High-resolution mass spectrometry of small molecules bound to membrane proteins

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Small molecules are known to stabilize membrane proteins and to modulate their function and oligomeric state, but such interactions are often hard to precisely define. Here we develop and apply a high-resolution, Orbitrap mass spectrometry–based method for analyzing intact membrane protein–ligand complexes. Using this platform, we resolve the complexity of multiple binding events, quantify small molecule binding and reveal selectivity for endogenous lipids that differ only in acyl chain length.

Integral membrane proteins act as biological gatekeepers, controlling the flow of biomolecular substrates across membranes in the cell1. Membrane proteins are key protagonists in mediating cellular function, responding to stress and maintaining homeostasis. Consequently, understanding their interactions with other biomolecules is essential to developing therapeutic interventions. Membrane protein assemblies remain, however, challenging targets to study because of their hydrophobic nature and enigmatic interactions with lipid bilayers.

Recently, non-denaturing or native mass spectrometry (nMS) has emerged as a powerful technique to probe intact membrane protein assemblies2. Specifically, nano-electrospray quadrupole time-of-flight (Q-ToF) MS has been used to define lipid binding partners and to assess their effects on stability3. However, the lack of spectral resolution inherent to existing instrumentation has restricted the interactions that can be measured to those with large mass differences between proteins and substrates (>200–300 Da)4. High-resolution MS has previously been used to interrogate individual membrane proteins, but under the experimental conditions used quaternary interactions are lost5. It has therefore not been possible to identify endogenous lipid ensembles, or combinations of similar-sized lipids, peptides and drugs bound directly to membrane proteins. Methods exist to quantify and identify lipids that co-purify with membrane proteins in solution6,7, but do not define classes or families of lipids in direct contact with the proteins themselves.

To study such interactions, higher resolution nMS is required. Modified Orbitrap mass analyzers have been shown to provide the necessary resolving power8, and the successful analysis of high molecular weight complexes has recently been demonstrated for soluble proteins9,10. We therefore set out to develop an Orbitrap platform that enables unambiguous distinction among lipids, detergents, peptides and drugs in complex with membrane protein assemblies.

To maintain such interactions, micelles or other vehicles are required to protect the membrane protein complex on transfer to the gas phase11–13. Previously, we used increased backing pressure (2–12 mbar) in the initial stages of Q-ToF mass spectrometers and subsequently removed the micelle using activation in an online collision cell14. However, we found that with the Q Exactive instrument (Supplementary Fig. 1), only nominal pressures (1.4–1.6 mbar) were required in the source region in addition to a gentle voltage gradient that we applied to ensure optimal transmission. The intact membrane protein complex is released from the detergent micelle following collisional activation in either the higher energy collisional dissociation (HCD) cell, source region or both (Supplementary Figs. 2 and 3 and Online Methods).

To investigate the general applicability of this approach, we selected a wide range of membrane proteins with different masses (from 26 to 186 kDa), structural features and stoichiometries. They included the monomeric G-protein-coupled receptor chemokine receptor 5 (CCR5); the dimeric glycan transporter semiSWEET; two trimeric channels, the outer membrane porin OmpF and the ammonia transporter AmtB; and a pentameric ligand-gated ion channel (ELIC) (Fig. 1a). All spectra revealed the anticipated stoichiometry, narrow charge state distributions, and baseline resolution with narrow peak widths (~2–3 m/z) even at modest transient times (64 ms). At increased HCD pressures, and in contrast to previous results14, the pentameric ion channel ELIC was released intact from the micelle without appreciable dissociation (Supplementary Fig. 3). The Orbitrap analyzer also allowed greater precision in mass measurement than in our previous studies (Supplementary Table 1); the measured mass of semiSWEET suggests N-terminal processing and the small
molecular adducts previously convolved with the apo ELIC peak were identified as bound lipids.

