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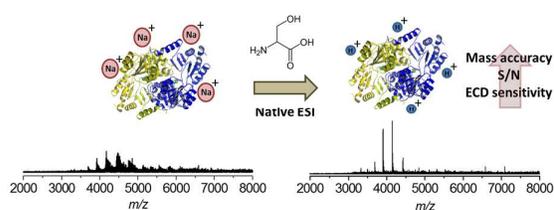
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14 A simple method for mitigating the adverse effects of salt adduction during native protein mass
15 spectrometry by addition of amino-acids.
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3 **Desalting Large Protein Complexes during Native Electrospray Mass Spectrometry by**
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5 **Addition of Amino Acids to the Working Solution.**
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ABSTRACT

Native mass spectrometry is a rapidly emerging field for characterising the structure of proteins and protein assemblies. The technique relies on electrospray ionisation (ESI) to efficiently ionise the protein analyte and transmit it into the gas phase with retention of protein structure, non-covalent protein-ligand and protein-protein interactions. In native ESI, both the ionisation efficiency and the resulting mass spectral signal is adversely effected by the presence of non-volatile inorganic salts, such as sodium chloride, which form extensive adducts with the protein ions. Consequently, there is great interest in finding experimental strategies that mitigate these phenomena.

Here we report our findings that the addition of 10 mM L-serine to the ESI spray solution reduces the adverse effects of sodium adduction to proteins. In the analysis of bovine serum albumin (BSA; 66 KDa), 10 mM serine increased signal to noise ratio (S/N) ~4 fold. This increase in sensitivity was accompanied by peak narrowing (~10 fold), which allowed more precise assignment of molecular mass. Similar effects were observed when analysing protein complexes - serine palmitoyl transferase (SPT, a 92 KDa homodimer), enolase (a 93 KDa homodimer;) and alcohol dehydrogenase (ADH, a 148 KDa tetramer). Reduction in sodium ion adduction occurs with no loss of the non-covalent protein-protein interactions, and with little effect on the overall observed charge state-distribution. As a consequence of increasing signal intensity, the addition of serine to the ESI spray solution greatly improved the quality of the data obtained from native top-down electron-capture dissociation (ECD) experiments. In ECD analysis of native BSA, we observed an increasing in the S/N of all ECD fragments upon addition of 10 mM L-serine. The number of ECD fragments we observed with $S/N > 1.5$ increased from 15 to 44 and the number of assigned

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3 c and z ions increased from 5 to 16. Finally we show that this phenomenon is not specific to
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6 L-serine, and occurs with several amino acids such as L-alanine. Our findings suggest that
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9 desalting may occur via binding of sodium ion to the amino acid in solution. This simple and
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11 inexpensive strategy has broad utility for improving the mass spectra obtained in a range of
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13 MS-based structural proteomic studies.
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INTRODUCTION

Electrospray ionisation (ESI) mass spectrometry (MS) is routinely used to measure the molecular mass of intact proteins.¹ In addition, it is now well established that, by careful control of the ESI conditions and solution composition, it is possible to retain protein-ligand and protein-protein, non-covalent interactions during ionisation and transmission into the gas phase. Consequently, protein complexes and assemblies can be directly monitored by ESI MS to yield insights into the composition, stoichiometry, and dynamics of multiprotein assemblies, a process now termed native MS.²⁻⁶ This technique has matured over the last decade, and it has proven itself a valuable technique for the analysis of: protein oligomerisation,⁷ protein-ligand complexes,⁸ heteromultimeric protein complex topology,^{9,10} membrane proteins;¹¹ and intact analysis of virus capsids complexes up to several million Daltons in mass.¹²

The preservation of protein higher order structure during native ESI relies on precise control of the solution conditions, which are usually required to be aqueous and at physiological pH. Achieving these conditions is hampered by the fact that non-volatile salts are detrimental to the performance of ESI mass spectrometry, even at micromolar concentrations. Their presence in solution can cause salt clusters, adduct formation and severe ion suppression, leading to a dramatic decrease signal to noise ratio (S/N).^{13,14} This is particularly pronounced with metal cations, such as Na^+ ; and ESI mass spectra recorded in the presence of Na^+ salts often display multiple nonspecific Na^+ adducts;¹⁵ most prominently on low charge state ions.¹³ Thus, many traditional biological buffer solutions (Tris, phosphate, phosphate-buffered saline (PBS) etc.) are incompatible with native ESI. Therefore, volatile buffer solutions are most often employed – typically ammonium acetate

