as van der Waals forces and hydrogen bonding, and repulsive forces such as steric interactions, electrostatic forces of charged lipids and the entropy gain due to fluctuations in the bilayer. It is possible that the introduction of the highly hydrated and bulky saccharide head group in our new lipids increased the flexibility of the bilayer in comparison with PC, and consequently the fluctuations and the repulsive forces increased, which prevented the formation of multi-layered structures [72].

Negatively charged PLs are moreover known to cause more swelling of the lipid bilayer compared to zwitterionic lipids such as PC [83, 84]. The hydroxyl groups form multiple hydrogen bond with each other, but also with surrounding water and thus cause swelling of the bilayer. This swelling will also prevent formation of multi-layered structures. This hypothesis was further strengthened by the fact that we did not see any dramatic effects of increased ionic strength. If the separation of the multilamellar structures was primarily caused by the net negative charge on the phosphatidyl saccharides, the shielding effect of higher electrolyte concentration would screen the electrostatic repulsion and promote formation of MLVs as was the case for PC lipids. However, as the salt solution was added after preparation of the vesicles, and any transportation of charges species across the bilayer is expected to be slow, the electrolyte concentration inside the vesicles was potentially lower than the bulk concentration. This could diminish their shielding effect. In that case the separation could be driven by charge repulsion, which has previously been reported for PC vesicles with addition of a small amount of anionic lipid [85]. Most probably, both mechanisms contribute to different extent to the observed vesicles morphologies. For some structures the head bulkiness dominates, and for others the negative charge is more prevalent for the breakage of multilamellar structures.

