Probing folded proteins and intact protein complexes by desorption electrospray ionization mass spectrometry

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ABSTRACT

Native mass spectrometry (Native MS) enables the study of intact proteins as well as non-covalent protein-protein and protein-ligand complexes in their biological state. In this work we present the application of a prototype Waters DESI source for rapid surface measurements of folded and native protein structures. Ions with narrow charge state distribution (CSD), i.e. folded structures are observed in the spectra of protein samples with the molecular weight ranging from 8.6 kDa up to 66.4 kDa. Intact protein complexes of holo-myoglobin and tetrameric hemoglobin are also successfully detected from a surface. These results reveal that DESI could be gentle enough to detect compact structures and noncovalent bond interactions. We also examine whether unfolded proteins and protein complexes can refold during transient spray solvent-sample interactions during DESI. Our results from ion mobility experiments of standards of ubiquitin, cytochrome c and protein complex myoglobin indicate that such phenomenon may occur, presenting artificial native-like spectra. Nevertheless, the observation of hemoglobin tetramer is promising as it demonstrates the capability of DESI to maintain truly native structures.

INTRODUCTION

As a powerful and versatile method in structural biology, native mass spectrometry can transfer large biomolecules and complexes from the condensed liquid phase to the gas phase while maintaining their structural integrity under physiological conditions. Since the first pioneering studies on noncovalent complexes in the early 1990s,[1, 2] most native mass spectrometry has been performed in solution phase by the electrospray ionization (ESI) method.

Extensive efforts from the Cooper group have explored the opportunity for performing native mass spectrometry experiments directly from surfaces, using liquid extraction surface analysis (LESA)[3] and

continuous-flow liquid microjunction surface sampling probe (LMJ-SSP).[4] Over the last few years they have shown that proteins and protein complexes as large as 800 kDa can be extracted and measured in native forms from various surface substrates using LESA.[5-9] However, because of its discrete sampling property and relatively large sampling regions, LESA inevitably suffers from the drawbacks of slow analysis speed and poor spatial resolution (usually a few hundred micrometers diameter of each sampled region[10-12]). Nonetheless, it is arguably the best approach for top-down and native protein analysis from a surface.

Since its introduction,[13] DESI has been widely used for imaging of small molecules including drugs, metabolites and lipids.[14-17] Several articles present DESI analysis of proteins, the majority of which have been performed under denaturing solvent conditions.[18, 19] Efforts to improve sensitivity for peptide and protein analysis have included a variety of strategies such as including additives of serine, or ammonium salts, or acids in the spray solvent,[20-22] modifying ambient conditions by the introduction of organic vapors,[23] and optimizing the delay between desorption and ionization.[24] Coupling DESI surface analysis with ion mobility separations has been shown to improve signal to noise ratio of protein ions and imaging contrast in DESI-MS imaging of proteins from tissue samples.[25, 26]

In 2006 it was shown that folded protein structures could be probed,[27] but the first intact protein complex surveyed by DESI was not reported until 2017 by Robinson's group[28] with a modified DESI source. In their set-up the fitted ion transfer inlet was removed to allow the DESI stage directly underneath the sampling cone of MS. It was shown that DESI could preserve the native structure of both soluble and membrane proteins and their complexes. In addition, native DESI also demonstrated its robust functionality to capture rapid turnover enzyme–substrate complex and perform detergent screening and ligand screening studies for membrane proteins.

In this article we sample standards of proteins, dried on glass surface, using the common DESI configuration

of a long external ion transfer inlet. We demonstrate that it is possible to retain folded structures and intact complexes with noncovalent interactions. We also sampled denatured proteins and protein complexes using native DESI conditions and provide evidence for protein refolding, despite the inlet conditions. Our results show that traditional DESI inlet systems can be used for both native experiments, and the study of protein refolding.

Experimental Section

Sample preparation

The protein standards bovine ubiquitin (8.5 kDa), equine cytochrome c (12.3 kDa), equine myoglobin (Mb, 17.5 kDa), human hemoglobin (Hb, 64.4 kDa) and bovine serum albumin (BSA, 66.4 kDa) as well as MS grade salt ammonium acetate were purchased from Sigma-Aldrich (Gillingham, UK). Optima™ LC/MS grade water, methanol, and formic acid were obtained from Fisher Scientific (Loughborough, UK). A solution containing each protein at 50 μM was prepared in three conditions: 1) water; 2) 200 mM ammonium acetate and 3) 1:1 water/methanol (v:v) with 0.1% formic acid. Ten-microliter aliquots of each protein solution were spotted onto plain glass microscope slides and left to air dry.

