Analytical Characterization and Biological Impacts of Multi-Lipid Nanodiscs

by

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Dedication

This dissertation is dedicated to my Avô, you always knew I would be a doctor.

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First, I would like to thank my family. Mom and Dad, thank you for always supporting me, believing in me, and encouraging me to be my best. Thank you teaching me accountability, that hard work pays off, how to advocate for myself, and how to speak the truth always (even if it means less people like me). Perhaps most importantly, thank you for teaching me that sometimes things just suck, there are things we cannot control, and that it is okay if I am not good at everything, I would not have made it through grad school without it. Sorry I keep moving slightly farther away every few years, I miss you, but thank you for always picking up the phone (plus, now I can visit more!). Paul and Matt, thank you for being the best brothers ever and for being my longest tenured best friends. I always look forward to us being together, and despite being the oldest, I look up to both of you and wish I could live my life more like you (sometimes).

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Abstract

Membrane proteins participate in numerous biological processes such as signal transduction and metabolism. Consequently, they have become popular therapeutic targets, comprising about 60% of current drugs despite making up only 30% of the proteome. This necessitates their characterization, but membrane proteins only make up about 3% of structures deposited in the Protein Data Bank. This is due to their propensity to aggregate and misfold in aqueous solution which poses a significant challenge for *in vitro* analyses. To overcome this challenge, membrane mimetics have been developed to solubilize and stabilize membrane proteins in solution. One such mimetic is the Nanodisc which is a discoidal lipid bilayer encircled by an amphipathic helical belt protein termed membrane scaffold protein (MSP). Nanodiscs self-assemble from a mixture of lipids, MSP, and membrane protein(s) upon detergent removal into relatively monodisperse particles.

Nanodiscs have become a popular membrane mimetic system offering a well-defined bilayer environment to stabilize membrane proteins for *in vitro* analyses; however, lipid compositions common in their deployment are simplistic and often fail to model native membrane complexity. Additionally, our understanding of how Nanodisc synthesis conditions impact the final lipid composition is still very limited, primarily resulting from the lack of rigorous analytical and biophysical characterization of Nanodiscs comprised of more than one lipid. We sought to address these challenges through the development of LC-MS/MS strategies to quantify and profile lipid incorporation into Nanodiscs.

This thesis comprises the development and application of two LC-MS/MS methods to interrogate lipid incorporation into Nanodiscs. The first is a targeted LC-MS/MS approach to quantify Nanodisc lipid compositions of 2-6 lipids. The second is an untargeted LC-MS/MS assay to profile the lipid landscape in Nanodiscs synthesized with natural lipid extracts and varied detergent, temperature, MSP sizes, and synthetic lipid additives. We utilized the targeted lipidomics approach to determine that lipids do not always incorporate stoichiometrically into Nanodiscs depending on their physical properties like curvature and fluidity (Chapter 2). The untargeted lipidomics approach was used to discover that Nanodisc synthesis parameters can enrich or deplete the incorporation of specific lipid species into Nanodiscs (Chapter 3).

We then sought to utilize this enhanced understanding of Nanodisc synthesis parameters to selectively enrich different membrane protein classes. Using a bottom-up proteomics approach, we found that similarly to how detergents and neighboring lipids could influence lipid incorporation, these conditions could be tuned to enrich different protein classes (Chapter 4).

Overall, these studies were among the first to quantitatively confirm Nanodisc lipid compositions as well as to profile the Nanodisc lipid and membrane protein environment upon modulation of synthesis parameters. We hope these studies serve as a foundation to better understand how multi-lipid Nanodiscs are formed and to promote more robust analytical characterization for necessary quality control experiments. These studies are essential to enable the use of Nanodiscs in structural and/or functional characterization of membrane proteins in more native-like environments.

Chapter 1 An Introduction to Nanodiscs and a Critical Review of Their Characterization

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1.1 Introduction

Membrane proteins are essential to many biological processes such as signal transduction and metabolism, which has led to their importance and prevalence as therapeutic targets. Despite making up 30% of the proteome, membrane proteins currently encompass about 60% of pharmaceutical targets.[1,2] Thus, characterization of membrane protein structure and function is necessary. However, their analysis lags behind soluble proteins, where approximately 3% of Protein Data Bank (PDB) structures belong to membrane proteins.[3,4] This results from membrane proteins aggregating and misfolding once removed from the native bilayer environment, making them increasingly difficult to study *in vitro* in aqueous solutions.

To combat this challenge, there has been extensive research into developing membrane mimetics to solubilize and stabilize membrane proteins in aqueous solutions.[5–10] These mimetics have enabled characterization of intact membrane proteins and elucidated their involvement in signal transduction, molecular transportation, cell-cell interactions, and enzymatic activity.

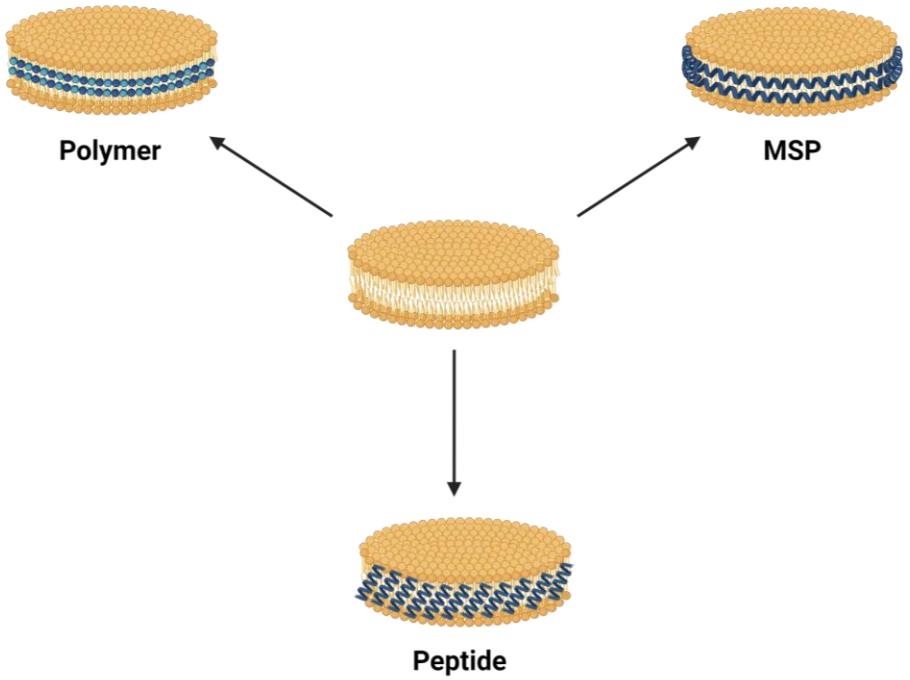

Figure 1-1 Nanodisc scaffolds. Polymers, peptides, and membrane scaffold protein (MSP) can be used to synthesis SMALPs, peptidiscs, and Nanodiscs, respectively.

Further, the surrounding lipid environment has been shown to alter protein function[11–14]; however, many membrane mimetics utilize detergents to stabilize membrane proteins (e.g., DDM, OG) which do not provide a native-like environment for characterization. Contrarily, a few membrane mimetics surround the membrane proteins within lipids (e.g., liposomes, bicelles), which provide a more native-like environment for membrane protein characterization.

Here, we focus on the Nanodisc, a membrane mimetic consisting of a lipid bilayer stabilized by a scaffold material wrapped around the lipid tails. Since their development, many unique scaffolds have been developed to synthesize these mimetic structures, such as proteins, peptides, and polymers (**Figure 1-1**).[15–20] These scaffolds allow for better control over the size and homogeneity of the Nanodisc, since the size of the scaffold generally determines the bilayer diameter. Further, Nanodiscs provide access to both sides of the bilayer, making the directionality of membrane protein insertion unimportant, but this limits their usability for assays that take advantage of an inner compartment (as in liposomes), like ones to study ion channels for example.

Since their development nearly two decades ago, Nanodiscs have become a premier tool for membrane protein solubilization and stabilization for *in vitro* studies, especially for structural biology. They have been used to characterize numerous membrane protein structures within different classes using X-ray crystallography, NMR, cryo-electron microscopy, all of which have been reviewed extensively. In addition to structural measurements, Nanodiscs have been used to study membrane protein activity and their interactions with other proteins, lipids, and other ligands using tools such as mass spectrometry, fluorescence assays, and surface sensitive techniques.[15,16,21]

Despite their frequency of use and broad applicability to a wide range of proteins and analytical and biochemical techniques, there has been limited work in the characterization of Nanodiscs themselves, not the proteins they encapsulate. This is an oversight in our field considering there are numerous examples that show mimetic systems influence membrane protein structure and function.[22–30] It is not improbable that variations in Nanodisc physical properties like size and dispersity as well as lipid composition could contribute to variations in membrane protein measurements. Additionally, with the recent push to increase the lipid complexity of Nanodiscs to better replicate the complexity of native membranes, characterization tools are necessary to judge the success and variation of Nanodisc synthesis with complex lipid mixtures. This chapter aims to highlight Nanodisc characterization approaches, identify gaps of knowledge, and propose further directions in Nanodisc characterization needed to create a robust and well-characterized mimetic platform.

1.2 Nanodiscs: Classes, formation, and applications

This section aims to introduce different Nanodisc classes with scaffolds spanning proteins, peptides, and polymers. Recent advancements, applications, and challenges will be discussed.

1.2.1 Membrane Scaffold Protein-Based Nanodiscs

Nanodiscs, developed by Sligar and coworkers, are discoidal lipid bilayers encircled by an amphipathic helical belt protein, membrane scaffold protein (MSP).[31–33] MSP was engineered based on the template sequence of human apolipoprotein A1, the major component of high density lipoprotein (HDL) particles that solubilize and transport lipids in aqueous environments. To synthesize Nanodiscs with larger diameters to incorporate larger membrane proteins or complexes, additional helical regions are added to the MSP to extend the diameter of the Nanodisc. These extensions have enabled Nanodiscs to be made between 9 and 17 nm.[34] Further, MSP can be engineered with affinity tags such as histidine tags and FLAG tags for downstream purification to isolate Nanodiscs from unused components such as lipids or unincorporated membrane proteins.

MSP Nanodiscs are formed by incubating lipids, MSP, and a recombinantly expressed membrane protein or cell lysate in a carefully optimized ratio in a detergent solution.[31–33] Nanodiscs self-assemble once detergent is removed from this mixture by the addition of detergent removal beads or dialysis (**Figure 1-2**). Nanodisc synthesis generally takes between 2 and 18 hours depending on the components used. The temperature used to form Nanodiscs is dependent on the lipid composition, as a temperature near the transition temperature of the lipid or lipid mixture is optimal. The synthesis of MSP Nanodiscs is amenable to many different buffer and ionic conditions and allows for better control of the lipid composition than other Nanodisc scaffolds. However, MSP Nanodiscs are generally made with synthetic lipids, whereas other scaffolds (e.g.,

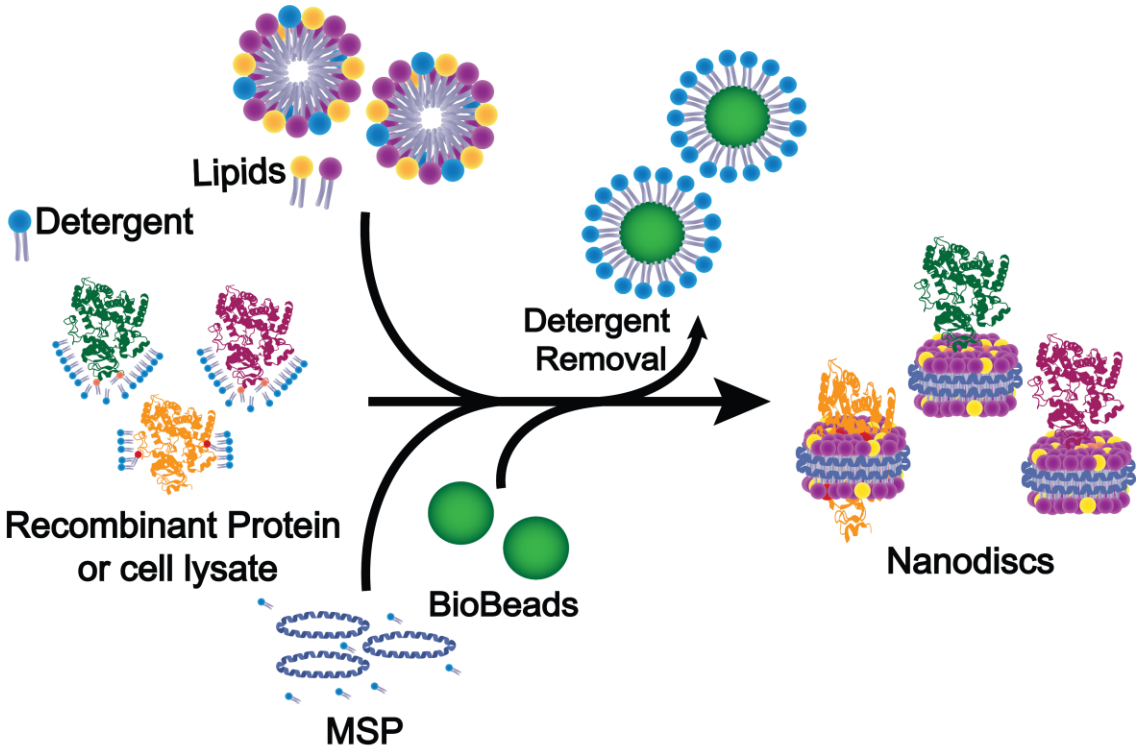

Figure 1-2 Overview of MSP Nanodisc synthesis. First, lipids, MSP, membrane protein(s), and detergent are mixed together to form a component mixture. Next, detergent adsorbing beads are added to remove detergent and initiate Nanodisc self-assembly.

styrene maleic acid copolymers) can be added directly to native cell membranes to encapsulate native lipids and membrane proteins directly. This has been partially addressed with the development of "soluble membrane protein libraries" or "library Nanodiscs" in which Nanodiscs are made with a cell lysate or membrane extract instead of a recombinantly expressed protein.[35–38] This allows for all membrane proteins to be incorporated and has the potential to incorporate some native lipids from the local membrane environment of the proteins; however, they still require supplementation with synthetic lipids to meet the correct lipid:MSP ratio for proper Nanodisc formation.

Since their development, MSP Nanodiscs have been used to solubilize and stabilize many different membrane proteins of diverse classes.[15,16,21] The homogeneity and limited aggregation of MSP Nanodiscs has enabled their use in structural analyses using X-ray crystallography, cryo-electron microscopy, and nuclear magnetic resonance. The ability to tune their lipid composition

has allowed MSP Nanodiscs to be used to study membrane associated proteins, particularly to determine the impact of specific lipids on membrane binding. Additionally, MSP Nanodiscs have been used to probe membrane protein activity, ligand binding, and protein interactions.

Despite MSP Nanodiscs' broad utility in membrane protein research and many strengths over other mimetic platforms, they lack throughput due to lengthy preparation time and require large material inputs which hinders analysis of low abundance membrane proteins. Additionally, lengthy preparation times can be detrimental to membrane protein stability, especially if the lipid transition temperature is suboptimal for proteins over long time periods. To address these challenges, Wade et al. developed a microfluidic device to synthesize Nanodiscs in minutes and with less material input.[39] The microfluidic device is composed of a bead bed packed with detergent removal resin that enables Nanodisc self-assembly when a Nanodisc component mixture is flown across the device. Initial work with this device demonstrated the incorporation of a functional cytochrome P450, CYP3A4, into Nanodiscs. Unpublished work has also demonstrated the ability to incorporate other functional membrane proteins, including Epidermal Growth Factor Receptor (EGFR) and Viperin, into Nanodiscs directly from whole cell lysate using the microfluidic device.[40]

Recent efforts have focused on expanding the Nanodisc diameter even more, between 50 and 100 nm, to enable studies of large proteins, protein complexes, and events at the membrane surface. One way this has been accomplished is by creating covalently circularized Nanodiscs in which the termini of the scaffold protein are linked using a sortase or split-intein method.[41,42] Another method created to stabilize these larger diameter Nanodiscs uses DNA origami barrel scaffolds instead of covalently linked MSP.[43–45] All of these methods enable the formation of larger Nanodiscs with improved stability and proteolytic resistance. However, these alternatives still

require detergents for formation, result in variable circularization yield for covalently-linked MSP, and can cause instability from weak interactions between the DNA-corral and lipids, especially for lipids that do not form planar bilayer structures (although this lack of interaction can be a positive as it does not perturb the lipid environment as much as conventional MSP Nanodiscs). Other types of Nanodiscs using scaffolds other than MSP, such as peptides and polymers, have recently gained popularity due to distinct advantages and will be discussed further.

1.2.2 Peptidiscs

Peptides are an alternative to scaffold proteins that have gained popularity for membrane protein solubilization and therapeutics due to their ease of assembly and particle size modulation. Further, peptidiscs result in less detergent exposure compared to MSP Nanodiscs. Peptergents, lipopeptides, nanostructured beta-sheet peptides, and derivatives of truncated Apo-A1 sequences, termed beltides, are all reported examples of peptides used for these applications.[46–49] These particles are typically made by adding the solubilized peptide with reconstituted membrane proteins, without the addition of extra lipids.[17] The 18A (18 amino acids long) peptide, also known as 2F (2 phenylalanines), and its derivatives are the most common peptide scaffolds for peptidisc formation. 18A has been shown to have ideal lipid binding characteristics and to form DMPC peptidiscs encapsulating bacteriorhodopsin.[49,50]

The 18A peptide has since been modified to create new peptide scaffolds, initially to alter the length of the peptide. A truncated version of 18A, called 14A, was developed for NMR structure analysis of disc formation.[51] 18A peptide derivatives varying in hydrophobic phenylalanine residue content were screened to find one peptide with four phenylalanines, 4F, that showed the most stable lipid interactions, second highest solubility in water, and most effective in

clarifying lipid vesicles and mimicking apoA-I activity *in vivo*.[52] These lipid interactions are from orthogonal peptide orientation compared to lipid tail groups observed with NMR.[53]

A combination of 18A and 4F with a proline linker, NSP, was developed as a scaffold peptide.[54] The NSP showed better thermal stability and comparable solubilization of natural vesicles to 4F. To improve the solubility of NSP in water, the sequence was reversed to make NSP_r, along with the substitution of two leucine residues to phenyalanines to increase lipid affinity.[17] The reversal of the sequence generated a peptide that acted more like an amphipol than a HDL since no supplemental lipids are added when incorporating membrane proteins with their annular lipids. Since its development, NSP has been used to stabilize various membrane proteins and protein assemblies for various applications including mass spectrometry-based proteomics and interactomics.[55,56]

A hybrid chain of 18A, known as 37pA, was tested to look at peptide chain length[57] and showed the same lipid affinity and incorporated protein activity as 18A.[58] Further comparisons of 18A and 37pA were done by modifying the proline linker by deletion, double glycine replacement,[49] or alanine replacement.[59,60] The linker changes displayed that the more flexible sequence created more adaptable sizes of HDLs,[49] but a proline linker displayed better *in vivo* HDL association.[60] The downfall to the sequence of 18A and 37pA was the preferred peptide-peptide interactions over peptide-lipid which can generate aggregates instead of discs.[61] Mutagenesis of the 37pA sequence to increase the number of alanine residues increased the peptide solubility in water.[62] This new peptide with five alanines, 5A, was used to create peptidiscs with varying lipid composition to target cholesterol incorporation, which could be leveraged for membrane protein incorporation.[63]

Another peptide gaining traction is 22A due to its ability to withstand several freeze thaw cycles. [64,65] This heightened stability makes them a promising candidate for therapeutics, but also would be beneficial for membrane protein incorporation as the peptidiscs could be stored prior to downstream analyses. Recent studies have shown the incorporation of modified lipids to 22A peptidiscs, which would enable studies examining lipid-protein or lipid-lipid interactions.

Some peptides have been developed separately from the original 18A scaffold, mainly to modulate lipid remodeling and cholesterol efflux via changes in orientation of the peptides in the disc structure. One example are ELKs which are comprised mostly of glutamate, leucine, and lysine with varying amounts of alanine.[66] Another peptide was designed to generate multivalent branched helices at 21 and 16 amino acids long with N-terminal cysteines.[67] On top of promoting cholesterol efflux and lipid remodeling, the peptide was shown to generate peptidiscs using less peptide than 4F, which could be beneficial for downstream applications utilizing functionally-tagged peptides, but could also limit the size range of the peptidiscs formed.

Peptidiscs are advantageous in that they are easy to assemble, do not always require the use of detergent, and their size can be easily modified by changing the peptide to lipid ratio during formation. However, peptidiscs have variable stability and solubility based on pH, salt content, and temperature which can be challenging for membrane protein applications but leveraged for other applications like therapeutic release. Inability to control the number of peptides used to form the peptidisc can hinder downstream applications that utilize tagged peptides. Recent advancements focus on bolstering peptidiscs as therapeutic vehicles, but many of these peptide modifications could facilitate *in vitro* membrane protein solubilization and downstream characterization. Overall, peptidiscs are the least characterized in terms of both their lipid composition and other physical properties.

1.2.3 Polymer Nanodiscs

Despite their monumental contribution to membrane protein research as the first approach to encapsulate a membrane protein in its native conformation in a lipid bilayer particle, MSP-Nanodiscs still require solubilizing components, including the membrane protein, in detergent during the self-assembly process. Peptidiscs also at times require detergent addition for synthesis. However, detergents disrupt the local lipid environment around membrane proteins and can cause membrane proteins to adopt non-native conformations and lose activity. Therefore, methods that eliminate the use of detergents are attractive to membrane protein researchers. A recently developed approach by Overduin and coworkers utilizes an amphipathic polymer (similar to the amphipathic MSP), styrene maleic acid (SMA) copolymer, to directly solubilize membrane proteins from membranes without the use of detergents.[68,69] These styrene maleic acid lipid particles, or SMALPs, are stabilized by the intercalation of the hydrophobic styrene groups with the lipid acyl chains while the hydrophilic maleic acid groups face the solution. SMALPs have been shown to retain many of the physical properties of native lipid bilayers such as lipid composition, structural organization, and phase behavior. The size of the particles can be adjusted by modulating the ratio of polymer to lipid up to a maximum of 15 nm, so larger proteins or protein complexes greater than ~400 kDa are likely to be excluded from these preparations. Similar to MSP in that it can be functionalized with tags for downstream purification, the original SMA copolymer can be modified was the addition of cysteamine for conjugation of functional groups.[70]

SMALP formation is predicted to occur first via initial styrene intercalation into the membrane driven by hydrophobic interactions and electrostatics. This then enables the styrene to insert further to destabilize the membrane, where the insertion efficiency is driven by acyl chain packing of the lipid species. This process results in membrane destabilization and the formation of

vesicular intermediates that bud off the initial membrane that then form into the final SMALP. The exact method of the membrane destabilization depends on the copolymer ratio. This process was shown to be dependent on the physical properties of the lipid membrane as well as number of consecutive styrene units for efficient interaction with the membrane. [71,72] A recent study has been performed on *E. coli* membranes to observe the effects of SMA concentration, temperature, incubation time, and salt dependence on this mechanism.[73] Characterization of SMALPs using ^{1}H ssNMR showed close proximity of styrene to the lipid acyl chains as the mechanism for stabilization.[74] The polymer scaffold exerts lateral pressure on the lipid tails, but not near the terminal methyl groups.

The initial SMA copolymer is not water soluble below pH 6.5, so SMALP formation is restricted to above this pH and ideally above pH 7, which prevents applications requiring acidic pH. [75] Preliminary work showed this could be tuned by adjusting the ratio of styrene to maleic acid (less styrene content) and molecular weight to increase the stability of the polymer at lower pH. [76–79] Another limitation is that SMA polymer is a chelator of divalent ions (Mg^{2+}, Ca^{2+}) and the chelate is insoluble, meaning that experiments requiring high concentrations of divalent cations (above 5 mM) likely disrupt SMALP formation.

Due to the ability of SMALPs to solubilize membrane proteins while retaining their native lipid environments, optimization of the copolymer structure has been an attractive direction to overcome the pH and divalent cation instability. [75,76,80] Initial modifications were made to the maleic acid moiety due to its intrinsic metal chelating behavior and its charge state modulation with pH, both of which contribute to aggregation. Modification of the maleimide with an ethyleneamine (SMAd-A) made the copolymer stable below pH 6 and tolerant to divalent cations.[81] Comparatively, the open-ring structure with the addition of ethylenediamine (SMA-ED)

directly to the maleic acid presented stability below pH 5 and above pH 7; however, divalent cations destabilized the accessible carboxylic acid at basic pH.[81] A variant of SMA-ED with an alcohol in place of the amine (SMA-EA) has been shown to reconstitute a net negatively charged membrane protein.[82] Another modification close to the open-ring structure of SMA-ED utilized a methylamide addition (SMA-MA) which showed a slightly increased tolerance of divalent cations at near physiological concentrations.[83]

The SMAd-A copolymer has been further modified to a quaternary ammonium (SMA-QA) shown to be tolerant to pH 2-10 and of divalent cations at 100x the concentration of SMA.[84] The basic pI of SMA-QA was leveraged to reconstitute a net positively charged membrane protein due to the repulsion from the polymer belt preventing aggregation between the polymer and protein.[82]

A styrene maleimide copolymer with a tertiary amine (SMI) showed divalent cation stability and preference for pH below 7.8, making SMI a good candidate for native membranes with its pH tolerance being near physiological levels.[85] A limitation of SMI is the small size of the Nanodiscs formed, 5-10 nm, resulting in it acting more like an amphipol than a Nanodisc. New work into zwitterionic SMA copolymers (zSMA) have modified the maleic acid to resemble a phosphatidylcholine headgroup that increased pH stability range and enabled Nanodiscs to be made with different sizes.[86]

A similar copolymer design utilizing maleic acid is the use of diisobutylene-maleic acid (DIBMA) copolymers to form Nanodiscs with less intercalation into the lipid bilayer and with increased cation tolerance.[87,88] In fact, it was shown that physiological concentrations of divalent cations improve the efficiency of membrane protein extraction with DIBMA.[89] More recently, studies have been performed comparing protein extraction efficiencies and lipid extraction

preferences of different Nanodisc-forming copolymers.[90,91] These preferences should enable the development of new polymers and fine-tuning of which polymers to use for specific applications.

With increased research efforts in the polymer Nanodisc space, new polymers that are not functionalized versions of SMA have been developed. Polymers without styrene, poly(methyl methacrylate) copolymers (PMMAs), have been developed for Nanodisc formation to eliminate background in UV and fluorescence spectra and the direct lipid interactions with styrene.[92] New copolymers composed of poly(acrylic acid-co-styrene) were developed for Nanodisc formation to mimic the functionalities of SMA but to allow for more control over monomer distribution and better control over the molecular weight and dispersity of the polymer through reversible addition-fragmentation chain-transfer (RAFT) polymerization; however, the polymer is sensitive to divalent cation-induced aggregation.[77,79] Non-ionic inulin-based polymers were developed to be able to analyze differently charged membrane protein complexes, as demonstrated with the oppositely charged CYP450-CPR complex.[93]

Polymer Nanodiscs have the distinct advantage of being able to natively extract lipids and proteins from their environment without the use of detergent. Depending on the polymer in use, pH and salt conditions can destabilize the bilayer, but there have been many recent advancements into new polymer modalities that mitigate these challenges while still providing efficient membrane protein and lipid extraction. The polymer synthesis process can result in polydisperse mixture of polymers and by default, a disperse mixture of Nanodiscs, but advancements in more controlled polymerization strategies are promising steps to reduce the dispersity. Uncertainty around how many polymers form the disc structure however could cause changes in the lipid environment per particle. Overall, this is a promising and rapidly growing field for membrane protein solubilization. New approaches that create libraries of copolymers with well-defined

composition and lengths will continue to provide new insights into parameters that result in efficient protein extraction while remaining tolerant to cations and will allow precise tuning of polymer conditions for specific membrane protein applications.[94]

1.3 Nanodisc characterization approaches to determine lipid composition

Membrane protein localization and function is affected by the surrounding lipid environment.[11] Despite this, the characterization of the lipid composition within Nanodisc has been limited, where most characterizations primarily access membrane protein structure and function. Recently, Nanodisc synthesis conditions were shown to alter the incorporation of lipids into these mimetics; thus, it is essential for us to characterize the lipid membrane composition following synthesis (see Chapter 3).[91] This will enable us to design Nanodisc synthesis conditions to produce the desired lipid composition and/or membrane protein incorporation for downstream analyses. Further, lipid membrane characterization will provide an additional control and metric to ensure sound membrane protein measurements. Here, we describe the current capabilities to characterize the lipid membrane composition of Nanodiscs mimetics.

1.3.1 Scintillation Counting

Early characterization of Nanodisc lipid composition relied on scintillation counting in which a known amount of tritiated lipid is doped into the Nanodisc component mixture for subsequent incorporation. Based on the radioactive response, MSP concentration (usually determined by A_{280} measurement), and total phospholipid content (via inorganic phosphate analysis), the number of lipids per Nanodisc can be determined.[31,32] In addition to characterizing

100% POPC, DMPC, and DPPC Nanodiscs, this method was used to determine that POPS and POPC were incorporated stoichiometrically by doping in traces of ^{3}H-POPS during the self-assembly process.[95] However, scintillation counting cannot be highly multiplexed to characterize complex mixtures analogous to native membranes.

1.3.2 Thin Layer Chromatography

Thin layer chromatography (TLC) is one of the most popular methods for determining lipid composition in Nanodiscs, particularly for polymer Nanodiscs. Typically for TLC analysis of lipids from Nanodiscs, lipids are first extracted from the Nanodisc samples using established solvent systems for lipid extraction from biological samples (e.g., Bligh and Dyer, Folch, and others). Separation of the lipids is achieved on a stationary phase, typically silica gel, due to polarity differences of lipid species. When analyzed alongside a mixture of lipid standards, various lipid species in a sample can be identified and lipids can be relatively quantified across samples.[96]

The first example of TLC lipid analysis with MSP Nanodiscs was used to compare the phospholipid content of three major phospholipid types (PC, PE, PI) of starting membrane preparations to the content in the final Nanodiscs.[97] More recently, TLC has been applied to lipid analysis of polymer Nanodiscs most commonly to determine the effectiveness of SMA in solubilizing lipids representative of various starting membrane compositions (**Figure 1-3**).[98–101]

However, the analysis is typically limited to lipid class information and lipid identification is impossible without comparison to a synthetic standard. This hinders the application of TLC for discovery-based lipid analyses or very complex Nanodisc lipid compositions. There have been some examples of TLC being coupled with mass spectrometry, which facilitates the identification

of what lipids are present. However, this method provides less resolution than HPLC based lipid separation coupled to mass spectrometry.

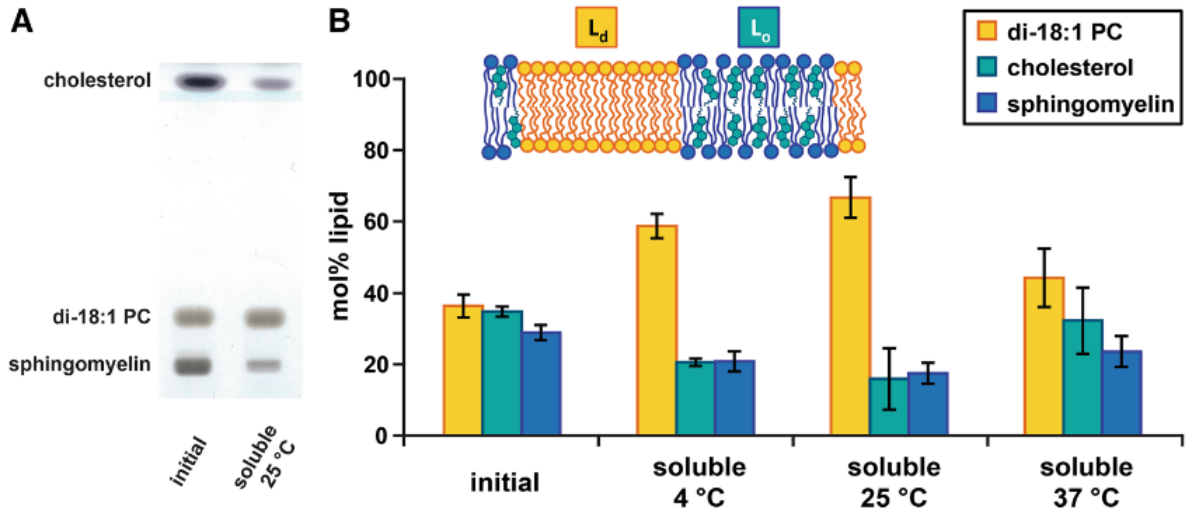

Figure 1-3 TLC for investigation of lipid incorporation into SMALPs. Figure reproduced from Dominguez-Pardo et al. Eur Biophys J. 2017.

1.3.3 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a powerful biophysical tool capable of generating atomic resolution details of a molecule. NMR can differentiate chemically identical atoms, experiencing small variations in their magnetic environment influenced by its neighboring atoms. This allows for the assignment of each peak in the spectrum to a specific atom within a molecule of interest.

To date, NMR is typically applied to lipid order and protein dynamics in Nanodiscs. Recently, one method was developed to identify and quantify the lipid composition of SMALPs using ^{1}H-^{31}P NMR.[102] This solution state NMR method allowed for the identification and quantification of phospholipid headgroup composition around membrane-associated systems in their natural lipid environment incorporated in a SMALP by comparing to spectra of a library of common phospholipids. However, this method is insensitive to different acyl chains or to lipids

not containing phosphorus groups which limits its utility in Nanodiscs made with complex lipid mixtures, especially if looking for species-specific identification and/or non-phospholipids. It could be useful in confirming the presence of different phospholipid classes and for confirmation of lipid ratios in Nanodiscs made with simple lipid mixtures.

1.3.4 Native Mass Spectrometry

Over the last decade, Nanodiscs have been increasingly utilized by the native MS community as a vehicle for membrane protein structural analysis in the gas phase.[103] Along with these advancements, Nanodisc stoichiometry and lipid composition have been subsequently probed. Native mass spectrometry (native MS) uses nondenaturing ionization conditions, typically with nano-electrospray ionization, to preserve noncovalent complexes in the mass spectrometer. Typical denaturing MS experiments utilize organic solvents and low pH whereas native MS experiments use volatile aqueous buffers near physiological pH as well as lower temperatures and voltages to preserve noncovalent interactions.