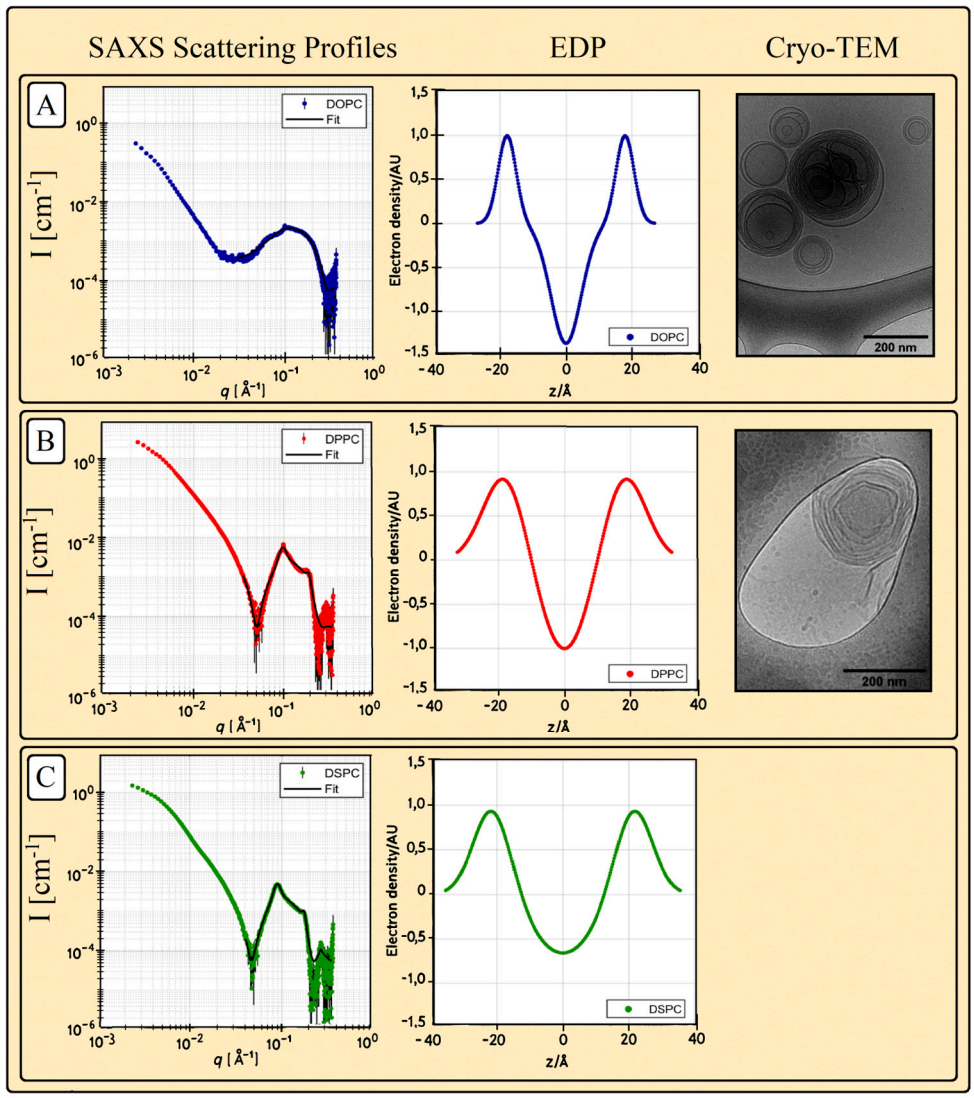

Figure 11. SAXS scattering profiles, electron density profiles and cryo-TEM images of reference lipids; Panel A: DOPC, Panel B: DPPC and Panel C: DSPC in water, 25°C.

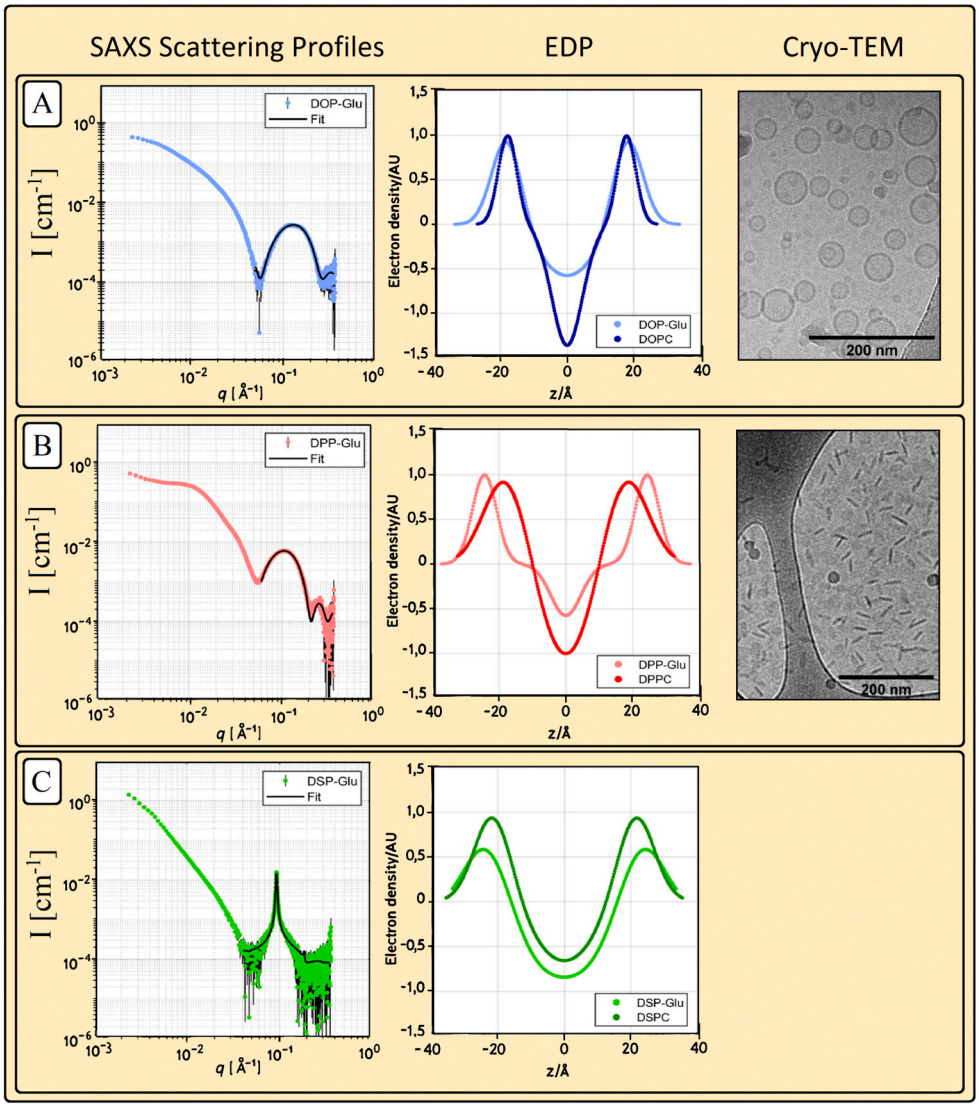

Figure 12. SAXS scattering profiles, electron density profiles and cryo-TEM images of phosphatidylglucose with various acyl chain compostion; Panel A: DOP-Glu, Panel B: DPP-Glu and Panel C: DSP-Glu in water, 25°C.