Instrument setup

Experiments were performed using a 2D-DESI source (Prosolia, Indianapolis, USA) equipped with a Waters developed prototype DESI sprayer, mounted to an ion mobility enable Q-ToF mass spectrometer (SYNAPT G2-Si, Waters, Manchester, UK). 200 mM ammonium acetate in 1:1 water/methanol (v:v) and 1:1 water/methanol (v:v) with 0.1% formic acid were used as DESI solvent for native and denaturing MS respectively. A high voltage of 1.2 kV and a flow rate of 3 μL/min was applied to the spray solvent. Nitrogen was used as the nebulization gas with pressure set at 1.5 bar. A heated MS-capillary inlet (details described

elsewhere[25]), which was designed to facilitate the desolvation of organic solvent, was employed for ion transmission with the temperature heated to 100 ˚C. The MS setup was operated in positive ion mode and 'resolution mode' with the scan rate of 1 scan per second.

Data were collected as line rasters across 5 sample spots at the rate of 100 μm/s. Waters Masslynx software V4.1 was used to record the data and OriginPro (OriginLab, Northampton, MA, USA) was used to plot figures.

RESULTS AND DISCUSSION

Solvent filtering for native DESI

Commonly, buffered aqueous solution at the neutral pH is used in native MS studies, regardless of samples in the form of solution phase or solid surface. As the prototype DESI sprayer was designed and optimized with a solvent mixture containing a large organic proportion, here 200 mM ammonium acetate in 1:1 water/methanol rather than in pure water was used for native DESI-MS studies. The efficacy of this uncommon solvent system for native MS studies was first evaluated in LESA (TriVersa NanoMate, Advion Inc., Ithaca, NY). From Fig. 1(a-c), for the native condition prepared ubiquitin, essentially identical spectra were obtained when the LESA solvent was changed from 200 mM ammonium acetate in water (Fig. 1a) to that in 1:1 water/methanol (Fig. 1b). The narrow distribution at low charge states (4+ to 6+) and spectra dominated by 5+ indicate that ubiquitin was detected as native-like folded structures. However, when the organic percentage was increased to 80% (Fig. 1c), protein unfolding did occur which resulted in two peak envelops and a broad charge state distribution ranging from 4+ to 11+. The relative ion intensities of detected peaks were more uniform compared with that from native MS.

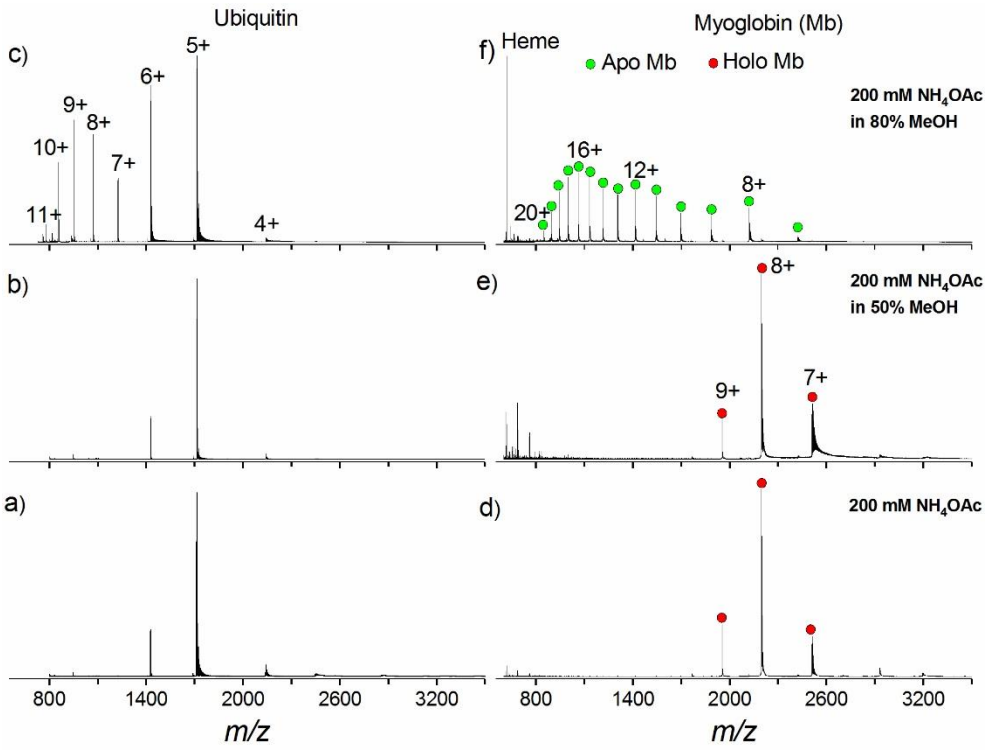

Figure 1. LESA mass spectra of ubiquitin (a-c) and myoglobin (d-f) prepared as 50 μM aqueous solution buffered with 200 mM ammonium acetate and air dried on plain glass slides. The sampling solvent used is 200 mM ammonium acetate in water for a, d), in 50% methanol for b, e) while in 80% methanol for c, f).