The first example utilizing native MS for Nanodisc characterization was able to discern the lipid distribution (dispersity) and stability of POPC and DMPC Nanodiscs.[104] Analysis of the native mass spectra concluded that each peak represented a Nanodisc with a different number of lipids, with adjacent peaks differing by one lipid. With this observation, it was shown that POPC and DMPC Nanodiscs varied around ±5 lipids per Nanodisc which was remarkably homogenous. Additionally, the Nanodiscs were found to be stable up to 70V of activation energy before lipids began to be stripped from the Nanodisc, likely due to constraints from the MSP belt. This initial work was expanded further to binary lipid mixtures of POPC with either POPG or POPS to discern the composition of lipids in the Nanodiscs agreed closely with the starting mixture.[105] The lipid

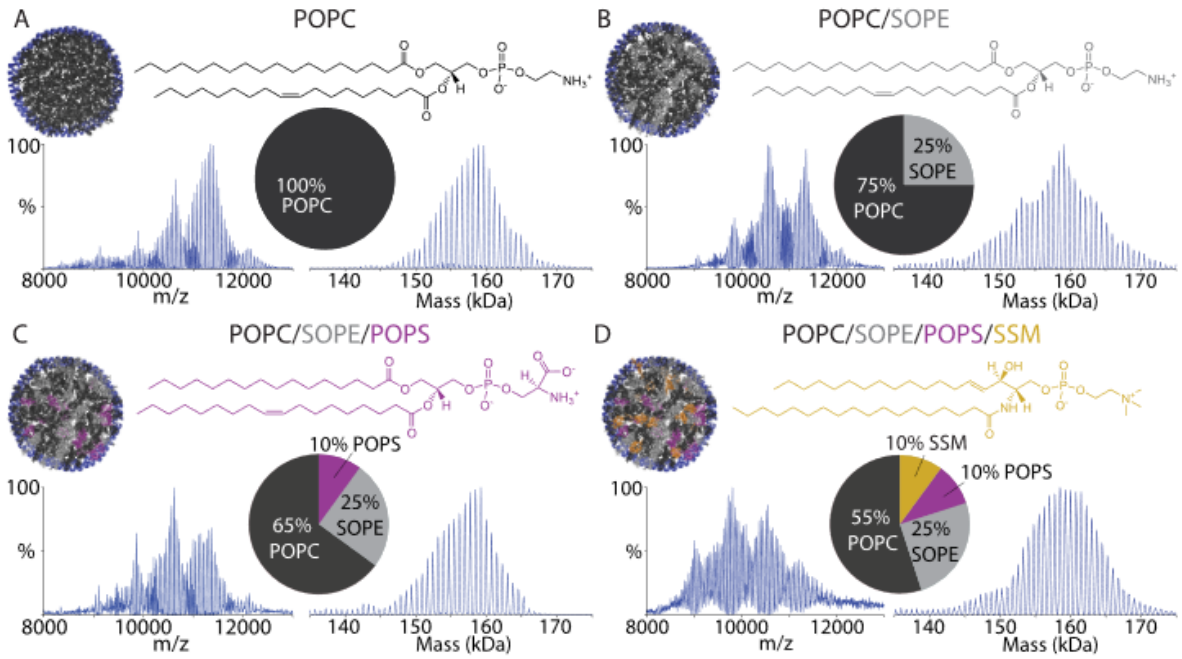

Figure 1-4 Native MS for investigation of lipid incorporation into MSP Nanodiscs made to model the plasma membrane. Figure reproduced from Kostelic et al. Anal. Chem. 2021.

composition was interpolated from the average lipid mass obtained from the spacing between the peaks. These studies have been expanded upon further to use this native MS characterization approach to design specific lipid compositions that could be incorporated into Nanodiscs including cardiolipin, glycolipids, and sterols as well as model membrane compositions reflective of mammalian, bacterial, and mitochondrial membranes (**Figure 1-4**).[106,107]

In addition to measuring lipid composition, native MS has been used to observe lipid exchange between Nanodisc populations with and without embedded membrane proteins.[108] Empty 50% POPC/50% POPG Nanodiscs made with untagged MSP were mixed with His-tagged Nanodiscs containing AmtB at varying POPG percentages and were incubated for 2.8 days to allow for lipid exchange to reach equilibrium. A native MS method was developed to eject the incorporated AmtB with bound lipids, and the average bound lipid mass was used to determine

that POPG was enriched in the AmtB Nanodiscs after they were removed and analyzed separately from the total mixture using Ni-NTA columns.

Thus far, native MS has been a valuable tool for intact analyses of Nanodiscs to provide information about lipid composition, MSP:Nanodisc stoichiometry, and lipid exchange. However, this technique can be limited to lipids of similar mass to enable deconvolution of complex spectra. Additionally, this technique requires specialized instrumentation and deconvolution analysis that is not accessible to all Nanodisc users.

1.3.5 Liquid chromatography coupled to tandem mass spectrometry

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) presents an opportunity to analyze Nanodiscs containing any lipid species analogous to targeted and untargeted lipidomics. These lipidomics methods typically separate lipids that previously underwent a liquid-liquid extraction via reverse phase and/or hydrophilic interaction liquid chromatography and subsequently analyze them via targeted or untargeted tandem mass spectrometry methods. Targeted methods typically involve multiple reaction monitoring with a triple quadrupole mass spectrometer to quantitate lipids of interest. Untargeted methods require higher resolution instrumentation and database searching to get high confidence assignments of lipid species present (**Figure 1-5**).

Recently, Bailey and coworkers developed a targeted LC-MS/MS lipidomics method to quantify lipids in MSP Nanodiscs and used this method to discover lipid incorporation disparities in Nanodiscs that correspond with lipid physical parameters (see Chapter 2). For instance, lipids with high intrinsic curvature like PE species were depleted in purified Nanodisc samples relative to the starting component mixture. Additionally, lipids that impart rigidity in the membrane were

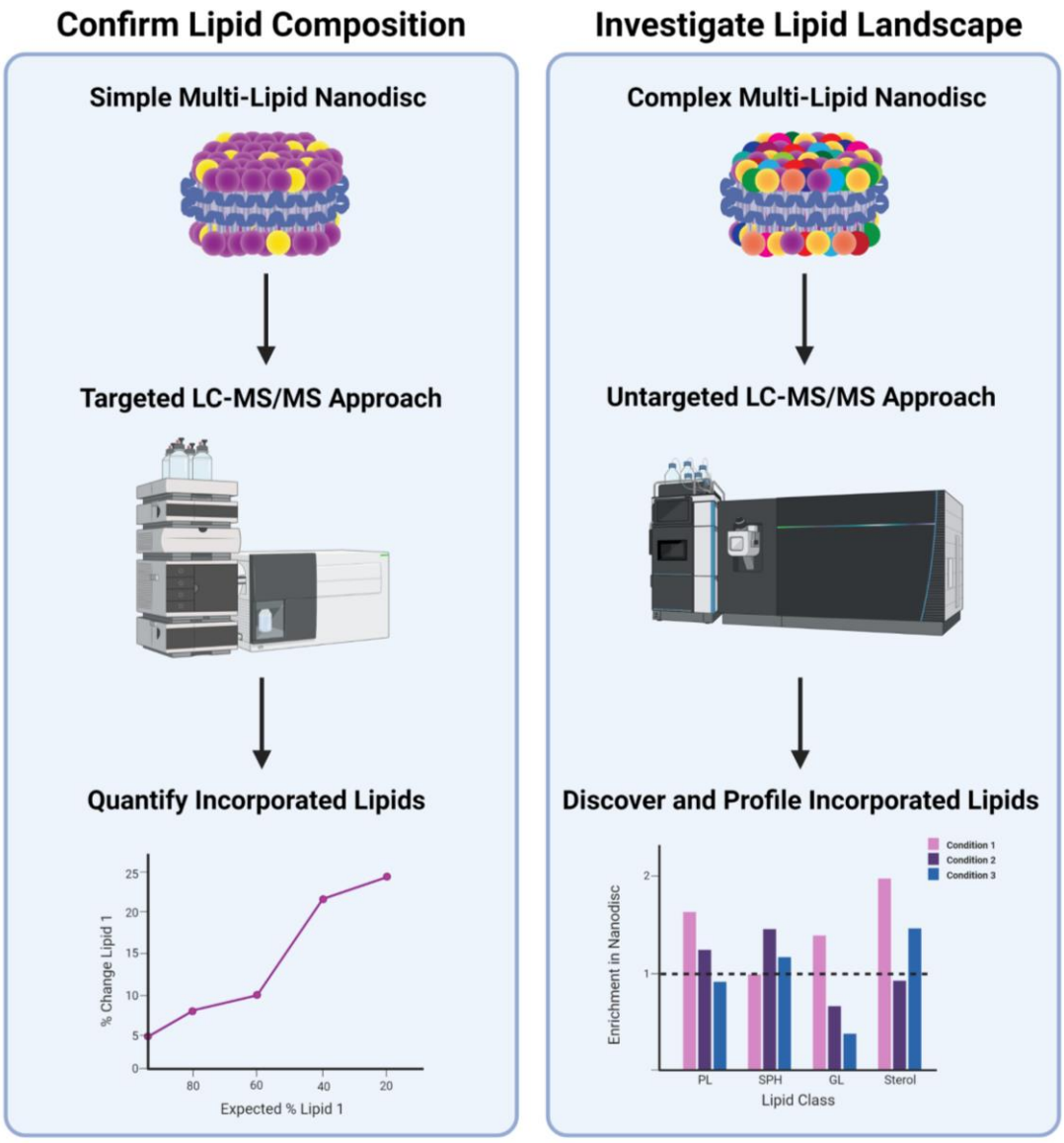

Figure 1-5 Depiction of targeted and untargeted lipidomics approaches to determine Nanodisc lipid composition. . Targeted approaches are valuable for simpler lipid mixtures to confirm compositions. Untargeted approaches are beneficial for complex lipid mixtures to profile the lipid landscape under different synthesis conditions.

similarly depleted. Other work by this same group utilized an untargeted lipidomics method to observe how changes in Nanodisc synthesis conditions like detergent selection, temperature, and MSP size affected lipid incorporation into Nanodiscs (see Chapter 3). They found that the detergent selected impacted how lipids were solubilized, and consequently, which lipids were available for incorporation into the Nanodiscs. Temperature and belt size also selectively enriched

for certain lipid species. A similar study was done for polymer Nanodiscs which observed preferences in lipid solubilization by different maleic acid-based copolymers, all of which generally favored less curved lipids and less ordered domains.[91]

Further, LC-MS or direct infusion mass spectrometry can be used to measure lipid exchange in MSP Nanodiscs.[108] Empty Nanodiscs made with untagged MSP were mixed with membrane protein-filled Nanodiscs with His-tagged MSP for 2.8 days to allow for lipid exchange equilibration. The mixture was able to be analyzed together via direct infusion MS on a triple quadrupole, and then His-tagged Nanodiscs were analyzed separately after being enriched with Ni-NTA immobilized metal affinity chromatography. Using AmtB as a model system, they showed that membrane proteins could remodel the lipid environment, as tagged Nanodiscs were observed to be enriched in POPG relative to POPC. Control studies with both the untagged and tagged Nanodiscs having no membrane protein incorporated, generally showed that the populations of Nanodiscs equilibrated to the same POPC/POPG composition.

To date, LC-MS/MS has provided significant insights into the lipid membrane composition of Nanodiscs. However, its ability to quantify the direct composition of Nanodiscs is limited to simplistic mixtures that contain synthetic standards to produce calibration curves. For more complex mixtures (e.g., native lipid membranes or membrane extracts), untargeted lipidomics provides the ability to identify what lipids are incorporated into the Nanodisc. However, its quantitative capabilities are primarily limited to relative alterations between samples preparations.

1.4 Integration with Nanodisc physical property characterization

In addition to characterizing the lipid composition of Nanodiscs, these studies would benefit greatly from the integration with physical property characterization (**Figure 1-6**). Previous

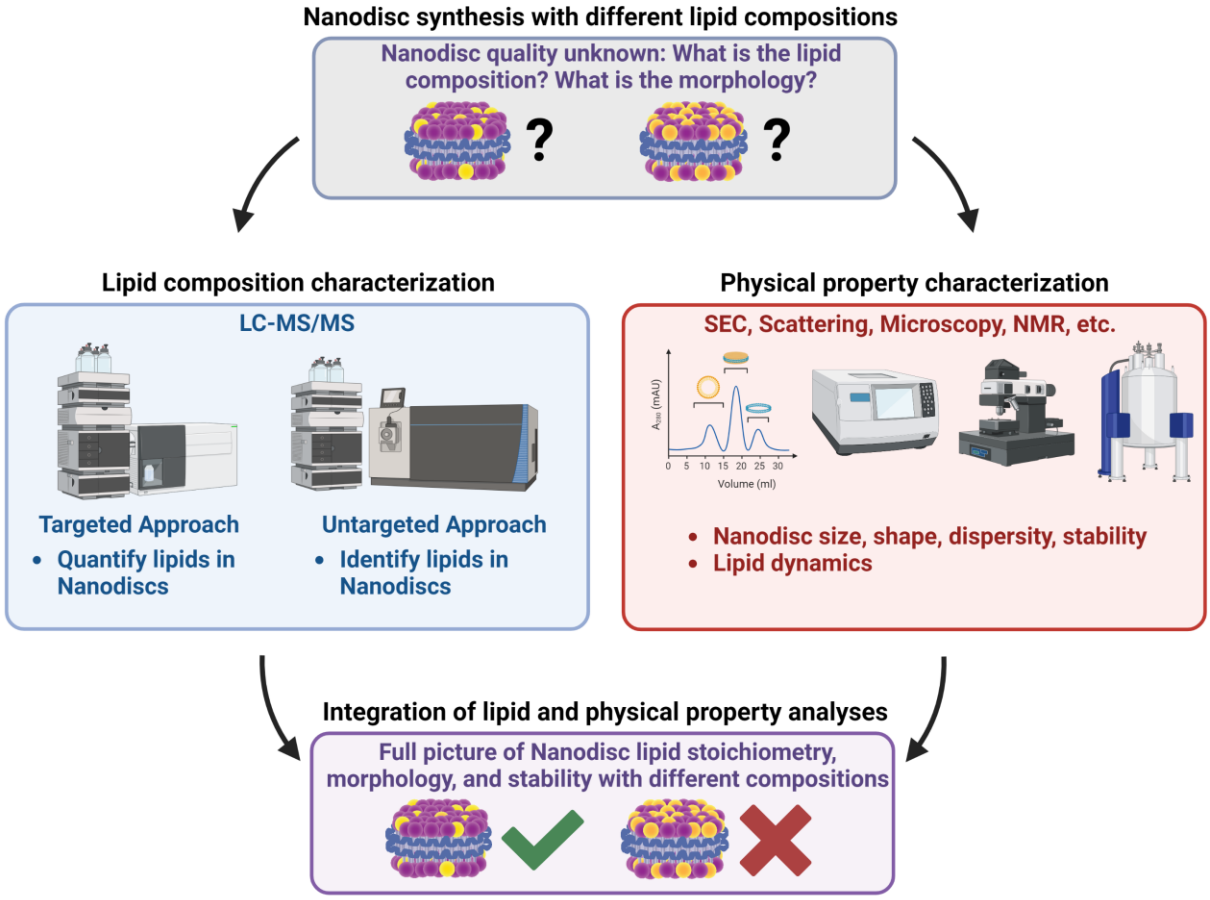

Figure 1-6 Integration of Nanodisc lipid composition analysis with physical property characterization. Merging these analyses results in a complete picture of how different lipids can impact Nanodisc composition, morphology, and stability to create more native-like and robust vehicles for membrane protein analyses.

research has demonstrated that membrane mimetic structures themselves, in addition to lipid composition, impact membrane protein structural and functional integrity. Therefore, characterization of Nanodisc lipid composition and physical properties is essential to elucidate the roles of membrane proteins in native membranes.

The most common technique utilized is size exclusions chromatography (SEC), which separates molecules by their hydrodynamic radius. SEC is already employed in Nanodisc synthesis pipelines, as it is commonly used for Nanodisc purifications.[21] Since Nanodisc diameter is predetermined by the scaffold length or the ratio of scaffold to lipid, SEC provides an initial

assessment of Nanodisc quality by correlating their retention time (hydrodynamic radius) to the theoretical size provided by the scaffold. Similarly, the retention time and peak width provide information regarding the Nanodisc diameter and dispersity, respectively. Chapter 2 presents an example of ways in which this technique can be integrated with lipid composition characterization, as it was shown that diameter and dispersity of Nanodiscs is modulated with lipid composition. Specifically, smaller and more disperse Nanodiscs often correlate with lipids that did not incorporate stoichiometrically. An interesting aspect of SEC is that it can allow for fractionation of the Nanodisc peak, so measurements can be obtained across the Nanodisc distribution and provide more information than just an average measurement. This was utilized by Arleth and coworkers to determine the range of lipid:scaffold in Nanodiscs[109], but further analysis by LC-MS/MS lipid quantification could provide more insights into lipid membrane composition variability. A limitation to SEC assessments is the inability to know the Nanodisc stoichiometry (MSP:Nanodisc), as these different particles can coelute with the Nanodisc peak. Marty and coworkers were able to deconvolute this with native MS.[107]

Scattering techniques such as dynamic light scattering (DLS), small angle X-ray scattering (SAXS), and small angle neutron scattering (SANS) have been used to characterize Nanodisc shape and bilayer thickness in response to different single-lipid systems and synthesis temperatures.[31,32] These techniques involve applying an incident beam of radiation to a sample and measuring the intensity and angle of the scattered beam. The scattering arises from the interaction of the radiation with regions of different refractive index (DLS), electron density (SAXS) or nuclear composition (SANS). SAXS has been used to determine the overall elliptical shape of Nanodiscs and how this shape is set more by the scaffold protein and temperature than lipid when two PC lipids were compared.[110] It has also been used to observe changes in bilayer thickness with

the incorporation of cholesterol, which could have implications for how much a membrane protein is buried in the membrane.[111] SANS has been used to observe conformational rigidity of MSP in Nanodiscs with more PG relative to PC (more anionic lipids).[112] In addition to scattering techniques, various microscopy techniques, such as scanning probe, transmission electron, atomic force microscopy, and electron microscopy have also been used for characterization of MSP and polymer Nanodisc size, dispersity, and morphology.[31,39,70,79,113]

In addition to morphological physical properties, analyzing the internal lipid dynamics is advantageous to compare to native-like dynamics as this could impact how membrane proteins interact with the lipid environment. Nuclear Magnetic Resonance (NMR) and Electron Paramagnetic Resonance (EPR) have been popular techniques for making these measurements. These techniques measure nuclear spins (NMR) or electron spins (EPR) as a result of an applied magnetic field. NMR and EPR analyses on MSP Nanodiscs have shown differences in lipid bilayer dynamics compared to other mimetics or native membranes.[114,115] Nanodisc lipid bilayers are more fluid over wider temperature ranges, and the lipids in the Nanodiscs behave differently at the boundary near MSP (more disordered) versus at the center of the Nanodisc (more ordered). Similar effects have been seen with polymer scaffolds as well.[116,117] These dynamic properties could be influenced by surrounding lipids, but many different compositions have not been studied.

Overall, many techniques are available to pair with lipid composition analyses to determine both which lipids incorporate stoichiometrically and the effects of these lipids on Nanodisc physical properties. Although most examples discussed here involve MSP Nanodiscs, many of these studies can be applied to Nanodiscs made with other scaffold materials. Additionally, further expansion of these methodologies with increased lipid complexity could be assisted with the

integration of computational approaches that can model lipid dynamics in Nanodiscs made with different lipid components.

1.5 Conclusions and outlook

Nanodiscs are a valuable membrane mimetic platform that enable membrane protein solubilization for *in vitro* biochemical, biophysical, and analytical studies. Since their initial development, extensive research has been conducted into scaffolding material for Nanodisc stabilization and advantages for downstream applications. MSP Nanodiscs are advantageous in that they can be engineered to different lengths and with functional tags, allowing for control over Nanodisc size and downstream isolation, respectively. However, they still require the use of detergent in the initial component mixture which can cause some membrane proteins to adopt non-native structures or become inactive. Peptidiscs are easy to assemble and have easy size modulation via changing the lipid:peptide ratio. They suffer from variable stability and solubility due to pH, salt content, and temperature, all of which can limit downstream applications and membrane protein compatibility. Polymer Nanodiscs do not require any detergent for synthesis and are thought to better model native membrane environments since they form directly from native membranes. Both present great advantages to other scaffolds, but polymer scaffolds are sensitive to pH and salt content, like peptides. Extensive research into new polymer scaffolds has recently expanded their stability in these environments markedly. Taken together, scaffold selection generally depends on the desired membrane protein system and analysis tool.

Despite the vast number of membrane protein analyses achieved with Nanodiscs, there have been few studies that characterize the Nanodiscs themselves. These studies typically utilize a combination of spectroscopic, microscopic, and computational approaches to measure Nanodisc

physical properties such as diameter, dispersity, morphology, scaffold-lipid interactions, and lipid dynamics. Very few studies have characterized the lipid composition of Nanodiscs despite many studies attributing membrane protein structural and functional measurements to the surrounding lipid environment. It has generally been assumed that lipids incorporate into Nanodiscs stoichiometrically (the final lipid composition matches the input composition), but recent results suggest this is not always the case and it is often dependent on physical properties of the lipids. There are only a few methods developed to determine lipid composition of Nanodiscs: scintillation counting, which has limited multiplexability; NMR, which is insensitive to acyl chain differences and non-phospholipids; native MS, which can only be applied to certain lipid combinations to enable deconvolution and requires specialized instrumentation; and LC-MS/MS, which loses the ability to be as quantitative in complex lipid mixtures. Even with these limitations, the few methods developed have provided great insights into both MSP Nanodiscs and polymer Nanodiscs. Particularly, LC-MS/MS has shed light on non-stoichiometric lipid incorporation into Nanodiscs and how synthesis conditions effect lipid incorporation in MSP and polymer Nanodiscs. These studies will need to be expanded to include different synthesis conditions, lipid environments, and new polymer and peptide scaffolds as they continue to be rapidly developed. Overall, integration of lipid membrane characterization into Nanodisc synthesis pipelines is essential to elucidate how the lipid environment affects membrane protein structure and function.

Analogous to how the lipid membrane alters membrane protein structure, the physical properties of Nanodiscs are similarly affected, such as size, dispersity, shape, and lipid dynamics. Here, we introduce various techniques capable of elucidating these physical properties, but these foundational studies generally lack the lipid complexity that mimics native cell membranes. Together, the integration of Nanodisc physical property and lipid membrane characterizations will

provide a robust platform to interrogate how increasing lipid complexity affects Nanodisc and membrane protein structure and function. Collectively, these foundational studies will expand our knowledge regarding how the neighboring lipid environment affects membrane protein structure, function, and localization *in vivo*.

1.6 Thesis overview

The work in this thesis acknowledges the utility of multi-lipid Nanodiscs as a native-like construct for downstream analyses of membrane proteins and recognizes that these vehicles require characterization as a complex and dynamic entity separate from the membrane protein they house. To accomplish this, the work presented here aimed to develop and apply analytical methodologies to better characterize MSP Nanodiscs, particularly their lipid composition, to better understand how the variables in Nanodisc synthesis impact lipid incorporation into Nanodiscs. With this information, users can ultimately tune variables such as lipids and detergents to obtain a desired lipid composition relevant for membrane protein activity, structure, and enrichment.

Chapter 2 describes the development of a targeted quantitative LC-MS/MS assay to quantify lipids in Nanodiscs. This method was used to discover lipid incorporation disparities dependent on lipid physical properties such as curvature and membrane rigidity. *Chapter 3* expands upon this initial work to much more complex lipid mixtures, natural lipid extracts. In this work, an untargeted LC-MS/MS approach was utilized to ascertain the effects of detergent, synthetic lipid additives, MSP size, and temperature on lipid incorporation into Nanodiscs made with natural lipid extracts. *Chapter 4* builds on the knowledge gained in chapters 3 and 4 to intentionally construct Nanodiscs that can enrich different classes of membrane proteins for

proteomics analyses. Also highlighted in this thesis are efforts to optimize high throughput microfluidic technologies to synthesize Nanodiscs with less material input, and a sampling of collaborative projects involving structural and functional assays of cytochrome P450s and G-protein coupled receptors, respectively. Overall, this body of work relays advances in analytical characterization of Nanodiscs to better understand how variables in their synthesis impact the resulting lipid composition and membrane protein incorporation as well as biological ramifications of multi-lipid Nanodiscs involved in downstream membrane protein analyses.

Chapter 2 Lipid Curvature and Fluidity Influence Lipid Incorporation Disparities in Nanodiscs

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2.1 Introduction

Membrane proteins are important therapeutic targets since they are essential to a multitude of cellular processes such as signal transduction and metabolism.[1,118] Thus, *in vitro* membrane protein characterization is essential, but their propensity to misfold or aggregate in aqueous solutions makes them challenging to analyze. This has led to the development of membrane mimetic systems to stabilize membrane proteins *in vitro* and preserve their structure and activity.[5–10] One such mimic is the Nanodisc, a discoidal lipid bilayer encircled by an amphipathic helical belt protein, membrane scaffold protein (MSP), that provide membrane proteins a stable, native-like bilayer that is structurally and compositionally defined.[15,16,21] Initially developed by Sligar and colleagues, this valuable model membrane construct self-assembles upon the removal of detergent

from a solubilized component mixture containing a defined ratio of lipids, MSP, and membrane protein.[31,33,119]

To date, many studies utilizing Nanodiscs have relied on simplistic bilayer compositions, primarily those solely containing phosphatidylcholine (PC) lipids. To better mimic the native membrane environment for membrane protein analyses, increased lipid complexity is needed.[11] This is especially true since lipids can allosterically modulate membrane protein activity.[12,13] There have been recent efforts toward this goal, but better characterization tools to quantify the lipid composition in Nanodiscs are required to better inform the rational synthesis of multi-lipid Nanodiscs. This is particularly important as certain phospholipid physical properties, such as the propensity to induce membrane fluidity and/or curvature, could hinder incorporation, resulting in Nanodisc content that does not accurately reflect the input lipid stoichiometry. Additionally, knowledge of lipids that do not yield intended compositions will help provide a better understanding of compositions that are predictable and amenable to Nanodiscs. Recently, Marty and coworkers created Nanodiscs modelling various native membranes with unique combinations of lipids that incorporate stoichiometrically and are amenable to native mass spectrometry.[105–107] We sought to create a more straight-forward characterization approach to analyze any abundant lipids found in phospholipid membranes commonly used in Nanodisc synthesis that does not require access to the expensive instrumentation and specialized training necessary to perform native mass spectrometry.

To accomplish these goals, we combined a Nanodisc formation and purification workflow with a targeted lipidomics method to quantify the lipids in Nanodiscs of varying composition. We surveyed mixtures of lipids with different headgroups and acyl chains to determine impacts of curvature and fluidity on the lipid composition in Nanodiscs and their physical properties (e.g.,

diameter and dispersity). The incorporation of just one additional type of lipid significantly changed the diameter and dispersity of the resulting Nanodiscs compared to 100% PC Nanodiscs. We identified that lipids capable of inducing higher membrane curvature were present in lower, sub-stoichiometric quantities compared to their expected levels. Additionally, PCs that impart greater membrane rigidity were also present in lower quantities than expected based upon the starting composition. We further expanded beyond binary lipid mixtures and created Nanodiscs to model the endoplasmic reticulum membrane and observed dynamics of cholesterol levels that reflect native membrane biology. These insights provide a foundation for deeper understanding of lipid incorporation into Nanodiscs using established targeted LC-MS/MS techniques. Furthermore, this type of Nanodisc characterization will likely be essential for further advances of this construct to enable the structural and/or functional characterization of membrane protein systems of increasing complexity.

2.2 Materials and Methods

2.2.1 Materials

Amberlite XAD-2, cholesterol, HPLC grade chloroform, LC-MS grade 2-propanol, and LC-MS grade ammonium formate were purchased from Sigma Aldrich. Sodium cholate, LC-MS grade acetonitrile, LC-MS grade formic acid, acetic acid, LC-MS grade methanol, potassium chloride, sodium chloride, sodium azide, Tris Base, and ethylenediaminetetraacetic acid were purchased from Fisher. 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-

glycero-3-phosphate (POPA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), N-palmitoyl-D-erythro-sphingosylphosphorylcholine (SM), 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (CL), 1,2-distearoyl-sn-glycero-3-phosphoinositol (PI) were purchased from Avanti Polar Lipids. Membrane Scaffold Protein 1E3D1 (MSP) was purchased from Sigma Aldrich (M7074) or expressed and purified as previously described.[33]

2.2.2 Nanodisc assembly.

Binary lipid Nanodiscs were synthesized with 20, 40, 60, or 80% POPS, POPA, POPE, DOPE, SM, and CL (only 20 and 40%) balanced with POPC, or 20, 40, 60, or 80% DSPC, DPPC, DMPC, POPC, and DOPC balanced with POPS. Mitochondrial membrane Nanodiscs were made with 50% POPC, 30% POPE, and 20% CL. Endoplasmic reticulum-inspired Nanodiscs were made with 58% POPC, 20% POPE, 7% POPS, 7% PI, 4% SM and 4% cholesterol.[120] In the case without cholesterol, 62% POPC was used. For the DPPC composition, it replaced POPC at 58% and all other lipids were kept the same. Nanodiscs were assembled as previously described.[31,119] Briefly, chloroform-dissolved lipid mixtures in defined ratios were dried under nitrogen and stored in a desiccator overnight. The dried lipids were then solubilized at a concentration of 50 mM total lipid with 100 mM sodium cholate, except for DSPC Nanodiscs where 200mM cholate was used to solubilize lipids to 25mM. Nanodiscs were assembled by adding MSP to the solubilized lipids diluted to 225 µL in standard disc buffer (20 mM Tris pH 7.4, 0.1 M NaCl, 0.5 mM EDTA, 0.01% NaN_3) and supplemented with sodium cholate to a final concentration of 20 mM. The final lipid concentration in the mixture was 5 mM and the lipid:MSP was 160:1 for POPC discs, 185:1 for DMPC discs, 210:1 for DPPC discs, and 235:1 for DSPC discs to ensure Nanodiscs were fully

lipidated.[121] The component mixture was incubated on an end over end mixer at room temperature for POPC (POPC Nanodiscs with SM were made at 37℃) and DMPC discs for 30 minutes. For DPPC and DSPC discs, an incubator was used at 41℃ and 55℃, respectively. The mitochondrial membrane Nanodiscs were made at room temperature and the endoplasmic reticulum Nanodiscs were made at 41℃. To the solubilized lipid/MSP mixture, 112 mg of Amberlite beads were added before incubating at their respective temperatures for 6 hours before the solution was removed from the beads using a gel-loading pipette tip. Nanodiscs were then immediately purified with Ni-NTA spin columns (NEB). They were then further purified by size exclusion chromatography (SEC) using a Superdex Increase 200 3.2/300 column (Cytiva) on a Waters 2695 liquid chromatograph by injecting 50 µL of Nanodiscs onto the column and pooling the fractions that contained the Nanodisc peak **(Figure 2-1)**. The mobile phase was standard disc buffer.

2.2.3 Characterization of Nanodisc size and dispersity using size exclusion chromatography.

A 50 µL volume of purified Nanodisc sample was reinjected on the same column for size and dispersity analysis **(Figure A-III-1-3)**. The Stokes diameters of the Nanodiscs were determined using a calibration created from a protein standard solution (BioRad). For each Nanodisc composition, the diameters were compared to that of a Nanodisc containing 100% of the PC lipid. Percent change in dispersity was calculated by determining the diameter of the Nanodiscs at FWHM and normalizing to Nanodiscs containing 100% of the PC lipid.

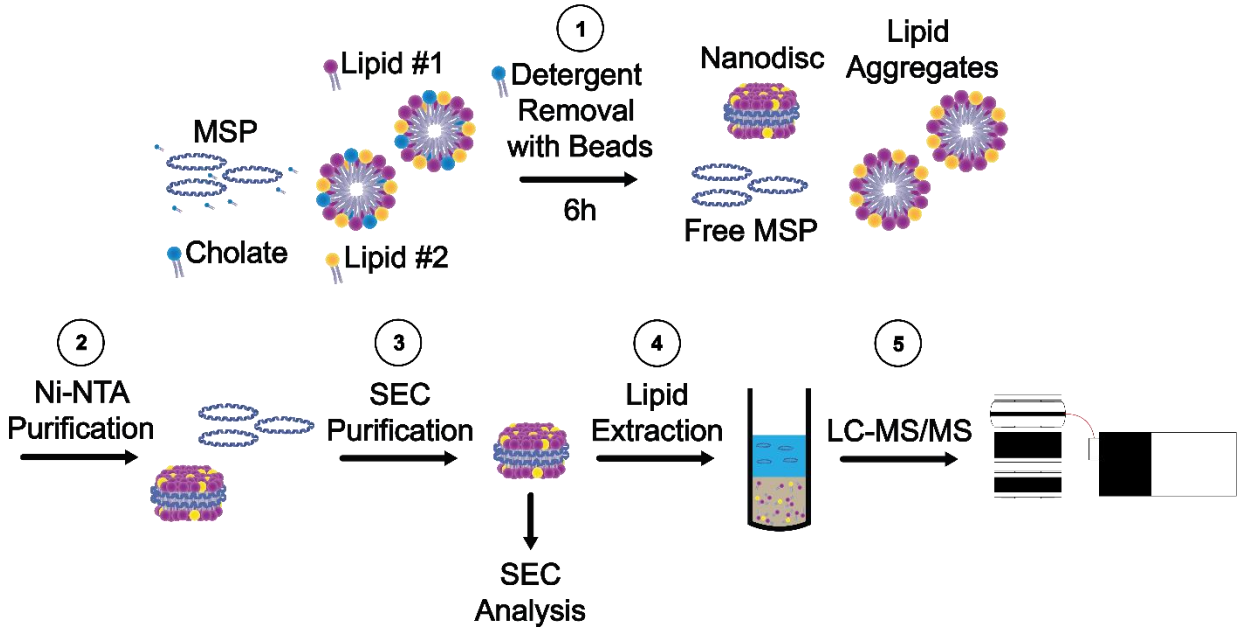

Figure 2-1 Nanodisc formation, purification, and analysis workflow. (1) Nanodiscs self assemble upon detergent removal. (2) Nanodiscs are purified with Ni-NTA. (3) Nanodiscs are purified with SEC. From here, Nanodisc samples are reinjected and analyzed via SEC. (4) For quantitative analysis, lipids are extracted from Nanodiscs using a modified Bligh and Dyer protocol. (5) Lipids are separated with reverse phase liquid chromatography and quantified using multiple reaction monitoring on a triple quadrupole mass spectrometer.

2.2.4 Sample preparation and LC-MS/MS analysis

Lipids were extracted from the dual-purified Nanodiscs using a modification of the Bligh and Dyer extraction protocol (**Figure 2-1**).[122,123] To 25 µL of Nanodisc sample in an Eppendorf tube, 100 µL of 0.15 M KCl in water, 200 µL methanol, 100 µL chloroform, and 0.5 µL acetic acid (all cold) were added and vortexed. An additional 100 µL of water and 100 µL chloroform (both cold) were then added and vortexed. The tubes were shaken at 4°C for 10 minutes and then centrifuged at 12,100 x g for 5 minutes at 4°C. A 150 µL volume of the organic layer was collected and transferred to an HPLC vial, dried under nitrogen gas, and reconstituted in 150 µL of mobile phase B. Samples were diluted within range in mobile phase B.

The resulting lipids were separated using a Phenomenex Onyx Monolithic C18 column with a guard column at 400 µL/min on an Agilent 1290 Infinity II liquid chromatograph interfaced to an Agilent 6410 triple quadrupole mass spectrometer. Mobile phase A was 60/40 (v/v) water/acetonitrile with 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B was 85/10/5 (v/v/v) 2-propanol/acetonitrile/water with 10 mM ammonium formate and 0.1% (v/v) formic acid. The gradient is displayed in **Table A-III-1**. The autosampler was held at 15°C, and 5 µL was injected for each sample. The eluting lipids were quantified using multiple reaction monitoring and ionized using electrospray ionization in positive mode at 4 kV or negative mode at -3.5 kV **(Table A-III-2)**. The gas temperature was 350°C, the gas flow rate was 11 L/min, and the nebulizer gas pressure was 35 psi.

To quantify the lipids, calibration curves were created for each lipid species. DMPC (10 nM positive mode, 500 nM negative mode) was used as the internal standard for all analyses, unless DMPC was used for the Nanodisc sample, in which case POPC (10 nM positive mode, 500 nM negative mode) was used as the internal standard. Automated peak integration was performed using the Agilent MassHunter Workstation Quantitative Analysis Software. All peaks were visually inspected to ensure proper integration. The calibration curves were plotted as the $\log_{10}$[Response Ratio] versus the $\log_{10}$[Concentration (pM)] and the lipids were quantified using the resulting linear regression. Percent change was calculated using the following equation, where impure Nanodisc refers to lipids extracted from the crude Nanodisc mixture before dual-purification (after Step 1 in **Figure 2-1)** and pure Nanodisc refers to lipids extracted from the Nanodiscs after dual-purification (after Step 3 in **Figure 2-1)**:

$$\%\ change\ \%\ PC = \frac{\%\ PC\ in\ impure\ Nanodisc - \%\ PC\ in\ pure\ Nanodisc}{\%\ PC\ in\ impure\ Nanodisc}\ x\ 100\%$$

2.3 Results and Discussion

2.3.1 Binary lipid Nanodiscs are larger and more disperse.

Previous reports have demonstrated that changes in Nanodisc synthesis components can alter their physical properties.[31,110,119,121,124] To ascertain whether the addition of a second lipid to PC Nanodiscs changes the physical properties of Nanodiscs, we made Nanodiscs containing POPC with increasing amounts of POPS, POPA, POPE, DOPE, SM, or CL to survey the effects of different headgroups. Purified Nanodiscs were analyzed via SEC, which was calibrated with protein standards to determine Nanodisc diameter. Change in dispersity was measured by determining the diameter range of the Nanodiscs at FWHM and comparing that to Nanodiscs made with 100% PC. In almost all cases (POPE, POPA, POPE, DOPE, and CL), the addition of a second lipid in any amount significantly increased the dispersity of Nanodiscs relative to the 100% POPC Nanodiscs, with higher percentages for most lipids ranging between 1 and 2.5 nm more disperse. **(Figure 2-2A)**. This is likely due to differences in lipid shape leading to altered lipid incorporation and packing, allowing for a wider variety of particles with different numbers of lipids. This is supported by previous work showing that large quantities of anionic and PE lipids can alter the Nanodisc populations produced.[105,107] Contrarily, SM containing Nanodiscs were less disperse than the 100% POPC **(Figure 2-2A)**, perhaps due to SM's propensity to strongly hydrogen bond and associate with raft domains.[125,126]

Additionally, adding in a second lipid to create a binary Nanodisc changes the resulting diameter of the particles **(Figure 2-2B)**, either increasing or decreasing it depending on the identity and amount of the lipid added. In many cases, the 20% and 40% compositions had little change in diameter, but for some lipids, 60% and 80% significantly increased (like POPS, CL) or decreased (POPE, DOPE, POPA) the size of the Nanodiscs relative to Nanodiscs made with 100% POPC. In

most cases (e.g., PA and PE), the Nanodiscs trended smaller as the dispersity increased. This is likely due to these lipids not incorporating fully into Nanodiscs resulting in a wider range of lipidation, with many Nanodiscs underlipidated when highly curved lipids are incorporated.