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3 or ammonium bicarbonate; and protein sample preparation for native ESI often involves
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5 stringent removal of metal ion salts by dialysis, liquid chromatography or buffer exchange
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7 prior to analysis. However, complete removal of salts is not always possible and salt-induced
8
9 ion suppression in native ESI is a commonly encountered problem.
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14 Several strategies have been proposed to suppress sodium ion addition in native ESI.
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16 Lowering the pH to ~3 units lower than the pI of the protein has been shown to lower salt
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18 adduction.¹³ However, the manipulation of pH to such an extent will often affect protein
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20 higher order structure in solution, and may result in loss of physiologically relevant
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22 structural information.¹⁶ Williams and co-workers found that high concentrations (>2M) of
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24 ammonium acetate can reduce sodium adduction,¹⁵ presumably by displacement of Na⁺
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26 with NH₄⁺ in solution, followed by loss of NH₃ in the gas phase.¹⁷ However, the success of
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28 this technique has been shown to be protein-dependant, and poorly resolved mass spectra
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30 have been reported for some large proteins analysed in high concentrations of ammonium
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32 acetate.¹¹ The most promising strategies seem to involve inclusion of specific small molecule
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34 chemical additives to reduce the formation of non-specific metal-adducts. Konermann and
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36 co-workers demonstrated that calcium adduction could be reduced by addition of the
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38 solution phase chelator L-tartrate or citrate.¹⁸ More recently, promising results have been
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40 achieved by the addition of selected low proton affinity (PA) anions in native ESI solutions.¹⁹
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42 Flick *et al.* demonstrated that dramatic increases in *S/N* could be achieved when analysing
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44 small proteins in the presence of 25 mM ammonium bromide or ammonium iodide.²⁰ In
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46 addition, supercharging reagents (e.g. m-nitrobenzyl alcohol (m-NBA)) have been
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48 demonstrated to reduce salt adduction.²¹ When added to aqueous solutions (typically at
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50 <2% v/v) prior to ESI, these high-boiling point additives can be used to increase the charge
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3 state distribution observed in protein mass spectra.^{22,23} The exact mechanism of this
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6 phenomenon is still being investigated;²⁴⁻²⁷ However, Cassou et al. recently reported that
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8 this supercharging phenomenon is accompanied by a decrease in salt adduction, and can
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10 effectively be used to increase *S/N* in native protein mass spectra. It is important to note
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12 that, using this approach, care must be taken in controlling the solution conditions and
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14 exact concentration of supercharging reagents, as labile, non-covalent interactions can be
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16 lost during this process.²⁸ Finally, a recent report has highlighted that the exposure of
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18 aqueous nanoelectrospray droplet to organic vapours (such as volatile alcohols) can
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20 dramatically reduce Na-adduction without inducing protein denaturation.²⁹ This
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25
26 phenomenon is attributed to ion evaporation of clusters of the organic species with sodium
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28 cations during the nESI process; thereby depleting the remaining (protein containing)
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30 droplet of sodium ions. This approach holds great promise, although requires specific
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32 instrument modification to allow the introduction of organic vapour into the ESI source.
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37 Here we report a serendipitous finding that the addition of free amino acids, at low
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39 millimolar concentrations, can efficiently reduce sodium adduction to protein ions formed
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41 during native ESI. In recent studies of the pyridoxal 5'-phosphate (PLP)-dependant enzyme
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43 serine palmitoyl transferase (SPT),³⁰⁻³² we noted the surprising observation that addition of
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45 the enzyme substrate, L-serine, prior to native MS analysis significantly mitigated the
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47 adverse effect of sodium adduction. Here we show that this surprising observation also
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49 occurs with a range of amino acids additives and we demonstrate these findings by
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51 analysing a range of large protein systems. This phenomenon occurs with little observable
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53 change in charge state distribution, and without any apparent dissociation of protein-
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55 protein and protein-ligand interactions. Finally, we highlight the potential of using amino
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3 acid additives, and similar 'desalting' additives, to improve the efficiency of native top-down
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6 fragmentation experiments.
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9 10 **EXPERIMENTAL**

11 12 **Materials**

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16 Yeast alcohol dehydrogenase (ADH) and enolase were purchased as lyophilized powders
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18 from Sigma-Aldrich (St. Louis, MO, USA); bovine serum albumin (BSA, ultrapure) was
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20 purchased from Gerbu Biotechnik (Gaiberg). Serine Palmitoyl Transferase (SPT) from the
21
22 bacterium *S. paucimobilis*, was expressed and purified as described previously.^{31,32}
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26 Ammonium acetate and sodium chloride were obtained from Sigma-Aldrich; all amino acids
27
28 were acquired from Novabiochem. Ammonium acetate was prepared in LC-MS grade water
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30 (Chromasolv; Sigma-Aldrich).
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33 34 **Sample Preparation for Native ESI MS**

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38 Lyophilized proteins were dissolved in 200 mM ammonium acetate, pH 7.0. To ensure the
39
40 removal of trace salts, protein samples were buffer exchanged into 200 mM ammonium
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42 acetate, pH 7.0 using Micro-biospin 6 columns (Bio-Rad) immediately prior to MS analysis
43
44 (following the protocol outlined in the manufacturer's instructions). If required, sodium
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46 chloride and amino acids were added from 10 fold stock solutions prior to MS analysis.
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50 Protein samples were typically analysed at an assembly concentration of 5 μ M.
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53 54 **Mass Spectrometry**

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57 Mass spectrometry data was acquired on a Solarix FT-ICR mass spectrometer equipped with
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59 a 12 Tesla superconducting magnet (Bruker Daltonics). Nanoelectrospray (nESI) was
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3 performed using a Nanomate running in positive ion infusion mode and equipped with a
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5 HD_A_0 ESI Chip (Advion Bioscience). Typically, ionisation was achieved by applying a
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7 potential of 1.5-1.7 kV to the nESI Chip and the drying gas was set to 120 °C. The ion-
8
9 transmission RF frequencies and the time-of-flight were tuned based on the size of the
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11 protein assembly analysed. Native mass spectra were typically acquired between m/z 500
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13 and 10,000 to yield a broadband 1MW time-domain transient, ion accumulation was set at
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15 1000 ms, and each spectrum was the sum of 100 acquisitions. The resulting mass spectra
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17 were externally calibrated using ES tuning mix (Agilent) and analysed using DataAnalysis
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19 software (Bruker Daltonics).
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27 Top-down fragmentation was performed on a Solarix FT-ICR mass spectrometer using
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29 electron capture dissociation (ECD). No quadrupole isolation was performed prior to ECD,
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31 therefore all charge states of the protein assemblies were fragmented simultaneously.
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34 Typically, 1.6 A was applied to the dispenser cathode filament (Heatwave Technologies), 12
35
36 V to the lens, 0.6 V to the bias, and a pulse of between 5 and 14 ms was employed.
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39 Fragmentation data was the sum of 1000 acquisitions and data analyses were performed
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41 using DataAnalysis software (Bruker Daltonics). The SNAP 2.0 algorithm was used for
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43 automated peak picking. The resulting top-down fragment mass lists were searched against
44
45 the protein primary sequence using ProSight-PTM software packages.³³ Mass error
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47 tolerances were set for all searches at 15 ppm.
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51 52 53 **RESULTS AND DISCUSSION**

54 55 56 **The effect of sodium ion adduction in native protein mass spectra.** 57 58 59 60

