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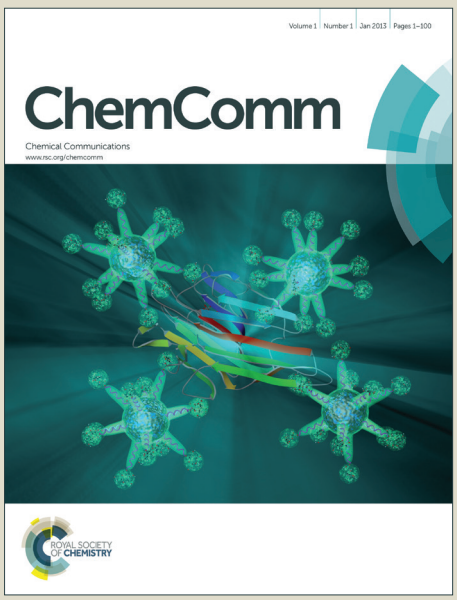

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Controlling release, unfolding and dissociation of membrane protein complexes in the gas phase through collisional cooling†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Mass spectrometry of intact membrane protein complexes requires removal of the detergent micelle by collisional activation. We demonstrate that the necessary energy can be obtained by adjusting the degree of collisional cooling in the ion source. This enables us to extend the energy regime for dissociation of membrane protein complexes.

Detergents and lipids protect membrane protein complexes during the transition from solution to the gas phase.[1-4] To enable the study of intact membrane proteins by mass spectrometry, this protective layer has to be removed by collisional activation. This is usually achieved by raising the collision voltage in the ion trap or the collision cell of the instrument (Fig. 1 A). However, most detergents require significant amounts of energy to dissociate,[5] and the release of membrane proteins from lipid environments has to date only been achieved using customized high-energy mass spectrometers.[6] Only limited energy can be applied once micelle removal is achieved, which often precludes collision-induced dissociation studies of membrane proteins that can provide valuable information about complex stoichiometry and stability.[7]

Ions can acquire energies in the electronvolt range during the passage from atmospheric to low pressure in the source of the mass spectrometer.[8] Maintaining a high pressure at the first pumping stage of the instrument (*i.e.* the source or "backing" pressure) between the sample cone and extractor cone increases the amount of low-energy collisions and reduces ion velocity (see Supplementary Methods). As a result, the ions avoid collisional activation and their trajectories are focused to facilitate efficient transmission.[9-13] This process, termed "collisional cooling", is widely used for native MS of protein complexes. This raises the question as to whether reduced collisional cooling could be used to facilitate more efficient detergent removal. We find that the high-energy collisions at low source pressure provide a similar level of activation as achieved in the collision cell. By controlling the amount of collisional cooling, we are able to increase the energy range available for collisional unfolding and dissociation studies of membrane protein complexes.

Using the 82 kDa dimer of the Na+/H+ antiporter NapA[14], we evaluated whether reduced source pressure results in elevated ion activation while keeping all other instrument settings constant. NapA - phospholipids complexes in the detergents C8E4 or n-dodecyl-β-D-maltoside (DDM) were subjected to nESI-MS at a range of collision voltages and source pressures. At a constant collision voltage of 50 V, a reduction of the source pressure from the commonly used 7.5 mbar to 1.8 mbar, the lowest value at which we can still achieve sufficient ion transmission, clearly shows progressive removal of C8E4 while keeping the interactions with added phospholipids intact (Figure 1 B). The source pressure-driven protein release closely resembles the detergent removal by activation in the collision cell (Figure 1 B). Similarly, lowering the source pressure drastically reduces the energy required to strip the mild detergent n-dodecyl-β-D-maltoside (DDM) from NapA (Figure S 1).

Next, we performed ion mobility measurements to compare the effects of gradual pressure reduction and increasing collisional activation on the conformation of NapA.[15] Both strategies give rise to nearly identical unfolding trajectories (Figure 1C). Under the conditions employed here, a pressure reduction by 5.5 mbar roughly corresponds to a 60 V increase in collision energy. This supports the observation that reduced collisional cooling can release intact membrane protein complexes in the same manner as in the collision cell. Similarly, compact conformations of NapA at low source pressure are only observed when the collision voltage is reduced (Supplementary Figure 2).

Being able to activate membrane proteins in the source significantly increases the energy range available for collisional

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† Electronic Supplementary Information (ESI) available: Supplementary Methods and Supplementary Figure 1. See DOI: 10.1039/x0xx00000x