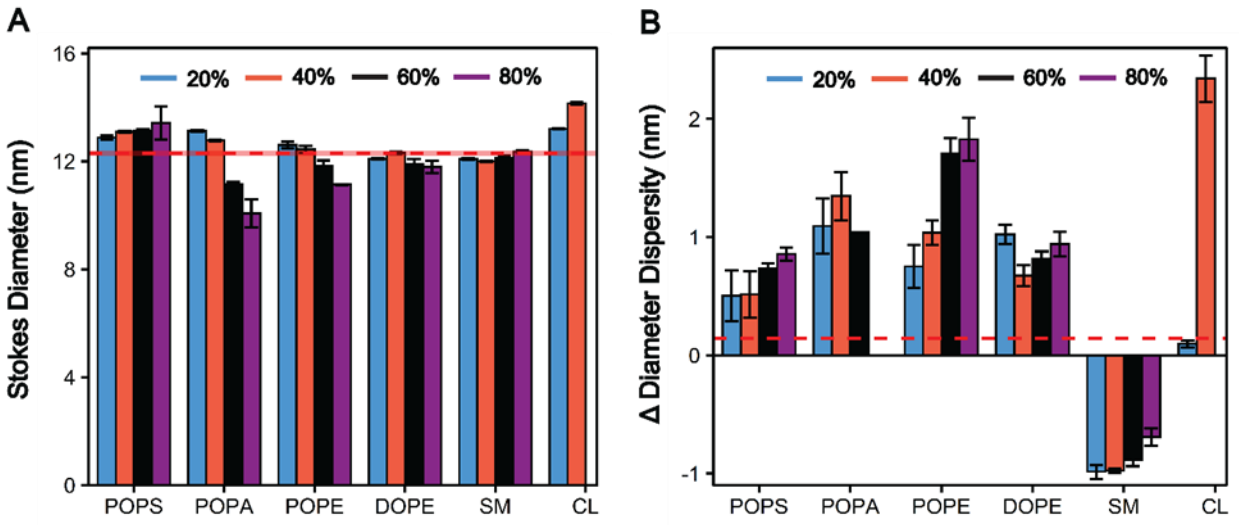

Figure 2-2 Diameter and dispersity of binary lipid Nanodiscs with phospholipids with varying headgroups and a sphingolipid balanced with POPC. (A) Stokes diameter of Nanodiscs made with different lipid compositions. The red dash indicates the average diameter of 100% POPC Nanodiscs and the shaded region is the standard deviation of three replicate Nanodisc preparations. (B) Change in diameter dispersity of Nanodiscs made with different lipid compositions. Error bars are shown as the standard deviation of three replicate Nanodisc assemblies.

In addition to determining the effect of lipid headgroups, phospholipid fluidity could further alter the synthesis of binary Nanodiscs. To identify this effect, Nanodiscs were made containing POPS with varying amounts of DMPC, DPPC, DSPC, POPC, and DOPC to determine the effects of different acyl chains. In nearly all cases, adding POPS to Nanodiscs made with PCs with varying acyl chains led to similarly increased Nanodisc dispersity **(Figure 2-3A)**. Only with DSPC were the Nanodiscs less disperse relative to 100% DSPC, likely due to the challenges in making 100% DSPC Nanodiscs due to its high transition temperature. All Nanodiscs made with varying acyl chains showed increased diameters relative to the 100% PC. Additionally, it is evident that the different PCs used to make the Nanodiscs result in different diameter particles, with PCs that impart more membrane rigidity having smaller diameters **(Figure 2-3B)**, likely due to tighter

packing capabilities of these lipids. Taken altogether, small changes in the lipid composition can lead to altered physical properties of Nanodiscs, which in turn could affect both membrane protein incorporation and the structure and/or function of membrane proteins after incorporation.

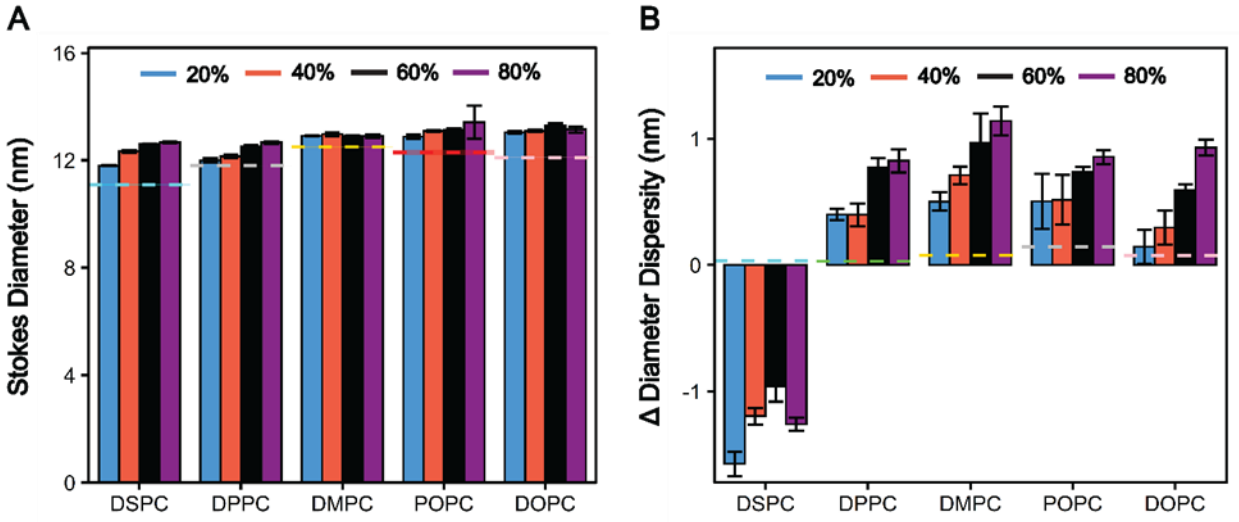

Figure 2-3 Diameter and dispersity of binary lipid Nanodiscs with POPS balanced with PCs that impart varying membrane fluidity. (A) Stokes diameter of Nanodiscs made with different lipid compositions. The dashed line indicates the average diameter of Nanodiscs made with 100% of the respective PC lipid and the shaded region represents the standard deviation of three replicate Nanodisc assemblies. (B) Change in diameter dispersity of Nanodiscs made with different lipid compositions. Error bars are shown as the standard deviation of three replicate Nanodisc assemblies.

2.3.2 Nanodiscs containing curved lipids show disparities in lipid incorporation.

While multi-lipid Nanodiscs have been produced for membrane protein analyses by combining different lipids in the pre-assembly component mixture, the actual lipid composition of the resulting Nanodiscs is typically not characterized. Since lipid headgroup and fluidity can affect Nanodisc physical characteristics, we sought to identify whether the resulting Nanodiscs contained the expected lipid composition reflective of the initial component mixture. To investigate the addition of other lipids into PC-based Nanodiscs, we surveyed lipids with varying headgroups: PS, PA, PE, CL, and a sphingolipid, SM. We first established a Nanodisc synthesis and dual purification workflow to ensure there were no excess lipids present to convolute our analyses

(Figure 2-1, Figure A-III-1-3). While many Nanodisc analyses only utilize SEC purification, we incorporated a Ni-NTA purification to enrich His_6-tagged Nanodiscs and remove excess lipids. We then developed a targeted LC-MS/MS method to quantify lipids in purified Nanodiscs to determine any disparities in lipid incorporation when these other lipids were added to the component mixture **(Figures A-III-4-6)**.

We compared the percentage of POPC in the impure and pure Nanodiscs samples for multiple compositions for each binary mixture of lipids. Comparing to the percentage in the impure mixture allowed us to account for slight deviations in the starting composition away from the 20, 40, 60 and 80% and any lipid loss during solubilization or incubation, as only the lipids in the impure Nanodisc mixture had the opportunity to incorporate into the Nanodiscs. We found that in nearly all cases, there is enrichment of POPC relative to the intended amount of POPC added to the component mixture **(Figure 2-4A)**. This enrichment was generally greater as the percentage of the second lipid was increased in the component mixture and could be as high as 15-30%, as was the case for POPA, POPE, DOPE, and CL. Nanodiscs made with PE lipids showed the greatest POPC enrichment between 25 and 30%. Some lipids like POPS and SM showed little to no change from the input composition. These results are correlated with lipid shape, as conical lipids (POPA, DOPE, POPE, CL) that impart more curvature on the membrane were depleted in the purified Nanodiscs when present in greater percentages of the total lipid composition. These results trend with lipid intrinsic curvature values determined in supported lipid bilayers.[127,128] For each lipid headgroup, the POPC enrichment for the 20% POPC condition was compared to the spontaneous curvature of each lipid. Lipid headgroups with greater curvature, particularly negative curvature like PE and PA, had the greatest disparity in lipid incorporation **(Figure AIII-7)**. As these lipids are usually added to Nanodiscs to ascertain their effect on membrane protein structure and

function, it is critical to confirm these compositions to enable correlation of these changes with specific lipid compositions. While all lipids tested can be incorporated, we found their resulting compositions do not fully represent the input. Thus, analytical characterization of multi-lipid Nanodisc composition will be extremely important to identify effects of lipids associated with membrane proteins.

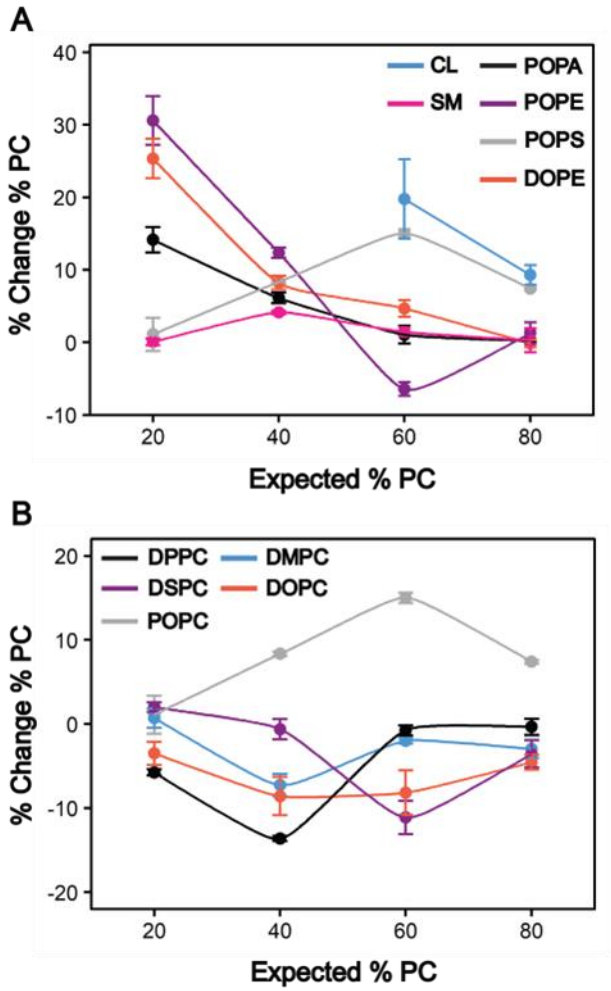

Figure 2-4 Quantitation of lipids in Nanodiscs made with binary lipid mixtures. (A) Percent change of POPC measured in Nanodiscs made with POPC and lipids of varying headgroups. (B) Percent change of PC measured in Nanodiscs made with POPS and PCs of varying fluidity. Error bars are shown as the standard deviation of three replicate Nanodisc assemblies.

2.3.3 Nanodiscs containing lipids with varying acyl chains show disparities in lipid incorporation

In previous work with multi-lipid Nanodiscs, it has been more common to add lipids with different headgroups but not different chain types. In addition to associating with various lipid headgroups, membrane proteins also interact with areas of the membrane that are more rigid or fluid.[11] To draw correlations between these interactions and changes in membrane protein structure or function, it is important to evaluate these compositions as well. We surveyed Nanodisc synthesis containing PCs with different chain length and unsaturation and made Nanodiscs with POPS, a generally robust and popular secondary lipid for Nanodiscs. We found that lipids that produce more rigid membranes showed an opposite trend to POPC, where the POPS was generally enriched **(Figure 2-4B)**. Comparing DMPC to DPPC and DSPC, it is evident that PS is enriched with increasing PC rigidity. For DSPC, the PS enrichment was greater at higher percentages of DSPC, likely because the high transition temperature of the lipid makes formation of higher percentage DSPC Nanodiscs less favorable. For DOPC, which has two degrees of unsaturation, POPS was also enriched, likely showing that lipids with multiple kinks do not incorporate as favorably into Nanodiscs as lipids with a single degree of unsaturation like POPC. These results show that despite the lipids having the same headgroup, they incorporate differently based on their acyl chains, as lipids that create more rigid membranes were depleted in the purified Nanodisc samples, perhaps to modulate the fluidity of the bilayer.

2.3.4 Increased lipid complexity in Nanodiscs models native organelle membrane interactions

Most published studies using multi-lipid Nanodiscs have focused on binary mixtures, though some studies have included compositions with three or more lipids. It is important to

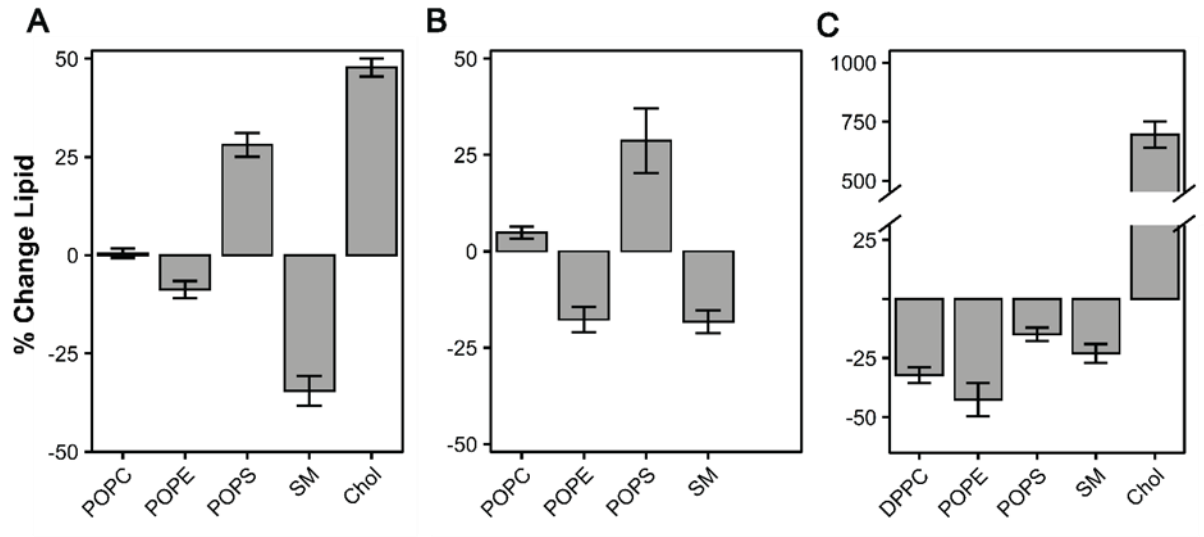

Figure 2-5 Quantitation of lipids in ER lipid mixture Nanodiscs. (A) Nanodiscs made with ER mixture containing POPC. (B) Nanodiscs made with same mixture as in (A), but without cholesterol. (C) Nanodiscs made with same mixture as in (A), but with DPPC instead of POPC. Error bars are shown as the standard deviation of three replicate Nanodisc assemblies.

increase the complexity of the lipid composition in Nanodiscs to study membrane proteins in more native-like environments. To increase the complexity of the lipid composition beyond binary mixtures, we synthesized Nanodiscs to model the inner mitochondrial membrane and endoplasmic reticulum membrane. The mitochondrial membrane Nanodiscs contained a mixture of 50% POPC, 30% POPE, and 20% CL to model the native mitochondrial membrane composition,[129] but it is evident that there was significant depletion of CL compared to the intended composition, likely due to the large size and shape of CL **(Figure A-III-8)**. The endoplasmic reticulum Nanodiscs contained a mixture of 58% POPC, 20% POPE, 7% POPS, 7% PI, 4% SM, and 4% cholesterol to model the native endoplasmic reticulum membrane as previously described.[120] We observe marked enrichment of cholesterol and depletion of SM compared to the input compositions, perhaps to equilibrate to an intermediate liquid ordered phase **(Figure 2-5A)**.[130] When we made the same Nanodisc mixture without cholesterol, we see less depletion of SM **(Figure 2-5B)**. Similar trends

were seen in styrene maleic acid copolymer Nanodiscs made with an equimolar ternary mixture of PC, SM, and cholesterol.[98]

We also investigated how incorporating a more rigid PC lipid into the ER mixture would affect lipid incorporation, so we chose to introduce the more rigid DPPC. Under these conditions, we saw astonishingly high enrichment of cholesterol in these Nanodiscs **(Figure 2-5C)**. Due to the increased rigidity of DPPC relative to POPC, this DPPC Nanodisc lipid mixture is more raft-like. Rafts are cholesterol-rich domains in native membranes,[126] and cholesterol has been shown to associate more with highly saturated PCs in other model membrane systems.[131] We also found generally that there was greater variability across replicates when we made Nanodiscs with more than two lipid components. This means our method would be less able to distinguish smaller changes in lipid composition with increasing complexity. More importantly, this could have greater ramifications for membrane protein study replicates as they could show greater variability depending on lipid environment changes in the Nanodisc. This is essential for the field to consider as more complex lipid compositions are created and studied.

2.4 Conclusions

With the increased need and interest in *in vitro* membrane protein characterization, increased lipid complexity that better models the native membrane in mimics such as Nanodiscs is vital. Since Nanodiscs have mainly been synthesized using single PC lipids, not much is known about the changes in physical properties or lipid composition upon the addition of other lipids to the component mixture. This work provides foundational analytical and biophysical characterization of multi-lipid Nanodiscs. We surveyed lipids with different headgroups and acyl chains in binary mixtures with PC and identified that the resulting Nanodiscs were often larger and

more disperse via SEC analysis. Further, we developed and applied a targeted LC-MS/MS assay that enables facile determination of lipid ratios in the Nanodiscs, allowing comparison against the composition of the starting lipid mixture. We found that the Nanodisc composition often deviated from the input mixture, especially when containing lipids with greater intrinsic curvature and with increased membrane rigidity. We further found that when increasing Nanodisc complexity to model ER membrane mixture, that the lipid incorporation into Nanodiscs was more variable, but we detected native-like cholesterol dynamics when modeling a less fluid membrane. Both changes in physical properties and disparities in lipid composition could influence membrane protein incorporation and activity, so it is critical we understand and expect these changes and work to assess their impact on membrane proteins. We also hope this work serves as a foundation to better understand how multi-lipid Nanodiscs are formed and for better characterization and quality control in Nanodisc studies.

Chapter 3 Tuning Nanodisc Synthesis Parameters Alters The Lipid Landscape

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3.1 Introduction

Membrane proteins make up most therapeutic targets due to their vital role in many cellular processes.[1,118] This necessitates *in vitro* characterization of membrane proteins to better understand their structure and function, but this is often difficult due to their tendency to aggregate or misfold in aqueous solution. This promoted the development and implementation of membrane mimetic platforms to solubilize and stabilize membrane proteins in solution to maintain their native structural integrity.[5–10] One of these mimetic structures is the Nanodisc, a discoidal lipid bilayer encircled by an amphipathic helical belt protein – membrane scaffold protein (MSP). These lipoprotein particles were developed by Sligar and coworkers and self-assemble upon detergent removal from a mixture of components that include lipids, MSP, and membrane proteins.[31–33] Since their discovery, they have been used to incorporate a diverse set of membrane proteins in a native-like bilayer shown to be stable and monodisperse relative to other mimetic systems.[15,21,132]

Most studies involving Nanodiscs utilize very simplistic lipid compositions primarily containing phosphatidylcholine lipids that do not fully model the complexity of native membranes. Recent studies have aimed to produce compositions that better model native membranes as well as to develop analytical tools to characterize the lipid composition of Nanodiscs for better quality control. Previously, we found that the formation of more complex mixtures of Nanodisc membranes is highly dependent on lipid physical properties such as lipid curvature and membrane fluidity (See Chapter 2). Specifically, curved lipids and lipids imparting more membrane rigidity were depleted in Nanodisc membranes following synthesis. Further, more complex Nanodisc lipid membranes (3-4 lipids) that model various bacterial and mammalian membranes were developed for analysis by native mass spectrometry.[107] However, lipids with desired headgroups were selected by modulating the chain length and unsaturation until Nanodiscs formed reliably, which limits this approach to a select subsection of lipids.

While these works provide significant advances in our understanding of producing more complex Nanodisc lipid compositions, native lipids membranes are much more complex.[11] Additionally, we still know very little about how the input materials used for Nanodisc synthesis impact the final lipid composition, especially for native-like membranes. Furthermore, in the case of synthesizing library Nanodiscs (made by incorporating membrane proteins directly from cell lysate), it is unknown which native lipids end up incorporated into the Nanodisc along with the supplementary synthetic lipids. A recent study showed preferences in lipid solubilization by maleic acid-based copolymers used to form native Nanodiscs.[91] Polymers with variable chemical properties showed differences in lipid solubilization, but all generally favored less curved lipids and less ordered domains. This matches a previous study for binary lipid mixtures within MSP-based Nanodiscs, but a similar study has not been done with complex lipid compositions in MSP-

based Nanodiscs that use detergents to solubilize lipids prior to Nanodisc formation. Some detergents have been shown to preferentially solubilize certain lipid species.[133] This, coupled with lipid incorporation disparities previously shown in MSP-Nanodiscs, makes it increasingly likely that these variables could be tuned to tailor specific lipid environments.

To address this fundamental gap in knowledge, we surveyed detergents, temperature, MSP belt size, and synthetic lipid supplements to determine their effects on lipid incorporation in Nanodiscs made with natural lipid extracts. We synthesized Nanodiscs under these different conditions and analyzed the lipid landscape via an untargeted LC-MS/MS method. We found that lipid incorporation generally depended on the lipids available after solubilization. Many lipids were solubilized similarly across all detergents, but in some cases such as for glycerolipids, detergent selection altered the lipids available after solubilization significantly. Temperature had marginal effects on lipid incorporation aside for increasing the incorporation of glycerolipids and PI at higher temperatures. MSP belt size also implicated lipid incorporation, as smaller Nanodiscs had more sphingolipids and less glycerolipids relative to larger Nanodiscs. The most important determinant of preferential lipid incorporation came from the Nanodiscs supplemented with synthetic lipids – DPPC, POPC, and POPS. POPC and POPS additives were able to greatly enrich glycerolipids and sphingolipids relative to non-supplemented Nanodiscs. DPPC supplemented Nanodiscs depleted both glycerolipids and sphingolipids relative to non-supplemented Nanodiscs, but interestingly it markedly improved saturated phospholipid incorporation over all other conditions. Overall, these insights provide a foundation for understanding how modulating Nanodisc synthesis conditions can aid in tailoring Nanodisc lipid compositions, particularly for incorporation of specific membrane proteins, while more accurately modelling the native cell membrane.

3.2 Materials and Methods

3.2.1 Materials

Membrane Scaffold Protein E3D1 (MSPE3D1), Membrane Scaffold Protein 1D1 (MSP1D1), CHAPS hydrate, Amberlite XAD-2, chloroform, 2-propanol, and ammonium formate were purchased from Sigma Aldrich. Sodium cholate, n-Octyl-β-D-glucopyranoside (OG), Triton X-100, acetonitrile, and formic acid were purchased from Fisher. Liver polar lipid extract, soy polar lipid extract, yeast polar lipid extract, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids. n-Dodecyl-B-D-maltoside (DDM) was purchased from Gold Biotechnology.

3.2.2 Nanodisc Assembly

Mixed lipid extract Nanodiscs were synthesized with 40% liver polar lipid extract, 40% soy polar lipid extract, and 20% yeast polar lipid extract to provide a rich selection of lipid classes. Mixed extract and synthetic lipid Nanodiscs were made with 20% liver polar lipid extract, 20% soy polar lipid extract, 10% yeast polar lipid extract, and 50% either POPC, DPPC, or POPS. Nanodiscs were assembled as previously described.[31,32] Briefly, lipid mixtures in defined ratios solubilized in chloroform were dried under nitrogen and stored in a desiccator overnight. Lipids were solubilized to 12.5 mM with 100 mM sodium cholate, CHAPS, or OG, or 150 mM DDM or Triton X-100. Nanodiscs were assembled by adding MSP to the solubilized lipids diluted to 450 µL in standard disc buffer (20 mM Tris pH 7.4, 0.1 M NaCl, 0.5 mM EDTA, 0.01% NaN_3) and supplemented with detergent if necessary to a final concentration of 40 mM for cholate, CHAPS,

and OG, or 60 mM for DDM and Triton X-100. The final lipid concentration in the mixture was 5 mM and the lipid:MSP was 110:1 for cholate and CHAPS discs, 100:1 for DDM and OG discs, and 90:1 for Triton X-100 discs. The component mixture was incubated on an end over end mixer at room temperature for 30 minutes aside for discs made with cholate at 4 degrees and 30 degrees, and discs made with DPPC which were incubated at 40 degrees. 225 mg of beads were added, and the component mixtures were incubated at their respective temperatures for 4 hours before the beads were removed using a gel-loading pipette tip. Three replicates were synthesized for each condition. Immediately after, Nanodiscs were purified by size exclusion chromatography on a Superdex Increase 200 3.2/300 column (Cytiva) on a Waters 2695 liquid chromatograph by injecting 50 µL of Nanodiscs onto the column and pooling the fractions that contained the Nanodisc peak. 2 injections were done per sample. 40 µL of the purified Nanodisc samples were then reinjected for quality control analysis (**Figure 3-1**).

3.2.3 Sample preparation and LC-MS/MS analysis

Lipids were extracted from the purified Nanodiscs using a modification of the Bligh and Dyer extraction protocol (**Figure 3-1**).[122] To 100 µL of impure Nanodisc sample, 400 µL of 0.15 M KCl in water, 800 µL methanol, 400 µL chloroform, and 2 µL acetic acid (all cold) were added to an Eppendorf tube and vortexed. An additional 400 µL of water and 400 µL chloroform (both cold) were added and vortexed. The tubes were shaken at 4°C for 10 minutes and then centrifuged at 12,100 x g for 5 minutes at 4°C. 700 µL of the chloroform layer was collected and transferred to and HPLC vial, dried under nitrogen gas, and reconstituted in 88 µL of mobile phase B to ~3 mg/mL. For pure Nanodisc samples, 300 uL was extracted and other components were scaled by a factor of 3. 2100 µL of the chloroform layer was collected and dried under N_2. The samples were

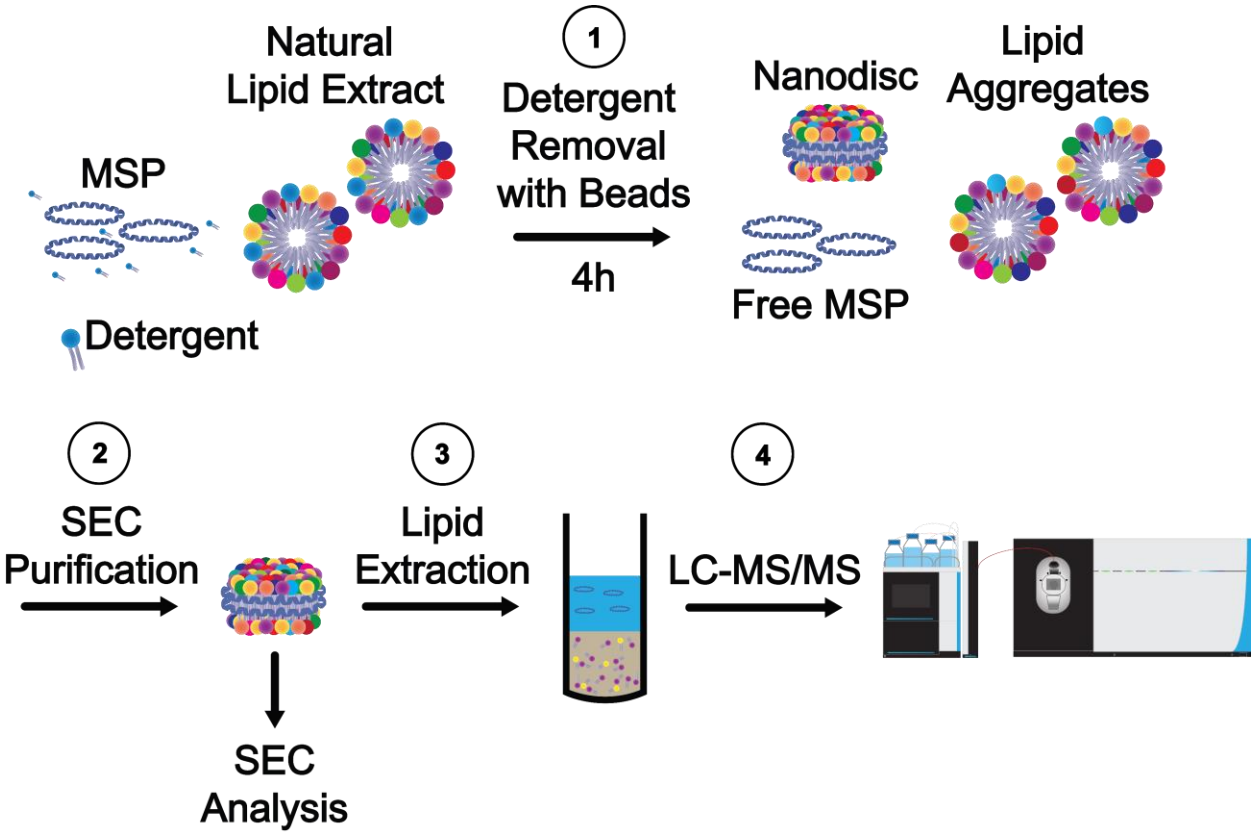

Figure 3-1 Nanodisc formation, purification, and analysis workflow. 1) Nanodiscs self assemble upon detergent removal. 2) Nanodiscs are purified with SEC and are reinjected for quality control analysis. 3) Lipids are extracted from Nanodiscs using a modified Bligh and Dyer extraction. 4) Lipids are separated by reverse phase liquid chromatography and analyzed using an orbitrap mass spectrometer.

reconstituted in 40-150 µL mobile phase B depending on their lipid:MSP ratio and SEC peak intensity to inject similar amounts of lipid per sample (~ 3 mg/mL). Three samples of the starting lipid extract mixture were also dried under N_2 and reconstituted to ~3 mg/mL in mobile phase. Three separate starting lipid extracts underwent lipid extraction the same as the impure samples, and these samples were dried and reconstituted the same as the starting extract to serve as an internal standard since every possible peak analyzed in the Nanodisc samples is present in this starting mixture.

The resulting lipids were separated using a Waters ACQUITY Premier HSS T3 Column column at 400 µL/min on a Thermo Scientific Vanquish Horizon liquid chromatograph interfaced

to a Thermo Scientific Orbitrap ID-X Tribrid mass spectrometer (**Figure 3-1, Figure A-IV-4**). Mobile phase A was 60/40 (v/v) water/acetonitrile with 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B was 85/10/5 (v/v/v) 2-propanol/acetonitrile/water with 10 mM ammonium formate and 0.1% (v/v) formic acid. The gradient is displayed in **Supplemental Table A-IV-1**. The autosampler was held at 4°C, and 5 µL was injected for each sample. The eluting lipids were ionized using electrospray ionization in positive mode at 3.6 kV. The ion transfer tube temperature was 275°C, vaporizer temperature was 300°C, sheath gas flowrate was 60, auxiliary gas flowrate was 25, and sweep gas flowrate was 2. For full scan only runs, the Orbitrap was scanned from 150 – 1700 m/z at 120,000 resolution with RF lens at 40%. For data dependent acquisition runs, the Orbitrap was scanned from 150 – 1700 m/z at 120,000 resolution with RF lens at 40%. Ions with signal above 10,000 were subsequently selected for fragmentation using assisted HCD at 20%, 30%, and 40% collision energies. The Orbitrap at 30,000 resolution was used to collection the fragmentation specta with a maximum injection time of 54 ms. Each selected ion was dynamically excluded for 3 s following fragmentation. Each instrument cycle lasted 1.2 s. To maximize the number of lipid identifications, five iterative injections were performed using AcquireX to create an exclusion list for lipids fragmented in prior injections.

3.2.4 Feature identification and alignment

A list of features from the pooled lipid extract mixture was loaded into a custom software tool, *MetIDTracker,* which searches experimental spectra against multiple libraries. Identifications were manually reviewed to be sure the top scoring hit matched the experimental spectrum without missing key fragment ions as well as a precursor ion abundance of at least three times the signal in the blank. Features that had high-quality spectral matches, a precursor ion match, and at least

two fragment ion matches were counted as metabolomics standard initiative level 2 identifications (MSI2). Features with identity, in-source, and hybrid search results that demonstrated good fragmentation alignment without acceptable precursor ion agreement were counted as MSI level 3 (MSI3) identifications.

For the starting extract, impure Nanodisc, and pure Nanodisc samples, MS1 features were extracted in a targeted manner to include only features corresponding to MSI2 and MSI3 level identifications from the pooled lipid extract mixture using MZmine3 with parameters: intensity tolerance, 50%; m/z tolerance, 0.008 m/z or 10.000 ppm. Chromatograms were aligned with the "Join aligner" function with the following parameters: m/z tolerance, 0.008 m/z or 10.000 ppm; weight for m/z, 50; retention time tolerance, 0.700 min; weight for RT, 50; mobility weight, 1.000. Gap filling was done with the "Peak finder" function with the following parameters: intensity tolerance, 50%; m/z tolerance, 0.0080 m/z or 10.0000 ppm; retention time tolerance, 0.700 min; minimum data points, 1.

Identified features from the pooled lipid extract mixture were aligned with the features from the starting extract or Nanodisc samples using *metabCombiner*, an R-based package for alignment of LC-MS data. In the case of duplicate features, the highest intensity feature was kept. Using a custom R script, all peaks that contained gaps in the starting extract samples and all peaks with an average peak intensity below 20,000 in the starting extract were filtered out. Lipid abbreviations can be found in **Table A-IV-2**.