To quantify protein-ligand interactions, instrument conditions need to be carefully controlled. Spectra recorded for the interaction of OmpF with a peptide mimic of colicin OmpF binding sequence 1 (OBS1) revealed three successive binding events (Supplementary Fig. 4). Intensity values for each state were fit to a multisite binding model using UniDec deconvolution software and yielded a dissociation constant (KD = 0.69 μM) for total OBS1-OmpF binding (Supplementary Figs. 4 and 5a) in close agreement with previous measurements (Q-ToF nMS, 1.4 ± 0.1 μM; isothermal calorimetry, 1.0 ± 0.1 μM). Individual microscopic KD values for successive ligand binding events were also extracted, highlighting the potential to investigate cooperativity (Supplementary Fig. 5b,c) and confirming the suitability of this Orbitrap platform for investigating multiple binding events well separated by mass.

Concomitant drug and lipid binding often involves species of similar mass and has proven challenging, restricting the type of interactions that can be studied to those with large mass differences. Concomitant binding of the OBS1 peptide to OmpF, for example, in the presence of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG) lipids cannot be resolved on existing instrumentation because of the small mass difference between OBS1 and two POPG lipids (Δmass = 55.5 Da). In the case of the ABC transporter P-gp, the immunosuppressant drug cyclosporin A (CsA) can affect the binding affinity of long chain cardiolipin (CDL) (Δmass = 452 Da). However, the lack of spectral resolution abrogated similar studies using shorter chain CDL (CDL 14:1(3)–15:1) (Δmass = 45.0 Da). To investigate whether these small mass differences could be resolved with our method, we added OBS1 to OmpF in the presence of POPG lipid. We found that the enhanced resolution allowed the OBS and 2POPG binding peaks to be clearly separated (Fig. 1b). Similarly, for P-gp, we observed concomitant binding of CsA and CDL 14:0 (Δmass = 39.0 Da) as clearly separated peaks (Fig. 1c and Supplementary Fig. 6). Notably, the spectrum of P-gp revealed three species separated by 76–80 Da within the main protein peak. These proteoforms are likely post-translationally modified forms of the protein. Orbitrap-based nMS can therefore distinguish multiple binding events of lipids with similar masses.

To identify binding of multiple endogenous lipids to membrane protein complexes, however, requires resolution of even smaller mass differences (12–14 Da) that arise from the natural distribution of fatty acid lipid chain lengths. To assess this, we first separately added phosphoglycerol lipids differing in their acyl chain composition (14:0, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG); 16:0, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG); 18:1, POPG) to OmpF to confirm lipid binding (Supplementary Fig. 7). Next we created a model system and prepared an equimolar mixture all three lipids, incubated this with OmpF and observed peaks corresponding to binding of up to six lipids. Closer examination of the singly bound peak revealed three separate peaks, one each for binding of DMPG, DPPG and POPG (Fig. 2). We were able to distinguish binding of multiple lipid combinations with increasingly small mass differences. Intriguingly, the relative abundance of POPG rose in the higher lipid bound states, suggesting that this longer chain, unsaturated lipid binds preferentially. Thus, even when they were attached to relatively large proteins (>100 kDa),
we were able to distinguish lipids from the same class with different chain lengths and degrees of unsaturation, in line with those in natural membranes. Our method therefore provides a substantial improvement on existing nMS approaches that indicate the presence of lipids but have not been able to resolve homologous series directly bound to membrane protein complexes.

