

Interfacing membrane mimetics with mass spectrometry

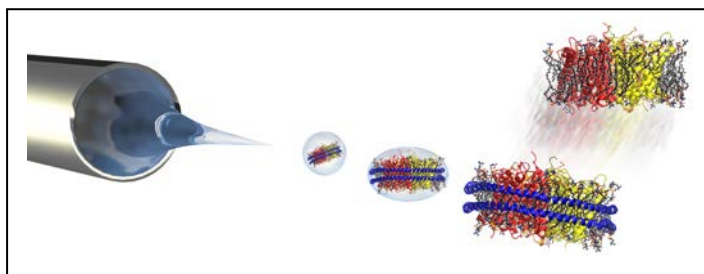
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CONSPECTUS

Membrane proteins play critical physiological roles and make up the majority of drug targets. Due to their generally low expression levels and amphipathic nature, membrane proteins represent challenging molecular entities for biophysical study. Mass spectrometry offers several sensitive approaches to study the biophysics of membrane proteins.



By preserving noncovalent interactions in the gas phase and using collisional activation to remove solubilization agents inside the mass spectrometer, native mass spectrometry (MS) is capable of studying isolated assemblies that would be insoluble in aqueous solution, such as membrane protein oligomers and protein-lipid complexes. Conventional methods use detergent to solubilize the protein prior to electrospray ionization. Gas-phase activation inside the mass spectrometer removes the detergent to yield the isolated proteins with bound ligands. This approach has proven highly successful for ionizing membrane proteins. With the appropriate choice of detergents, membrane proteins with bound lipid species can be observed, which allows characterization of protein-lipid interactions. However, detergents have several limitations. They do not necessarily replicate the native lipid bilayer environment, and only a small number of protein-lipid interactions can be resolved.

In this Account, we summarize the development of different membrane mimetics as cassettes for MS analysis of membrane proteins. Examples include amphipols, bicelles, and picodiscs with a special emphasis on lipoprotein Nanodiscs. Polydispersity and heterogeneity of the membrane mimetic cassette is a critical issue for study by MS. Ever more complex datasets consisting of overlapping protein charge states and multiple lipid-bound entities have required development of new computational, theoretical, and experimental approaches to interpret both mass and ion mobility spectra. We will present the rationale and limitations of these approaches.

Starting with the early work on empty Nanodiscs, we chart developments that culminate in recent high-resolution studies of membrane protein-lipid complexes ejected from Nanodiscs. For the latter, increasing collision energies allowed progressive removal of Nanodisc components, beginning with the scaffold proteins and continuing through successive shells of lipids, allowing direct characterization of the stoichiometry of the annular lipid belt that surrounds the membrane protein.

We consider future directions for the study of membrane proteins in membrane mimetics, including the development of mixed lipid systems and native bilayer environments. Unambiguous assignment of these heterogeneous systems will rely heavily upon further enhancements in both data analysis protocols and instrumental resolution. We anticipate that these developments will provide new insights into the factors that control dynamic protein-lipid interactions in a variety of tailored and natural lipid environments.

Detergents have driven membrane protein MS

Lipid membranes are a critical component of cellular architecture because they define the boundaries of cellular compartments. Membrane proteins have evolved to function within the amphipathic lipid bilayer and play a number of important biochemical roles, including controlling the flow of chemicals, information, and energy across the membrane barrier.

Membrane proteins present several challenges for biophysical study. First, because the membrane accounts for a relatively small fraction of the cell volume, expression is generally low and can present a critical bottleneck.¹ Second, biological membranes are insoluble in aqueous solution and generally very heterogeneous. Because many biophysical techniques are tailored for purified soluble proteins, membrane proteins often need to be solubilized and purified for characterization.² However, there is a growing appreciation of the importance of the lipid bilayer in modulating membrane protein structure and function.³⁻⁵ Thus, it is important to find a balance between effective solubilization and preserving the important lipid surroundings.

Mass spectrometry (MS) can provide a unique solution to these challenges. Relatively small amounts of material are required, and by removing the solvent inside the mass spectrometer, amphipathic complexes can be isolated that would be impossible to study in solution. While electrospray ionization (ESI) requires soluble complexes prior to ionization, gas-phase dissociation can be used to isolate and study insoluble complexes post-ionization in the absence of solubilization agents. Conventionally, detergents are used to solubilize the membrane protein prior to nano-ESI (nESI). Under the appropriate MS conditions, it is possible to preserve noncovalent interactions inside the mass spectrometer in a so-called

native MS experiment. Because solvent and many detergents can be removed by gas-phase activation of the membrane protein, the naked membrane protein complex can be isolated and studied with native-like tertiary and quaternary structure preserved.⁶⁻⁹ The mass of the complex reports on the oligomeric state,^{6,7} and the dissociation pattern can be used to piece together the connectivity of complex subunits.⁸

As detergents are removed, lipids that interact tightly with the protein may remain bound to the membrane protein, which allows analysis of isolated protein-lipid complexes.^{10,11} Gas-phase isolation of protein-lipid complexes enables a number of experiments. For example, the extra mass from bound lipid species can be used to identify the presence and stoichiometry of endogenous lipids and combined with lipidomics to confirm the identity of structural lipids.^{7,8,12} Coupling of ion mobility spectrometry with native MS has allowed structural analysis of protein-lipid complexes^{13,14} and exploration of the effects of lipids on gas-phase unfolding stability.⁹

While detergents are successful in presenting membrane proteins for native MS, there are several limitations of detergents as a membrane mimetic. First, detergents are generally used in high concentrations, which can destabilize protein structure, protein-protein interactions, and protein-ligand interactions.^{15,16} Second, given the wide chemical diversity of detergents, screening is often required to find the “sweet spot,” a detergent or mixture of detergents that is capable of efficiently solubilizing the membrane protein, effectively releasing in the gas phase, and preserving noncovalent interactions. Although some general rules apply, the optimal detergent for a given protein species is hard to predict.¹⁷ Third, detergent micelles are polydisperse and compete with lipids for lipid-binding sites. Thus, only a small number of protein-lipid interactions may be observed.^{11,18} Finally, detergent micelles do not

necessarily replicate the native lipid bilayer environment of membrane proteins.¹⁵ Given the importance of the membrane environment on protein structure and function, alternative membrane mimetics (Figure 1) offer a promising approach to create biologically-relevant approaches for MS of membrane proteins.