3.3 Results

3.3.1 Optimization of multi-lipid MSP Nanodisc synthesis for LC-MS/MS analysis

Previous work (see Chapter 2) suggests that lipids do not always incorporate stoichiometrically given their properties such as curvature and fluidity. This study provided the first quantification of Nanodiscs lipid composition by targeted LC-MS/MS, but these analyses are limited to the number of lipid synthetic standards available. Similarly, most other multi-lipid Nanodisc studies generally use between 2-4 lipids, which does not accurately reflect the complexity of native membrane. To facilitate their implementation, we sought to identify how different Nanodisc synthesis conditions (e.g., detergent, temperature, scaffold, supplemented synthetic lipid) affect the lipid landscape following synthesis. Here, we utilized a 4:4:2 mixture of natural lipid extracts (liver, soy, and yeast, respectively) to provide a wide variety of lipid classes (PC, PE, PS, PA, PI, etc.), since we desired to gauge how synthesis conditions could enrich these different lipid classes. To optimize our synthesis conditions, we initially based our conditions on previously published work that synthesized POPC containing MSP1D1 Nanodiscs using different detergents.[109] Here, we utilized MSP1E3D1 which needs a 130:1 POPC:MSP ratio compared to the smaller MSP1D1 which requires 65:1 POPC:MSP ratio when synthesized in cholate. Generally, we found that our LSY lipid extract Nanodiscs require 10-20 fewer lipids than the 100% POPC condition made with the same detergents in both MSP3ED1 and MSP1D1 (**Figures A-IV-1,2,3**). For Nanodiscs made at different temperatures, the same lipid:MSP ratio produced similar quality Nanodiscs. The resulting Nanodiscs were subsequently purified using SEC to ensure the removal of contaminating unincorporated lipids following the Nanodiscs synthesis (**Figure 3-1** and **Figures A-IV-1,2,3**). Together, this process provides high quality and highly purified Nanodiscs to identify how lipid incorporation is broadly affected by Nanodisc synthesis conditions.

3.3.2 Detergent solubilization alters lipids available for Nanodisc synthesis

Most Nanodisc synthesis studies utilize cholate since it enables the robust Nanodisc synthesis. However, detergents used in Nanodisc synthesis could highly modulate the incorporation of lipids into Nanodiscs, since dried lipid films are resuspended in a detergent prior to synthesis. Therefore, detergents can affect both what lipids are solubilized for incorporation into Nanodisc and what lipids are incorporated once they are solubilized. To test the impact of detergents on Nanodisc synthesis, we synthesized Nanodiscs in five different detergents – Cholate, CHAPS, DDM, OG, and Triton X-100. These detergents span multiple classes, molecular weights, and critical micelle concentrations, and are often used in Nanodisc synthesis and/or cell lysis or membrane protein solubilization. Here, the effect of detergent on lipid solubilization and lipid incorporation into Nanodiscs was identified by LC-MS/MS.

Specifically, we measured lipids in three different samples for each condition, the starting extract mixture, the impure Nanodisc (after detergent removal and before purification), and the pure Nanodisc (post-SEC purification). Since lipids could be lost during the detergent solubilization step, differences between the pure Nanodisc lipid landscape and the starting lipid extract mixture could be caused by non-stoichiometric lipid solubilization or incorporation into Nanodiscs. To differentiate discrepancies caused by lipid solubilization and incorporation, impure Nanodisc samples were compared directly to the starting extract to observe solubilization differences, and pure Nanodisc was compared directly to impure Nanodisc to broadly identify preferential lipid characteristics for incorporation into Nanodiscs. Initially, we identified how detergent solubilization affects the available lipid pool for incorporation into Nanodiscs.

In general, we find that broad lipid classes present within our lipid extract (glycerolipids, phospholipids, and sphingolipids) are solubilized and at similar extents no matter the detergent

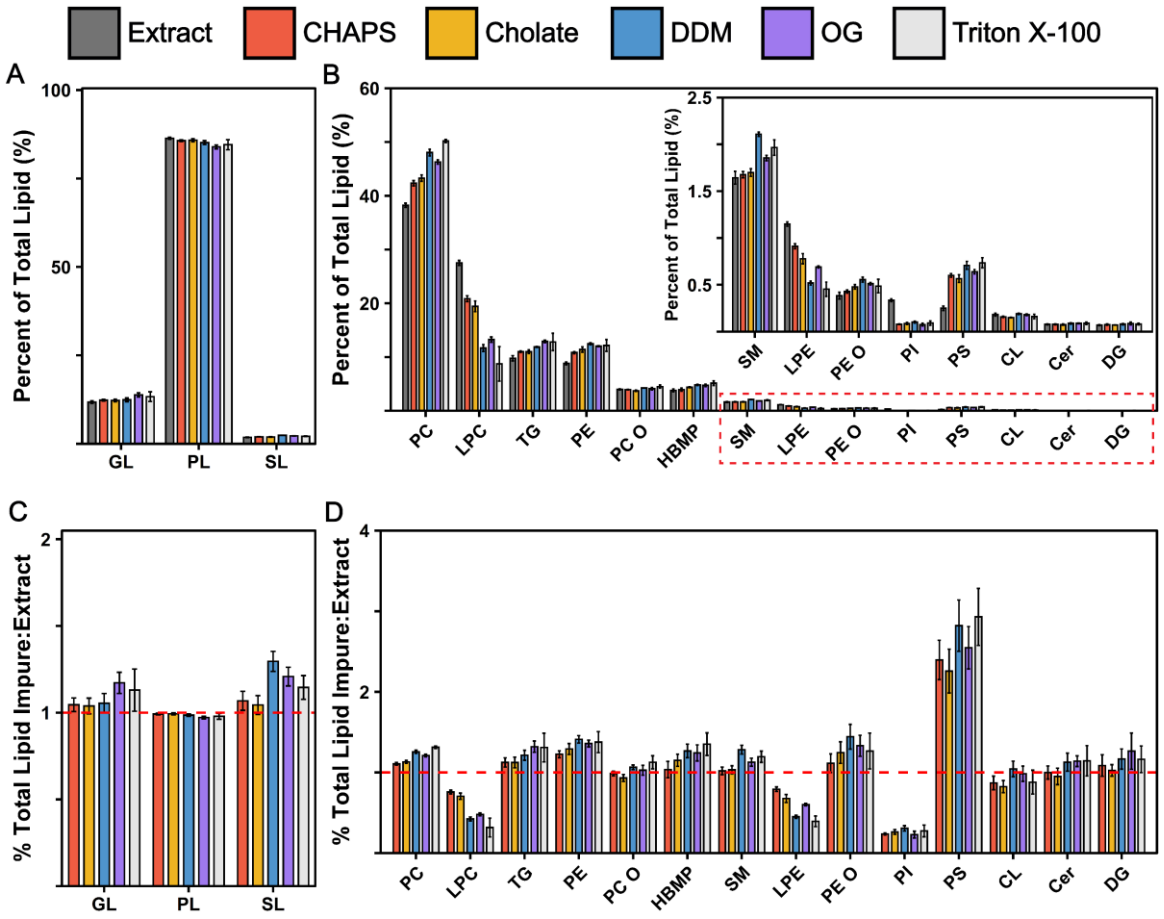

Figure 3-2 Effect of detergent selection on lipid solubilization. A) Percent of each class of lipids relative to all lipids identified in the starting lipid extract and impure Nanodiscs made in each detergent. B) Percent of specific lipid species relative to all lipids identified in the starting lipid extract and impure Nanodiscs made in each detergent. C) Ratio of percent total lipid in the impure Nanodisc sample over the starting lipid extract for broad lipid classes. The red dashed line represents a fold change. D) Ratio of percent total lipid in the impure Nanodisc sample over the starting lipid extract for different lipid species. The red dashed line represents a fold change.

used (CHAPS, cholate, DDM, OG, and Triton X-100, **Figure 3-2A,C**). However, sphingolipids are modestly increased when the lipid extract is solubilized in DDM, OG, and Triton X-100 (**Figure 3-2A,C)**. Despite the limited alterations in broad lipid classes, we found that specific lipid headgroup classes are significantly altered during lipid solubilization by detergents. For example, we find that PS is substantially enriched during detergent solubilization, but certain phospholipid

species were de-enriched relative to the starting lipid extract including LysoPCs, LysoPEs, and PIs in all detergents as well as CL in cholate and CHAPS (**Figure 3-2B,D**). Sphingolipids were well solubilized and in some cases were enriched relative to the starting extract as was the case for SMs (DDM, OG, Triton X-100) and ceramides (DDM, OG, Triton X-100) (**Figure 3-2B,D**). These results match the overall detergent solubilization trends for sphingolipids broadly as these detergents were better at solubilizing sphingolipids compared to cholate and CHAPS (**Figure 3-2A,C**). Further, glycerolipid species were modestly enriched during solubilization, such as TGs (**Figure 3-2B,D**).

Additionally, certain detergents solubilized lipids species similarly, such as CHAPS and cholate. For example, CHAPS and cholate solubilized lysophospholipids better than the other detergents tested, but comparatively struggled to solubilize most other lipids species (e.g., PCs, TGs, Pes, ether-linked phospholipids, HBMPs, SMs, and ceramides) (**Figure 3-2B,D**). Interestingly, cholate, the most common detergent utilized in Nanodisc synthesis, provides the most representative lipid pool relative to the starting extract mixture compared to the other detergents tested (**Figure 3-2B,D**).

In addition to species level lipid analysis, we sought to ascertain if detergents selectively solubilize different chain lengths (total acyl chain carbons in entire lipids). Here, we analyzed lipids within their broad lipid class since phospholipids make up over 80% of lipid signal detected. Interestingly, we find that short phospholipids (<32) and glycerolipids (<48) are depleted for all detergents (**Figure A-IV-5A,C**). This is perhaps unsurprising since LysoPC and LysoPE species are similarly depleted during solubilization by all detergents. Interestingly, only even chain phospholipids (32, 34, 36, 38) are enriched during solubilization, where glycerolipids are generally increased no matter the chain number (**Figure A-IV-5A,C**). DDM, OG, and Triton X-100

(especially OG and Triton X-100) were generally better at solubilizing glycerolipids of most chain lengths relative to cholate and CHAPS. This difference was most significant for larger glycerolipids (>56) where cholate and CHAPS depleted these longer chains relative to the starting mixture (**Figure A-IV-5A,C**). Contrarily, short and long chain SMs (<35 and >37) were enriched while moderate chain length SMs (35-37) were depleted (**Figure A-IV-5B**). Overall, we find there is not a solubilization preference for specific lipid chain lengths based on detergent selection, but certain chain lengths are solubilized differently.

Lastly, we sought to determine the effects of the number of double bonds on lipid solubility. Similar to chain length, we find that varying detergent selection does not alter phospholipid, sphingolipid, or glycerolipid solubilization based on the number of double bonds (**Figure A-IV-5D-F**). Instead, all detergents tend to trend toward enrichment or de-enrichment similarly. Specifically, phospholipids with zero, three, and six double bonds are depleted while two, four, and five double bonds are enriched (**Figure A-IV-5D**). Further, sphingolipids with few double bonds (<4) were depleted, while many double bonds were enriched (>5) during solubilization (**Figure A-IV-5E**). Oddly, DDM greatly enriched sphingolipids with two double bonds. Contrarily, glycerolipid solubilization based on double bonds was generally unaffected, except for two double bonds which showed an overall decrease in abundance for all detergents (**Figure A-IV-5F**). Similar to lipid chain length, there was minimal dependence on detergent selection with regards to lipid double bond count, except for phospholipids with four or five double bonds being enriched when solubilized in DDM, OG, and Triton X-100 and glycerolipids with greater than four double bonds being enriched when solubilized in OG and Triton X-100 compared to other detergents (**Figure A-IV-5D,F**). Likely the alterations in the lipid landscape are arising from differential capabilities of lipids to be incorporated into the lipid-detergent micelles.

Overall, we found that lipids were selectively solubilized, but this generally was not dependent on the detergent selected, as lipid species, chain lengths, and double bonds were enriched or depleted broadly across all detergents. This was not always the case, sometimes cholate and CHAPS and DDM, OG, and Triton X-100 displayed different trends, like for glycerolipids. These results have implications for lipid incorporation into Nanodiscs since the lipids available for Nanodisc synthesis depend on the initial solubilization step. Therefore, detergent selection is not trivial and could alter the lipid landscape accessible for Nanodisc incorporation.

3.3.3 Lipid incorporation into Nanodiscs is mostly based on availability after solubilization

Nanodisc synthesis occurs through the interaction of the membrane scaffold proteins with the detergent micelles, where detergent removal causes the spontaneous formation of the stable membrane mimetic.[134] Since the Nanodisc directly interactions with the detergent micelle, the composition and size of the micelle could affect lipid incorporation into Nanodiscs. Thus, we sought to test whether detergent selection affects the incorporation of lipids into Nanodiscs following solubilization.

Similar to lipid solubilization, we found that detergent selection does not drastically affect lipid incorporation into Nanodiscs, except for the depletion of glycerolipids in CHAPS and cholate Nanodiscs (**Figure 3-3A**). Interestingly, only TGs are depleted while DGs incorporate relatively efficiently (**Figure 3-3B**). This is perhaps unsurprising since the bulkiness of glycerolipids likely hinder their incorporation, so the larger TGs would be hindered more than DGs. However, this depletion is not detected in all detergents assayed, suggesting certain detergents can remove these steric effects regarding lipid incorporation.

Further, lipid incorporation efficiency is highly independent of lipid solubilization. For example, PIs are the least soluble lipid in all five detergents tested, but they are incorporated at the highest efficiencies into Nanodiscs (**Figure 3-3B**). Similarly, lysophospholipids and PSs are

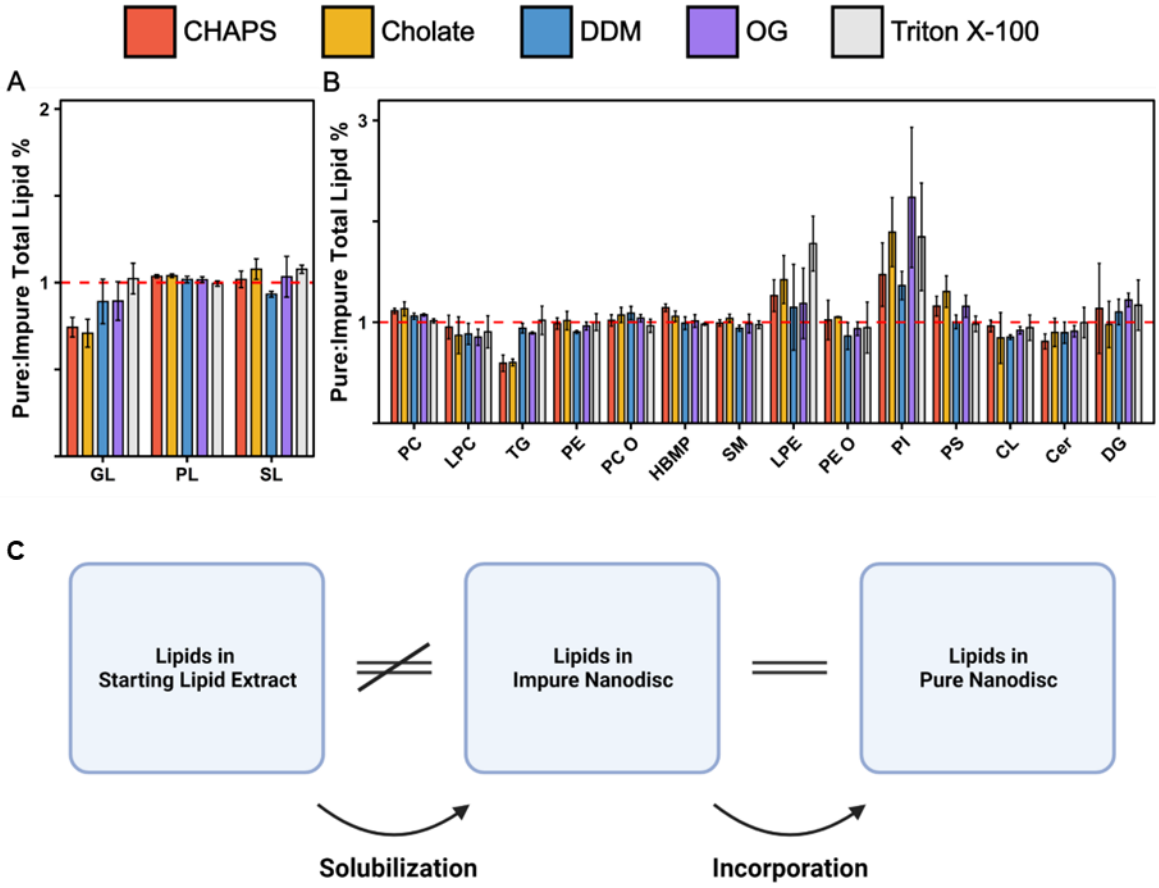

Figure 3-3 Effect of detergent selection on lipid incorporation into Nanodiscs. Effect of detergent selection on lipid incorporation into Nanodiscs

generally depleted and enriched during solubilization, respectively, but are incorporated stoichiometrically into the Nanodiscs (**Figure 3-3B**). In general, we find that phospholipid, sphingolipid, and glycerolipid incorporation into Nanodiscs is independent of lipid chain length and number of double bonds, besides for the general enrichment of sphingolipids with two double bonds (**Figure A-IV-6A-F**). Additionally, fully saturated lipids are not favored during Nanodiscs synthesis, which correlates well with previous studies that found that lipids that reduce membrane

fluidity are not incorporated at high efficiencies into Nanodiscs (see Chapter 2, **Figure A-IV-6D-F**). Overall, we found that the Nanodisc lipid landscape is highly representative of the lipids solubilized by detergents prior to synthesis. This signifies that the solubility of lipids by detergents used for Nanodisc synthesis does not correlate well with lipids that incorporate efficiently into Nanodiscs, suggesting that lipid incorporation is mainly dependent on lipid availability rather than lipid properties (**Figure 3-3C**).

3.3.4 Temperature does not affect lipid incorporation into Nanodiscs

In addition to detergent, there are multiple other Nanodisc synthesis parameters that could alter lipid incorporation into the membrane mimetic – synthesis temperature, MSP belt size, and the addition of supplemental synthetic lipid species. We investigated these parameters using cholate detergent, since it is the most common detergent utilized in Nanodisc synthesis. Specifically, the temperature of Nanodiscs synthesis alters the fluidity of the lipids based on the lipid melting temperature. Therefore, altering this condition could influence the lipids incorporated.

Here, we screened three different temperatures (4℃, 20℃, and 30℃), which can be used for maintaining membrane protein activity for downstream analyses. In general, we find that Nanodisc synthesis temperature only modestly alters the broad lipid distribution incorporated into Nanodiscs (**Figure 3-4A**). However, we do detect a slight increase in glycerolipid abundance in the 30℃ condition (**Figure 3-3A**), primarily occurring from the minor enrichment of TGs and DGs in Nanodiscs (**Figure 3-3B**). Interestingly, PIs were significantly enriched during Nanodisc synthesis at 30℃, suggesting that PIs require a higher temperature to be stably incorporated into Nanodiscs (**Figure 3-3B**). Further, we find that LysoPCs are depleted in the higher temperature

conditions (**Figure 3-3B**). Lysophospholipids are cone-shaped lipids that promote the formation of curved lipid membrane, suggesting that LysoPC induced membrane curvature could be

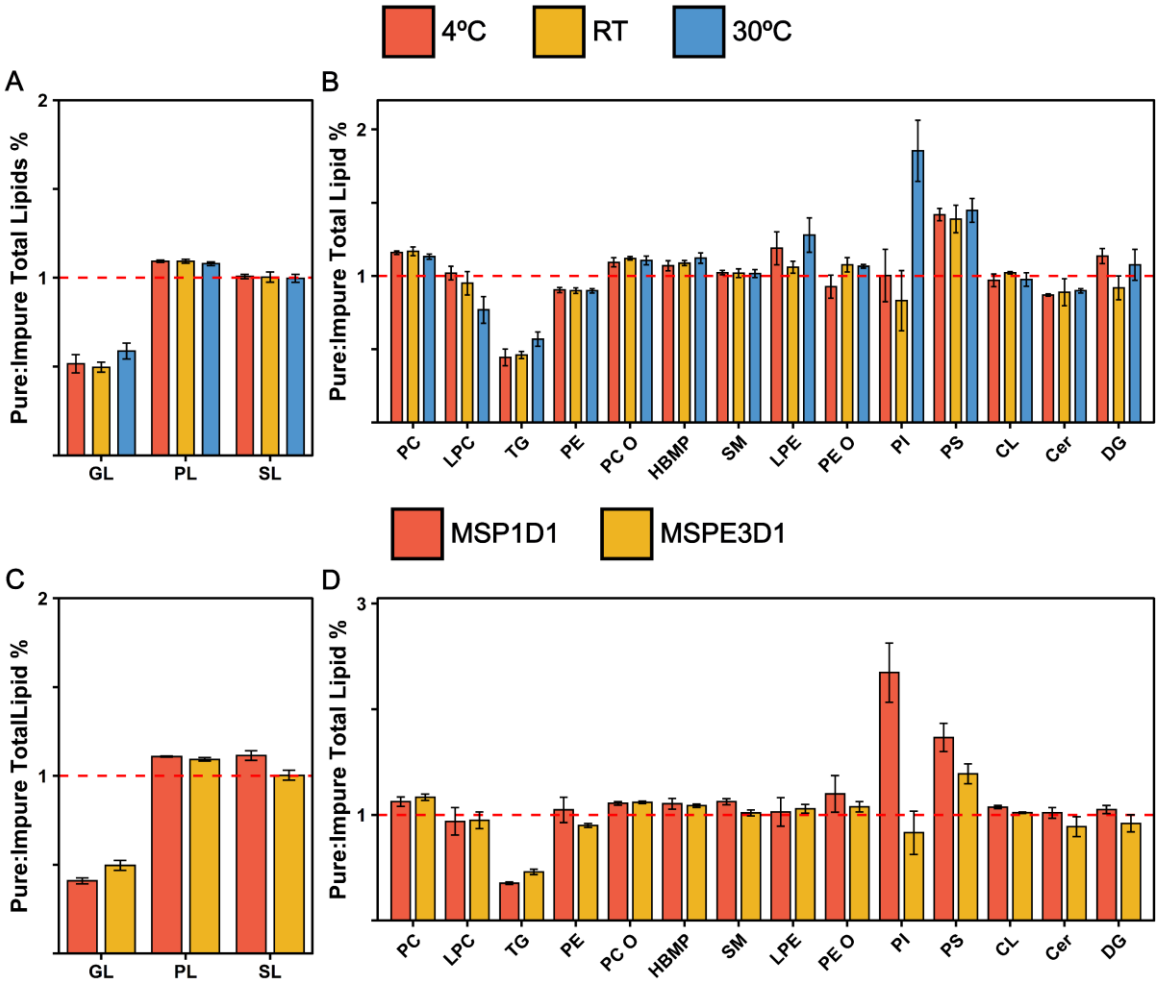

Figure 3-4 Effect of temperature and MSP size on lipid incorporation into Nanodiscs. A) Effect of temperature on broad lipid class incorporation depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change. B) Effect of temperature on specific lipid species incorporation depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change. C) Effect of MSP size on broad lipid class incorporation depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change. D) Effect of MSP size on specific lipid species incorporation depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change.

unfavorable at higher temperatures.[135] Interestingly, we do not see a depletion in LysoPE at higher temperature, possibly since LysoPE induced membranes curvature is not as severe as LysoPC.

Similar to detergent selection, the incorporation of lipids into Nanodiscs is primarily independent of lipid chain length and number of double bonds no matter the broad lipid class (**Figure AIV-7A-F**). While Nanodisc synthesis temperature provides some variability regarding what lipid headgroup class is incorporated, lipid incorporation into Nanodiscs remains primarily dependent on availability of lipids following solubilization.

3.3.5 MSP1D1 Nanodiscs have more phospholipids and sphingolipids and less glycerolipids than MSP1E3D1 Nanodiscs

In addition to Nanodisc synthesis temperature, the MSP belt utilized to stabilize the lipid bilayer is commonly modulated. Primarily, different MSP belts enable the modulation of the membrane mimetic diameter, enabling the incorporation of a range protein and protein complex sizes into these mimetics for downstream characterization. Similar to how these MSP belts could alter protein incorporation, we posited that they could influence the incorporation of specific lipids species into the membrane mimetic. Here, we compared the lipid incorporation into two different MSP belts – MSP1D1 (10 nm diameter) and MSP1E3D1 (13 nm diameter). We figured the smaller surface area of D1 Nanodiscs could affect how lipids incorporated, and it has been shown that D1 Nanodiscs have different internal lipid dynamics than E3 Nanodiscs.[136]

Perhaps unsurprising, we find that glycerolipids are depleted in the smaller D1 Nanodiscs (**Figure 3-3C**), where steric interaction of TGs likely hinder incorporation into D1 Nanodiscs compared to E3D1. Further, we find that sphingolipids are broadly increased in the smaller D1 Nanodiscs (**Figure 3-3C**), where both SM and Cer species are enriched (**Figure 3-3D**).

Sphingolipids are known to cause tighter packed membranes than other lipid classes[137], suggesting that tighter packed membranes may be favorable for Nanodiscs made with smaller MSP belts. Interestingly, PI and PS species are known to be enriched in membranes containing higher levels of sphingolipids[138], and we found that these phospholipids are enriched in smaller D1 Nanodiscs with sphingolipids (**Figure 3-3D**). This suggests that there is an interplay between different lipids classes that could influence incorporation of lipids into Nanodisc.

Similar to previous results, we find that lipid incorporation into Nanodiscs is highly independent of lipid chain length and number of double bonds (**Figure A-IV-8A-F**). However, we do find that only glycerolipids with even number of chains are depleted in the smaller D1 Nanodiscs (**Figure A-IV-8C**). Further, sphingolipids with smaller number of double bonds (zero, one, two, and three) are enriched in Nanodisc, while more double bonds is generally unfavored besides for six (**Figure A-IV-8E**). Likely, these alterations occur from how the specific lipid characteristic affects the ability of the lipid membrane to form tightly packed membranes that efficiently organize within the smaller MSP1D1 lipid bilayer scaffold. Together, MSP belts size does influence the lipid landscape incorporated into Nanodiscs, where smaller scaffolds enrich lipids that promote tightly packed membrane with significant interactions between incorporated lipid species.

3.3.6 Supplemental synthetic lipid addition leads to preferential lipid incorporation in Nanodiscs

Library Nanodiscs are a subclass of Nanodiscs that are synthesized using a cell lysate instead of recombinantly-expressed membrane proteins. It is thought that native lipids from the cell lysate get incorporated into the Nanodiscs, but usually synthetic lipids are added to supplement

the native lipids to fill out the entirety of the Nanodisc. To model this phenomenon, we chose three synthetic lipid additives (DPPC, POPC, and POPS) to supplement our lipid extract Nanodiscs by synthesizing them with 50/50 mixtures of synthetic/natural lipids. The selected lipids represent

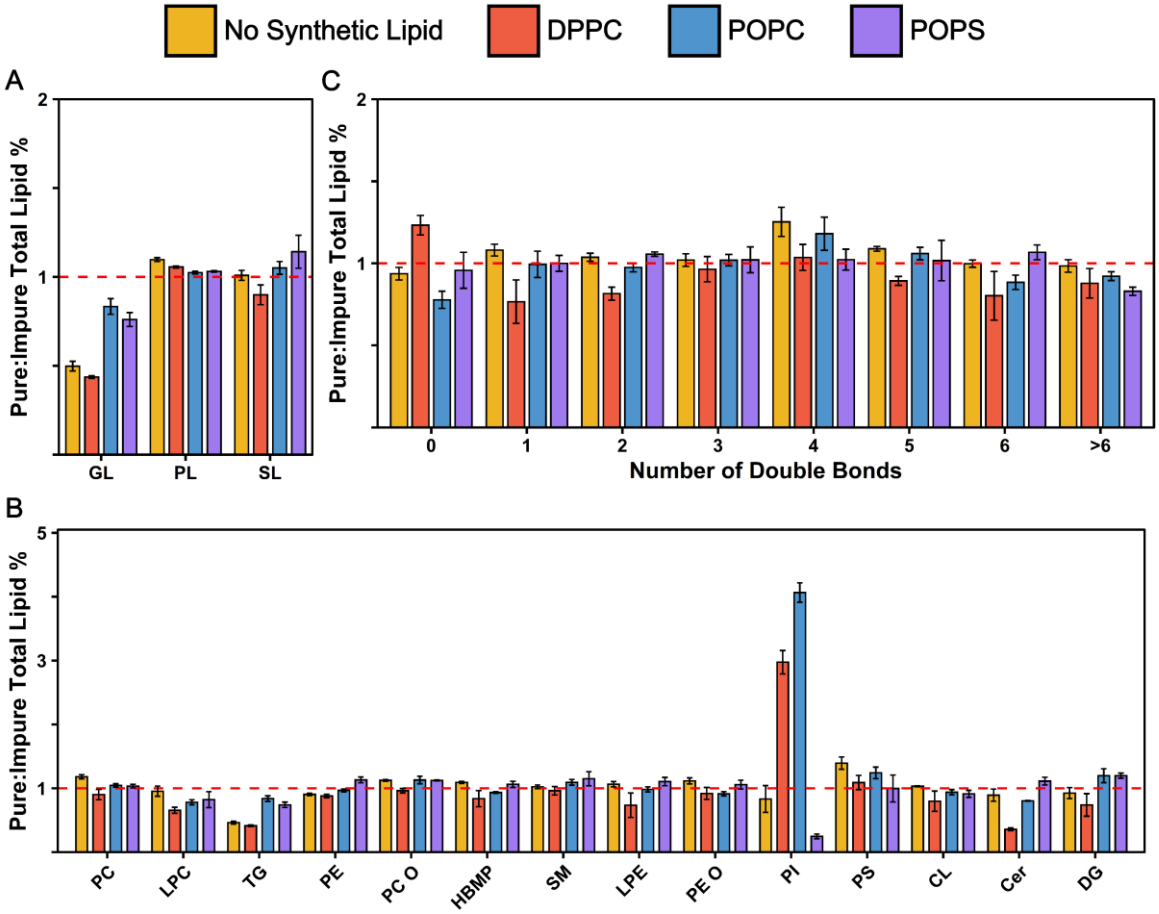

Figure 3-5 Effect of synthetic lipid additives on lipid incorporation into Nanodiscs. A) Effect of synthetic lipid additives on broad lipid class incorporation depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change. B) Effect of synthetic lipid additives on specific lipid species incorporation depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change. C) Effect of synthetic lipid additives on phospholipids with specific degrees of unsaturation incorporated depicted as the ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample. The red dashed line represents a fold change saturated, unsaturated, and charged lipid species to investigate the effect of these different properties.

In general, we find that the addition of a synthetic lipid alters the broad lipid classes incorporated more than detergent selection, temperature, and MSP belt (**Figure 3-5A**). Perhaps

unsurprisingly, we find that the addition of synthetic phospholipids decreases the extent of other phospholipids incorporated into the Nanodiscs, likely due to the synthetic lipid being incorporated into the Nanodsics. Specifically, we find that PC and LysoPC are the most depleted when the PC synthetic lipids (DPPC and POPC) were utilized for synthesis (**Figure 3-5B**). Similarly, we find that native PS lipids are the most depleted when POPS is used for synthesis (**Figure 3-5B**), suggesting there are limitations on the percentage of lipids of a specific class incorporated into the Nanodisc. Interestingly, we found that PIs are drastically enriched in DPPC and POPC doped Nanodiscs, while was depleted in POPS doped Nanodiscs (**Figure 3-5B**). This suggests that there is some synergy between PIs and PC lipids, while PI incorporation is unfavorable under high PS lipid conditions, likely due to electrostatic interactions between anionic lipids.

Further, we find that glycerolipids are enriched in POPC and POPS doped Nanodiscs and depleted in DPPC doped Nanodiscs (**Figure 3-5A**). We detect this alteration for both TG and DG species (**Figure 3-5B**), suggesting that glycerolipid incorporation into Nanodiscs is more preferential when high levels of monosaturated phospholipids are present. Monosaturated lipids reduce the rigidity of membranes, possibly suggesting that glycerolipids require more fluid membranes to incorporate efficiently into Nanodiscs. Additionally, we find that sphingolipids (SM and Cer species) are enriched in POPS doped Nanodiscs (**Figure 3-5A,B**), further suggesting a synergistic relationship between these lipids species.[138]

Similar to other conditions, we found that there is minimal relationship between lipid chain length and efficiency of incorporation when during synthesis with doped synthetic lipids. We see a similar trend for the number of double bonds for all lipids subclasses (**Figure 3-5C, Figure A-IV-9A-E**). However, fully saturated phospholipids are more enriched than any unsaturated lipids in Nanodiscs doped with DPPC (**Figure 3-5C**). Saturated lipids provide very compact and rigid

membranes, which likely provides unfavorable conditions for unsaturated lipid incorporation into Nanodiscs. We do not detect a similar trend for the Nanodiscs doped with POPC and POPS, which does not affect phospholipid incorporations based on the number of double bonds.

Together, we find that doping native lipid extracts with synthetic lipids during Nanodisc synthesis provides the widest variations in lipid incorporation into Nanodiscs. Particularly, we find that lipid incorporation is primarily altered by preferential interactions with the doped lipid species. This is especially important since library Nanodiscs commonly utilize synthetic lipids during Nanodisc synthesis. Thus, this process could alter the incorporation of native lipids from the cellular extracts, which could alter membrane protein function based on the surrounding lipid landscape present within the membrane mimetic.

3.4 Discussion

Nanodiscs have become an increasingly useful tool for solubilizing membrane proteins for *in vitro* analyses, as they provide a relatively monodisperse and controllable native-like lipid bilayer. Traditionally, these studies utilize a single phosphatidylcholine lipid, a belt size dependent on the size of the protein of interest, and cholate as the detergent. Increased lipid complexity in Nanodiscs would provide more native-like lipid environment for membrane protein enrichment and measurements, but recent studies have found that lipids do not always incorporate into Nanodiscs stoichiometrically. These findings led us to further investigate Nanodisc lipid incorporation and determine how we could tune lipid compositions for membrane proteins of interest. We thought to survey a variety of detergents, MSP belt sizes, temperatures, and synthetic lipid supplements since these variables are often untouched in Nanodisc synthesis and could alter lipid incorporation.

To assess lipid incorporation differences, we generated Nanodiscs with a mixture of natural lipid extracts to survey a variety of lipid classes in a complex membrane environment. We synthesized Nanodiscs in five detergents – cholate, CHAPS, DDM, OG, and Triton X-100, and chose cholate to evaluate temperature (4℃, 20℃, 30℃), MSP size (MSP1D1 vs. MSP1E3D1), and synthetic lipid supplementation (DPPC, POPC, POPS). We discovered that detergents alter lipid solubility, but lipid incorporation into Nanodiscs was based on the available lipids after solubilization, not on detergent selected for synthesis. This is important, as different detergents are utilized for cell lysis and membrane protein stabilization, where we find these detergents can affect lipid solubility and the resulting lipid pool available for incorporation. This points to the need for optimizing detergents best for both the membrane protein and lipid environment when synthesizing Nanodiscs, perhaps with a mixture of two detergents.

We also determined that temperature had marginal effects on lipid incorporation into Nanodiscs aside for enrichment of TG and PI lipids. Often, library Nanodiscs are synthesized at low temperatures to preserve membrane protein activity, so it is important to understand how this could alter the Nanodisc lipid environment. Additionally, more robust membrane protein systems could possibly be synthesized at higher temperatures to facilitate incorporation of these lipid species, especially if they are known ligands that affect the membrane protein's structure and/or activity.

Additionally, we found that MSP size changed lipid incorporation into Nanodiscs with the smaller D1 able to enrich sphingolipids, while the larger E3 was better for incorporating larger glycerolipids. MSP belt size is typically selected with the target membrane protein in mind and is usually the smallest possible to avoid multimerization. Often, the larger E3 is selected for library

Nanodiscs to incorporate a larger selection of proteins and complexes of different sizes, so these Nanodiscs would have a different lipid profile compared to smaller Nanodiscs.

Most apparent in the data was the large effect of supplemental synthetic lipid addition. Nanodiscs with added DPPC were depleted in both glycerolipids and sphingolipids relative to non-supplemented Nanodiscs, but interestingly they had markedly increased levels of saturated phospholipid incorporation over all other conditions. Nanodiscs with POPC and POPS additives were greatly enriched in glycerolipids and sphingolipids relative to non-supplemented Nanodiscs. These results are critical as library Nanodiscs are synthesized with supplemental synthetic lipids to fill out the rest of the Nanodisc, meaning the native lipids incorporated could be drastically altered based on the synthetic lipid selection. Resulting, this could modify membrane protein incorporation efficiency, structure, and activity if a known lipid ligand is not incorporated efficiently with a specific doped synthetic lipid.