The three solvent conditions were also evaluated for the study of myoglobin (Fig. 1, d-f), a protein complex containing a non-covalent bond. Similarly, using 200 mM ammonium acetate, increasing to 50% methanol content did not induce any unfolding of the protein complex. A narrow CSD (7+ to 9+) was also observed for peaks relating to ions of the intact complex formed by the apo-Mb protein combined with its prosthetic heme group. Although the relative ion intensity of heme (*m/z* of 616) increased from ~ 5% to ~20%, no obvious apo-Mb peak was detected indicating that 200 mM ammonium acetate in 1:1 water/methanol is also capable of preserving the folded states of protein complexes. As expected, when the organic proportion was increased to 80%, the resulting spectrum was dominated by ions from heme (Fig. 1f), with broad charge state distribution from 7+ to 20+ of apo-Mb ions, indicating that the hydrogen bond connecting heme

to apo-Mb was lost.

Furthermore, the suitability of the solvent of 200 mM ammonium acetate in 50% methanol for native MS study was investigated by direct infusion ESI (TriVersa NanoMate, Advion Inc., Ithaca, NY) of 25 μM hemoglobin in solution. The nano-ESI spectra was shown as Fig. 2. Compared with the obvious evidence of dimers in the form of $\alpha\beta^{H}$ and $\alpha\beta^{2H}$, plus the intact hemoglobin tetramer of $\alpha_2\beta_2{}^{4H}$ in the native MS (bottom panel), the relative intensities of those complex ions are much lower when hemoglobin is prepared in 50% methanol. This was not surprising as precipitation of hemoglobin over time was observed in 50% methanol. Nevertheless, the detection of dimers and tetramers demonstrated that non-covalent interactions, which are critical to stabilize protein complexes, can be preserved in the solvent containing 50% methanol. Hence, 200 mM ammonium acetate in 1:1 water/methanol was selected for evaluation as the spray solvent for native DESI studies discussed below.

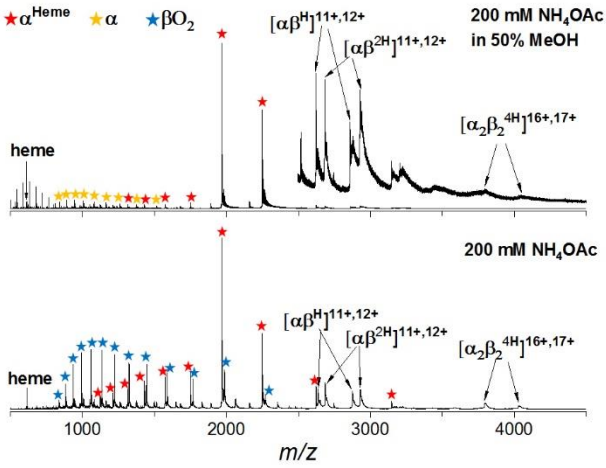

Figure 2. Direct infusion ESI mass spectra of hemoglobin prepared as 25 μM solution in 200 mM ammonium acetate in water (bottom panel) and 200 mM ammonium acetate in 1:1 water/methanol (top panel).

DESI of folded proteins

DESI mass spectra of dried droplets of protein standards dissolved in water are presented in Figure 3. Using

200 mM ammonium acetate in 1:1 water/methanol as the DESI spray solvent allows surface sampled proteins to be detected as compact folded structures. Narrow charge state distributions are observed for a 76-amino acids protein (ubiquitin), but also cytochrome c (a covalent assembly) and the large single peptide protein BSA (583 amino acids).