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4 In order to ascertain the effect that sodium cations has on native mass spectra of intact
5
6 proteins, we began by analysing 5 μ M Bovine Serum Albumin (BSA) by nESI from a solution
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8 of 200 mM ammonium acetate, pH 7.0 with the addition of varying concentration of NaCl
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10 (Fig. 1). In similar experiments performed by Williams and co-workers on small proteins
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12 (ubiquitin, cytochrome C and myoglobin), differing sodium adducts ($[M+nNa+mH]^{(n+m)+}$) are
13
14 easily mass resolved and each individual adducted form can observed. Thus an average
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16 number of sodium adducts on each charge state can easily be calculated.²⁰ However, for
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18 analytes larger than 25 KDa, the isotope distributions of the individual adducted forms
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20 overlap. Consequently, salt adduction on larger proteins appears in the mass spectrum as a
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22 broadening of each charge state. Therefore we assessed the extent of salt adduction by
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24 monitoring the peak width of individual charge states.
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32 The native mass spectrum of BSA presents in 5 major charge states, which
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34 correspond to the +14 to +18 forms of monomeric BSA (Figure 1A-D; highlighted in red). In
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36 addition, a second charge state distribution is observed which corresponds to the +24 to
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38 +26 charge states of a BSA dimer (Fig. 1A-D; highlighted in blue); presumably formed by
39
40 non-specific association in the gas phase. From the native spectrum of BSA with no added
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42 NaCl (Fig. 1A) an average molecular mass of $66,485 \pm 26$ Da can be measured; which is in
43
44 close agreement with the calculated theoretical value of 66,459 Da (the red line indicates
45
46 calculated theoretical m/z for the 15+ species assuming 18 disulfide bonds, and retention of
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48 the proposed Zn^{2+} ligand during native ESI). Increasing the concentration of NaCl in the
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50 native ESI solution has a detrimental effect on S/N ratio (Figure 1A-D, *left hand side*). In our
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52 hands addition of 1 mM NaCl (Fig.1D) resulted in a \sim 4-fold reduction in S/N . This may seem
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54 modest; however, native MS of large proteins and protein complexes is a comparatively low
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3 sensitivity technique, the user is often required to accumulate scans for several minutes to
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5 obtain adequate spectral data. S/N increases proportionally with the square root of the
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7 number of scans summed. Therefore, in order to obtain comparable data, the addition of 1
8
9 mM NaCl requires a ~16 fold increase in the acquisition time w.r.t. a spectrum acquired in
10
11 the absence of salt. Furthermore, the presence of NaCl also results in peak broadening, due
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13 to sodium adduction (Fig. 1A-D, *right hand side*). By estimating the full width at half maximal
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15 height (FWHM) for the 15+ charge state of BSA (red bar) the extent of peak broadening can
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17 be estimated as ~8-fold by the addition of 1 mM NaCl. This broadening results in a reduction
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19 in the mass accuracy obtainable from the analysis; here the average molecular mass can be
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21 calculated to be 66878 ± 289 Da. In addition, peak broadening potentially obscures the
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23 presence of different proteoforms of the analyte (e.g. post translationally modified (PTM)
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25 forms, ligand-bound forms etc).
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38 **Addition of L-serine can reduce sodium adduction in native protein mass spectra.**

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41 In recent studies of the pyridoxal 5'-phosphate (PLP)-dependant enzyme serine palmitoyl
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43 transferase (SPT), we noted that addition of the enzyme substrate (L-serine), prior to native
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45 MS analysis mitigated the adverse effect of sodium adduction.³² Interestingly, this
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47 phenomenon was also observed when analysing BSA by native ESI (Fig. 2A-D). By titrating L-
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49 serine from 0-10 mM there was a clearly observable sharpening of all the peaks in the
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51 charge state distribution. The effect was dependent on the amino acid concentration, with
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53 near complete removal of Na^+ adducts observed when MS analysis was performed in a
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55 solution that contained 200 mM ammonium acetate (pH 7.0), 1 mM NaCl and 10 mM L-
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57 serine (Fig. 2D). Native MS analysis of this BSA sample resulted in a spectrum with ~4 fold
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3 increase in overall S/N and a peak-width reduction of ~ 10 fold (Fig. 2D, *right hand side*).

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5 Thus, with the addition of 10 mM L-serine, it is possible to assign the average mass of the
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7 BSA species with far higher mass accuracy - 66439 ± 32 Da (theoretical value 66,459 Da)
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9 despite the presence of 1 mM NaCl. We found that a ratio of $\sim 10:1$ (L-serine:NaCl) was
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11 optimal for reduction in sodiation; and the strategy appeared to be less effective for
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13 concentrations of NaCl over 2 mM.
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19 We subsequently extended our study to include multimeric proteins (Fig. 3A-D) – the
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21 homodimeric proteins SPT (92 KDa), yeast enolase (93 KDa); and the homotetramer yeast
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23 alcohol dehydrogenase (ADH; 148 KDa). This apparent ability of L-serine to reduce sodium
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25 adduction to protein analytes was observed for all of the native proteins analysed. On
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27 addition of L-serine, peak narrowing was evident in all of the systems studied, and S/N was
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29 increased, albeit by varying amounts (~ 8 fold for SPT, ~ 4 fold for enolase, ~ 2 fold for ADH).
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31 Crucially, this effect occurred without dissociation of protein multimers and without evident
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33 disruption of the higher order structure of the gas phase protein assemblies. Furthermore,
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35 the charge state distribution observed for each protein assembly remained predominantly
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37 unchanged – suggesting that the addition of L-serine has no significant effect on the
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39 structural conformation of the protein multimers.^{34,35} Thus, it seems that this additive is a
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41 potentially powerful strategy for improving the quality of the mass spectra obtained during
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43 structural interrogations of proteins by native MS. Such studies, for example - ion mobility
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45 experiments^{6,36,37} or protein topology/disassembly studies by gas phase dissociation^{38,39},
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60 rely on retaining solution-phase, higher order protein structure during MS analysis.

