EXPANDING THE APPLICATION OF NANODISCS FOR NATIVE MASS SPECTROMETRY TO STUDY BIOMOLECULES

By

CIARA KATHRYN ZAK

_____

A Thesis Submitted to The W.A. Frank Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Molecular and Cellular Biology

THE UNIVERSITY OF ARIZONA

M A Y 2 0 2 2

TABLE OF CONTENTS

ABSTRACT

Membrane proteins mediate critical physiological roles which establishes them as attractive therapeutic targets. However, membrane protein interactions have proven difficult to study in a native lipid environment given their structural lability, transient nature, as well as the lack of lipid bilayer platforms to study protein-lipid interactions. Therefore, nanodiscs were developed for use in conjunction with native mass spectrometry to improve membrane protein analysis. Nanodiscs serve as a tool that provide a stable, biologically relevant lipid bilayer platform to solubilize membrane proteins. They are favorable given their more native state than detergent micelles and bicelles. Native mass spectrometry of membrane protein-nanodisc complexes allows for further investigation and characterization of membrane protein-lipid interactions.

Previously, native mass spectrometry of nanodiscs was limited by the lack of nanodisc controllability. The occasional instability of nanodiscs makes it difficult to preserve entire nanodisc complexes with embedded membrane proteins and the inability to significantly destabilize nanodisc complexes limits ejection of biomolecules. We demonstrate that electrospray ionization conditions as well as charge manipulation chemical additives modulate nanodisc stability during native mass spectrometry. The significant difference in stability of nanodiscs with various chemical additives and electrospray ionization conditions expands the application of native mass spectrometry of nanodiscs and opens new avenues for study.

Former studies have synthesized nanodiscs containing one or two prominent phospholipids to study membrane proteins and other peptide interactions. Unfortunately, these nanodiscs are lacking the complexity of a natural polydisperse lipid bilayer. To better model biological membranes and provide a more biologically consistent environment for the study of membrane

proteins, we developed nanodiscs which mimic mammalian, bacterial and mitochondrial lipid compositions, with as many as four different phospholipids, whose structure was then determined with native mass spectrometry. We applied our approach to successfully characterize the incorporation of the human antimicrobial peptide LL-37 in single lipid versus bacterial nanodiscs. The development of model membrane nanodiscs provides a more in depth understanding of the assembly of complex nanodiscs and expands the available tools for studying lipoproteins in model biological membranes.

Additionally, native mass spectrometry allows for the characterization of high-mass complexes and evaluation of molecular interactions but usually requires resolution of the different charge states produced by electrospray ionization, a process that is difficult to accomplish for extremely polydisperse samples. These samples tend to have overlapping charge states which leads to unresolvable spectra. Charge detection mass spectrometry (CD-MS) aims to address these challenges posed by conventional native mass spectrometry by simultaneously measuring the charge and *m/z* of isolated ions. There is occasional charge state uncertainty that limits the resolution of spectra, but this is addressed by the development of UniDecCD software for computational deconvolution of CD-MS data. UniDecCD improves the resolution of large heterogeneous samples including megadalton viral capsids and heterogeneous nanodiscs made from natural lipid extracts. Therefore, we provide a new computational tool for CD-MS data analysis as well as expand the application of nanodiscs to studying natural lipid extracts and extremely large, polydisperse biomolecular complexes.

Overall, we discuss various ways to expand the application of nanodiscs for native mass spectrometry and the study of biomolecular structures.

CHAPTER 1: INTRODUCTION

1.1 **Challenges of Membrane Protein Analysis**

Biological membranes are an essential component of life as all organisms are encompassed by a form of lipid bilayer which serves to define the cell, separates internal components from the outside, and provides an interface in which organisms can interact with their environment.[1] Membrane proteins are an integral component of these cellular membranes and help mediate their functionality. The role of membrane proteins is vast and critical for biological function and survival. Membrane proteins allow cells to communicate and interact with each other in order to take part in greater tissue-wide and system-wide action. This type of communication and interaction is typically accomplished by membrane protein receptors which relay signals between the external and internal cellular environment. Without such communication, the physiologic functions of the body would fail. Membrane proteins also serve as channels to allow for the import and export of various molecules including ions which regulate osmotic pressure, enable the flow of electrical signals and regulate cell membrane potential; molecules needed for metabolism such as glucose; cellular building blocks such as proteins for synthesizing organelles; as well as other molecules necessary for cellular activity.[1] A lack of membrane protein channels would result in the cell becoming more of a closed system that is not self-sustainable and eventually resulting in cell death. Additionally, membrane proteins can aid in cell adhesion which is necessary during an organism's development, for further cellular regulation and communication, and maintenance of tissues and body systems.[2] Loss of cell adhesion would be detrimental to survival due to a loss of the ability for tissues to adapt, grow and communicate. Aside from cell signaling, molecular transport, and cell adhesion,

membrane proteins retain other activities including supporting cell shape and size for proper tissue function, acting as enzymes to catalyze key chemical reactions, taking part in defense mechanisms, and more.[3]

The form and function of membrane proteins establishes them as key drug targets and clinical biomarkers. For one, alterations in membrane protein function have the potential to cause disease on a large scale as it can impair a wide range of critical cellular functions. The class of membrane proteins is estimated to comprise nearly 30% of the cellular proteome, further owing to their prevalence due to their abundance, and their availability makes them easier to target.[4] Additionally, due to their location in the cellular membrane, this allows easy accessibility for drugs which need to only make it to the exterior of the cell in order to initiate a response. Drugs which interact with membrane proteins can modulate cellular signaling, influence chemical reactions, effect transporters, or inhibit the proteins amongst other mechanisms that can take place on a systemic scale.[5] Given this, membrane proteins are estimated to comprise at least 40-60% of current drug targets.[1] Therefore the study and characterization of membrane proteins is important for therapeutic development and clinical understanding.

Membrane proteins have historically proven challenging to analyze. For one, there is relatively low expression of membrane proteins when compared to other protein classes which has resulted in their underrepresentation in structural biology and proteome analyses.[4] Low expression levels not only have limited our current understanding of membrane protein complexes, but it also makes it difficult to express and purify large quantities of protein for analysis. Therefore, the development of more sensitive technology or higher yield methods are

necessary. Membrane proteins are also difficult to solubilize given their heterogeneity and numerous hydrophobic domains that leads them to aggregate and precipitate during sample preparation. This has limited the study of membrane proteins in their native state. Another issue is that the lipid environment membrane proteins reside in typically requires exchange using detergents which can disrupt protein structure and function. Detergents are not compatible with various types of purification and analysis technologies. Overall, membrane proteins are difficult to study because of their hydrophobic properties, low abundance, limited solubility, and resistance to extraction which complicates their isolation, solubilization and identification.[5]

Current technologies that aim to analyze membrane proteins include X-ray crystallography, cryo-electron microscopy, and NMR spectroscopy. X-ray crystallography is a standard method for analyzing proteins by determining their three-dimensional structure from a crystallized form. Briefly, a desired protein sample is purified in high concentration, crystallized, and exposed to an X-ray beam to produce diffraction patterns. These are then processed to yield a map of electron densities that provide structural information regarding the protein.[6] Unfortunately, membrane proteins present several challenges for X-ray crystallography. Membrane proteins must be removed from their native lipid environment and placed in an artificial environment prior to analysis. This can disrupt the natural structure and function of membrane proteins because their interactions are different in a non-native environment. This may also yield inaccurate data regarding membrane protein structure due to a loss of its normal environment. Additionally, X-ray crystallography looks at static structures

and can only provide limited information regarding membrane protein dynamic states and interactions which are essential to understanding the function of the protein.

Another technology aimed at visualizing protein structures is cryo-electron microscopy (cryo-EM). Cryo-EM removes the need for organized crystals used in X-ray crystallography which is very difficult to obtain for membrane proteins.[7] Instead, proteins can be visualized in vitreous ice with near-atomic resolution.[7] However, for cryo-EM analysis, proteins need to be pure and highly concentrated which is difficult with membrane proteins due to their low expression rates. Additionally, the proteins are still being visualized in a more static state instead of being able to observe their molecular dynamics which provides more information regarding protein function. They are also removed from their native environment which can result in a loss of normal structure and function.

NMR spectroscopy is another method for protein visualization and analysis which involves placing proteins in solution in a magnetic field so that the magnetic dipole moment of each nucleus aligns with the field and then the sample undergoes radio frequency electromagnetic radiation that causes nuclei to resonate at characteristic frequencies.[8] This information is then used to derive a three-dimensional structure of the protein. NMR spectroscopy overcomes the need for crystal formation in order to analyze protein structure. NMR also provides more information regarding protein dynamics through investigating relaxation times.[8] For membrane proteins, NMR spectroscopy is limited due to the need to solubilize membrane proteins in detergents which can be denaturing.[9] Samples also need to be highly concentrated which is difficult to achieve with membrane proteins.

Overall, for improved membrane protein analysis, there is a need for the development of more sensitive technology that produces higher protein yields and can analyze membrane proteins in a dynamic state within a native environment.

1.2 Native Mass Spectrometry for Membrane Protein Analysis

Mass spectrometry (MS) is a major analytical tool that is highly utilized for analyzing biomolecules by measuring the mass of a given molecule after conversion to a gas-phase ion. It can provide information regarding structure, binding interactions, and molecular dynamics and has been extended to investigating membrane proteins. Mass spectrometry offers an alternative approach for membrane protein analysis when conventional methods prove difficult as it aims to overcome some of the challenges of membrane protein analysis including low expression levels and the need for a dynamic state. Mass spectrometry allows for the characterization of protein dynamic states and can provide accurate data from small sample quantities that are not necessarily homogenous. By studying membrane protein dynamic states, more functional information can be derived as well as broader interactions between the protein and lipids or other ligands. Also, the ability to analyze smaller quantities of less pure samples reduces the need to express high concentrations of membrane proteins.

One example of MS targeted at membrane proteins includes shotgun MS which focuses on proteolytic cleavage of membrane proteins and then uses identification and quantification by mass spectrometry with high sequence coverage. This has provided useful information regarding membrane protein sequences and identification.[4] Other MS methodologies include chemical cross-linking, hydrogen-deuterium exchange, and foot printing that provides further

insight regarding membrane protein dynamics, measurement of residue distances, binding site information, and solvent exposure.[3,10,11]

This information is important because it can be used to develop therapeutics by providing more specific and accurate information on membrane proteins to be targeted by these drugs. Membrane proteins already encompass a significant amount of drug targets due to their accessibility in the plasma membrane and wide range of functions such as cellular signaling, molecular transport and catalyzing chemical reactions. By improving drug efficacy through more thorough and accurate data with MS, it can allow for derivation of more specific mechanisms of action and more targeted therapy which has the potential to reduce drug-related side effects. Additionally, through better understanding of membrane proteins, we can better understand how the body functions on a cellular level that can better guide how we understand and treat pathologic conditions.

Over the past few decades, native mass spectrometry (MS) has emerged in the MS field as a technology to better study membrane proteins because it eliminates the need for labelling, provides a more native environment for study, and is a more direct method for protein analysis. Labeling involves creating a specific mass tag introduced into a given protein metabolically, chemically, or enzymatically. Eliminating the need for the labeling means experimentation is no longer limited by the chemistry required to tag a given peptide as well as reduces the number of steps involved in sample preparation.[12] Additionally, most native MS employs electrospray ionization (ESI) which works to help preserve non-covalent interactions of membrane proteins upon their ionization and transfer to the gas phase.[6] This prevents denaturation of tertiary and quaternary structures that can then be investigated *in vacuo* and provides information regarding

their dynamics.[6] Analyzing membrane proteins in a more native environment, through preserving non-covalent interactions, preserves their more natural structure, function and lipo-protein interactions as compared to a synthetic environment. This makes the resulting data more applicable to a cellular environment in a living system. Native MS is also a more direct method for protein analysis since it allows for samples to be analyzed directly as intact proteins and analyzes dynamic quaternary structures in real time.[13] Native MS can aid in determining membrane protein stoichiometry by observing the measured mass of the protein as it is dissociated. Native MS also can investigate ligand binding in a similar manner. If a molecule is bound to the protein, the resulting mass spectrum will show an additional peak corresponding to the protein-ligand complex. Overall, native MS offers a highly sensitive method of protein analysis more compatible with membrane proteins.

Typically, native MS starts with sample ionization using electrospray ionization (ESI) where a voltage is applied across a tapered capillary containing a sample in a non-denaturing solution. Charged droplets containing the given analyte causes ions to enter the gas-phase and travel to the vacuum region of the mass spectrometer for analysis.[4] ESI creates several charged analyte ions and each species of the given protein is represented by a mass spectrum with a series of peaks that each corresponds to a specific mass-to-charge ratio (m/z) referred to as a charge-state distribution.[3] These distributions usually represent a form of Gaussian distribution.[7] The membrane protein complexes also can also be dissociated by applying a collision voltage via gas molecules within the mass spectrometer to eject ligands or subunits.[14] Overall, native mass spectrometry has the potential to provide valuable information regarding

membrane protein components, stability, ligand preferences, connectivity, and oligomerization.[14]

1.2 **Conventional Membrane Protein Solubilization Methods**

Given their amphipathic nature, membrane proteins need to be interfaced with membrane mimetics to aid in solubilization before purification and analysis by native MS. Membrane proteins are often initially expressed in a system such as *Escheria coli* or mammalian or insect cells. The cells expressing the membrane protein are homogenized in a buffer with added protease inhibitors and then a buffer commonly containing detergent, milder non-ionic detergent, or bile acid salts extracts the protein from the plasma membrane. Following this process, the membrane proteins can be interfaced with various cassettes. Common membrane protein cassettes include amphipols, liposomes, bicelles and detergent micelles which exist to help promote membrane protein functionality and stability, but they also can have negative effects on the natural lipoprotein environment or mass analysis.[15]

Amphipols are amphipathic copolymers that contain monomers with hydrophobic and hydrophilic units that then are combined with isolated membrane proteins which are often solubilized in detergent due to their hydrophobic nature.[16] Amphipols will then bind the membrane proteins in a relatively stable manner. Amphipols containing protein then undergo ESI and are stripped from the membrane protein complex after application of collisional activation in the gas-phase.[16] Membrane protein stabilization seems to vary across the type of protein and amphipol used. Liposomes are spherical vesicles that contain a phospholipid bilayer that surrounds a liquid interior. They can be of varying sizes and contain a variety of lipids. Liposomes can trap the membrane protein of interest following membrane protein

extraction and then are purified and undergo ESI. Bicelles are disc-like membranes that are assembled from phospholipids which compose the lipid bilayer with short-chain phospholipids or detergents that form the rim. Following formation, membrane proteins can then be incorporated and analyzed as described above.

More traditionally, detergent micelles have been used as vehicles for solubilizing and delivering membrane proteins to the gas phase for analysis by native MS.[17] Detergent micelles provide a protective amphipathic environment to help membrane proteins retain their natural structure and biological functions.[17] Typically, the detergent micelle-membrane protein complex is desolvated and ionized as an entire entity via ESI. The bound detergent then can be dissociated from the membrane protein complex through the application of high collision energy with buffer gas to leave behind solely the protein for mass analysis.

1.3 **Nanodisc Development for Native Mass Spectrometry**

Unfortunately, detergent micelles present other challenges for membrane protein analysis despite improving the solubility and stability of membrane proteins for native MS interrogation. One challenge is that detergent micelles can be denaturing and disrupt membrane protein function which can yield inaccurate data because proper folding is needed for proper membrane protein function. Alternatives to detergent micelles such as liposomes, amphipols and bicelles provide more native lipid environments which aim to mimic the plasma membrane that membrane proteins naturally reside in because they decrease the amount of detergent required for sample assembly, can include more natural versus synthetic lipids, and more closely resemble lipid bilayers. But, these also have limitations given their large size or structure.

Therefore, nanodiscs have been developed to help address the limitations of current technology including the need for detergent, large size of molecules like liposomes, and need for more native environments. Nanodiscs are disc-shaped nanoscale lipid bilayers composed of lipids surrounded by two membrane scaffold protein belts and they self-assemble during incubation.[18] Nanodiscs provide more biologically relevant lipid bilayer environments in which to study membrane proteins and better preserve membrane protein structure and function when compared to traditional detergent micelles.[19] Nanodisc complexes are also monodisperse which makes them more ideal for various characterization methods.[19] Other favorable properties include the ability to modulate nanodisc size from 8 to 16 nm in diameter by varying the MSP belt, the ability to control lipid composition, as well as compatibility with diverse chromatography purification methods given MSPs can be engineered to have different affinity tags.[19, 20] Nanodiscs are much smaller than liposomes whose extensive size poses challenges for native MS because of the need for resolution of complex spectra. Amphipols help solubilize membrane proteins without detergent but only scarcely resemble the natural lipid bilayer in which membrane proteins are found. Nanodiscs, on the other hand, provide nanoscale lipid bilayers in which to incorporate membrane proteins and more closely resemble these bilayers than bicelles and micelles.

Some disadvantages of nanodiscs include the requirement for some detergent or membrane-active polymers for direct solubilization and incorporation of membrane proteins into nanodisc complexes.[20] However, this detergent is removed prior to analysis by native MS unlike with detergent micelles. Nanodiscs also produce complex mass spectra that requires deconvolution software, but other membrane mimetics also produce complex mass spectra.[19,20]

Overall, nanodiscs provide a promising resource for membrane protein studies given the need for less detergent, resemblance to native lipid bilayers, and easy modulation. These advantages can help to further expand the applications for native MS by addressing challenges posed by membrane proteins and allow for more extensive biomolecular characterization.