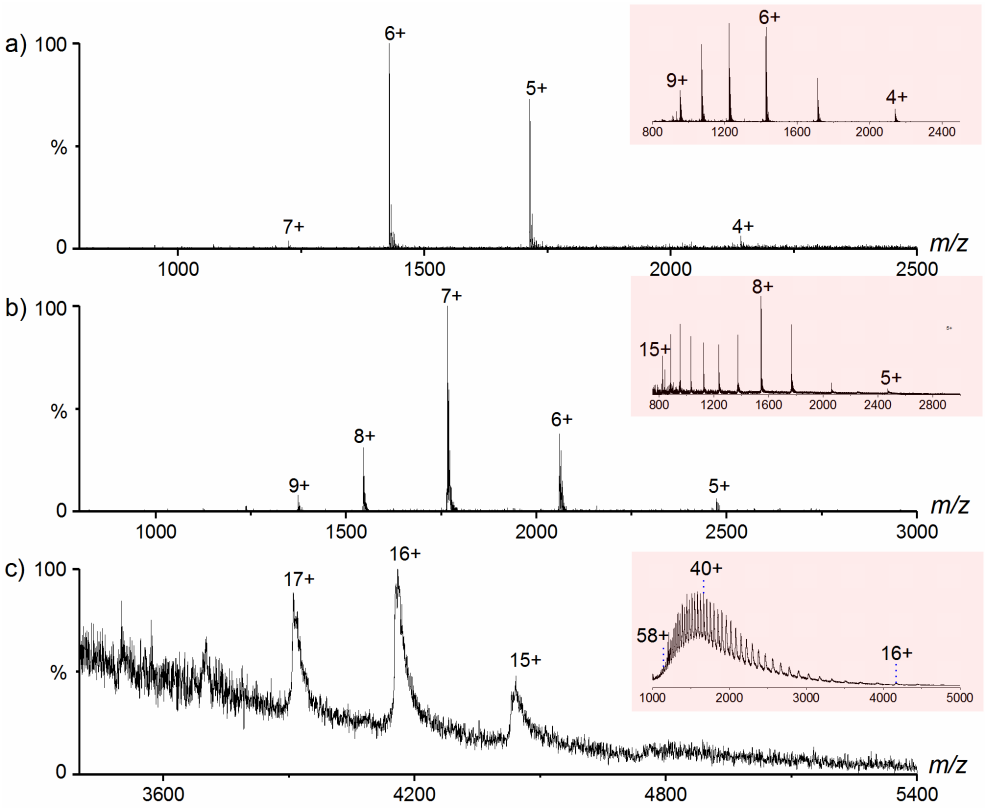

Figure 3. Native DESI mass spectra of dried droplets of proteins of ubiquitin (a), cytochrome c (b) and bovine serum albumin (c) prepared in water by using the spray solvent of 200 mM ammonium acetate in 1:1 water/methanol. The inlet graphs show the spectra acquired with the denaturing spray solvent of 1:1 water/methanol with 0.1% formic acid

The DESI mass spectrum of ubiquitin (Fig. 3a) is dominated by peaks of 5+ and 6+, with smaller peaks relating to 4+ and 7+, indicating a compact folded protein structure. With the spray solvent changed to denaturing conditions of 0.1% formic acid in 1:1 water/methanol, the CSD of ubiquitin ions spans 4+ to 9+

(see the inlet graph in Fig. 3a). Compared with typical denaturing ubiquitin mass spectra,[29, 30] here high charge states 10+ to 12+ were not detected, suggesting that very few ions of completely unfolded protein were detected. This may be attributed to the very brief, transient, interaction time between the harsh solvent and the protein within DESI analysis as well as very stable folded structure of ubiquitin[31] leading to an incomplete unfolding in this experiment.

The mass spectrum from analysis of cytochrome c is presented in Fig. 3b. It is noted that peaks relating to ions in charge states 6+ to 8+ dominate the spectrum with highest ion counts observed for 7+. The results show good agreement with previous findings from ESI studies of native cytochrome c as folded structures.[32] However, small peaks from ions of 5+ and 9+ charge states were detected, and it seems probable that inclusion of 50% methanol leads to some of the ion population being detected in a less folded state. From the DESI mass spectra of cytochrome c acquired using denaturing DESI spray conditions (0.1% formic acid in 1:1 water/methanol), a dramatic change was observed in the number of peaks detected. A bimodal distribution of ion charge states is observed with maxima at 8+ and 13+. These results indicate the presence of both unfolded structures and native-like folded conformers.

Results from the 66 kDa monomeric protein BSA are presented in Fig. 3c. Albeit with poor signal to noise, a narrow CSD can still be identified from the detected peaks representing compact folded structures of BSA. In a separate acquisition of the *m/z* region below 3500 (data not shown), no peak was detected. The recorded spectrum is dominated by 15+ to 17+ BSA ions, which is consistent with the finding of the native DESI work from Robinson's group.[28] However, the width of ion peaks in our native BSA study is found to be broader. Apart from the differences in resolving power afforded by a Q-ToF compared to an Orbitrap mass analyzer, we suggest that the broad peak width may also be a result of the non-desalting protein sample preparation method employed in our study. When the denaturing solvent is used in DESI spray, a very broad

CSD (16+ up to 58+) is observed for BSA ions, indicating extensive protein unfolding has occurred.