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3 **Addition of L-serine increases the sequence coverage obtained during native top-down**
4 **fragmentation of proteins.**
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9 Native MS techniques are becoming increasingly important approaches for investigating
10 protein structure, particularly in the analysis of protein targets which prove problematic, or
11 which are still not tractable, using traditional structural biology approaches (e.g. intrinsically
12 disordered proteins,⁴⁰ membrane proteins,¹¹ and heterogeneous protein populations due to
13 PTMs). For the most part, native MS studies are performed on quadrupole/time-of-flight (Q-
14 ToF) instruments, often with ion mobility capability, due to the instruments inherent high
15 upper mass limit – required for analysis of native proteins and large protein assemblies.
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19 However, recent studies have demonstrated that high resolution Fourier transform
20 instruments also have great potential in this field. Heck and co-workers have developed a
21 platform for native protein MS based on an Orbitrap instrument, and recently demonstrated
22 that in-house modifications allowed efficient detection of capsid assemblies up to 4.5 MDa
23 in mass.^{41–43} In contrast, several groups have utilised Fourier transform ion cyclotron
24 resonance (FT-ICR) instruments for native protein MS studies (see below). Although the
25 inherent upper mass limit of FT-ICR MS is yet not comparable to studies using Q-ToF and/or
26 Orbitrap analysers, future developments in instrumentation and magnet design will increase
27 their range in the upcoming years. However, the use of FT-based instrumentation has
28 allowed protein complexes to be interrogated using the suite of top-down fragmentation
29 techniques available on these instruments.
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33 Of all these techniques, electron-capture dissociation (ECD), developed in the McLafferty
34 labs in 1998,^{44,45} has garnered the most interest; and perhaps has the most potential.⁴⁶ The
35 exact mechanism of ECD is still under investigation.⁴⁷ However, it is thought to be a
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3 nonergodic process with backbone fragmentation occurring before energy randomization
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6 and without disruption of non-covalent interactions.⁴⁸ Thus, it has been postulated that ECD
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8 fragmentation of native proteins and protein complexes could be utilised to report on
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10 protein higher order structure and protein-ligand binding.
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14 Loo and co-workers first demonstrated that ECD could be used to locate ligand-
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16 binding sites when interrogating the non-covalent complex between alpha-synuclein and
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18 spermine.⁴⁹ Subsequent work from our research group demonstrated that this technique
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20 could be extended to the study of protein-peptide interactions.⁵⁰ More recently, a series of
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22 studies by Gross and colleagues have demonstrated ECD of native proteins and protein
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24 complexes may provide important structural information – particularly in the technique’s
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26 ability to highlight regions of structural flexibility.^{51–53} These findings are supported by
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28 reports from the Loo group, who have demonstrated ECD of alcohol dehydrogenase (148
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30 KDa) and aldolase (158 KDa) tetramers under native conditions.^{54,55} Their findings suggest
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32 that, in addition to regions of flexibility, regions located on the surface topology of the
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34 complex are prone to ECD cleavage.
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42 It is clear therefore that ECD of native protein complexes shows great potential as a
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44 structural biology technique. However, ECD is inherently a low efficiency process. Therefore,
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46 in order to acquire ECD spectra with fragment ion intensities of sufficient *S/N* for accurate
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48 mass assignment, the precursor signal must be relatively high intensity (compared to top-
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50 down fragmentation using higher efficiency techniques, such as CID). Furthermore, spectra
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52 often have to be the sum of several hundred scans, resulting in long experimental
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54 acquisition times. Unfortunately, ECD efficiency is known to increase quadratically with
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56 increasing charge.⁴⁴ Therefore the fact that the conditions required for native MS result in
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3 protein charge state distributions of low charge further compounds these issues. In order to
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5 mitigate these drawbacks, it is apparent that the success of native ECD experiments is
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protein charge state distributions of low charge further compounds these issues. In order to mitigate these drawbacks, it is apparent that the success of native ECD experiments is reliant on the production of high intensity precursor ions. Therefore we postulated that the use of L-serine as an additive in native protein nESI solutions, to reduce the detrimental effects of Na⁺ adduction, could be beneficial and increase the quality of ECD fragmentation data. Thus, we performed ECD of BSA under native conditions, with and without the addition of 10 mM L-serine to the ESI solution (Fig. 4A-C). A typical ECD spectrum is shown in Fig. 4A, which clearly shows that the majority of electron capture during ECD results in no dissociation (EC no D). The positive protein ion captures an electron, reducing the ion's charge; as no backbone fragmentation occurs we observe a shift in the observed charge state distribution to higher *m/z*. This observation is well documented and highlights the inefficiency of native ECD experiments.⁵² However, after summing several hundred scans, we do observe ECD fragmentation products in the region 500 -2500 *m/z*. Fig. 4B shows an expansion of this region of the mass spectrum and the ECD spectra are compared between nESI solutions with (*bottom*) and without (*top*) 10 mM L-serine. It is clear that addition of the L-serine results in ECD fragments of higher abundance. This is most pronounced with higher mass fragments Automated peak picking was performed using the SNAP 2.0 algorithm (Bruker Daltonics), and ECD peak lists were compiled (see supplementary tables). These peak lists were then searched against the primary amino acid sequence of BSA using the Prosight PTM software,³³ taking into account the known PTM profile of BSA (removal of the signal-peptide and propeptide sequence, aa1-24, and including 18 disulfide bonds). Although ECD fragments are present in both spectra, it is clear that the addition of L-serine improved the quality of the fragmentation data obtained under identical instrument conditions. 15 ECD peaks were observed in the spectrum analysed without addition of