Historically, lipid incorporation into Nanodiscs was believed to occur stoichiometrically; however, we find that this is not always true. Overall, these results show how critical it is to assess and optimize every variable of Nanodisc synthesis including detergent, temperature, MSP size, and synthetic lipid supplements, as these are able to change the lipid environment in Nanodiscs. The lipid environment is important for incorporating membrane proteins that are structurally and actively sound. These results also have broader implications for other membrane mimetics. For example, bicelles are mixtures of detergents and lipids, and our work suggests that the detergent selection could alter lipids in the final bilayer structure. Additionally, other lipid bilayer mimics such as liposomes and topically relevant lipid nanoparticles for therapeutic delivery are synthesized from mixtures of synthetic lipids, which have been shown in this work and previously to impact the incorporation of other lipids in the mixture. This could alter the final lipid

composition as specific lipid species could be enriched or de-enriched which has the potential to alter localization as well as cargo stability and release. Taken together, synthesis conditions, particularly for Nanodiscs or other lipid bilayer mimics with complex lipid compositions, are extremely important and are worth careful optimization and characterization.

Chapter 4 Detergent and Lipid Selection in Nanodisc Synthesis for Membrane Protein Enrichment

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4.1 Introduction

Membrane proteins participate in many biological processes such as signal transduction and metabolism. Consequently, innovative approaches to interrogate membrane proteins are vital to their characterization. Current chemical and biophysical techniques require solubilized membrane protein removed from the native cell membrane, but membrane protein misfolding and aggregation under non-native conditions limit the capability of these assays. This has prompted the development of membrane mimetics, such as Nanodiscs, to preserve the native structure and function of membrane proteins *in vitro*. Nanodiscs are composed of a discoidal phospholipid bilayer encircled by a membrane scaffold protein (MSP) belt and are formed via self-assembly once detergent is removed from the solubilized components.[31,139]

One specific application that would benefit from improved membrane protein enrichment strategies is proteomics. Despite advancements in sample preparation strategies, separations technology, MS instrumentation, and informatics which have allowed for great strides in proteomics and interactomics, membrane proteins provide inherent challenges for these experiments. They are expressed in relatively low levels compared to their cytosolic counterparts, so they are often underrepresented in proteome analyses. Their hydrophobicity, especially for integral membrane proteins, makes solubilization at multiple stages in sample preparation pre-MS challenging. Additionally, they often lack many charged amino acid residues for common enzymatic digestions resulting in low sequence coverage.[140–142] Therefore, membrane proteomics involves specific extraction, enrichment, solubilization, and digestion techniques for representative proteome analyses.[143] Detergents are typically used to solubilize membrane proteins, but their presence is not compatible with enzymatic digestion and MS. Therefore, solubilization strategies that are detergent free and retain membrane protein integrity are needed. Nanodiscs are one potential solution to this and have previously been used in MS studies of membrane proteins.[36,144] Nanodiscs made from whole cell lysate have been shown to be representative of the membrane proteome via proteomics analyses, and Nanodiscs made with tailored lipid compositions have been previously shown to enrich different membrane protein classes as a function of lipid composition.[36,144]

To expand on previous work that would benefit all applications and assays regarding membrane protein libraries, we assessed the ability of different detergents and lipids utilized in Nanodisc synthesis to enrich different membrane protein classes. Given our previous work on how different detergents can enrich different lipid classes (see Chapter 3) and previous work showing Nanodisc lipid compositions can incorporate different membrane proteins, we expected these

detergents to have similar effects on membrane protein enrichment. To test this, we made Nanodisc libraries with HeLa cell membrane extracts with detergents commonly used in membrane protein solubilization and stabilization.[145] We also synthesized the same library Nanodiscs with complex natural lipid extracts to vary the lipid environment. We then performed bottom-up proteomics experiments to determine how these variables impacted which membrane proteins were extracted under the different conditions. We found that these different synthesis conditions identified unique proteins.

4.2 Materials and Methods

4.2.1 Materials

Amberlite XAD-2 and membrane scaffold protein (MSP1E3D1) were purchased from Sigma Aldrich. Sodium cholate, Triton X-100, ammonium bicarbonate, and sodium deoxycholate SDC) were purchased from Fisher. RapiGest (RG) was purchased from Waters. Sequencing grade modified trypsin was purchased from Promega. n-Dodecyl-B-D-maltoside (DDM) was purchased from Gold Biotechnology. 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and liver polar lipid extract were purchased from Avanti Polar Lipids.

4.2.2 Cell Culture

Human cell line HeLa was cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 in a humidified incubator.

4.2.3 Extraction of plasma membranes

Membranes were extracted from cells using a modified version of the Mem-PER Plus Membrane Protein Extraction Kit (Thermo). Briefly, the cell pellet harvested previously was thawed on ice. The cells were resuspended in 1.5 mL of Cell Wash Solution and centrifuged at 300 x g for 5 minutes. The supernatant was then discarded. 0.75 mL of Permeabilization Buffer supplemented with 10 µL/mL of Halt Protease and Phosphatase Inhibitor Cocktail (Thermo) was added to the cell pellet and the mixture was briefly vortexed to obtain a homogenous cell suspension. The mixture was incubated on an end over end shaker for 10 minutes at 4°C. The permeabilized cells were centrifuged for 15 minutes at 16,000 x g at 4°C. The supernatant containing cytosolic proteins was removed. 0.25 mL of Solubilization buffer supplemented with 20 µL/mL of Halt inhibitor was added and the pellet was resuspended by pipetting up and down. The mixture was incubated on an end over end shaker for 30 minutes at 4°C. The tube was then centrifuged at 16,000 x g for 15 minutes at 4°C. The supernatant containing solubilized membrane proteins was transferred to a new tube. The concentration of the membrane proteins was determined using Pierce BCA Protein Assay Kit (Thermo) and the µg/mL concentration obtained was converted to µM via an estimated average membrane protein molecular weight of 40 kDa. The membrane proteins were then immediately used for Nanodisc synthesis.

4.2.4 Nanodisc Assembly

Nanodiscs were made with 100% POPC in cholate, Triton X-100, or DDM. Additional lipid compositions of 80% POPC/20% POPE and Liver polar lipid extract were made with cholate.

Nanodiscs were assembled as previously described.[31,32] Briefly, lipids solubilized in chloroform were dried under nitrogen and stored in a desiccator overnight. POPC was then solubilized to 50 mM with 100 mM sodium cholate, 25 mM with 150 mM Triton X-100, or 12.5 mM with 150 mM DDM. POPC/POPE was solubilized to 50 mM in 100 mM cholate and liver extract was solubilized to 25mM in 100 mM cholate. Nanodiscs were assembled by adding MSP and membrane protein extract to the solubilized lipid diluted to 1 mL in standard disc buffer and supplemented with detergent if necessary to a final concentration of 20 mM for sodium cholate, 30 mM for Triton X-100, or 40 mM for DDM. The final lipid concentration in the mixture was 5 mM, the MSP:MP was 4:1, and the lipid:MSP was 90:1 for cholate, 60:1 for Triton X-100, or 70:1 for DDM. The component mixture was incubated on an end over end mixer at 4°C for 1 hour. 500 mg of beads were added, and the component mixture was incubated at 4°C overnight before the beads were removed. 20 µL of Nanodisc sample was injected on a Superdex Increase 200 3.2/300 column for quality analysis **(Figure A-V-1A-C)**.

4.2.5 Nanodisc purification and disassembly

Nanodiscs were simultaneously purified and disassembled (**Figure 4-1, Figure A-V-2**). The crude Nanodisc mixture was loaded on a Ni-NTA spin column (Thermo) and washed to remove unincorporated membrane proteins. 25 mM sodium cholate was then added to the column and incubated for 30 minutes to break up the Nanodiscs and release incorporated membrane proteins while retaining most of the MSP on the column. Proteins liberated from the Nanodiscs underwent a methanol–chloroform–water precipitation to remove lipids, salts, and detergents for downstream analyses. The protein pellet was resuspended in 1% SDC or 0.01% RG/10% Methanol by agitation at 37°C for 30 minutes followed by 10 minutes of sonication on ice.

4.2.6 SDS-PAGE Analysis

Samples incubated in 1X Laemmli buffer at room temperature for 1 hour. Samples were then separated on 4-12% gradient gels at 200V for 35 minutes. Gels were stained in either Coomassie Brilliant Blue or Coomassie Fluor Orange. For Coomassie Blue, gels were stained for 1 hour and destained in water for 1 hour before imaging. For Coomassie Fluor Orange, gels were stained for 1 hour, followed by a 1 minute rinse with 7.5% acetic acid and 5 minute rinse with water before imaging.

4.2.7 Sample preparation and LC-MS/MS analysis

Samples underwent disulfide reduction/alkylation with DTT/IAA followed by trypsin digestion overnight at 36℃. TFA was added to samples and the supernatant was collected by centrifugation. The peptide samples were cleaned with C18 SPE tips (for SDC samples) or SCX tips (for RG samples). The resulting peptides were analyzed by liquid chromatography tandem mass spectrometry using a Thermo Ultimate 3500 coupled to a Thermo Orbitrap Fusion Lumos. Peptides were separated via reverse phase with an Acclaim PepMap 100 C18 Column over a 90 minute gradient. The eluting peptides were ionized using nano electrospray ionization in positive mode. Data was acquired using a data dependent acquisition method.

4.2.8 Data Analysis

Raw data collected by LC-MS/MS was searched using Proteome Discoverer (v 2.2) against UniProt-human protein data base (for lysate vs. Nanodisc in SDC samples). For all other samples, the data was searched with MSFragger and FragPipe using default proteomic workflow settings.

Protein enrichment analysis was performed using a GO tool.

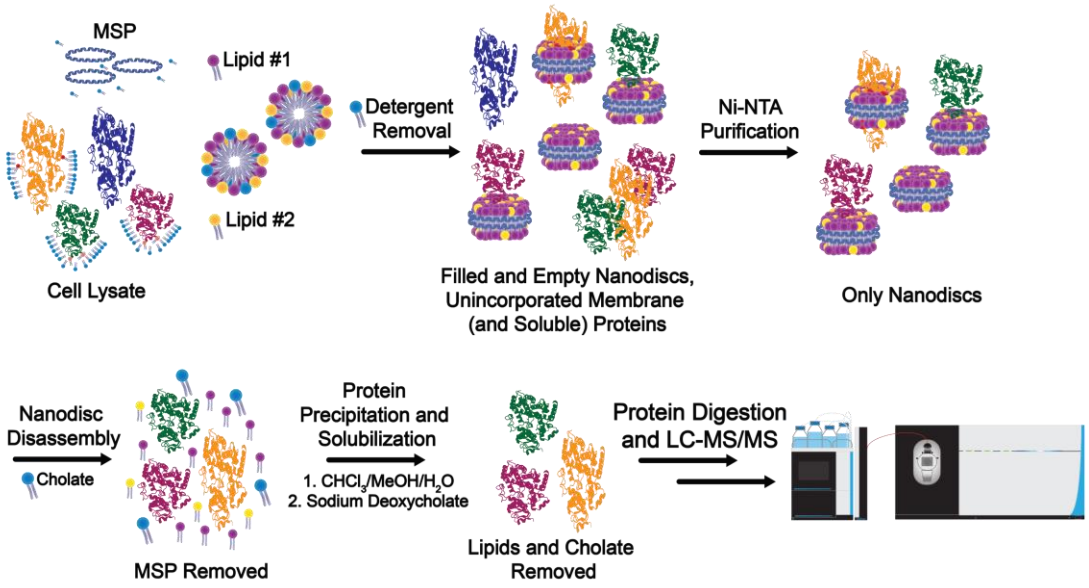

Figure 4-1 Workflow displaying Nanodisc synthesis and sample preparation prior to bottom-up proteomics analysis. First Nanodiscs are synthesized with different lipid compositions or detergents. Next, Nanodiscs are simultaneously purified and disassembled using a Ni-NTA column. The eluted membrane proteins are then precipitated to remove lipids and cholate. Finally proteins are digested and analyzed by LC-MS/MS.

4.3 Results and Discussion

4.3.1 Optimization of library Nanodisc workflow to improve protein quantity and MSP depletion

Library Nanodiscs require optimization of multiple component ratios since they have an extra layer of complexity with the potential for native lipid incorporation from the membrane extract. This essentializes the optimization of both the MSP:lysate and lipid:MSP. Additionally,

the library Nanodiscs desired were made with different detergents and more complex lipid compositions than conventional library Nanodiscs. To maximize the membrane protein concentration and minimize extraneous MSP that would be a contaminant for proteomics experiments, we chose a 4:1 MSP to membrane protein ratio. For the varied detergent and lipid compositions, we modified the lipid to MSP ratios that were previously optimized in chapter 3 by lessening the ratio proportionally to the 100% POPC condition (90:1). We settled on 60:1 for Triton X-100 and 70:1 for DDM (**Figure A-V-1A-C**).

Next, MSP depletion prior to bottom-up proteomics is required to avoid ion suppression due to it being present in excess relative to our proteins of interest. Taking advantage of a His-tag present on MSP, we bound Nanodiscs to a Ni-NTA resin and then added a cholate solution to disassemble the Nanodiscs, releasing the incorporated membrane proteins while keeping MSP mostly bound. Previous studies that have used this methodology have used up to 200 mM cholate to disassemble the Nanodiscs, though we found that this was not depleting the MSP enough from our samples. We tested guanidine hydrochloride and urea as alternative methods, but this resulted in MSP elution and/or membrane protein loss (**Figure A-V-3A lanes 5,6,8,9**). Ultimately, we settled on a new Ni resin and a less concentrated cholate solution, 25mM to significantly deplete MSP while recovering a large number of proteins (**Figure A-V-3B, lanes 5 and 8**).

After precipitating the resulting membrane proteins to eliminate excess cholate and lipids from the solution, we tested different solubilization conditions (SDC and RG, both removable with acid addition) prior to the protein digestion step and compared them to using Laemmli buffer via SDS-PAGE. SDC has been previously shown to outperform RG and identify more membrane proteins. We found that SDC and RG worked similarly, but SDC resulted in marginally better solubilization relative to the starting non-pelleted lysate (**Figure A-V-4B, lanes 1 and 2**).

4.3.2 Nanodiscs identify unique proteins and enrich membrane proteins relative to starting lysate

We first aimed to analyze a single Nanodisc condition to confirm Nanodiscs could enrich membrane proteins relative to the starting membrane extract and that membrane proteins would be in large enough quantities to get a large number of protein identifications. We isolated membrane proteins from HeLa cells and used them to synthesize library Nanodiscs in cholate and 100% POPC. The Nanodiscs underwent purification and disassembly to liberate membrane proteins that were incorporated into the Nanodiscs. These proteins were analyzed by bottom-up proteomics following trypsin digestion and compared between the starting membrane extract and the Nanodiscs. We found that Nanodiscs identified unique proteins relative to the starting membrane extract (**Figure A-V-5**). We also compared the two surfactants utilized in the solubilization of the membrane protein pellet prior to digestion and found they had a lot of overlapping protein identifications, but each were able to selectively enrich unique proteins. For following studies, we selected SDC due to ease of access and a larger number of unique identifications. In addition to identifying unique proteins, Nanodiscs were able to enrich for membrane proteins relative to the starting membrane extract (**Figure A-V-6**). These findings propelled us to elucidate whether different Nanodisc synthesis conditions such as detergent selection and lipid composition could enrich different membrane proteins.

4.3.3 Detergent selection results in unique protein identifications, but minimal changes in identified protein function or cellular location

Detergents are commonly used in lysis buffers to compromise the integrity of cell membranes and subsequently solubilize membrane proteins.[146,147] Previously, we have shown that detergent selection also impacts the lipid composition of Nanodiscs (see Chapter 3). We sought to determine if in addition to modulating the lipid landscape, detergent selection could alter which membrane proteins were enriched in Nanodiscs. To do this we synthesized Nanodiscs in cholate, DDM, and Triton X-100. Cholate is the most common detergent utilized in Nanodisc synthesis, and DDM and Triton X-100 are both common non-ionic detergents in lysis buffers and some of the most common detergents used for membrane protein stabilization.[145] Preliminary results show unique protein identifications between the three detergent conditions (**Figure 4-2A**). Preliminary analyses were done to group identified proteins by primary function and cellular location, but there were minimal changes in these broad categories across different detergent conditions (not shown). More analysis is required to determine which proteins or protein classes are enriched specifically. These results point to detergent selection as an important variable for membrane protein enrichment, perhaps due to improved or reduced structural integrity of different membranes in various detergents.

4.3.4 Lipid complexity results in unique protein identifications, but minimal changes in identified protein function or cellular location

Membrane proteins are known to localize to different lipid environments, so it is likely that Nanodiscs with different compositions would enable membrane protein enrichment. This has been shown previously in Nanodiscs with four different lipid compositions – 100% POPC, 80%

POPC/20% POPS, 80% POPC/20% Cholesterol, and 72% POPC/20% POPS/8% Cholesterol.[36] These compositions were shown to enrich different membrane protein classes as well as result in different activity levels two classes tested, Na^+/K^+ ATPases and kinases. We thought a more complex lipid environment could enrich more proteins than 1-3 lipid mixtures. We compared Nanodiscs made with 100% POPC and 100% Liver extract, along with a less complex lipid mixture

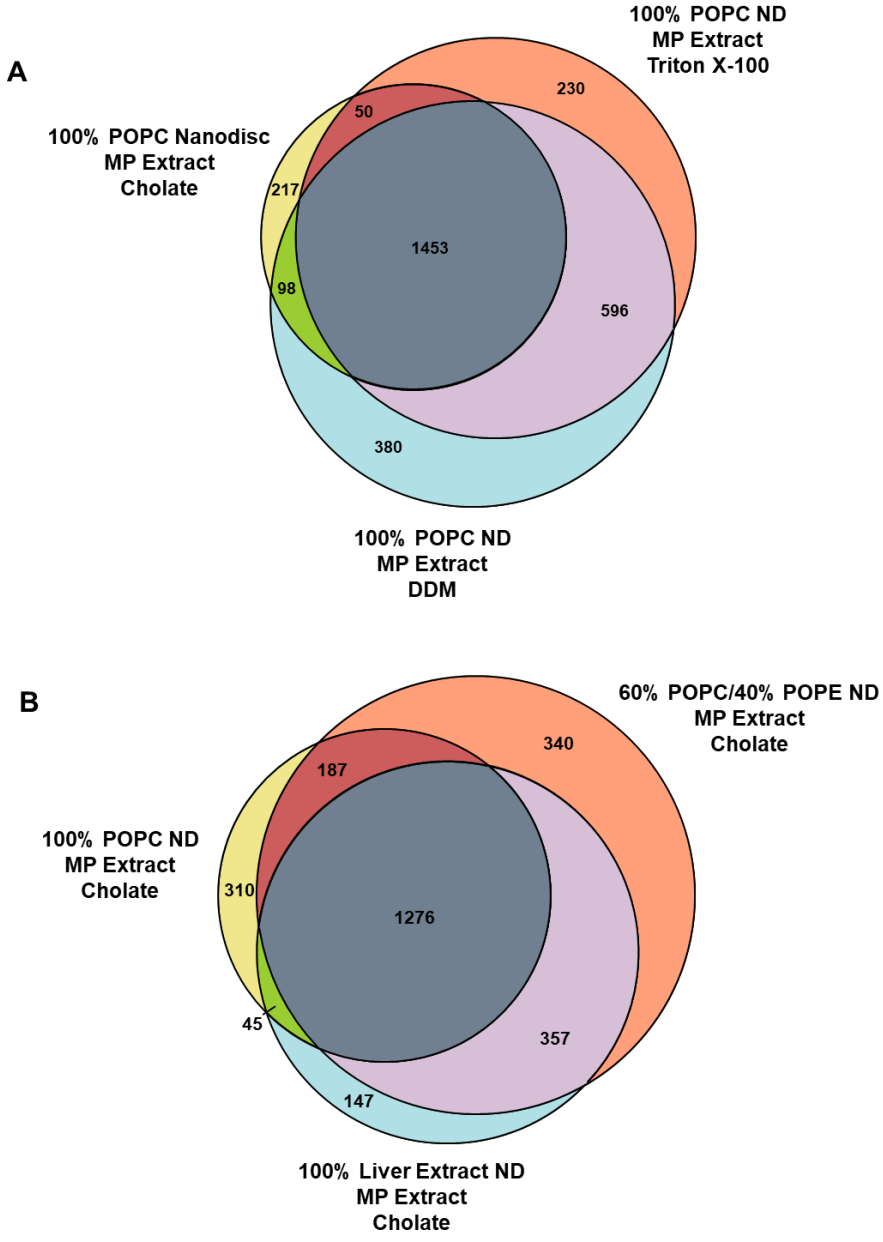

Figure 4-2 Nanodiscs made with different detergents and lipids result in unique protein identifications. Protein identification landscape in A) Nanodiscs synthesized with different detergents and B) Nanodiscs synthesized with different lipid compositions. Changing the detergent and lipid composition results in unique protein identifications.

in between the PC and liver condition – 60% POPC/40% POPE. Preliminary results depict all three compositions enrich unique proteins (**Figure 4-2B**), but it was the PC/PE that had the greatest number of unique protein identifications, even compared with the most complex liver extract composition, likely due to the liver extract representing an average membrane composition. Preliminary analyses were done to group identified proteins by primary function and cellular location, but there were minimal changes in these broad categories across different Nanodisc lipid compositions (not shown).

4.3.5 Nanodiscs made with whole cell lysate enrich membrane proteins similarly to membrane extract Nanodiscs

Most studies that synthesize library Nanodiscs, including those that have done proteomics analyses, have started with a membrane extract rather than whole cell lysate.[36,144] This enriches the membrane protein fraction to improve incorporation efficiency and membrane protein detection. However, it would be advantageous to skip the membrane isolation step to save time in the synthesis and facilitate native lipid incorporation. Library Nanodiscs have been synthesized directly from whole cell lysate previously.[37] We sought to evaluate if we could enrich similar classes of membrane proteins once starting from whole cell lysate instead of a membrane extract. Preliminary results show that Nanodiscs made with the membrane extract and the whole cell lysate contained unique proteins (**Figure A-V-7**); however, more analysis is required to determine how many of the proteins in the whole cell lysate condition are potential soluble protein contaminants. Interestingly, preliminary analyses of identified proteins from Nanodiscs made with the membrane extract versus whole cell lysate showed more enrichment of membrane proteins in the whole cell

lysate Nanodiscs, perhaps suggesting that an initial membrane enrichment step is not necessary (not shown), but more work needs to be done at the individual protein level to ascertain what information or proteins would be lost without the enrichment step.

4.4 Conclusions

This worked aimed to discover if modulating Nanodisc synthesis conditions such as the detergent selection and lipid composition could alter the membrane proteins enriched in the resulting Nanodiscs. We developed a workflow to synthesize Nanodiscs from either membrane enriched fractions or whole cell lysate from HeLa cells. These Nanodiscs were then bound to a Ni-NTA column and disassembled to release incorporated membrane proteins while depleting MSP from the samples. A bottom-up proteomics approach was then used to analyze the incorporated proteins across these different Nanodisc conditions. We found that these different conditions identified unique sets of proteins, but preliminary results that grouped identified proteins by function or cellular conditions did not show many global changes in protein classes across conditions. More analyses that look at the individual proteins identified across these samples or relative quantitation could shed light on more significant changes between these samples.

Of note, we found significant cytosolic protein contamination in all samples, even when starting with the enriched membrane fraction. Additionally, we found significant nonspecific absorption to the Ni-NTA columns as there were many proteins identified in membrane fraction or whole cell lysate samples that were not incorporated into Nanodiscs. A more specific binding step, like FLAG or biotin-streptavidin, is required to deconvolute which proteins are enriched due to the Nanodiscs or nonspecific interactions with the nickel resin. With these improvements, these

findings could potentially serve to enrich different membrane protein types for various downstream analyses or to improve membrane proteome coverage.

Chapter 5 Conclusions and Future Work

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5.1 Dissertation Summary

The goal of the thesis work presented here was to increase our understanding of lipid incorporation into Nanodiscs. Previously, there were few studies that analyzed the lipid composition in Nanodiscs, and the tools available are either limited in mutliplexability or ease of use. To remedy this, we developed targeted and untargeted LC-MS/MS methods to profile lipids in Nanodiscs. Using the targeted approach, we quantified lipids in Nanodiscs and found that lipids do not always incorporate stoichiometrically, and this often depends on lipid properties such as curvature and fluidity. We then utilized the untargeted approach to observe how the lipid landscape in Nanodiscs was altered upon modification of synthesis parameters such as detergent, temperature, MSP size, and synthetic lipid supplements. We found that detergents alter the lipid pool available to be incorporated into Nanodiscs. Additionally, temperature and MSP size alter specific lipid species that are incorporated. The most important determinant were synthetic lipid additives that greatly affected lipid species and saturation levels incorporated into Nanodiscs.

Lastly, we attempted to use these findings to tailor Nanodisc synthesis conditions and lipid compositions to enrich specific membrane protein classes into Nanodiscs. We found that both detergents and lipid compositions can incorporate different membrane protein. Taken together, this thesis provided much needed analytical characterization tools to better understand Nanodisc lipid environments and how to use this knowledge to better design Nanodiscs for membrane protein enrichment.

5.2 Future Directions

5.2.1 Expand lipid and detergent screens and evaluate the effect of membrane protein incorporation

Chapters 2 and 3 detailed both lipids that did not incorporate into Nanodiscs stoichiometrically and synthesis conditions that could enrich different lipid classes, respectively. Both studies only utilized a few lipids and detergents, but future work can evaluate more of them for a more comprehensive overview of their effects on Nanodisc physical properties and lipid composition. For example, the targeted method in chapter 2 could be expanded to include lipids of interest discovered with the untargeted approach in chapter 3. The detergents initially screened in chapter 3 were meant to represent diverse detergent classes common in cell lysis buffers and membrane protein solubilization. Future work could analyze more detergents either within the same class(es) to uncover Nanodisc physical property and/or composition changes, or across other detergent classes and properties such as molecular weight. Integration of current and future results with other Nanodisc measurements such as structure, stability, dispersity, diameter, etc. would be beneficial to link lipid composition to physical property alterations. A more comprehensive analysis that includes more lipids and/or detergents would allow for a library of synthesis

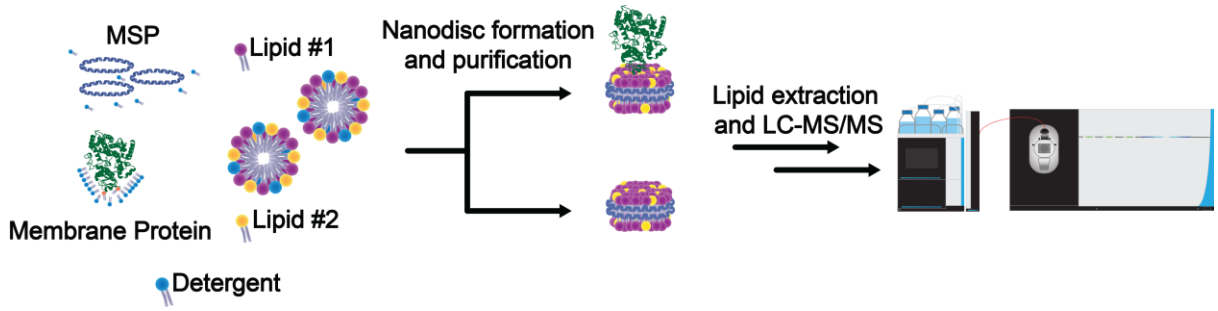

Figure 5-1 Examining the effects of membrane protein incorporation on lipid incorporation into Nanodiscs. Nanodiscs can be made with recombinantly expressed membrane proteins or cell lysate and lipidomics analyses can be performed on empty and filled Nanodiscs for comparison.

conditions to follow to achieve desired Nanodisc compositions. Interestingly, this has the potential to be combined with a machine learning approach that could utilize lipid physical properties and outcomes from Nanodisc syntheses to predict conditions to use for a desired Nanodisc composition. This would immensely improve the utility and robustness of Nanodisc experiments.

Perhaps more important than only few lipids and detergents being analyzed, these studies were only done on empty Nanodiscs, but most of the time Nanodiscs are filled with membrane proteins. It would be interesting to evaluate whether the trends discovered hold when membrane proteins are incorporated into Nanodiscs. For example, utilizing a targeted approach, one could compare the lipid compositions in filled versus empty Nanodisc lipid compositions by separating filled Nanodiscs (via a tag on a membrane protein for example) from empty Nanodiscs in the same mixture (**Figure 5-1**). Multiple questions could be answered such as does this composition match our results when empty Nanodiscs were used? Can membrane proteins selectively incorporate different lipids? Previous research suggests membrane proteins can remodel their lipid environment.[108] A similar approach could be used to look at more complex lipid mixtures with the untargeted lipidomics method.

5.2.2 Synthesize "designer" Nanodiscs that match expected lipid compositions

Chapter 2 of this thesis discussed lipid incorporation disparities in Nanodiscs based on lipid physical properties. Lipids that were more conical in shape and lipids that induced more membrane rigidity were incorporated into Nanodiscs less than expected considering the input lipid ratios. These studies were performed utilizing lipids common in Nanodisc literature. We can imagine instead, carefully crafting the lipid mixtures and ratios to produce Nanodiscs in which the final lipid composition matches the intended composition. For example, the work in Chapter 1 discusses different endoplasmic reticulum mimic compositions (7 lipid mixture) comparing two different PC's and the final compositions were very different. This lays the groundwork for modulating lipid physical properties such as chain length and unsaturation to be able to get a desired composition. Chapter 3 detailed the effects of synthesis conditions on lipid incorporation and despite it not being a quantitative study, it provides a starting point for lipids to use for these "designer" Nanodiscs.

5.2.3 Integration of other membrane mimetic structures into targeted lipidomics workflows to achieve desired lipid compositions

Based on our findings in Chapters 2 and 3, it is likely that some lipid compositions are not feasible in a planar Nanodisc structure. One example of this is for compositions that need high percentages of PE lipids that do not incorporate as well due to their curved structure. A possible approach is to integrate other membrane mimetic structures into our targeted LC-MS/MS pipeline from chapter 2 to get coverage of a wide range of lipid compositions. For example, mimetics that are more curved like liposomes would be beneficial for compositions requiring PE. Comparison studies could be performed on different mimetic structures like bicelles, liposomes, MSP

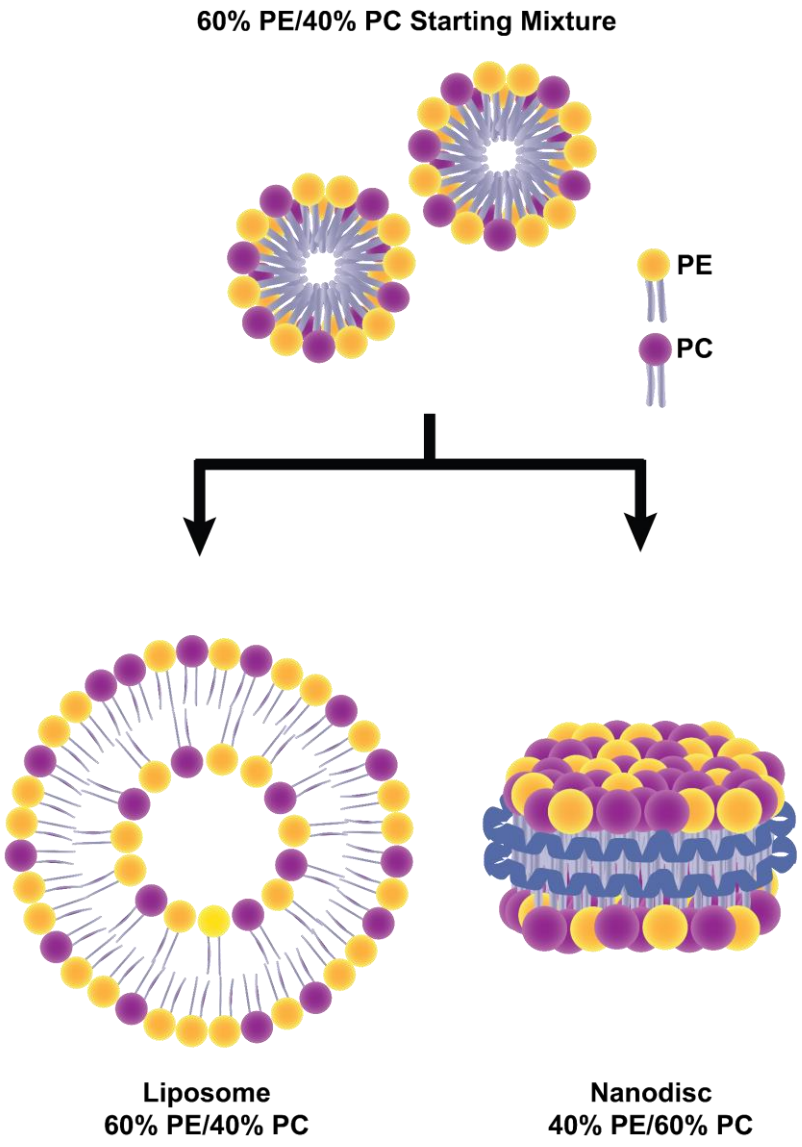

Figure 5-2 Depiction of stoichiometric incorporation of PE into a liposome versus nonstoichiometric incorporation of PE into Nanodiscs.

Nanodiscs, and polymer Nanodiscs, to observe how efficiently lipids are incorporated across these systems (**Figure 5-2**). Additionally, the changes in lipid composition upon incorporating a membrane protein could be discerned. This can be paired with studies being performed that look to evaluate the effect of different membrane structures on membrane protein structure as well activity. Altogether, this approach would allow membrane protein researchers to select mimetic

structures that would reflect desired lipid compositions, with the knowledge that certain mimetics can perturb membrane protein measurements in predictable ways.

5.2.4 Identification of native lipids incorporated into Nanodiscs from lysate

Chapter 3 discussed the impact of Nanodisc synthesis conditions on the resulting Nanodisc lipid composition using an untargeted lipidomics approach. In this work, a mixture of natural lipid extracts was used to model the complexity of native membranes and served as the groundwork for eventual expansion of this work to library Nanodiscs made from cell lysate. In library Nanodiscs, there is the potential for native lipids to be incorporated into the Nanodiscs in addition to the supplemental synthetic lipids. These lipids are important to identify as it has been shown that lipids can modulate proteins both allosterically and via bulk environment effects on membrane protein geometry and localization.[11–13,148] Future work would apply this method to library Nanodiscs to evaluate which native lipids are enriched under specific conditions. This work can be extended to specific membrane proteins of interest. Membrane protein-filled Nanodiscs can be isolated with a dual purification workflow and utilizing a lipidomics approach, we could evaluate which native lipids are incorporated with different membrane proteins (**Figure 5-3**). This could be useful for evaluating their impact on structure and/or function. Another option to facilitate studying these native lipid-membrane protein interactions is to apply a cross-linking approach to evaluate protein-lipid interactions.[149,150]

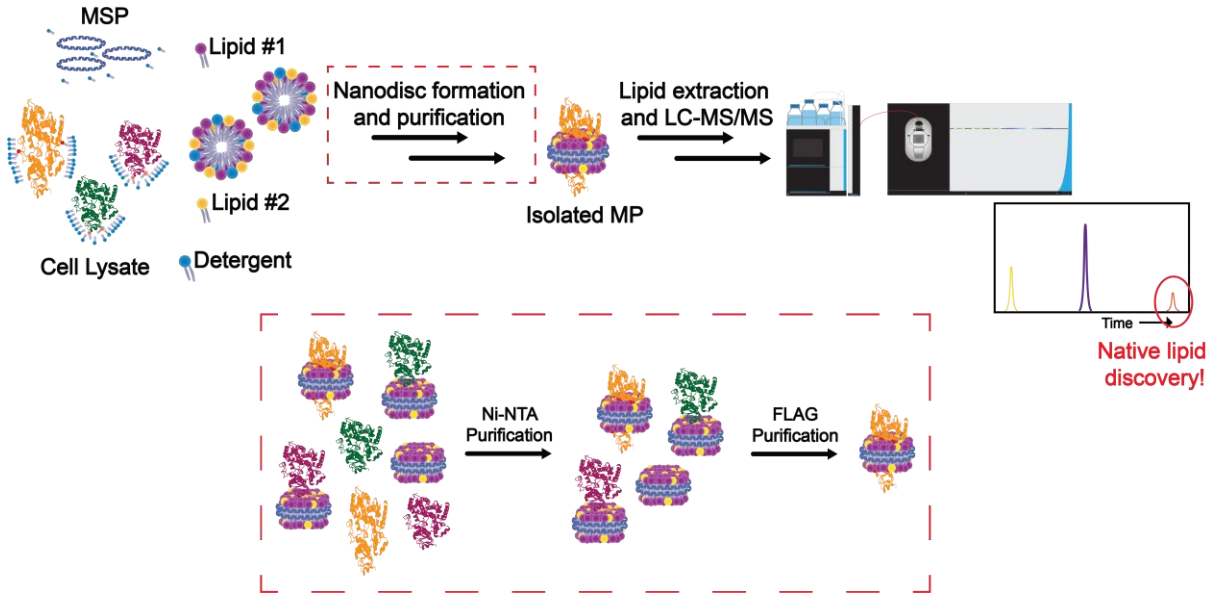

Figure 5-3 Untargeted lipidomics analysis to discover native lipids incorporated into Nanodiscs. Library Nanodiscs are synthesized and a membrane protein-filled Nanodisc of interest can be isolated via a dual purification workflow. Lipids are analyzed with LC-MS/MS to discover native lipids associated with membrane protein of interest.