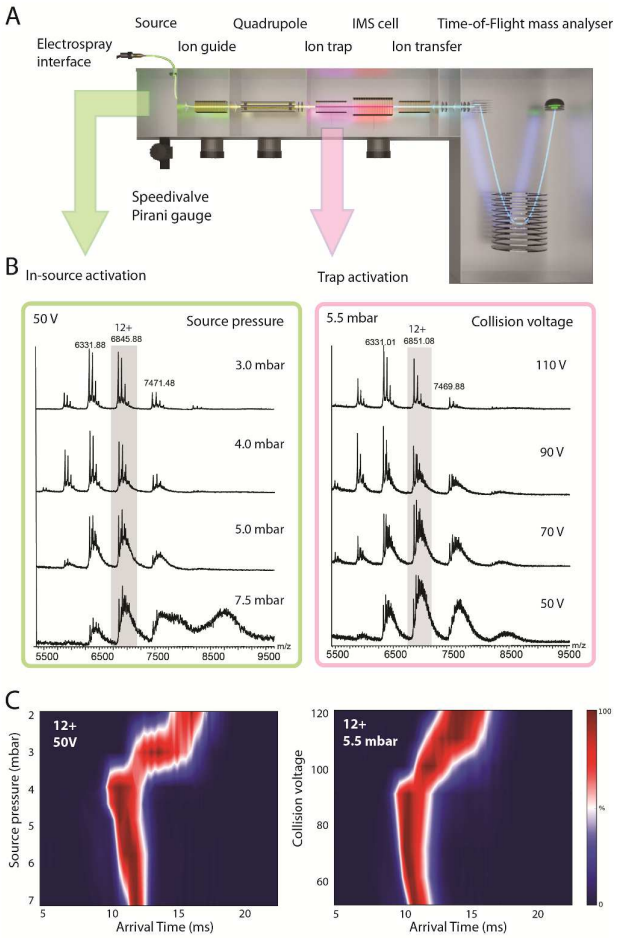

Fig. 1. Reduced source pressure enables the efficient release of NapA from detergent micelles at low activation energy. (A) Geometry of a quadrupole-ion mobility-time-of-flight mass spectrometer. The pressure in the source (green) can be regulated by a speedivalve. Complex activation is usually performed by increasing the collision energy in the ion trap (pink). (B) Intact NapA dimers with bound PG can be released from C8E4 micelles by adjusting the source pressure at fixed collision energy (green) or by adjusting the collision energy at fixed source pressure (pink). (C) Decreasing the source pressure or increasing the collision energy yield similar unfolding trajectories of the 12+ charge state of NapA.

activation in the ion trap (the collision cell of the IMMS instrument, Figure 1 A) as it allows us to detect free protein at low collision voltages. In previous studies, we found that lipids can remain bound to membrane protein complexes across the entire activation energy range.[2] We therefore tested whether controlled collisional cooling enables us to strip bound lipids from NapA. By increasing the collision voltage from 40 to 100 V at a source pressure of 2.0 mbar, we are able to dissociate 1-palmitoyl-2-oleoyl-glycerophospho-ethanolamine (PE) and 1-palmitoyl-2-oleoyl-phosphatidyl-glycerol (PG) from NapA (Figure 2 A). Plotting the fraction of intact protein-lipid complexes as a function of charge state-normalized collision energy (E_{Lab}) reveals that complexes with negatively charged PG exhibit higher gas phase stability than with the zwitterionic PE (Figure 2 B). The differences in stability can be rationalized by the high number of positive residues that line the intracellular side of the transmembrane region of NapA and mediate interactions with negatively charged lipid headgroups.[14] This shows that reducing collisional cooling extends the available activation energy range to compare relative gas phase stabilities of protein-lipid interactions. Additionally, we observed that increasing the collision energy beyond 140 V at a source pressure of 2.0 mbar dissociates the native NapA dimer (Figure 2 C). A comparison of the unfolding trajectories reveals that the onset of dimer dissociation coincides with an extra unfolding step not observed at high source pressure (Figure 2 D).

We confirmed these findings with the significantly larger ammonia channel AmtB (Figure.3), which forms a 130 kDa trimer, and found that detergent release and lipid dissociation are not significantly affected by molecular weight, although the less effective ion transmission at low source pressure may limit the application to very large complexes.