CHAPTER 2: CHEMICAL ADDITIVES ENABLE NATIVE MASSS SPECTROMETRY MEASUREMENT OF MEMBRANE PROTEIN OLIGOMERIC STATE WITHIN INTACT NANODISCS

2.1 Introduction

Membrane proteins have diverse and important physiological roles. They currently comprise nearly 60% of drug targets due to their ability to influence cellular signaling.[21] A significant amount of these proteins function in extensive complexes which makes it challenging to characterize their oligomeric state and stoichiometries within natural lipid environments because of the need break up these complexes but not destroy important interactions. Membrane proteins are also difficult to study due to the transient nature of their lipo-protein interactions which is difficult to visualize with current technology due to the need to study dynamic states. Significant advances in the understanding of membrane proteins have been made possible by native mass spectrometry (MS) which can better capture and characterize the protein-protein, protein-ligand and lipid-protein interactions of these membrane protein complexes.

To analyze membrane proteins with native mass spectrometry, they typically are solubilized in detergent micelles prior to electrospray ionization (ESI). Then they undergo gas-phase activation which results in collision induced dissociation (CID) in the mass spectrometer to remove the associated detergent and yield the bare membrane protein with any attached ligands or lipids.[22] Mass analysis of the complexes reveal the stoichiometry of the membrane proteins.[20] However, the use of detergent-based approaches can lead to over-delipidation, destabilization, and loss of biological activity of membrane protein complexes.[23]

To overcome these obstacles and study membrane proteins in a more native environment, we use nanodiscs as a tool for native MS of membrane proteins.[24] An empty nanodisc structure is shown in Figure 2.1 below.

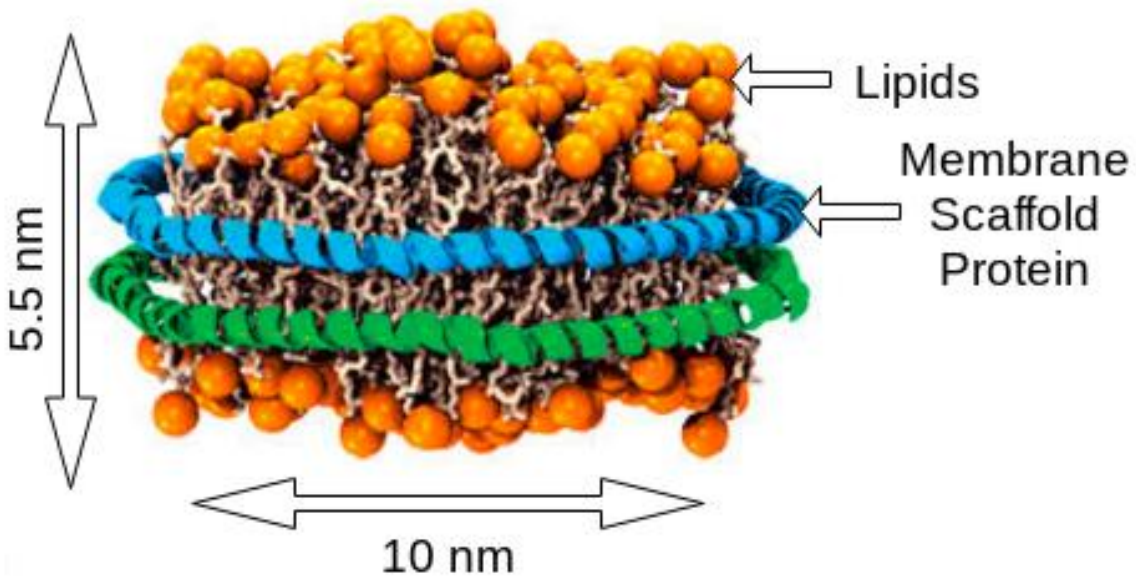


Figure 2.1.[25] Schematic of a nanodisc with two different alpha-helical membrane scaffold protein (MSP) belts shown in green and blue. Lipid head groups are shown in bright orange and their tails are detailed in tan. The average nanodisc measures 10 nm in diameter and 5.5 nm in height but this can be slightly altered based on lipid and MSP belt composition. (The nanodisc portion of this figure appeared in Nano Lett., 2007, vol. 7, No 6. 1692-1696. © ACS).

Nanodiscs mimic cellular membranes in that they are comprised of a nanoscale lipid bilayer held together by two membrane scaffold protein (MSP) belts in the shape of a disc. In this sense, nanodiscs offer an advantage over amphipols and micelles due to more closely

mimicking a lipid bilayer structure. Following assembly, peptides and other biomolecules of interest can be inserted into the center of the lipid bilayer.

Nanodiscs are optimal for native MS over liposomes, amphipols, bicelles and picodiscs due to their homogeneity, monodispersity and size.[13] The planar bilayer structure and size of nanodiscs are favorable over these membrane mimetics because their 10 nm diameter provides enough space for the incorporation of a few membrane proteins with access to each side of the environment.[16] Nanodiscs can be precisely controlled regarding composition and size which allows for the investigation of specific molecular recognition events on a membrane surface.[16] Nanodiscs are also completely soluble which helps streamline biochemical techniques. Numerous studies have also detailed the benefits of nanodiscs over detergent in providing a more native lipid environment that does not compromise the structural integrity or function of membrane protein complexes. [23]

Nanodiscs have some limitations including occasional inefficient release of bare membrane proteins during conventional native MS conditions, occasional instability, and complex mass spectra.[16] To address these limitations, we hypothesized that the addition of charge manipulation reagents to nanodiscs in solution would allow us to modulate the stability of nanodisc complexes during native MS and yield more resolvable mass spectra. The instability may be due to suboptimal charge retained on the nanodisc complexes in the gas-phase. Excessive charge can be destabilizing to the nanodisc and any incorporated biomolecules that results in unfolding and loss of interactions. Not enough charge may make the sample difficult to analyze as mass spectrometry relies on the mass to charge (m/z) ratio to determine information regarding the analyte. Additionally, with charge manipulation, analysis still takes

place in an environment comparable to a native state. This is because it has been demonstrated that that if charge manipulation additives are kept at a low concentration, such reagents have an insignificant impact on the protein structure in solution and therefore do not severely alter the near-native environment.[28] We added charge manipulation reagents to nanodiscs in solution at low concentrations prior to electrospray ionization. Charge manipulation reagents modulate the stability of a given compound by either increasing (or supercharging) or decreasing (charge reducing) the overall charge it acquires. The exact mechanisms of supercharging and charge reduction are still unclear, but they have been demonstrated to stabilize protein complexes.[28]

We discovered that nanodiscs are responsive to charge manipulation reagents in ways that allow us to modulate nanodisc stability. Preserving the nanodisc-membrane protein complex and yielding more resolvable spectra will enable better characterization of membrane proteins. However, despite these discoveries, the mechanisms of charge manipulation of nanodiscs are complex and elude simple explanation.

2.2 Methods

Materials. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphsphocholine (POPC) and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phospho-(1'-*rc*-glycerol) (POPG) lipids were purchased from Avanti Polar Lipids. Amberlite XAD-2, ammonium acetate and sodium cholate were purchased from Sigma-Aldrich. Membrane scaffold proteins were expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography (IMAC) as described previously.[25] TEV protease was added to remove the polyhistidine tags on all MSPs.

Nanodisc Assembly. Nanodiscs were assembled as described previously by solubilizing POPC, POPG or a mixture of both in sodium cholate.[25] POPC was chosen because it is

zwitterionic and POPG was chosen because it is anionic, and they may be influenced in a different manner by charge manipulation additives. Such phospholipids are also relatively abundant in mammalian membranes and were chosen due to their relevance.[33] MSPD1T1(-) was added to the lipid mixture and the detergent was removed through the addition of Amberlite XAD-2 hydrophobic beads. MSPD1T1(-) is based off the standard MSPD1T1 and the (-) indicates that the polyhistidine purification tag has been removed. The T1 variant has an additional threonine residue which has been inserted near the N-terminus. This MSP variant was chosen because of its thorough and extensive characterization with native MS. The T1 variant has been engineered so it is not isobaric with 29 POPC molecules which significantly complicates the mass assignment with traditional MSP1D1(-). The nanodiscs were purified by size exclusion chromatography with a Superose 6 Increase 10/300 column (GE Healthcare) into 0.2 M ammonium acetate at pH 6.8.

Supercharging and Charge Reduction.

For charge reduction of nanodiscs, samples were mixed with 400 mM imidazole (IM) with the pH adjusted to 7 using acetic acid to produce a final concentration of 40 mM. The same process was repeated with triethylamine (TEA). Controls were performed with the addition of ammonium acetate to show that the effects of charge reduction were not caused by the slight increase in ionic strength or slight dilution of analyte. IM proved superior to TEA in charge reduction ability and therefore was selected over TEA for further experimentation. For supercharging, samples were mixed with 19:1 v/v neat propylene carbonate (PC) or 4-vinyl-1-3-dioxoln-2-one (4V) to give a final concentration of 5% reagent by volume.

Native Mass Spectrometry. Nanoelectrospray ionization (nESI) was performed using borosilicate needles pulled in-house with a P-1000 micropipette puller (Sutter Instrument, Novato, CA). Mass spectrometry was performed using a Q-Exactive HF quadropole-Orbitrap mass spectrometer equipped with ultrahigh mass range research modifications (Thermo Fischer Scientific).[26] General instrumental parameters were used as described previously.[14] Important instrumental parameters included a capillary voltage of 0.9-1.3 kV, a capillary temperature of 200 °C for running samples in positive ion mode and 175-200 °C for running samples in negative ion mode.[31] Scan collection was from 2000-25000 *m/z* with a goal resolution of 15000 with 10 microscans summed into a single scan.[20] In-source trapping voltage was applied in the injection flatpole and increased from 0 to 200 V in 20 V increments with 11 min acquisitions.

Mass Spectrometry Data Analysis. MetaUniDec was used to deconvolve native mass spectra as previously described.[26] The mass range was extended up to 350 kDa and charge range was 1-35. The average mass was measured as a weighted average of peaks above a 50% relative abundance. The deconvolved spectra were summed across all collision voltage steps as described previously to provide a better picture of all species present.[27]

2.3 Results and Discussion

Charge Modulation of Empty Nanodiscs. Previous literature demonstrates that supercharging reagents significantly increase the average charge state of the analyte which leads to partial denaturation of the noncovalent protein complexes during native MS.[28] Although other factors influence charge state, including the voltage applied for activation in the mass spectrometer, the overall effect is due to the supercharging additive since voltage

must be applied to activate any sample analyzed by native MS.[28] Partial unfolding is attributed to Coulombic repulsion between the charged residues. Charge-reducing reagents, on the other hand, have been shown to stabilize protein complexes due to the reduction of Coulombic repulsion between charged residues which promotes a more compact protein state.[28,29] Therefore, we predicted we would see similar effects on nanodiscs, with supercharging reagents causing instability and a higher charge state, and charge-reducing reagents stabilizing the nanodisc complexes.

Nanodiscs were assembled with MSP1D1T1 (-) belts and zwitterionic POPC, anionic POPG or a 50/50 mix of both POPC and POPG. We collected native mass spectra with no additives, IM, 4V and PC. Mass spectra were then deconvolved as shown in Figure 2.2 below.

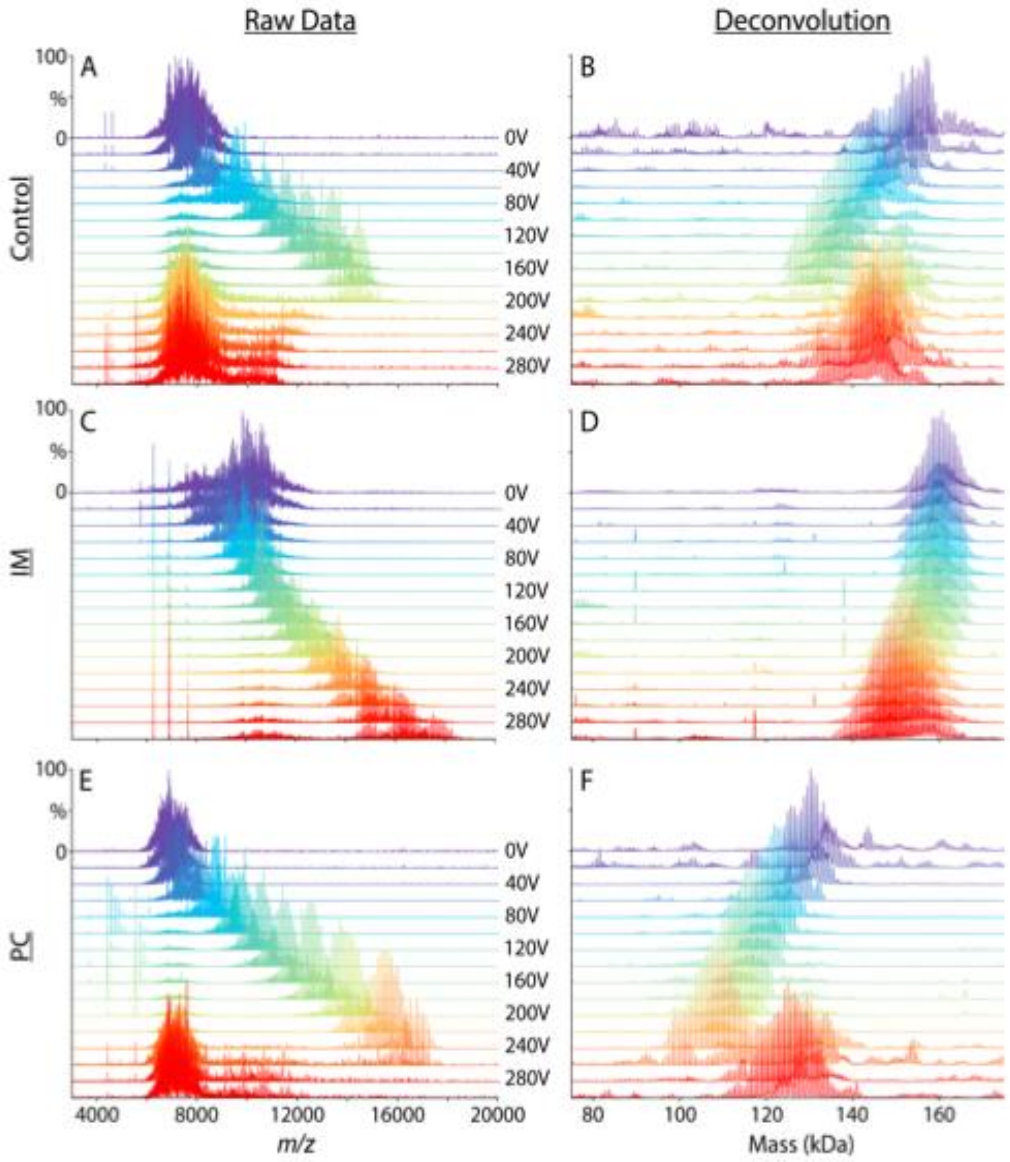


Figure 2.2. Representation of various positive ion mode mass spectra (A, C, E) and zero-charge deconvolved spectra (B, D, F) of empty POPC nanodiscs with no additives (A and B), 40 mM IM (C and D) and 5% PC (E and F). Spectra are shown for increasing collision voltages from 0 V (purple) to 300 V (red) in 20 V increments using in-source trapping. The variation in spectra at higher voltages is thought to be due to insufficient trapping and therefore incomplete activation of ions.

Comparing the mass spectra of nanodiscs with no additives to that with 40 mM IM, nanodisc distributions were less broad and polydisperse, nanodiscs fragmented less, and retained more mass. Spectra also appeared less noisy and more resolvable. The addition of supercharging reagent PC appeared to have the opposite effect, owing to an overall lower mass and less clean of spectra. Therefore, addition of IM or other charge reducing reagents can help provide higher quality data for nanodisc analysis with native MS and increase the utility of nanodiscs for the study of biomolecules.

We also investigated the stability of the nanodiscs in the gas phase by plotting the average mass and charge of nanodiscs as a function of collision voltage which is shown in Figure 2.3 below. This allows us to observe the impact of charge manipulation reagents on nanodiscs in the presence of differently charged ions as they are destabilized to determine, if any reagents, help stabilize nanodiscs even as increasing voltages targeted at dissociating the complexes are applied, or do the opposite.