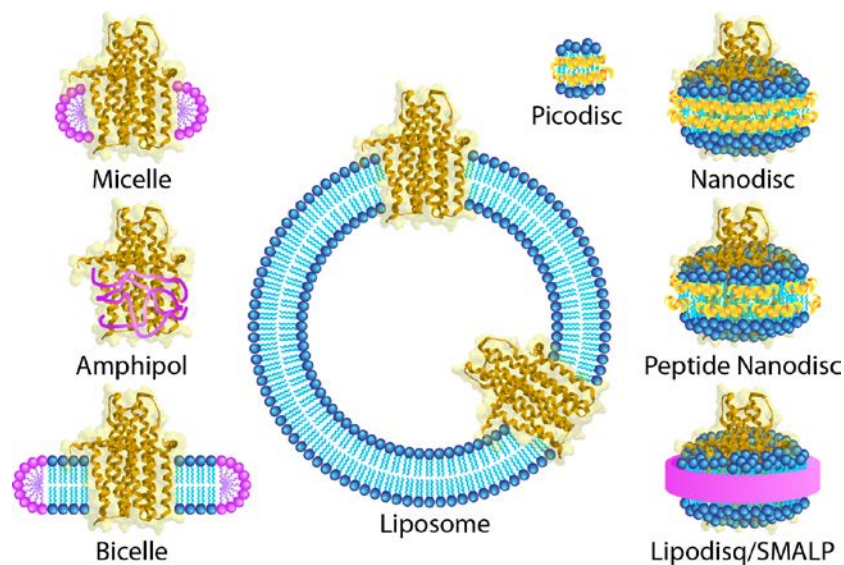


Figure 1. Schematic of various solubilization cassettes for membrane proteins. Structures of proteins and peptides are shown in *yellow*, the lipid bilayer in *blue*, and synthetic constructs (detergents, amphipols, and styrene-malic acid copolymers) are in *magenta*.

Amphipols as a detergent alternative

Amphipols were the first alternative membrane mimetic to be applied to native MS. Amphipols are amphipathic copolymers composed of both hydrophobic and hydrophilic monomer units.¹⁹ Because amphipols bind to the membrane protein in a quasi-irreversible

manner, they can be used at a much lower concentration than detergents. Moreover, membrane proteins can be more stable in amphipols than micelles.^{16,19}

Amphipols were first applied to monomeric outer membrane proteins OmpT and PagP.²⁰ Similar to micelles, collisional activation was used to strip the amphipols in the gas phase and release the bare membrane proteins for ion mobility-mass spectrometry (IM-MS), as shown in Figure 2A. OmpT, which was folded in solution, showed a mostly compact state by IM-MS with some unfolding at higher charge states. PagP, which was partially unfolded in solution, showed a mixture of folded and unfolded states by IM-MS with greater unfolding at higher charge states. In both cases, the collision cross section (CCS) of the compact states agreed with the predicted CCS from the crystal structure, showing that the membrane proteins can be released from the amphipol in a native-like state. However, the unfolding of PagP in both the solution and gas phase suggests that the stabilizing effects of amphipols may vary depending on the specific combination of protein and amphipol used.

Further work²¹ sought a more direct comparison between these proteins solubilized in detergent micelles and amphipols. While the CCS values were similar for PagP, a close examination of collision-induced unfolding showed that PagP solubilized in amphipols was more stable, remaining folded at higher collision energies than when solubilized in dodecyl-maltoside (DDM) micelles. A similar trend was observed for OmpT, which showed overall lower charge states and more compact CCS values when in amphipols. Similar to OmpT, α -helical membrane proteins Mhp1 and GalP showed lower charge states and more compact structures with amphipols than DDM micelles. Moreover, several spectra showed bound lipid species, demonstrating the ability of amphipols to preserve lipid-bound states in the gas phase.²¹

Similar to detergents, there are protein-dependent differences in utility of different amphipols.²² Enzymatic activity of outer membrane proteins can be highly dependent on the molecular composition of the amphipol, although these are manifested through relatively small structural changes. The gas-phase release of membrane proteins from amphipols is also dependent on the amphipol composition, with different charging behaviors and release efficiencies depending on the protein and amphipol structure. In general, smaller and less-charged amphipols are more universally effective for ESI-MS. As with detergent, the CCS vs. charge relationship is very similar irrespective of the molecular composition of the amphipol.²² It remains to be seen how amphipols will translate to oligomeric complexes as initial studies have shown poor performance of amphipols for preservation of trimeric DgkA (see below).²³

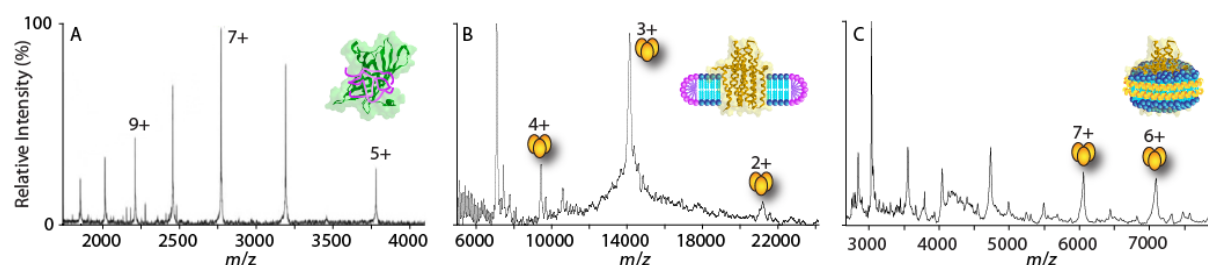


Figure 2. Representative mass spectra of membrane proteins ejected from different solubilization cassettes. (A), (B), and (C) show the mass spectra and structures of PagP in amphipols,²¹ DgkA in bicelles,²³ and DgkA in Nanodiscs²³ respectively. (A) adapted with permission from ref. 25. Copyright 2014 American Chemical Society. (B) and (C) adapted with permission from ref. 27. Copyright 2013 Nature Publishing Group.