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3 serine (Fig. 4B, top), and 5 of these were assigned as specific ECD c and z ions (see Table 1).
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6 In contrast, addition of 10 mM L-serine to the nESI solution resulted in 44 ECD peaks, 16 of
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8 which could be assigned as specific c and z ions (Fig. 4B, bottom, see Table 1; a full list of
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10 ECD ions can be found in supplementary data). The observed S/N ratio of all fragments was
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12 higher in the spectrum acquired with L-serine in the nESI solution; and of the 5 fragment
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14 ions common in both spectra, the S/N ratio was improved by an average of 187%.
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20 Figure 4C highlights the improvement observed in S/N for several of these ions. The
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22 most striking example is observed in the region between m/z 1700 and 2300. For the
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24 spectrum acquired with L-serine, in this region a series of 9 z-ions (z_{76} to z_{90}) are observed
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26 with $S/N > 1.5$, which allows clear determination of the charge-state for each ion and
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28 confident fragment assignment. In contrast, in the spectrum acquired without L-serine these
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30 peaks are not fully resolved and are not observed with sufficient S/N to assign with
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32 confidence. It is interesting to note that this set of z ions, used as a sequence tag, is
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34 sufficient to allow identification of the protein from the NCBI database.
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41 As expected we also noted that Na^+ adduction to fragment ions is far more prevalent
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43 in the spectrum acquired without L-serine. For example, the z_{88}^{5+} species is observed
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45 primarily in the $[\text{z}_{88}+5\text{H}]^{5+}$ form in the spectrum acquired with L-serine (Fig. 4C, lower
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47 spectrum, *gold box*). In contrast, the same ion is observed as several multiply-sodiated
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49 species ($[\text{z}_{88}+4\text{H}+\text{Na}]^{5+}$, $[\text{z}_{88}+3\text{H}+2\text{Na}]^{5+}$ etc) in the spectrum without L-serine (Fig. 4C, upper
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51 spectrum, *gold box*).
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3 **Reduction in sodium adduction is observed upon addition of a range of amino acids and**
4 **may be due to direct binding of sodium ions.**
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9 Finally, we tested a range of amino acids (glycine, L-alanine, L-serine, L-histidine, and L-
10 lysine) for their ability to reduce sodium adduction to proteins (Fig. 5A-F). It was apparent
11 that all five amino acids tested did reduce sodium adduction to some extent, demonstrating
12 that this phenomenon isn't specific to L-serine, and is largely independent of the side chain
13 chemistry of the amino acid. The physical basis for this phenomenon remains unclear, and
14 will be the basis of future investigations. However, it is interesting to note that the
15 effectiveness of these amino acid additives at reducing sodium adduction seems to trend
16 with the gas phase sodium affinities of the amino acids. Based on empirical findings, the
17 amino acids have been ordered in increasing Na⁺-affinities as follows: Gly, Ala, Cys, Val,
18 (Leu,Ile), Ser, Met, Thr, (Phe, Pro), Asp, Tyr, (Glu, Lys), Trp, Asn, Gln, His, Arg.⁵⁶ It is
19 particularly noticeable that glycine and, to some extent, alanine (the two amino acids with
20 the lowest sodium affinities) are less effective than L-serine as additives (Fig. 5B and C). This
21 correlation suggests a mechanism whereby the free amino acid, which is present in the nESI
22 solution at relatively high concentrations, directly sequesters Na⁺ in solution. In addition, we
23 noted that addition of L-lysine or L-histidine resulted in a significant change in the charge
24 state distribution of the protein, with a noticeable shift to lower charge (Fig. 5E and F). A
25 possible explanation for this observation may be proposed based on the recent exploration
26 of the ESI mechanism by Ogorzalek Loo et al.²⁵ and the high proton affinities of these amino
27 acids. As these amino acids are present in such high concentrations and are sufficiently
28 ionisable, it may be that they consume a significant portion of the gas phase charge emitted
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3 by the ESI source; thus reducing the charge available to the protein during ESI and driving
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5 the shift in charge state distribution of the protein.
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10 11 12 **Conclusions**

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15 Here we report the observation that addition of millimolar concentrations of amino acids
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17 can mitigate the adverse effects of sodium adduction in native protein mass spectrometry.
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19 Addition of 10 mM L-serine to 200 mM ammonium acetate (pH 7.0) in native ESI spray
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21 solution containing 1 mM NaCl results in almost complete removal of Na⁺ adducts in the
22
23 resulting mass spectra; enhancing the *S/N* for protein ions and allowing more accurate
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25 intact mass measurement of large proteins and protein assemblies. This effect occurs
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27 without disruption of non-covalent protein assemblies, and with little effect on the protein
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29 charge state distribution. We demonstrate that by using this additive to improve the signal
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31 generated in native protein MS analyses, we can increase the information gained in ECD
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33 fragmentation experiments. This strategy of removing detrimental sodium adducts in
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35 protein mass spectra by inclusion of specific additives to the ESI solution has widespread
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37 utility in MS-based structural proteomic studies; and offers a readily available, inexpensive
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39 method for improving analytical MS data without the need for instrument modification.
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3 reviewers for helpful comments and suggestions concerning the mechanism of this reported
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6 phenomenon.
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FIGURES