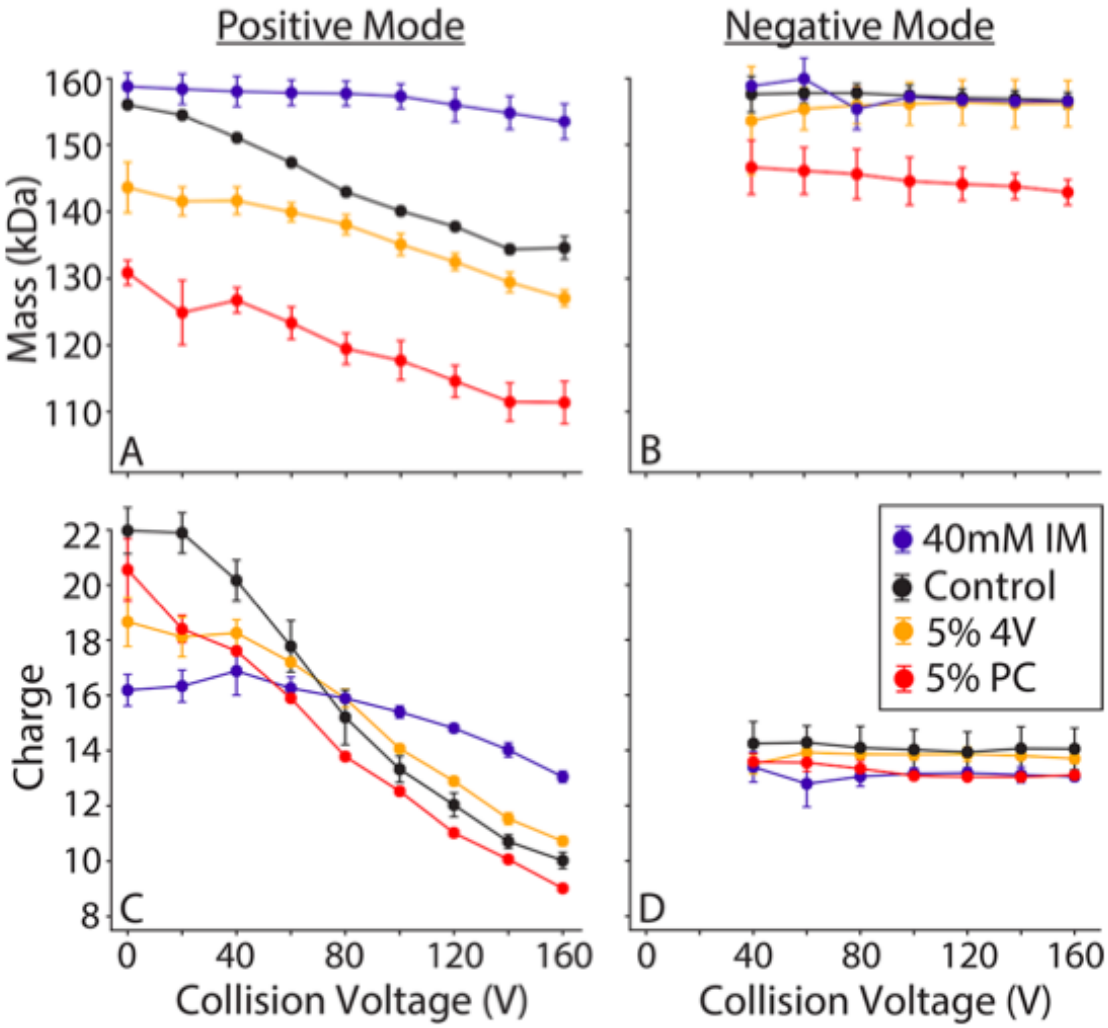


Figure 2.3. The average mass (A, B) and charge (C, D) plotted as a function of collision voltage for empty POPC nanodiscs with 40 mM IM (shown in blue), zero additives (shown in black), 5% 4V (shown in orange) and 5% PC (shown in red) for positive (left) and negative (right) modes. The error bars indicate the standard deviation for triplicate replicate nanodisc experiments.

We found that in positive ion mode, the addition of charge-reducing reagent IM significantly stabilized the nanodisc as its mass was almost entirely conserved over the course of increasing the collision voltage from 0 to 160 V. Very few lipids were ejected during the process. There was also a lower initial charge on the nanodisc complex, and this charge was relatively preserved over the course of increasing collision voltage from 0 to 160 V. This helps establish IM as a candidate for future use in nanodisc experiments for native mass spectrometry as it helps stabilize the nanodisc complexes. In positive mode with 4V and PC, the initial mass of the nanodisc complexes were much lower than that of the control and with IM. As the collision voltage was increased, a large amount of lipids were lost. The initial charge of the nanodisc complexes were also much higher than that of nanodiscs with added IM and much more charge was lost as the collision voltage was increased from 0 to 160 V. PC appeared to be the most destabilizing. This establishes PC and 4V as candidates for destabilizing nanodiscs with incorporated peptides in future experiments for native mass spectrometry as extreme destabilization may allow for the ejection of the incorporated peptides from the nanodiscs so the peptides can then be studied in their bare state. These results are also consistent with the predicted effect of charge-reduction and supercharging reagents on lipoprotein complexes which supports the use of charge manipulation reagents for manipulating nanodisc complexes.

In negative ion mode, the masses of the nanodiscs were preserved across all additives as collision voltage was increased. The initial nanodisc mass was lowest in the presence of PC. Very similar charge states existed for all conditions and no loss of charge was observed as the collision voltage was increased. This may be due to loss of charged lipids upon the addition of

supercharging reagents before ESI as the overall nanodisc mass and charge was lower than that of the control in negative ion mode. This introduces an interesting use for super-charging reagents for stabilizing nanodiscs in negative mode as long as they do not negatively impact any incorporated biomolecules. These charge states were all lower than that in positive ion mode. This is consistent with past observations.[32]

To investigate how different lipids effect the supercharging and charge-reducing of nanodiscs, we synthesized discs consisting entirely of POPG, POPC and a 50% mix of POPG/POPC. POPG is negatively charged at a neutral pH while POPC is zwitterionic.[33] Anionic lipids comprise around 10-20% of biological membranes and POPC is the most abundant zwitterionic lipid found in such membranes, adding to the biological relevance of the chosen lipids.[22] We ran triplicate replicates of each nanodisc type with no additives, IM, PC and 4V and then plotted the average mass and charge of nanodiscs as a function of collision voltages which are shown in Figure 2.4 and 2.5 below.

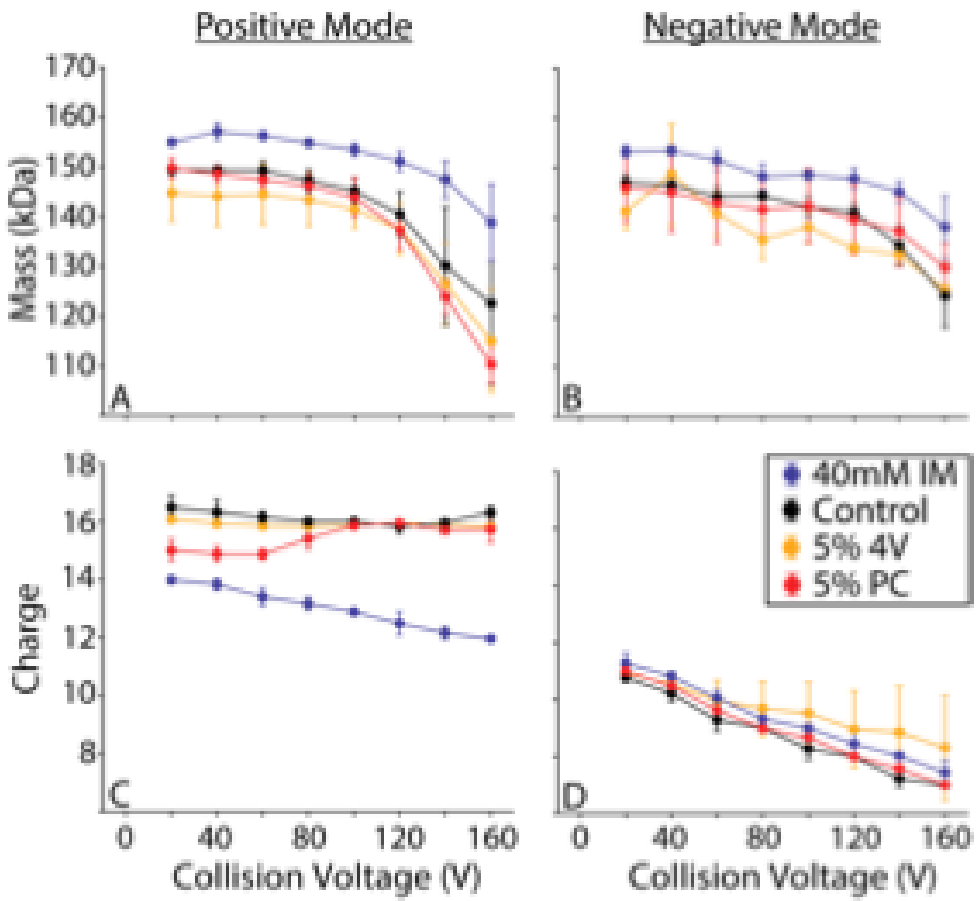


Figure 2.4. Charge manipulation of POPG nanodiscs. The average mass (A, B) and charge (C, D) plotted as a function of collision voltage with 40 mM IM (shown in blue), zero additives (shown in black), 5% 4V (shown in orange) and 5% PC (shown in red) for positive (left) and negative (right) modes. The error bars indicate the standard deviation for triplicate replicate nanodisc experiments.

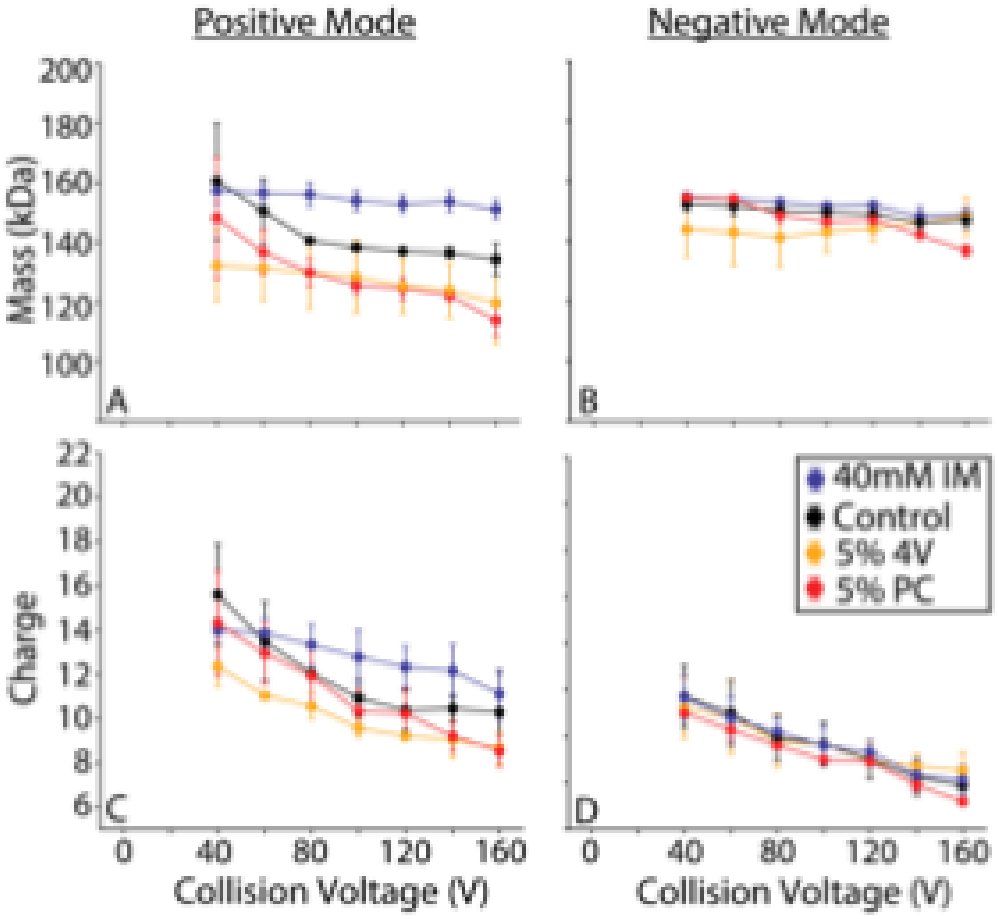


Figure 2.5. Charge manipulation of 50% POPG/POPC nanodiscs. The average mass (A, B) and charge (C, D) plotted as a function of collision voltage with 40 mM IM (shown in blue), zero additives (shown in black), 5% 4V (shown in orange) and 5% PC (shown in red) for positive (left) and negative (right) modes. The error bars indicate the standard deviation for triplicate replicate nanodisc experiments.

We found that in positive ion mode, IM had significant charge-reducing effects on 100% POPG nanodiscs but for the 50% POPG/POPC mixed lipid nanodiscs IM had no stabilizing effect and instead appeared destabilizing. IM allowed for greater lipid retention for both POPG and 50% POPG/POPC nanodiscs but was only effective for reducing the overall charge of 100% POPG nanodiscs. 4V and PC destabilized the 100% POPG nanodiscs. However, for the 50% POPG/POPC nanodiscs, there was limited lipid retention and an overall lower charge on the nanodiscs in the presence of each supercharging reagent.

Negative ion mode was more stabilizing than positive ion mode for all nanodisc mixtures. There were minimal effects elucidated by the charge manipulation reagents for each nanodisc type which was also consistent with the minimal effects the charge manipulation reagents had on POPC nanodiscs. Given the charge states were all lower in negative ion mode, the stabilizing effect is likely not due to the chemistry of the lipid headgroups but the lower charge of negative ionization.

Comparing our results to 100% POPC nanodiscs, in positive ion mode, the charge state of the 100% POPG nanodiscs was lower regardless of which charge manipulation reagent was added. The overall charge state of the 50% POPG/POPC nanodiscs was even lower across all reagent conditions than the 100% POPG nanodiscs. It is not clear why POPG lipids lower the charge state of nanodiscs, but they did not eliminate the stabilizing effects of IM. POPC nanodiscs may be more destabilized by charge reducing reagents due to their zwitterionic nature and higher likelihood to carry positive charges when compared to POPG given differences in headgroup chemistry. If a greater number of lipids within the nanodisc can become charged during positive ion mode, then they are more prone to

dissociation. These differences in nanodisc stability depending on lipid composition demonstrates that the chemistry of lipid headgroups can affect the stability of nanodiscs. We can greatly destabilize nanodiscs and induce significant lipid loss with supercharging reagents in positive ion mode.

Overall, supercharging and charge reduction techniques demonstrate new ways to modulate nanodisc stability and open new avenues for native MS of nanodiscs. The next step will to be to examine charge manipulation of nanodiscs with embedded membrane proteins. Charge reducing reagents may prove useful in expanding the application of nanodiscs to more difficult to analyze structures. They also allow for more stabilized nanodiscs that yield more resolvable spectra.

2.4 Conclusions

We have shown that charge manipulation reagents modulate the stability of nanodiscs during native MS and effect the overall quality of mass spectra. Nanodiscs were stabilized by charge reduction with IM and negative ion mode while supercharging reagents allowed for ejection of lipids. This work will be crucial for retaining labile lipids, membrane proteins, and peptides in nanodiscs during native MS analysis. This technique opens new avenues for applying nanodiscs to study more unstable biomolecular complexes and may prove useful for improving the analysis of membrane proteins.

CHAPTER 3: ASSEMBLY OF MODEL MEMBRANE NANODISCS FOR NATIVE MASS SPECTROMETRY

3.1 Introduction

Membrane proteins have been historically difficult to study in their natural lipid environment. Native mass spectrometry (MS) has aimed to analyze the complex lipo-protein interactions of membrane proteins in a more biologically relevant state. Nanodiscs have been employed in conjunction with native MS to better characterize their oligomeric state and lipo-protein interactions in a more native lipid environment.[31]

Natural cell membranes consist of an extremely heterogenous mixture of over a thousand lipids. [42] Nanodiscs containing natural lipid extracts would provide the most native state possible, but these complexes cannot yet be analyzed effectively by native MS in a controllable manner given their extreme heterogenity.[42] Most conventional nanodiscs are composed of only one or two phospholipid types which is not characteristic of cellular membranes. Nanodiscs commonly are assembled using one type of synthetic phospholipid because they provide a clean and easily resolvable spectra. These spectra have a pattern-like distribution with each peak in the series separated by lipid mass.[37] When adding an additional new phospholipid to the nanodisc complex, achieving resolution of the mass spectra is much more difficult because of the addition of new overlapping peaks to the mass spectra with each new lipid, and this often renders mixed-lipid nanodisc samples unresolvable.[38]

To expand the use of nanodiscs that are more biologically relevant, but still suitable for analysis by native MS, we created nanodiscs consisting of up to four different

phospholipid types instead of only one. We demonstrated that careful selection of lipids is necessary to be able to resolve nanodiscs with more than one phospholipid type. Incorporating lipids similar in mass allowed for resolution of mixed-lipid nanodiscs by native MS.[39] Resolution is possible because of the resulting peaks that can be separated by the average weighted mass of the two lipids.[40] Recently it has also been demonstrated that combining lipids with masses that are a simple integer multiple of each other can enable resolution of mass spectra of nanodiscs with numerous lipid types including glycolipids, sterols and cardiolipin.[41] We were able to use this method to resolve intact mixed-lipid nanodiscs and analyze their stability.

We selected combinations of lipids that serve to help better model mammalian, mitochondrial and bacterial membranes based off their lipid composition. Lipids of different headgroups but similar mass were selected, and various combinations were explored to investigate which lipids not only mimicked the composition of mammalian, mitochondrial and bacterial membranes but correctly formed nanodiscs. For mammalian-lipid nanodiscs, we chose the four most common species found in mammalian cell membranes: phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), and phosphatidylserine (PS) and explored the incorporation of saturated and unsaturated lipids.[35] Regarding bacterial-lipid nanodiscs, we incorporated various combinations of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) as these are the most abundant lipid types found in bacterial cell membranes.[36] For model mitochondrial nanodiscs we selected a combination of PC/PE/CL lipids as these compose the greatest proportion of lipids in the inner mitochondrial

membrane.[23] We successfully synthesized a variety of nanodiscs that incorporated up to four different lipid types common to mammalian, bacterial or mitochondrial lipid bilayers that were resolvable with native MS. We also observed some lipid combinations that formed nanodiscs with three lipoprotein belts which are not viable for study by native MS.