Lipid nanostructures as membrane mimetics

Although amphipols offer a detergent-free approach to solubilize membrane proteins, they are heterogeneous, unnatural, and struggle to preserve oligomeric membrane protein complexes. To better mimic the natural lipid environment, membrane proteins can be solubilized in a variety of lipid nanostructures.^{24,25} Liposomes are the simplest form of lipid nanostructures and are simply aqueous suspensions of lipid bilayers, which curve into large vesicle structures. However, liposomes have not yet been successfully employed for MS, likely due to their large size and polydispersity. Bicelles are a combination of lipids with a detergent or short-chained lipid (Figure 1).^{26,27} The lipids form discoidal bilayer structures that are capped by the detergent. Depending on the ratio of lipids to detergent, bicelles can be formed in a range of sizes, but they are generally much smaller than liposomes. Nanodiscs are discoidal lipid bilayers similar to bicelles.²⁸⁻³⁰ While bicelles cap the bilayer with detergents or short-chain lipids, Nanodiscs cap the bilayer with two amphipathic membrane scaffold proteins (MSP). Because MSP can be engineered with a precise size, Nanodiscs are less polydisperse and more homogeneous than alternative membrane mimetic platforms.³¹

Pioneering work²³ examined membrane protein complexes solubilized in amphipols and several lipid nanostructures with native ESI MS. For DgkA, both detergent micelles and amphipols predominantly showed fragmentation of native trimers while bicelles and Nanodiscs showed a higher fraction of intact trimers (Figure 2B and C). For pSRII protein, larger bicelles with a more extensive lipid bilayer showed an enhanced ability to preserve the native dimers. Similarly, Nanodiscs showed a higher fraction of dimers. Interestingly, much higher collision energies were required to eject the membrane protein complexes from

lipidic structures than from detergent micelles or amphipols. Thus, more energy is required to strip the lipid molecules from the membrane protein surface.

Intact Nanodiscs in the gas phase

The extraordinary gas-phase stability of Nanodiscs was first discovered on “empty” Nanodiscs without membrane proteins.³¹ Initial experiments were performed on a simple system consisting of a homogeneous phosphatidylcholine lipids and the canonical scaffold protein, MSP1D1. Nanodiscs were ionized by nESI, and spectra were acquired on a Fourier transform ion cyclotron resonance mass spectrometer. Two main features were observed in the spectra. At low m/z , several sharp peaks appeared for isolated lipid monomer, dimers, and trimers. At higher m/z , a complex series of peaks was observed.

The complexity of these higher m/z peaks presented a challenge for interpretation and assignment of the spectra. The pattern contained two primary features, a series of sharper peaks superimposed over a series of broader peaks, as shown in Figure 3. The first key insight was that the sharp peaks were Nanodiscs with a defined charge state and lipid stoichiometry. Neighboring peaks differed by the mass of a single lipid but had roughly the same charge state. Because the lipid mass was known, the charge state of a peak could be estimated by dividing the lipid mass by the m/z difference between adjacent peaks. From the charge state, it was then possible to calculate the masses.

Armed with the masses, interpretation of the rest of the features fell into place. The broader peaks appeared to correspond to adjacent charge states. A crude fitting of each charge state revealed a relatively narrow lipid distribution, demonstrating the relatively low polydispersity of Nanodiscs. Moreover, the measured masses agreed closely with data for

intact Nanodisc complexes in solution. Thus, it was possible to preserve the intact Nanodisc complexes in the gas phase, a remarkable fact given the number of molecules in the complex, 2 MSP proteins and around 150 lipids, and the importance of hydrophobic interactions in maintaining this complex ensemble.

Further experiments³² investigated the dissociation of gas-phase Nanodisc complexes. As the energy in either collision-induced dissociation or infrared multiphoton dissociation was increased, Nanodisc ions shifted to higher m/z values. As charge states began to spread out at these higher m/z values, part of the initial naïve interpretation began to break down. The broader peaks superimposed over the lipid distribution could no longer be caused by the charge state distribution. Strangely, they were regularly-spaced and positioned at either integer or half-integer multiples of the lipid mass. In fact, this complex pattern is caused by constructive overlap between adjacent charge states. Each charge state will “resonate” at integer and half-integer multiples of the lipid mass, causing increased intensity at these positions and a broad peak. In Figure 3B, the m/z value for peak 1 could be created from any of a number of combinations of lipid count (mass) and charge, which are marked by black rectangles in Figure 3C. Intensity from these combinations resonates by constructively overlapping at peak 1, which is nearly an integer multiple of the lipid mass. This interpretation exposed a new layer of complexity and necessitated an improved approach to quantitative interpretation of the spectra.

To extract the underlying charge and lipid count, we developed a probability-based deconvolution approach, which relied on information from adjacent charge and oligomeric states to assign peaks in the spectrum. This revealed that Nanodisc complexes lose both mass and charge continuously as they are activated. Although the initial deconvolution

approach was applied to a small number of extracted intensities,³² we generalized the approach to continuous m/z spectra and even two-dimensional IM-MS spectra. We called this new generalized deconvolution program UniDec, short for Universal Deconvolution.³³

Equipped with a quantitative deconvolution approach, we sought to answer an outstanding question: are intact Nanodiscs still discoidal in the gas phase? Using a linear drift cell Q-IM-TOF instrument, we applied UniDec to IM-MS spectra and monitored the mass, charge, and CCS of Nanodisc ions under increasing collision energies. At low collision energies, the Nanodisc was largely intact, and the CCS agreed with a modeled discoidal structure.³⁴ Interestingly, at higher collision energies, Nanodiscs undergo a significant collapse, likely due to the transition from a more extended discoidal shape into a more compact sphere. This suggests that empty Nanodiscs are largely compositionally and conformationally intact at lower collision energies but undergo collapse and dissociation as collision energy is increased.