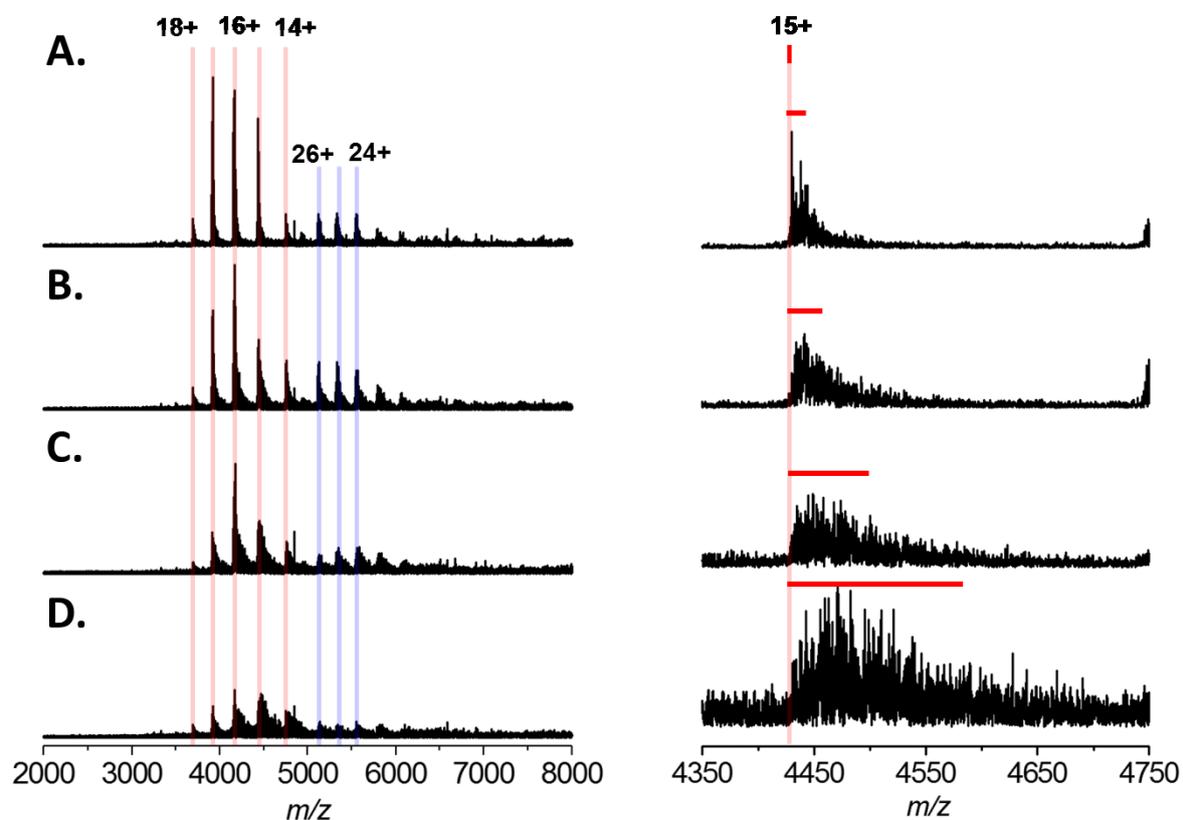


Fig. 1 Native mass spectra of Bovine Serum Albumin (66 KDa) analysed by nESI from aqueous solution containing 200 mM ammonium acetate, pH 7.0 with (A) 0 μM NaCl (B) 200 μM NaCl (C) 500 μM NaCl (D) 1 mM NaCl. *Left*, The calculated charge states are indicated and monomer charge states are highlighted by red lines; dimeric charge states by blue lines. The spectra displayed are the sum of 100 scans and the intensity scales are equivalent for each spectrum. *Right*, Expansions of the 15+ charge state of monomeric BSA; for clarity the intensities are normalised. The red bars indicate the FWHM peak width for each charge state.

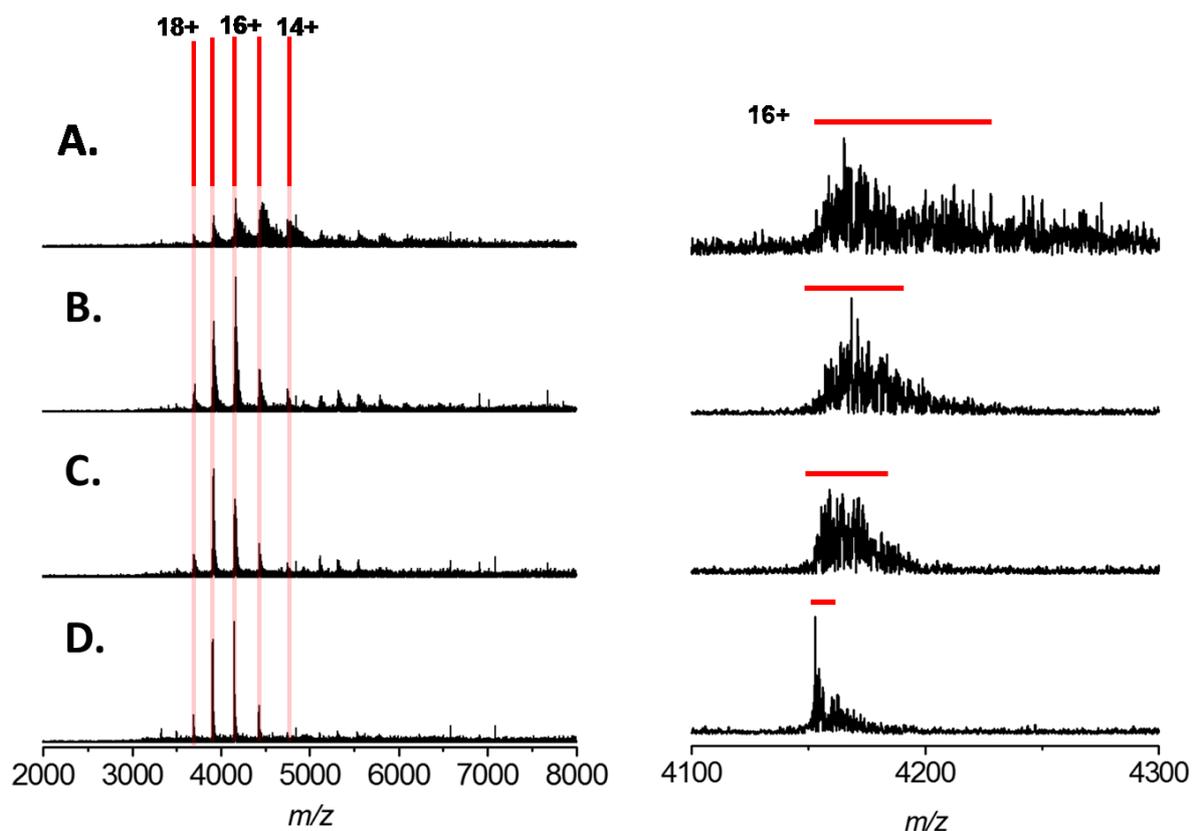


Fig. 2 Native mass spectra of Bovine Serum Albumin (64 KDa) analysed by nESI from aqueous solution containing 200 mM ammonium acetate, pH 7.0, 1 mM NaCl with (A) 0 mM L-serine (B) 2 mM L-serine (C) 5 mM L-Serine (D) 10 mM L-serine. *Left*, The calculated charge states for monomeric BSA are indicated and the spectra displayed are the sum of 100 scans. The intensity scales are equivalent for each spectrum. *Right*, Expansions of the 16+ charge state of BSA; for clarity the intensities are normalised. The red bars indicate the FWHM peak width for each charge state.