Additionally, we tested how these different mixtures of lipids affected the incorporation of LL-37, an antimicrobial peptide, into nanodiscs to show that model lipid nanodiscs allow for the incorporation of biomolecular compounds into more heterogenous nanodiscs and to characterize LL-37 in various lipids environments as its oligomeric state and pore mechanisms are not well understood.[45] Overall, these new types of nanodisc complexes provide controllable lipid mixtures that more closely model various biological membranes and allow for further exploration of how lipid bilayers effect biomolecules.

3.2 Methods

Materials. 1-Palmitoyl-2-oleoyl-sn-glycero-3- phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphoglycerol (POPG), 1-stearoyl-2-oleoyl-sn-glycero-3- phosphoethanolamine (SOPE), 1-palmitoyl-2-oleoyl-sn-glyc- ero-3-phospho-L-serine (POPS), N-stearoyl-D-erythro-sphingosylphosphorylcholine (SSM), 1,2-dipalmitoyl-sn-glycero-3- phosphocholine (DPPC), 2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG), 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphoethanolamine (POPE), 1,2-distearoyl-sn-glycero-3- phosphoethanolamine (DSPE), 1′,3′-bis[1-palmitoyl-2-oleoyl- sn-glycero-3-phospho]-glycerol (POCL), 1′,3′-bis[1,2-dioleo- yl-sn-glycero-3-phospho]-glycerol (TOCL), and 1′,3′-bis[1,2- distearoyl-sn-glycero-3-phospho]-glycerol (TSCL) were purchased from Avanti Polar Lipids.[40] Imidazole was

purchased from Arcos Organics. Ammonium acetate, Amberlite XAD-2, and 2 methylimidazole were purchased from Sigma Aldrich. Sodium cholate was purchased from Alfa Aesar. LL-37 was purchased from Bachem. Membrane scaffold protein (MSP), MSP1D1 was expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography (IMAC) as described previously.[43] TEV protease was used to cleave the polyhistidine tag of MSP1D1 to form MSP1D1(-) which was incorporated into nanodiscs as described previously.[31]

Nanodisc Preparation. Nanodiscs consisting of a single lipid, POPC, DPPC or DPPG, were prepared as describe previously.[41] For mixed-lipid nanodisc assembly, individual lipid stocks were dissolved in chloroform. Given difficulty solubilizing some lipids, a 5:1 chloroform: methanol mixture was used to solubilize DPPC, DPPG, TSCL and POCL. Lipid stocks were mixed prior to drying under nitrogen to remove organic solvent. The drying of multiple lipids together in chloroform allowed for re-solubilization as a mixed membrane with the addition of detergent.[44] For effective nanodisc assembly, we found that the solutions of mixed-lipids and chloroform contained in a test tube need to be completely clear in color instead of opaque and fully solubilized. For effective formation of DPPC and DPPG based nanodiscs, mixed-lipid chloroform solutions usually required the addition of heat (around 60 °C) for complete solubilization. POPC/ SOPE, DPPC/SOPE, and DPPG/SOPE mixtures were prepared at a molar ratio of 75/25.[40] POPC/SOPE/POPS and DPPC/SOPE/POPS compositions were prepared at a molar ratio of 65/25/10.[40] POPC/SOPE/POPS/SSM and DPPC/SOPE/ POPS/SSM nanodiscs were

prepared at a ratio of 55/25/10/10. DPPG/SOPE/POCL and DPPC/SOPE/TSCL nanodiscs were prepared at a 65/25/5 molar ratio.[40]

After drying for four hours, lipids were resolubilized in mild 0.1 M sodium cholate detergent. MSP1D1(-) was then added to the resuspended lipids in detergent. The optimal lipid: MSP ratios were determined empirically by testing several different molar ratios and selecting those that resulted in the most stable, monodisperse nanodiscs. For single-lipid POPC nanodiscs, MSP was added at a 1:65 MSP: lipid molar ratio. For POPC-based mixed-lipid nanodiscs, MSP was added at a 1:80 MSP: lipid molar ratio. For DPPC and DPPG single-lipid and mixed-lipid nanodiscs, MSP was added at a 1:1120 MSP: lipid molar ratio.[40] POPC-based nanodisc mixtures consisting of lipids, cholate and MSP were first incubated at 4°C for 30 min.[40] Amberlite XAD-2 beads were adjusted to match the reconstitution temperature and then added at a ratio of approximately 0.5g of beads per 1mL of volume. (Amberlite XAD-2 beads help to remove detergent prior to nanodisc purification and analysis to help maintain a more native state and prevent disruption of added peptides). POPC reconstitution mixtures were then incubated with beads at 4°C for at least 12 h but no more than 24 h. In a similar manner, DPPC and DPPG based nanodisc mixtures were initially incubated at 41°C for 30 min prior to the addition of Amberlite XAD-2 beads. The mixtures then incubated at 41°C for 4 h. DPPG-based nanodiscs were incubated for 12 h or longer to improve the nanodisc quality.[40] After Amberlite XAD-2 beads were removed by filtration, nanodiscs were purified using a Superose 6 Increase 10/300 column (GE Healthcare) into 0.2 M ammonium acetate at pH 6.8.[29] Final concentration of nanodiscs following fractionation and collection was 2-10 μM.

Peptide Nanodisc Preparation. Peptide nanodiscs were prepared as described previously.[37] After purification, nanodiscs were diluted to 2.5 μM.[29] LL-37 was dissolved in methanol and then added to DPPG or SPPG/SOPE/POCL nanodiscs at a molar ratio of 3:1 LL-37:nanodisc using a Hamilton syringe.[40] Imidazole was added to the LL-37 nanodiscs for stabilization during ionization.[40] Imidazole was dissolved in water at a concentration of 400 mM with a pH of 6.8.[40] The final volume composition of the LL-37 nanodisc mixtures were 19 μL of 2.5 μM nanodiscs, 1.5 μL of 400 mM imidazole, and 3 μL of 50 μM peptide.[40] The final nanodisc concentrations were 2 μM, imidazole was 25 mM, and LL-37 was 6.38 μM.[31] Control nanodisc replicates without any added peptide had 3 μL of methanol added instead.[40]

Native Mass Spectrometry. Nanoelectrospray ionization (nESI) was performed using borosilicate needles pulled in-house with a P-1000 micropipette puller (Sutter Instrument, Novato, CA). Mass spectrometry was performed using a Q-Exactive HF quadropole-Orbitrap mass spectrometer equipped with ultrahigh mass range research modifications (Thermo Fischer Scientific).[26] Instrumental parameters were used as described previously.[25] Important instrumental parameters included a capillary voltage of 1.1 kV, a capillary temperature of 200 °C, and 50 V source fragmentation for positive ion mode and 30 V for negative ion mode.[40] Mass spectra scan collection was from 2000-25000 or 30000 *m/z* with a goal resolution of 15000 with 10 microscans.[29] For analysis of LL-37 nanodiscs, data were acquired for 5 min for each replicate using 0 V collision voltage in the high collisional dissociation (HCD) cell. Nanodisc stability was tested by increasing the collision voltage in the HCD cell from 0-100 V in 20 V increments with 1

min acquisitions for each step for a total of 6 min. Collision voltage ramp experiments with empty nanodiscs were also performed using 40 mM of charge reducing reagents imidazole and 2-methylimadazole respectively. Experiments were conducted in triplicate with tree separate nanodisc assemblies analyzed for each, and data is shown for a single representative replicate. Uncertainties are indicated using the standard deviation of single measurements of the replicate assemblies.

Mass Spectrometry Data Analysis. Mass spectra were analyzed using UniDec and MetaUniDec as described previously.[25] The mass range was extended from 20 to 250 kDa and charge range was 5-25. Peak width was set to 5 *m/z* for empty nanodiscs and for peptide nanodiscs, the peak width was set to 1 *m/z*. Charge smooth width was -0.5 and mass smooth width was 1, with the theoretical average lipid mass used as the mass difference.[40] Average lipid mass analysis was employed to confirm the lipid compositions of the empty nanodiscs.[45] Mass defect analysis was used for the peptide nanodiscs in order to determine the stoichiometry of LL-37 in DPPG and DPPG/SOPE/POCL nanodiscs as described previously.[37, 40]

3.3 Results and Discussion

Model Mammalian Nanodiscs. We first focused on creating model mixed-lipid mammalian nanodiscs that were resolvable by native MS. Mammalian cell membranes are extremely complex with thousands of lipid species but they primarily consist or phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA).[35] We chose the 4 most common species for an accurate representation of mammalian membrane

composition that can still accurately be analyzed by native MS: PC, PE, PS and SM. All lipid structures are included in Figure 3.2 below.

We synthesized nanodiscs with varying combinations of palmitoyl-oleoyl-phosphatidylcholine (POPC), 1-stearoyl-2-oleoyl-sn-glycero-3- phosphoethanolamine (SOPE), 1-palmitoyl-2-oleoyl-sn-glyc- ero-3-phospho-L-serine (POPS), and N-stearoyl-D-erythro-sphingosylphosphorylcholine (SSM). POPC was chosen as the primary eukaryotic phospholipid because it is one of the most abundant phospholipids found in mammalian membranes.[47] We chose SOPE because phosphatidylethanolamines (PE) are the second most abundant class of phospholipids found in mammalian cell membranes.[37] PE lipids are generally difficult for lipid membrane mimetics because they have a negative curvature which influences the overall shape of the lipid bilayer and this is not ideal for incorporation into flat nanodiscs.[46] Therefore, we screened a variety of PE lipids prior to our selection. SOPE was ideal because its mass was close to that of POPC which helps resolution of mass spectra, it formed the most stable PE nanodiscs, and it formed the most consistent nanodiscs in regard to size.[40] We observed that SOPE was preferentially ejected from POPC/SOPE mixed-lipid nanodiscs at high collision voltages so we trialed the addition of various charge reducing reagents to these nanodiscs as they have previously been shown to stabilize nanodisc complexes.[31] We found that 2-methylimidazole had the greatest positive effect on POPC/SOPE nanodiscs as it best stabilized the nanodiscs and prevented dissociation because the nanodisc complexes had a higher mass distribution and required much higher collision voltages to eject SOPE.[31] Therefore, 2-methylimidazole

was added to all subsequent POPC-based nanodiscs before analysis with native mass spectrometry.

When compared to POPC nanodiscs, 75/25 POPC/SOPE nanodiscs with added 2-methylimidazole had a similar mass distribution and polydispersity. The average lipid mass of POPC nanodiscs is higher than that of the POPC/SOPE mixed lipid nanodiscs. POPC nanodiscs have an expected lipid mass of 760.08 Da and based off our data we calculated the average lipid mass of POPC to be 760.24 ± 0.03 Da. SOPE lipids are lighter with an expected lipid mass of 746.05 Da and this was reflected by the calculated average lipid mass of the POPC/SOPE mixed lipid nanodiscs which was 757.02 ± 0.29 Da. This again, is very close to the expected value of 756.57 Da for 75/25 POPC/SOPE nanodiscs. Given the measured average lipid mass of the mixed lipid nanodiscs, the percent ratio for lipid incorporation was 78.2/21.8 ± 2.0% POPC/SOPE. All expected and measured lipid masses are reported in Table 1 below.

Model	Lipid Combination	Lipid Ratio (%)	Expected Mass (Da)	Measured Mass (Da)
Mammalian	POPC	100	760.08	760.24±0.03
	POPC/SOPE	72/25	756.57	757.02±0.29
	POPC/SOPE/POPS	65/25/10	756.76	757.28±0.20
	POPC/SOPE/POPS/SSM	55/25/10/10	753.86	753.13±0.86
	DPPC	100	734.04	734.17±0.09
	DPPC/SOPE	75/25	737.04	736.16±0.06
	DPPC/SOPE/POPS	65/25/10	739.84	739.25±0.76
	DPPC/SOPE/POPS/SSM	55/25/10/10	739.54	739.84±0.49
Bacterial	DPPG	100	722.97	723.18±0.11
	DPPC/SOPE	75/25	728.74	727.35±0.08
	DPPG/SOPE/POCL	65/25/5	726.74	725.54±0.42
Mitochondrial	DPPC/SOPE/TSCL		736.94	736.46±0.05

Table 1. The expected and measured average lipid masses for mixed lipid nanodiscs.

In increasing the complexity of the model mammalian nanodiscs, the third phospholipid we chose to incorporate was 1-palmitoyl-2-oleoyl-sn-glyc- ero-3-phospho-L-serine (POPS). PS lipids are required in many mammalian membranes for the targeting and function of intracellular proteins and are the most abundant of the anionic lipids in eukaryotic membranes.[48] POPS was chosen specifically because its mass of 762.00 Da is very close to that of POPC (760.08 Da) and SOPE (746.05 Da) to aid in resolution of mass spectra. We created POPC/SOPE/POPS mixed lipid nanodiscs with a ratio of 65/25/10

and 2-methylimidazole was added for stabilization prior to analysis with native mass spectrometry. The average lipid mass of the POPC/SOPE/POPS nanodisc complexes were calculated to be 757.28 ± 0.20 Da. The average lipid masses of the POPC/SOPE and POPC/SOPE/POPS were extremely close and difficult to differentiate but small-molecule MS was employed to confirm POPS incorporation.[40]

Finally, we incorporated SSM as the fourth and final lipid of the model mammalian nanodiscs. SSM plays a role in the regulation of transmembrane signaling in mammalian membranes, is a component of microdomains such as lipid rafts, has a role in human disorders and is relatively abundant in mammalian membranes.[49] We created POPC/SOPE/POPS/SSM mixed lipid nanodiscs with a ratio of 55/25/10/10 and 2-methylimidazole was added for stabilization prior to analysis with native mass spectrometry. These mixed lipid nanodiscs produced a well-resolved native mass spectra with a measured average lipid mass of 753 ± 0.86 Da which correlated with the expected average lipid mass of 753.86 Da. The successful addition of different lipid types that are close to the expected mass suggests that each lipid type was incorporated at the expected ratio. Given the significant stability and easily resolvable mass spectra of these model mammalian nanodiscs, it is expected that additional lipids similar in mass to POPC can be incorporated without difficulty to create more complex nanodiscs which better resemble a more native lipid bilayer environment. Additionally, this establishes these nanodiscs as another tool for native MS to study lipo-protein interactions in a more mammalian-type lipid bilayer to preserve native states and interactions of any incorporated peptides.

Saturated Model Mammalian Nanodiscs. To expand the utility of model mammalian nanodiscs we also applied our mixed lipid nanodisc recipe to saturated lipids. We selected dipalmitoyl-phosphatidylcholine (DPPC) as the starting lipid component. DPPC plays an important role in pulmonary cell tissue and is a common phospholipid found in mammalian membranes.[50] Consistent with POPC nanodiscs, we trialed incorporating PE lipids. Unfortunately, we were not able to maintain saturated lipid tails in the nanodisc complexes with the incorporation of distearoyl-phosphatidyl-ethanolamine (DSPE) as DPPC/DSPE mixed lipid nanodiscs were insoluble and did not readily assembly in nanodisc complexes.[40] This lack of compatibility of DPPC and DSPE in forming mixed lipid nanodiscs may be due to their diverse properties and different phase transition temperatures.[51] Therefore, we incorporated SOPE with DPPC lipids and successful nanodisc assembly occurred after optimization experiments to determine the ideal lipid ratios, MSP to lipid ratios, and phase transition temperatures. We found that a molar ratio of 75/25 DPPC/SOPE was ideal. Assembling nanodiscs at the phase transition temperature of the most abundant lipid (41° for DPPC) as detailed by prior studies, led to the formation of reproducible nanodiscs.[52] This provides successful baseline methods for synthesizing nanodiscs containing more complex levels of saturated mammalian lipids that can be used for future studies, including those to compare differences between saturated and unsaturated lipid complexes and to interrogate peptide lipid specificity.

Similar to POPC/SOPE nanodiscs, SOPE was preferentially ejected at high collision voltages, so we again trialed the addition of charge reducing reagents. Imidazole was shown to best stabilize the mixed lipid nanodiscs and therefore was added to each

sequential DPPC-based nanodisc recipe. DPPC-only nanodiscs had a measured average lipid mass of 734.17 ± 0.07 Da which is very close to the expected average lipid mass of DPPC at 737.04 Da that indicates proper incorporation of DPPC into the nanodisc complex. DPPC/SOPE mixed lipid nanodiscs have a measured average lipid mass of 736.16 ± 0.05 Da which is less than the expected average lipid mass of 737.04 suggesting differences in incorporation of DPPC and SOPE than expected. The ratio of DPPC/SOPE was calculated to be 81.0/19.0 ± 0.4% indicating preferential incorporation of DPPC. This suggests that DPPC may be better suited for incorporation into nanodiscs and is important to keep in mind when synthesizing various nanodiscs as DPPC incorporation may help facilitate easier assembly. Greater incorporation may also be due to different lipid-lipid interactions between DPPC and SOPE.

Next, we added POPS for the increasingly complex saturated model mammalian nanodiscs. DPPC/SOPE/POPS nanodiscs were assembled with a ratio of 65/25/10 and yielded well-resolved mass spectra in positive ionization mode with a measured average lipid mass of 739.25 ± 0.49 Da which correlates with the expected average lipid mass of 739.84 Da and suggests that lipids were incorporated in a manner close to the anticipated ratio.