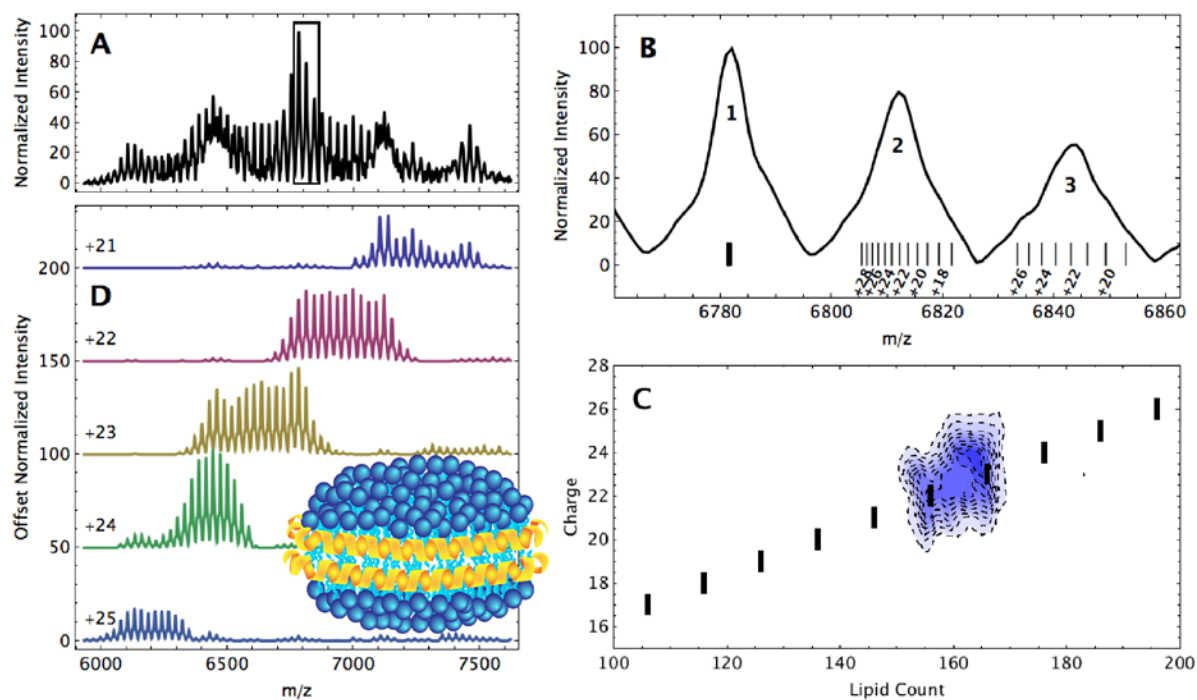


Figure 3. Spectrum and analysis of empty Nanodiscs.³² (A) shows the mass spectrum of a Nanodisc with the deconvolved contributions (D) from each charge state. The boxed region, expanded in (B), highlights the “resonance” at peak 1. Potential charge states are denoted below the data. (C) shows the deconvoluted lipid count and charge states from (B) with the intensity shown by contours. The rectangles mark the location of potential charge state and lipid count combinations overlapping at peak 1. Adapted with permission from ref. 35. Copyright 2013 Springer.

Mixing it up: Heterogeneous lipid Nanodiscs

Nanodiscs offer the ability to create a tailored heterogeneous lipid bilayer composition. The first application of this approach to MS relied on a catch-and-release experiment to study peripheral membrane proteins interactions with glycolipids in Nanodiscs (Figure 4). Here, glycolipid Nanodiscs were combined with the soluble protein of

interest in solution and ionized by nESI. Although it was not possible to resolve the mass spectrum of the protein-Nanodisc complex, gas-phase activation released the protein with bound glycolipids.³⁵ Quantifying the number of glycolipids bound to the soluble protein enabled investigation of the mechanisms and affinities of protein-glycolipid interactions.³⁶ It was also possible to further dissociate the protein-glycolipid complex to release the individual glycolipids for mass analysis. In this case, a heterogeneous pool of glycolipids in the Nanodisc was used to screen for glycolipids that bind competitively to the protein.³⁷

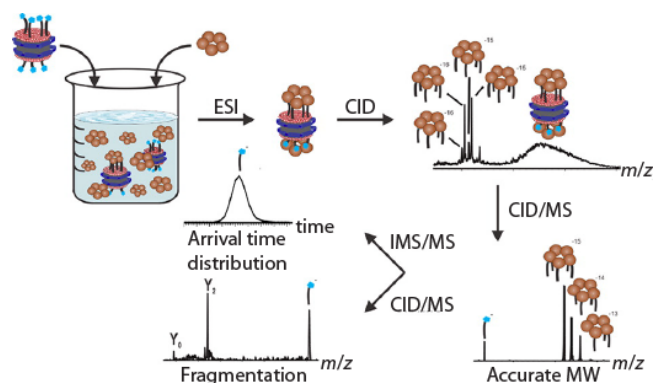


Figure 4. Protein-glycolipid interaction screening using Nanodiscs.³⁵ The protein is incubated with Nanodiscs containing glycolipids and then ionized with ESI. After activation by CID, protein-glycolipid complexes dissociate from the Nanodisc to allow determination of stoichiometry of glycolipids. Adapted with permission from ref. 38. Copyright 2012 American Chemical Society.

The same approach was also applied to picodiscs. Similar to Nanodiscs, picodiscs are lipoprotein complexes, but they use a different scaffold protein, saposin A, to form smaller complexes, typically with hydrodynamic radius of 3.2 nm (Figure 1).³⁸ Despite a few

differences, picodiscs showed similar performance to Nanodiscs for catch-and-release assays³⁹ and screens.⁴⁰

For glycolipids, the intact Nanodisc complex was too heterogeneous and polydisperse to be resolved by native MS. A distribution in the total number of lipids per complex combined with the statistical distribution in the loading of each lipid created essentially a continuum of microstates with different masses. To take full advantage of heterogeneous Nanodiscs for native MS, we needed a strategy to address this level of complexity.

Our strategy⁴¹ to determine the lipid composition in heterogeneous Nanodiscs was to employ lipids of slightly different mass. Because the mass difference was small compared to the total mass of the complex, differences in microstates were not resolved (Figure 5). A single peak was observed for all microstates with the same total number of lipids, regardless of the loading of each lipid within the complex. To retrieve the global lipid composition of these microstates, we used the spacing between the peaks. Because each peak differs by a single lipid, we reasoned that the mass difference between peaks would report on the average lipid mass and hence average composition of the bilayer. Assuming the lipid composition is uniform, each charge state produces a series of peaks evenly spaced by the average lipid mass, m_l , divided by the charge, z . The Fourier transform of that charge state leads to a frequency peak at z/m_l . Because there were several charge states, frequency peaks appeared in an evenly-spaced pattern at z/m_l , $(z+1)/m_l$, $(z+2)/m_l$, and so on. A second Fourier transform yielded a primary frequency at m_l , giving a direct measurement of the average lipid mass (Figure 5E). A similar Fourier-based approach was applied not only to extracting the lipid mass but also to deconvolving overlapping charge states in homogeneous Nanodiscs with a number of different lipid species.⁴²

Applying the dual Fourier transform approach to Nanodiscs prepared with palmitoyl-oleoyl-phosphatidylcholine (POPC), palmitoyl-oleoyl-phosphatidylglycerol (POPG), and palmitoyl-oleoyl-phosphatidylserine (POPS) in different ratios, we were able to measure the average lipid mass with startling accuracy, which allowed determination of the POPG:POPC ratio to within 3% (Figure 5G). Examining the lipid composition as a function of collision voltage, Nanodiscs showed a fairly uniform composition at low collision energy. However, at elevated collision energies, we observed a polarity-dependent depletion of certain lipid species.