With the ability to resolve unique lipid species, we hypothesized that the Orbitrap-based approach would allow us to resolve and identify endogenous lipids. To test this, the Vibrio species glycans transporter semiSWEET was expressed in Escherichia coli, where the endogenous bilayer lipids essentially comprise cardiolipin (~5%), phosphoglycerol (~20%) and phosphoethanolamine (~70%)19. Orbitrap-based nMS of monomeric semiSWEET released from n-dodecyl-β-D-maltoside (DDM) micelles revealed peaks assigned to binding of endogenous CDL (Fig. 3a). At increased transient times, isotopic resolution of the protein confirmed a previously unreported N-terminal methionine cleavage (error 0.9 ppm). Accurate mass measurement of the lipid adducts confirmed their identities as different forms of CDL. The lipid acyl chain length was assigned by comparison with a database of reported E. coli lipids20 (Fig. 3b). CDL molecules bound to semiSWEET were then compared with the total distribution of CDL in the E. coli membrane from cells cultured under the same conditions. (Fig. 3c and Supplementary Fig. 8). Unexpectedly, a marked shift to higher chain length was discerned for CDL in complex with semiSWEET, showing clear selectivity for binding longer chain lipids. Such membrane remodeling may arise from the need to stabilize the rocking motion associated with the function of this transporter. Our direct lipid identification strategy overcomes the shortcomings of procedures based on lipid extraction and lipidomics, which typically identify only copurified lipids, not necessarily those that are in direct contact with the protein.

In conclusion, we describe the development and application of a general method using a modified Orbitrap platform for high-resolution nMS of membrane protein complexes. We show that careful selection of instrument conditions, particularly the voltage gradient, allowed the controlled release of membrane proteins from detergent micelles with retention of stoichiometry and small molecule binding quantitatively similar to that of both conventional (Q-ToF) nMS and solution measurements. The high resolution afforded by this approach exposed unexpected proteoforms and enabled multiple substrates to be distinguished, such as homologous series of endogenous lipids and drug binding in the presence of similarly sized lipids that comprise the natural membrane. Direct identification of individual protein-bound lipid species represents a significant advance over previous methods and showcases the applicability of this technique to more complex membrane proteins, which undergo multiple ligand binding events, or where an abundance of glycoforms and other post-translational modifications are anticipated.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

Figure 2 | nMS spectrum (main panel) of trimeric OmpF bound to an equimolar ratio of DMPG, DPPG and POPG lipids (cartoon inset). The 22+ charge state is shown in an expanded view (right), with peaks showing up to three lipids bound. Theoretical distributions corresponding to different combinations of lipids are shown by colored lines and correlate with the spectrum. | 100 Relative abundance 5+ 4+ 3+ 2+ 1+ m/z 2,889 2,892 3,230 3,240 3,250 5,100 5,150 Mass (Da) 0 2,000 2,500 3,000 3,500

Figure 3 | nMS of membrane protein semiSWEET in direct contact with bound lipids. (a) Isotopically resolved mass spectrum of semiSWEET released from DDM micelles obtained at an increased transient time of 256 ms. Monoisotopic mass measurement of the main protein peak (blue) gives an error of 0.9 ppm once N-terminal methionine cleavage is taken into account. Inset shows a zoom of the apo protein peak (blue) and of the satellite peaks (orange), revealing a distribution of homologous series of lipids. (b) Deconvolved spectrum of semiSWEET with bound CDL adducts showing monoisotopic mass. Acyl chain length assignment is indicated. (c) Comparison of CDL bound to semiSWEET (orange bars) with the distribution of CDL in the total membrane (open bars); error bars, s.d.
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AUTHOR CONTRIBUTIONS
J.G. and C.V.R., with assistance from J.T.S.H., designed the research. M.B., E.D., A.M., T.M. and J.G. modified the Q Exactive mass spectrometer and optimized the MS experiment for membrane protein complexes. J.G., J.A.C.D., I.L., K.G., J.T.S.H. and W.B.S. expressed and purified membrane proteins in appropriate conditions for nondenaturing MS. J.G., J.A.C.D. and I.L. performed MS experiments. J.G. and J.A.C.D. performed lipidomics experiments and data analysis. Y.Z. and B.W. provided a sample of the CCR5 protein. N.G.H. and C.K. provided samples of the OmpF protein and OBS1 peptide. C.B. set up the lipidomics platform. M.T.M. modified the UniDec software for use in this work. J.G. and C.V.R. wrote the manuscript with contributions from all other authors.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