Finally, we incorporated SSM to form DPPC/SOPE/POPS/SSM mixed lipid nanodiscs with a molar ratio of 55/25/10/10. These nanodiscs also were well-resolved in positive ionization mode. The average measured lipid mass was 739.84 ± 0.49 Da which closely matched the expected average lipid mass of 739.54 Da which suggests proper lipid incorporation into the nanodisc complex. SSM incorporation was validated by small-

molecule MS.[40] An interesting difference between the POPC and DPPC based nanodiscs was that the mixed lipid DPPC complexes had lower mass distributions than the 100% DPPC nanodiscs. Therefore, incorporation of unsaturated lipids likely affects the lipid packing of the nanodiscs and have the potential to be used to modulate the lipid packing of nanodiscs as desired for various experiments.[42] Again, given the relative stability and resolvable mass spectra of these saturated model mammalian nanodiscs, it is expected that additional lipids similar in mass to DPPC can be incorporated without difficulty. Successful formation and resolution of these complex nanodiscs shows that increasingly complex nanodiscs can be synthesized which offers new, more native-like tools for investigating biomolecules with native mass spectrometry. These different nanodiscs can also be employed to investigate lipoprotein interactions in more saturated and unsaturated nanodiscs with mammalian lipids.

Model Bacterial Nanodiscs. We next focused on creating model mixed-lipid bacterial nanodiscs that were resolvable by native MS. Bacterial membranes consist of a wide array of lipids with extremely diverse structures but some of the most common bacterial phospholipids include phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL).[36] We chose PG, PE, and CL lipids for an accurate representation of bacterial membrane composition that can still accurately be analyzed by native MS.

We first selected palmitoyl-oleoyl-phosphatidylglycerol (POPG) given its importance in Gram-negative and Gram-positive bacteria and lipid tail similarity to POPC which was the primary component of our model mammalian nanodiscs.[52] We trialed incorporating SOPE as the second phospholipid component of the model bacterial

nanodiscs because of the high abundance of PE lipids in bacterial membranes and successful incorporation of SOPE with the mixed lipid mammalian nanodiscs.[36] Unfortunately, assembly of these POPG based nanodiscs was not feasible due to the frequent formation of POPG/SOPE nanodiscs with three MSP belts regardless of lipid ratio, MSP:lipid ratio, incubation time and temperature for assembly.[40] We experienced similar issues when trying to incorporate POPE in place of SOPE.

Due to the properties of SOPE phospholipids which creates a negative curvature of the lipid bilayer, we hypothesized the formation of the triple-MSP belt nanodiscs may have been influenced by SOPE. We trialed replacing POPG with DPPG to try and offset the negative curvature caused by the incorporation of SOPE. DPPG nanodisc assemblies produced resolvable spectra after the addition of charge-reducing reagent imidazole which was added to every sequential DPPG based nanodisc recipe. The improvement in stability and spectra resolution with imidazole suggests that the addition of charge reducing reagents are a favorable strategy for synthesizing complex lipoprotein nanodiscs. DPPC-only nanodiscs had a measured average lipid mass of 723.18 ± 0.11 Da which correlates with the expected average lipid mass of 722.97 Da and suggests that DPPC was incorporated well into the nanodisc complex. We were able to successfully assemble DPPG/SOPE nanodiscs with a molar ratio of 75/25 which offers another different type of nanodisc recipe for investigating the incorporation and characterization of various biomolecules. The measured average lipid mass was 727.35 ± 0.08 Da which is less than the expected average lipid mass of 728.74 Da and correlates with a ratio of 82.3/17.7 ± 0.5% DPPG/SOPE. It is unclear why there is slight preferential incorporation of DPPG. We also observed

preferential ejection of SOPE from DPPG/SOPE mixed lipid nanodiscs. This means that DPPG/SOPE nanodiscs offer another tool for biomolecular study. It is important to keep in mind this behavior of SOPE ejection as it is seen in the absence of any additional biomolecules and is not a result of their incorporation.

Finally, we incorporated cardiolipin as the third component of the model bacterial nanodiscs. Cardiolipin is important for maintaining the shape of the bacteria and influences the organization and activity of bacterial proteins, especially those involved in oxidative phosphorylation.[43] We initially trialed incorporating tetra-oleoyl-cardiolipin (TOCL) that has fully unsaturated tails, but we observed the formation of more triple-MSP belt nanodiscs which are unfavorable for study because of the loss of normal lipid bilayer structure. With palmitoyl-oleoyl-cardiolipin (POCL) we were able to successfully assemble DPPG/SOPE/POCL nanodiscs. Though the mass of POCL is much greater than that of DPPG or SOPE, it is approximately equal to the mass of two DPPG or SOPE lipids which allows for resolvable mass spectra. Therefore, lipids demonstrating mass resonance or that are similar in mass to the other lipids incorporated in the nanodiscs are optimal for selection and this is a strategy that can be employed when adding further lipids. DPPG/SOPE/POCL mixed lipid nanodiscs were synthesized with a molar ratio of 65/25/5 given the mass of POCL is twice that of DPPG and SOPE. DPPG/SOPE/POCL had a measured average lipid mass of 725.54 ± 0.42 Da which is a bit less than the expected average lipid mass of 726.74 Da but still similar enough to suggest incorporation of each lipid close to the expected ratio of 65/25/5. Overall, DPPG/SOPE/POCL mixed lipid nanodiscs provide a simple model of bacterial membranes that is resolvable by native MS.

They offer an additional tool for interrogating biomolecular structures and interactions and can be used to compare interactions between unsaturated mammalian lipids, saturated mammalian lipids, and bacterial lipids contained in nanodisc complexes. We hypothesize that additional lipids maybe be incorporated after careful selection to model the complex native lipid environment of plasma membranes more closely.

Model Mitochondrial Membranes. Mitochondria are crucial for cell survival as they are the primary generator of ATP and influence complex cell signaling events.[44] Mitochondrial membranes are composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), as well as phosphatidylglycerol (PG) and cardiolipin (CL) which are exclusive to mitochondria.[14] The inner membrane of mitochondria is primarily composed of PC/PE/CL.[34] CL has diverse and important functions including stabilizing the respiratory chain, mitochondrial import, ceramide synthesis, aging, apoptosis and translation of electron transport chain components which made it an important component for our model mitochondrial nanodiscs.[45] We mimicked the primary mitochondrial lipid types by synthesizing nanodiscs with DPPC/SOPE/POCL but these again formed unfavorable triple MSP belt nanodiscs and therefore are not ideal candidates for creating nanodiscs with mitochondrial lipids. Trials with tetra-stearoyl-cardiolipin (TSCL), which is a fully saturated cardiolipin we considered earlier for model bacterial nanodiscs, allowed for the formation of stable mixed lipid nanodiscs with resolvable mass spectra after the addition of imidazole, offering another type of mixed-lipid nanodisc. The mass of TSCL is close to double that of DPPC so mass resonance allowed for well-resolved mass spectra.

DPPC/SOPE/TSCL nanodiscs were synthesized with a 65/25/5 molar ratio and had a measured average lipid mass of 736.46 ± 0.05 Da which is slightly bellowed the expected average lipid mass of 736.94 Da suggesting the actual ratio of incorporated lipids is slightly different than anticipated but still allows for successful nanodisc formation. Overall, these simplified model mitochondrial membrane nanodiscs open avenues for further studies with the incorporation of additional lipids when selected carefully. They also offer further nanodisc recipes for observing different biomolecular complexes in different lipid environments.

Peptide Incorporation in Bacterial Nanodiscs. We considered the incorporation of LL-37 into model bacterial nanodiscs to show that model lipid nanodiscs allow for the incorporation of biomolecular compounds into more complex nanodiscs and to characterize LL-37 in various lipids environments. LL-37 is a human antimicrobial peptide that plays a role in the innate immune response including microbial eradication, inflammation, angiogenesis, and wound healing which establishes the relevance of LL-37 for studies with native mass spectrometry.[56] LL-37 is thought to interact with bacterial cell walls to form a pore and ultimately cause cell death, but the oligomeric state and mechanisms of pore assembly is not thoroughly understood.[56] Therefore, we aimed to investigate how bacterial phospholipids affect LL-37 incorporation into membranes.

To further characterize LL-37 lipid specificity, we incorporated LL-37 in DPPG and DPPG/SOPE/POCL model bacterial nanodiscs. We found that LL-37 has primarily a trimeric state (3:1 peptide to nanodisc ratio) in 100% DPPG lipid nanodiscs, as shown in Figure 3.1 below, though other stoichiometries were transiently observed. Similarly in the

model bacterial nanodiscs, LL-37 was observed in its trimeric form but less often with more incorporation of four and five peptides when compared to single DPPG nanodiscs. These results suggest that PG is the primary lipid component needed for LL-37 incorporation in model bacterial nanodisc bilayers but other lipids play a subtle role in LL-37 membrane interactions. Overall, our data supports the utility of model membrane nanodiscs for characterizing incorporated biomolecules.

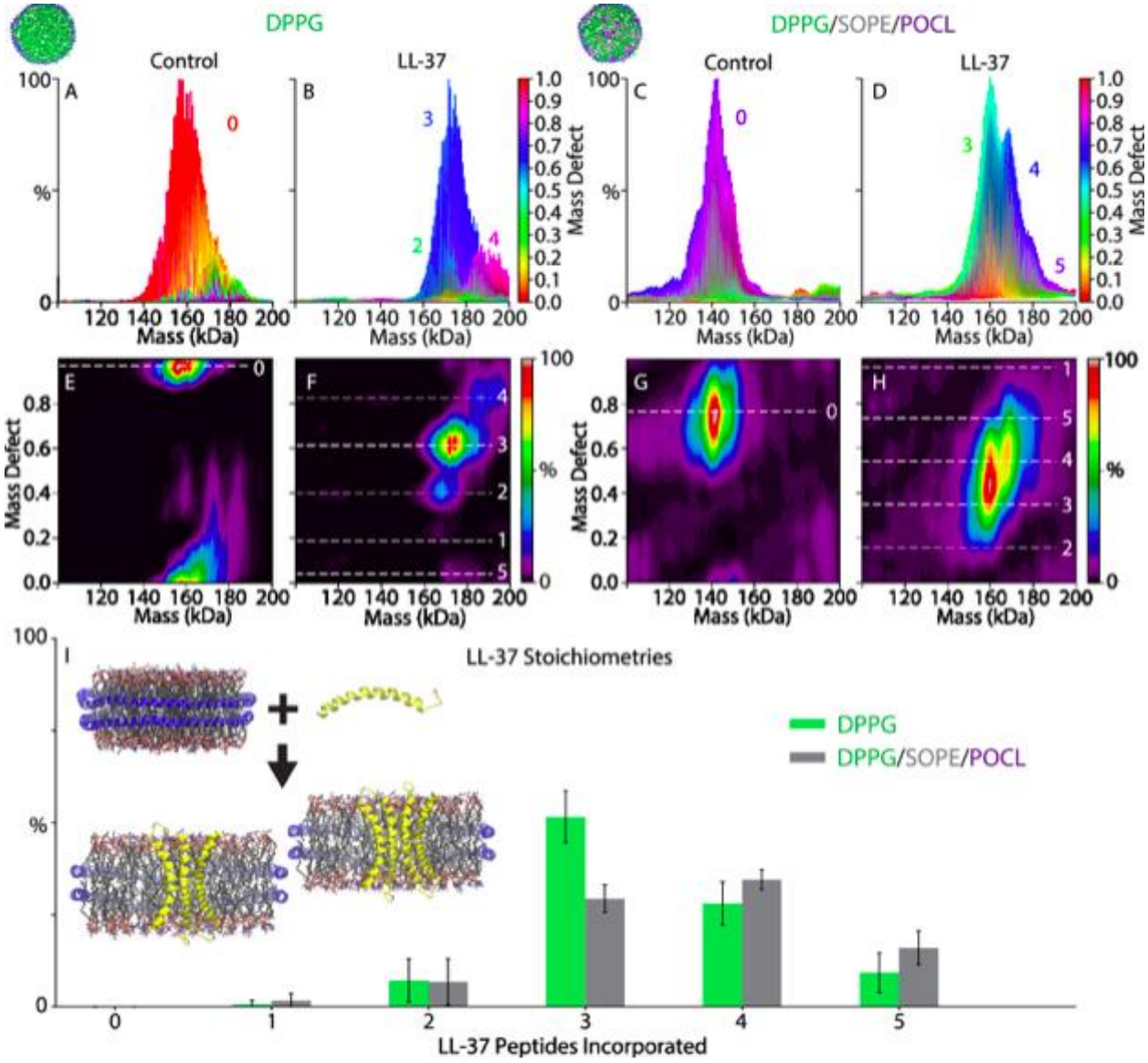
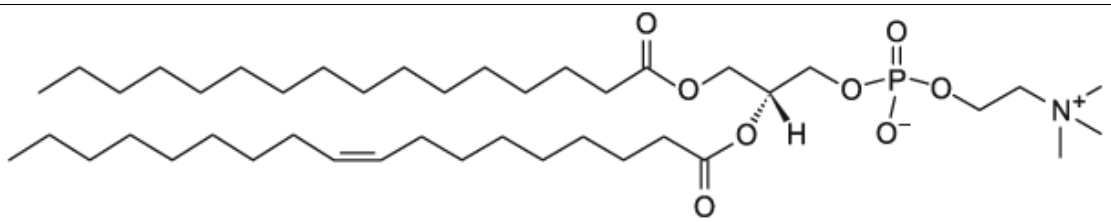
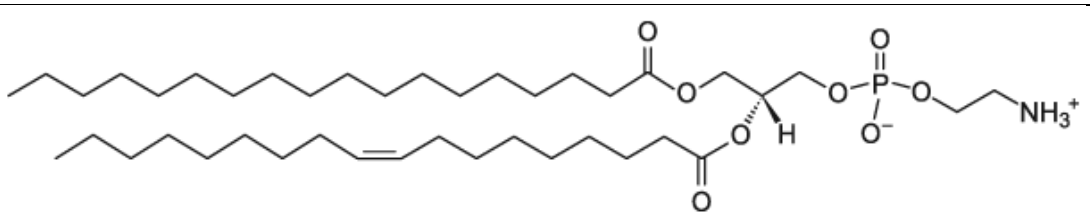
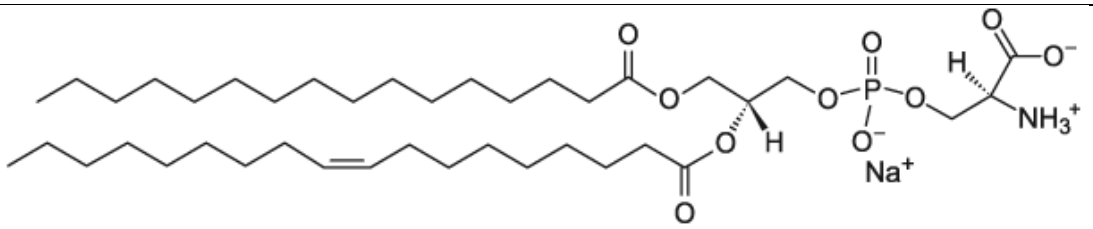
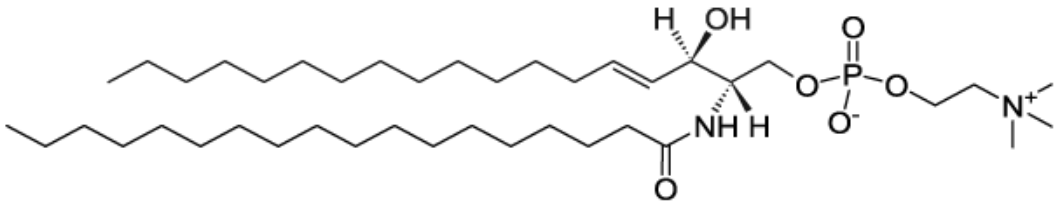
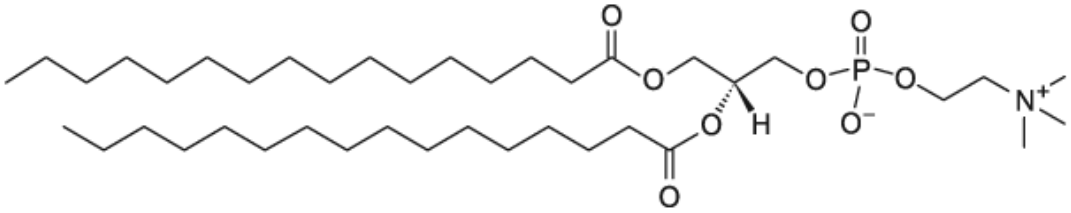
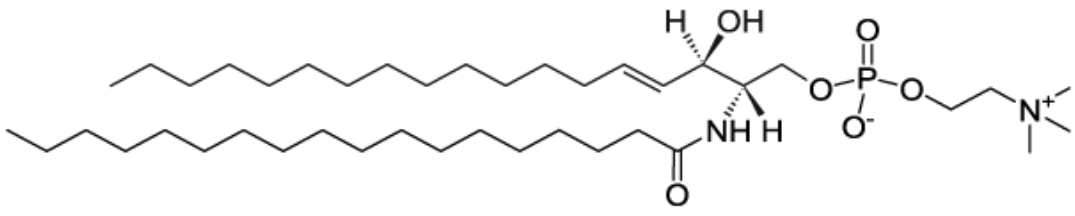


Figure 3.1. LL-37 incorporation in DPPG and DPPG/SOPE/POCL nanodiscs. Deconvolved mass spectra for (A) single lipid DPPG nanodiscs, (B) 3:1 LL-37:DPPG nanodiscs. (C) DPPG/SOPE/POCL nanodiscs, and (D) 3:1 LL-37:DPPG/SOPE/POCL nanodiscs. The mass peaks are colored by the mass defect with labeled stoichiometries. The corresponding mass peaks are colored by mass defect plots with the mass defect on the *y* axis and mass on the *x* axis with the predicted stoichiometries shown as dotted whit lines for (E) single lipid DPPG nanodiscs (control),

(F) 3:1 LL-37:DPPG nanodiscs, (G) DPPG/SOPE/POCL nanodiscs and (H) 3:1 LL-37:DPPG/SOPE/POCL nanodiscs. (I) Indicated bar charts with average relative intensities of LL-37 stoichiometries in single lipid DPPG (green) and DPPG/SOPE/POCL nanodiscs (gray). Error bars are shown as the standard deviation of triplicate nanodisc assembles. LL-37 PDB: 2K6O.