DESI of protein complex holo-myoglobin

To investigate suitability of the method to examination of intact protein assemblies including non-covalent bonds, the monomeric complex myoglobin was analyzed. The native DESI spectrum recorded for myoglobin is presented in Figure 4a. In the *m/z* region above 1500, a narrow CSD (6+ to 9+) was observed for the ion peaks of intact Mb (holo-Mb) with heme group bonded, indicated successful preservation and detection of the compact folded structure. Surprisingly, evidence of collapse of the intact complex with noncovalent bond breakage was also observed. Peaks relating to a series of high charge state (15+ to 19+) ions, corresponding to heme loss apo-Mb were detected in the *m/z* region below 1200. Furthermore, free heme at m/z *616* was also detected.

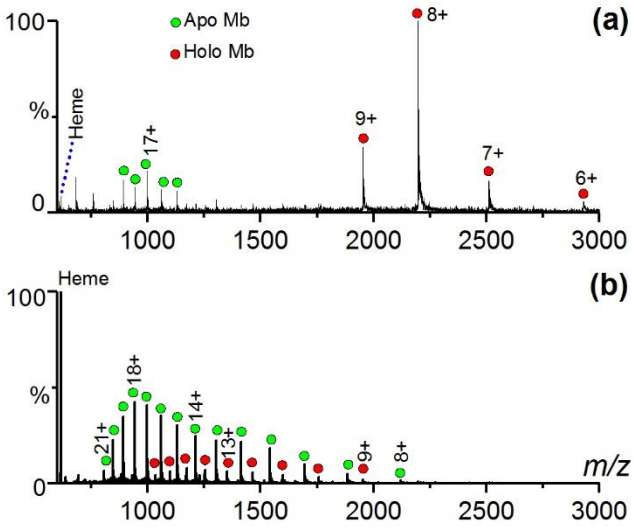

Figure 4. DESI mass spectra of dried droplets of myoglobin prepared in water by using DESI solvent systems of 200 mM ammonium acetate in 1:1 water/methanol (a) and 1:1 water/methanol with 0.1% formic acid (b).

Interestingly, this structure collapse of myoglobin was not observed in the LESA study using the same sampling solvent. It is probable that these features observed in the DESI experiments may be attributed to the heated ion transfer tube, in which both desolvation and activation of ions could occur. Nevertheless, comparing the relative abundance of apo-Mb to that of holo-Mb, more than 70% was detected as intact form with a compact folded structure. Further work will examine inlet temperature conditions to optimize relative populations of folded structures, and to examine whether altering inlet temperatures permits study of protein unfolding as a dynamic process.

Using denaturing solvent, the resulting DESI mass spectrum (Fig. 4b) was dominated by an abundant peak relating to the heme group. A broad CSD was observed from 8+ to 21+ relating to apo-Mb with loss of heme, indicating conversion of the intact globular entity into an extended structure. A broad CSD of holo-Mb from 9+ to 17+ was also noted, indicating that the protein complex was kept intact using this acidic solvent condition, but adopting an elongated conformation.

Refolding of unfolded proteins and complexes in native DESI

Results presented in figures 3 and 4 showed that folded protein structures and intact protein complexes can be detected using DESI with a standard inlet capillary. In previous LESA studies,[29, 33] structure collapse was observed during room temperature drying of droplets of proteins and protein complexes prepared in native conditions. Furthermore, refolding of unfolded proteins and protein complexes has been shown to occur when using native sampling solvent conditions for analysis of denatured samples.[29, 33] As a result, during native protein surface analysis, the sampling solvent seems to play a vital role in determining the observed spectra features. In these experiments we investigate whether refolding can also be observed in native DESI-MS. Ubiquitin, cytochrome c and the protein complex myoglobin were analyzed. We sampled

denatured preparations using denaturing and native DESI solvents. We also analyzed native preparations using native DESI sampling solvent condition. The mass spectra obtained are shown in Figure 5.

