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Constructing Lipid Tools for Biochemical Applications

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# Constructing Lipid Tools for Biochemical Applications

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Chemistry

by

Judith Flores

Committee in charge:

Professor Neal K. Devaraj, Chair
Professor Geoffrey Chang
Professor Alexis C. Komor
Professor Brian Leigh
Professor Navtej Toor

2022

The dissertation of Judith Flores is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

# DEDICATION

I dedicate this dissertation to my incredibly supportive family.
My parent's sacrifice to move to a different country has been
the sole purpose of why I can now explore my full potential.

# EPIGRAPH

“Science, and our society, are stronger when the people doing science reflect our society as a whole.”

Lydia Villa-Komaroff

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# ACKNOWLEDGEMENTS

This dissertation would have not been completed without the help and support of my own personal village. My parent’s highest level of education stopped at primary school, but that did not stop them from knowing the value of academic education. I owe many thanks to my mom and dad for never discouraging me from perusing higher education, even when the road seemed long. As the youngest of four daughters, I have my sisters to thank for paving the path for me throughout the years. A special thanks to my sister Lilibeth for introducing me to the Office of Training, Research, and Education in the Sciences (OTRES); an organization that deserves a thanks of its own. I would like to thank the rest of my family such as my grandparents, aunts, uncles, and cousins for being such amazing cheerleaders.

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## Notes About the Chapters

Chapter one was in part (with co-author permissions) published as **J. Flores**, B. M. White, R. J. Brea, J. M. Baskin, N. K. Devaraj "Lipids: Chemical Tools for their Synthesis, Modification, and Analysis," *Chem. Soc. Rev*., 2020, 49, 4602-4614. The dissertation author was the primary researcher and author of this material.

Chapter two, in full, is a reprint (with co-author permission) of the material as it appears in the publication: **J. Flores**, R.J. Brea, A. Lamas, M. Salvador-Castell, C. Xu, C.R. Baiz, S.K. Sinha, N.K. Devaraj "Rapid and Sequential Dual Oxime Ligation Enables De Novo Formation of Functional Synthetic Membranes from Water-soluble Precursors," *Angew. Chem. Int. Ed.* 2022, e202200549.The dissertation author was the primary researcher and author of this material.

Chapter four, in part, is currently being prepared for submission for publication. Flores, Judith; Khanal, S; Devaraj, Neal K. I would like to thank the Rapoport laboratory for providing the genes of both Sey1 and Yop1. The dissertation author was the primary researcher and author of this material.

# VITA

## Education

| | |
|---|---|
| **2022** | Doctor of Philosophy, Chemistry, UC San Diego |
| **2019** | Master of Science, Chemistry/Biochemistry, UC San Diego |
| **2017** | Bachelor of Science, Biochemistry, CSU San Marcos |

## Publication

**J. Flores**, B. M. White, R. J. Brea, J. M. Baskin, N. K. Devaraj “Lipids: Chemical Tools for their Synthesis, Modification, and Analysis,” *Chem. Soc. Rev*., 2020, 49, 4602-4614.

**J. Flores**, R.J. Brea, A. Lamas, M. Salvador-Castell, C. Xu, C.R. Baiz, S.K. Sinha, N.K. Devaraj “Rapid and Sequential Dual Oxime Ligation Enables De Novo Formation of Functional Synthetic Membranes from Water-soluble Precursors,” *Angew. Chem. Int. Ed.,* 2022, e202200549.

## Awards

| | |
|---|---|
| **2017** | National Science Foundation Graduate Fellowship |
| **2017** | Alfred P. Sloan Student Fellowship |
| **2017** | San Diego Fellowship |

# ABSTRACT OF THE DISSERTATION

Constructing Lipid Tools for Biochemical Applications

by

Judith Flores

Doctor of Philosophy in Chemistry

University of California San Diego, 2022

Professor Neal K. Devaraj, Chair

Synthetic membranes have long been a powerful platform to reconstruct life's functions and shed light on the origin-of-life. More recently, they have been employed as convenient model systems to reconstitute membrane proteins, allowing their biochemical characterization and use in applications such as nanopore sequencing. While several techniques rely on commercially available phospholipids to form functional membranes, a grand challenge is the de novo construction of synthetic membranes in a manner that more closely mimics native lipid membrane generation in cells. Chemoselective coupling reactions have been used to generate cell-like synthetic lipid membranes. These methodologies suffer from distinct limitations, including the use of considerable amounts of micelle forming amphiphilic precursors that can interrupt lipid bilayer formation.

Here we describe a novel *de novo* phospholipid synthesis strategy to rapidly form biomimetic membranes at physiological conditions in the micromolar concentration range, without the use of amphiphilic starting materials. The methodology takes advantage of oxime dialkylation via the condensation of two simple water-soluble precursors, a short-chain aldehyde and a dihydroxylamine-containing phosphocholine to afford a dual oxime phospholipid. Our strategy enables the chemoselective synthesis of phospholipids, which spontaneously self-assemble into membrane-bound vesicles. Membrane formation takes place in biologically relevant aqueous solution, and is stable in the presence of a variety of biomolecules and small polar molecules. Advantageously, we were able to reconstitute a membrane protein via *in situ* oxime phospholipid formation without the use of initial detergent solubilization. The oxime-based reconstitution technology can be used as a powerful tool for the straightforward fabrication of proteoliposomes, enabling thus to investigate membrane protein structure and function.

We believe our novel chemoselective phospholipid synthesis approach will aid in creating functional artificial cells. Additionally, we envision our work will aid the study of membrane proteins within synthetic membranes or organelles.

# 1 Introduction

## 1.1 Motivation

Phospholipids provide structure for biological membranes, defining the boundaries of life.[1] These boundaries function as separation and protection from the environment, compartmentalization of functions, energy production, storage, protein synthesis and secretion, and cell-cell interaction. The phospholipids that construct these membranes are highly diverse in structure, and their distribution varies between organisms. This immense diversity arises from the biosynthesis of various combinations of these building blocks and results in a wide range of functional implications.[2]

Despite their essential functions and significance in cells, lipids remain less studied than other equally important biomolecules, such as proteins and nucleic acids. Relative to other disciplines of study, lipid research lacks the breadth of techniques required to interrogate, manipulate, and visualize their functions. Furthermore, given the high structural diversity of lipid families, it is not possible to accommodate all classes with common methods of extraction, purification, characterization, and analysis. Because of the vast diversity, much of the knowledge we have about lipids comes from the study of synthetic membranes with specific lipid compositions in a bottom-up approach. These model membranes consist of a few select lipid species, enabling a better understanding of the specific properties and functions of the selected lipids in a controlled setting. Bottom-up model membrane research also allows for the study of other cellular properties by increasing the complexity *in vitro*. Over the last few decades, the field of cell-free synthetic biology has grown rapidly, providing fruitful incite and vivid demonstration of large-scale biomimetic behaviors.

Lipids are generally synthesized in two ways in eukaryotic cells.[3] In the Kennedy pathway, phospholipids are synthesized *de novo* via several enzymatic reactions.[4] Alternatively, the Lands cycle is a route in which a pair of enzymes carry out diacylation/reacylation reactions to modify fatty acid composition of phospholipids produced in the Kennedy pathway.[5] Efforts to mimic these reactions have included the reconstitution of the recombinant enzymes into synthetic membranes and *de novo* lipid formation *in vitro* using non-enzymatic reactions.[2,6–14]

Over the past two decades, many methods for creating and utilizing synthetic membranes have been published. These methods have been valuable in understanding fundamental biological concepts, and used for a variety of applications in drug delivery, microreactor design, and origin of life studies.[10,15,16] Therefore, there is significant interest to widen the tools that create synthetic membranes. In this chapter, I will cover recently developed technologies employed for chemical synthesis and modification of lipids *in vitro*.

## 1.2 Biorthogonal and chemoselective strategies

A major goal of biomimetic membrane chemistry is to develop methods to synthesize cell-like membranes in the absence of enzymes. The general strategy is to find synthetic routes to produce membrane-forming lipids from simple non-membrane forming precursors in aqueous solution to promote lipid self-assembly. Given the importance of mimicking biological systems, several research groups have recently explored chemoselective coupling reactions for the *de novo* generation of synthetic membranes. The result has been an increasingly robust and diversified toolbox for chemical lipid synthesis. The evolution of bilayer membrane formation by *in situ* lipid synthesis has been set forth through the efforts of several groups. For example, pioneering work by Zepik et al. demonstrated that chemical reactions that form a sulfur bridge between surfactant precursors led to vesicle-forming amphiphiles which resembled naturally occurring phospholipids.[17] In this section, I will highlight the most prominent strategies that have been employed for *in situ* formation of lipids capable of driving the self-assembly of biomimetic membranes.

### 1*.2.1 Copper-catalyzed click chemistry*

Budin and Devaraj demonstrated the use of a copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction for the efficient synthesis of phospholipids, paving the way for additional bioorthogonal reactions to be employed for lipid synthesis.[18] In this study, a simple alkyne-functionalized lysolipid and an oleyl azide were coupled using a copper(I) catalyst, which afforded a biomimetic triazole-containing phospholipid. Remarkably, the CuAAC approach has been utilized to design a self-reproducing system that can drive the repeated synthesis and growth of phospholipid membranes.[19] The regeneration of membrane bound autocatalysts continuously induces the formation of triazole phospholipids, mimicking natural membrane generation.

Employing a similar biomimetic approach, Enomoto et al. added spatiotemporal control to phospholipid synthesis using intramolecular photoinduced electron transfer to generate copper(I) from a photosensitizer dyad, triggering the CuAAC reaction and the subsequent *in situ* phospholipid membrane formation.[20]

### *1.2.2 Native chemical ligation*

NCL (native chemical ligation) is one of the most popular tools for the synthesis and derivatization of large peptides, small proteins, and nucleic acids. Recently, Brea et al. utilized NCL to couple long-chain acyl thioesters to cysteine-functionalized lysolipids in a highly specific and chemoselective way to form the corresponding phospholipids.[21] These lipids are capable of self-assembly into vesicles that can grow to several microns in diameter. The versatility NCL was further employed for the incorporation of functional integral membrane proteins into biomimetic membranes. For instance, Brea et al. developed an *in situ* lipid synthesis for protein reconstitution technology (INSYRT) for the construction of adenosine A2A receptor (A2AR) proteoliposomes.[22] INSYRT proceeds in the absence of dialysis and/or detergent absorbents, which make it an excellent tool for the assimilation of G protein-coupled receptors (GPCRs) into synthetic liposomes.

The Devaraj group has also shown that a reversible NCL reaction can used to achieve *in situ* remodeling of both lipid acyl chains and head groups, triggering changes in vesicle spatial organization, composition, and morphology.[23] The application of this novel approach allowed the construction of dynamic membranes that can spontaneously generate and subsequently modulate their physical and chemical properties by using nonenzymatic chemoselective reactions.

### *1.2.3 Dynamic imine chemistry*

Another relevant chemoselective strategy for the *in situ* formation of lipids is the combination of aldehyde and amine amphiphiles to form an imine linkage. One of the most attractive features of this reaction is its reversibility depending on environmental factors. This strategy is advantageous when engineering synthetic cells with dynamic features. Although the ligation is reversible, Sugawara showed that combination of two single chains containing the necessary moieties afforded a kinetically stable product.[24] This reaction results in the release of a negatively charged molecule, which aids in the *in situ* formation of giant vesicles. Continuing the efforts to develop vesicles that resemble simple cell-like features, Matsuo et al. generated an imine based self-reproducing protocell formed from a synthetic oleyl phospholipid (V ole).[25] The unsaturation of the lipid facilitated the fluidity of the structure, resulting in the budding and replication of the vesicles by the continuous addition of the phospholipid precursor. Further, the resulting vesicles can serve as microreactors, as shown by the encapsulation and amplification of DNA. In a similar study, Seoane et al. used the reversibility of the imine formation approach to develop liposomes that respond to external stimuli and showed controlled release of encapsulated cargo.[26]

### *1.2.4 Other ligations*

Histidine ligation (HL) has also been employed to form phospholipids *in situ*.[27] The catalytic role of the imidazole ring of a histidine-containing lysolipid was used to drive its coupling to a long-chain acyl thioester, affording a new class of biomimetic phospholipids. These synthetic lipids have physical properties similar to their natural counterparts and spontaneously self-assemble into micron-sized vesicles. The chemoselectivity, high reactivity, and biocompatibility of the HL approach make it a powerful tool for the efficient encapsulation of relevant biomolecules such as proteins. Zhao and coworkers also reported the formation of novel synthetic phospholipids using a two-step coupling consisting of a sulfur transfer and a subsequent [2,3]-sigmatropic rearrangement.[28] The ligation afforded an ortho-sulfiliminyl phenol product by combining an oxyactamide moiety with a thiophthalimide group. Fluorescent microscopy confirmed that these phospholipids formed biomimetic vesicles.

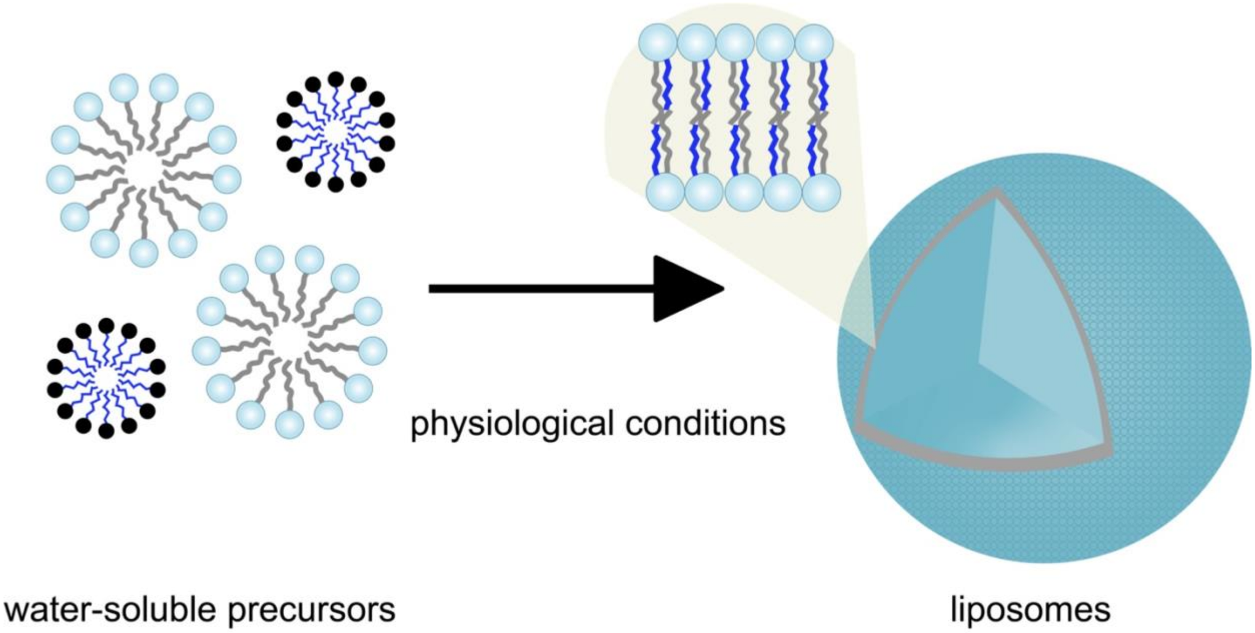

**Figure 1.1: Simplified schematic of in situ phospholipid formation.**
The micelle-like structures represent the water-soluble starting materials since most techniques require amphiphilic precursors that arrange into micelles in an aqueous environment. The goal is to only use simple chemical reactions without the use of enzymes in physiological conditions to produce phospholipid-like structures that self-assemble into liposomes

## 1.3 Incorporation of membrane proteins into artificial cells

Integral and membrane proteins play vital roles in biological processes, yet they have been traditionally difficult to study due to several factors.[4] Primarily, membrane protein expression and purification yields are low due to the toxicity of high expression levels to the host.[29] In addition, the detergents used to isolate and purify membrane proteins from the cellular milieu in common reconstitution methods do not reflect the protein's native cellular environment, most noteably the phospholipid bilayer. Using alternative methods to avoid the use of traditional detergents in membrane protein reconstitution provides a solution to such problems. Brea et al. have previously developed a synthetic strategy to use reactive detergent precursors that form membranes *in situ* to solubilize proteins and subsequently reconstitute them into artificial phospholipid membranes. *In situ* lipid synthase for protein reconstruction technology (INSYRT) was used to reorganize GPCRs into proteoliposome in our lab.[22] The INSYRT method uses NCL-promoted incorporation of the membrane protein into synthetic liposomes by using n-dodecyl-β-D-maltoside (DDM) thioester derivatives. Unfortunately, this method still requires detergents for protein purification. As they can often interfere with protein function, a detergent free reconstitution method would be of great utility for recombinant membrane protein synthesis.

In developing the dual oxime ligation strategy, we introduce a simple chemoselective method to form phospholipids *in situ* around membrane protein extracts, eliminating the need for harsh detergents in recombinant protein reconstitution. Chapter two focuses on the characterization of the novel phospholipid to decipher how well it mimics a natural phospholipid. Chapter three explores the technique in relation to those that have been previously developed in our lab. Finally, Chapter four focuses on showcasing the applications of dual oxime ligation by attempting to create a synthetic organelle.

### 1.4 Acknowledgements

Chapter one was in part (with co-author permissions) published as **J. Flores**, B. M. White, R. J. Brea, J. M. Baskin, N. K. Devaraj “Lipids: Chemical Tools for their Synthesis, Modification, and Analysis,” *Chem. Soc. Rev*., 2020, 49, 4602-4614. I would like to thank Roberto Brea for his contribution in drafting and editing the manuscript. I would like to thank Neal Devaraj for his oversight and assistance in preparing the manuscript. The author of the dissertation is the primary author of this manuscript.

# 2 Dual oxime ligation for de novo formation of functional synthetic membranes

## 2.1 Introduction

Generating functional lipid membranes can provide compartments for artificial cells and may suggest mechanisms of cellular evolution at the origin of life.[30] Membranes formed from phospholipids can also be used to reconstitute transmembrane proteins, facilitating their biochemical and structural characterization, as well as enabling applications such as nanopore sequencing.[10,31–35] While a number of techniques rely on preformed phospholipids to form functional membranes, a grand challenge is the *de novo* construction of synthetic membranes in a manner that more closely mimics native lipid membrane generation in cells. Living cell membranes are primarily composed of phospholipids.[9] Natural phospholipid synthesis involves a large number of enzymes and requires the generation of activated fatty acids that are then coupled to polar head groups, eventually yielding dialkyl lipids that can self-assemble to form planar bilayers.[36,37] Reconstituting these enzymatic processes remains challenging, though recent efforts have shown that lipid generation is feasible through careful enzyme reconstitution.[12,14,38] An alternative approach is to create membranes composed of non-canonical lipids using a variety of biomimetic chemistries in the absence of enzymes.[2] These strategies have relied on micellar assembly of two single-chain reactive precursors to accelerate the coupling reaction and form synthetic phospholipids.[21,22,25,27,28,39] For instance, it was recently shown that dynamic imine bond formation between a single-chain lysosphingomyelin and several single-chain aldehydes yields membrane-forming lipid analogs in water, leading to the spontaneous assembly of vesicles.[26] Unfortunately, relying on micellar assembly to initiate reaction has several drawbacks. The amphiphilic and membrane-disrupting nature of the precursors used can negatively affect the function and structure of the desired lipid membrane as well as embedded biomolecules such as proteins.[40] Furthermore, the formation of non-canonical phospholipids from soluble polar head groups and two single-chain precursors is not feasible, as the polar head group would not be expected to assemble into micelles.[8] Chemistry that could enable rapid dialkylation of polar head groups to form lipid analogs would better mimic cellular *de novo* phospholipid synthesis and would decrease the chance of perturbing the structure and/or function of membranes and other associated biomolecules.

To achieve this goal, we envisioned a system where two alkyl chains could be chemoselectively attached to a phosphocholine (PC) head group possessing two reactive functional groups. By tuning the composition, length, and functional groups of the head group and reactive alkyl moieties, we hypothesized that all precursors could be made non-amphiphilic and water soluble, while the resulting product would still assemble into lipid membranes. With these parameters in mind, we decided to explore leveraging oxime couplings to generate synthetic phospholipids.[41,42] There has been a recent surge of interest in using oxime ligation for bioconjugation.[43] Oxime formation between aminooxy moieties and aldehydes has been shown to be highly rapid, compatible with biological functional groups, and chemoselective.[42,44–46] Additionally, due to their favorable kinetics, oxime ligations are highly suitable for multivalent labeling of biomolecules, a necessity if we aim to install two alkyl chains onto a single-charged lipid head group. Also, compared to imines and hydrazones, oximes are far more stable.[42] Here we demonstrate that oxime ligations can rapidly lead to the formation non-canonical phospholipid membranes at physiological conditions using micromolar concentrations of water soluble precursors. Our methodology uses two sequential oxime ligations via the condensation of two simple water-soluble precursors: two short-chain aldehydes and a diaminooxy-containing PC head group. Finally, we use *de novo* membrane formation via oxime ligation to directly incorporate an overexpressed transmembrane protein [diacylglycerol kinase (DAGK)] from bacterial extracts, without the addition of detergents. To our knowledge this is the first example of biomimetic phospholipid synthesis from non-amphiphilic precursors, avoiding the requirement of excess detergent-like molecules during *de novo* membrane formation.

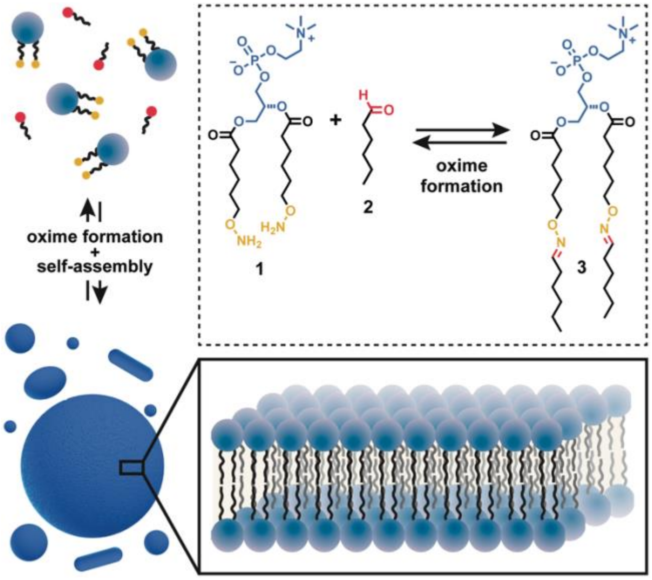

**Figure 2.1: De novo synthesis of dioxime-based phospholipid**
De novo synthesis of dioxime-based phospholipid 3 and subsequent self-assembly into membrane-bound vesicular structures. Top right, Schematic representation of the dual oxime bond formation leading to phospholipid 3 by reaction between the diaminooxy-containing phosphocholine 1 and hexanal (2). Note, only one product isomer (trans) is depicted.