3.4 Conclusions

Model mammalian, bacterial and mitochondrial nanodiscs of complex lipid composition can be well-resolved with native MS when the incorporated lipids are similar in mass or demonstrate mass resonance. Successful mixed lipid nanodisc assembly requires balancing lipid head group, saturation, and tail length as well as consideration of phase transition temperature, molar ratios amongst other variables. The recipes we constructed provide a starting point for preparing other complex nanodiscs for analysis by native MS and may help guide the construction of liposomes or polymer nanodiscs amongst other lipid nanoparticles. These nanodisc complexes demonstrated stability with the addition of imidazole derivatives and additional lipids likely can be incorporated into the models when carefully selected to create more biologically relevant, heterogenous lipid bilayers. Successful incorporation and characterization of the LL-37 peptide in model bacterial membranes supports the use of these model membranes for incorporating and analyzing other biomolecules to gain a greater understanding of lipoprotein interactions in their native environment.

POPC	
SOPE	
POPS	
SSM	
DPPC	
DPPG	

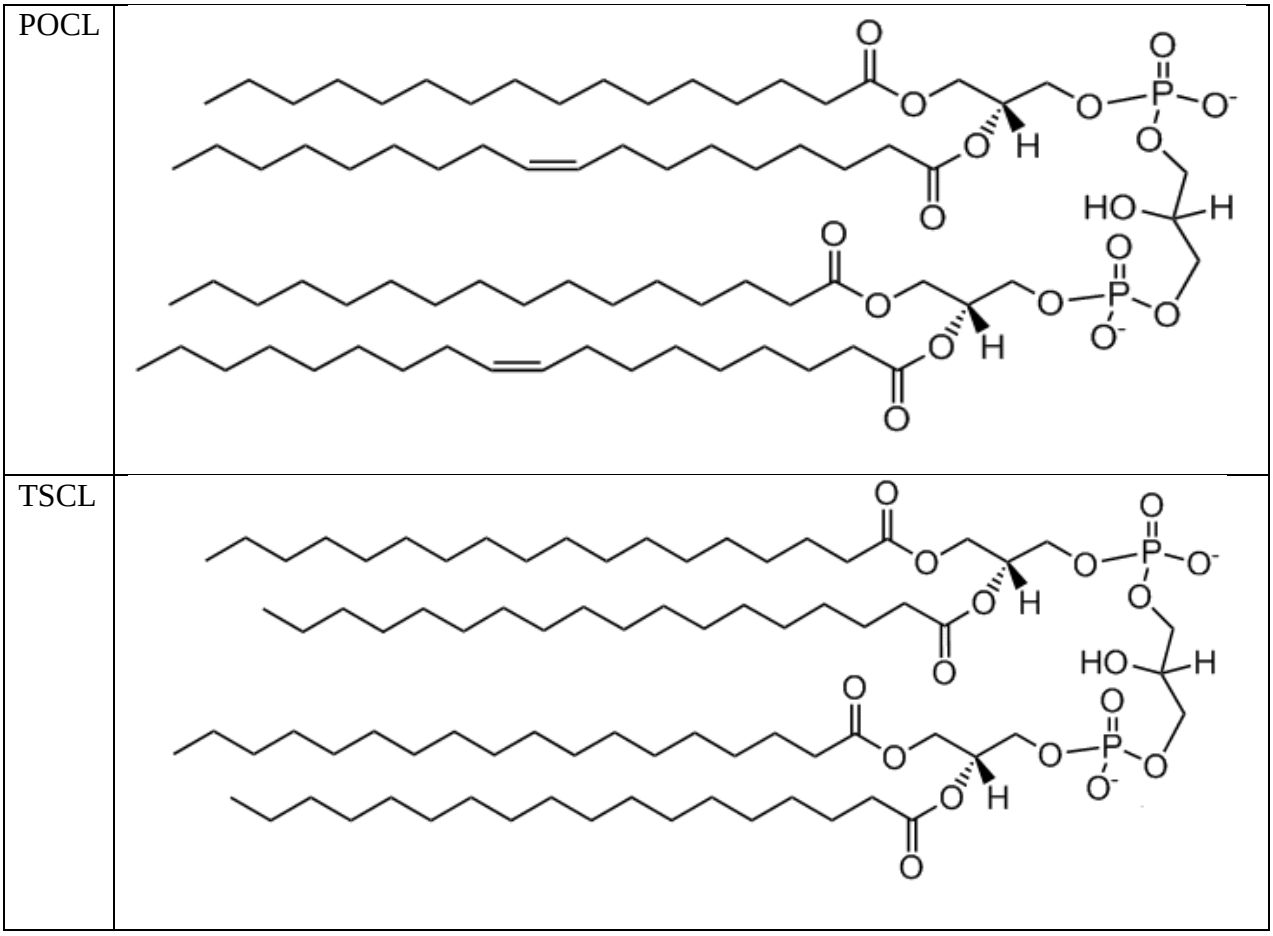


Figure 3.2. Molecular line structures of lipids used in assembling nanodiscs.

CHAPTER 4: UniDecCD: DECONVOLUTION OF CHARGE DETECTION-MASS SPECTROMETRY DATA

4.1 Introduction

Electrospray ionization mass spectrometry (ESI-MS) has emerged as an important technique for analyzing biomolecules such as membrane proteins.[57] ESI-MS can be used in conjunction with nanodiscs to study biomolecules in a more native state as they provide a monodisperse lipid bilayer without the drawbacks of detergents. The utility of nanodiscs for ESI-MS has been expanding to encompass increasingly complex samples as well as provide more biologically relevant conditions in which to study lipoprotein interactions. These complexes can be employed to characterize the oligomeric state and interactions of various peptides, and nanodisc lipid composition can be modulated to model that of various biological membranes.[31, 40]

An important component of data analysis of nanodisc mass spectra following ESI-MS is charge deconvolution which infers the mass from the mass over charge (*m/z*) state.[58] Charge states are typically assigned based off isotope distribution that uses the known mass between various isotopologues, or the charge state distribution pattern can be used to infer charge states.[50] However, deconvolution of native mass spectra can be difficult given adduction, low resolution, and overlapping charge states. [58] Large, polydisperse samples like intact viral capsids and natural lipid extract nanodiscs yield mass spectra that are uninterpretable due to too many mass peaks that reconstructively overlap. This limitation of ESI-MS has made it difficult to analyze various biomolecules in their most native state, including nanodiscs containing natural lipid extracts.

We aim to address this challenge with charge detection-mass spectrometry (CD-MS) which can measure the *m/z* and charge states of single ions [60] CD-MS is becoming a more widely used technology given its ability to analyze the intact mass of large polydisperse samples and complexes including vaccines and gene delivery vehicles, and is no longer limited to custom built mass analyzers.[59, 60] Unfortunately, despite the compatibility of CD-MS with Orbitraps, custom instruments are still capable of higher charge resolution as there is relatively high charge uncertainty that results from CD-MS with Orbitraps.[61] In order to overcome such charge uncertainty, we use UniDecCD (UCD) which is the first deconvolution algorithm for CD-MS data.[59] UCD expands upon UniDec which is currently used for deconvolution of ESI-MS mass spectra and applies the UniDec algorithm to CD-MS.[59] UCD enables efficient deconvolution of two-dimensional CD-MS data that computationally reduces the uncertainty of charge assignment to improve the accuracy of CD-MS for Orbitrap mass analyzers.[59] UCD of CD-MS data also allows analysis of adeno-associated viral (AAV) capsids and nanodiscs containing natural lipid extracts which further expands the utility of nanodiscs for studying biomolecules in their most native state and increases the capability of native MS to analyze complex samples.

4.2 Methods

Materials. Ammonium acetate, Amberlite XAD-2 biobeads and sodium cholate detergent were purchased from Sigma Aldrich. Dipalmitoyl-phosphatidylcholine (DPPC) lipids, brain polar lipid extract (porcine), and *Escherichia coli* were purchased from Avanti Polar lipids. Lipid concentrations were determined via phosphate analysis.

Capsid Preparation. The membrane scaffold protein, MSP1D1(-), was expressed and purified from *Escherichia coli* and purified by immobilized metal affinity chromatography (IMAC) as described previously.[35] Adeno-associated viral 8 (AAV8) capsids were produced by triple transfection HEK293 cells as described elsewhere and then purified by affinity and ion-exchange chromatography.[59, 62] Fractions of only empty capsids were consolidated and resuspended in phosphate-buffered saline supplemented by 0.001% poloxamer 188. AAV8 capsids were buffer exchanged by resuspension in 200 mM ammonium acetate and concentrated with a 100 kDa molecular weight filter two or more times prior to analysis with native MS.

Nanodisc Preparation. DPPC nanodiscs were synthesized as described previously.[40] DPPC lipids were dissolved in chloroform and dried under nitrogen and then vacuum for 4 hours. DPPC lipids were resuspended in 0.1M cholate before the addition of MSP1D1(-). The lipid/MSP solution was incubated for 30 minutes at 41°C. SM-2 biobeads were added next at a ratio of 0.5 mg/1 mL MSP and the mixture was incubated for 4 hours at 41°C. The nanodiscs were purified by size-exclusion chromatography with a Superose 6 Increase column (Cytiva).[59]

For the *E. coli* lipid extract nanodiscs, *E. coli* polar lipids were dried under nitrogen and then vacuum for 4 hours. *E. coli* polar lipids were then resuspended in 0.1M sodium cholate before the addition of MSP1D1(-). The final molar ratio of lipid to MSP was 50:1 and the final concentration of cholate in the *E. coli* polar lipids, MSP, cholate mixture was 25 mM.[59] This mixture was incubated for 30 minutes at room temperature before XAD-2 biobeads were added and then the mixture incubated for another 4 hours at room

temperature. For brain nanodiscs, brain polar lipids were dried under nitrogen and then vacuum for four hours. The brain lipids were then resuspended in 0.1M sodium cholate before the addition of MSP1D1(-). The final molar ratio of lipid to MSP was 40:1 and the final concentration of cholate in the brain lipids, MSP, cholate mixture was 35 mM.[59] This mixture was incubated for 30 minutes at room temperature before XAD-2 biobeads were added and then the mixture incubated for another 4 hours at room temperature. Following incubation with biobeads, the nanodiscs were filtered by a 0.2-micron spin filter and purified using a Superose 6 Increase column. Charge reducing reagent imidazole was then added to all nanodiscs for a final concentration of 40mM prior to native MS and CD-MS.

Native Mass Spectrometry. Nanoelectrospray ionization (nESI) was performed using borosilicate needles pulled in-house with a P-1000 micropipette puller (Sutter Instrument, Novato, CA). Mass spectrometry was performed using a Q-Exactive HF quadropole-Orbitrap mass spectrometer equipped with ultrahigh mass range research modifications (Thermo Fischer Scientific).[6] Important instrumental parameters for nanodiscs included injection flatpole voltage 10V, inter-flatpole lens 10V, bent flatpole voltage 1V, transfer multipole 0V and C-trap entrance lens 6V.[59] For the empty AAV8 capsids, argon was used for the collision gas and trapping gas pressure was set to 10.[59] Important instrumental parameters included bent flatpole voltage 4V, and C-trap entrance lens 9V.[59]

CD-MS Data Analysis. CD-MS was performed using a Q-Exactive HF quadropole-Orbitrap mass spectrometer equipped with ultrahigh mass range research modifications (Thermo Fischer Scientific).[34] For each sample analyzed with CD-MS,

resolution was set to 240,000, or a transient time of 512 ms, and the noise threshold was set to 0.[59] Data was collected for 2-15 mins. Optimal replicates contained at least 5000 ions following filtering.

For CD-MS analysis of DPPC, *E. coli,* and brain nanodiscs, important settings included a trapping gas pressure of 1, high *m/z* detector optimization, high *m/z* transfer optics, *m/z* range of 2000-25,000 for natural lipid extract nanodiscs and 6000-25,000 for DPPC nanodiscs, and source fragmentation was 30 V for natural lipid extract nanodiscs and 50 V for DPPC nanodiscs.[50]

For CD-MS analysis of AAV8 particles, important settings included an *m/z* range of 5000-45,000, HCD voltage of 200 V, spray voltage of 2200-2600 V, and trapping gas was set to 8-19. Detailed deconvolution settings were used as described previously.[59]

UCD Software, Data Processing and Deconvolution Algorithm. UCD software, data processing and deconvolution was developed by Marty and co-workers and extensive details can be found elsewhere.[59] Briefly, UCD is available as a module of the UniDec software package in version 5.0.0 (https://github.com/michaelmarty/UniDec). UCD is programmed in Python 3 and uses a Model-Presenter-View architecture.[59] The UCD algorithm includes a data processing and deconvolution part. For data processing, data is first imported from the provided file path and must be provided in centroid mode. UCD then saves *m/z*, intensity, and scans to a NumPy array as a NumPy compressed binary file. Noise level is then measured. The data array is filtered to remove unwanted ions, the intensity values of the ions are converted to charge states, and then the list of ions with *m/z* and *z* values are pooled with a two-dimensional histogram to produce a 2D array of ion

counts as a function of the *m/z* and *z*.[59] The histogram is processed, converted into a resampled mass vs. charge array, and then the array is summed along the charge axis to produce a final mass distribution.[59] CD-MS data then can be deconvolved with a Bayesian deconvolution algorithm that has a Richardson-Lucy deconvolution algorithm at its core.[48,53] The user defines a 2D "point spread function" that specifies how much an ideal delta function of ions would spread in *m/z* and *z* dimension in the instrument and this is determined by measuring a well-resolved protein. Apart from this primary component of deconvolution software, other additional deconvolution tactics are available including smoothing of charge state distribution, a SoftMax function and point smoothing to further improve resolution of data.[59]

4.3 Results and Discussion

Orbitrap CD-MS Data Calibration. Our primary goal was to employ computational deconvolution to reduce charge uncertainty of CD-MS data and expand the utility of nanodiscs to include natural lipid extracts through applying CD-MS with UniDecCD to nanodiscs containing natural lipid abstracts. We wanted to demonstrate that UCD is capable of deconvolving and improving the resolution of CD-MS data obtained from large, polydisperse samples that are not readily resolvable with traditional native MS. We used the signal-to-noise (S/N) ratio instead of raw intensity to measure the charge of various ions.[59] Noise is defined as the peak of the second derivative of the intensity distribution above the median noise level.[50] Our calibration yielded a slope of 0.2083 S/N per charge, but it is best for users to pursue their own calibration given variability across instruments.[59] The UCD deconvolution algorithm was employed after calibration for

converting S/N to charge through transferring raw data to UniDecCD. Raw data was notable for charge inconsistencies but after UCD deconvolution, we were able to remove the charge uncertainty from CD-MS spectra and reliably assign charge states.

Deconvolution of AAV Capsids. AAV8 capsids are promising vehicles for delivery of gene therapeutics.[63] However, they are very complex with small variations between capsids proving difficult to analyze. Recently, progress has been made by CD-MS to analyze filled, partially filled, and empty capsids so we aimed to employ CD-MS and UCD deconvolution to a basic AAV capsid system as well.[49] We began by measuring the mass of empty AAV capsids using raw CD-MS data. We found that the average mass of the AAV capsids was 3.81 ± 1 MDa which correlated with the expected mass of 3.78-3.84 MDa. With UCD we found the average mass to be 3.82 ± 0.02 MDa. An F-test found no statistical difference in the standard deviation of the mean, so the UCD algorithm did not significantly alter the accuracy of the measured mass.[59] However, UCD did improve the resolution of CD-MS measurement. We found the mass distribution for the AAV capsids form raw CD-MS data had an average full width half maximum (FWHM) value of 274 kDa but UCD deconvolution lowered this to 144 kDa.[50] This data demonstrates that UCD is capable of deconvolving and improving the resolution of CD-MS data obtained from large, polydisperse samples that are not readily resolvable with traditional native MS.

CD-MS of Nanodiscs. We used CD-MS to analyze DPPC nanodiscs to test UCD with a sample that produces well-resolved yet overlapping charge states in order to show that CD-MS with UCD can deconvolve mass spectra of traditional nanodisc structures that are resolvable by traditional native MS. The mass smoothing function from UniDec was

added to UCD and allowed for the deconvolution of DPPC nanodiscs with resolved lipid mass peaks. This was similar to traditional native MS data and demonstrated significant mass resolution as shown in Figure 4.1 below. This shows us that CD-MS with UCD for nanodiscs is successful and allows for resolution of lipoprotein complexes.