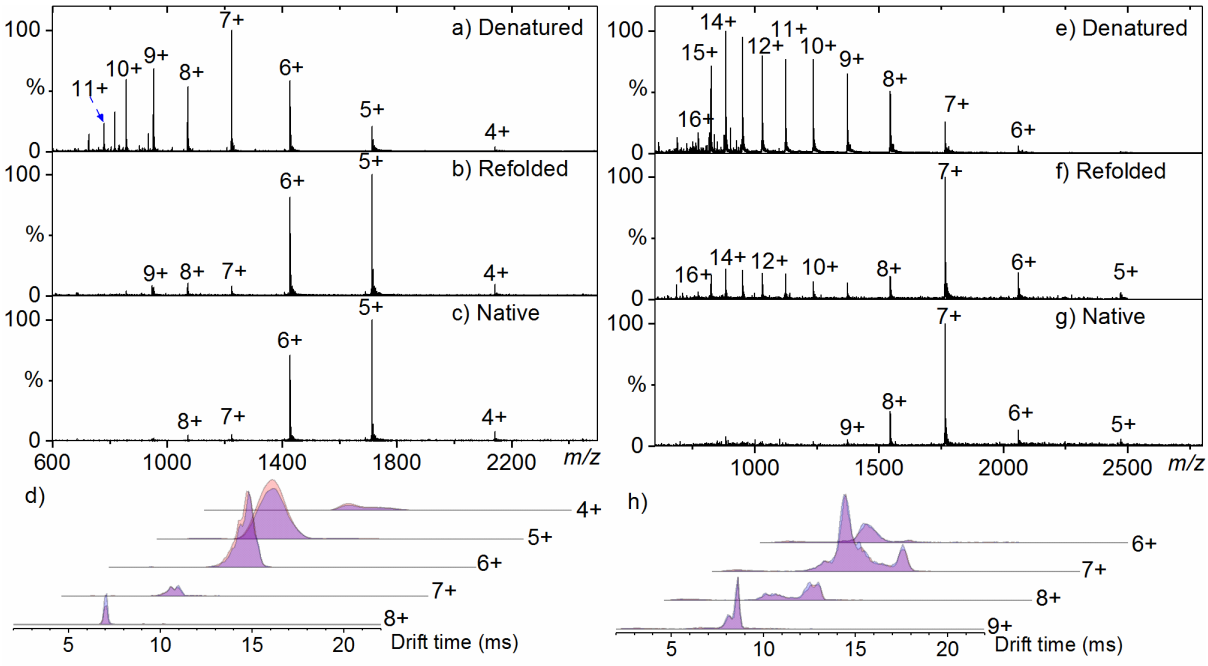

Figure 5. DESI mass spectra of dried droplets of proteins of ubiquitin (a-c) and cytochrome c (e-g) prepared in 1:1 water/methanol with 0.1% formic acid (a, b, e, f) and 200 mM ammonium acetate in water (c, g) using the spray solvent of 1:1 water/methanol with 0.1% formic acid (a, e) and 200 mM ammonium acetate in 1:1 water/methanol (b, c, f, g). The comparisons of drift time profiles of commonly detected protein ion charge states under refolded (pink color) and native (purple color) conditions are presented as d) and h).

The DESI spectra of natively prepared ubiquitin sampled using native solvent is shown in Figure 5c. The spectra was dominated by peaks relating to 5+ and 6+ ions, with 5+ being most abundant. The results mirror those obtained in the native LESA measurement (see in Fig. 1a). In contrast, a broad CSD of protein ions was observed when the denatured ubiquitin was analyzed using denaturing spray solvent (Fig. 5a). The bimodal distribution of charge states from 4+ to 11+ indicates that a mixed population of ions, was detected, as both elongated structures and compact folded structures. Similar to the observation of

refolding in native LESA studies, the same phenomenon was also apparent in native DESI analysis of denatured ubiquitin (Fig. 5b). A dramatic reduction of the relative intensities of ions with high number of charges was observed. Ions in 10+ and 11+ charge states were barely detectable and relatively few 7+ to 9+ ions were observed. The relative abundances of 5+ and 6+ were found to increase and were the most abundant ions detected. The CSD and relative ion intensities from native DESI-MS of denatured ubiquitin were very similar to those from native DESI of native ubiquitin, suggesting that the extended structures of denatured protein refolded into compact native-like structures, induced by soft, native DESI spray solvent. Results from ion mobility studies are presented in Figure 5d. No obvious differences in drift time profiles of commonly detected protein ions from native and refolded DESI spectra were found, suggesting a very high similarity between the structures of refolded ubiquitin and that of natively prepared, captured by using native DESI technique.

The spray solvent induced protein refolding was also investigate for cytochrome c. As seen in the spectrum of denatured sample measured by using native-like DESI condition (Fig. 5f), though a broad CSD of cytochrome c ions was observed, the relative intensities of high charge states from 8+ to 16+ to the 7+ charge state were dramatically reduced compared with that in the denatured spectrum (Fig. 5e) indicating a much smaller amount of unfolded structures for the former case. The refolded spectrum was notably dominated by the peak of 7+ ion, which agrees well with that in the native spectrum presented in Fig. 5g. A comparison of drift time distributions (Fig. 5h) of commonly detected charge states in refolding and native spectra suggests that no obvious difference was found between the refolded structures and preserved native structures. Therefore, due to intense refolding effect caused by the sampling solvent, a mixture of major refolded structures and minor unfolded structures were detected when the denatured cytochrome c was analyzed by native DESI.