## 2.2 Phospholipid synthesis

We first synthesized a diaminooxy PC head group by double esterification of *L*-α-glycerylphosphorylcholine (GPC) with *N*-Boc-protected (aminooxy)hexanoic acid. After acidic deprotection, the resulting free diaminooxy-containing PC derivative (**1**) was resuspended in phosphate-buffered saline solution, where it was readily soluble. In deciding on a single-chain aldehyde to react with **1**, we were attracted to the use of hexanal, which has been shown to have a water solubility of 50 mM.[47] Reaction of **1** with two equivalents of hexanal by oxime ligation would create a non-canonical phospholipid mimicking 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), which should readily form vesicles.

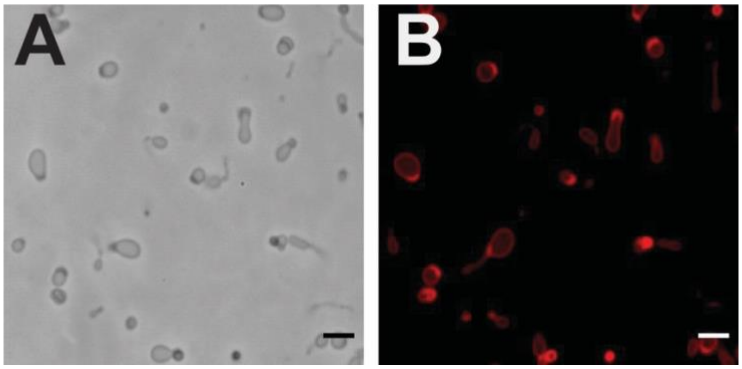

**Figure 2.2: Oxime phospholipids microscopy**
Phase contrast *(*A) and fluorescence microscopy (B) images of membrane vesicles formed by hydration of a thin film of **3**. Membranes were stained using 0.1 mol% Nile Red dye. Scale bar denotes 10 μm.

Taking this into account, we decided to pursue our initial studies using hexanal as a model aldehyde. Precursor **1** (1 mM) was reacted with two equivalents of hexanal (**2**) (2 mM) in 1x PBS buffer (pH 7.4). The formation of dioxime-based phospholipid **3** was analyzed over time using high performance liquid chromatography (HPLC), mass spectrometry (MS), and evaporative light-scattering detection (ELSD) measurements. Complete conversion to **3** was observed in less than 30 min. Surprisingly, only traces of the monooximated PC (**M-3**) intermediate were detected, and only during the first minute of reaction. Additionally, equimolar mixtures of **1** and **2** showed the exclusive formation of **3.** We believe these results are due to the partitioning of **2** into micelles of **M-3**, bringing the reactants into proximity, and leading to an extremely rapid formation of the second oxime after the first ligation. **M-3** would be expected to have detergent-like properties since it has a single chain**.** However**,** our data suggest that **M-3** formation is rate-limiting, and once formed, is nearly immediately converted to dioxime phospholipid **3**.

The ability of hexanal to react rapidly with **1** is supported by a previous seminal study by Kool and coworkers, where they identified that simple alkyl aldehydes would have structural features that enhance the rate of hydrazone/oxime ligation compared to aryl aldehydes.[44] Finally, shorter and longer chain-length aldehydes were also screened for reaction with **1**. We found that oxime ligation occurred rapidly despite the aldehyde chain length.

However, couplings using shorter aldehydes [butanal (**8**)] led to compounds (phospholipid **10**) that did not form membranes, while longer aldehydes [decanal (**9**)] were poorly soluble in water, hindering the formation of the corresponding phospholipid (**11**).

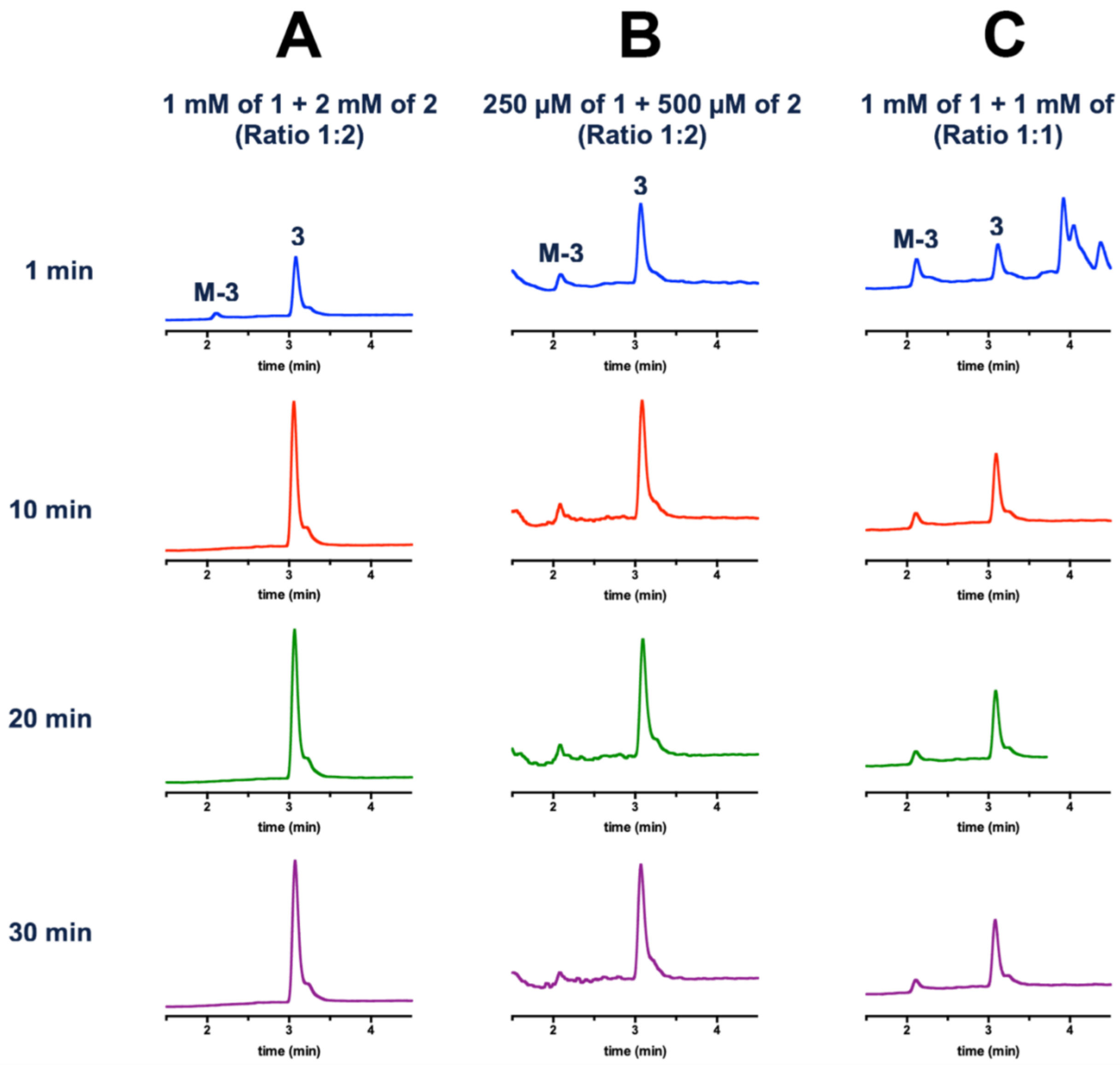

**Figure 2.3: Formation kinetics**
Kinetics of phospholipid **3** formation for different mixtures of diaminooxy-containing PC derivative **1** and hexanal (**2**) [A: 1:2 (1 mM); B: 1:2 (250 µm); C: 1:1 (1 mM)]. Complete conversion to **3** for mixtures 1:2 of **1** and **2** was observed in less than 30 min (A; 1 mM of compound **1**), even at millimolar concentrations (B; 250 µM of compound **1**) via HPLC-ELSD analysis. Surprisingly, only traces of monooximated PC (**M-3**) intermediate were detected during the reaction. Moreover, equimolar mixtures of **1** (1 mM) and **2** (1 mM) showed the exclusive formation of **3**.

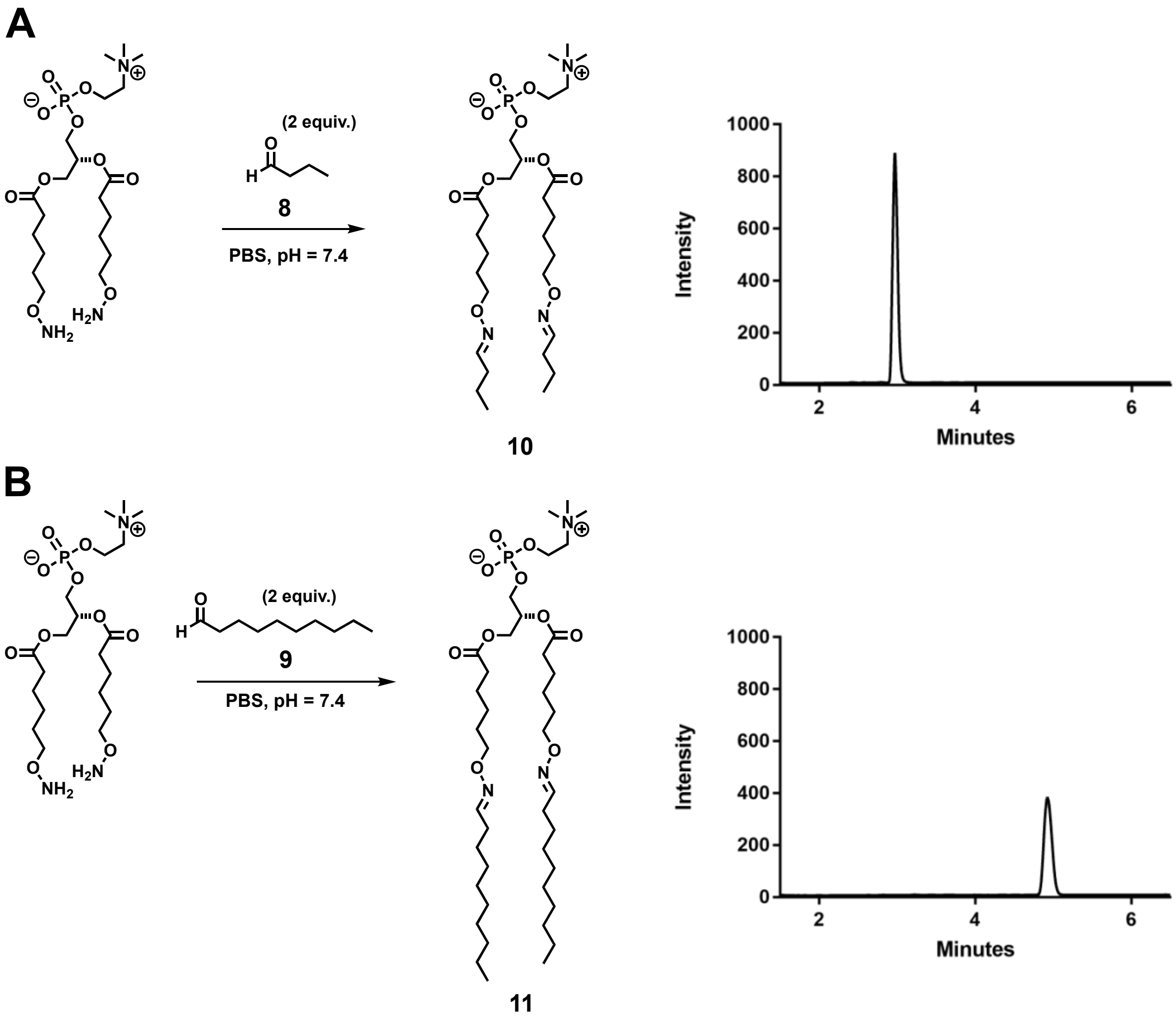

**Figure 2.4: Synthesis of different length dioxime-based phospholipids**
A) Precursor **1** and butyraldehyde [1:2] mixture resulted in phospholipid **10** after 30 min, confirmed by HPLC-ELSD-MS. The resulting phospholipid did not result in observable membranes. B) Precursor **1** and decanal [1:2] combination resulted in phospholipid **11** after 30 min. The product peak by HPLC-ELSD-MS suggests a lower yield of phospholipid conversion compared to hexanal or butyraldehyde. The resulting phospholipid showed vesicle formation.

Hydrating a thin film of purified phospholipid **3** results in the formation of micrometer-sized membrane-bound vesicles. Vesicular structures were identified by phase-contrast microscopy and fluorescence microscopy using the membrane-staining dye Nile Red. We also demonstrated that dioxime-based liposomes were capable of encapsulating and retaining polar molecules, including 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), Alexa Fluor 546, and proteins such as sfGFP.

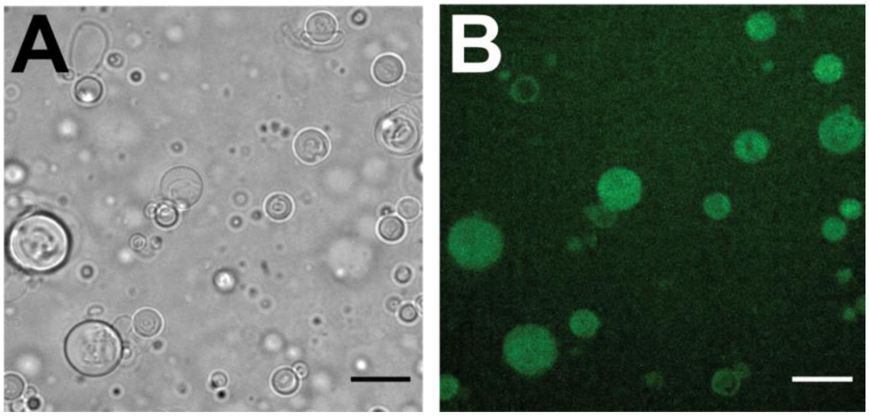

**Figure 2.5: HPTS encapsulation**
Phase-contrast (A) and fluorescence microscopy (B) images demonstrating the encapsulation of HPTS in 3 vesicles. Scale bar denotes 10 µm.

Steady-state fluorescence spectrometry measurements using standard Laurdan assays were performed to establish the minimal concentration in which the dioxime phospholipid **3** starts to aggregate into bilayer liposomes.[48] The critical aggregation concentration (cac) was estimated to be 230 µM, demonstrating that, despite the inclusion of polar oximes in the middle of the acyl chains, phospholipid **3** still self-assembles into membranes in the micromolar concentration range.

## 2.3 Characterization

To ascertain structural information about the bilayers formed from dioxime-phospholipid **3**, X-ray diffraction (XRD) studies were performed at 25 ºC and 98% relative humidity (RH) adapting previously published protocols.[49,50] Lipid multilamellar structures were formed, which give rise to Bragg peaks distanced at intervals of 2π/Q. From the one-dimensional I(Q) profile, we accurately obtained the membrane repeat distance (D = 43.2 Å), which includes the thickness of the lipid bilayer and its surrounding water layer. From the electron density profile (EDP), we extracted the lipid bilayer thickness ($D_{hh}$ = 29.2 Å), and the water layer thickness ($D_w$ = 14 Å). The EDP of the lipid bilayers presented two characteristic maxima attributable to the glycerol backbone of the lipid head groups and an intensity minimum that corresponds to the methyl terminal groups of the lipid chains. For convenience, 0 Å represents the midplane of the lipid bilayer. Dioxime phospholipid **3** has a particularly thin bilayer thickness, probably due to a higher tilt on the oxime bonds, as is observed for unsaturated lipids. This tilt is likely due to the mixture of *cis*/*trans* isomers formed during the oxime ligation, which we experimentally observed using $^{1}$H-NMR. For instance, the fully saturated phospholipid DMPC presents a lamellar distance of 51 Å at 98% RH and 30 $^{o}$C, and its bilayer thickness is 35.3 Å.[51] Finally, the shape of the EDP corresponds to a phospholipid in a liquid disordered phase.[52]

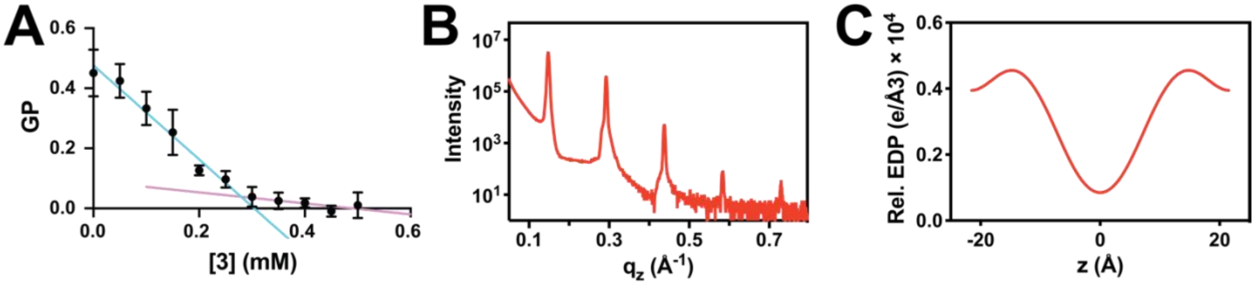

**Figure 2.6: Structural characterization of bilayer membrane**
A) Critical aggregation concentration (cac) for phospholipid **3**. Laurdan (0.1 µM) generalized polarization (GP) was determined at different concentrations of **3**, and by intersecting the two straight lines corresponding to monomer and vesicle regions a cac of 230 µM was estimated. B) X-ray diffraction (XRD) intensity profile of a phospholipid **3** multilayer film at 25 $^{o}$C with 98% relative humidity (RH). C) Relative electron density profile (EDP) of a multilayer film of phospholipid **3** at 25 $^{o}$C and 98% RH.

We characterized the oxime C=N stretch vibration using two-dimensional IR (2D IR) spectroscopy, a technique that amplifies weak vibrations compared to IR absorption spectroscopy.[53] The spectrum shows the strong ester carbonyl feature around 1720-1750 cm$^{-1}$ and the weak oxime peak around 1680 cm$^{-1}$. Electronic structure calculations confirmed the assignment and showed that the C=N stretch transition dipole moment is 160x lower than the carbonyl stretch. In addition, we characterized the interfacial H-bond dynamics by measuring time-dependent 2D IR spectra of the carbonyls, which are precisely located within the ~1nm interface between the hydrophilic headgroup and the hydrophobic tails. In brief, 2D IR directly measures the timescales of the frequency-fluctuations at the carbonyl positions.[54–56] The frequency fluctuation correlation decay is 0.96 ps in dioxime-based lipids, which is identical, within measurement uncertainty, to the value of 1.19 ps measured for DMPC.[57] These measurements indicate that the interfacial H-bond networks remain largely unperturbed by the presence of the oxime groups in the lipid tails. Temperature-dependent spectra in the $CH_2$ stretching region also confirmed that oxime lipids remain in the liquid phase between 5 to 50 °C, showing that the presence of the double-bond decreases packing order resulting in a phase transition temperature below 5 °C.[58]

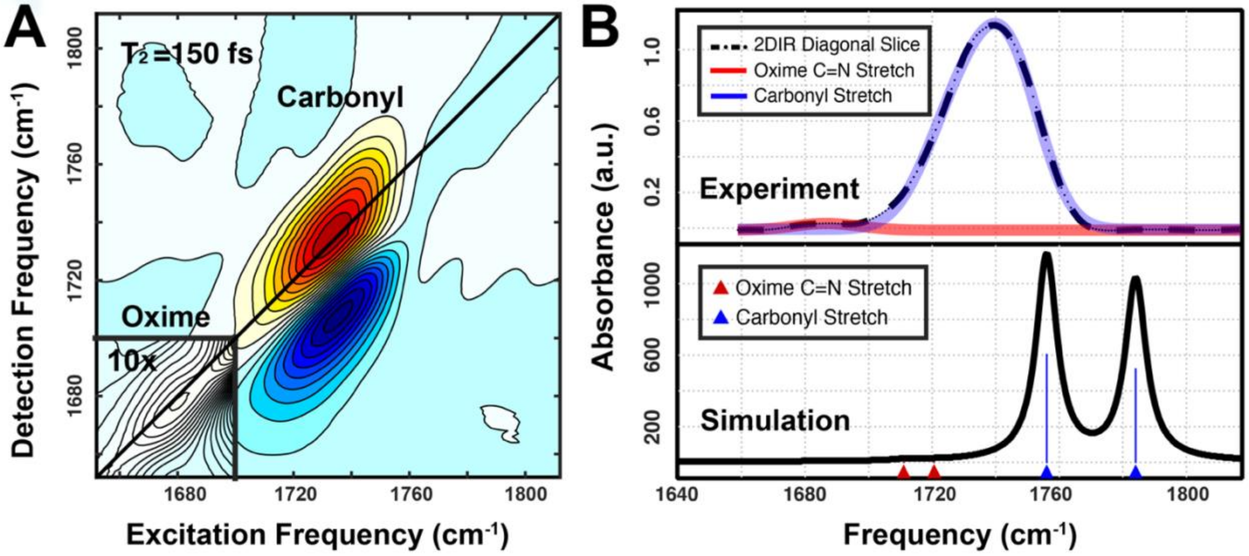

**Figure 2.7: Spectroscopic characterization of dioxime-based phospholipids**
A) 2D IR spectrum of dioxime-based lipids showing the oxime C=N stretch centered around 1680 $cm^{-1}$ and the ester carbonyl C=O around 1720-1750 $cm^{-1}$. B) Diagonal slice of the experimental 2D IR spectrum showing the two peaks. Simulated IR spectrum of a single oxime lipid based on vibrational mode analysis performed using electronic structure.