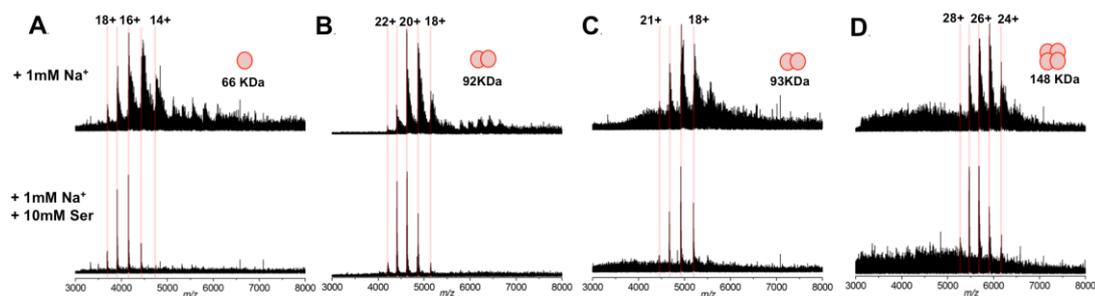


Fig. 3 Native mass spectra of protein assemblies analysed by nESI from aqueous solution containing 200 mM ammonium acetate, pH 7.0, 1 mM NaCl; without (*top*) and with (*bottom*) the addition of 10 mM L-serine. (A) monomeric BSA (66 KDa); (B) dimeric serine palmitoyltransferase, SPT (92 KDa); (C) dimeric yeast enolase (93 KDa); (D) tetrameric yeast alcohol dehydrogenase, ADH (148 KDa). The calculated charge states for each protein assembly are indicated and the spectra displayed are the sum of 100 scans, for clarity the intensities of each spectra are normalised.

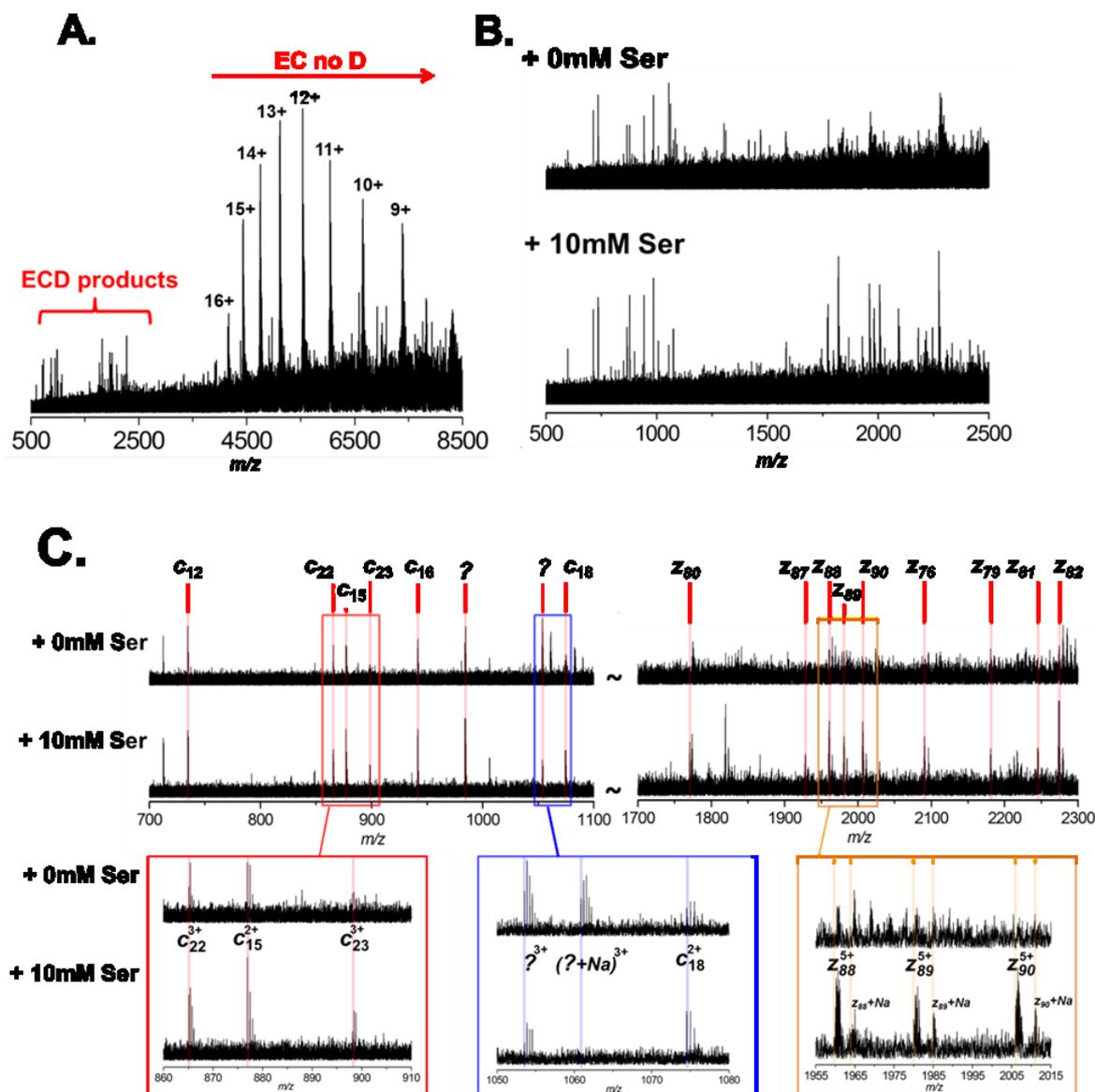


Fig. 4 Electron-capture dissociation of BSA under native conditions. BSA was analysed from 200mM ammonium acetate (pH 7.0), 1 mM NaCl with or without the addition of 10 mM L-serine. No precursor ion selection was performed, and entire BSA charge state was subject to ECD. (A) A typical ECD spectrum of BSA, the region where fragmentation products are observed is highlighted. (B) Expansion of the ECD spectrum in the region between m/z 500 to 2500. Here the ECD products of BSA with (B, *bottom*) and without (B, *top*) the addition of 10mM L-Serine are compared. (C) Further expansion of two regions (m/z 700 to 1100 and m/z 1700 to 2300) of the ECD spectrum. Here specific fragment ions are annotated; again