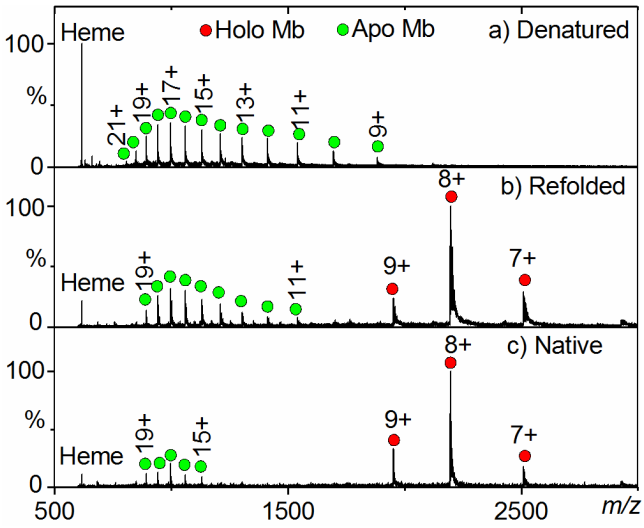

Figure 6. DESI mass spectra of dried droplets of protein complex of myoglobin in 1:1 water/methanol with 0.1% formic acid (a, b) and 200 mM ammonium acetate in water (c) using the spray solvent of 1:1 water/methanol with 0.1% formic acid (a) and 200 mM ammonium acetate in 1:1 water/methanol (b, c)

Considering the wide application of surface native MS in studying protein-ligand bindings, it is of vital importance to determine whether structure refolding occurs as well in native DESI analysis of such analyte systems. Previous solution phase studies of the noncovalent ligand binding holo-myoglobin demonstrated that the collapsed structure of acid denatured protein could be reconstructed when the pH was increased to around neutral value.[34, 35] However, refolding of holo-myoglobin was not observed in native LESA studies of surface dried sample, although adjusting native sampling solvent of 25mM ammonium acetate by 2.5% ammonium hydroxide led to ~ 3% of myoglobin detected as holo-protein.[33] Surprisingly, an extraordinary amount of refolding was observed during our native DESI studies of denatured myoglobin. As seen in Fig. 6a, in addition to an intense ion peak of heme group, only apo-protein ions were detected in denaturing DESI analysis of denatured sample, with a broad CSD from 9+ to 21+. However, when the spray solvent was changed to native-like condition (Fig. 6b), a narrow CSD of holo-protein ions was observed in the refolded spectrum with the 8+ ion peak being the most abundant. This indicates that some unfolded apo-myoglobin

refolds into compact structures, followed by incorporation of heme group to form intact holo-myoglobin. The relative intensity of free heme group ion was dramatically reduced, resulting in an overall very similar spectrum to that from native DESI analysis of natively prepared holo-myoglobin (see in Fig. 6c).

Truly native DESI of protein complex holo-hemoglobin

To fully establish whether DESI with an inlet configuration is capable of preserving truly native structures, the study was also performed on a larger protein complex - holo-hemoglobin, which consists of four subunits (two α-chains and two β-chains), each bound to a heme moiety. The Hb tetramer is very unstable outside red blood cells and spontaneously dissociates to dimers in diluted solution. Refolding of denatured hemoglobin requires several extreme conditions, i.e. low temperature and chemical treatment to inhibit β-globin aggregation.[36]

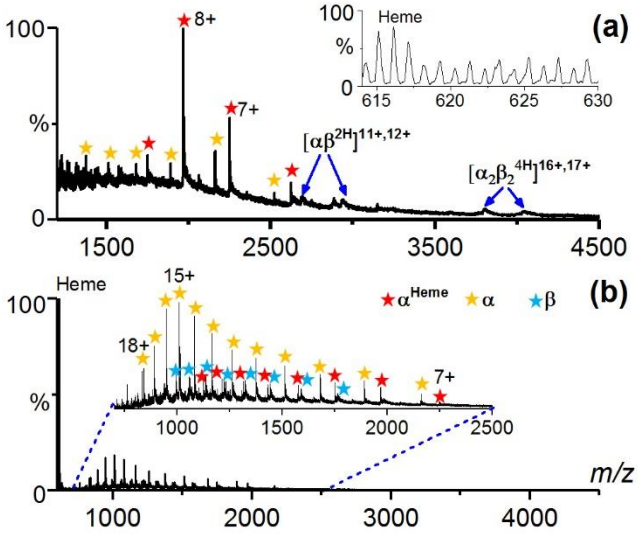

Figure 7. DESI mass spectra of dried droplets of hemoglobin prepared in water by using DESI solvent systems of 200 mM ammonium acetate in 1:1 water/methanol (a) and 1:1 water/methanol with 0.1% formic acid (b).