We next explored whether *de novo* vesicle assembly could take place spontaneously, without an intermediate purification step. HPLC-MS-ELSD measurements were employed to verify the *in situ* synthesis of the self-assembling dioxime-based phospholipid **3**. Liposome formation was monitored by time-lapse phase contrast and fluorescence microscopy. As expected, no observable aggregates were detected immediately after the combination of precursors **1** (1 mM) and **2** (2 mM) in the presence of Nile Red (1 µM) as a membrane staining dye. Both the head group containing aminooxy groups **1** and the hexanal **2** were soluble in water at these concentrations.[47] Minutes after mixing, tubular structures began to appear, which were rapidly converted to micron-sized spherical vesicles. This morphological transformation is similar to previous descriptions of micelle to vesicle transitions driven by coupling reactions.[21,24] Furthermore, we characterized vesicle samples using dynamic light scattering (DLS) and cryogenic electron microscopy (Cryo-EM) to analyze the basic membrane morphology and particle distribution. DLS data suggests the absence of micelles since there were no observable particles below 10 nm; the formed structures were predominantly 100 nm diameter-sized particles. Cryo-EM results showed the presence of liposomes, while other morphologies, such as tubes and disordered aggregates, were not observed.

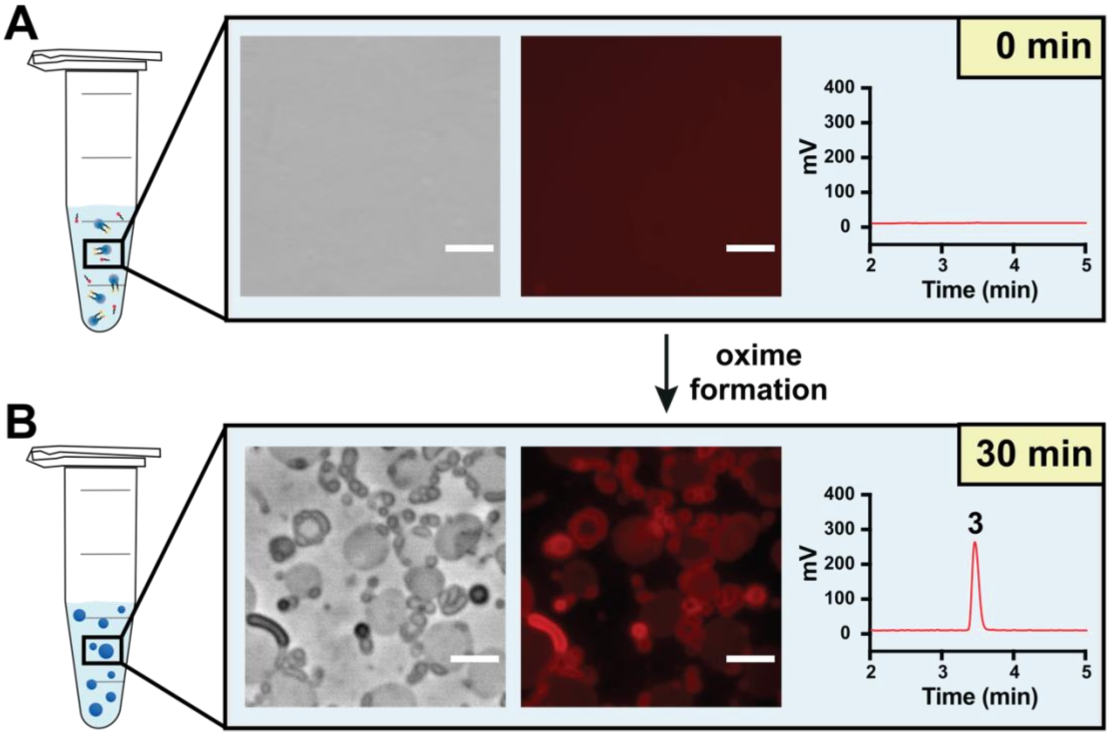

**Figure 2.8: Spontaneous vesicle formation**
Spontaneous generation of phospholipid **3** vesicular structures. Phase contrast and fluorescence images corresponding to in situ **3** vesicle formation. An aqueous buffer solution of diaminooxy-containing PC **1** (1 mM) and hexanal **2** (2 mM), in the presence of Nile Red (1 µM), was imaged at different times after initial mixing. No observable **3** membranes were found immediately after the combination of both precursors (A). After 30 min, large fields of vesicles were detected (B). Scale bars denote 10 µm. Phospholipid **3** formation was verified by HPLC-MS-ELSD measurements.

## 2.4 Biochemical applications for in situ phospholipid formation

Having shown the suitability of the dual oxime formation approach for fabricating biomimetic liposomes from non-amphiphilic precursors, we performed preliminary studies on the use of this *in situ* lipid synthesis strategy for detergent-free transmembrane protein reconstitution. Reconstitution of membrane proteins into synthetic liposomes enables functional studies, this might include, determining how specific lipid species cause curvature stress and consequently affect function.[43] We hypothesized that our detergent-free synthesis of phospholipids via dual oxime bond formation could be useful for spontaneous membrane protein reconstitution.

The reconstitution of transmembrane proteins during *de novo* lipid synthesis was demonstrated by *in situ* formation of the dioxime phospholipid **3** in the presence of crude diacylglycerol kinase (DAGK) as a model protein. DAGK is a small 13.2-kDa inner membrane protein containing three transmembrane helices which oligomerize to form a homotrimer structure.[22,^44] This enzyme catalyzes the ATP-dependent phosphorylation of diacylglycerols (DAGs) and monoacylglycerols (MAGs) to phosphatidic acids (PAs).[44--48] *E.^^coli* DAGK has been widely studied and shown to not feature structural motifs that are common among other similar bacterial and eukaryotic kinases; furthermore, prokaryotic DAGK is the smallest known kinase to date.[44,^49] Its unique structure has made it a target for further investigation. Given DAGK's structural, biological, and clinical importance, we decided to evaluate its assimilation into synthetic liposomes using our dual oxime bond formation strategy. To visualize the localization of the DAGK protein within the corresponding vesicles, we employed an sfGFP fused DAGK (sfGFP-DAGK). The fluorescently fused protein was initially expressed and prepped using standard protocols (see Supporting Information).[50] The membrane fraction containing overexpressed sfGFP-DAGK was then resuspended with 20^^mM of hexanal (**2**) in 1× PBS (pH^^7.4). The resulting mixture was subsequently treated with 10^^mM diaminooxy PC **1**. The reaction was then incubated for 2^^h at room temperature. HPLC-ELSD-MS analysis corroborated the production of the dioxime phospholipid **3**. Light microscopy confirmed the presence of vesicles, which remained stable after two weeks of incubation, similar to oxime vesicles in the absence of protein. Spinning-disk confocal microscopy also showed colocalization of sfGFP-DAGK to the synthetic membrane, suggesting reconstitution of this fluorescently fused protein in the dioxime-based liposomes. Cryo-

EM images confirmed liposome formation after DAGK lysate incubation, with predominant observation of multilamellar structures.

Once we confirmed that DAGK could be spontaneously reconstituted in liposomes, we next sought to determine whether the enzyme retained its catalytic activity. Therefore, we carried out standard assays[32] to establish the activity of the DAGK protein reconstituted into the dioxime-based phospholipid **3** vesicles. sfGFP-DAGK/**3** proteoliposomes were incubated with 1-oleyl-*rac*-glycerol (**12**), ATP, and $MgCl_2$ to monitor the phosphorylation of the monoacylglycerol. HPLC-ELSD-MS analysis showed the formation of the phosphorylated product 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid (**13**) over time. Alternatively, analogous experiments using DAGK solubilized simply in detergent [protein solubilized in 0.1^% *n*-dodecyl-β-D-maltoside (DDM)] did not lead to the generation of lysophospholipid **13**. Our results highlight the importance of the lipid environment in membrane protein reconstitution and demonstrate the retention of functionality for DAGK reconstituted into synthetic liposomes via dual oxime bond formation.

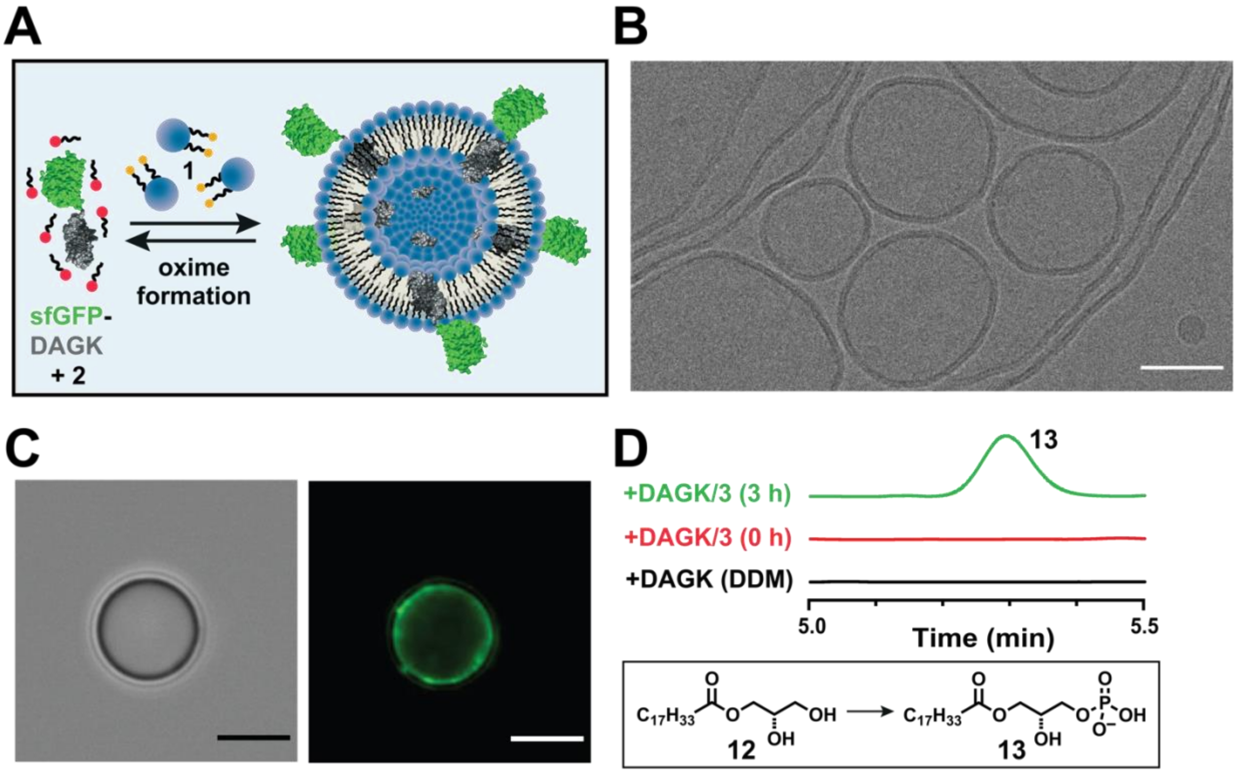

**Figure 2.9: Detergent-free reconstitution of membrane protein**
Detergent-free reconstitution of membrane protein sfGFP-DAGK using the *in situ* dual oxime bond formation approach. A) Schematic representation of the incorporation of sfGFP-DAGK into dioxime-based phospholipid **3** vesicles. B) Cryo-EM images of sfGFP-DAGK/**3** proteoliposomes. Scale bar denotes 50 nm. C) Phase contrast *(left)* and fluorescence *(right)* microscopy images of sfGFP-DAGK/**3** proteoliposomes, showing the localization of the membrane protein into the lipid bilayer. Scale bars denote 10 µm. (D) HPLC-ELSD-MS traces corresponding to an assay testing the activity of in situ reconstituted sfGFP-DAGK in phospholipid **3** vesicles over time. The activity assay is based on the production of the lysophospholipid 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid (**13**) from 1-oleyl-*rac*-glycerol (**12**) in the presence of sfGFP-DAGK/**3**, ATP, and $MgCl_2$. The red line represents the reaction at 0 h and the green line represents the reaction after 3 h of incubation at 37 ˚C. Analogous experiments using detergent-solubilized sfGFP-DAGK [protein solubilized in 0.1% *n*-dodecyl-β-D-maltoside (DDM); black line] did not generate the phosphorylated product.

## 2.5 Conclusion

In summary, we have explored oxime bond formation for the *in situ* synthesis of a new class of dioxime-based phospholipids, which spontaneously self-assemble into micron-sized giant vesicles. Unlike previous approaches using alternative conjugation chemistries, the rapid reaction rate of oxime ligation enables dialkylation of polar head groups without the need to use amphiphilic precursors that self-assemble into micelles. The orthogonality, the high reaction rate, and the biocompatibility of this non-enzymatic, detergent-free approach are key features that make it a powerful tool for membrane synthesis applications, including the efficient reconstitution of transmembrane proteins. The oxime-based strategy was used for direct DAGK incorporation into synthetic liposomes from bacterial extracts, with retention of functionality. We foresee future applications of our technology in enabling the investigation of functional artificial cells, the construction of functional proteoliposomes, and facilitating studies on membrane protein structure and function.

## 2.6 Materials and methods

### *2.6.1 Experimental section*

**Encapsulation experiments**: First, 60.0 µL of a 20 mM solution of phospholipid **3** in $CHCl_3$ were added to a 1 mL vial, placed under $N_2$, and dried for 15 min to prepare a lipid film. Then, 240.0 µL of a 100 µM dye (HPTS, Alexa Fluor 546) solution in 1x PBS buffer or 0.5 mg/mL of sfGFP stored in 1x PBS were added and the mixture was tumbled at 25 ºC for 1 h. Afterward, the solution was transferred to a 100 KDa spin filter and centrifuged for 10 min at 9,000-10,000 rcf to remove the non-encapsulated dye or protein. The lipid-containing solution was finally examined by phase contrast and fluorescence microscopy, observing vesicles containing the desired dye or protein.

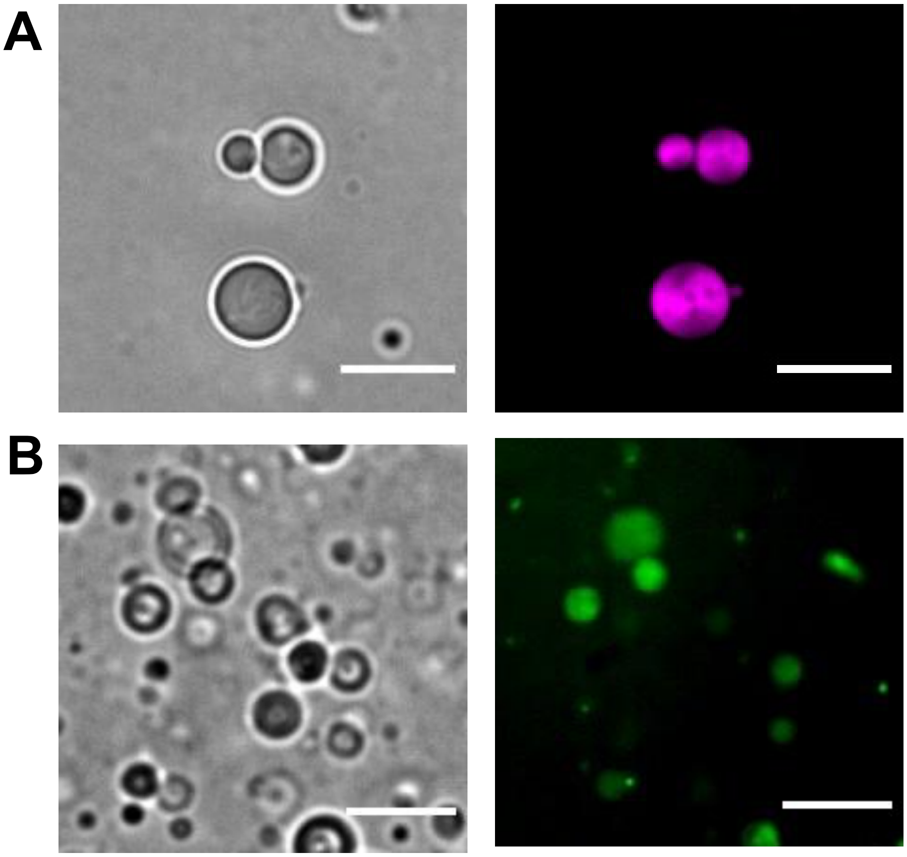

**Figure 2.10: Additional encapsulation experiments**
Phase-contrast (left) and fluorescence (right) spinning-disk confocal microscopy images demonstrating the encapsulation of Alexa Fluor 546 (A) and sfGFP (B) in dioxime-based 3 vesicles. Scale bars denote 10 μm.

**Critical aggregation concentration (cac) determination**: The cac of the phospholipid **3** was estimated using a method based on the solvatochromic fluorescent dye Laurdan.[48] Various solutions (20 μL each) of the dioxime lipid in milliQ $H_2O$ at different concentrations (ranging from 0 mM to 0.5 mM) were prepared. The samples were kept at rt for 1 h, following the addition of 0.25 μL of Laurdan (100 μM in EtOH). The solutions were transferred to a 384-well plate and analyzed on a Tecan Infinite plate reader. Samples were excited at 364 nm, and emission spectra were acquired over 430-500 nm.

**X-ray diffraction (XRD) studies**: XRD experiments were performed on multistacks of oriented lipid bilayers deposited on freshly cleaned hydrophilic silicon [100] wafers. Silicon substrates, cut to 18x20 mm, were sonicated three times for 15 min in MeOH followed by another 15 min in deionized $H_2O$ (18 M-Ohm cm-1, Milli-Q; Millipore, Billerica, MA). Substrates were then nitrogen-dried and exposed to short-wavelength UV radiation for 30 min to make the surface hydrophilic.

The wafers were placed on an accurate leveled platform for lipid deposition. 0.002 mol of the dioxime-phospholipid **3** were dissolved in 200 µL of a $CHCl_3$:TFE (1:1) solution and deposited drop by drop on the silicon substrate. The wafer was left about 2 h covered at the fume hood for slow evaporation. Then, it was placed under high vacuum for 24 h to remove trapped solvents. The lipid-dried film was equilibrated under 96% relative humidity (RH) at a temperature of 50 ºC for 48 h. Finally, it was equilibrated 24 h at rt under different RH (i.e. 98%, 93.5%, 83%, and 75%), which was achieved by a reservoir of different saturated salt solutions ($K_2SO_4$, $K_2NO_3$, KCl, and NaCl, respectively).

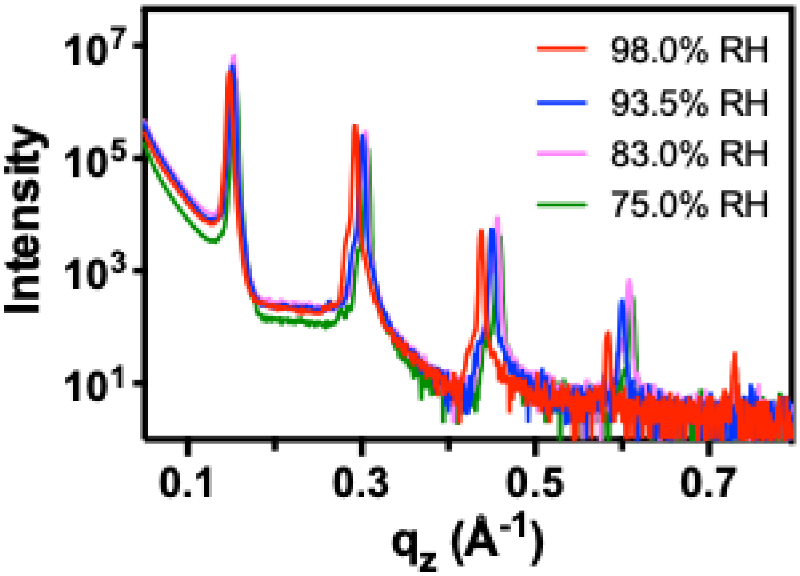

**Figure 2.11: XDR intensity profile**
X-ray diffraction (XRD) intensity profiles of a phospholipid 3 multilayer film at 25 ºC and different relative humidity (RH).

**Fourier-transform IR (FTIR) spectroscopy**: FTIR spectra were collected at 1 $cm^{-1}$ resolution using a Bruker Vertex 70 spectrometer. Samples were held between two $CaF_2$

windows using a 50 µm PTFE spacer. A total of 32 scans are averaged to generate the final FTIR spectrum. Temperature-dependent spectra were measured between 5 and 50 °C using a custom-build sample cell connected to a recirculating water chiller which provides a temperature accuracy of 0.1 °C.