5.2.5 Membrane protein analyses

This thesis discussed the development of methods to better characterize Nanodisc lipid compositions for quality control as well as for tailoring Nanodisc synthesis conditions to enrich specific lipid and protein classes. The next logical extension of this work is to apply this knowledge to membrane protein analyses. With new knowledge of how lipid physical properties influence Nanodisc lipid composition and how different detergents can enrich specific lipid classes in Nanodiscs, we can strategically synthesize Nanodiscs with lipid compositions that increase the incorporation efficiency of membrane proteins. This is specifically important for studies that aim to isolate Nanodiscs filled with a specific membrane protein from a library of Nanodiscs, since it would not be present in as high of abundance as when starting with a recombinantly expressed protein.

Previous work in our group aimed to isolate Epidermal Growth Factor Recpetor-filled Nanodiscs from library Nanodiscs for downstream activity assays (commercialized phosphate assays).[40] This proved to be very challenging because after Nanodisc synthesis and multiple rounds of purification to isolate the target protein, there was too little functional protein left for reliable activity assay results. Pairing what we know about detergent and lipid selection (especially if with an overexpression system and/or expressing the protein in lysate with a high-efficiency affinity tag) should increase the amount of protein available for downstream analyses. This should apply to any membrane protein system of interest.

In addition to increasing the incorporation efficiency of specific membrane protein targets, the methods developed here, particularly in Chapter 2, can confirm lipid compositions in Nanodiscs. This means we can determine the effects of lipid composition of the Nanodisc on membrane protein structure and activity. For example, when attempting to develop "designer" Nanodisc systems as discussed above, these conditions could differ in filled Nanodisc systems relative to empty Nanodiscs. We could measure structural and functional changes in the membrane protein with differences in the lipid composition, whether these are intended lipid compositions or test conditions. This would facilitate better membrane protein research as we would know how sensitive proteins can be to very subtle changes in local lipid environment.

5.2.6 Utilization of a microfluidic platform for Nanodisc synthesis

Despite their broad utility, Nanodiscs suffer from long preparation times and large component inputs which can be problematic for membrane proteins that express at low levels. Our group previously developed a microfluidic platform that could produce Nanodiscs on the minute timescale (instead of hours) with less material input. This device operated by flowing the Nanodisc

component mixture through a channel packed with detergent removal resin. The initial iteration was modified with a 3-port mixing channel to allow for Nanodiscs of different lipid composition to be generated via a temporal gradient by tuning the flow rates at the inlets. This platform and some limitations are discussed in greater detail in Appendix I. Briefly, size exclusion chromatography analysis of Nanodiscs produced on the microfluidic device compared to conventional methods showed two different Nanodisc populations, the ones made on device being of smaller size likely due to underlipidation. This was partially remediated by increasing the concentration of Nanodisc components used on device, but ultimately this uses more material which eliminates one of its advantages.

A microfluidic platform would be advantageous for many of the directions discussed above that would require many iterations of Nanodiscs made under different conditions, especially since all of these require extensive optimization (detergent concentration, ratio of lipids to membrane scaffold protein, etc.) which requires large amounts of material. Additionally, a microfluidic platform could be interfaced directly with mass spectrometry for applications that do not require a separation. Keeping the current iteration of the device would require using coatings or biocompatible materials that would limit adsorption of Nanodisc components which is difficult due to all components being amphipathic. One example is a PDMS-PEG block copolymer that when blended with PDMS during device manufacture, spontaneously segregate in the presence of aqueous solutions to create a more hydrophilic surface.[151] Another is to consider utilizing 3D-printed materials, though it would be challenging to make a channel small enough to keep the resin inside, but large enough to allow the removal of the support material.

A complete device redesign would address some other limitations of a resin-packed device such as the uneven flow path that causes more longitudinal mixing and the limited detergent

removal capacity before the resin needs to be regenerated which greatly limits the amount of sample outputted (See **Figure A-I-1**). One possibility is to design a dialysis-based device[152] that would have an unlimited detergent removal capacity, even flow path, and would be operated at flow rates more compatible with mass spectrometry interfacing (**Figure 5-4**).

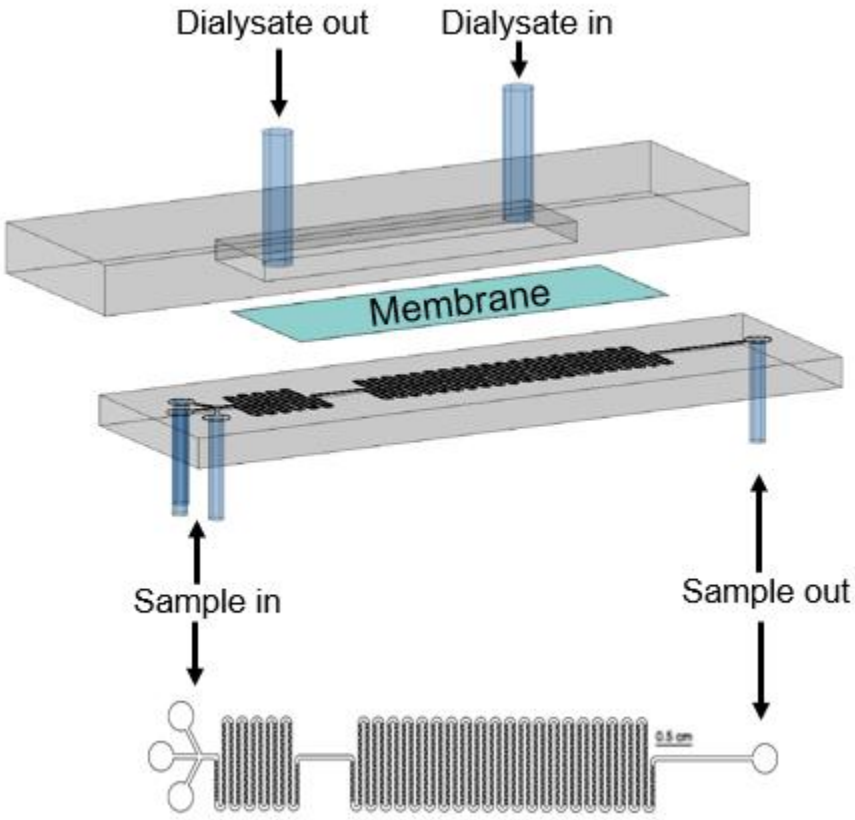

Figure 5-4 . Example of a microfluidic device utilizing dialysis to generate Nanodiscs. Example of a microfluidic device utilizing dialysis to generate Nanodiscs

5.3 Concluding Remarks

Overall, this dissertation took measurable strides toward applying established targeted and untargeted LC-MS/MS approaches to better understand lipid incorporation into Nanodiscs due to physical properties of lipids and Nanodisc synthesis conditions. This work set the stage to develop libraries of synthesis conditions to use for desired lipid compositions as well as to promote the

utilization of analytical techniques in quality control of traditional Nanodisc experiments. Future efforts should work to further integrate lipid composition analysis with Nanodisc physical property measurements as Nanodiscs made with complex lipid compositions continue to gain popularity. This is the only way to ensure an extensive understanding of how the local Nanodisc environment influences membrane protein structural and functional assessments which will improve the robustness of Nanodiscs as a membrane mimetic platform.

Appendix I Optimization of a Microfluidic Platform for Nanodisc Synthesis

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I.1 Introduction

Membrane proteins (MPs) participate in many biological processes such as signal transduction and metabolism. Consequently, innovative approaches to interrogate MPs are vital to characterization. Current chemical and biophysical techniques require solubilized MP removed from the native cell membrane, but MP misfolding and aggregation under non-native conditions limit the capability of these assays. This has prompted the development of membrane mimetics, such as Nanodiscs (NDs), to preserve the native structure and function of MPs in vitro. NDs are composed of a discoidal phospholipid bilayer encircled by a membrane scaffold protein (MSP) belt and are formed via self-assembly once detergent is removed from the solubilized components. Detergent is typically removed via dialysis, a 1-3 day process, or beads, a 2-18h process. Currently, NDs provide an effective method to incorporate a wide range of MP classes that are purified or from whole cell lysate for biochemical characterization.

More recently, NDs have been employed in MS studies, but typical preparation strategies lack the throughput and tunability necessary to comprehensively interrogate the membrane

proteome and the surrounding lipid environment. Previously, we demonstrated a microfluidic device (**Figure A-I-1A**) for on-chip ND self-assembly with structurally sound and active MP incorporation.[39] The PDMS device is fabricated using standard photolithography, includes a 60 μL bed packed with a detergent removal resin, and is operated at 30 μL/min. A purification device was also created with the same dimensions and is packed with Ni-NTA resin (for the His-tag on MSP). One rendition of the ND assembly device includes a 3-port mixing channel (**Figure A-I-1C**) prior to the packed bed and allows for the screening of different component mixtures, such as the ability to tune the lipid composition of resulting NDs. The lipid environment impacts which MPs are incorporated and the structural integrity of these MPs once in the ND. While this device proved the viability of microfluidic ND formation, further optimization is necessary to transition to new applications. A unique challenge for this microfluidic platform lies in monitoring and characterization of resultant NDs at the rate in which they are produced. Therefore, interfacing a newly optimized ND assembly device to proper characterization elements, such as nESI-MS, is highly desired.

I.2 Results and Discussion

I.2.1 Optimization of a microfluidic device did not lead to better Nanodisc formation

Initial work was done with the previously developed microfluidic device. Currently, sub-optimal ND formation, in the form of both aggregation and inconsistent ND distributions in comparison to bulk (**Figure A-I-1I**), has led to the imperative need for an optimized device platform. These issues were especially apparent when utilizing POPC as the lipid, which is integral as a natively produced lipid. Initial steps were to optimize empty ND formation with this device in ways that did not require a device redesign. This included changing operational flow rate,

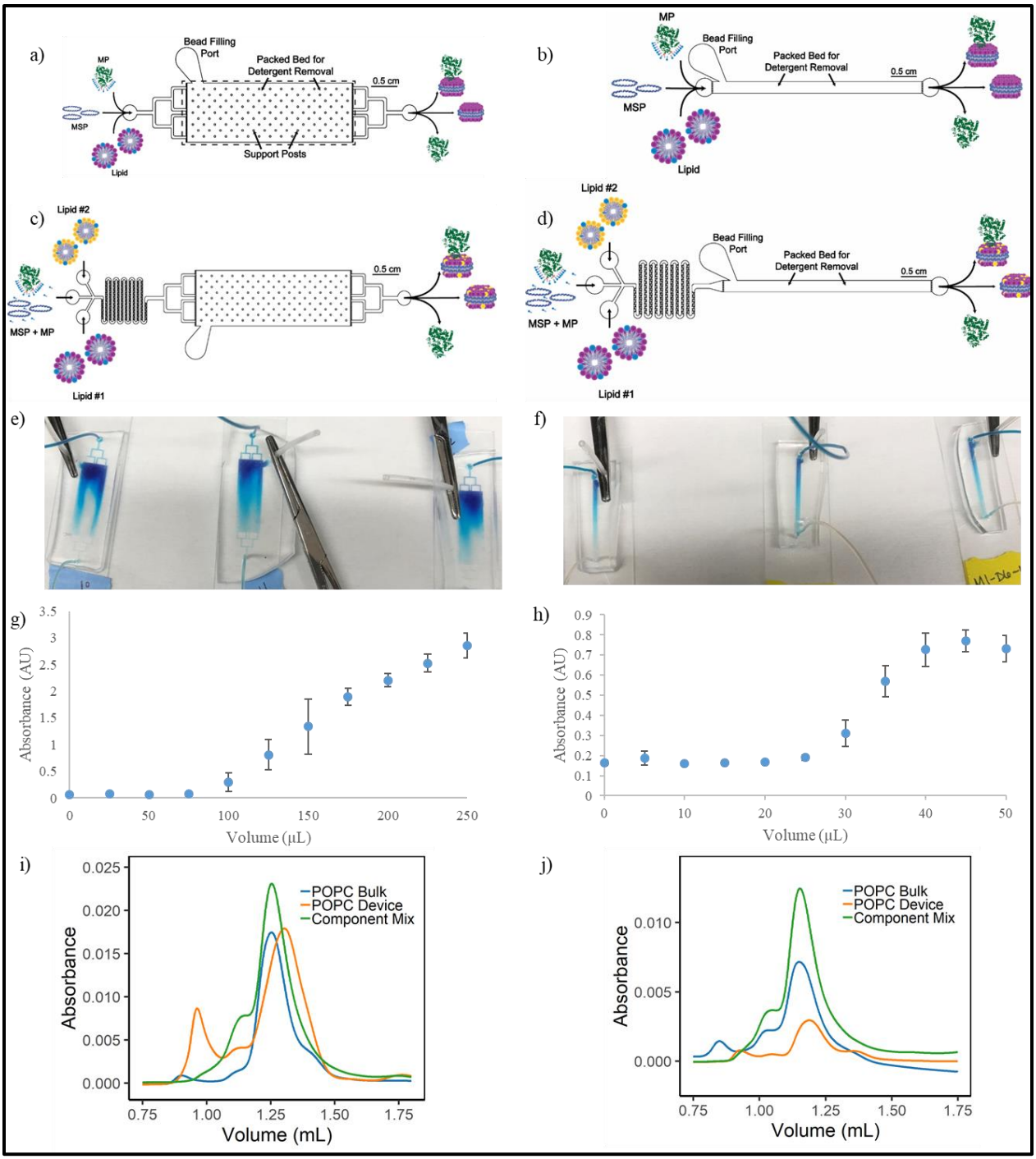

Figure A-I-1 Microfluidic device comparison. Microfluidic device comparison.

MSP:lipid ratios, detergent concentration, temperature, component mix incubation time, and reagent stocks and age. Since none of these changes noticeably improved ND formation, a reimagined device became the most viable solution.

This challenge, coupled with uneven and irreproducible flow patterns in the original device (likely caused by non-uniform bead packing) that would not be compatible with lipid ratio gradient experiments, prompted me to redesign the initial device to a narrower channel approximately 1/6th the initial device volume (**Figure A-I-1B,D**). This volume was chosen by scaling down the expected detergent removal capacity and maintaining a high enough volume to characterize ND formation with SEC. Additionally, it would be operated at lower flow rates to facilitate potential interfacing with complementary characterization techniques, such as MS. Based on initial experiments in which dye was flowed through the device, a much more even flow pattern could be visualized in comparison to the original device (**Figure A-I-1E,F**). Additionally, the smaller device had a higher than expected detergent capacity (**Figure A-I-1G,H**), which was initially attributed to better flow patterns that allowed the component mix to contact more of the beads in the packed bed. Unfortunately, this did not translate to better ND formation based on our current metrics (**Figure A-I-1I,J**).

I.2.2 Bulk Nanodisc experiments help inform microfluidic Nanodisc challenges

The challenges with synthesizing Nanodiscs on device compelled us to investigate Nanodisc fundamentals in bulk. We sought to determine how to make the best and most consistent Nanodiscs in bulk to hopefully translate this information about which variables in synthesis are most important to address the challenges with the microfluidic device. We determined that variation in lipid and MSP stocks, detergent removal bead equilibration, component mixture volume, tube size, and method of mixing played little roles in replicate variation for Nanodiscs. It was not until we compared varying the amount of detergent removal beads (Amberlite) or resin (Pierce) relative to the volume of the Nanodisc component mixture. The recommended ratio of

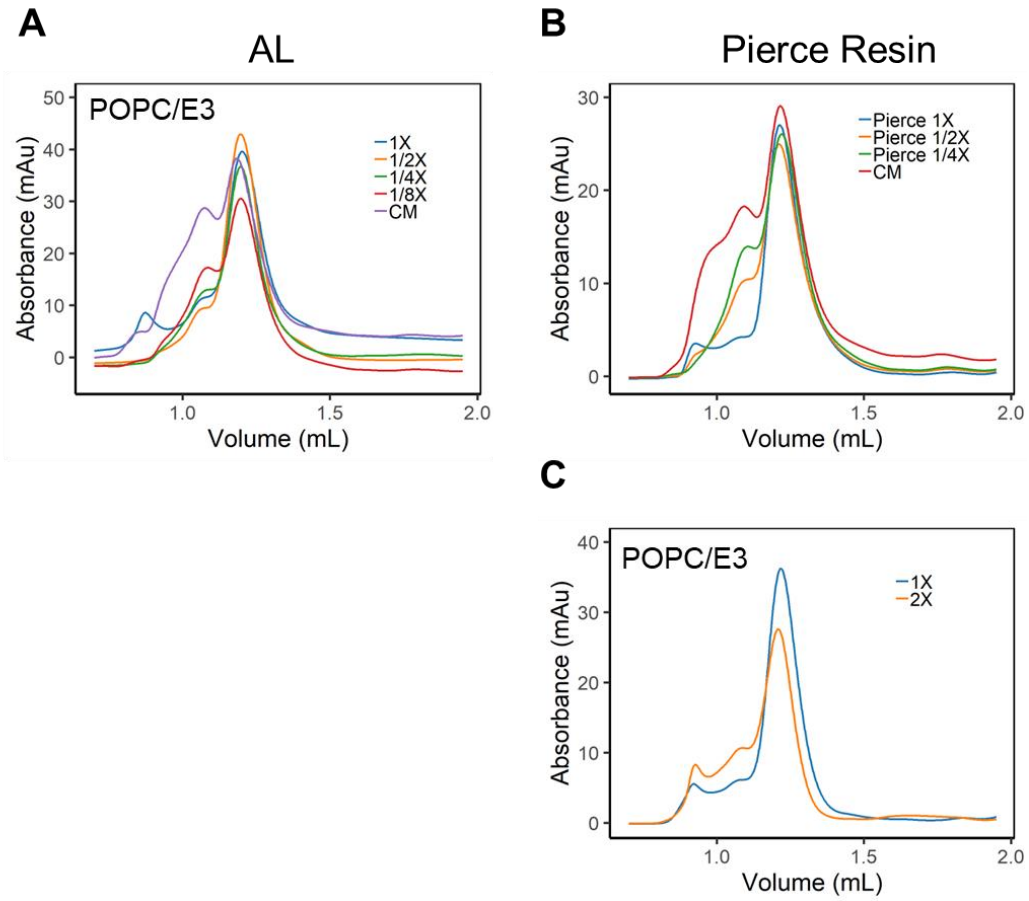

Figure A-I-2 Comparison of Nanodiscs made with detergent removal beads or resin at varying amounts. A) Amberlite beads between 0.125 and 1 g/mL. B and C) Pierce resin between 0.25 and 2 g/mL. Nanodiscs made with less beads or resin resemble the component mixture, while 1-2 g/mL induces more aggregation.

beads to use is between 0.5 and 1 g/mL of component mixture. We found that increasing this beyond 0.5 g/mL lead to an increase in aggregates for POPC/MSP1E3D1 Nanodiscs for both the beads and resin (the resin required 2 g/mL) (**Figure A-I-2**). We realized that considering the 25 µL detergent capacity of the resin and that 55 mg of resin is added on average to each device, that we are on average around 2 g resin/mL of component mixture on device, which could point to why we were observing poorer Nanodisc formation. Additionally, we generally were at lower component concentrations (MSP and lipid) than recommended to conserve materials. These results match the proposed Nanodisc formation mechanism by Sligar and coworkers.[134]

We sought to observe the effects of increasing the component concentration and found that when we compared a 2 mM POPC sample and 10 mM POPC sample (MSP scaled appropriately), that the 2 mM showed a lot more variability depending on the bead or resin amount and the microfluidic Nanodiscs were still shifted and contained more aggregates. In contrast, the 10 mM samples were indistinguishable for all conditions (**Figure A-I-3**). The 2 mM bulk condition was still a high quality Nanodisc, so this points to the concentration being less on the microfluidic device, likely due to material loss since all Nanodisc components are amphipathic making them able to stick to both hydrophobic and hydrophilic surfaces. We concluded either a coating would need to be optimized or the device material changed to something biocompatible (see Chapter 5), since it was not useful to have to increase the concentration of the components that high to achieve good quality Nanodiscs, as this contradicts the goals of using this device to use less material than Nanodiscs in bulk.

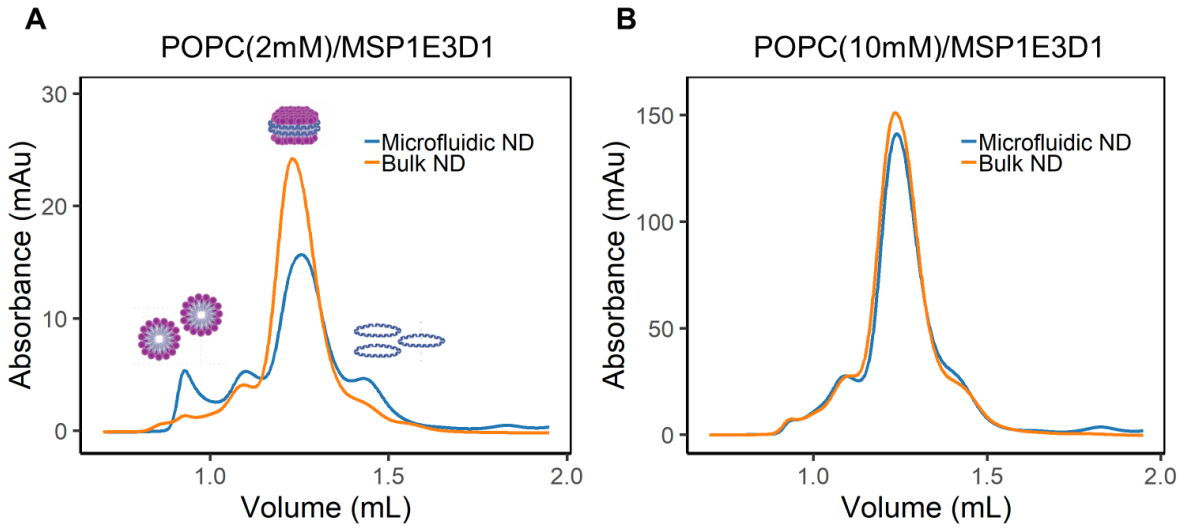

Figure A-I-3 Comparison of microfluidic and bulk Nanodiscs made with different component concentrations. A) 2 mM POPC. Microfluidic Nanodiscs are shifted and have more aggregation. B) 10 mM POPC. Both Nanodiscs match. MSP was scaled appropriately to maintain lipid:MSP ratio.

Appendix II Investigating Methods for Membrane Scaffold Protein Quantitation

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Quantifying MSP is particularly important for downstream applications that require knowledge of Nanodisc concentration as well as for determination of MSP concentration to ensure correct reagent addition depending on the optimized lipid:MSP ratio. Typically, MSP is measured using absorbance at 280 nm or BCA assay, but it has been previously shown that lipids and detergents can interfere with these measurements, hindering the accuracy of the determined MSP concentration. We sought to develop an approach for MSP quantitation in Nanodiscs with the intention of pairing it with lipid quantitation to aid in optimizing lipid:MSP ratios for novel Nanodisc compositions.

To address the challenges with quantifying MSP in its typical matrix, we examined multiple techniques to measure MSP concentration. First was switching to micro-BCA with SDS as an additive, which had been previously shown to be successful in lipoprotein concentration determination. Still we saw inconsistent results between replicates. Next, we tried LC-UV and while we were able to generate a successful method, it was low-throughput (1 hour per sample) and still suffered from needing an accurate concentration for the starting MSP standard. Lastly, we

tried SDS-PAGE with a fluorescent stain, Coomassie Fluor Orange, utilizing Apolipoprotein A-1 as a standard. Apo-lipoprotein A-1 is the native protein that served as the model for MSP sequence engineering. Additionally, unlike stains like Coomassie Blue, Coomassie Fluor Orange shows less dependence on protein molecular weight due to its mechanism of staining. Using Apo-A1 standards between 10 and 500 ng, we were able to make a well-correlated calibration curve and quantify MSP in Nanodiscs (**Figure A-II-1**). Our results showed consistent concentration across Nanodisc replicates, even when loaded in different quantities, which was better than all BCA methods tested.

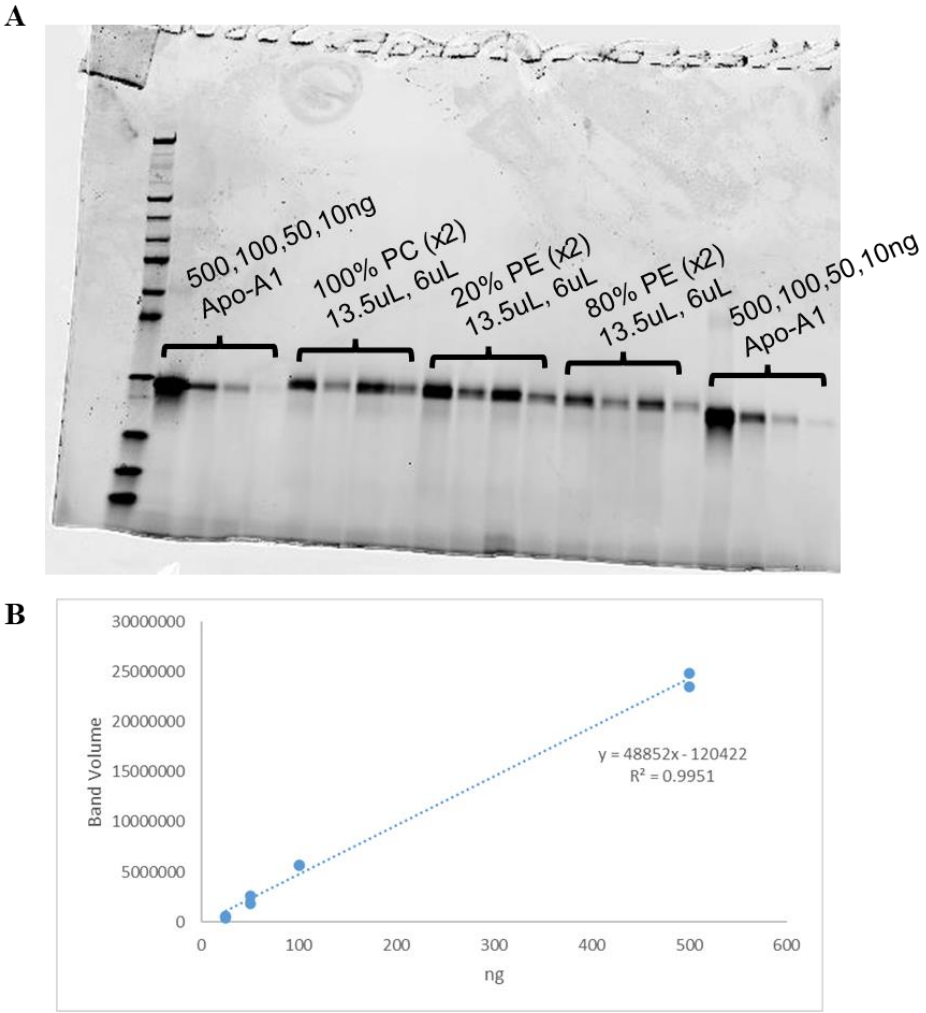


Figure A-II-1 MSP quantitation with Nanodiscs. A) SDS-PAGE gel with Nanodisc samples and Apo-A1 standards. B) Corresponding calibration curve.

Appendix III Chapter 2 Supplemental Materials

Table A-III-1 Gradient method utilized for lipid separation.

	Time (min)	**%A**	**%B**
1	0	40	60
2	6	0	100
3	8.5	0	100
4	8.8	40	60
5	11	40	60

Table A-III-2 MRM conditions utilized for lipid analysis.

Lipid	Prec. m/z	Prod. m/z	Frag (V)	CE (V)	Cell Acc (V)	Polarity
POPC	760.6	184.1	120	20	2	Pos
DMPC	678.6	184.1	122	27	2	Pos
POPS	762.6	577.4	120	20	2	Pos
POPE	718.6	577.4	120	20	2	Pos
DOPE	744.6	603.6	120	30	2	Pos
DPPC	734.6	184.1	90	35	5	Pos
DSPC	790.6	184.1	90	35	4	Pos
DOPC	786.6	184.1	120	19	2	Pos
16:0 SM	703.6	184.1	110	25	1	Pos
18:1 CL	1458	603.5	130	25	1	Pos
Chol	369.3	369.3	60	0	3	Pos
POPA	673.5	255.2	180	40	3	Neg
POPC	804.5	744.7	170	25	1	Neg
DMPC	722.5	662.4	160	20	2	Neg

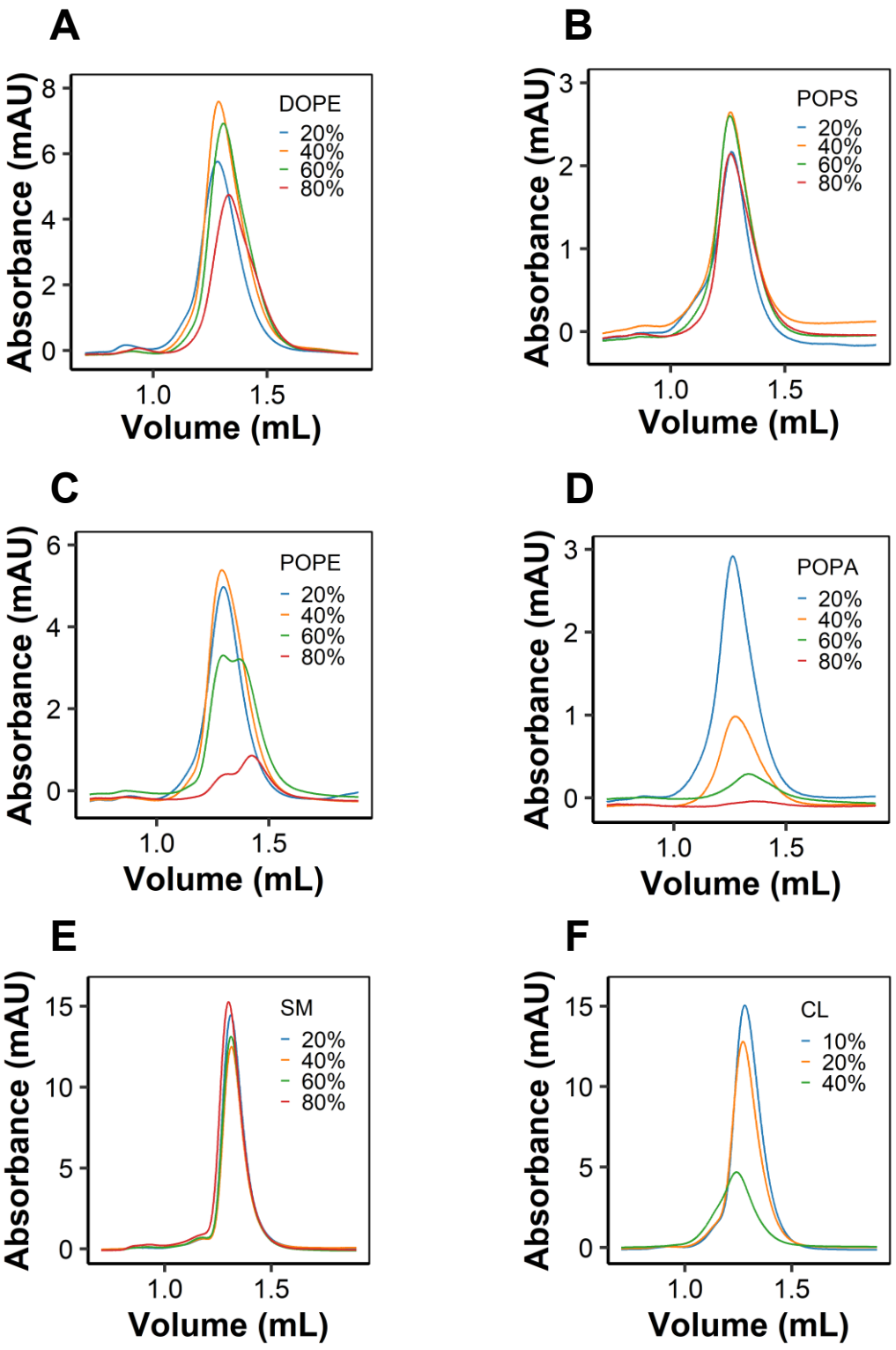


Figure A-III-1 Representative size exclusion chromatograms of Nanodiscs synthesized with binary POPC lipid mixtures and purified with Ni-NTA and SEC. Nanodiscs made with POPC and varying amounts of DOPE (A), POPS (B), POPE (C), POPA (D), SM (E), and CL (F).

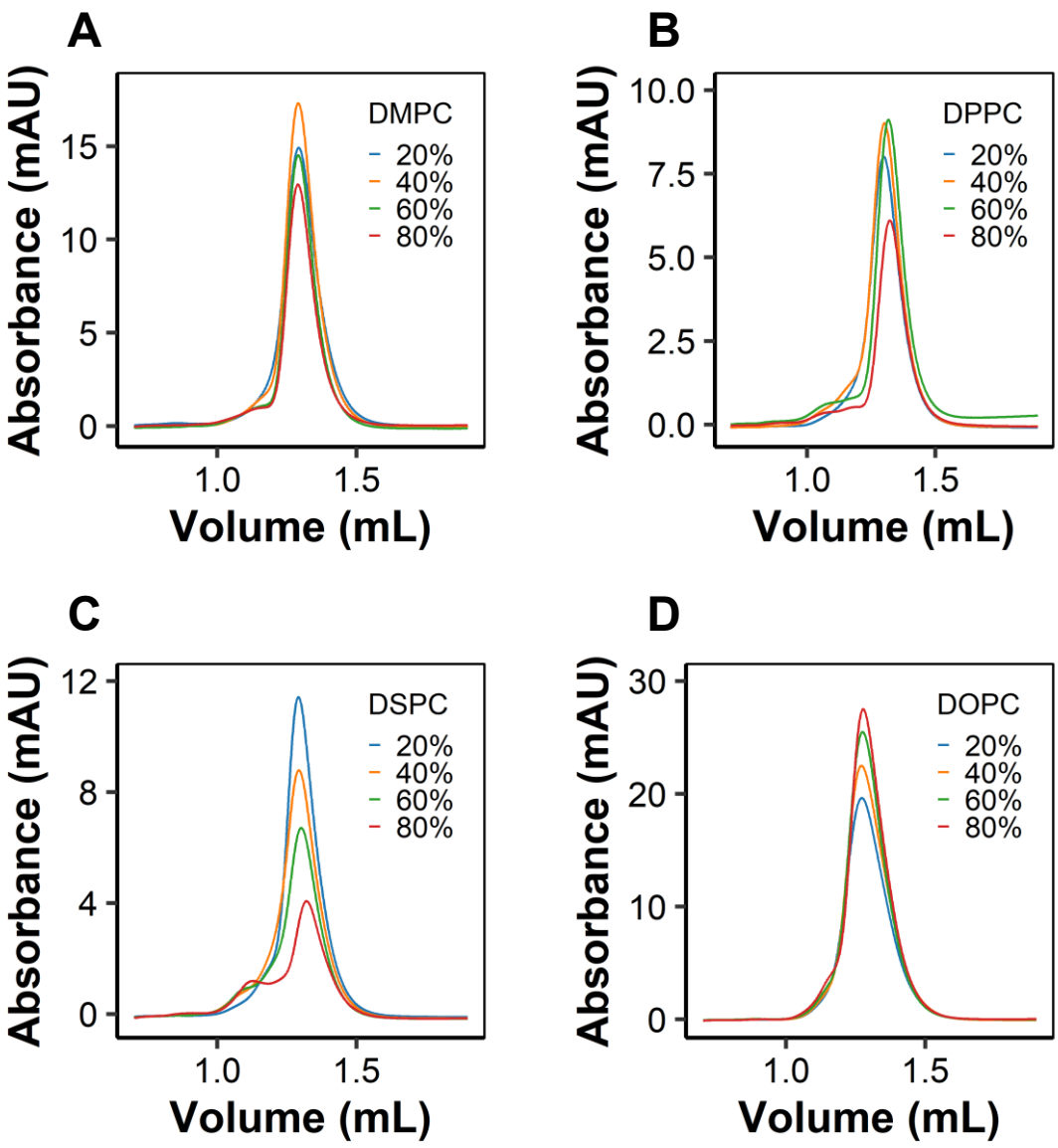


Figure A-III-2 Representative size exclusion chromatograms of Nanodiscs synthesized with binary POPS lipid mixtures and purified with Ni-NTA and SEC. (A) Nanodiscs made with POPS and varying amounts of DMPC. (B) Nanodiscs made with POPS and varying amounts of DPPC. (C) Nanodiscs made with POPS and varying amounts of DSPC. (D) Nanodiscs made with POPS and varying amounts of DOPC.