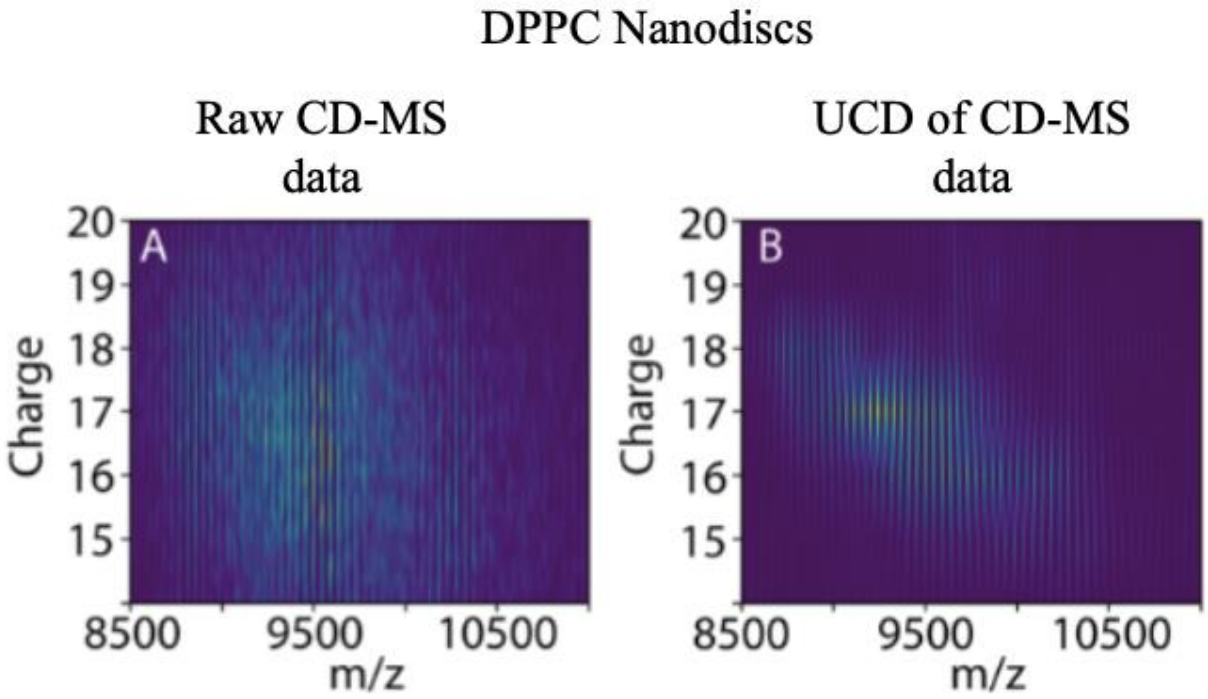


Figure 4.1. Charge versus *m/*z plots for raw CD-MS data of DPPC nanodiscs (left) and UCD deconvolution of CD-MS data of DPPC nanodiscs (right).

Next, we sought to analyze nanodiscs synthesized using natural lipid extracts. We have shown that mixed lipid nanodiscs can be resolved by native MS when the lipids are close in mass or their mass is resonant of other lipids.[40] However, natural lipid membranes can contain over 1000s lipid types all of varying mass which makes them nearly impossible

to resolve with traditional native MS. We performed conventional native MS of the *E. coli* polar lipid extract and porcine brain polar lipid extract nanodiscs which produced unresolvable spectra as expected. We then conducted CD-MS of these nanodiscs which produced spectra with charge and mass values similar to that of the well-resolved DPPC nanodiscs, but the mass distributions remained rather broad. We applied UCD using only the Richardson-Lucy deconvolution (so there was no charge smoothing) and this significantly reduced the broad mass distribution, allowing for a polydispersity that was very similar to DPPC nanodiscs (see Figure 4.2).

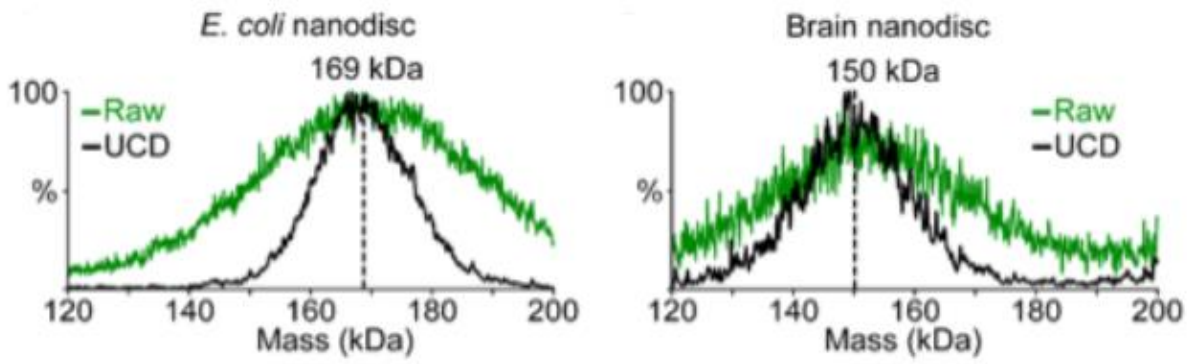


Figure 4.2. Replicates of *E. coli* nanodiscs (left) and brain nanodiscs (right) with raw CD-MS data plotted in green and UCD deconvolution of CD-MS data in black.

Despite being unable to resolve the individual lipid mass peaks of the natural lipid extract nanodiscs completely, as shown by the elevated baseline of the peaks indicated in Figure 4.2, the mass distribution provided useful information regarding size and polydispersity that has helped to optimize nanodisc assembly.[59] Using the standard deviation of the average mean from three nanodisc replicates, we found that *E. coli*

nanodiscs had a center of mass of 166.0 ± 4.1 kDa and the brain nanodiscs had a center of mass od 152.8 ± 3.8 kDa. The brain lipid extract nanodiscs had a lower center of mass than the DPPC nanodiscs (161.9 ± 3.6 kDa) which demonstrated looser lipid packing than the DPPC nanodiscs. The average FWHM of the mass distributions of bath natural lipid extract nanodiscs matched that of the DPPC nanodiscs which demonstrates the capability of UCD to improve the precision of CD-MS data and reveal a similar polydispersity between natural and synthetic lipid nanodiscs.[59]

Ultimately, UCD deconvolution significantly improved the resolution of natural lipid extract nanodiscs. These nanodiscs provide a more native environment than previously synthesized nanodiscs using synthetic lipids or mixed lipid compositions for the study of biomolecules such as membrane proteins. The addition of UCD to CD-MS is a useful tool for more precise characterization of the polydispersity and mass distribution of natural lipid nanodiscs and aiding in optimizing nanodisc assembly.

4.4 Conclusions

We have shown that computational deconvolution by UCD of CD-MS data greatly reduces charge uncertainty and allows for the assignment of charge states of single ions. We used UCD for characterizing AAV capsids that are not resolvable with ESI-MS. UCD also allowed for the resolution of nanodiscs synthesized with *E. coli* and brain polar lipid extract which provides the first native MS measurement of natural lipid extracts and expands the utility of nanodiscs for providing near-native environments for characterizing biomolecules.

CD-MS technology is quickly advancing with the development of new methods such as selective temporal overview of resonant ion (STORI) plots that will further improve the resolution of CD-MS data with Orbitrap mass analyzers and further reduce charge uncertainty. We anticipate UCD will prove beneficial for deconvolution of this type of data as well.

Overall, UCD provides CD-MS data analysis and deconvolution software that will expand the use of CD-MS for studying very complex, polydisperse and high mass samples that will further the capabilities of native MS.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Biological membranes are extremely complex and heterogeneous, owing to their numerous important physiological roles. A key component of biological membranes that helps distinguish their function are membrane proteins. Membrane proteins are favorable pharmaceutical targets given their accessibility and role in cellular signaling. Additionally, nearly a quarter of all proteins are membrane proteins as they represent 20-30% of the proteome for most organisms.[65] However, membrane proteins themselves are difficult to analyze due to their variable stability, flexibility, and complexity which presents challenges for expression, solubilization, purification and analysis.[65] This is reflected by lack of characterized membrane protein structures as only 1% of the entries in the protein repository data represent membrane protein complexes.[65] Despite the challenges presented by membrane proteins, technological advances have allowed for some structures to be captured by X-ray crystallography and analyzed by NMR. These provide limited static interpretations of membrane protein structure and relay little information regarding their dynamic interactions. Native MS more recently has allowed for membrane protein analysis in a more native lipid environment and can be used to determine lipo-protein interactions.[13] Detergent micelles have been traditionally employed for membrane protein analysis with native MS due to helping facilitate membrane protein solubility in solution, but the use of detergent can alter membrane protein structure and function. Nanodiscs provide a more native platform for studying membrane proteins with native MS given their monodispersity, controllability and ability to provide specific combinations of phospholipids needed for active membrane protein function.[66]

The advantages of nanodiscs make them more suitable for native MS than traditional detergent micelles, liposomes and amphipols. We have demonstrated that charge reducing and

supercharging reagents allow for further control of nanodisc stability. Charge reducing reagents allowed for more stable nanodiscs that had a narrower mass distribution with cleaner spectra. The addition of charge reducing reagents to nanodisc complexes also provides higher quality data and improves the application of nanodiscs for native MS, allowing for more complex studies to be conducted. Such reagents can also allow for the incorporation of various biomolecules including membrane proteins for further characterization of these understudied complexes. Supercharging reagents, on the other hand, allowed for the ejection of lipids from nanodisc complexes and may be useful for ejecting other biomolecules of interest from nanodiscs with bound lipids.[32] In negative mode, some supercharging reagents were seen to be stabilizing instead of disruptive and can also be employed for manipulating nanodiscs under specific ionization conditions. Overall, we demonstrated various ways to modulate nanodisc stability which has the potential to be extended to improve the use of other lipid nanoparticle complexes and increase the range of biomolecules capable of being characterized by native MS with nanodiscs.

These advances in nanodisc technology provide better lipoprotein vehicles for further experimentation. Our initial studies were limited to a small range of charge manipulation reagents. Analyzing nanodisc complexes with a variety of other reagents may allow for further optimization of nanodisc samples that confers greater stability and allows for better incorporation of biomolecules for analysis by native MS. They may also improve the accuracy of characterizing such biomolecules and better modulate ejection from nanodisc complexes. The mechanism of stabilization and destabilization by given reagents is also not thoroughly understood. With further advances in technology, we may be able to better characterize the mechanism by which various reagents manipulate nanodiscs. CD-MS has recently become more available for native MS in

instruments that are not custom built and can more closely investigate lipo-protein interaction through the assignment of charge states of single ions that allows for better resolution of complex mass spectra.[59] Incorporating additional biomolecules into charge-manipulated nanodiscs may also provide further insight. Our approaches were also limited to a few lipid types. We used only anionic and zwitterionic lipids that are not applicable to a wide range of biological membranes. With the advancement of more complex nanodiscs consisting of two to four synthetic phospholipids, the effects of charge manipulation reagents on more complex lipid bilayers may prove useful in our understanding of their effects on nanodiscs.[40] Additionally, nanodiscs even containing natural lipid extracts are now more resolvable with native MS using CD-MS and charge manipulation reagents may be useful in modulating these more natural nanodiscs.[59]

Advances in nanodisc manipulation expanded the application of nanodiscs for native MS. However, even with the increased stability, improved spectra resolution and data quality, as well as controllability of nanodiscs with charge manipulation reagents, traditional nanodiscs only consist of one or two synthetic phospholipid types which is not characteristic of biological membranes that can contain thousands of different types of phospholipids.[42] In Chapter 3, we discuss how we address this challenge and create more native-like nanodiscs containing up to four different phospholipids that mimic bacterial, mammalian and mitochondrial membranes based on the chosen lipid class. By carefully selecting lipids similar in mass or resonant in mass, we were able to resolve mass spectra of these more complex nanodiscs and the addition of the same charge reducing reagents as used in Chapter 2 (IM) further improved the resultant mass spectra and kept nanodiscs more intact. We also demonstrated how these different types of model nanodiscs effect the incorporation of human antimicrobial peptide LL-37. We found specificity for PG in LL-37

incorporation into model bacterial membranes which supports the use of model membrane nanodiscs for analyzing biomolecules. Overall, we have synthesized nanodiscs of increasing complexity that can serve as tools for studying biomolecules in more natural environments which further increases the application of nanodiscs for native MS. These nanodiscs can provide promising vehicles for investigating the specificity of various peptides. They also are the next step towards creating natural lipid nanodiscs resolvable with native MS. Successful synthesis and resolution by native MS of more complex nanodiscs can also help in creating liposomes amongst other lipid nanoparticles.

The primary issue with mixed-lipid nanodiscs is the difficulty in resolving nanodisc complexes that do not contain lipids close in mass. Therefore, developing new methods for data analysis will prove useful in better understanding these complex structures and improve the resolution of data when other biomolecules are incorporated for more accurate characterization. We also only included a small range of the available phospholipids that provided a few select models. The usefulness of mixed-lipid nanodiscs may be improved by incorporating a wider range of other phospholipids that are specifically suitable for the given biomolecules of choice. Further investigation of different phospholipids for mixed-lipid nanodiscs may also allow for the optimization of nanodiscs in serving as models for bacterial, mitochondrial and mammalian membranes. The use of different MSP belts may allow for further tailoring of nanodiscs to meet the needs of various models as we exclusively used MSP1D1(-). Regarding future uses of model membrane nanodiscs, one application may involve using more heterogeneous nanodiscs to help investigate how membrane proteins remodel their environment. Another interesting prospect would be to investigate the effects of temperature on these model membrane nanodiscs to gain

further insight into the unfolding and destabilization process, especially if other biomolecules were inserted into the nanodisc complexes.

As we have stabilized nanodisc complexes and carefully synthesized more heterogeneous samples to mimic a natural membrane environment, the next logical step was to improve the resolution of mass spectra that results from the use of nanodiscs containing natural lipid extract which would provide for the most native possible nanodisc state. However, unless the lipids selected for in any given nanodisc prep are similar or resonant in mass, the mass spectra are difficult to resolve, which is not possible for natural lipid extracts given their extreme polydispersity.[59] We strived to overcome this limitation by using CD-MS, which can analyze large, polydisperse samples on the same level of complexity as vaccines and gene delivery systems. We paired CD-MS with the first ever deconvolution algorithm for CD-MS known as UniDecCD. With this technology we were able to improve accuracy and overall data analysis using CD-MS for native mass spectrometry of nanodiscs containing natural lipid extracts and AAV capsids. We not only have significantly improved the resolution of natural lipid nanodiscs but have furthered the capabilities of native MS for extremely complex samples.

We anticipate further optimization of CD-MS for native MS with technology such as STORI plots that allow for the complete correction of intermittent signals and ion differentiation.[67] This may help provide even more complete resolution of natural lipid extract nanodiscs as well as for other high-mass samples including AAV capsids. Our research can also be expanded upon by investigating other natural lipid extracts such as that of mitochondria to further our model membrane nanodiscs. These may also provide further insights into the capabilities of CD-MS.

We have engineered nanodiscs to provide stable and extremely biologically relevant conditions in which to study lipo-protein interactions, membrane proteins and drug binding effects. We have also improved mass spectra resolution and data deconvolution to characterize various nanodiscs complexes more accurately with and without the addition of biomolecules. UniDecCD paired with CD-MS also expands the capabilities of native MS to resolve increasingly convoluted mass spectra. Taken together, this opens new horizons for structural biology as well as pharmaceutical development. Native MS of nanodiscs helps bridge the gap for existing analytical techniques and addresses numerous limitations. Overall, these new stabilization and charge manipulation techniques, synthesis of near-native state environments, data analysis approaches and instrument advancement have the potential to solve the exceedingly complex biological and analytical challenges facing the world.

REFERENCES

(1) Helbig A, Heck A, Slijper M. Exploring the membrane proteome – Challenges and analytical strategies. Journal of Proteomics 2010, 73 (5) 868-878. https://doi.org/10.1016/j.jprot.2010.01.005

(2) Rosendo-Pineda MJ, Moreno CM, Vaca L. Role of ion channels during cell division. Cell Calcium. 2020 Nov;91:102258. doi: 10.1016/j.ceca.2020.102258. Epub 2020 Jul 24. PMID: 32736154.

(3) Griffin, N. M., & Schnitzer, J. E. (2011). Overcoming key technological challenges in using mass spectrometry for mapping cell surfaces in tissues. Molecular & cellular proteomics: MCP, 10(2), R110.000935. https://doi.org/10.1074/mcp.R110.000935

(4) Cournia, Z., Allen, T. W., Andricioaei, I., Antonny, B., Baum, D., Brannigan, G., Buchete, N. V., Deckman, J. T., Delemotte, L., Del Val, C., Friedman, R., Gkeka, P., Hege, H. C., Hénin, J., Kasimova, M. A., Kolocouris, A., Klein, M. L., Khalid, S., Lemieux, M. J., Lindow, N., … Bondar, A. N. (2015). Membrane Protein Structure, Function, and Dynamics: a Perspective from Experiments and Theory. The Journal of membrane biology, 248(4), 611–640. https://doi.org/10.1007/s00232-015-9802-0

(5) Gong J, Chen Y, Pu F, Sun P, He F, Zhang L, Li Y, Ma Z, Wang H. Understanding Membrane Protein Drug Targets in Computational Perspective. Curr Drug Targets. 2019;20(5):551-564. doi: 10.2174/1389450120666181204164721. PMID: 30516106.