High-resolution Orbitrap nondenaturing MS. Experiments were performed using a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer21 (Thermo Fisher Scientific) modified for the transmission and detection of high m/z ions. Hardware alterations included a modified quadrupole RF board that allowed a lower RF frequency to be applied to the selection quadrupole (284 kHz), a modified preamplifier with lower high-pass filter cut-off and several modifications to the HCD gas inlet, including replacing the standard PEEKSil capillary from the diverter valve to the HCD cell, which is 100 µm internal diameter, with one of 530 µm internal diameter and replacing the metal restrictor tubing from the manual valve to the diverter valve with a 0.01-inch internal diameter metal tubing. These modifications allowed the selection quadrupole to operate as a mass selector up to 20,000 m/z, allowed the high m/z-low frequency signals to be detected, and allowed higher pressures to be achieved in the HCD cell (~1 × 10⁻⁹ mbar rather than the standard operating pressures of 10⁻¹¹–10⁻¹⁰ mbar). This allows better capture of ions in the HCD cell, as described elsewhere22. Ions were generated in the positive ion mode from a static nanospray source using gold-coated capillaries prepared in-house, then passed through a temperature controlled (40–60 °C) transfer tube, RF-only S-lens, injection flatapole and bent flatapole. The instrument was operated under Tune 2.4 instrument control software in native mode, in which the RF applied to the multipoles (injection flatapole, bent flatapole (950 Vp-p) and C-trap (2,950 Vp-p) was increased to the maximum allowed by electronic boards. After traversing the selection quadrupole, which was operated with a wide selection window (2,000–20,000 m/z) in these experiments, ions were trapped in the HCD cell before being transferred to the C-trap and Orbitrap mass analyzer for detection.

The key transformative step to successfully performing these experiments was to move away from the pressure gradients used previously11,14,23 and to apply a gentle voltage gradient throughout the instrument and especially on the ion transfer optics (injection flatapole, inter-flatapole lens, bent flatapole, transfer multipole: 8, 7, 6, 4 V respectively) to avoid collisional activation of the ions while they are still protected by the detergent micelle and before micelle removal in the HCD cell. Particular care was taken when tuning the C-trap entrance lens as this can act as a mass filter if operated at too high a voltage. To ensure better capture of high-m/z ions in the Orbitrap analyzer, the initial central electrode voltage was adjusted from −3.7 kV to −3.2 kV, while the setting during detection remained unchanged (−5 kV). Transient times were 64 ms (corresponding to resolution setting 17,500 at m/z 200) unless otherwise stated and AGC target was 1 × 10⁶. Spectra were acquired with ten microscans and averaged with a noise level parameter of 3 rather than the 4.68 default. Removal of the micelle was achieved through increased acceleration voltages applied in the HCD cell. Additional energy could be applied to the S-lens (in source activation) if required. The collision gas was either nitrogen or argon and pressure in the Orbitrap cell was maintained at around 1 × 10⁻⁹ mbar unless otherwise stated. Calibration was performed up to 11,304 m/z using clusters of C₁₁ in infusion mode. Data was viewed using Xcalibur 2.2 SP1.48 (Thermo Fisher Scientific); no further smoothing or baseline subtraction needed to be applied to the raw data. For nonisotopically resolved data, masses were calculated using an in-house software tool available online (http://benesch.chem.ox.ac.uk/resources.html) and based on a minimization of error calculated from different charge state assignments24,25. For isotopically resolved data, the Xtract algorithm, licensed as part of Qual Browser in Xcalibur 2.2 SP1.48, was used for peak picking and deconvolution. For all proteins presented in this work, once optimal instrument conditions had been established for efficient transmission and release of membrane proteins from the micelle, measurements were found to be entirely reproducible, with only minimal variation in parameters and spectral quality. Solutions of purified membrane protein complexes were buffer exchanged into 200 mM ammonium acetate (Sigma-Aldrich) supplemented with 2 × critical micelle concentration (CMC) detergent (Anatrace, Ohio, USA) using P6 Biospin columns (Bio-Rad) immediately before MS analysis. All proteins were prepared for MS at approximately 10 µM complex concentration before buffer exchange. If the signal intensity was very high (very short ion trap fill time), the concentration was adjusted accordingly.