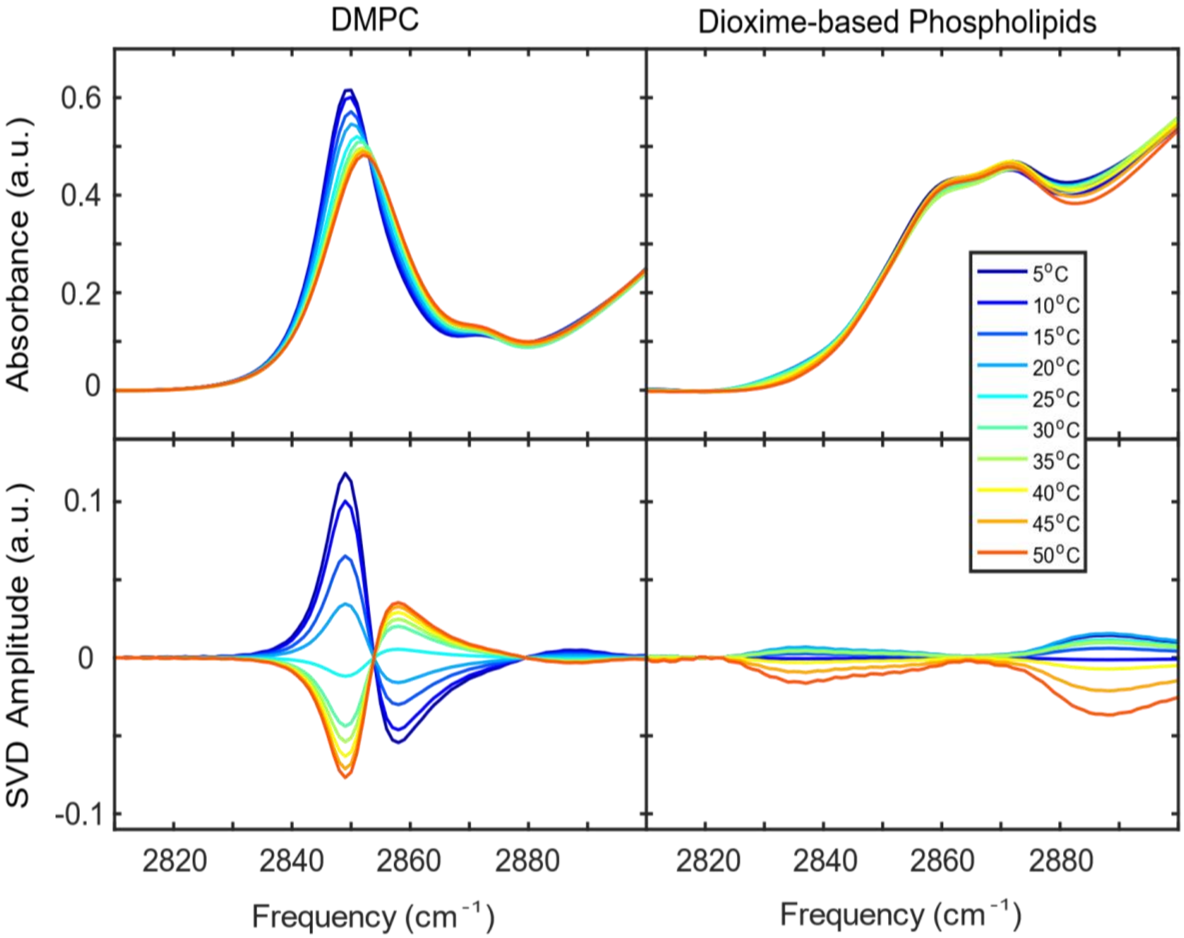

**Figure 2.12: FTIR spectra**
Gel-to-liquid phase transition. Temperature-dependent FTIR spectra of the $CH_2$ symmetric stretch measured for both DMPC and dioxime-based phospholipids. For DMPC, the stretch band centered around 2849 cm$^{-1}$ shows a clear blue shift at higher temperatures. This shift represents the melting transition between gel liquid crystalline phases. The lower plots show the second component of the singular-value-decomposition (SVD). In the dioxime-based phospholipids, the $CH_2$ symmetric stretch does not show a strong temperature dependence, indicating that the dioxime-based phospholipids remain in the liquid crystalline phase throughout this temperature range, suggesting that the phase transition is below 5 °C for these lipids.

**Two-dimensional IR (2D IR) spectroscopy**: 2D IR spectra were measured using a pulse-shaper-based spectrometer, which has been previously described in detail.[59] In brief, three laser pulses, two pumps, and one probe pulse, create a nonlinear polarization in the sample. The time delay between pump pulses ($t_1$) was scanned to 3 ps in 15 fs steps to generate the excitation frequency axis by Fourier transformation. After a selected waiting time, $t_2$, a third pulse (probe) interacts with the sample and is routed into a grating spectrometer. The spectrum of the probe is measured directly in the frequency domain and used to generate the vertical detection frequency. Pump and probe polarization were perpendicular, and phase cycling was used to remove contributions from scattering.[60] A total of 500,000 laser shots were collected to produce each 2D IR spectrum. Slices were taken along the diagonal axis, where the excitation and detection frequency are equal, and ground-state bleach is the dominant feature.[61] The diagonal slices are approximately equivalent to the conventional FTIR spectrum and can be compared with the vibrational analysis using electronic structure theory. All 2D IR spectra and diagonal slices were normalized to the maximum intensity of the main carbonyl peak.

**Center-line slope (CLS) analysis**: A 2D IR spectrum can be interpreted as a correlation map between excitation and detection frequencies: a diagonally elongated 2D IR peak indicates a high correlation, whereas a loss of elongation indicates a loss of correlation. Here we used center-line slope (CLS) analysis to quantify the evolution of the lineshapes as a function of waiting time and extract a frequency-frequency correlation function, which directly reports on the timescales of the frequency fluctuations of the C=O bonds.[62] The CLS values were plotted against the waiting time, $t_2$, and an exponential fit reveals the timescale of the frequency fluctuations.[63] These frequency fluctuations are primarily driven by the H-bond dynamics at the interface.[54] The 95% confidence interval of each linear fit was used to define the standard deviation of a normal distribution around each point. For each iteration, a random point within this distribution was generated for each $t_2$ value, and the resultant decay is fit to a single exponential function. This process was repeated 1,000 times, and the reported value is the bootstrap error estimate of the time constant.

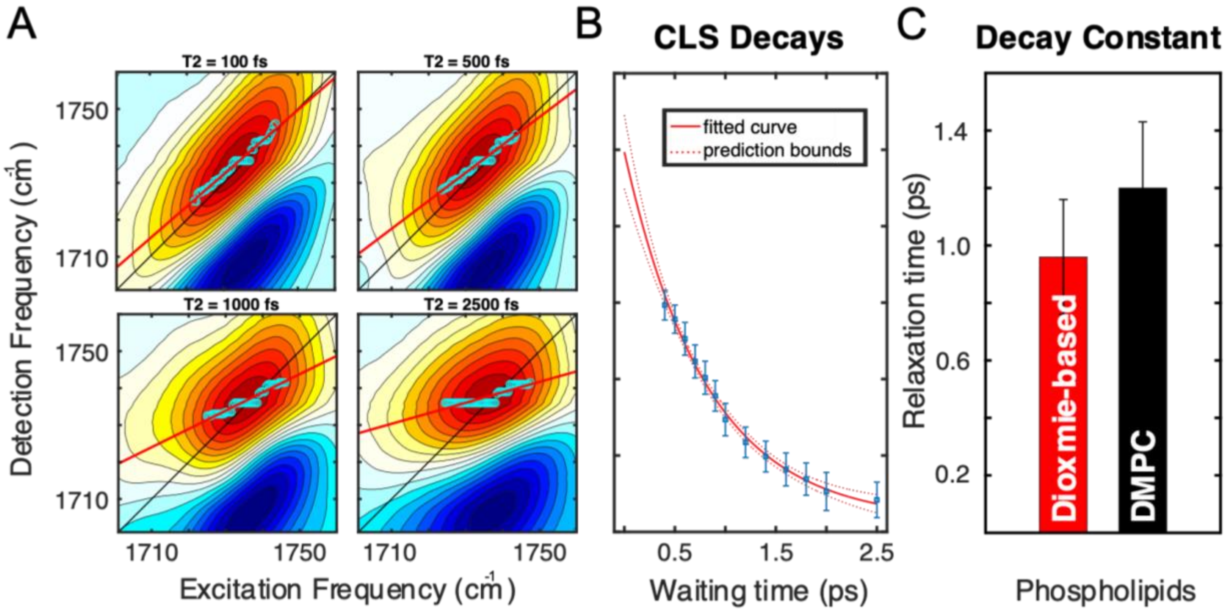

**Figure 2.13: 2D IR spectra**
Interfacial H-bond dynamics in the dioxime-based phospholipid measured using 2D IR spectroscopy. A) Ester C=O 2D IR spectra of dioxime-based lipids at waiting time $t_2$ of 100, 500, 1000, and 2500 fs, shown as an example. The solid red lines represent the linear fits from which the center-line slope (CLS) is extracted. B) Center-line slope (CLS) at measured waiting times along with a monoexponential fit (solid red line). The error bars and prediction bounds represent the 95% confidence interval of the CLS fit. C) Comparison between the decay constant of dioxime-based phospholipid and DMPC showing that the two lipids exhibit similar interfacial dynamics.[56]

**Vibrational analysis using density-functional theory (DFT):** The structure of the dioxime-based phospholipids is based on a structure of DMPC downloaded from the CHARMM-GUI website.[64] The original structure was modified by replacing the respective Carbon atoms and bonds before performing a geometry optimization at the BP86/SDD level of theory.[65–67] Following optimization, a harmonic computation of the normal modes at the same level of theory was run using the Gaussian16 package of programs.[68]

**Cryo-TEM of vesicles:** Samples were applied to either Quantifoil Multi-A grids or 300 mesh Lacey carbon grids on copper (Electron Microscopy Services) to provide a wide range of hole sizes to capture large vesicles in ice. The Multi-A grids were treated by plasma cleaning using the "Quantifoil Carbon" present on a Solarus II (Gatan), while the Lacey grids were glow discharged at 20 mA for 45 s in a K100X instrument (Emitech) Immediately after applying samples to the grid, they were vitrified using a Vitrobot Mark IV (ThermoFisher) using 4 s blot times and a 3 blot-force setting at 4 ºC and 100% humidity by plunge-freezing into liquid ethane cooled by liquid nitrogen. 3.5 uL of samples were used for the Multi-A grids, while Lacey grids used 4 uL. Samples were stored in liquid nitrogen until use.

Cryo-EM data were collected on a Talos Arctica (FEI) operated at 200 kV, equipped with a K2 Summit direct electron detector (Gatan). Data collection was performed with Leginon.[69] Grids were manually screened, and vesicles were always found near thick ice areas in both samples. Acquisition images were collected at 36,000 magnification (pixel size of 1.16 Å) with a 12 s exposure resulting in 60 frames (200 ms frames) at an average total dose of 54 e/Å$^2$. Micrograph frames were aligned with Cryosparc v3.3.1 patch alignment tool.[70]

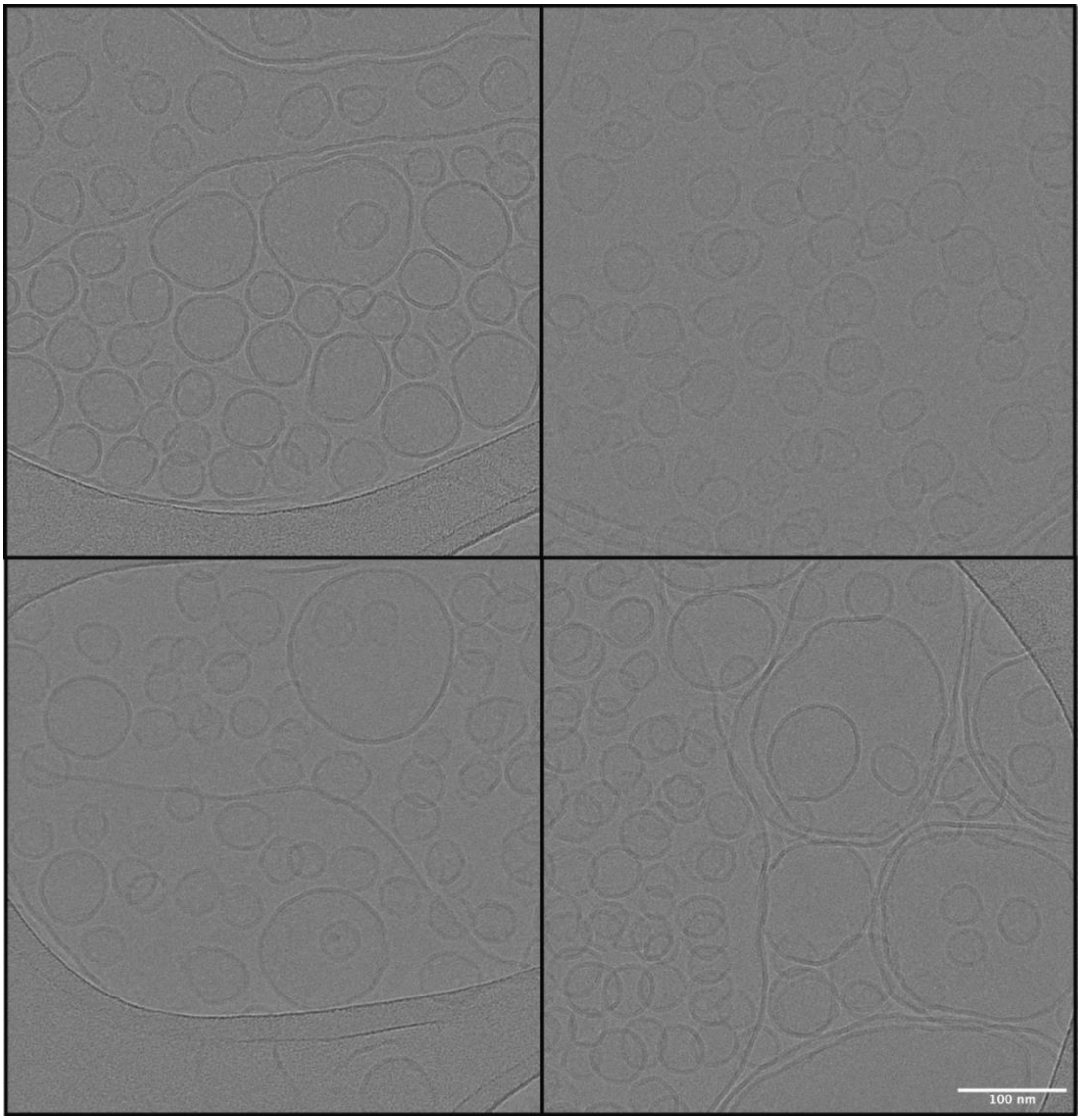

**Figure 2.14: Liposomes under Cry-EM**
Compilation of Cryo-EM images of oxime phospholipid liposomes containing no protein. Scale bar denotes 100 nm.

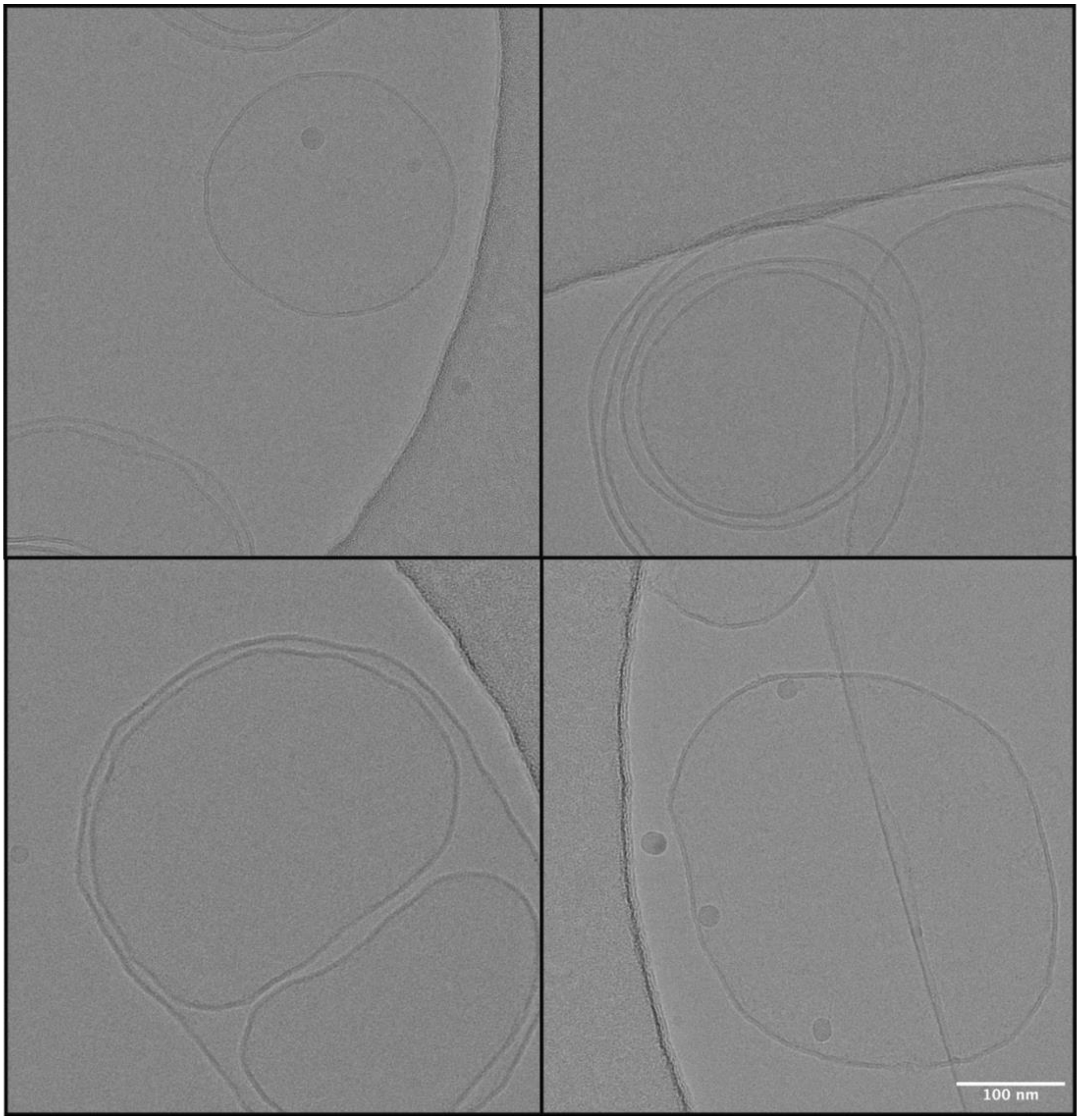

**Figure 2.15: Protein containing liposome under Cryo-EM**
Compilation of Cryo-EM images of oxime phospholipid liposomes containing DAGK. Scale bar denotes 100 nm.

**Protein expression and membrane preparation**: The pTNT-sfGFP-DAGK plasmid with a polyhistidine-tag and an ampicillin resistant gene was transformed in Bl21 competent cells. One colony was cultured in 5 mL of LB broth with carbenicillin overnight at 37 ºC. Then, 2 mL of starter culture along with carbenicillin was grown in 200 mL LB media and induced with 200 ng/µL of IPTG at a 1.0 OD absorbance, following a previously published protocol.[71] After incubation for 4 h at 37 ºC, the cells were split into four 50 mL falcon tubes, harvested by centrifugation, and stored frozen at -80 °C. For crude membrane preparation, the pellets were resuspended in 2 mL 1x PBS buffer containing 1 mM PMSF, and the cells were lysed by an ultrasonicator probe. The resulting mixture was centrifuged at 5,000 x g's for 30 min. The supernatant was aliquoted in 1 mL tubes and stored at -80 ºC for later use.

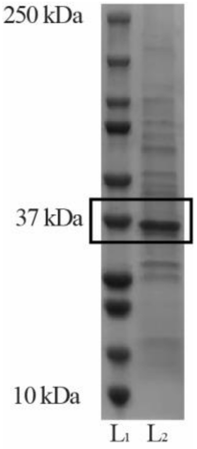

**Figure 2.16: sfGFP SDS-PAGE gel**
SDS-PAGE analysis of the purified non-reconstituted sfGFP-DAGK [protein solubilized in 0.1% *n*-dodecyl-β-D-maltoside (DDM)]. Lane 1 ($L_1$): Ladder, Lane 2 ($L_2$): sfGFP-DAGK.

**Reconstitution of sfGFP-DAGK**: The aliquoted sfGFP-DAGK crude protein lysate was thawed on ice and spun at 75,000 x g for 45 min. The resulting green pellet was treated with a 10 mM solution of hexanal (**2**) in 1x PBS buffer, gently tapped, and then incubated until the pellet was mostly resuspended. Afterward, the dual oxime ligation was carried out by the addition of a 5 mM solution of diaminooxy-containing PC derivative **1** in 1x PBS buffer. The reaction was gently spun for several hours at rt. The resulting vesicles were observed under optical and confocal microscopy, and dioxime-based phospholipid **3** formation was followed via HPLC-ELSD-MS.

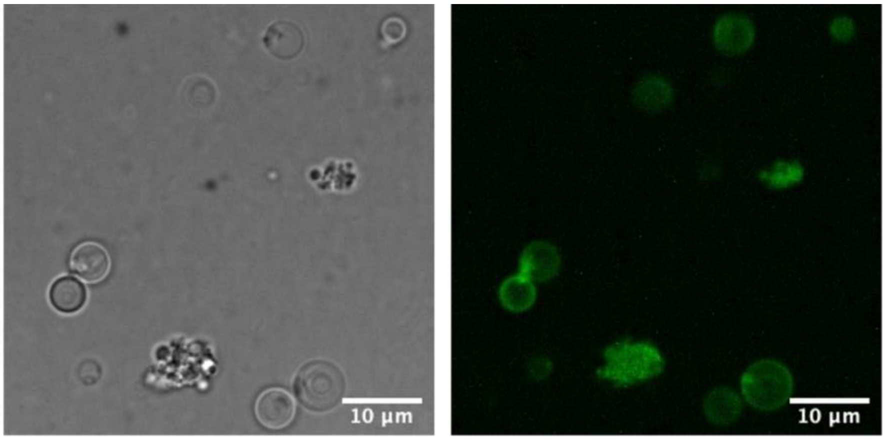

**Figure 2.17: Reconstitution microscopy**
Phase-contrast *(left)* and fluorescence *(right)* spinning-disk confocal microscopy images of sfGFP-DAGK/**3** proteoliposomes formed in situ using our dual oxime reconstitution methodology. Scale bars denote 10 µm.

**Activity assays of reconstituted protein**: The activity of the reconstituted sfGFP-DAGK was tested by combining sfGFP-DAGK/**3** proteoliposomes with a solution containing 5 mM 1-oleyl-rac-glycerol (**12**), 0.5 mM ATP, and 0.5 mM MgCl2 in 1x PBS buffer (pH 7.4), followed by incubation for 4 h at 37 ºC. Afterward, the reaction was quenched with 10 mM EDTA and 60% MeOH. The solution was centrifuged, and the supernatant was injected directly into the HPLC-ELSD-MS system for analysis.