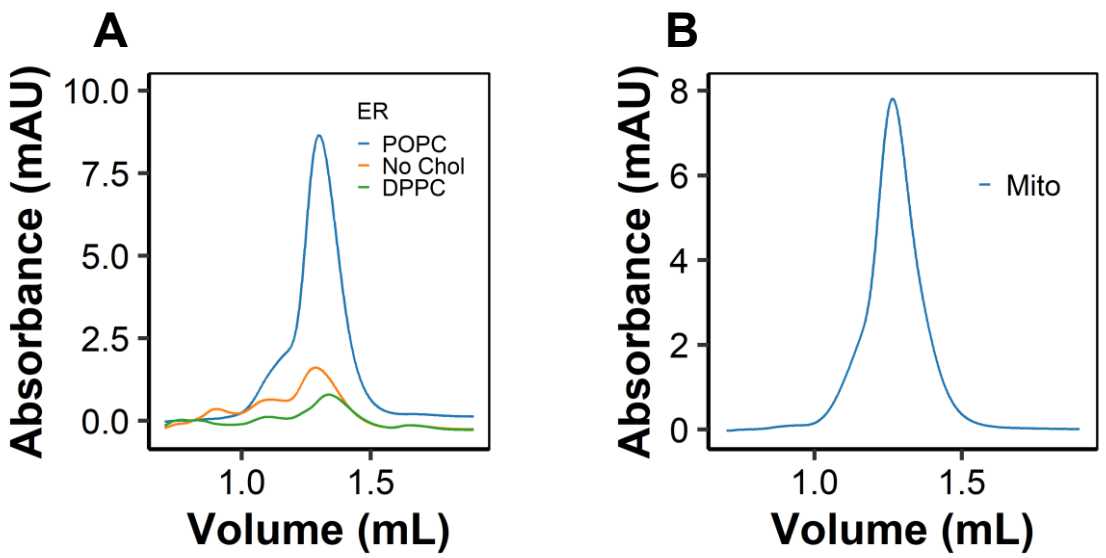


Figure A-III-3 Representative size exclusion chromatograms of endoplasmic reticulum-inspired and mitochondrion-inspired Nanodiscs synthesized and purified with Ni-NTA and SEC. (A) Endoplasmic reticulum membrane Nanodiscs made with POPC, POPC without cholesterol, and DPPC. (B) Mitochondrial membrane Nanodiscs.

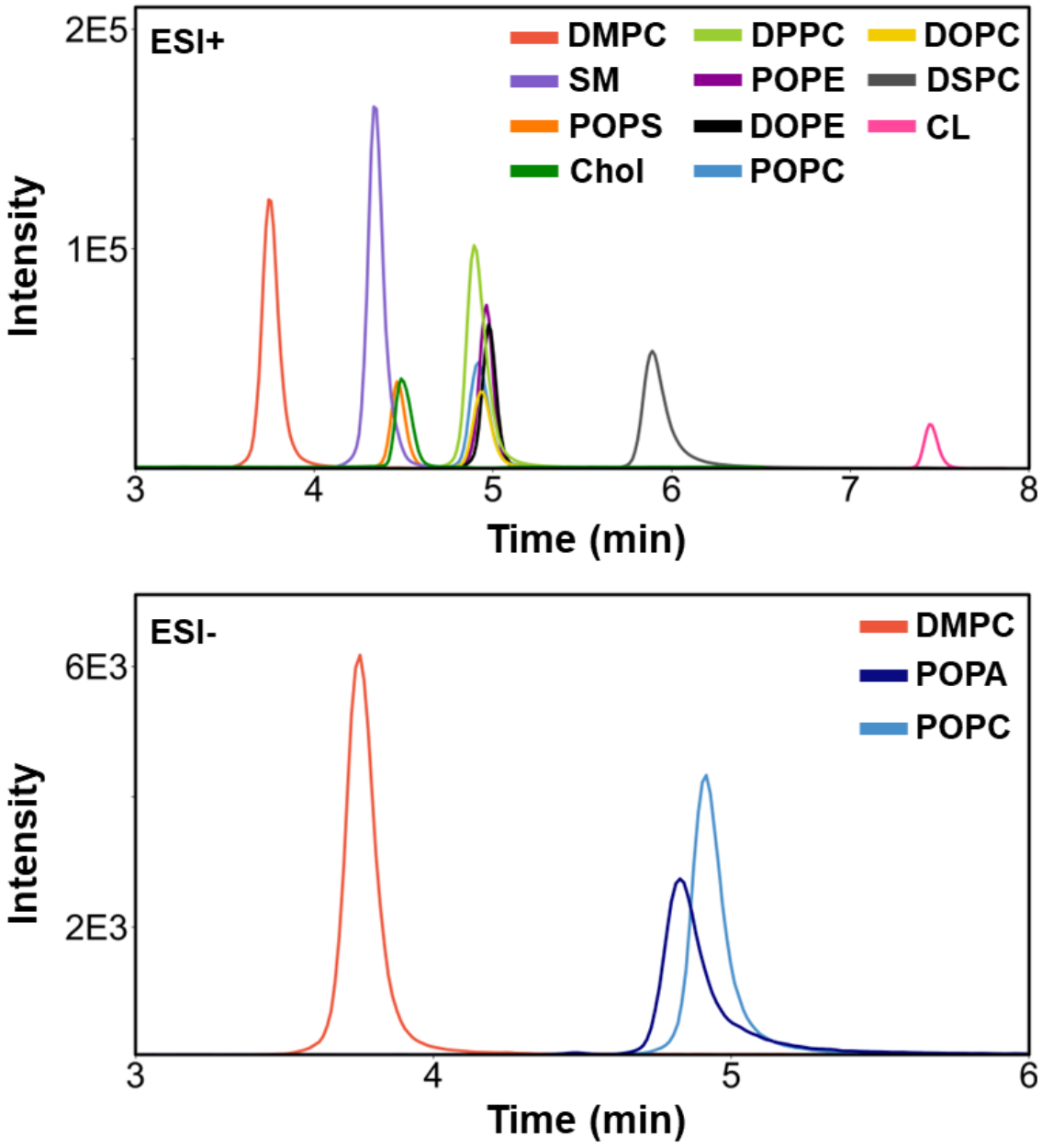


Figure A-III-4 LC-MS/MS method development to quantify lipids in Nanodiscs. (A) Positive mode extracted ion chromatogram. (B) Negative mode extracted ion chromatogram.

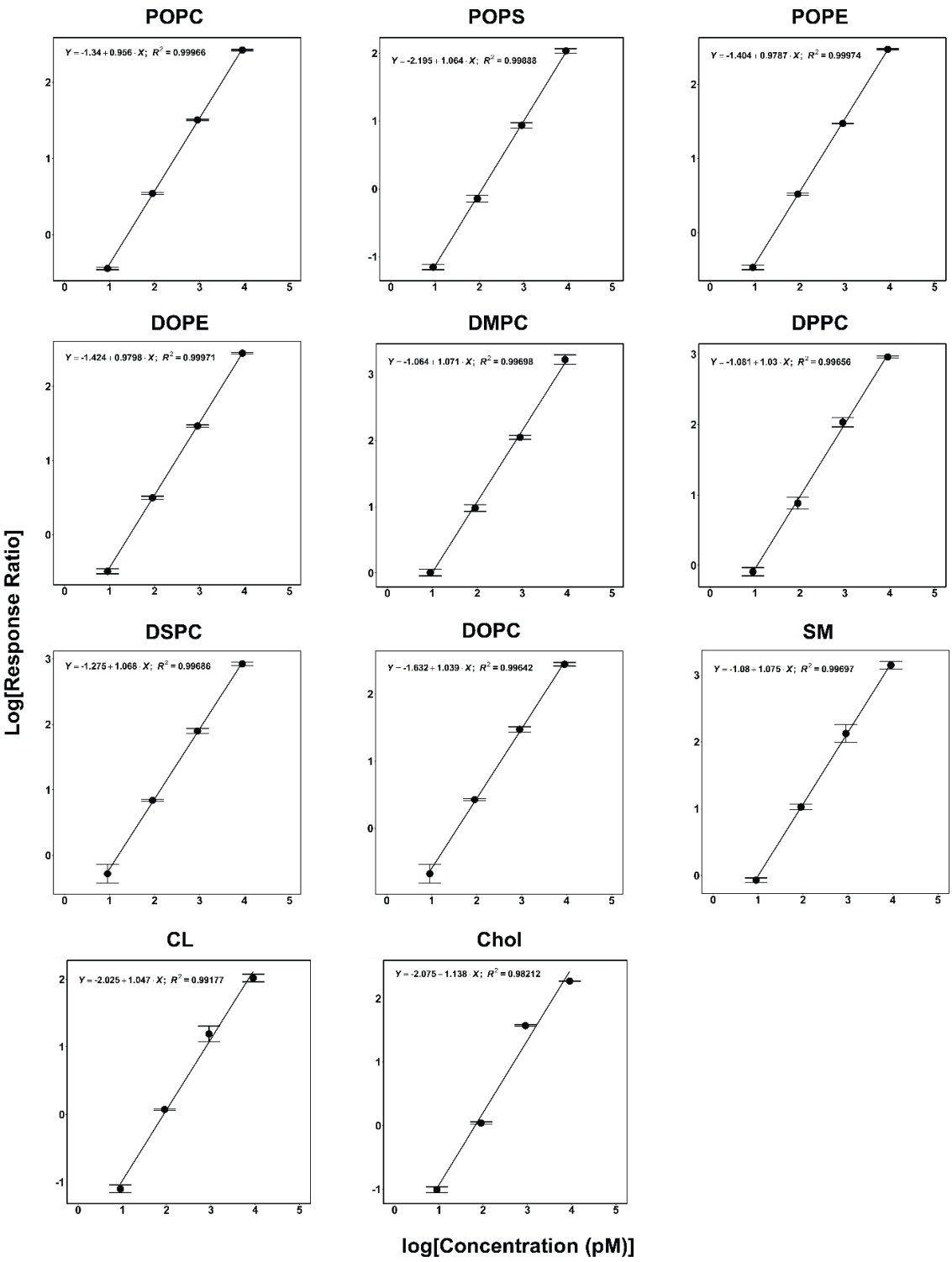


Figure A-III-5 Positive mode calibration curves used to quantify lipids in Nanodiscs.

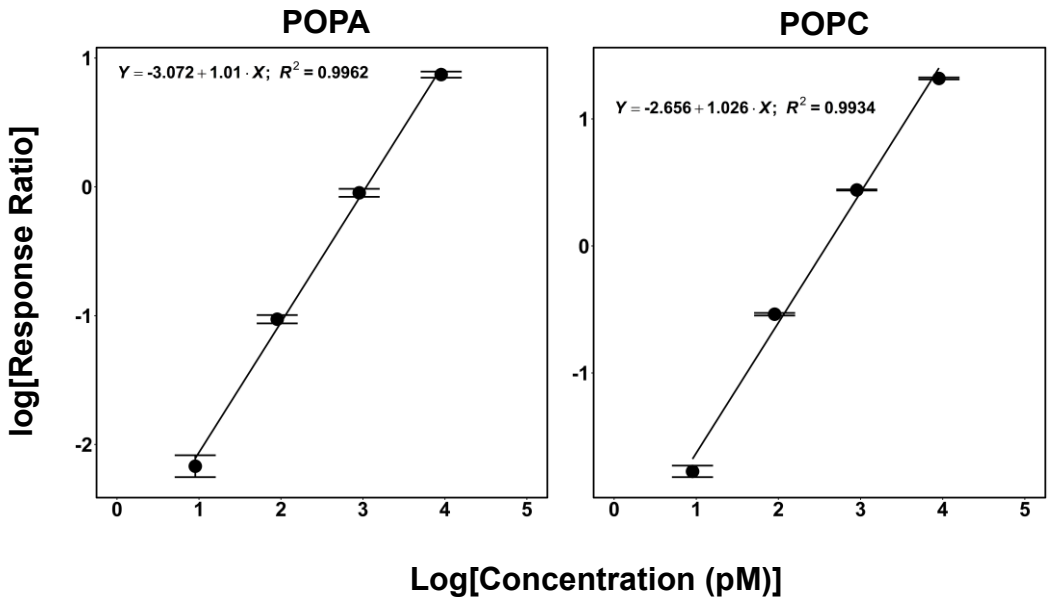


Figure A-III-6 Negative mode calibration curves used to quantify lipids in Nanodiscs.

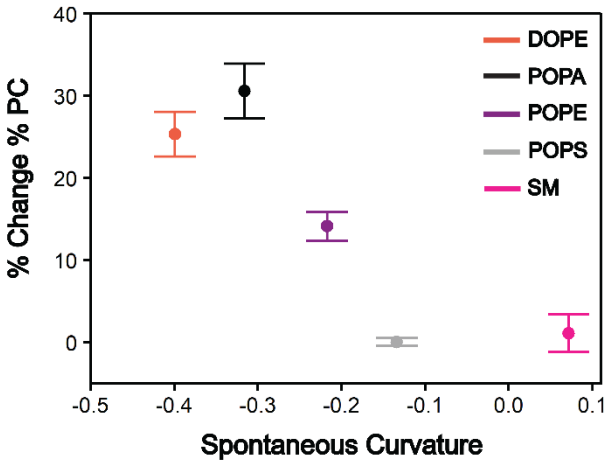


Figure A-III-7 Correlation between POPC enrichment for the 20% POPC condition and lipid spontaneous curvature for different lipid headgroups as found in the literature.[1,2] Error bars are shown as the standard deviation of three replicate Nanodisc assemblies.

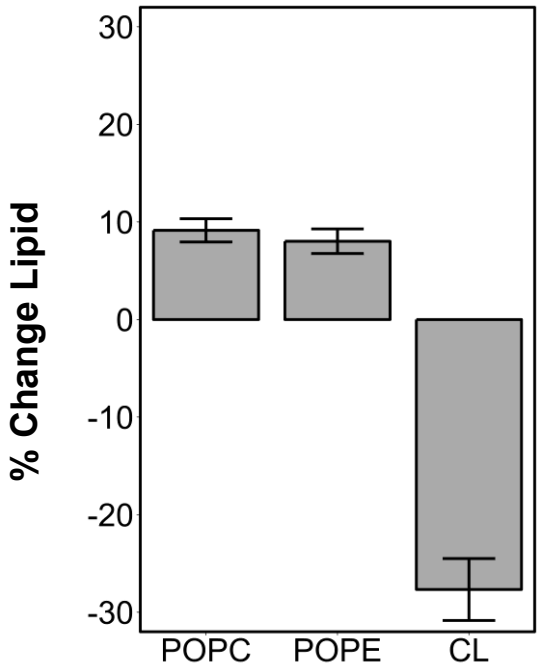


Figure A-III-8 Quantitation of lipids in mitochondrion lipid mixture Nanodiscs. Error bars are shown as the standard deviation of three replicate Nanodisc assemblies.[127,128]

Appendix IV Chapter 3 Supplemental Material

Table A-IV-1 Gradient method utilized for lipid separation.

	Time (min)	**%A**	**%B**
1	0	45	55
2	40	25	75
3	50	0	100
4	55	0	100
5	55.5	45	55
6	62	45	55

Table A-IV-2: Lipid abbreviations.

Abbreviation	Definition
GL	Glycerolipid
PL	Phospholipid
SL	Sphingolipid (including glycosphingolipids)
PC	Phosphatidylcholine
LPC	Lyso-PC
TG	Triglyceride
PE	Phosphatidylethanolamine
PC O	Ether-linked PC
HBMP	Hemibismonoacylglycerophosphate
SM	Sphingomyelin
LPE	Lyso-PE
PE O	Ether-linked PE
PI	Phosphatidylinositol
PS	Phosphatidylserine
CL	Cardiolipin
Cer	Ceramide
DG	Diacylglyceride

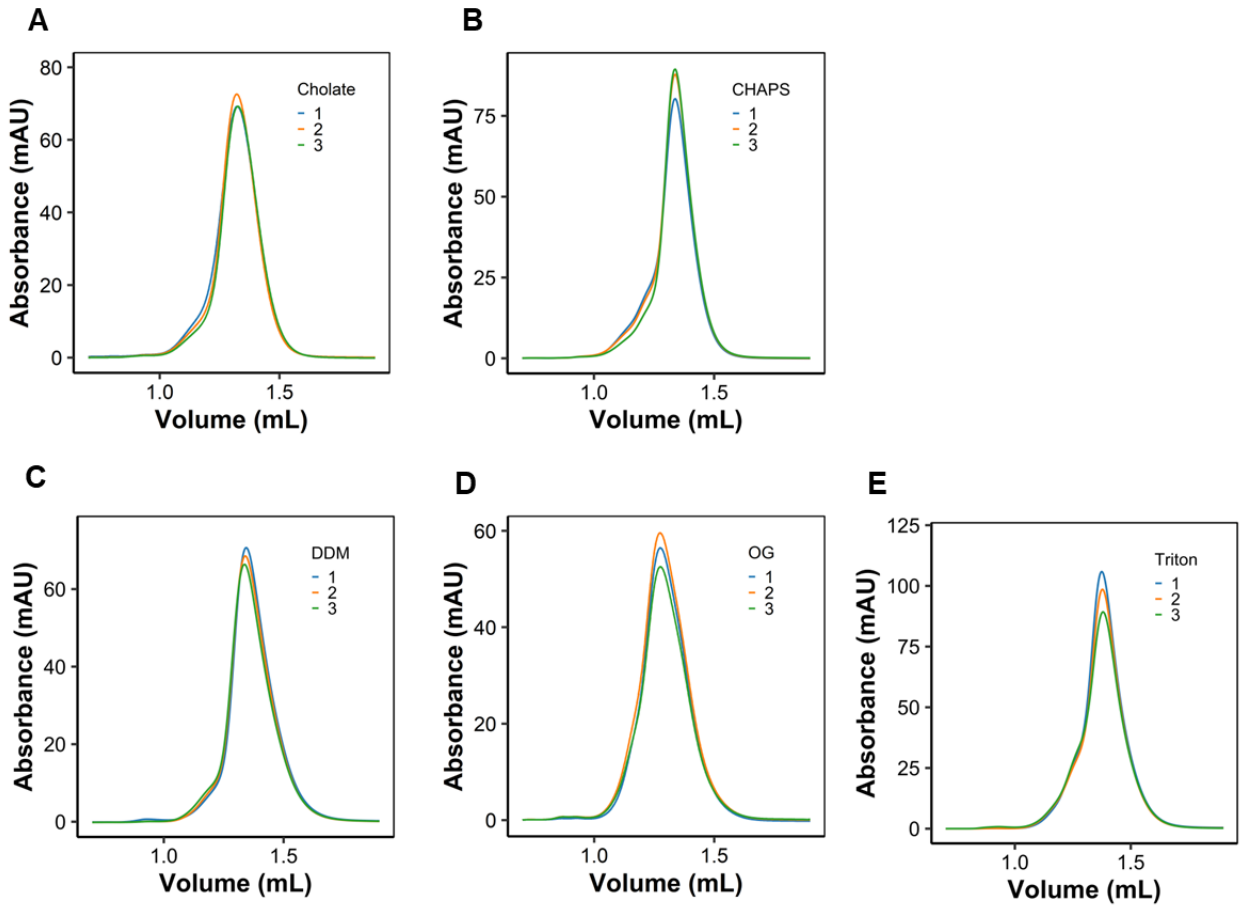


Figure A-IV-1 Representative size exclusion chromatograms of Nanodiscs synthesized with natural lipid extract mixtures and different detergents after SEC purification. Nanodiscs made with cholate (A), CHAPS (B), DDM (C), OG (D), and Triton X-100 (E).

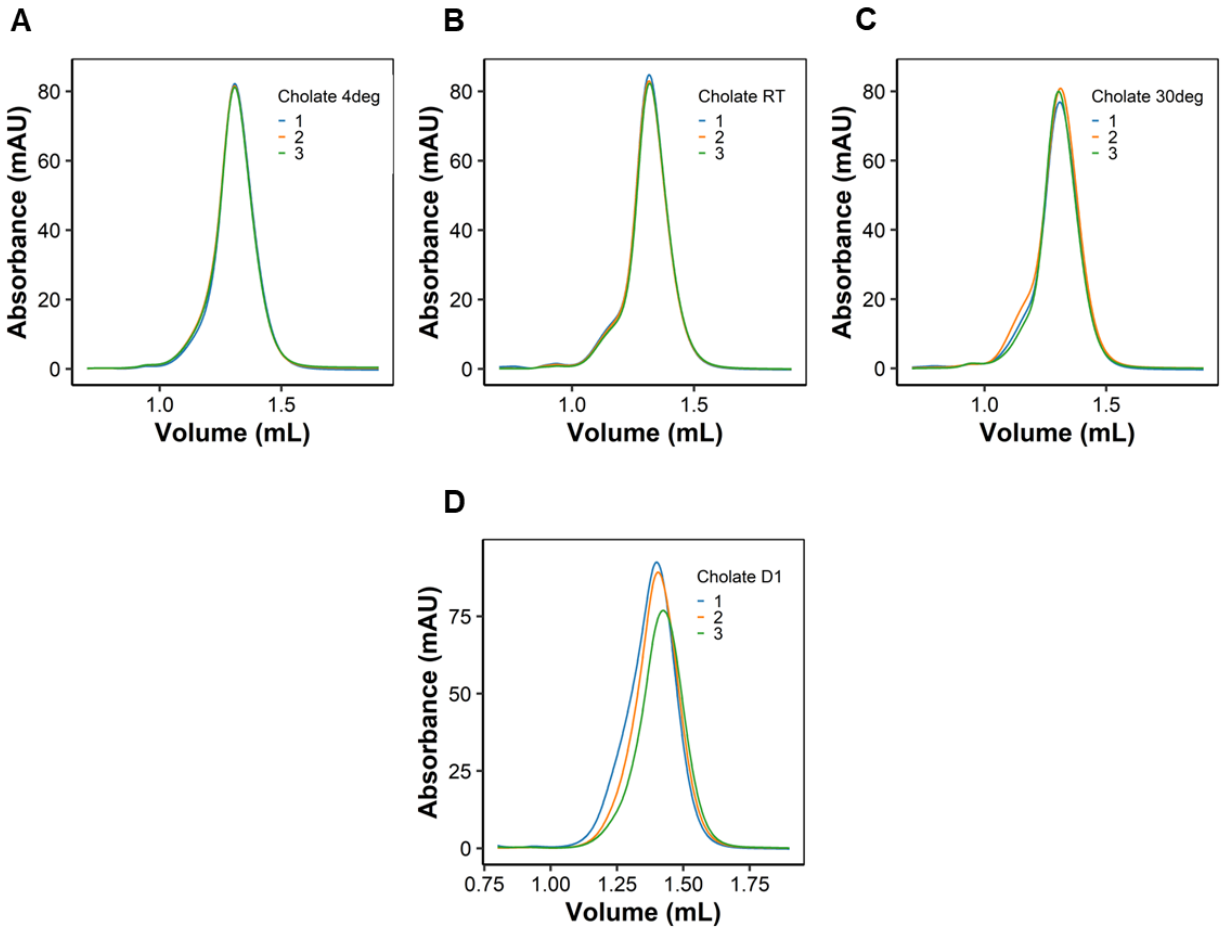


Figure A-IV-2 Representative size exclusion chromatograms of Nanodiscs synthesized with natural lipid extract mixtures with cholate and different temperatures or MSP belt sizes after SEC purification. Nanodiscs made at 4℃ (A), RT (B), 30 ℃ (C), or with MSP1D1.

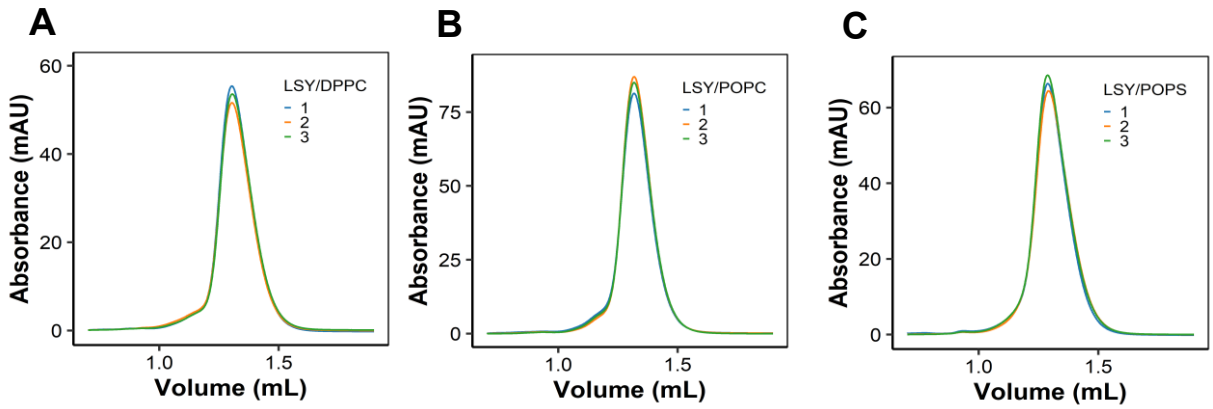


Figure A-IV-3 Representative size exclusion chromatograms of Nanodiscs synthesized with natural lipid extract mixtures with cholate and synthetic lipid supplements after SEC purification. Nanodiscs made with DPPC (A), POPC (B), or POPS (C).

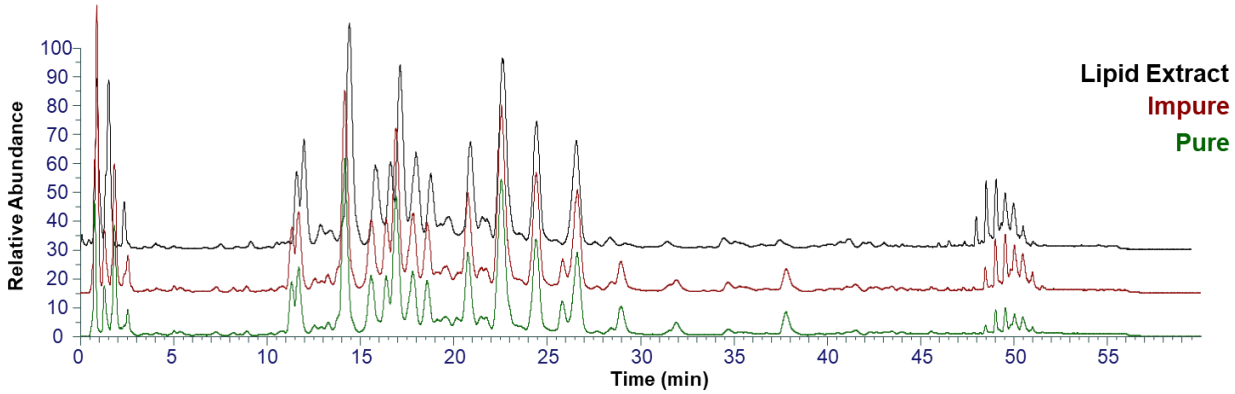


Figure A-IV-4 Representative chromatograms of lipid extracts from starting stock or Nanodisc samples. In black is the starting lipid extract stock. In red are the extracted lipids from the Nanodisc sample prior to purification. In green are the extracted lipids from the Nanodisc sample after SEC purification.

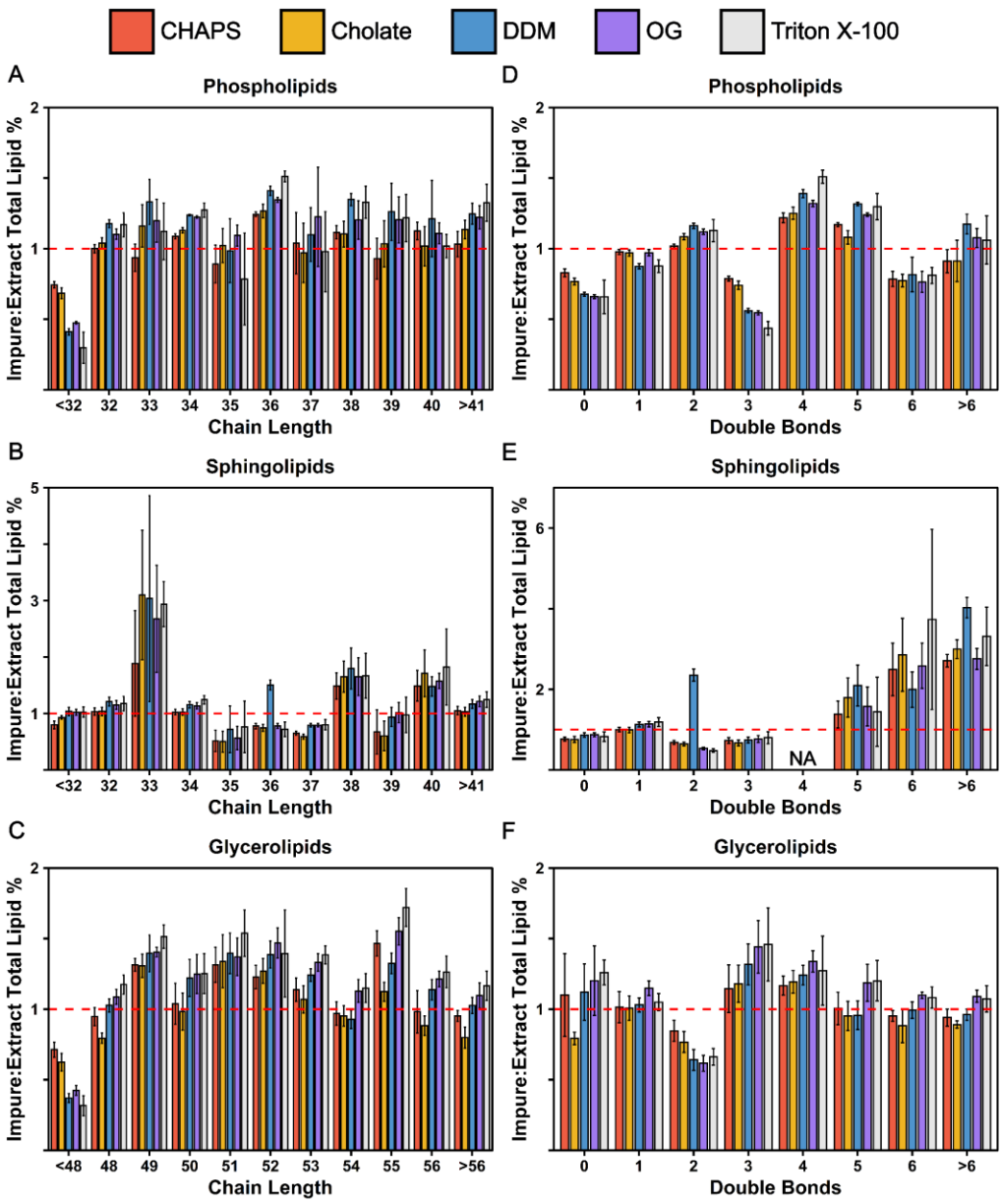


Figure A-IV-5 Effect of detergent selection on lipid solubilization. Ratio of percent total lipid in the impure Nanodisc sample over the starting extract mixture for lipids of different (A) phospholipid, (B) sphingolipid, or (C) glycerolipid chain lengths. The red dashed line represents a fold change. Ratio of percent total lipid in the impure Nanodisc sample over the starting extract mixture for lipids of different (D) phospholipid, (E) sphingolipid, or (F) glycerolipid double bonds. The red dashed line represents a fold change.

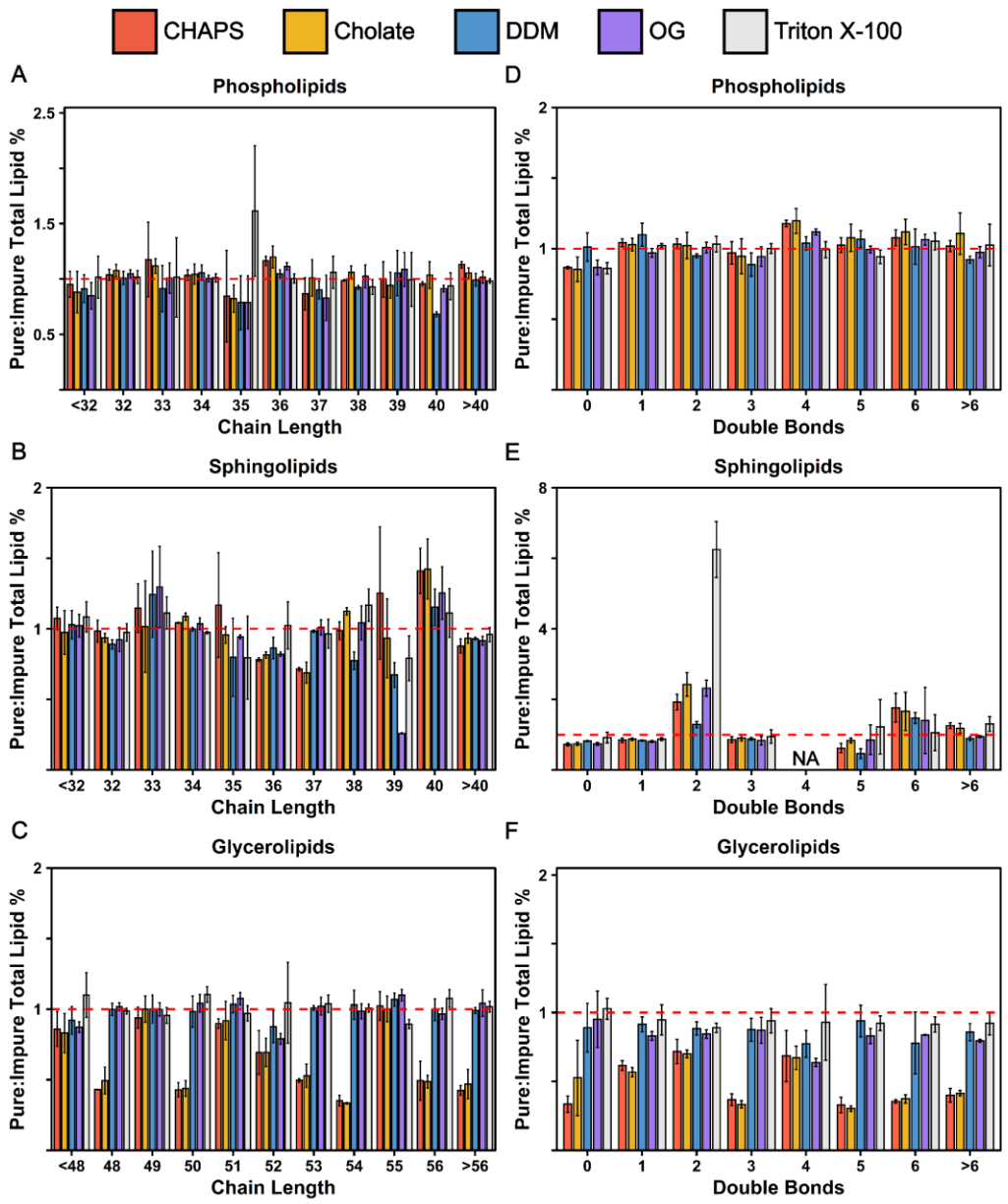


Figure A-IV-6 Effect of detergent selection on lipid incorporation into Nanodiscs. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (A) phospholipid, (B) sphingolipid, or (C) glycerolipid chain lengths. The red dashed line represents a fold change. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (D) phospholipid, (E) sphingolipid, or (F) glycerolipid double bonds. The red dashed line represents a fold change.