Exploring the polarity dependence further, we investigated the dissociation behavior of Nanodiscs with different lipid compositions under different instrumental polarities. While heterogeneous Nanodiscs behaved similarly in each polarity, homogeneous Nanodiscs showed polarity-dependent behavior. Based on their pK_a values we can classify the lipids as either cationic, preferring to be positively-charged, or anionic, preferring to be negatively-charged. Phosphatidylcholine lipids are cationic while phosphatidylserine and phosphatidylglycerol lipids are anionic. For homogeneous Nanodiscs, cationic lipids dissociate continuously in positive mode but discontinuously in negative mode. By contrast anionic lipids dissociate continuously in negative mode but discontinuously in positive mode. These results demonstrated that the chemistry of the lipid molecules and the instrumental polarity play critical roles in dissociation mechanisms.

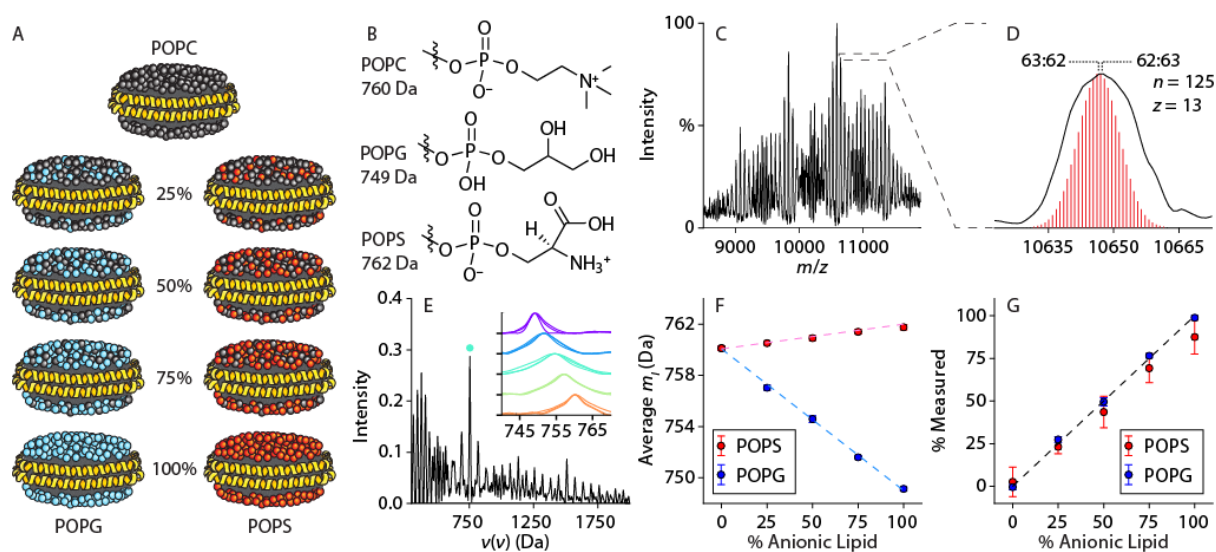


Figure 5. Heterogeneous lipid Nanodiscs.⁴¹ (A) shows Nanodiscs with lipids of different head groups (B) but similar masses. (C) shows the mass spectrum of Nanodisc containing 1:1 POPG:POPC. Prospective microstates (*red bars*) of 13+ (z) Nanodisc containing 125 lipids (n) are shown in (D). (E) is the dual Fourier transform of the Nanodisc spectrum with the inset showing the dual Fourier transform for various ratios of POPC:POPG. (F) and (G) show the average lipid mass and lipid composition where the *dashed lines* indicate the predicted values. Adapted with permission from ref. 44. Copyright 2016 American Chemical Society.

Starting at the top: Membrane protein-Nanodisc complexes

Having established that empty Nanodiscs can be resolved intact in the mass spectrometer, we next questioned if intact membrane protein-Nanodisc complexes could also be resolved. To explore this, we embedded two membrane protein oligomers, trimeric AmtB and tetrameric AqpZ, in several types of Nanodiscs.⁴³ Switching to a higher-resolution native Orbitrap instrument, membrane protein-Nanodiscs showed highly complex

spectra with several distinct features that varied as a function of collision energy. At high collision energy, the Nanodisc complex dissociated into its constituent parts: lipids, MSP monomers (Figure 6, *blue*), and membrane protein monomers (Figure 6, *green*). At intermediate collision energy, AmtB showed a clear charge state pattern convolved with a Gaussian distribution in the number of lipids per complex. From the masses, it was clear that these peaks came from AmtB trimer with around nine lipids bound.

At lower collision energies, spectra were dominated by a complex pattern similar to the constructive overlap pattern observed for empty Nanodiscs. The masses were lower than an intact complex, indicating that they were dissociation products. However, from a single spectrum, it was impossible to determine which of the Nanodisc components were lost and which remained. To assign the structures, we developed a high mass analog of Kendrick mass defect analysis. Conventional Kendrick analysis looks at the mass difference between the measured mass and an integer multiple of a reference mass. For hydrocarbon analysis, the reference mass is generally methylene, and hydrocarbons that are atomically identical except for the number of methylene units will have the same mass defect value. In our case, we used the mass of the lipid as the reference mass such that the mass defect is independent of the number of lipids and reports exclusively on the protein components in the complex.

Because MSP1D1(-) has a mass defect of nearly zero with POPC, we used Nanodiscs formed with these two components to discern the oligomeric state of the membrane protein component of the complex. In both cases, AmtB and AqpZ were present in their expected oligomeric state. We then used a larger scaffold protein with a different mass defect, which would change the measured mass defects significantly if the complex contained the scaffold protein. However, no change in mass defect was observed, indicating that the complex

contained only membrane protein oligomer and no scaffold protein. This conclusion was further supported by changing the lipid content, which changed the mass defects of all of the components.