### *2.6.2 General methods, materials, and instruments*

Commercially available 6-(aminooxy)hexanoic acid hydrochloride was obtained from Enamine. Sodium bicarbonate ($NaHCO_3$), di-*tert*-butyl dicarbonate ($Boc_2O$), 1,4-dioxane, 4-dimethylaminopyridine (DMAP), *L*-α-glycerylphosphorylcholine (GPC), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl), adenosine 5'-triphosphate (ATP), ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA), dichloromethane (DCM), Nile Red, tetrafluoroethylene (TFE), 1-oleyl-*rac*-glycerol (DL-α-monooleoin), *n*-dodecyl-β-D-maltoside (DDM), and 100kD spin filters were obtained from

Sigma Aldrich. 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), Alexa Fluor 546, 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), and 10x PBS were purchased from Thermo Fisher Scientific. Bl21 *E. coli* competent cells were obtained from New England Biolabs (NEB). Deuterated dimethylsulfoxide (DMSO-d6) was obtained from Cambridge Isotope Laboratories. All reagents obtained from commercial suppliers were used without further purification unless otherwise noted. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 $F_{254}$ plates. Compounds, which were not UV active, were visualized by dipping the plates in a ninhydrin or potassium permanganate solution and heating. Silica gel flash chromatography was performed using E. Merck silica gel (type 60SDS, 230-400 mesh). Solvent mixtures for chromatography are reported as v/v ratios. HPLC analysis was carried out on an Eclipse Plus C8 analytical column with *Phase A*/*Phase B* gradients [*Phase A*: $H_2O$ with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. HPLC purification was carried out on Zorbax SB-C18 semi-preparative column with *Phase A*/*Phase B* gradients [*Phase A*: $H_2O$ with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. Flash chromatography was performed in a CombiFlash EZ Prep system (Teledyne ISCO) via liquid introduction into a 4 g RediSep Rf Gold® Silica Gel Flash Column (20-40 µ). Proton nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a Varian VX500 MHz spectrometer and were referenced relative to residual proton resonances in DMSO (at δ 2.50 ppm). Chemical shifts were reported in parts per million (ppm, δ) relative to tetramethylsilane (δ 0.00). $^1$H NMR splitting patterns are assigned as singlet (s), doublet (d), triplet (t), quartet (q), or pentuplet (p). All first-order splitting patterns were designated based on the appearance of the multiplet. Splitting patterns that could not be readily interpreted are designated as multiplet (m) or broad (br).

Carbon nuclear magnetic resonance ($^{13}$C NMR) spectra were recorded on a Varian VX-500 MHz spectrometer and were referenced relative to residual proton resonances in d6-DMSO (at 39.51 ppm). Spinning-disk confocal microscopy images were acquired on a Yokogawa spinning-disk system (Yokogawa, Japan) built around an Axio Observer Z1 motorized inverted microscope (Carl Zeiss S-3 Microscopy GmbH, Germany) with a 63x, 1.40 NA oil immersion objective to an Evolve 512×512 EMCCD camera (Photometrics, Canada) using ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany). Absorbance measurements were performed in a NanoDrop 2000C spectrophotometer. Fluorescence measurements were carried out on a Tecan infinite F200 plate reader instrument.

### *2.6.3 Synthesis of diaminooxy-containing phosphocholines*

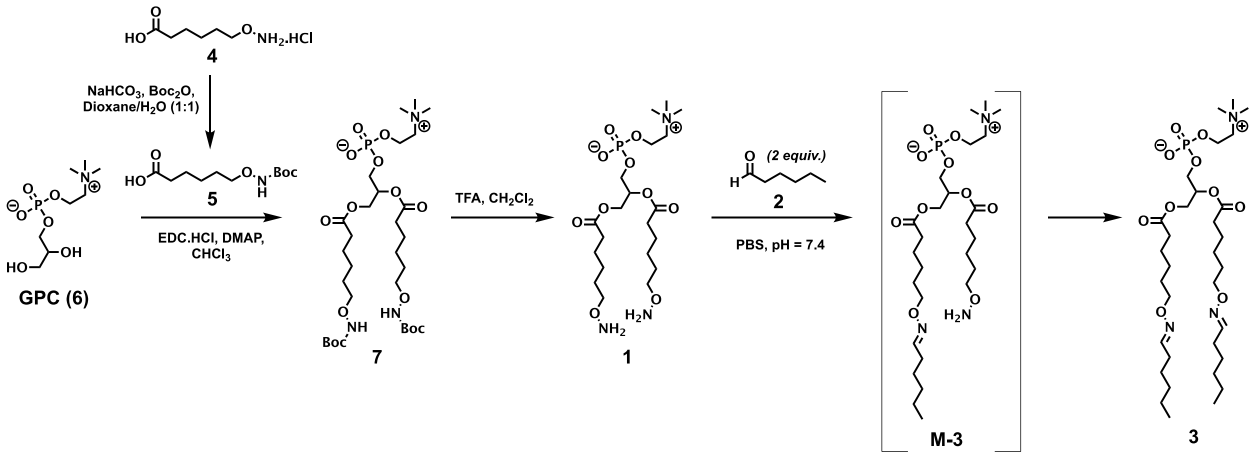

**Figure 2.18: Synthesis of precursor**
Synthesis of the diaminooxy-containing PC derivative **1** and subsequent treatment with hexanal (**2**) to form the dioxime-based phospholipid **3**.

*6-(((tert-butoxycarbonyl)amino)oxy)hexanoic acid (**5**).* 6-(Aminooxy)hexanoic acid hydrochloride (**4**; 100.0 mg, 544.6 mmol) and $NaHCO_3$ (171.0 mg, 2.0 mmol) were dissolved in milliQ water (4 mL). The mixture was then stirred at 0 ºC for 10 min. Afterward, a solution of $Boc_2O$ (333.0 mg, 1.5 mmol) in 1,4-dioxane (4 mL) was added dropwise. After stirring at rt for 2 h, the solution was acidified to pH = 3 by addition of HCl (1 M) and extracted with $CH_2Cl_2$ (3 × 6 mL). The combined organic layers were dried ($Na_2SO_4$), filtered, and concentrated. The crude was then purified by flash chromatography on silica gel (10% MeOH in $CH_2Cl_2$), affording 63.8 mg of **5** as a colorless oil [47%]. $^1H$ NMR (500 MHz, MeOD): δ 3.77 (t, *J* = 6.5 Hz, 2 H), 2.30 (t, *J* = 7.4 Hz, 2 H) 1.68-1.58 (m, 4 H), 1.47 (s, 9 H), 1.45-1.38 (m, 2 H); $^{13}C$ NMR (125 MHz, MeOD): δ 177.7, 159.2, 81.9, 77.2, 34.9, 28.8, 28.6, 26.6, 25.9; MS (ESI$^+$) *m/z*: $[M+H]^+$ calculated for $C_{11}H_{21}NO_5Na^+$, 270.1312; found, 270.1313.

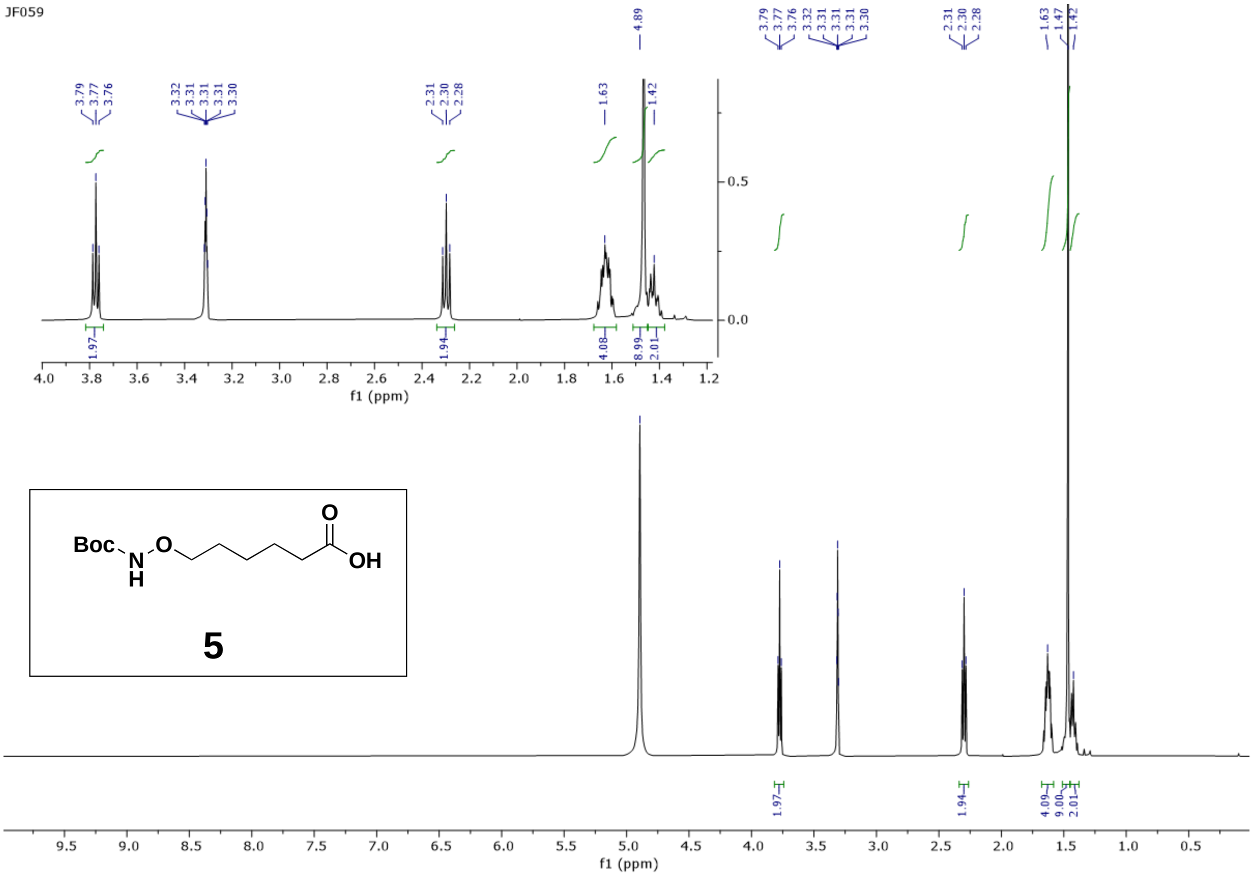

**Figure 2.19: $^1H$ NMR spectrum of 5 (in MeOD, 500 MHz).**

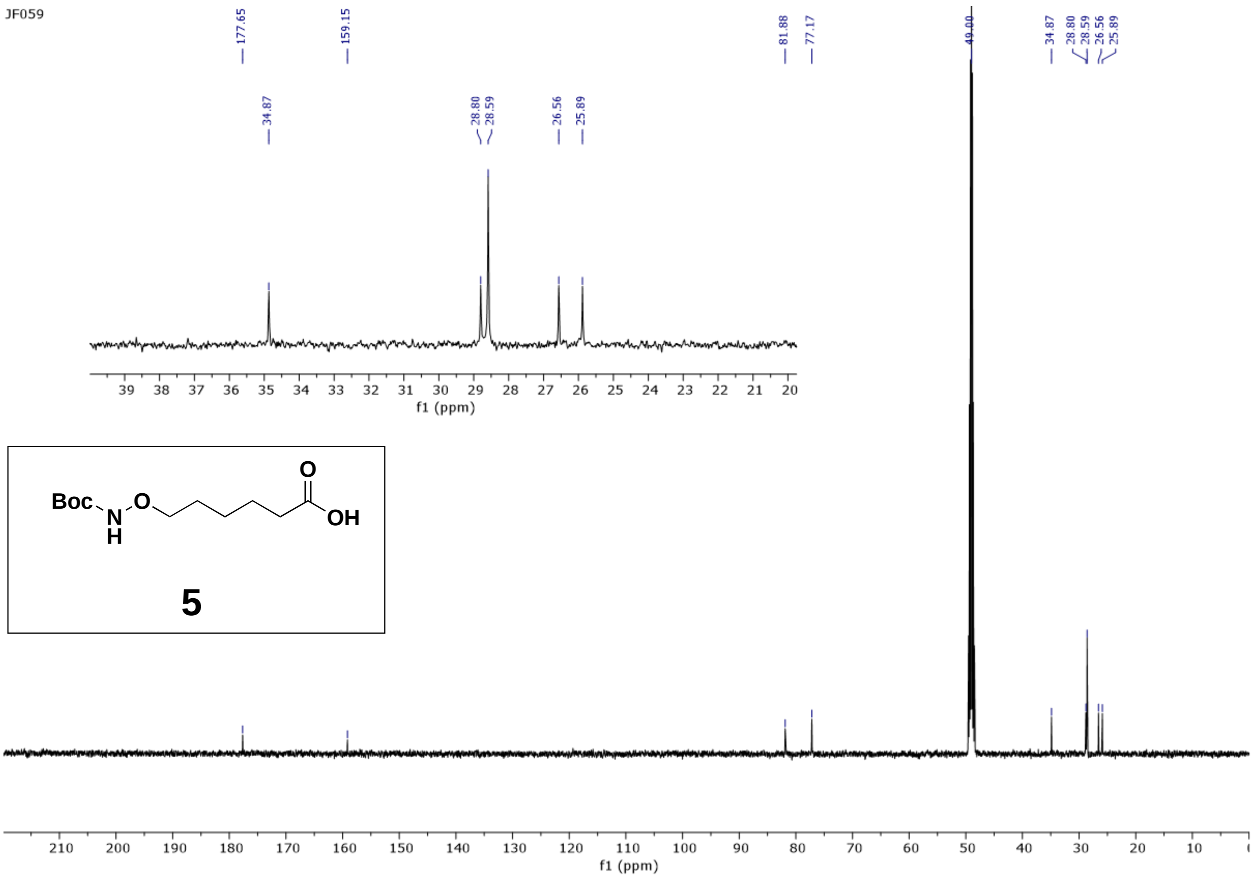

**Figure 2.20: $^{13}$C NMR spectrum of 5 (in MeOD, 125 MHz).**

*(R)-15-((6-(((tert-butoxycarbonyl)amino)oxy)hexanoyl)oxy)-2,2-dimethyl-4,12-dioxo-3,6,13-trioxa azahexadecan-16-yl (2-(trimethylammonio)ethyl) phosphate (***7***).* Compound **5** (63.8 mg, 258.0 µmol), GPC (**6**; 26.5 mg, 103.2 µmol), DMAP (12.6 mg, 103.2 µmol), and EDC.HCl (49.5 mg, 258.0 µmol) were dissolved in dry $CHCl_3$ (5 mL) under Ar. After stirring overnight at rt, the reaction mixture was concentrated under vacuum. Although the reaction was incomplete, the desired product was observed, and no major side products identified. The resulting crude product was purified by Prep HPLC, affording 13.6 mg of **7** as a colorless film [18%, $t_r$ =25.4 min (RediSep Prep C18 20x150mm column, 0-2 min 30% *Phase B*, 2-25 min 85% *Phase B*, 25-30 min 100% *Phase B*)]. $^1$H NMR (500 MHz, MeOD): δ 5.28-5.19 (m, 1 H), 4.47-4.39 (m, 1 H), 4.32-4.23 (m, 2 H), 4.22-4.14 (m,

1 H), 4.08-3.95 (m, 4 H), 3.78 (m, 4 H), 3.65 (m, 2 H), 3.23 (s, 9 H), 2.41-2.31 (m, 4 H), 1.69-1.59 (m, 8 H), 1.47 (s, 18 H), 1.45-1.38 (m, 4 H); $^{13}$C NMR (125 MHz, MeOD): δ 174.9, 174.6, 159.1, 81.9, 77.1, 71.9, 67.4, 64.9, 63.6, 60.5, 54.7, 39.9, 34.7, 28.8, 28.6, 26.5, 25.6; MS (ESI$^+$) *m/z*: $[M+H]^+$ calculated for $C_{30}H_{59}N_3O_{14}P^+$, 716.3729; found, 716.3728.

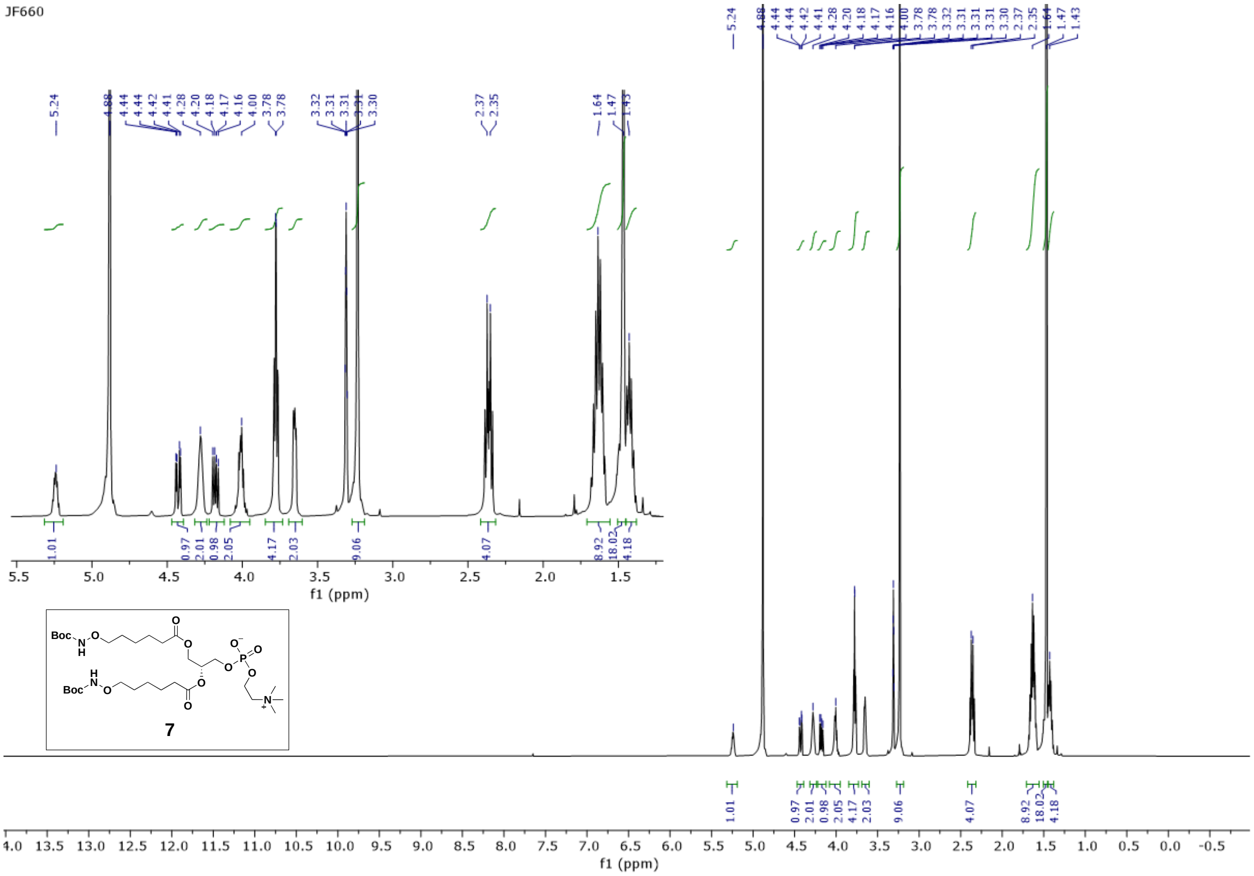

**Figure 2.21: $^1$H NMR spectrum of 7 (in MeOD, 500 MHz).**

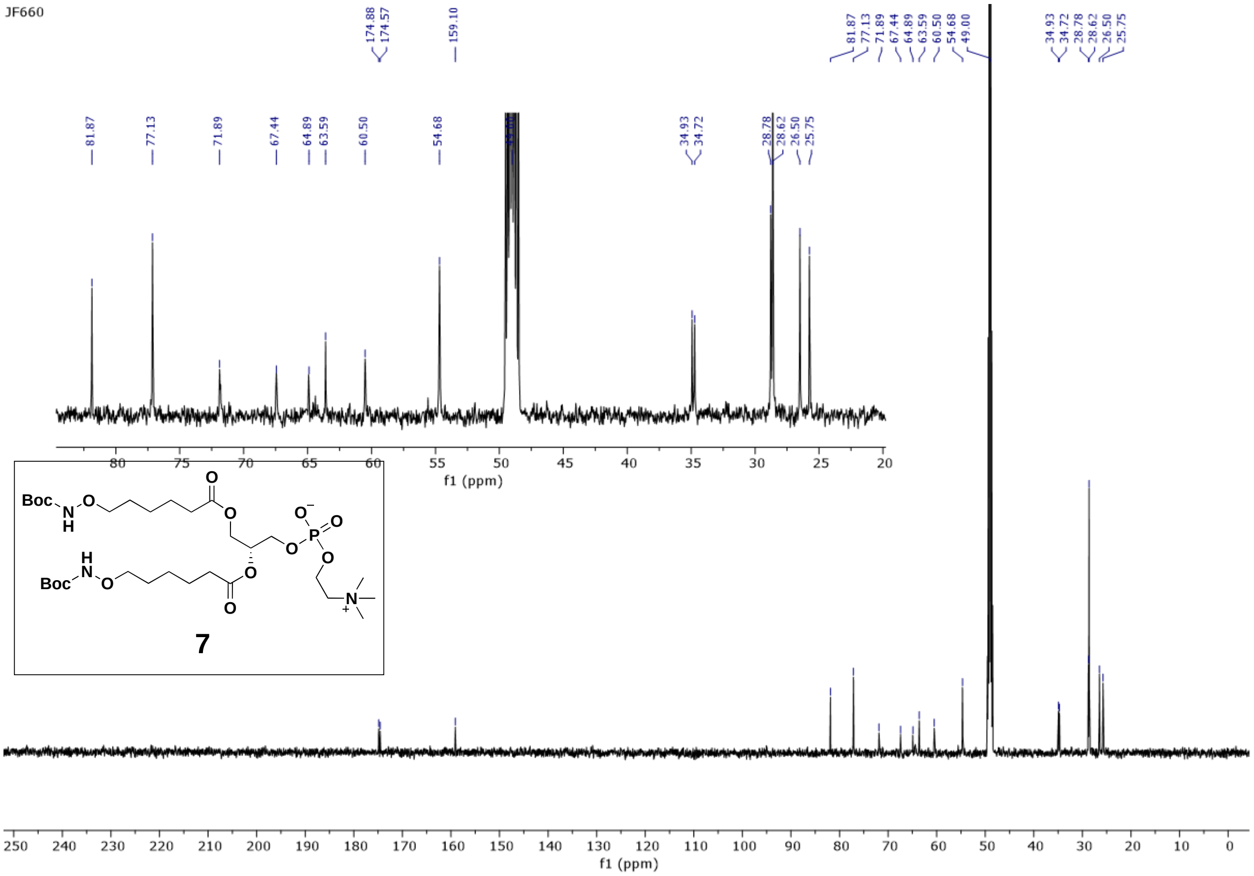

**Figure 2.22: $^{13}$C NMR spectrum of 7 (in MeOD, 125 MHz).**

*2,3-bis((6-(aminooxy)hexanoyl)oxy)propyl (2-(trimethylammonio)ethyl) phosphate (**1**).* Compound **7** (13.6 mg, 19.0 µmol) was dissolved in 500 µL of CH$_2$Cl$_2$. Then, 500 µL of TFA were added. The solution was gently stirred for 15 min at rt. Then, the reaction mixture was concentrated using a gentle stream of N$_2$, obtaining a colorless film. The resulting diaminooxy-containing PC Derivative (**1**) was stored at -20 ºC and used without any further purification [98% yield]. $^{1}$H NMR (500 MHz, MeOD): δ 5.23 (quint, $J$ = 4.8 Hz, 1 H), 4.41-4.34 (m, 1 H), 4.30-4.23 (m, 2 H), 4.21-4.14 (m, 1 H), 4.06-3.95 (m, 6 H), 3.68-3.63 (m, 2 H), 3.35 (m, 2 H), 3.23 (s, 9 H), 2.46-2.28 (m, 4 H), 1.75-1.60 (m, 8 H), 1.51-1.39 (m, 4 H); $^{13}$C NMR (125 MHz, MeOD): δ 174.7, 174.4, 76.0, 72.0, 71.9, 67.4, 65.1, 63.4, 60.5, 54.6, 34.8, 34.6, 28.4, 28.3, 26.2, 26.0, 25.5; MS (ESI$^+$) *m/z*: [M+H]$^+$ calculated for $C_{20}H_{43}N_3O_{10}P^+$, 516.2681; found, 516. 2683.