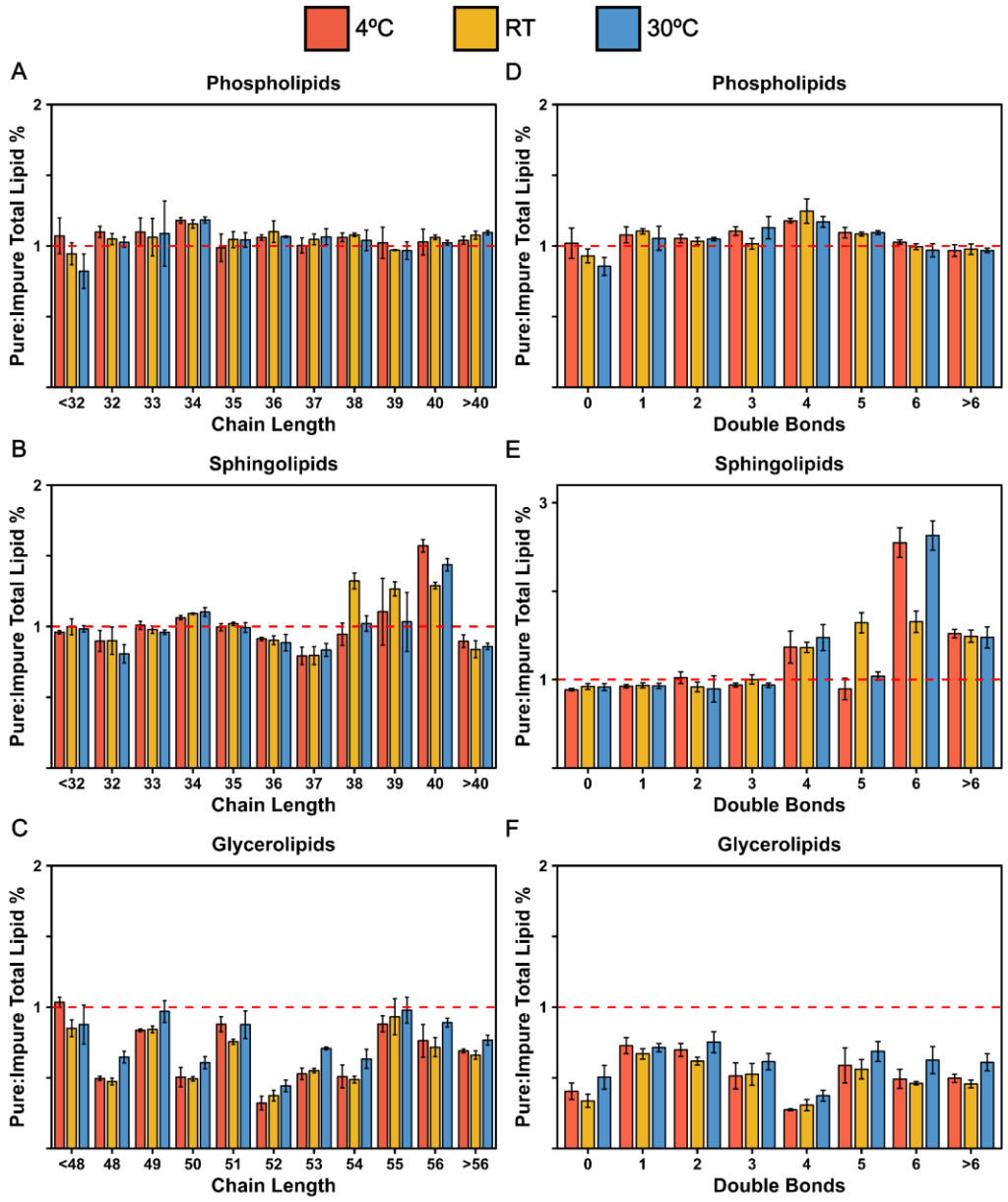


Figure A-IV-7 Effect of temperature on lipid incorporation into Nanodiscs. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (A) phospholipid, (B) sphingolipid, or (C) glycerolipid chain lengths. The red dashed line represents a fold change. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (D) phospholipid, (E) sphingolipid, or (F) glycerolipid double bonds. The red dashed line represents a fold change.

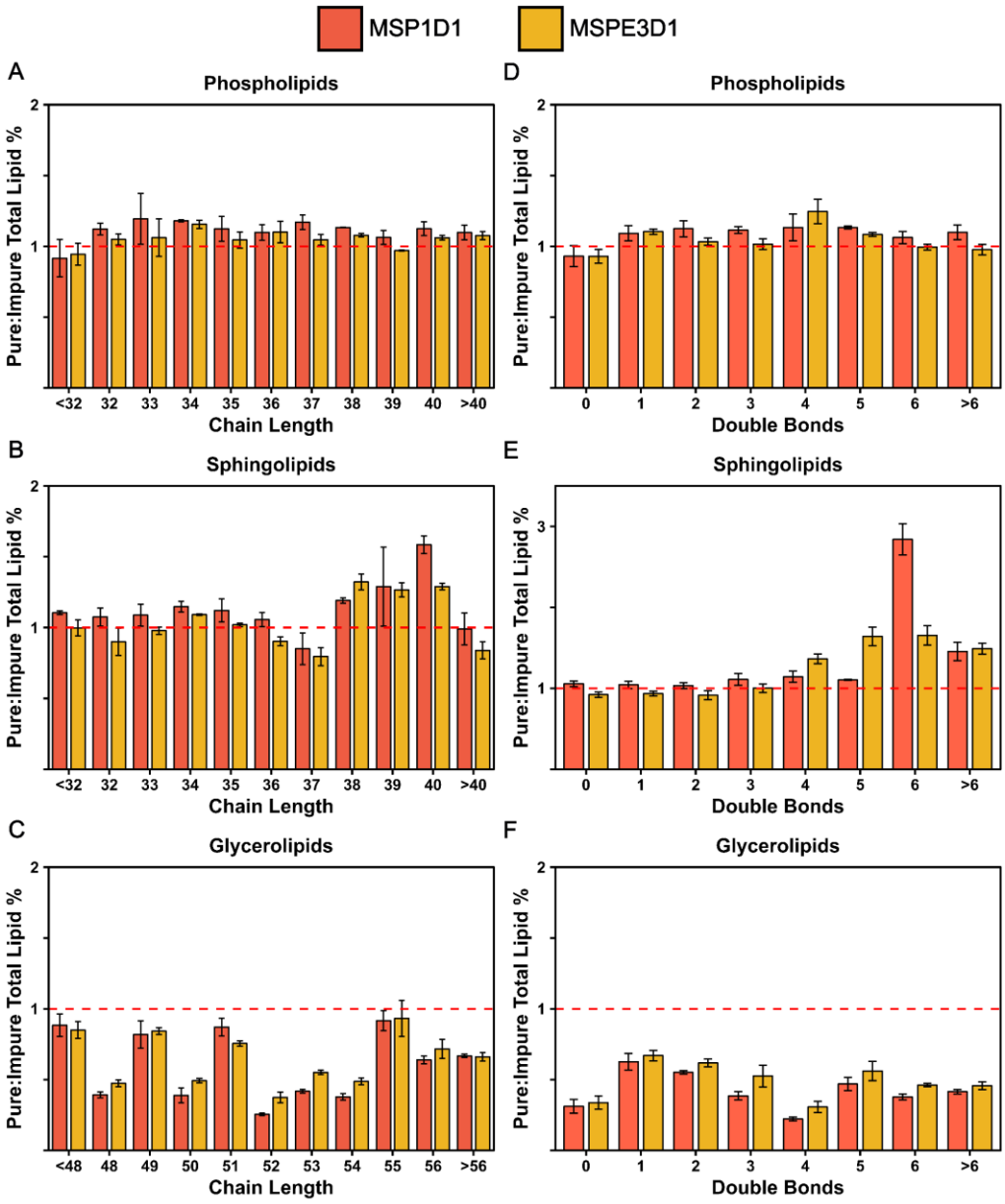


Figure A-IV-8 Effect of MSP belt sizes on lipid incorporation into Nanodiscs. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (A) phospholipid, (B) sphingolipid, or (C) glycerolipid chain lengths. The red dashed line represents a fold change. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (D) phospholipid, (E) sphingolipid, or (F) glycerolipid double bonds. The red dashed line represents a fold change.

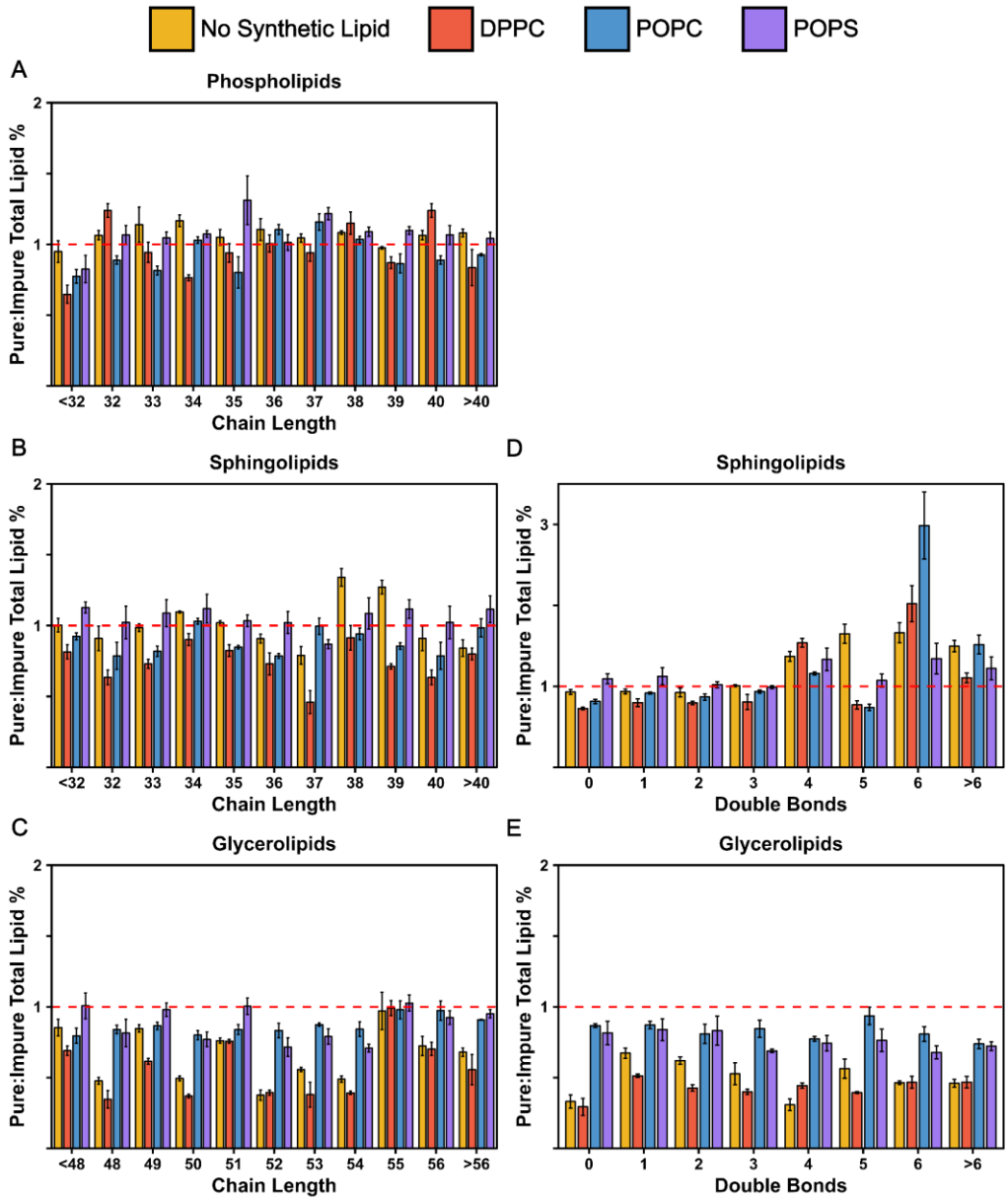


Figure A-IV-9 Effect of synthetic lipid additives on lipid incorporation into Nanodiscs. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (A) phospholipid, (B) sphingolipid, or (C) glycerolipid chain lengths. The red dashed line represents a fold change. Ratio of percent total lipid in the pure Nanodisc sample over the impure Nanodisc sample for lipids of different (D) sphingolipid or (E) glycerolipid double bonds. The red dashed line represents a fold change.

Appendix V Chapter 4 Supplemental Materials

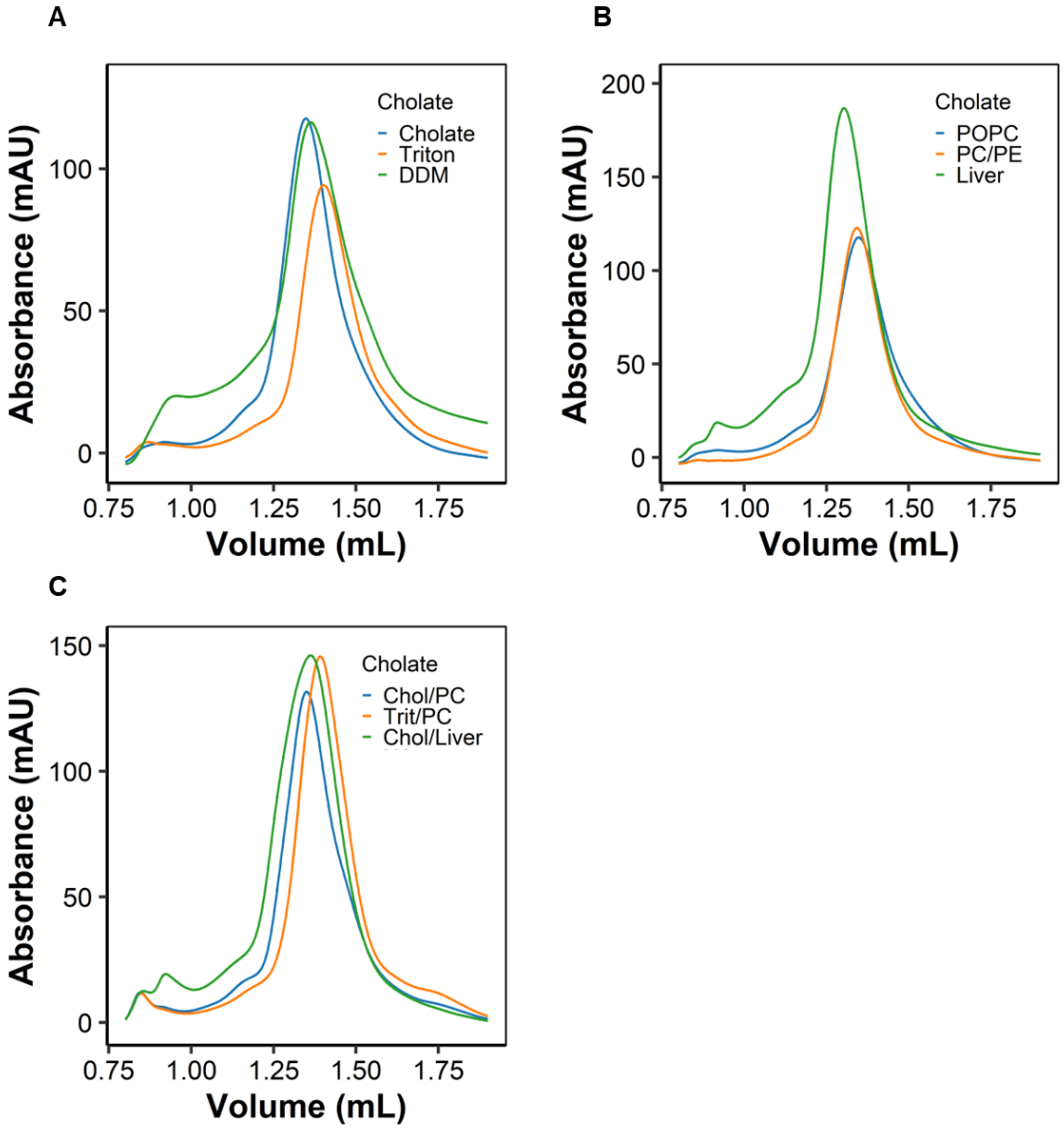


Figure A-V-1 Representative size exclusion chromatograms of Nanodiscs synthesized with different detergents, lipids, or lysate starting materials. Nanodiscs made with POPC and different detergents (A), cholate and different lipids (B), whole cell lysate instead of membrane fraction (C).

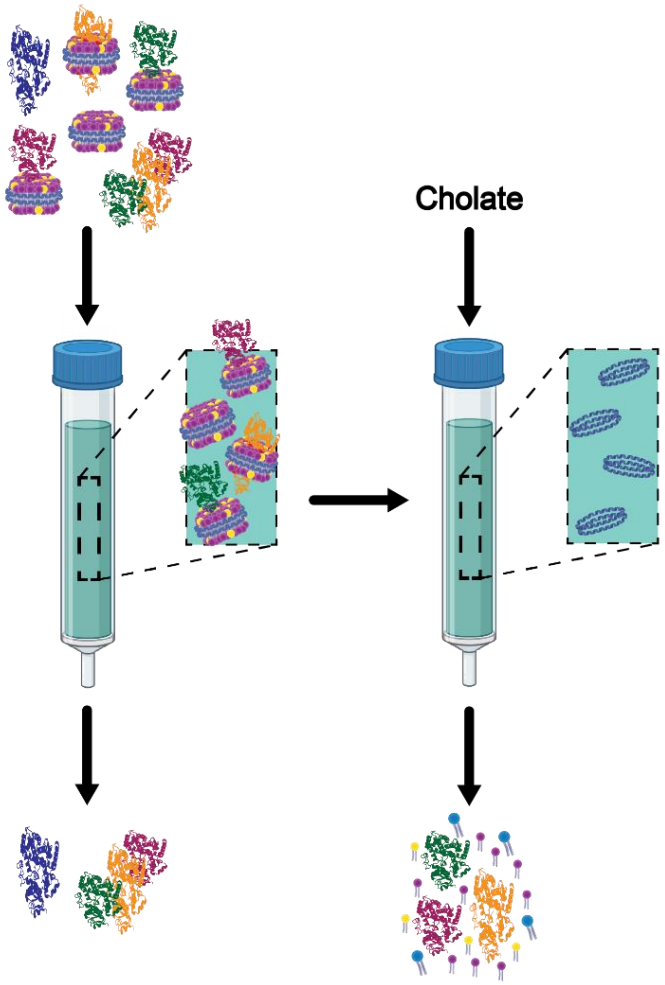


Figure A-V-2 Simultaneous Nanodisc purification and disassembly to liberate incorporated membrane proteins and deplete MSP. The impure Nanodisc mixture is loaded on a Ni-NTA spin column. Nanodiscs bind via a His-tag on MSP and the unincorporated membrane proteins are washed away. The resin is then incubated in a 50 mM cholate solution to disassemble the Nanodiscs, releasing incorporated membrane proteins while MSP remains on the Ni column.

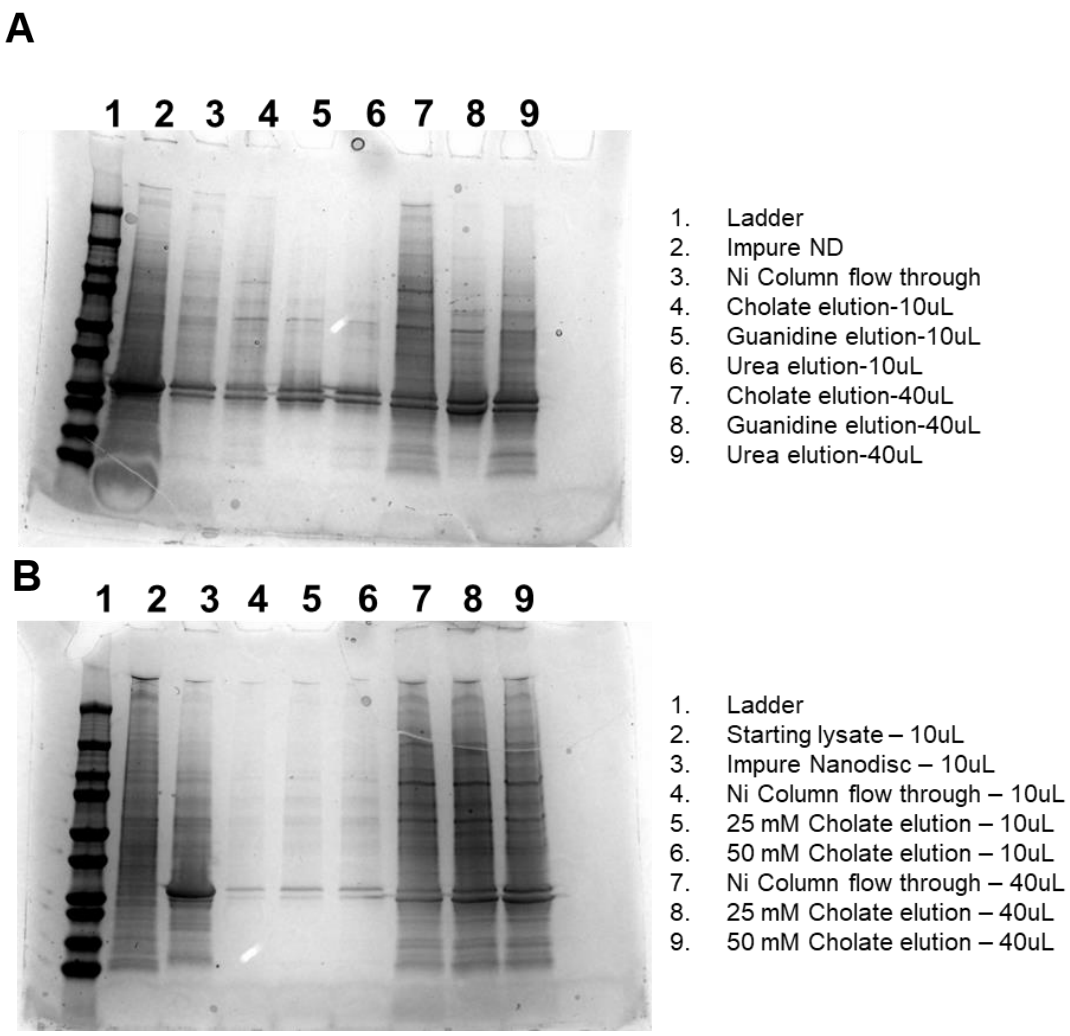


Figure A-V-3 Optimization of MSP depletion from library Nanodisc samples.

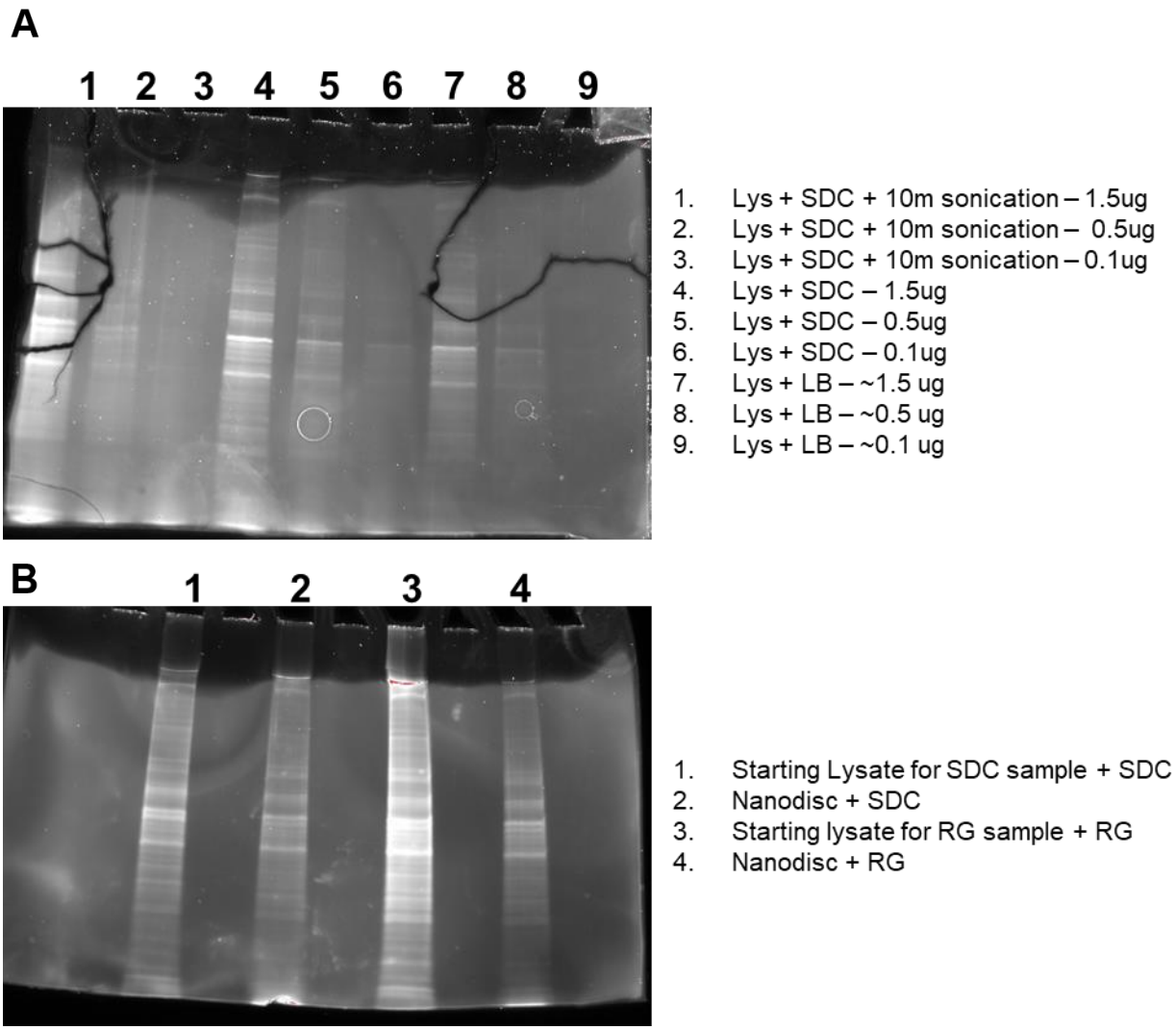


Figure A-V-4 Optimization of protein pellet solubilization conditions.

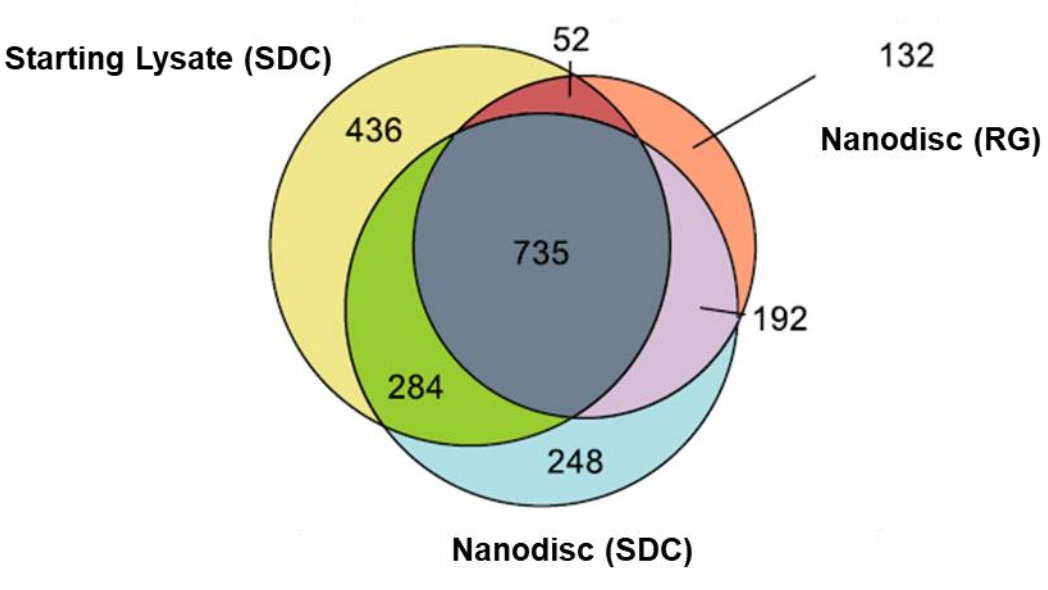
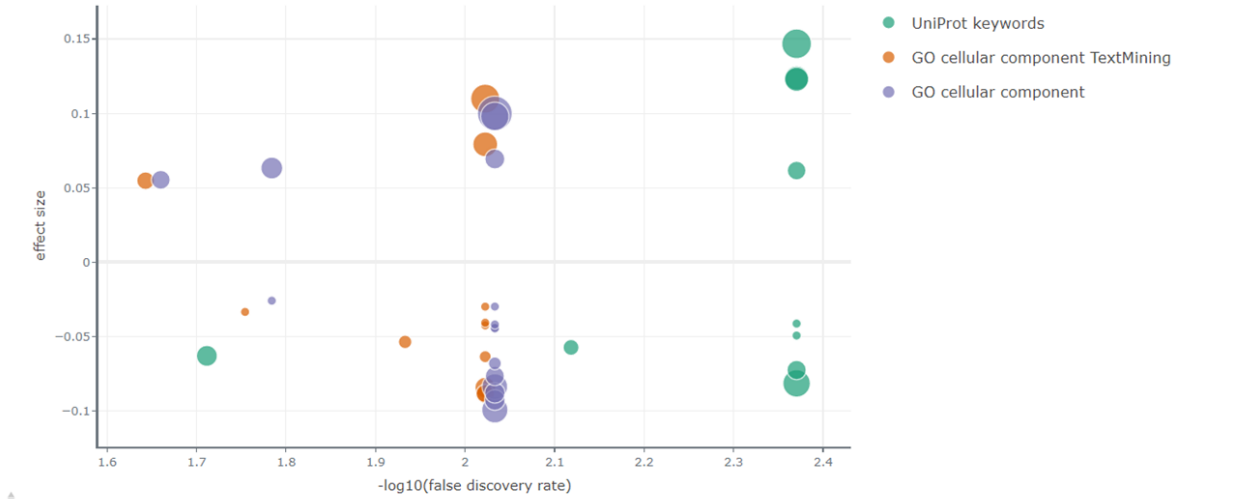


Figure A-V-5 Protein identification landscape in lysate and Nanodisc samples solubilized in either SDC or RG. The Nanodiscs show unique proteins not identified in the lysate. The two solubilization conditions also show distinct protein identifications.



s value	term	description
0.78	KW-0472	Membrane
0.66	KW-0812	Transmembrane
0.65	KW-1133	Transmembrane helix
-0.40	KW-0007	Acetylation
-0.36	KW-0539	Nucleus
0.29	KW-0325	Glycoprotein
-0.27	KW-0687	Ribonucleoprotein
-0.24	KW-1017	Isopeptide bond
-0.24	KW-0963	Cytoplasm
-0.22	KW-0689	Ribosomal protein

s value	term	description
0.57	GOCC:0016020	Membrane
-0.47	GOCC:0043228	Non-membrane-bounded organelle
-0.46	GOCC:0043232	Intracellular non-membrane-bounded organelle
-0.44	GOCC:0005634	Nucleus
0.39	GOCC:0031090	Organelle membrane
-0.35	GOCC:1990904	Ribonucleoprotein complex
-0.24	GOCC:0031981	Nuclear lumen
-0.24	GOCC:0005840	Ribosome
0.23	GOCC:0005886	Plasma membrane
-0.22	GOCC:0044391	Ribosomal subunit
-0.16	GOCC:0015934	Large ribosomal subunit
-0.14	GOCC:0005730	Nucleolus

s value	term	description
0.53	GO:0016020	membrane
-0.52	GO:0031974	membrane-enclosed lumen
0.51	GO:0031090	organelle membrane
-0.48	GO:0043228	non-membrane-bounded organelle
-0.46	GO:0031981	nuclear lumen
-0.42	GO:0005634	nucleus
-0.39	GO:0005654	nucleoplasm
-0.37	GO:1990904	ribonucleoprotein complex
0.35	GO:0098588	bounding membrane of organelle
0.25	GO:0005886	plasma membrane
-0.24	GO:0005840	ribosome
-0.23	GO:0044391	ribosomal subunit
-0.21	GO:0005730	nucleolus
0.21	GO:0031984	organelle subcompartment
-0.16	GO:0015934	large ribosomal subunit
-0.10	GO:0022626	cytosolic ribosome

Figure A-V-6 GO term analysis of the lysate and Nanodisc samples. The Nanodisc samples show enrichment of membrane-associated proteins and depletion of soluble proteins relative to the starting lysate.

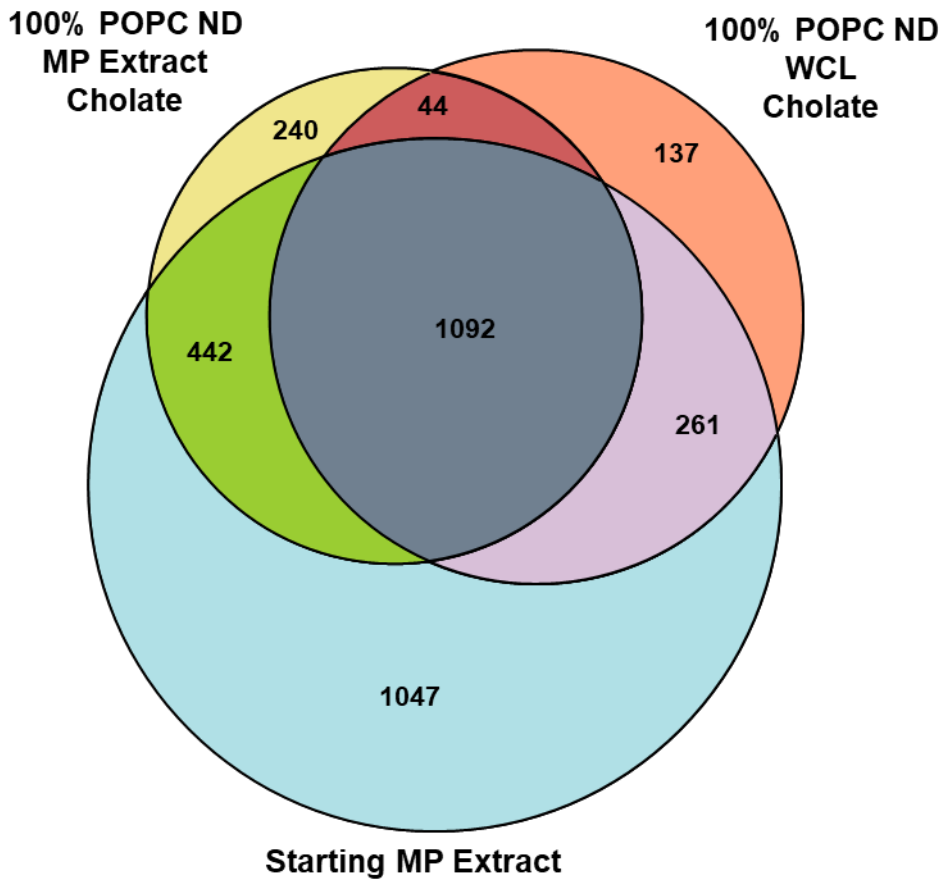


Figure A-V-7 Protein identification landscape in Nanodiscs made with the membrane extract or whole cell lysate relative to the starting membrane extract. The two Nanodisc samples show some overlap, but they both provide unique proteins identifications. Confirmation is still needed to determine if they are all membrane associated.

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