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3 ECD products of BSA with (C, *bottom*) and without (C, *top*) the addition of 10 mM L-serine
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6 are compared.
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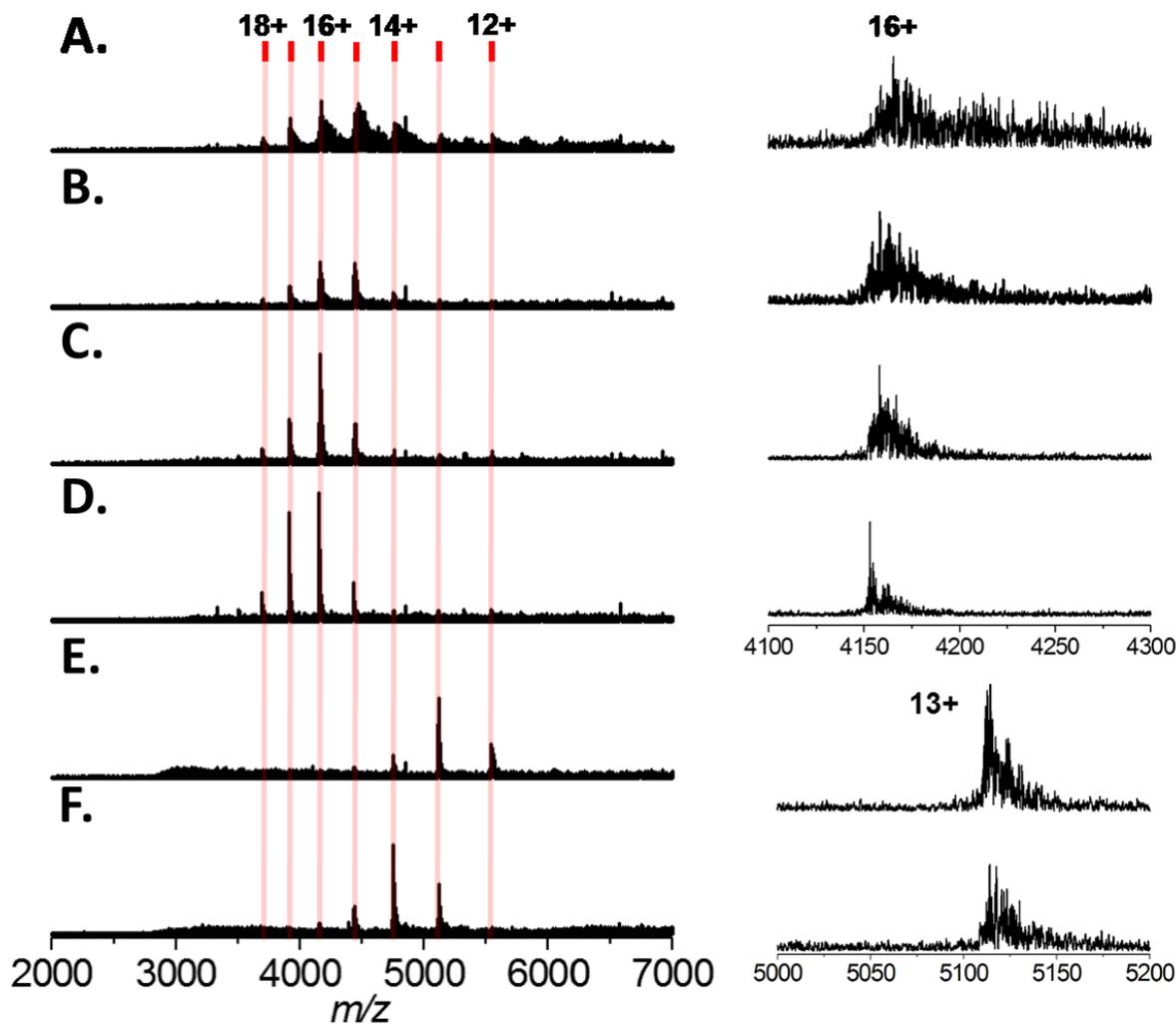


Fig. 5 Native mass spectra of Bovine Serum Albumin (64 KDa) analysed by nESI from aqueous solution containing 200 mM ammonium acetate, pH 7.0, 1 mM NaCl with (A) no additive (B) 10 mM glycine (C) 10 mM L-ala (D) 10 mM L-serine (E) 5 mM L-histidine (F) 5 mM L-lysine. *Left*, The calculated charge states for monomeric BSA are indicated. The spectra displayed are the sum of 100 scans and the intensity scales are equivalent for each spectrum. *Right*, The expansions of the 16+ or 13+ charge state of BSA is shown; for clarity the intensities are normalised.

Frag Ion	m/z	z	Mol. Mass (Da)	10mM Ser	0mM Ser
				S/N	S/N
c12	734.3894	2+	1467.7715	6.7	4.8
c15	876.9537	2+	1752.9001	5.5	3.6
c16	941.4735	2+	1881.9397	5.8	3.7
c18	1074.5235	2+	2148.0396	4.1	< 1.5
c22	865.1071	3+	2593.3066	4.8	2.8
c23	898.1285	3+	2692.3709	2.7	< 1.5
z76	2089.2917	4+	8354.145	2.9	N/O
z79	2179.8397	4+	8716.337	2.7	N/O
z80	1769.6961	5+	8844.4513	2	N/O
z81	2244.1284	4+	8973.4919	2.2	N/O
z82	2272.8797	4+	9088.497	5.7	< 1.5
z87	1926.7695	5+	9629.8183	1.8	N/O
z88	1959.3803	5+	9792.8723	4.7	1.5
z89	1979.5884	5+	9893.9127	3.2	< 1.5
z90	2005.5986	5+	10023.9639	4.5	N/O

Table 1. Fragment ions observed upon native ECD of BSA with and without addition of 10 mM serine. N/O indicated species is not observed. Confident assignment requires S/N > 1.5.