Effect of acyl chain composition of Phosphatidylcholine and Phosphatidylglucose

Bilayer thickness can directly impact vesicle properties such as their colloidal stability and deformability against e.g. shear. Thicker bilayers are commonly less flexible and less permeable compared to thinner ones, reducing leakage and enabling slow release. However, this rigidity can limit their adaptability to external conditions, controlled release, and fusion with other membranes. Thicker bilayers are also often associated with a higher lipid phase transition (chain melting) temperature from the more rigid gel phase to the flexible liquid crystalline state. For these reasons determination of bilayer parameters is an important part of the vesicle characterisation.

The bilayer thickness varied in the order of 18:1<16:0<18:0 for both the phosphatidylglucose lipids and their choline analogues, which is in agreement with their effective acyl chain lengths [86]. Longer acyl chains increase hydrophobicity, which leads to denser lipid packing due to more van der Waals interactions. This may promote more correlated structures and aligns with the higher number of stacked bilayers in the vesicles observed for DPPC and DSPC compared to DOPC as well as for DSP-Glu compared to DPP-Glu and DOP-Glu.

The modified phosphatidylglucose lipids consistently had a larger head-to-head spacing than their PC analogues, especially for the lipids with saturated acyl chains. This could potentially be a result of the combined effect of a larger, bulkier head group, and the high number of hydroxyl groups on phosphatidylglucose compared to PC. As the bilayer thickness increases, so does the bending modulus and the energy required to form curved structures [87, 88]. Since the bilayer thickness of DSP-Glu is much larger than DSPC, this could explain the formation of bilayer stacks rather than MLVs.

Effect of different carbohydrate head groups

The conjugation of different saccharides to the head group lead to significant variations in vesicle morphologies, highlighting the influence of carbohydrate structure on lipid self-assembly as discussed in **Paper IV**. The SAXS scattering profiles, extracted electron density profiles and cryo-TEM images of vesicles formed by the glycophospholipids conjugates are displayed in Figure 13. As previously noted, DOP-Glu formed small unilamellar vesicles (ULVs), which were characterized by their uniform size and shape (Fig 13A). Similarly, DOP-Fru produced ULVs, which were similar to DOP-Glu vesicles in both shape and size (Fig 13C). In contrast, DOP-Gal generated multilamellar vesicles (MLVs) with a large number of bilayers. This might be due to denser packing promoted by attractive interactions between saccharide head groups (Fig 13B). DOP-Xyl also resulted in MLVs, but with fewer stacked bilayers compared to DOP-Gal (Fig 13D).

Interestingly, the largest difference in vesicle morphology was exhibited by the glucose and galactose lipid conjugates. In their monomeric forms, these carbohydrates are structurally the most similar, and only differ in the stereochemistry at the C4 position. This observation suggests that even minor changes in hydroxyl group orientation can have a substantial impact on lipid self-assembly, likely by influencing intermolecular interactions and steric effects within the bilayer. The orientation of the C4 hydroxyl group may alter hydrogen-bonding capabilities and hydration, thereby affecting vesicle size, and bilayer organization. Furthermore, the stability and hydrogen-bonding potential of the saccharides themselves can play a critical role. Beta-glucose, with its hydroxyl groups organized in equatorial positions, represents the most energetically favourable hexose configuration and has the greatest capacity to form hydrogen bonds with surrounding water molecules. Deviations from this optimal configuration, whether due to changes in stereochemistry or ring form, could disrupt the hydrogen-bonding network and swelling of lipid bilayer, thereby influencing the self-assembly process of the lipid conjugates.

However, it is crucial to recognize that the final product compositions of the lipid-carbohydrate conjugates reflect the equilibrium of all possible carbohydrate tautomers in the head group. This includes the distribution of pyranose and furanose ring forms, as well as alpha and beta anomeric configurations. As a result, the product composition of DOP-Glu may differ significantly from that of DOP-Gal in terms of the relative proportions of these cyclic and anomeric forms. Such differences in tautomeric equilibria would significantly contribute to the widely varying vesicle morphologies observed between the two lipid conjugates.