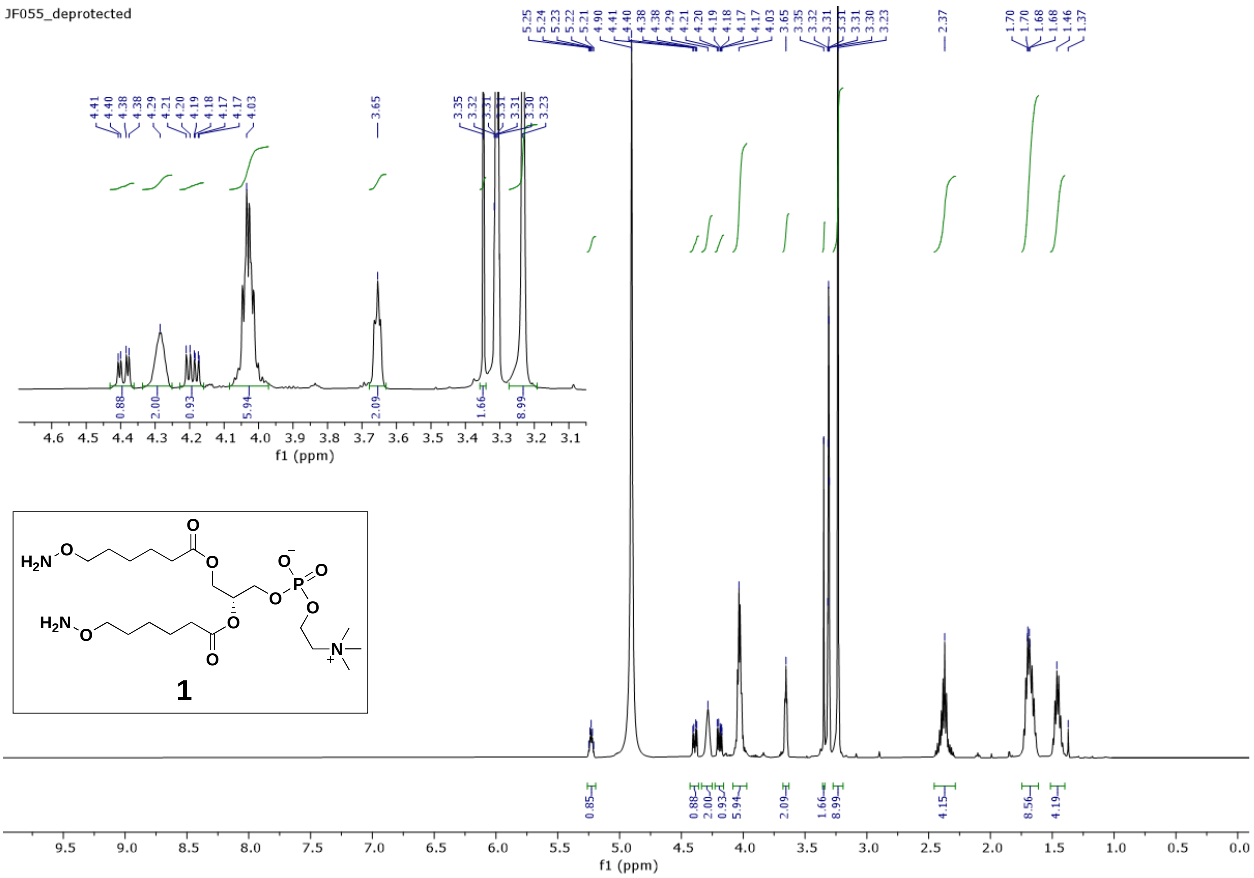

**Figure 2.23: $^1$H NMR spectrum of 1 (in MeOD, 500 MHz).**

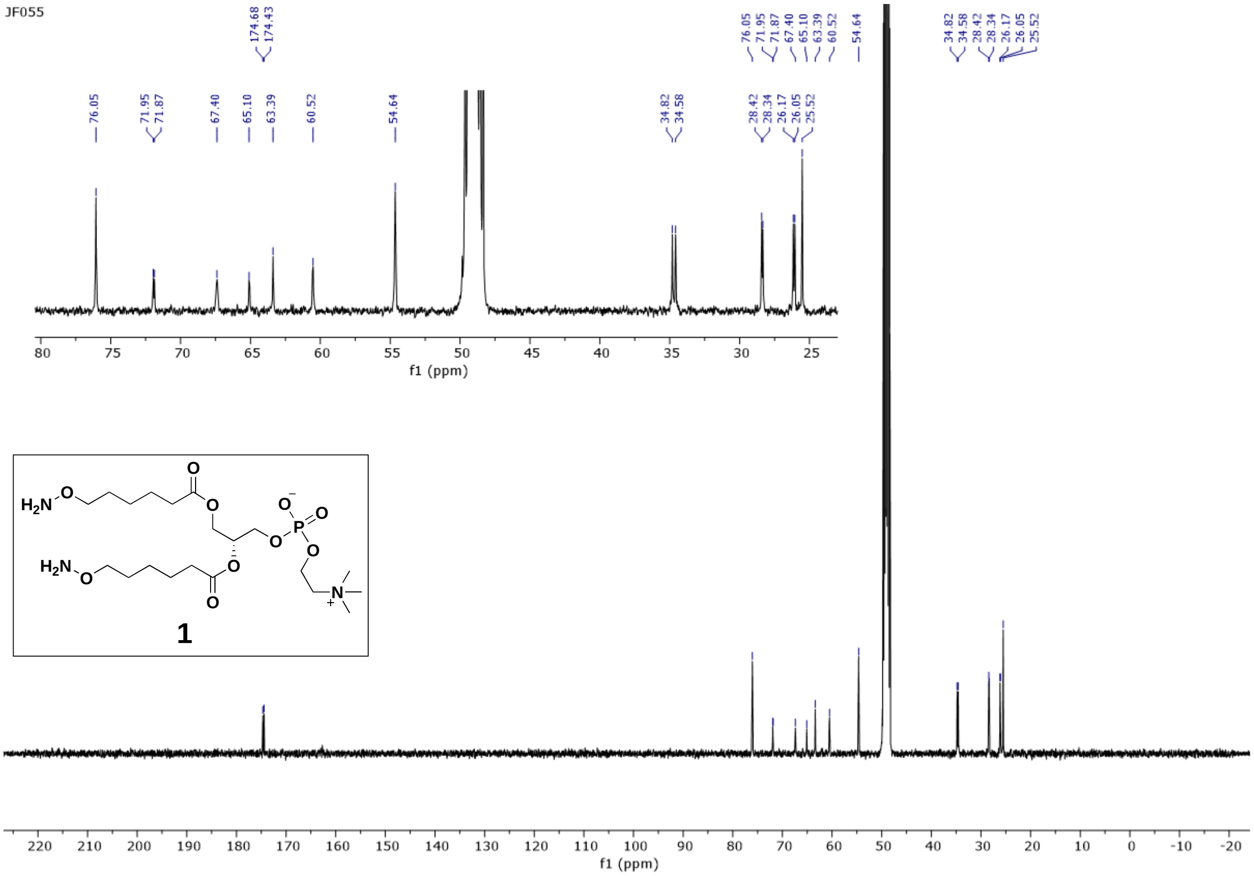

**Figure 2.24: $^{13}$C NMR spectrum of 1 (in MeOD, 125 MHz).**

### *2.6.4 Synthesis of dioxime-based phospholipids*

*Phospholipid **3** formation via dual oxime bond ligation.* Diaminooxy-containing PC Derivative (**1**, 10 mM solution in 1x PBS) was combined with hexanal (**2**, 20 mM solution in 1x PBS) in a 0.5 dram vial. Then, the mixture was slowly tumbled at rt. The sample was analysed over time using HPLC-ELSD-MS measurements. Complete conversion to phospholipid **3** was observed after 30 min of reaction. Afterward, compound **3** was purified using Prep HPLC [80%, $t_r$ =15.1 min (RediSep Prep C18 20x150mm column, 0-1 min 50% *Phase B*, 1-6 min 95% *Phase B*, 6-16 min 100% *Phase B*). $^1$H NMR (500 MHz, MeOD): δ 7.37 (t, *J* = 6.2 Hz, 1 H), 6.66 (t, *J* = 5.5 Hz, 1 H) 5.24 (quint, *J* = 4.8 Hz, 1 H), 4.47-4.39 (m, 1 H), 4.30-4.23 (m, 2 H), 4.21-4.14 (m, 1 H), 4.05-3.98 (m, 4 H), 3.98-3.94

(m, 2 H), 3.66-3.63 (m, 2 H), 3.23 (s, 9 H), 2.40-2.27 (m, 6 H), 2.19-2.13 (m, 2 H), 1.70-1.60 (m, 8 H), 1.43-1.27 (m, 12 H), 0.92 (t, *J* = 6.9 Hz 6 H); $^{13}$C NMR (125 MHz, MeOD): δ174.8, 174.6, 159.1, 81.9, 77.1, 71.9, 67.5, 64.9, 63.6, 60.5, 54.7, 34.9, 34.7, 28.8, 28.6, 26.5, 25.8; MS (ESI$^+$) *m/z*: [M+H]$^+$ calculated for $C_{32}H_{63}N_3O_{10}P^+$, 680.4246; found, 680.4249.

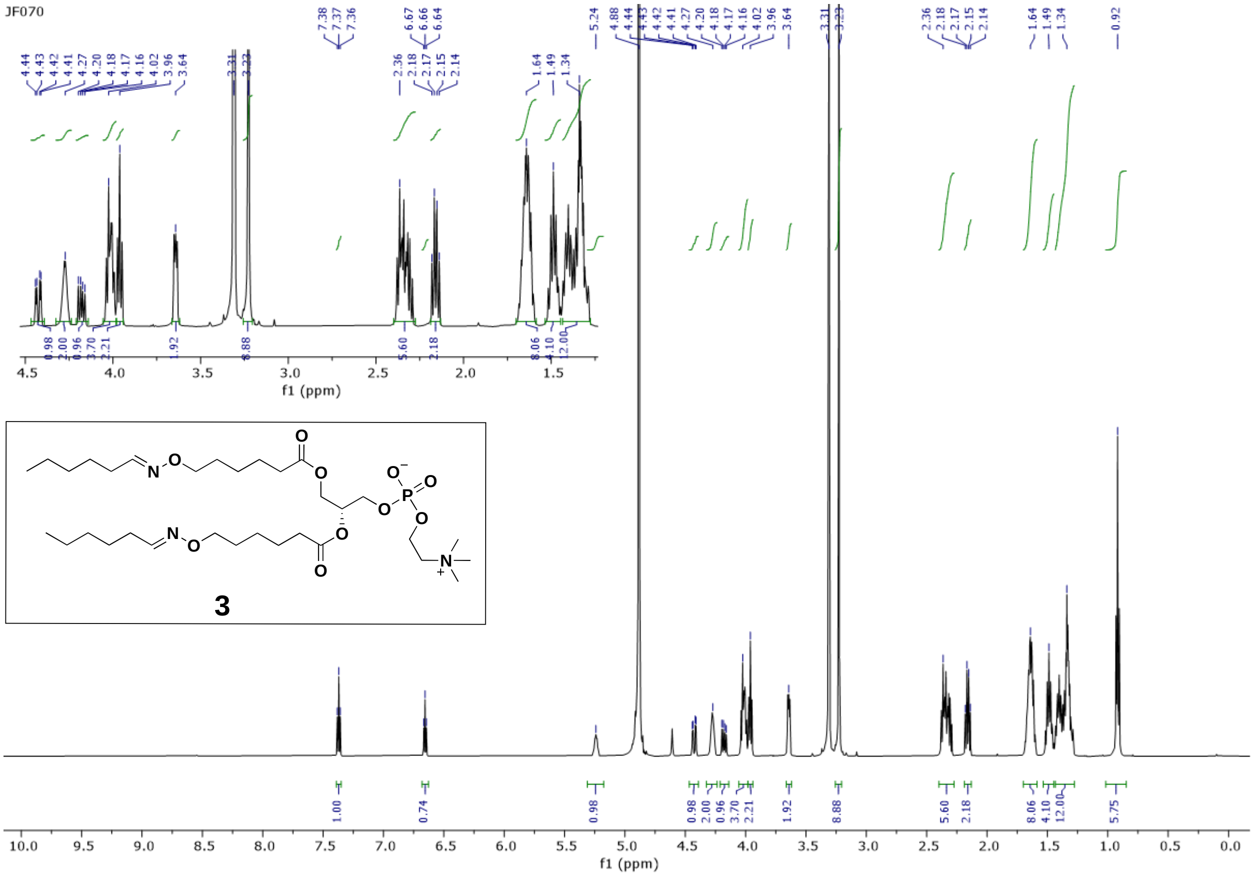

**Figure 2.25: $^1$H NMR spectrum of 3 (in MeOD, 500 MHz).**

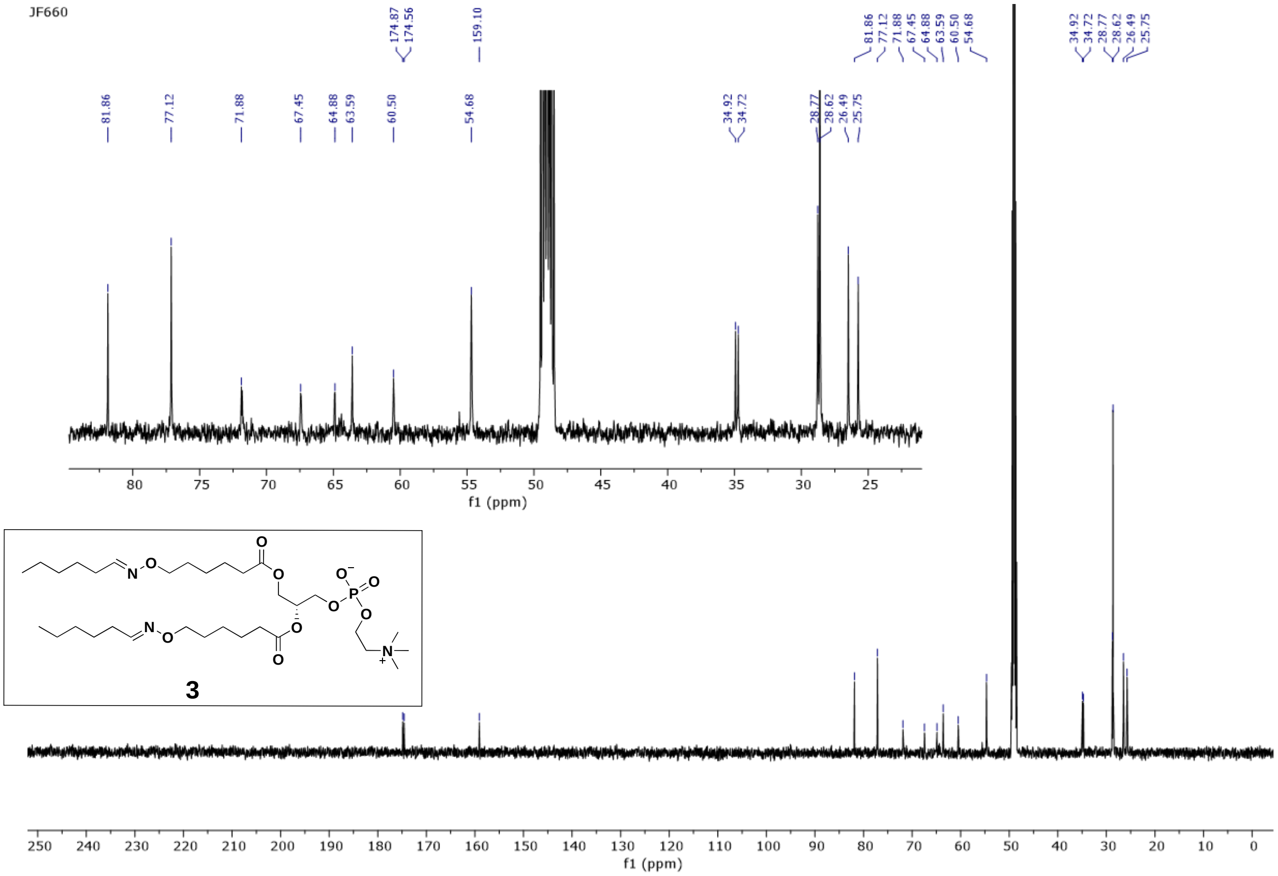

**Figure 2.26: $^{13}$C NMR spectrum of 3 (in MeOD, 125 MHz).**

### *2.6.5 Characterization of phospholipid membranes*

**Critical aggregation concentration (CAC) determination**: The CAC of the phospholipid **3** was estimated using a method based on the solvatochromic fluorescent dye Laurdan. Laurdan is a fluorescent, polarity-sensitive dye specifically designed to have a dipolar relaxation in response to changes in solvent penetration in phospholipid membranes.[48] Consequently, it has been extensively used to quantify lipid organization and determine critical aggregation concentration (CAC) in phospholipid membranes.[48,49,72–74] Monitoring the shift in Laurdan's fluorescence emission wavelength (440 nm-490 nm) based on water molecule interaction allows for the calculation of general polarization[49] with the following equation:

$$GP = \frac{\mathrm{I}_{440} - I_{490}}{\mathrm{I}_{440} + I_{490}}$$

where $I_{440}$ and $I_{490}$ stand for the fluorescence intensities at 440 and 490 nm, respectively.

**X-Ray Diffraction (XRD) studies**: The diffraction measurements were carried out using an in-house Cu $K_\alpha$ tube spectrometer with wavelength 1.54 Å operating in the horizontal plane. During the in-house X-ray diffraction measurements, we used a specially constructed humidity cell designed for high accuracy and sensitivity in RH.[75] The scattered intensity was plotted as a function of Q (the scattering vector), which is directly related to the scattering angle by $Q = 4\pi \sin(\theta)/\lambda$, where $\lambda$ is the wavelength of the X-rays. Therefore, we obtained one-dimensional I(Q) profiles for each RH. The X-ray diffraction pattern presented a series of sequential peaks positioned at an equal interpeak distance, characteristic of a lamellar phase. The diffraction peaks were fitted by a Gaussian distribution after background subtraction to determine their position and area under the peak. The repeat distance (or d-spacing) of the lamellar phase was calculated by determining the slope of a linear fit (i.e., y = a + bx), on a plot of peak location (q) vs. diffraction order (h) and using the following equation:

$$d = \frac{2\pi}{\Delta \mathrm{q}}$$

The integrated intensity $I_n$ of $n^{th}$ order peaks was then used to calculate the electron density profiles (EDPs) with the following equation[76]:

$$\rho_{bilayer}(z) = \frac{2}{D}\sum_{n=1}^{M} f_n \nu_n cos\left(\frac{2n\pi}{D} z\right)$$

where coefficients $f_n$ can be found due to the formula $I_n = \frac{|f_n|^2}{Q_z}$, $Q_z$ is the Lorentz correction factor equal to q for oriented bilayers, and $I_n$ is the integrated intensity of the $n^{th}$ Bragg peak; and $v_n$ corresponds to the phase of the structure factor.[77] Because of the mirror symmetry of the bilayers in the z-direction, it can be shown that the phase factors can only be ± 1. We used the swelling method to assign the following phases: -, -, +, -, + at 98% RH. For each phase, intensities of all diffraction orders are normalized by the sum of all peak intensities in that phase to account for the full-beam intensity normalization correction. Finally, the distance between the two characteristic maxima was attributed to the lipid headgroup to headgroup distance ($D_{hh}$) and the water layer thickness between lipid bilayers was defined as $D_w=D-D_{hh}$.

**Dynamic Light Scattering (DLS)**: FIltered and non-filtered starting materials and products were measured at the molar ratios that result in 1 mM of final phospholipid. Liposomes with sfGFP-DAGK were also tested to find the average particle size.

**Table 2.1: DLS results**
Average diameter and polydisperse index (PDI) of empty liposomes and reconstituted liposomes at 1 mM phospholipid concentration after filtering with a 0.22 µm PTFE filter. Results are shown as means and standard deviation (n=3).

| Type | Average diameter (nm) | PDI |
|---|---|---|
| 1 mM compound 1 | 0 | 0 |
| 2 mM hexanal | *n/a | *n/a |
| Empty liposomes | 144.9 ± 2.0 | 0.3 ± 0.019 |
| DAGK/liposomes | 176.9 ± 4.1 | 0.12 ± 0.020 |

*Data was uninterpretable

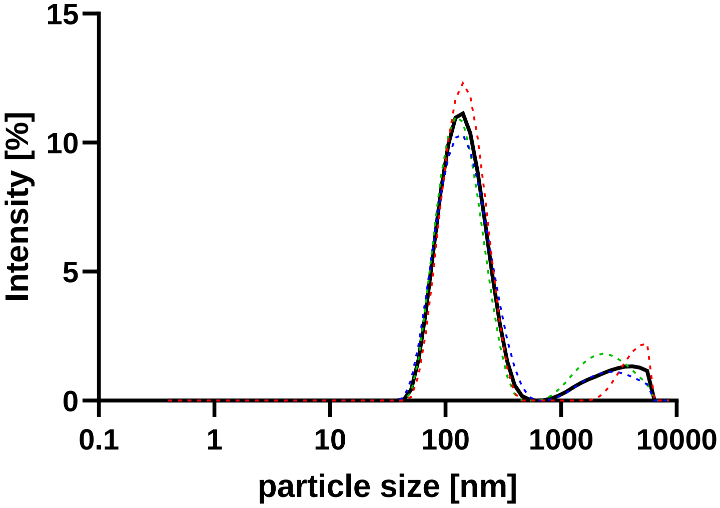

**Figure 2.27: Oxime ligation (no protein).**
The blue, red, and green dashed lines are three different measurements. The solid black line is the average of the three measurements.