(6) Calabrese, A. and Radford, S. (2018). Mass spectrometry-enabled structural biology of membrane proteins. Methods, 147(1), 187-205. https://doi.org/10.1016/j.ymeth.2018.02.020

(7) D'Jimprima, R., and Kulbrandt., W. (2021). Current limitations to high-resolution structure determination by single-particle cryoEM. Quarterly Reviews of Biopysics 52, E4. doi:10.1017/S0033583521000020

(8) Cavalli A, Salvatella X, Dobson CM, Vendruscolo M. Protein structure determination from NMR chemical shifts. Proc Natl Acad Sci U S A. 2007 Jun 5;104(23):9615-20. doi: 10.1073/pnas.0610313104. Epub 2007 May 29. PMID: 17535901; PMCID: PMC1887584.

(9) Tzitzilonis C, Eichmann C, Maslennikov I, Choe S, Riek R. Detergent/nanodisc screening for high-resolution NMR studies of an integral membrane protein containing a cytoplasmic domain. PLoS One. 2013;8(1):e54378. doi: 10.1371/journal.pone.0054378. Epub 2013 Jan 22. PMID: 23349867; PMCID: PMC3551814.

(10) Rozbesky, D., Man, P., Kavan, D., Chmelik, J., Cerny, J., Bexouska, K., Novak, P. Chemical Cross-Linking and H/D Exchange for Fast Refinement of Protein Crystal Structure. Analytical Chemistry 2012 84 (2), 867-870 DOI: 10.1021/ac202818m

(11) Liu, X. R.; Zhang, M. M.; Gross, M. L. Mass Spectrometry-Based Protein Foot printing for Higher-Order Structure Analysis: Fundamentals and Applications. Chem. Rev. 2020, 120, 4355– 4454, DOI: 10.1021/acs.chemrev.

(12) Liu, M., Van Voorhis, W.C. & Quinn, R.J. Development of a target identification approach using native mass spectrometry. Sci Rep 11, 2387 (2021). https://doi.org/10.1038/s41598-021-81859-4

(13) Barth M, Schmidt C. Native mass spectrometry-A valuable tool in structural biology. J Mass Spectrom. 2020 Oct;55(10):e4578. doi: 10.1002/jms.4578. PMID: 32662584.

(14) A.J. Borysik, S.E. Radford, A.E. Ashcroft. Co-populated conformational ensembles of β2-microglobulin uncovered quantitatively by electrospray ionization mass spectrometry. Biol. Chem., 279 (2004), pp. 27069-27077

(15) Song DH, Garcia G Jr, Situ K, Chua BA, Hong MLO, Do EA, Ramirez CM, Harui A, Arumugaswami V, Morizono K. Development of a blocker of the universal phosphatidylserine- and phosphatidylethanolamine-dependent viral entry pathways. Virology. 2021 Aug;560:17-33. doi: 10.1016/j.virol.2021.04.013. Epub 2021 May 10. PMID: 34020328; PMCID: PMC8673400.

(16) Marty, M. T.; Hoi, K. K.; Robinson, C. V. Interfacing Membrane Mimetics with Mass Spectrometry. Acc. Chem. Res. 2016, 49, 2459– 2467, DOI: 10.1021/acs.accounts.6b00379

(17) Denisov, I., Sligar, S. Nanodiscs for structural and functional studies of membrane proteins. Nat Struct Mol Biol 23, 481–486 (2016). https://doi.org/10.1038/nsmb.3195

(18) Lomeli, S. H.; Yin, S.; Ogorzalek Loo, R. R.; Loo, J. A. Increasing charge while preserving noncovalent protein complexes for ESI-MS. J. Am. Soc. Mass Spectrom. 2009, 20, 593– 596, DOI: 10.1016/j.jasms.2008.11.013

(19) Frick M, Schmidt C. Mass spectrometry-A versatile tool for characterising the lipid environment of membrane protein assemblies. Chem Phys Lipids. 2019 Jul;221:145-157. doi: 10.1016/j.chemphyslip.2019.04.001. Epub 2019 Apr 3. PMID: 30953608.

(20) (ALSO 46) Bayburt TH, Sligar SG. Membrane protein assembly into Nanodiscs. FEBS Lett. 2010;584(9):1721-1727. doi: 10.1016/j.febslet.2009.10.024

(21) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G., Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. Journal of the American Chemical Society 2004,126(11), 3477-3487.

(22) Denisov, I., Sligar, S. Nanodiscs for structural and functional studies of membrane proteins. Nat Struct Mol Biol 23, 481–486 (2016). https://doi.org/10.1038/nsmb.3195

(23) Yin, H., & Flynn, A. D. Drugging Membrane Protein Interactions. Annual review of biomedical engineering, 2016, 18, 51–76. https://doi.org/10.1146/annurev-bioeng-092115-025322

(24) Konijnenberg, A., Bannwarth, L., Yilmaz, D., Koçer, A., Venien-Bryan, C., & Sobott, F. (2015). Top-down mass spectrometry of intact membrane protein complexes reveals oligomeric state and sequence information in a single experiment. Protein science: a publication of the Protein Society. 2015, 24(8), 1292–1300. https://doi.org/10.1002/pro.2703

(25) Guo Y. (2021). Detergent-free systems for structural studies of membrane proteins. Biochemical Society transactions. 2021, 49(3), 1361–1374. https://doi.org/10.1042/BST20201080

(26) Reid, D. J.; Keener, J. E.; Wheeler, A. P.; Zambrano, D. E.; Diesing, J. M.; Reinhardt-Szyba, M.; Makarov, A.; Marty, M. T. Engineering Nanodisc Scaffold Proteins for Native Mass Spectrometry. Anal. Chem. 2017, 89, 11189– 11192, DOI: 10.1021/acs.analchem.7b03569

(27) van de Waterbeemd, M.; Fort, K. L.; Boll, D.; Reinhardt-Szyba, M.; Routh, A.; Makarov, A.; Heck, A. J. High-fidelity mass analysis unveils heterogeneity in intact ribosomal particles. Nat. Methods 2017, 14, 283– 286, DOI: 10.1038/nmeth.4147

(28) Marty, M. T.; Hoi, K. K.; Gault, J.; Robinson, C. V. Probing the Lipid Annular Belt by Gas-Phase Dissociation of Membrane Proteins in Nanodiscs. Angew. Chem., Int. Ed. 2016, 55, 550– 554, DOI: 10.1002/anie.201508289

(29) Sterling, H. J.; Daly, M. P.; Feld, G. K.; Thoren, K. L.; Kintzer, A. F.; Krantz, B. A.; Williams, E. R. Effects of supercharging reagents on noncovalent complex structure in electrospray ionization from aqueous solutions. J. Am. Soc. Mass Spectrom. 2010, 21, 1762– 1774, DOI: 10.1016/j.jasms.2010.06.012

(30) Mehmood, S.; Marcoux, J.; Hopper, J. T. S.; Allison, T. M.; Liko, I.; Borysik, A. J.; Robinson, C. V. Charge Reduction Stabilizes Intact Membrane Protein Complexes for Mass Spectrometry. J. Am. Chem. Soc.2014, 136, 17010– 17012, DOI: 10.1021/ja510283g

(31) Mitra, N. Nanodiscs: Membrane protein research in near-native conditions. Materials and Methods. 2013, 3(177). //dx.doi.org/10.13070/mm.en.3.177

(32) Keener, J. E.; Zambrano, D. E.; Zhang, G.; Zak, C. K.; Reid, D. J.; Deodhar, B. S.; Pemberton, J. E.; Prell, J. S.; Marty, M. T. Chemical Additives Enable Native Mass Spectrometry Measurement of Membrane Protein Oligomeric State within Intact Nanodiscs. J. Am. Chem. Soc. 2019, 141, 1054– 1061, DOI: 10.1021/jacs.8b11529

(33) Teo, C. A.; Donald, W. A. Solution Additives for Supercharging Proteins beyond the Theoretical Maximum Proton-Transfer Limit in Electrospray Ionization Mass Spectrometry. Anal. Chem. 2014, 86, 4455– 4462, DOI: 10.1021/ac500304r

(34) Dickey, A., & Faller, R. (2008). Examining the contributions of lipid shape and headgroup charge on bilayer behavior. Biophysical journal, 95(6), 2636–2646. https://doi.org/10.1529/biophysj.107.128074

(35) Vishal M. G., Miriam L. G. Mitochondrial membrane biogenesis: phospholipids and proteins go hand in hand. J Cell Biol 23 February 2009; 184 (4): 469–472. doi: https://doi.org/10.1083/jcb.200901127

(36) Shahane, G., Ding, W., Palaiokostas, M. et al. Physical properties of model biological lipid bilayers: insights from all-atom molecular dynamics simulations. J Mol Model 25, 76 (2019). https://doi.org/10.1007/s00894-019-3964-0

(37) Sohlenkamp C, Geiger O. Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev. 2016 Jan;40(1):133-59. doi: 10.1093/femsre/fuv008. Epub 2015 Apr 9. PMID: 25862689.

(38) Walker, L. R.; Marty, M. T. Revealing the Specificity of a Range of Antimicrobial Peptides in Lipid Nanodiscs by Native Mass Spectrometry. Biochemistry 2020, 59, 2135– 2142, DOI: 10.1021/acs.biochem.0c00335

(39) Li, J.; Han, L.; Li, J.; Kitova, E. N.; Xiong, Z. J.; Privé, G. G.; Klassen, J. S. Detecting Protein-Glycolipid Interactions Using CaR-ESI-MS and Model Membranes: Comparison of Pre-loaded and Passively Loaded Picodiscs. J. Am. Soc. Mass Spectrom.2018, 29, 1493– 1504, DOI: 10.1007/s13361-018-1936-8

(40) El-Hawiet, A., Shoemaker, G. K., Daneshfar, R., Kitova, E. N., Klassen, J. S. Applications of a catch and release electrospray ionization mass spectrometry assay for carbohydrate library screening. Anal. Chem. 2012, 84, 50– 58, DOI: 10.1021/ac202760e

(41) Kostelic, M.M.; Zak, C.K.; Jayasekera, H.S.; Marty, M.T. "Assembly of Model Membrane Nanodiscs for Native Mass Spectrometry" Anal. Chem. 2021, DOI: 10.1021/acs.analchem.1021c00735.

(42) Kostelic, M. M.; Ryan, A. M.; Reid, D. J.; Noun, J. M.; Marty, M. T. Expanding the Types of Lipids Amenable to Native Mass Spectrometry of Lipoprotein Complexes. J. Am. Soc. Mass Spectrom. 2019, 30, 1416– 1425, DOI: 10.1007/s13361-019-02174-x

(43) van Meer, G.; Voelker, D. R.; Feigenson, G. W. Membrane lipids: where they are and how they behave. Nat. Rev. Mol. Cell. Biol. 2008, 9, 112– 124, DOI: 10.1038/nrm2330

(44) Keener, J. E.; Zhang, G.; Marty, M. T. Native Mass Spectrometry of Membrane Proteins. Anal. Chem. 2021, 93, 583– 597, DOI: 10.1021/acs.analchem.0c04342

(45) Li, M. J.; Atkins, W. M.; McClary, W. D. Preparation of Lipid Nanodiscs with Lipid Mixtures. Curr. Protoc. Protein. Sci. 2019, 98, e100

(46) Zhang, G.; Keener, J. E.; Marty, M. T. Measuring Remodeling of the Lipid Environment Surrounding Membrane Proteins with Lipid Exchange and Native Mass Spectrometry. Anal. Chem.2020, 92, 5666– 5669, DOI: 10.1021/acs.analchem.0c00786

(47) Brown, M. F. Curvature forces in membrane lipid-protein interactions. Biochemistry 2012, 51, 9782– 9795, DOI: 10.1021/bi301332v

(48) van der Veen, J. N.; Kennelly, J. P.; Wan, S.; Vance, J. E.; Vance, D. E.; Jacobs, R. L. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Biochim. Biophys. Acta, Biomembr. 2017, 1859, 1558– 1572, DOI: 10.1016/j.bbamem.2017.04.006

(49) Vance J.E.; Tasseva G. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. Biochim Biophys Acta. 2013 Mar;1831(3):543-54. doi: 10.1016/j.bbalip.2012.08.016. Epub 2012 Aug 29. PMID: 22960354.

(50) Taniguchi M, Okazaki T. The role of sphingomyelin and sphingomyelin synthases in cell death, proliferation and migration-from cell and animal models to human disorders. Biochim Biophys Acta. 2014 May;1841(5):692-703. doi: 10.1016/j.bbalip.2013.12.003. Epub 2013 Dec 17. PMID: 24355909.

(51) Sheridan, A.J.; Slater, J.; Arnold, T.; Richard, A. C.; and Thompson C. J.; Changes to DPPC Domain Structure in the Presence of Carbon Nanoparticles. Langmuir 2017 33 (39), 10374-10384, doi: 10.1021/acs.langmuir.7b01077

(52) Curatolo, W.; Sears, B.; Neuringer, L. J. A calorimetry and deuterium NMR study of mixed model membranes of 1-palmitoyl-2-oleylphosphatidylcholine and saturated phosphatidylcholines. Biochim. Biophys. Acta, Biomembr. 1985, 817, 261– 270, DOI: 10.1016/0005-2736(85)90027-6

(53) Li, M. J.; Atkins, W. M.; McClary, W. D. Preparation of Lipid Nanodiscs with Lipid Mixtures. Curr. Protoc. Protein. Sci. 2019, 98, e10

(54) Strahl, H.; Errington, J. Bacterial Membranes: Structure, Domains, and Function. Annu. Rev. Microbiol. 2017, 71, 519– 538, DOI: 10.1146/annurev-micro-102215-095630

(55) El Khoury, M., Swain, J., Sautrey, G. et al. Targeting Bacterial Cardiolipin Enriched Microdomains: An Antimicrobial Strategy Used by Amphiphilic Aminoglycoside Antibiotics. Sci Rep 7, 10697 (2017). https://doi.org/10.1038/s41598-017-10543-3

(56) Giacomello, M., Pyakurel, A., Glytsou, C. et al. The cell biology of mitochondrial membrane dynamics. Nat Rev Mol Cell Biol 21, 204–224 (2020). https://doi.org/10.1038/s41580-020-0210-7

(57) Sancho-Vaello, E., Gil-Carton, D., François, P. et al. The structure of the antimicrobial human cathelicidin LL-37 shows oligomerization and channel formation in the presence of membrane mimics. Sci Rep 10, 17356 (2020). https://doi.org/10.1038/s41598-020-74401-5

(58) Ho, C. S., Lam, C. W., Chan, M. H., Cheung, R. C., Law, L. K., Lit, L. C., Ng, K. F., Suen, M. W., & Tai, H. L. (2003). Electrospray ionisation mass spectrometry: principles and clinical applications. The Clinical biochemist. Reviews, 24(1), 3–12.

(59) Bern, M., Caval, T., Kil, Y. J., Tang, W., Becker, C., Carlson, E., Kletter, D., Sen, K. I., Galy, N., Hagemans, D., Franc, V., & Heck, A. (2018). Parsimonious Charge Deconvolution for Native Mass Spectrometry. Journal of proteome research, 17(3), 1216–1226. https://doi.org/10.1021/acs.jproteome.7b00839

(60) Kostelic, M.M.; Zak, C.K.; Liu, Y.; Chen, V.; Wu, Z.; Sivinski, J.; Chapman, E.; Marty, M.T. "UniDecCD: Deconvolution of Charge Detection-Mass Spectrometry Data." Anal. Chem. 2021, DOI: 10.1021/acs.analchem.1c03181

(61) Miller M. L.; Bond M. K.; Draper B. E.; Jarrold M. F. "Characterization of Classical Vaccines by Charge Detection Mass Spectrometry." Analytical Chemistry. 2021 93(35), 11965-11972. DOI: 10.1021/acs.analchem.1c01893

(62) Wörner, T.P., Snijder, J., Bennett, A. et al. Resolving heterogeneous macromolecular assemblies by Orbitrap-based single-particle charge detection mass spectrometry. Nat Methods 17, 395–398 (2020). https://doi.org/10.1038/s41592-020-0770-7

(63) Clark, K. R.; Voulgaropoulou, F.; Fraley, D. M.; Johnson, P. R., Cell Lines for the Production of Recombinant Adeno-Associated Virus. Hum. Gene Ther. 1995, 6 (10), 1329-1341.

(64) Naso, M. F.; Tomkowicz, B.; Perry, W. L.; Strohl, W. R. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs 2017, 31, 317– 334, DOI: 10.1007/s40259-017-0234-5

(65) Marty, M. T.; Baldwin, A. J.; Marklund, E. G.; Hochberg, G. K.; Benesch, J. L.; Robinson, C. V. Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles. Anal. Chem. 2015, 87, 4370– 4376, DOI: 10.1021/acs.analchem.5b00140

(66) Carpenter, E. P., Beis, K., Cameron, A. D., & Iwata, S. (2008). Overcoming the challenges of membrane protein crystallography. Current opinion in structural biology, 18(5), 581–586. https://doi.org/10.1016/j.sbi.2008.07.00

(67) Bayburt TH, Sligar SG. Membrane protein assembly into Nanodiscs. FEBS Lett. 2010;584(9):1721-1727. doi: 10.1016/j.febslet.2009.10.024

(68) Kafader JO, Beu SC, Early BP, Melani RD, Durbin KR, Zabrouskov V, Makarov AA, Maze JT, Shinholt DL, Yip PF, Kelleher NL, Compton PD, Senko MW. STORI Plots Enable Accurate Tracking of Individual Ion Signals. J Am Soc Mass Spectrom. 2019 Nov;30(11):2200-2203. doi: 10.1007/s13361-019-02309-0. Epub 2019 Sep 11. PMID: 31512223; PMCID: PMC6852666.