Protein purification. G-protein-coupled receptor CCR5 in n-dodecyl-β-maltoside (DDM) was expressed and purified according to the published protocol26. His-tagged P-gp was expressed in Pichia pastoris and purified in DDM as described previously27. *Vibrio* sp. N418 semiSWEET was expressed in a pJexpress411 vector containing sequences for a 3C protease cleavage site and a histidine tag (DNA2.0)28 before exchange into DDM or C8E4 for MS analysis. The ELIC plasmid from I. Zimmerman and R. Dutzer (University of Zurich), was transformed into Rosetta 2 (DE3) cells (Novagen, Gibbstown, NJ) and expressed as previously described14 before exchange of the protein into DDM for MS analysis. AmtB was overexpressed as an N-terminal fusion to maltose binding protein preceded by a secretion signal peptide (pelB) and 10× His-tag in a modified pET15b vector and purified as previously described3 before exchange into C8E4 for MS. OmpF was purified in β-octyl-glucoside (β-OG) according to the published protocol29. Proteins were adjusted to approximately 10 µM complex concentration before buffer exchange and MS.

Measurement and calculation of OBS1-OmpF dissociation constant. Measurement of the dissociation constant for binding of OBS1 to OmpF was performed as described previously30. Briefly, OBS1 peptide (NH₂⁻⁵GgdGdrGHTAHStsc⁻¹⁸-COH₁₈) was diluted from a single stock to twice the desired concentration and mixed with equal volume of OmpF in 25 mM ammonium acetate with 1% (w/v) β-OG immediately before mass measurement. Conditions for nanoESI-MS were verified to generate spectra of sufficient quality to obtain resolved peaks without incurring ligand dissociation. 200 V was applied in the HCD cell with no additional in-source activation. Spectra were deconvolved using the UniDec software tool16.

The Data Collector module in UniDec was used to extract and normalize intensities summed over all charge states and for the computation of K_D values. For calculation of the overall OmpF-OBS1 K_D, peak intensities for all bound states (OmpF-OBS1, OmpF-2OBS1 and OmpF-3OBS1) were combined and data fit to the same binding model as described previously30, where the [OmpF] is considered to be the concentration of binding sites; that is, 3 × [OmpF trimmer]. For calculation of the individual binding site K_D values, extracted intensities for the *apo* and each
separate bound state were fit to $K_D$ binding models using either a single fixed $K_D$ for all binding sites or free $K_D$ values for each individual site. After fitting to both models, an $F$-test was used to determine the best model.

**OmpF-lipid binding.** Lipid stocks of 1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DPPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG) were prepared from powder (Avanti Polar Lipids Inc., Alabama, USA) at concentrations of $10 \text{ mg/mL}$ using the previously published procedure. Lipid stocks were diluted 50-fold in 1% (w/v) ammonium acetate containing 2 mM ammonium acetate and added to OmpF in an appropriate ratio for multiple lipid species to be bound. For multiple lipid binding, an approximately equimolar mixture of all three lipid species was prepared. The spectrum of concomitant binding of DMPG, DPPG and POPG to the OmpF trimer was acquired at an increased transient time of 128 ms ($35,000 \text{ resolution at } m/z 200$).

**P-gp binding.** Lipid stocks of 1’,3’-bis(1,2-dimyristoyl-sn-glycero-3-phospho)-sn-glycerol (Avanti Polar Lipids Inc., Alabama, USA) (CDL 14:0) were prepared at a concentration of $10 \text{ mg/mL}$ and diluted 50-fold in 200 mM ammonium acetate containing 2 × CMC DDM. Lipid was mixed with P-gp in an appropriate ratio to observe multiply bound species. Binding of cyclosporin A (CsA) (Sigma-Aldrich) and concomitant binding was performed in a similar fashion. Spectra of P-gp bound to cardiolipin 14:0, to cyclosporin A and to both cardiolipin and cyclosporin were acquired at an increased transient time of 128 ms ($35,000 \text{ resolution at } m/z 200$).