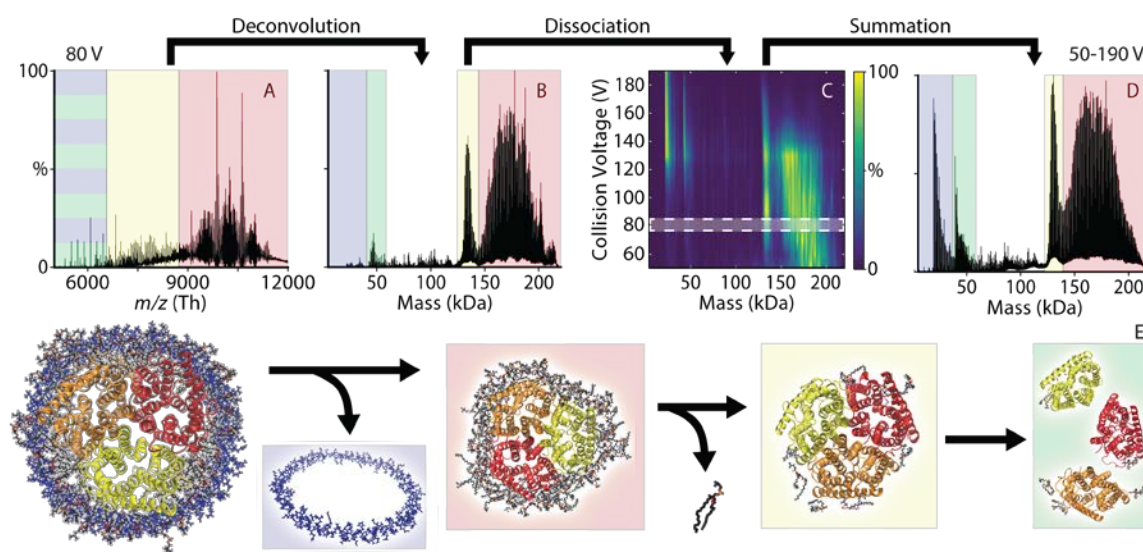


Figure 6. MS analysis of AmtB Nanodisc.⁴³ (A) and (B) show the representative mass spectrum and deconvoluted spectrum. Four species are identified and highlighted, including AmtB with large number of lipids (*red*), AmtB with ionic contact lipids (*yellow*), AmtB monomer (*green*), and MSP (*blue*). (C) combines the mass spectra at different collision voltages and (D) summation across all collision voltages. The proposed mechanism of Nanodisc dissociation is shown in (E). Reproduced with permission from ref. 45. Copyright 2015 John Wiley and Sons.

From the mass defect, we could then assign these higher molecular weight species to membrane protein oligomers with a large number of bound lipids. To help interpret this distribution in the number of bound lipids, we used molecular dynamics to simulate the lipid

bilayer around the membrane protein oligomers and count the number of lipids binding to the protein in different ways. The distributions for the high mass species agreed with the predicted stoichiometry of the full lipid annular belt and a subshell consisting of lipids interacting only through more polar interactions with the lipid head groups (Figure 6, *red*). Although not observed experimentally for AqpZ, the narrow lipid distribution for AmtB agreed with the number of lipids predicted to interact through ionic interactions (Figure 6, *yellow*).

From these results, we concluded that at low collision energies, the scaffold proteins and any bulk lipids are removed, leaving only the membrane protein oligomer and any lipids in contact with the protein surface. As the collision energy increases, the weakly-interacting lipids are removed, followed by the lipids interacting through polar contacts, leaving intact protein oligomers with lipids interacting through ionic contacts. Finally, at high collision energy, the complex can be completely dissociated into its constituent components.

Conclusions and outlook

Detergent-based membrane protein mass spectrometry has proven highly useful for a wide range of studies of membrane protein oligomers and protein-lipid interactions. Other membrane mimetics also show great promise to contribute to membrane protein native mass spectrometry. Amphipols offer an alternative to detergents that can be used at much lower concentrations and show improved stability. Bicelles and Nanodiscs provide the ability to eject membrane protein complexes from a more native-like lipid bilayer environment. However, the greatest potential strength of alternative membrane mimetics is not in replacing detergents but in enabling new experiments that were previously not possible. In

the case of membrane protein-Nanodisc complexes, we were able to see an unprecedented number of lipids bound to the membrane protein oligomer, pushing the limits of mass spectrometry further into the surrounding lipid bilayer environment.

The central challenge in analysis of protein-lipid complexes with a large number of lipids is addressing polydispersity and heterogeneity. Each step has required new data analysis approaches to first understand the complexity of the spectra and then quantitatively interpret the results. Thus far, experiments have focused on relatively simple systems of empty Nanodiscs and assemblies containing homo-oligomers. As we move towards more complex systems, the computational groundwork developed on homogeneous systems will continue to be critical, and experimental design will become ever more important. As seen for heterogeneous Nanodiscs, the right combination of lipid masses is vital for spectral resolution. Because the Nanodisc platform offers unique abilities to engineer the scaffold protein as well as to customize the bilayer, opportunities exist to improve the experimental design and tailor the Nanodisc for mass spectrometry studies.

As a rapidly developing field, emerging membrane mimetics offer promising platforms to explore. Instead of proteins, it is possible to form Nanodisc-like structures using smaller peptides in a variety of forms.⁴⁴⁻⁴⁶ These peptide-based nanodiscs could provide different dissociation mechanisms because the belts can be released one peptide at a time rather than as a concerted dissociation. Another approach utilizes styrene-malic acid copolymers to form a lipoprotein particle known as a SMALP⁴⁷ or Lipodisc.⁴⁸ The advantage of the SMALPS as well as with peptide-based nanodiscs is that they have surfactant-like properties and can thus be used to solubilize membrane proteins directly from the membrane without the need for initial solubilization with detergent. These new

membrane mimetics will introduce additional polydispersity and heterogeneity, requiring further biochemical, experimental, and computational approaches. Mass spectrometry is well-poised to unravel this complexity and in so doing enable deeper insights into the interactions between membrane proteins and their lipid environment.

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Notes

The authors declare no competing financial interest.

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Kin Kuan Hoi obtained his B.A. from Queensland University of Technology in 2011 and M.Sc. in 2013 at the University of Melbourne under the supervision of Richard O'Hair. He is currently working towards a D.Phil. at the University of Oxford under the direction of Carol Robinson.

Carol Robinson obtained her PhD from the University of Cambridge in MS in 1982 under the supervision of Dudley Williams. She has spent more than 25 years studying proteins in the gas phase introduced from their native state and the last 10 years studying membrane proteins.

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References

- (1) Zoonens, M.; Miroux, B.: Expression of Membrane Proteins at the Escherichia coli Membrane for Structural Studies. In *Heterologous Expression of Membrane Proteins*; Methods in Molecular Biology; Humana Press: New York, 2010; Vol. 601; pp 49-66.
- (2) Duquesne, K.; Sturgis, J. N.: Membrane Protein Solubilization. In *Heterologous Expression of Membrane Proteins*; Methods in Molecular Biology; Humana Press: New York, 2010; Vol. 601; pp 205-217.