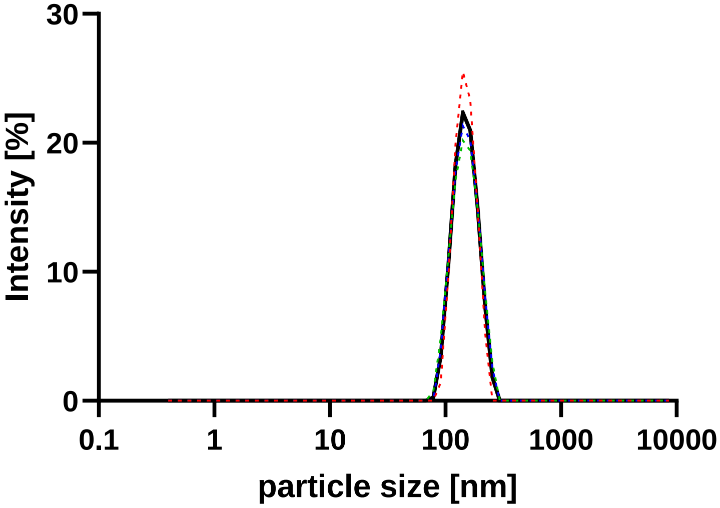

**Figure 2.28: Oxime ligation, filtered (0.22 µm).**
The blue, red, and green dashed lines are three different measurements. The solid black line is the average of the three measurements.

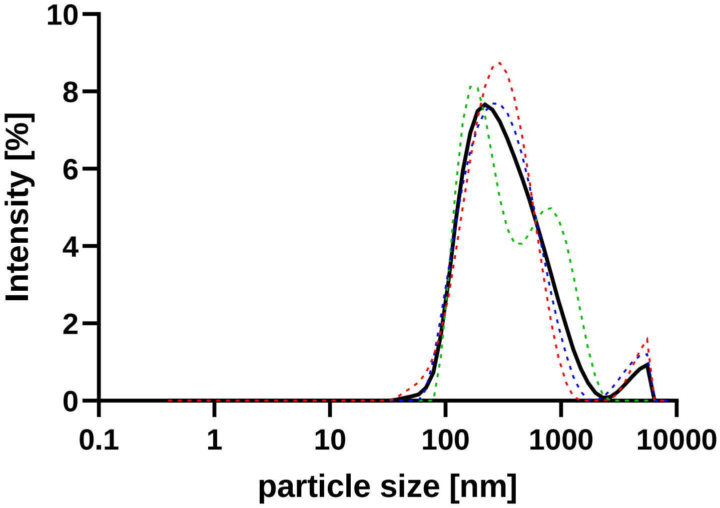

**Figure 2.29: Oxime ligation with protein**
The blue, red, and green dashed lines are three different measurements. The solid black line is the average of the three measurements.

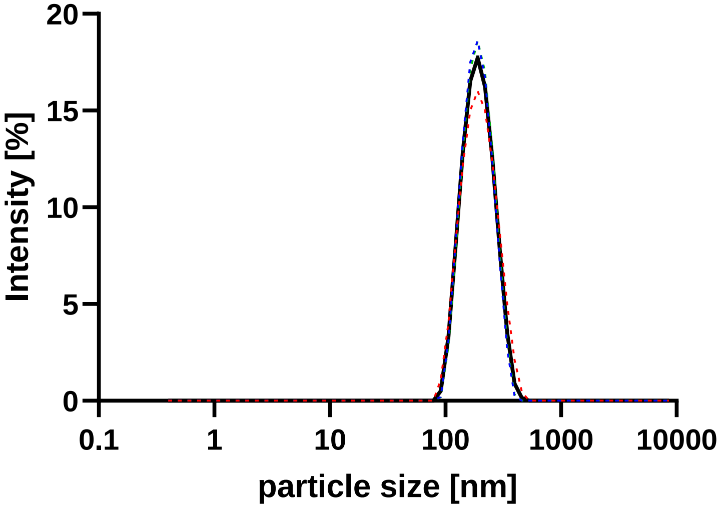

**Figure 2.30: Oxime ligation with protein, filtered (0.22 µm).**
The blue, red, and green dashed lines are three different measurements. The solid black line is the average of the three measurements.

## 2.7 Acknowledgments

Chapter two, in full, is a reprint (with co-author permission) of the material as it appears in the publication: **J. Flores**, R.J. Brea, A. Lamas, M. Salvador-Castell, C. Xu, C.R. Baiz, S.K. Sinha, N.K. Devaraj "Rapid and Sequential Dual Oxime Ligation Enables De Novo

Formation of Functional Synthetic Membranes from Water-soluble Precursors," *Angew. Chem. Int. Ed.* 2022, e202200549.

My contribution to this chapter was the design and execution of all biochemical experiments involving cloning, expression, and purification of genes. I also performed all the syntheses for the precursors and most of the drafting of the manuscript. I would like to thank Alejandro Lamas for his contribution to all the preliminary work and reaction kinetics. I would like to thank Roberto Brea for his contributions to drafting the manuscript and all the hours spent guiding me through the experiments. I would also like to thank Alessandro Fracassi for his contribution to the NMR analysis and characterization of precursors and products. I would also like to thank Cong Xu and Marta Salvador-Castell for their contribution to the 2D-IR and X-ray diffraction experiments, respectively. Lastly, I would like to thank Neal Devaraj for his guidance in the development and execution of this project and the preparation of the manuscript.

# 3 Applications for *In Situ* Lipid Synthesis for Protein Reconstitution Technology (INSYRT)

## 3.1 Introduction

About one-third of most genomes encode for membrane proteins.[29] They carry out essential functions in membranes and are at the forefront of cellular interactions with their environment. They carry out essential functions that, if disrupted, can lead to diseases and other difficulties for the organism.[78] Given the roles of membrane proteins, they encompass 60% of known drug targets,[79] thus emphasizing the importance of studying and understanding their fundamental roles. As mentioned earlier, several complications arise when studying membrane proteins that make them more difficult than typical cytosolic protein studies. Primarily, protein expression and purification yields are low because there is a limited amount the host organism can express before the high protein concentration becomes toxic. Additionally, as detergents are required for purification, the final buffer does not reflect the native cellular environment. This native lipid environment is fundamental for conserving the natural structures and activities of many membrane proteins. For example, it has been discovered that the conformation and activity of ABC (ATP-binding cassette) transporters are affected by the lipids surrounding them.[80] Therefore, reconstituting membrane proteins into synthetic membranes that resemble native membranes provides a solution to study them in vitro without losing native characteristics.

As briefly discussed in the previous chapter, reconstitution into synthetic membranes can be a powerful tool in analyzing the mechanics, structure, and function of a specific membrane protein. Several methods have become common practice for creating highly functional proteoliposomes. Generally, the reconstitution of membrane proteins in liposomes requires purified membrane proteins solubilized in detergent.[29] The sample is mixed with the desired phospholipid vesicles forming an isotropic solution of mixed phospholipid-protein-detergent micelles. The detergent is then removed slowly by dialysis, gel filtration, or Biobead adsorption. When the detergent concentration reaches a critical level, the protein will spontaneously associate with the phospholipid membrane to form biologically active proteoliposomes. Similarly, nanodisk and other methods for creating lipid bilayers have been used to successfully reconstitute proteins.[81] Although all the mentioned methods have provided valuable information about receptors,[82] ion channels,[83] transporters,[84] and many other types of proteins, a drawback remains; the methods utilize detergents that are removed during reconstitution, which can disturb the bilayer formation. Additionally, this methods is generally time-consuming, and residues of detergent often remain absorbed in the proteoliposome after detergent removal.[85]

The lab has developed INSYRT (*In Situ* Lipid Synthesis for Protein Reconstitution Technology), a synthetic strategy to use reactive detergent precursors that form membranes *in situ* to solubilize proteins and subsequently reconstitute them into artificial phospholipid membranes. We have reconstituted proteorhodopsin (pR) using this strategy. This method uses NCL-promoted incorporation of Adenosine $A_{2A}$ Receptor into synthetic phospholipid liposomes with reactive n-dodecyl-β-D- maltoside (DDM) thioester derivatives. To explore different possible proteoliposomes with this method, I sought to reconstitute proteorhodopsin (pR), a light-activated proton pump. The aim of this work was to reinforce the advantage of INSYRT while exploring possible artificial mitochondria systems.

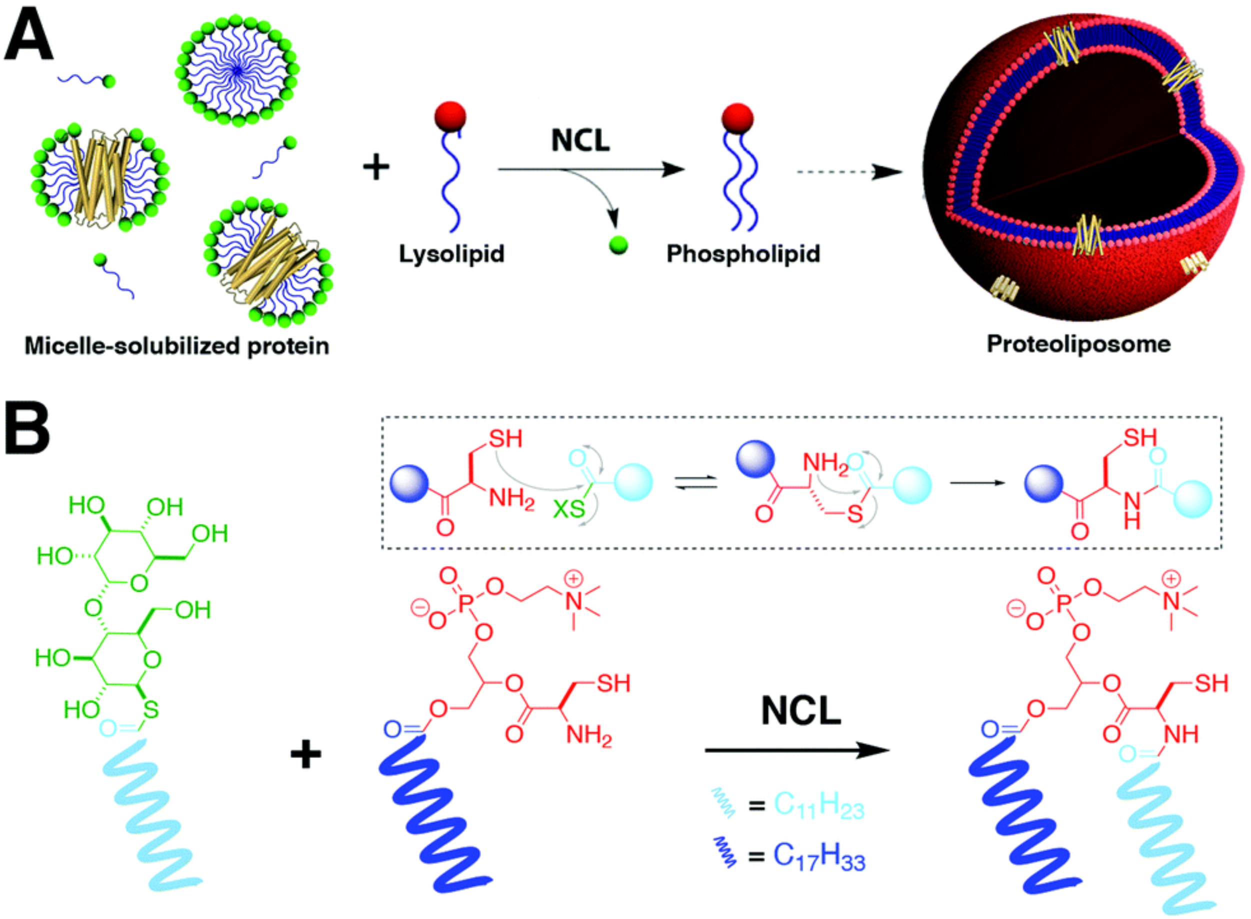

**Figure 3.1: *In situ* lipid synthesis for protein reconstitution technology (INSYRT).**
(taken from Flores. et al 2020) (A) Schematic representation of the NCL-based phospholipid membrane formation with embedded transmembrane proteins. The protein is initially solubilized with a synthetic surfactant to form micelle-solubilized protein complexes. Addition of the complementary reactive precursor and subsequent NCL results in the formation of the desired proteoliposomes. (B) Synthesis of phospholipids by NCL between acyl maltose thioesters and cysteine-functionalized lysophospholipids. (Note: color depicted in this published image differs from the rest of the color depictions in this document).

## 3.2 Proteorhodopsin reconstitution using INSYRT

Proteorhodopsin, pR, is a light-harvesting membrane protein that is globally abundant across various oceanic systems, including some of the most abundant bacteria in the ocean, such as SAR11 and SAR86.[86,87] It is a light-driven proton pump responsible for increasing membrane potential that leads to processes including ATP synthesis, substrate uptake, survival during starvation, and saline stress response.[88] Specifically, green-absorbing pR has been characterized to fold into a seven-transmembrane $\alpha$-helical bundle containing a covalently bound *all-trans*-retinal, which is conjugated to a conserved lysine residue via a characteristic Schiff base.[89] Given its significance, it has been extensively studied and used as a model protein for reconstitution methods, yet the first crystal structure was just published last year.[90] Therefore, efforts to continue studying pR are a prominent motivation to attempt to reconstitute it using our previously established method. Further, if successfully reconstituted, the INSYRT synthesized proteoliposomes could be functionalized with additional energy-producing proteins such as ATP synthase to progressively increase complexity in artificial cells.

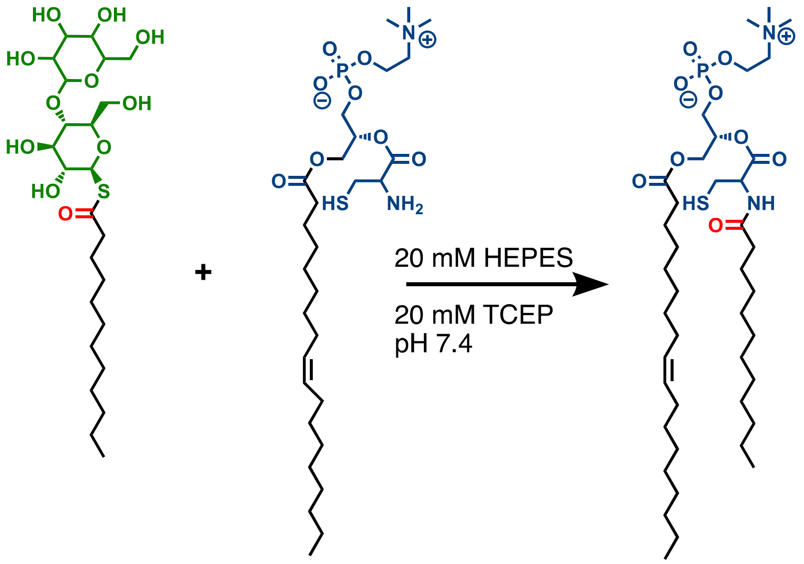

**Figure 3.2: Chemical synthesis of phospholipid.**
The Dodecyl-β-D maltoside (DDM) derivative was combined with cysteine functionalized lysolipid in 20 mM HEPES (20 mM TCEP, pH 7.4) to afford an amide phospholipid.

### *3.2.1 Protein preparation and phospholipid de novo formation*

Proteorhodopsin was expressed in bacteria and purified using DDM detergent as described in detail in the methods. The detergent was then thoroughly exchanged for the DDM thioester derivative. The phospholipid formation occurred in the presence of pR by the addition of cysteine-functionalized choline-based lysophospholipid in millimolar concentrations. The mechanism of the chemoselective formation occurs via a transesterification followed by an S-to-N transfer shift, resulting in an amide bond.

According to previous literature, each protein requires a different protein-to-lipid ratio to observe reconstitution. A series of different ratios were explored to produce a final lipid concentration of 1mM. Lipid synthesis was confirmed via LCMS.

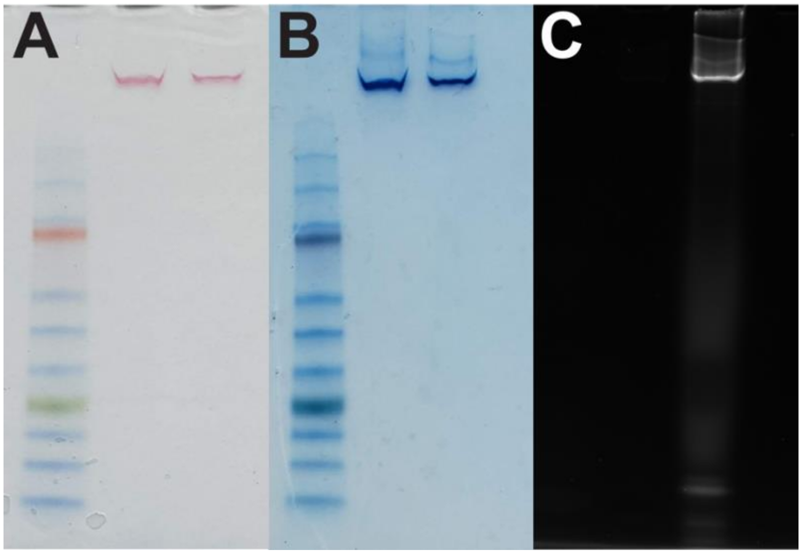

**Figure 3.3: SDS-PAGE gel of purified Proteorhodopsin**
A) Image before gel staining. pR did not run according to the molecular weight of the ladder so the pink color was used as an indicator of pR presence. B) After gel staining. Similar amounts of pR were analyzed to compare concentrations before and after labelling protein with A488 dye. C) Fluorescent scan of gel. The gel shows that pR was successfully labeled; the unlabeled control showed no fluorescence.

### *3.2.2 Microscopy of protein reconstitution*

Reconstitution was primarily confirmed using fluorescent microscopy. Prior to *in situ* reconstitution, proteorhodopsin was labeled with Alexa488 via a NHS ester conjugation (Figure 3.3) to track the localization of the pR. Confocal microscopy showed that different ratios resulted in either aggregation of both lipid and protein, vesicle formation with pR membrane localization, or coacervate-like structures with protein inside. Figure 3.4 confirms this structure both in brightfield and in the fluorescent channel. Liposome formation is confirmed as lipid staining shows a clear membrane. The coacervate-like formation shows a rich distribution of lipids throughout the structure, indicating a membrane-less coacervate with embedded protein.

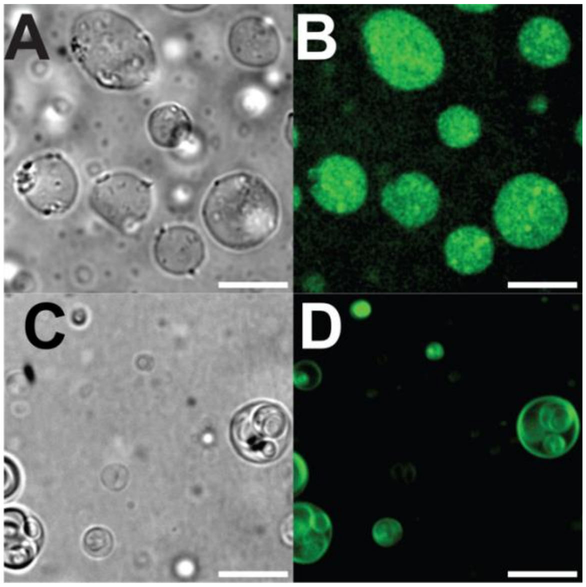

**Figure 3.4: Spinning-disk confocal microscopy**
A) A 1:1500 protein: lipid ratio resulted in sponge-like structures. The brightfield images showed different shaped structures with no clear membranes. B) Fluorescent microscopy showed localization of pR all over the structure. C) A 1: 20,000 Protein-to-lipid ration resulted in typical vesicles that are commonly observed with phospholipids. D) Fluorescent microscopy indicateses the protein localized in the membranes. Scale bars denote 10 µm.

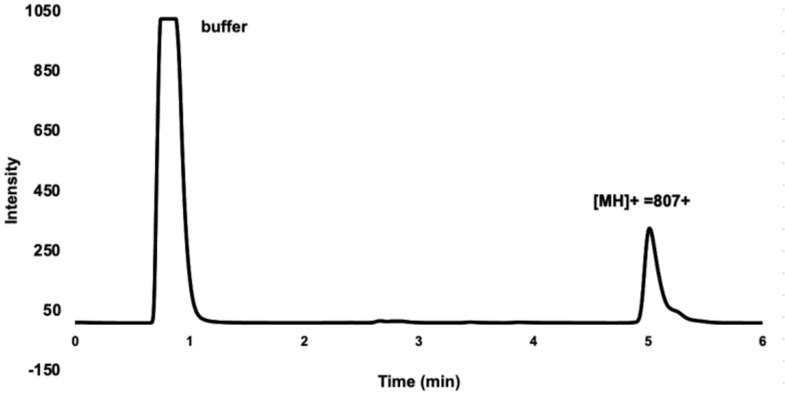

**Figure 3.5: LCMS ELSD trace of reaction with pR**
Two peaks were eluded, mass spectrometry correlated with molecular mass of phospholipid.

Further studies are needed to explore the characterizations of these structures. X-ray diffraction experiments would shed light on the lipid composition in both the vesicle and coacervate structures. Furthermore, a comparison of how embedding a membrane protein affects the presumed lipid bilayer and the protein itself can shed light on the importance of this reconstitution tool. This can be accomplished by testing the activity of pR using a pH gradient assay. Lastly, this technology may be applied to learn more about the structure of pR by utilizing crystallization methods mirroring techniques such as llipidic cubic phase crystallization.