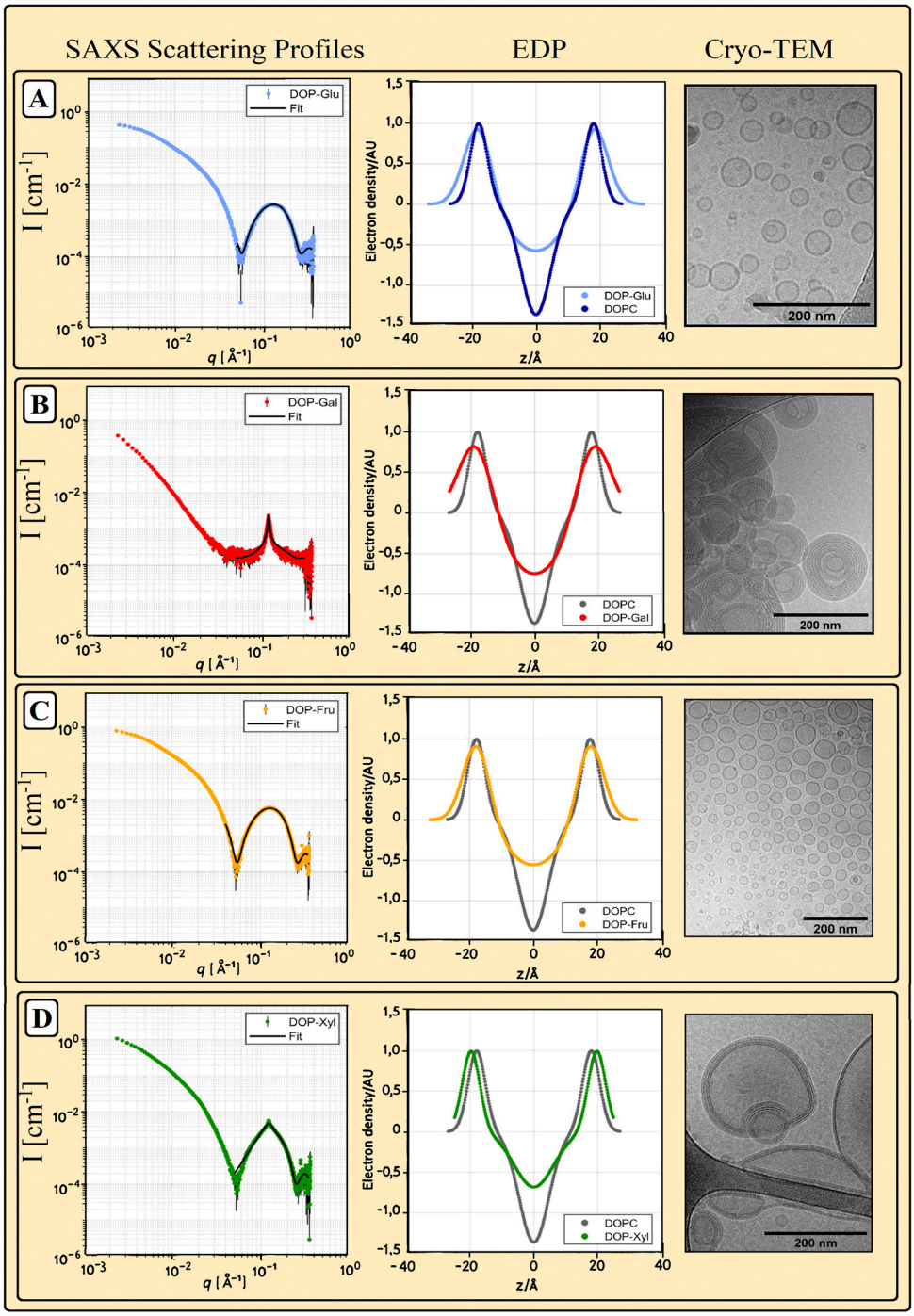

Figure 13. SAXS scattering profiles, electron density profiles and cryo-TEM images of glycophospholipids conjugates with different saccharide head groups; Panel A: DOP-Glu, Panel B: DOP-Gal and Panel C: DOP-Fru, Panel D: DOP-Xyl in water, 25 °C.