**Characterization of cardiolipin bound to semiSWEET.** Three separate preparations of semiSWEET were analyzed by native Orbitrap MS as described above but with extended transient times to achieve isotopic resolution ($140,000 \text{ resolution at } m/z 200$). Spectra were acquired with ten microscans for 50–100 scans before being averaged in the Xcalibur software. They were then deconvolved using the Xtract algorithm and the mass of the lipid adduct calculated by subtracting the mass of the apo protein measured in each case and normalized against the most intense lipid species. By comparison with a previously published list of *E. coli* cardiolipin species, the lipid adduct was assigned a chain length $n$ (where $n$ is the total number of acyl chain carbon atoms). Averages and s.d. (error bars are 1 s.d.) were then calculated for each species as a percentage of the total bound cardiolipin, for semiSWEET preparations from three biological replicates (three separate protein expressions), and represented in Figure 3.

**Profiling of cardiolipin in bulk *E. coli* membrane.** At the moment of cell harvest a 5-mL sample of cells was taken from four randomly chosen flasks of a 12-L semiSWEET expression. This ensured that the lipid composition of the total membrane extract was representative of the conditions during semiSWEET overexpression. Cells were pelleted and the supernatant removed and replaced with 100 μL lysis buffer (20 mM Tri-HCl, 300 mM NaCl, pH 7.4). After vigorous vortexing, the cell suspension was snap frozen to lyse cells, thawed and total lipid extracted using a modified Bligh and Dyer procedure. Briefly, to 100 μL cell suspension, 375 μL chloroform:methanol (2:1) was added, followed by 125 μL chloroform and 125 μL H2O. Samples were vortexed then allowed to phase separate. The bottom (organic) layer was removed and evaporated to dryness before being resuspended in 1,000 μL 68% solution A (ACN:H2O 60:40, 10 mM ammonium formate and 0.1% formic acid) and 32% solution B (IPA:ACN 90:10, 10 mM ammonium formate and 0.1% formic acid) for analysis by reverse phase liquid chromatography tandem MS (RF LC-MS/MS).

LC-MS/MS was performed using a Dionex UltiMate 3000 RSLC Nano system coupled to an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific). Total lipid extract resuspended in 32% solution B was loaded into a 1-μL loop using the autosampler and loading pump, then transferred onto a C18 column (Acclaim PepMap 100, C18, 75 μm × 15 cm, Thermo Fisher Scientific) at a flow rate of 300 nL/min. After 10 min solvent B was ramped to 65% over 1 min, then 80% over 6 min, before being held at 80% for 10 min, then ramped to 99% over 6 min and held for 7 min.

The column eluent was delivered to the LTQ Orbitrap XL system operated in negative ion mode via a dynamic nanospray source. Spray voltage was −1.6 kV and capillary temperature 160 °C. After a full MS scan acquired in the Orbitrap mass analyzer (AGC target 5e5, resolution 60,000 at m/z 400) tandem MS was performed in a data-dependent fashion using a top 5 method. Ions were selected, fragmented and detected in the linear ion trap (AGC target 1e4). Collision-induced dissociation (CID) energy was 38% NCE and activation time 30 ms. Ions were added to a dynamic exclusion list after being fragmented twice.

The proportion of the full scan chromatogram corresponding to cardiolipin (highlighted in Supplementary Fig. 8a) was then summed and mass spectra extracted in Xcalibur 2.2 software before being deconvolved using the Xtract algorithm to give monoisotopic neutral CDL masses. Ions corresponding to the $n$:2 peaks (where $n$ is the total number of acyl chain carbon atoms and the number the number of double bond equivalents) were taken as representative of the total lipid population with that chain length. Intensities of these ions were extracted and normalized to the most intense CDL species. The average percentage was then calculated along with the s.d. and represented in Figure 3.

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