- (3) Martfeld, A. N.; Rajagopalan, V.; Greathouse, D. V.; Koeppe II, R. E. Dynamic regulation of lipid–protein interactions. *Biochim. Biophys. Acta* **2015**, *1848*, 1849-1859.
- (4) Zhou, H.-X.; Cross, T. A. Influences of Membrane Mimetic Environments on Membrane Protein Structures. *Annu. Rev. Biophys.* **2013**, *42*, 361-392.
- (5) Hsia, C.-Y.; Richards, M. J.; Daniel, S. A review of traditional and emerging methods to characterize lipid-protein interactions in biological membranes. *Anal. Methods* **2015**, *7*, 7076-7094.
- (6) Barrera, N. P.; Di Bartolo, N.; Booth, P. J.; Robinson, C. V. Micelles protect membrane complexes from solution to vacuum. *Science* **2008**, *321*, 243-246.
- (7) Barrera, N. P.; Isaacson, S. C.; Zhou, M.; Bavro, V. N.; Welch, A.; Schaedler, T. A.; Seeger, M. A.; Miguel, R. N.; Korkhov, V. M.; van Veen, H. W.; Venter, H.; Walmsley, A. R.; Tate, C. G.; Robinson, C. V. Mass spectrometry of membrane transporters reveals subunit stoichiometry and interactions. *Nat. Methods* **2009**, *6*, 585-587.
- (8) Zhou, M.; Morgner, N.; Barrera, N. P.; Politis, A.; Isaacson, S. C.; Matak-Vinković, D.; Murata, T.; Bernal, R. A.; Stock, D.; Robinson, C. V. Mass Spectrometry of Intact V-Type ATPases Reveals Bound Lipids and the Effects of Nucleotide Binding. *Science* **2011**, *334*, 380-385.
- (9) Laganowsky, A.; Reading, E.; Allison, T. M.; Ulmschneider, M. B.; Degiacomi, M. T.; Baldwin, A. J.; Robinson, C. V. Membrane proteins bind lipids selectively to modulate their structure and function. *Nature* **2014**, *510*, 172-175.

- (10) Hopper, J. T.; Robinson, C. V. Mass spectrometry quantifies protein interactions--from molecular chaperones to membrane porins. *Angew. Chem. Int. Ed.* **2014**, *53*, 14002-14015.
- (11) Bechara, C.; Robinson, C. V. Different Modes of Lipid Binding to Membrane Proteins Probed by Mass Spectrometry. *J. Am. Chem. Soc.* **2015**, *137*, 5240-5247.
- (12) Bechara, C.; Nöll, A.; Morgner, N.; Degiacomi, M. T.; Tampé, R.; Robinson, C. V. A subset of annular lipids is linked to the flippase activity of an ABC transporter. *Nat. Chem.* **2015**, *7*, 255-262.
- (13) Konijnenberg, A.; Yilmaz, D.; Ingólfsson, H. I.; Dimitrova, A.; Marrink, S. J.; Li, Z.; Vénien-Bryan, C.; Sobott, F.; Koçer, A. Global structural changes of an ion channel during its gating are followed by ion mobility mass spectrometry. *Proc. Natl. Acad. Sci.* **2014**, *111*, 17170-17175.
- (14) Marcoux, J.; Wang, S. C.; Politis, A.; Reading, E.; Ma, J.; Biggin, P. C.; Zhou, M.; Tao, H.; Zhang, Q.; Chang, G.; Morgner, N.; Robinson, C. V. Mass spectrometry reveals synergistic effects of nucleotides, lipids, and drugs binding to a multidrug resistance efflux pump. *Proc. Natl. Acad. Sci.* **2013**, *110*, 9704-9709, S9704/9701-S9704/9713.
- (15) Cross, T. A.; Sharma, M.; Yi, M.; Zhou, H. X. Influence of solubilizing environments on membrane protein structures. *Trends Biochem. Sci.* **2011**, *36*, 117-125.
- (16) Popot, J. L. Amphipols, nanodiscs, and fluorinated surfactants: three nonconventional approaches to studying membrane proteins in aqueous solutions. *Annu. Rev. Biochem.* **2010**, *79*, 737-775.

- (17) Laganowsky, A.; Reading, E.; Hopper, J. T. S.; Robinson, C. V. Mass spectrometry of intact membrane protein complexes. *Nat. Protoc.* **2013**, 8, 639-651.
- (18) Landreh, M.; Marty, M. T.; Gault, J.; Robinson, C. V. A sliding selectivity scale for lipid binding to membrane proteins. *Curr. Opin. Struct. Biol.* **2016**, 39, 54-60.
- (19) Popot, J. L.; Althoff, T.; Bagnard, D.; Baneres, J. L.; Bazzacco, P.; Billon-Denis, E.; Catoire, L. J.; Champeil, P.; Charvolin, D.; Cocco, M. J.; Cremel, G.; Dahmane, T.; de la Maza, L. M.; Ebel, C.; Gabel, F.; Giusti, F.; Gohon, Y.; Goormaghtigh, E.; Guittet, E.; Kleinschmidt, J. H.; Kuhlbrandt, W.; Le Bon, C.; Martinez, K. L.; Picard, M.; Pucci, B.; Sachs, J. N.; Tribet, C.; van Heijenoort, C.; Wien, F.; Zito, F.; Zoonens, M. Amphipols from A to Z. *Annu. Rev. Biophys.* **2011**, 40, 379-408.
- (20) Leney, A. C.; McMorran, L. M.; Radford, S. E.; Ashcroft, A. E. Amphipathic Polymers Enable the Study of Functional Membrane Proteins in the Gas Phase. *Anal. Chem.* **2012**, 84, 9841-9847.
- (21) Calabrese, A. N.; Watkinson, T. G.; Henderson, P. J. F.; Radford, S. E.; Ashcroft, A. E. Amphipols Outperform Dodecylmaltoside Micelles in Stabilizing Membrane Protein Structure in the Gas Phase. *Anal. Chem.* **2015**, 87, 1118-1126.
- (22) Watkinson, T. G.; Calabrese, A. N.; Giusti, F.; Zoonens, M.; Radford, S. E.; Ashcroft, A. E. Systematic analysis of the use of amphipathic polymers for studies of outer membrane proteins using mass spectrometry. *Int. J. Mass Spectrom.* **2015**, 391, 54-61.
- (23) Hopper, J. T. S.; Yu, Y. T.-C.; Li, D.; Raymond, A.; Bostock, M.; Liko, I.; Mikhailov, V.; Laganowsky, A.; Benesch, J. L. P.; Caffrey, M.; Nietlispach, D.; Robinson,

C. V. Detergent-free mass spectrometry of membrane protein complexes. *Nat. Meth.* **2013**, *10*, 1206-1208.

(24) Bayburt, T. H.; Sligar, S. G. Self-assembly of single integral membrane proteins into soluble nanoscale phospholipid bilayers. *Protein Sci.* **2003**, *12*, 2476-2481.