Concluding remarks and future perspectives

This thesis focuses on the enzymatic synthesis of novel glycophospholipids, characterization of their chemical structures, and investigation of their self-assembly behaviour in aqueous solutions. A phospholipase D (PLD) catalysed transphosphatidylation reaction system was developed using DOPC and glucose as a model system. The reaction parameters were optimizes to maximize transphosphatidylation yield while minimizing the formation of hydrolytic by-product, phosphatidic acid (PA). This resulted in an adaptable method, which was further extended to synthesize various glycophospholipid conjugates, incorporating both monosaccharides and disaccharides. Isolation of pure lipid material through a combination of extraction and flash chromatography, provided material for the structural and initial physico-chemical characterization of these lipids.

Our studies revealed that replacing the common phosphatidylcholine (PC) head group with glucose had a dramatic effect on vesicle structures. While PCs typically forms multilamellar vesicles, the conjugation with glucose promoted the separation of bilayers, resulting predominantly in unilamellar vesicles. This separation is driven by altered intermolecular forces, where the net negative charge and the bulkiness of the carbohydrate head groups play a significant role. Furthermore, we discovered that the acyl chain composition of the lipid significantly affects the self-assembly. Shorter effective acyl chain length promoted more efficient bilayer separation, likely due to the reduced contribution of attractive van der Waals forces. These are essential for maintaining correlated bilayer structures.

Additionally, the type of carbohydrate in the head group had a profound effect on vesicle morphology. For instance, small differences in stereochemistry, such as the orientation of the hydroxyl group on C4 in DOP-glucose and DOP-galactose, resulted in remarkable variations: DOP-glucose predominantly formed unilamellar vesicles (ULVs), while DOP-galactose formed multilamellar vesicles (MLVs) with a high degree of bilayer correlation. This highlights the critical role of carbohydrate stereochemistry in lipid self-assembly behaviour. However, the detailed composition of the lipid material is fundamental for the resulting self-assembly characteristics, and more characterisation of the chemical composition of the lipids is essential to draw conclusions on how the chemical structure influence lipid self-assembly.

To address this, we employed a combination of mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. While mass spectrometry determined the

molecular formula and confirmed the chemical composition, it had limitations in resolving carbohydrate stereochemistry and the conjugation position. NMR spectroscopy confirmed the presence of multiple conjugation products. Glucose and galactose were revealed to conjugate via C6, which is the only primary hydroxyl available. Fructose, which depending on tautomer can have two primary hydroxyls, resulted in a mixed product with lipid conjugation via both C1 and C6. In addition, both pyranose and furanose form of DOP-fructose was detected in the lipid conjugate. The most surprising result was the conjugation of xylose via secondary hydroxyls, which usually are less accessible compared to primary hydroxyls for trans-phosphatidylation utilizing PLD as catalyst. Potentially, this can be attributed to its smaller size compared to the other carbohydrates. Ongoing work on this topic aims to fully resolve the chemical structures of our novel glycophospholipid structures and provide deeper insights into the chemical composition of the lipid conjugates.

Our findings underline that modifying the phospholipid head group with saccharides fundamentally alters the balance of attractive and repulsive forces within bilayers, leading to substantial changes in vesicle morphology. Furthermore, the acyl chain composition contributes with a surprisingly large influence on self-assembly behaviour. However, to fully understand the interplay between these forces and their impact on observed behaviours, additional experimental studies are required.

Looking ahead, this work lays the foundation for exploring the broad potential of glycophospholipids as molecular building blocks. The ability to finely tune the self-assembly and structural properties of lipids through head group and acyl chain modifications opens opportunities in fields such as drug delivery, nanotechnology, and biomedicine. Future research should aim to expand the diversity of carbohydrate-lipid conjugates, unravel their complex structural variations, and investigate their functional applications in greater depth.

Further investigation of PLD catalysed transphosphatidylation in regards to different donor and acceptor substrates would exploit the rich diversity of carbohydrates [89] and phospholipdis [6] and could provide a diverse portfolio of glycophospolipid conjugates with tailored properties.

A large step in linking the self-assembly behaviour to chemical structure would be unravelling the relative tautomeric composition of the lipids material. In depth determination of product conjugation and spatial arrangements of carbohydrate is a fundamental part of the lipid structural characterization as these pure compounds will behave like product mixtures when dispersed in solvent.

Further physico-chemical characterization could aim to document essential vesicle properties for drug delivery applications, including particle size, surface charge and phase transition temperature. Subsequent steps could involve loading these vesicles with relevant cargo and evaluate parameters such as storage stability, leakage rate, drug release rate and encapsulation efficiency [90, 91].

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