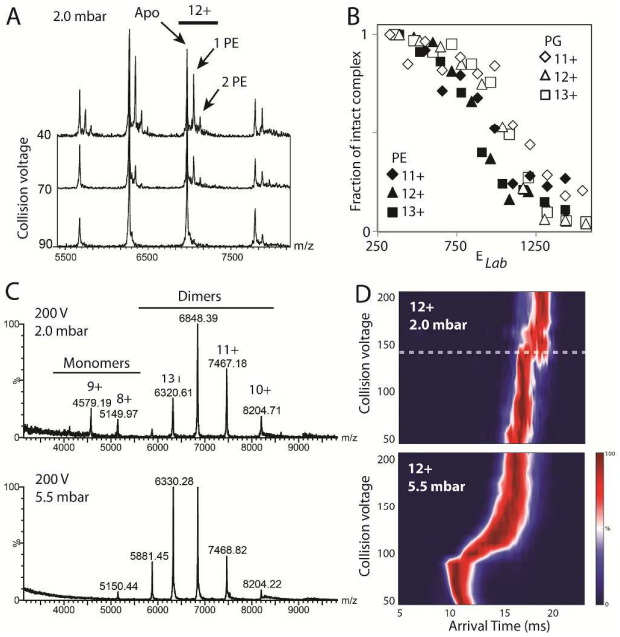

Fig. 2. Source pressure reduction extends the activation energy range. (A) Low source pressure enables lipid dissociation. The overlay of MS spectra of NapA with PE shows complex dissociation at increased collision voltages. (B) Plotting the amount of intact complexes of NapA with PE shown in (A) as well as with PG under the same conditions reveals a lower gas phase stability PE binding. (C) Low source pressure facilitates the detection of dimer dissociation within the collision energy range of the mass spectrometer. (D) The unfolding trajectory of the 12+ charge state of NapA at a source pressure of 5.5 mbar (lower panel) shows the onset of unfolding at the lowest activation energy. The unfolding trajectory obtained at a source pressure of 2.0 mbar (top panel) reveals a further unfolding step at the onset of dimer dissociation (dashed line).

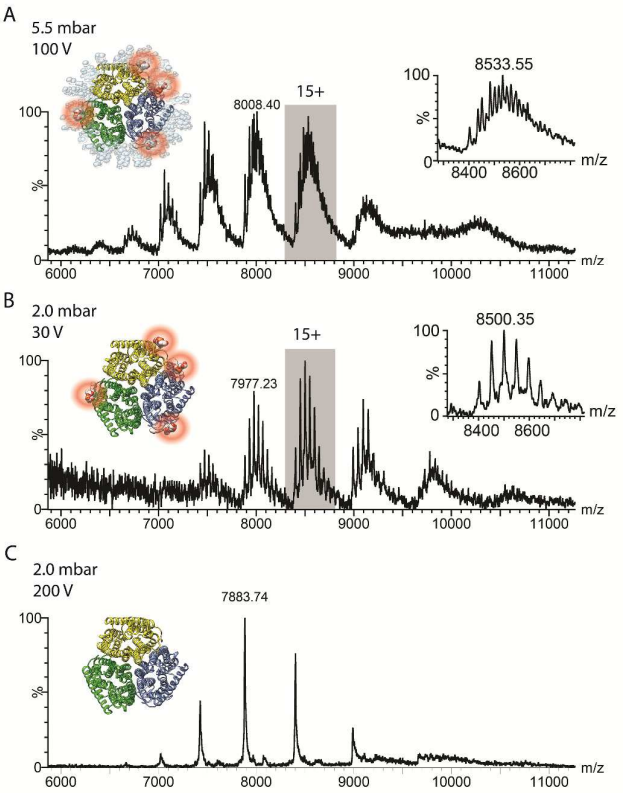

Fig. 3. Large membrane proteins can be released intact from C8E4 micelles. (A) ESI-MS spectra of AmtB at high source pressure and a collision voltage of 100 V show the trimer with detergent and lipid adducts (insert). (B) Reduced source pressure allows the detection of the intact AmtB trimer at low collision voltages with detergents but not lipids removed (insert). (C) Increasing the collision voltage to 200 V at reduced source pressure dissociates all adducts.

However, only the lower, but not higher charge states can maintain a compact conformation at low source pressure (Supplementary Figure 3). Taken together, controlled collisional cooling in combination with relatively volatile detergents extends the energy range sufficiently to allow complex dissociation.

In summary, we demonstrate that controlled collisional cooling is an effective way to tune the activation energy for the analysis of membrane protein complexes. We find that the extended collision energy range enables us to compare relative gas phase stabilities of protein-lipid interactions and observe unfolding steps associated with oligomer dissociation. The approach likely works best for small membrane proteins that can be transmitted even at low source pressures. However, there is a trade-off between transmission and in-source release, and both factors should be balanced when optimizing the MS conditions for each protein. Additionally, considering the activating effects of reduced source pressure, it is advisable to record calibration curves and CCS measurements of native-like states with maximised collisional cooling. Our results provide a rationale for the release and transmission of intact membrane protein assemblies in mass spectrometers without elevated source pressure or a collision cell. Considering the growing use of native MS for protein unfolding studies in general, these findings highlight the importance of closely monitoring the corresponding parameters when assessing gas phase stabilities. Interestingly, we find that release in the source at the front of the instrument can produce compact conformations similar to those observed when the protein is released in the trap, *i.e*. directly at the entrance to the ion mobility cell. This suggests that membrane proteins are able to adopt a stable conformation in the gas phase following detergent removal and maintain a native-like CCS for extended time periods. Our results therefore raise the possibility that the gas phase behaviour of detergent-free membrane proteins resembles that of soluble proteins.

ML holds a Marie Curie Early Career Development grant and a Junior Research Fellowship from St Cross College, University of Oxford. DD acknowledges the support from EMBO through the EMBO Young Investigator Program and the Knut och Alice Wallenberg foundation. CVR is supported by an ERC Advanced Investigator Award (26851) (IMPRESS), a Royal Society Research Professorship, and a Medical Research Council Program Grant (98101). Special thanks to Prof. A. Laganowsky (Texas A&M Health Science Center) for the rendering of the ion mobility mass spectrometer in Fig. 1 A.

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