(25) Milic, D.; Veprintsev, D. B. Large-scale production and protein engineering of G protein-coupled receptors for structural studies. *Front. Pharmacol.* **2015**, *6*, 82.

(26) Ujwal, R.; Bowie, J. U. Crystallizing membrane proteins using lipidic bicelles. *Methods* **2011**, *55*, 337-341.

(27) Durr, U. H.; Gildenberg, M.; Ramamoorthy, A. The magic of bicelles lights up membrane protein structure. *Chem. Rev.* **2012**, *112*, 6054-6074.

(28) Bayburt, T. H.; Grinkova, Y. V.; Sligar, S. G. Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. *Nano Lett.* **2002**, *2*, 853-856.

(29) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G. Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size. *J. Am. Chem. Soc.* **2004**, *126*, 3477-3487.

(30) Grinkova, Y. V.; Denisov, I. G.; Sligar, S. G. Engineering extended membrane scaffold proteins for self-assembly of soluble nanoscale lipid bilayers. *Protein Eng. Des. Sel.* **2010**, *23*, 843-848.

- (31) Marty, M. T.; Zhang, H.; Cui, W.; Blankenship, R. E.; Gross, M. L.; Sligar, S. G. Native Mass Spectrometry Characterization of Intact Nanodisc Lipoprotein Complexes. *Anal. Chem.* **2012**, *84*, 8957-8960.
- (32) Marty, M. T.; Zhang, H.; Cui, W.; Gross, M. L.; Sligar, S. G. Interpretation and Deconvolution of Nanodisc Native Mass Spectra. *J. Am. Soc. Mass. Spectrom.* **2014**, *25*, 269-277.
- (33) Marty, M. T.; Baldwin, A. J.; Marklund, E. G.; Hochberg, G. K. A.; Benesch, J. L. P.; Robinson, C. V. Bayesian Deconvolution of Mass and Ion Mobility Spectra: From Binary Interactions to Polydisperse Ensembles. *Anal. Chem.* **2015**, *87*, 4370-4376.
- (34) Shih, A. Y.; Denisov, I. G.; Phillips, J. C.; Sligar, S. G.; Schulten, K. Molecular Dynamics Simulations of Discoidal Bilayers Assembled from Truncated Human Lipoproteins. *Biophys. J.* **2005**, *88*, 548-556.
- (35) Zhang, Y.; Liu, L.; Daneshfar, R.; Kitova, E. N.; Li, C.; Jia, F.; Cairo, C. W.; Klassen, J. S. Protein-glycosphingolipid interactions revealed using catch-and-release mass spectrometry. *Anal. Chem.* **2012**, *84*, 7618-7621.
- (36) Han, L.; Kitova, E. N.; Li, J.; Nikjah, S.; Lin, H.; Pluvinaige, B.; Boraston, A. B.; Klassen, J. S. Protein–Glycolipid Interactions Studied in Vitro Using ESI-MS and Nanodiscs: Insights into the Mechanisms and Energetics of Binding. *Anal. Chem.* **2015**, *87*, 4888-4896.

- (37) Leney, A. C.; Fan, X.; Kitova, E. N.; Klassen, J. S. Nanodiscs and Electrospray Ionization Mass Spectrometry: A Tool for Screening Glycolipids Against Proteins. *Anal. Chem.* **2014**, *86*, 5271-5277.
- (38) Popovic, K.; Holyoake, J.; Pomès, R.; Privé, G. G. Structure of saposin A lipoprotein discs. *Proc. Natl. Acad. Sci.* **2012**, *109*, 2908-2912.
- (39) Leney, A. C.; Rezaei Darestani, R.; Li, J.; Nikjah, S.; Kitova, E. N.; Zou, C.; Cairo, C. W.; Xiong, Z. J.; Prive, G. G.; Klassen, J. S. Picodiscs for Facile Protein-Glycolipid Interaction Analysis. *Anal. Chem.* **2015**, *87*, 4402-4408.
- (40) Li, J.; Fan, X.; Kitova, E. N.; Zou, C.; Cairo, C. W.; Eugenio, L.; Ng, K. K. S.; Xiong, Z. J.; Prive, G. G.; Klassen, J. S. Screening Glycolipids Against Proteins in Vitro Using Picodiscs and Catch-and-Release Electrospray Ionization-Mass Spectrometry. *Anal. Chem.* **2016**, *88*, 4742-4750.
- (41) Hoi, K. K.; Robinson, C. V.; Marty, M. T. Unraveling the Composition and Behavior of Heterogeneous Lipid Nanodiscs by Mass Spectrometry. *Anal. Chem.* **2016**, *88*, 6199-6204.
- (42) Cleary, S. P.; Thompson, A. M.; Prell, J. S. Fourier Analysis Method for Analyzing Highly Congested Mass Spectra of Ion Populations with Repeated Subunits. *Anal. Chem.* **2016**, *88*, 6205-6213.
- (43) 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.

- (44) Zhao, Y.; Imura, T.; Leman, L. J.; Curtiss, L. K.; Maryanoff, B. E.; Ghadiri, M. R. Mimicry of High-Density Lipoprotein: Functional Peptide–Lipid Nanoparticles Based on Multivalent Peptide Constructs. *J. Am. Chem. Soc.* **2013**, *135*, 13414-13424.
- (45) Imura, T.; Tsukui, Y.; Sakai, K.; Sakai, H.; Taira, T.; Kitamoto, D. Minimum amino acid residues of an alpha-helical peptide leading to lipid nanodisc formation. *J. Oleo Sci.* **2014**, *63*, 1203-1208.
- (46) Kariyazono, H.; Nadai, R.; Miyajima, R.; Takechi-Haraya, Y.; Baba, T.; Shigenaga, A.; Okuhira, K.; Otaka, A.; Saito, H. Formation of stable nanodiscs by bihelical apolipoprotein A-I mimetic peptide. *J. Pept. Sci.* **2016**, *22*, 116-122.
- (47) Dörr, J.; Scheidelaar, S.; Koorengevel, M.; Dominguez, J.; Schäfer, M.; van Walree, C.; Killian, J. A. The styrene–maleic acid copolymer: a versatile tool in membrane research. *Eur. Biophys. J.* **2015**, 1-19.
- (48) Orwick-Rydmark, M.; Lovett, J. E.; Graziadei, A.; Lindholm, L.; Hicks, M. R.; Watts, A. Detergent-Free Incorporation of a Seven-Transmembrane Receptor Protein into Nanosized Bilayer Lipodisc Particles for Functional and Biophysical Studies. *Nano Lett.* **2012**, *12*, 4687-4692.