## 3.3 Possible applications with Cardiolipin Synthase B (ClsB)

The *Escherichia coli* phospholipidome is composed of approximately 70% phosphatidylethanolamine (PE), 20% phoshpatydylglycerol (PG), and 5-10% cardiolipin (CL).[4] The most unique of the major phospholipids is cardiolipin, which consists of two phosphatidic acids connected by glycerol.[4] This anionic phospholipid's function has been found to be essential for energy-producing membranes in eukaryotes, and in bacteria. Specifically, in *E. coli*, it has been linked to cell tasks involving translocation of proteins across the inner membrane, initiation of DNA replication, and division septum formation at mid-cell during replication, with additional roles being regularly discovered. [4,91,92] *E. coli* contains three genes encoding three Cardiolipin synthase (Cls) enzymes (A/B/C) which are responsible for the last step of CL synthesis from a biosynthesis pathway starting with phosphatidic acid (PA).[93]

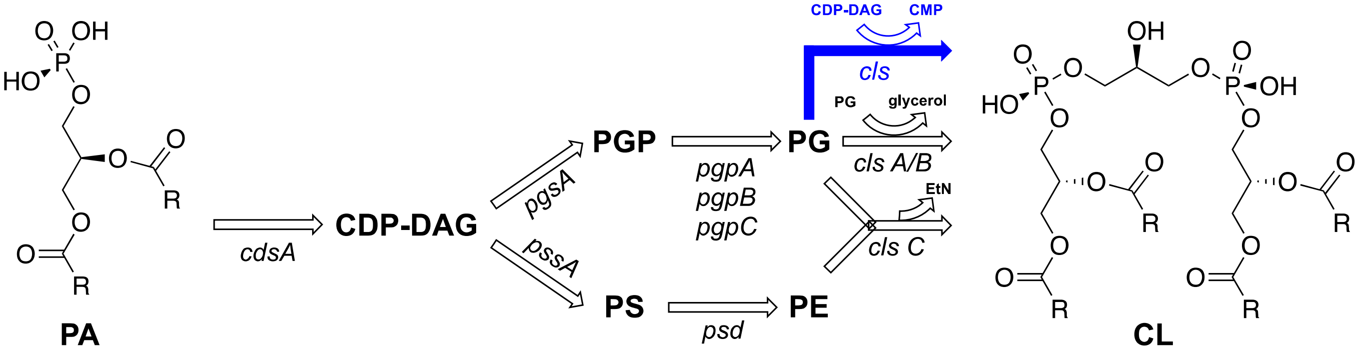

**Figure 3.6: Overview of the synthesis of cardiolipin (CL) and the enzymes involved**
The initial step in E. coli CL biosynthesis pathway consist of the condensation of phosphatidic acid (PA) with cytidine triphosphate to form CDP-DAG. The pathway diverts to phosphatidylglycerol (PG) or phosphatidylethanolamine (PE) synthesis. The blue, filled in arrow indicates the eukaryotic process for CL synthesis from PG and CDP-DAG.

The reasons for the existence of the three genes encoding CL enzymes remain unclear. However, it is known that all three enzymes exhibit HKD motifs that are characteristic of the phospholipase P (PLD) family to which they belong.[93] Of the three resulting proteins, the most recently discovered, and therefore least understood, are ClsB and ClsC. ClsB and ClsC have been shown to have different substrates and activity than that of ClsA—the main contributor of CL in stationary phase. Specifically, ClsB has additional PLD activity, and is shown to catalyze the formation of PG molecules in addition to from its normal CL synthesis role. Early studies of ClsB claimed that it did not exhibit in vivo Cl synthesis activity, however more recent studies have established that the formerly utilized methods did not take proper growth conditions into account. Therefore, having clear biochemical and structural analysis of the active sites of ClsB can illuminate the factors leading to the different enzymatic activity.

The mechanism of cardiolipin synthesis in *E. coli* was initially discovered in the early 1970’s using radioactive phosphatidylglycerol.[5] Despite multiple decades since the initial analyses, and methodological advances in the field, very few changes have been made in the way CL synthesis activity is studied. Since there have been few studies of cardiolipin synthase (Cls) from *E.Coli*, most *E. coli* ClsA and ClsB activity has been examined using harsh radioactive substrates, where CL production is detected using low precision Thin Layer Chromatography (TLC) imaging.[4] Therefore, there is a necessity for a practical technique that will take into consideration the complexity of membrane proteins and facilitate precise detection for accurate activity analysis. Fortunately, several protocols have been established for cardiolipin assays in mitochondrial studies, consisting primarily of three analyses: TLC phosphorus assays, High-Performance Liquid

Chromatography (HPLC), and Mass Spectrometry (MS).[94] In addition, a ClsC mechanistic study has shown that LCMS can be used to determine Cls activity.[95]

This project aimed to 1) explore reconstitution methods to study ClsB biochemically, and 2) clarify its role in the biosynthetic pathway by using non-radioactive LC-MS detection methods for CL synthesis. Artificial lipid membranes present a solution in studying proteins *in vitro* while taking into consideration their natural cell environment.

### *3.3.1 Expression and purification of ClsB*

Previous studies have successfully purified ClsB but have not fully established the activity. Li et al. have reported a purification and activity assay method [96] which was used to overexpress ClsB in *E.Coli* for this project. ClsB can be solubilized with Tween 20 detergent during purification. Thus, reconstitution into a DDM derivative using INSYRT[22] is a plausible method for solubilization for future exploration in the lab.

The ClsB gene was amplified from the *K12 E. Coli* strain genome. To achieve this, the gene was obtained from GeneBank ® to design PCR primers for general PCR amplification. Successively, the ClsB was cloned into a pET vector containing several tags. Several conditions were tested to determine the best overexpression conditions. To establish maximum protein yield, constructs with MBP or MISTIC tag were cloned to compare with the wild-type gene of interest. MBP and MISTIC tags have been previously reported to aid in the expression of membrane proteins by promoting proper folding.[97,98] Sample preparation and histidine tag purification were conducted following Li et. al protocol for ClsB and initial enzymatic assays conducted using the purified construct in Tween20 detergent.

The purified protein was tested for activity using an established normal phase HPLC method [99] and a calibration map of pure PG and CL created to assign peaks. Efforts test the activity of the purified ClsB in Tween 20 did not work. Given the results, a much more thorough investigation is needed to obtain the activity kinetics. For future studies, it would be important to assess how reconstitution might aid in protein activity retention.

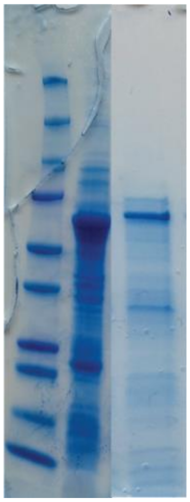

**Figure 3.7: Gel of overexpression and purification of MBP-ClsB**
Before and after purification with Ni-NTA column. Protein yield for 200 mL culture resulted in 0.8 mg of protein. The gel shows impurities after purification column.

## 3.4 Conclusion

Efforts to use INSYRT as a membrane protein reconstitution tool in our lab have yielded promising results. Nonetheless, more experimentation is needed to further develop the tool for membrane protein structural applications and/or to functionalize artificial cells. Results with proteorhodopsin indicate INSYRT can form various lipid phases, including coacervate-like structures. Coacervates have gained interest due to their application in artificial organelles[100], drug delivery[101], proteomic studies[102], and many more fields and industries.[103] Further, coacervates have the ability to contain a high concentration of membrane proteins in a small volume, providing the appropriate environment to explore protein crystallization options. Therefore, we decided to analyze cardiolipin synthase using reconstitution methods. To date, there are no elucidated structures of this essential protein. Here, I reported a method for overexpression of ClsB and confirmed past published purification methods worked on isolating the protein. The next stepin this work includes optimization of its reconstitution by using INSYRT or dual oxime ligation.

## 3.5 Methods and materials

### *3.5.1 General materials, and instruments*

Commercially available 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine was obtained from Avanti® Polar Lipids. Texas Red® 1,2-dihexa-decanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red® DHPE) and Alexa Fluor® 488 N-hydroxy-succinimide ester (Alexa Fluor® 488 NHS ester) were obtained from Life Technologies. Deuterated chloroform (CDCl3) was obtained from Cambridge Isotope Laboratories. Bl21 and Dh5α *E. coli* competent cells were obtained from New England Biolabs (NEB). Spinning-disk confocal microscopy images were acquired on a Yokogawa spinning-disk system (Yokogawa, Japan) built around an Axio Observer Z1 motorized inverted microscope (Carl Zeiss S-3 Microscopy GmbH, Germany) with a 63x, 1.40 NA oil immersion objective to an Evolve 512×512 EMCCD camera (Photometrics, Canada) using ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany). Absorbance measurements were performed in a NanoDrop 2000C spectrophotometer.

### *3.5.2 Experimental procedures*

**INSYRT reaction without protein:** The INSYRT reaction was performed as described by Brea et. al.[22] A lipid film of lysolipid was dissolved in 1x HEPES buffer containing 20mM tris(2-carboxyethyl)phosphine (TCEP) and adjusted to a pH of 7.4. The lipid concentration was adjusted to be 0.5mM. A 5mM stock concertation of DDM thioester was added at 0.5mM final concentration. The reaction tumbled at room temperature for 2 hours. Vesicle formation was confirmed with light microscopy.

**Expression and purification of Proteorhodopsin (pR):** The pET26b pR-his vector was transformed into BL21 (DE3) competent cells. An individual colony was picked and used to inoculate a 10mL LB culture containing 100µg/mL of carbenicillin and grown overnight at 37 $^{o}$C. The next morning, 1L of LB containing carbenicillin was grown to 0.8 $OD_{600}$. The pR overexpression was induced with the addition of 1mM IPTG anfd10µM all-trans-retinal. The cells were grown for an additional 4 hours at 37 $^{o}$C and centrifuged at 5,000 x g for 30 minutes.

The pink-colored pellet was resuspended with 10 mL of resuspension buffer (50 mM potassium phosphate, 150 mM KCl, 20 mM $MgCl_2$ and 0.2 mg/mL lysozyme, pH 8.2). The cells were lysed by sonication. The cells were centrifuged at 5,000 x g for 30 minutes and the lysate was transferred to an ultracentrifuge tube. The membrane fraction was obtained by centrifuging at 200,000 x g for 1 hour. A solubilization buffer (50 mM potassium phosphate, 150mM KCl, and 2 wt% dodecyl-β-D-maltoside (DDM) surfactant) was added and the sample was incubated overnight. The protein was then purified using Ni-NTA agarose His-tag affinity resin with 0.05 wt% DDM and 250mM Imidazole for elution. The pink-colored protein was then purified with size exclusion chromatography (SEC), concentrated, and stored in 5 µM aliquots.

**Labeling of pR**: Aliquots were labeled using Alexa Fluor® 488 dye in sodium bicarbonate buffer at pH 8.1. Using a 30k spin filter, the storage buffer was exchanged with the sodium bicarbonate buffer with 0.005 % wt DDM. Once exchanged, 2μL of 5 mM dye was added and tumbled for 2 hours at room temperature. The sample was spin filtered several times to remove excess dye and exchange buffer for storage buffer (50 mM potassium phosphate, 150 mM KCl, 0.05% wt DDM, pH 8.2). The labeled pR was stored at 4 $^{o}$C temporarily for subsequent experiments.

**INSYRT of pR:** The labeled proteorhodopsin (pR) buffer was exchanged using 30k spin filter with 1x HEPES and 5mM DDM thioester. Once fully exchanged, an equal molar ratio of the cysteine-functionalized oleoyl lysophosphatidylcholine and TCEP were added. The reaction was incubated overnight at room temperature. Phospholipid and liposome formation was tracked using LC-MS and spinning disk microscopy, respectively.

**Cloning, expression, and purification of Cardiolipin Synthase B (ClsB):** Primers were designed to amplify the ClsB gene from K12 *E. Coli* cells. The gene was then inserted in a pET28 plasmid with carbenicillin resistance containing an MBP and histidine tag using Gibson assembly and transformed into DH5α (DE3) competent cells. Once sequencing was confirmed, the protein was expressed and purified using a previously published protocol.[96]

**Synthesis of cysteine functionalized lysolipid:** Cysteine-functionalized oleoyl lysophosphatidylcholine was synthesized according to the Brea. et.al protocol.[22] Using a Yamaguchi esterification, the phosphorylcholine lysolipid was conjugated with a boc-protected L-cysteine in deuterated chloroform. The product was purified using HPLC. The product was deprotected with a TFA/DCM solution. The final product was dried and stored as a lipid film at -80 $^{o}$C.

## 3.6 Acknowledgments

Chapter three was contributed by **Flores, Judith**; Brea, Roberto J.; Gordon, Catriona; and Devaraj, Neal K. I would like to thank Roberto Brea for providing me with the derivatives for the INSYRT experiments and for all the guidance and patience he provided. I would like to thank Catriona Gordon for contributing to optimizing the expression of ClsB. I would like to thank Neal Devaraj for all the guidance. The dissertation author was the primary researcher and author of this material.

# 4 Applications for the construction of a synthetic Endoplasmic Reticulum (ER)

## 4.1 Introduction

Phospholipids are the major component in lipid bilayer membranes and are responsible for maintaining both the structural integrity of a cell and the spatial separation of subcellular compartments. These membranes contribute in many ways to maintaining the proper function of the organelles, some of which are still being discovered.[104] For example, mitochondria have a distinct inner membrane, which is directly responsible for the oxidative phosphorylation process.[105] It has been found that the activity of the membrane-associated proteins depends on the phospholipid composition of the membrane in order to fulfill functions such as mitochondrial respiration. Effects on this function are linked to human diseases such as Barth syndrome, ischemia, and heart failure.[105] Furthermore, the curvature of the endoplasmic reticulum (ER) membrane is of central importance for membrane trafficking and other cellular functions.[78] There has been tremendous progress over the decades to understand how proteins and lipids cooperate to form membrane-bound organelles, however, several outstanding questions remain.[104,106,107] For example, there are proteins containing domains or motifs that are specialized in sensing, generating, or stabilizing membrane curvature; some that act directly by changing lipids, and others that provide “scaffolding” by applying tension on membranes.[108] In all of this, it is unclear how organelle size is determined and maintained.[106] From an evolutionary biology perspective, it is unclear what physicochemical principles were essential for the de novo formation of membrane-bound organelles like the endoplasmic reticulum.[109] We believe bottom-up designer synthetic organelles using minimal and well-defined lipids and proteins would shed light on these questions. Specifically, the ER's particular form can shed light on how different shapes and sizes aid in its several functions.

The ER has three distinct regions: sheets of the nuclear envelope, peripheral ER sheets, and peripheral ER tubules. All regions are critical sites for protein synthesis, modification, and transportation.[110] In living cells, the ER is maintained through the constant synthesis of phospholipids and is shaped by the action of several membrane proteins. It has been discovered that Sey1, an integral membrane GTPase, has an important role in fusing membranes to form tubular ER networks.[111,112] The absence of Yop1, a conserved integral membrane, has shown disruption in tubular ER.[112] The Rapoport lab recently showed that reconstituting these two proteins into liposomes *in vitro* results in ER-like networks.[113] Given these results, we envisioned *in vivo* generation of both phospholipids and ER-like structures.

I attempted to generate a synthetic ER *de novo*, by combining the *de novo* dual oxime ligation phospholipid formation with recombinant proteins that influence membrane curvature and fusion. Here, I summarize the work I was able to achieve for this project and the future work needed in order to obtain a synthetic ER.

## 4.2 Reconstitution of Yop1 and Sey1

Yop1 and Sey1 containing plasmids were obtained from the Rapoport lab. To ensure localization, Yop1 and Sey1 were cloned with a sfGFP and mCherry tag, respectively. Both proteins were overexpressed BL21 (DE3) *E. Coli* cells according to previous protocols.[113,114] Before membrane extraction, the lysates were examined with Instant Blue$^{TM}$ stained SDS gels to confirm the protein was present in its desired oligomerization. The lysates were then spun at 25,000 x g to extract the membranes. The small fluorescent pellets were resuspended separately with 20mM hexanal in 1x PBS buffer. t10mM of compound **1** (from Chapter 2) were added dropwise and the reactions were incubated overnight at room temperature.

Fluorescent microscopy confirmed the formation of spherical bilayer liposomes displaying fluorescence on the membrane layer according to the tag for Yop1. Liposomes incubated with Sey1 did not yield any observable reconstitution. Attempts to combine liposomes containing Yop1 and Sey1 with the addition of GTP did not yield any network structures.

## 4.3 Conclusion

Our data showed that the dual-oxime phospholipid did not yield any synthetic ER networks. Optimization of the reconstitution for both proteins are needed for subsequent membrane fusion experiments. Further, some studies have shown that Sey1 needs specific lipid compositions for full activity.[115,116] It is possible that the composition of the novel oxime phospholipid will not allow for enough diversity in the bilayer membrane to form network structures. Our lab has designed and continues to find new ways to reconstitute proteins in synthetic membranes. Our goal for this project is to provide a tool for creating a synthetic ER that will create a minimalistic platform to study the shape and size of organelles. We believe that these tools have applications beyond the chemical biological world and bring the interface between biology and chemistry closer together.

## 4.4 Methods

### *4.4.1 Cloning, expression, and purification of membrane proteins*

The yop-1SBP gene was amplified using PCR and cleaned using a QIAquick PCR purification kit (QIAGEN®. The amplified insert was then cloned into PET21b vector with an sfGFP and histidine tag. Similarly, scSey1 was amplified, purified, and cloned into a pET28b vector with a mCherry and histidine tag. Separately, both genes were transformed in BL21 *E. Coli* competent cells and expressed in 200 mL LB broth. After centrifugation, cells were resuspended with cold buffer (1x PBS buffer, 300 mM NaCl, and 1 mM PMSF) and lysed via sonication. The lysate was centrifuged at 5,000 x g for 30 minutes and the pellet was discarded. The supernatant was aliquoted into small tubes (500 µL aliquots) and spun at maximum speed (25,000 x g) for 1 hour. The resulting pellets were flash-frozen and stored at -80 $^{o}$C for future use.

### *4.4.2 Reconstitution*

The frozen membrane fractions were thawed and washed with 1x PBS buffer making sure to not disrupt the pellet. The pellet was then lightly resuspended with 5 0µL 20mM hexanal in 1x PBS buffer and transferred to an 0.5-dram amber vial. 50µL of 10 mM solution of Compound 1 was added dropwise over a period of 1 hour and incubated in the dark overnight. All analysis was performed using the spinning-disk confocal microscope.

## 4.5 Acknowledgements

Chapter four, in part, is currently being prepared for submission for publication. Flores, Judith; Khanal, S; Devaraj, Neal K. I would like to thank the Rapoport laboratory for providing the genes of both Sey1 and Yop1. The dissertation author was the primary researcher and author of this material.

# 5 Conclusion

## 5.1 De novo formation of novel phospholipids

From answering the most fundamental questions of life to developing cancer therapeutics, lipid-based tools are elucidating new avenues of research. Recent advances in the preparation, derivatization, and analysis of lipids have created a toolbox for those studying lipids. In this work we aim to continue the development of technologies employed for chemical synthesis and modifications of lipids *in vitro* and in live cells, as well as explore their associated biological applications. Here, we specifically focus on chemoselective strategies for *in situ* formation of phospholipids for biochemical applications. The general strategy was to find synthetic routes to produce membrane-forming lipids from simple non-membrane forming precursors, all in the presence of water. Furthermore, physiological conditions were employed to create biocompatible tools for future applications. Our aim for this work was to contribute to the effort to understand membrane behavior and lipid biology.

## 5.2 Biochemical applications of reconstitution biology

In this work we explored two tools for reconstituting membrane proteins in the prospects of using them to study the function and structure of proteins and creating synthetic organelles. The main aim of this work was to explore options that would reduce or eliminate the use of detergents, which are known to be a shortcoming when studying membrane proteins. Various proteins were effectively reconstituted within the synthetic lipid bilayers. Specifically, dual oxime ligation has proven to be a versatile technique for reconstituting non-purified membrane proteins. In the future, it would be advantageous to optimize the technique to subsequently purify the protein of interest. Because of the broad range of membrane proteins that are shown to reconstitute in novel phospholipid membranes, we believe the full extent of applications are yet to be discovered.

# Appendix for Chapter 2: Plasmid for DAGK

## His-sfGFP-DAGK

TGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCC
AACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAA
CATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCC
ATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGC
GCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACT
GGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTG
GCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATT
GCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGG
GGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTC
ACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA
TTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTC
ATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGA
AAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCA
AACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAA
CTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTT
CTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATA
CCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGT
CTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCT
GAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAAC
TGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAA
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TTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAA
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TTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAA
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TGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTG
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GCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTA

GCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCC

CCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGC

ACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCC

CTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGAC

TCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATA

AGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATT

TAACGCGAATTTTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCC

TTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGC

TCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGC

CCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTC

CGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGA

AAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCT

TAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATT

TTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT

CAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATT

CCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAA

GTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATC

TCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATG

AGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCA

AGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCAC

CAGTCACAGAAAAGCATCTTACGGATGGCA

## Appendix Chapter 3: Plasmids for pR and ClsB

### pR- His

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT

TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCT

TTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCG

GGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAAC

TTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGC

CCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACA

ACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG

GCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAA

ATATTAACGTTTACAATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCC

TATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAATTAATTCTTAG

AAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATA

CCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTT

CCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAA

TACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCAT

GAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGACT

TGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACC

GTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAG

GACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATC

AACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCC

GGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTG

ATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGT

AACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGG

GCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCC

CATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAA

GACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCA

GACAGTTTTATTGTTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGC

GTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCG

TAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCG

GATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGAT

ACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTG

TAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGT

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CGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCT

TCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGG

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GGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGC

GGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTG

CTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCG

TATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGC

AGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTA

CGCATCTGTGCGGTATTTCACACCGCATATATGGTGCACTCTCAGTACAATCTGCT

CTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCA

TGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTC

TGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTG
TCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCA
TCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCT
CGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTA
AGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTT
CATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACT
GATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTAT
GGATGCGGCGGGACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAA
TACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGG
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GCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCC
GCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGGG
CCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGAC
CAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACA
GGCCGATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGA
GCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGC
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**Mistic-ClsB-His**

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CTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTT

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# Appendix in chapter 4: Plasmids for Yop1 and Sey1

## Yop1-sfGFP-his

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ACAAA

**mCherry-Sey1-his**

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TGCCGATTTCGGCCTATTGGTTAA

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