Under the soft spray condition, the dominant peaks in the spectrum relate to ions of isolated subunits (see in Fig. 7a). Both heme group attached α-globin and apo α-globin were detected, with the CSD from 6+ to 9+ and 6+ to 11+, respectively. In addition, peaks of oligomer ions, which include 11+ and 12+ ions of dimers $\alpha\beta^{2H}$ and the ionized intact molecule, i.e., 16+ and 17+ tetramers $\alpha_2\beta_2^{4H}$, were also detected. Detection of hemoglobin tetramer ions reveal that the truly native protein complex can be preserved in these DESI studies. Despite low abundance, this is the first indication that the intact form of this biologically important heme protein can be detected using DESI. In contrast, under harsh spray conditions, the result presented in Fig. 7b shows neither tetramer nor dimer is detected. Substantial unfolding occurred for Hb, leading to the detection of peaks corresponding to a large amount of free heme group as well as that relating to the respective subunits, i.e., 7+ to 18+ α-globin and 9+ to 16+ β-globin, both in heme-loss states.

Discussion

Protein refolding in DESI analysis

Experimental measurements[37, 38] and theoretical calculations[39] suggest that the average folding time of ubiquitin is a few milliseconds. Though the refolding of acid denatured cytochrome c forms several intermediate states possessing extensive α-helical structures (up to 70% of native level) within several hundred microseconds, it takes about 15 ms to form the complete native structure.[40] The time required for the reconstitution of ligand binding protein complex is much longer. Previous studies reveals that it takes milliseconds for refolding of unfolded apo-myoglobin[41] and a further several hundred milliseconds to seconds for reincorporation of the heme group into the folded apo-protein to form holo-myoglobin.[42] As a result, the protein (complex) refolding observed in this study implies that the interaction time between the spray solvent and the protein samples should be longer than hundreds of milliseconds. However, it is

reported that the residence time for DESI primary droplets on the surface is in the range of microseconds[43] while the lifetime of secondary charged droplets carrying analyte molecules is also microseconds,[44] which means that most protein refolding could not occur during such short timescale.

The ionization mechanism of DESI technique includes several possibilities,[45] of which 'droplet pickup' is believed to be most applicable for large analytes such as proteins.[43, 45, 46] In this mechanism, an initial spray impacts the sample surface to form a wet, thin, and localized film, into which analyte molecules are dissolved and subsequently extracted. Charged droplets colliding with the pre-wetted surface layer results in the ejection of secondary droplets containing analyte molecules, followed by an electrospray like ionization process. According to the above description, protein refolding is unlikely to occur inside the secondary droplets, and we suggest that it takes place inside the wet liquid layer on the surface. This is supported by the fact that with a typical spray coverage between 100 and 200 μm on the surface and the DESI stage moving speed at 100 μm/s in our studies, the analyte molecules could stay in the wet film for a duration of up to 1-2 seconds. During this timescale, not only protein standards such as ubiquitin and cytochrome c could refold from denatured extended structures to compact structures, but also folded apo-myoglobin has plenty of time to noncovalently bind to free heme groups to reform holo-myoglobin. Theoretically, refolding of most proteins can be minimized by reducing the interaction time between analyte and wet film down to below 10 ms, i.e. increasing the DESI stage moving speed up to 10 mm/s. However, it may inevitably bring extra challenges to effectively lift enough protein analytes up from the same pixel area.

Relative sensitivity of DESI

Unlike LESA, in which the events of sampling and ionization are decoupled, in DESI the time frame for extraction and solvation of surface associated molecules is extremely short before the droplet desolvation and generation of gas phase ions. These are positive attributes for rapid mass spectrometry imaging

experiments for easily extracted molecules but present challenges for extraction of proteins from complex surfaces.

Having demonstrated the capability of DESI using a common inlet design for detecting protein folded structures, further work will seek to optimize these measurements for sampling complex biological surfaces. Using the collected experimental data of ubiquitin presented in this manuscript, some simple calculations can be made (see supplementary Figure 1). Taking into account sampling 'footprints', raster speeds (DESI) and microjunction dwell time (LESA), along with MS scan times, a basic comparison of 'relative sensitivity' can be made between LESA and DESI.

As summarized in the supporting material Table 1, for each fmol analyte ~891 counts were detected using LESA while only ~157 counts were recorded by DESI. However, the microjunction sampling dwell time was 15 sec for LESA while only 1 sec in DESI analysis. These are encouraging results. While not intended as a full comparison and conceding that LESA sampling times can be shorter than those used here, this basic estimation indicates that further optimization of DESI may provide both the improvements in throughput and spatial resolution which are currently lacking in native protein measurements directly from complex surfaces.

CONCLUSIONS

In summary, we have demonstrated the application of a prototype DESI source for probing folded proteins and protein complexes directly from solid surfaces. For protein standards including 8.5 kDa ubiquitin, 12.3 kDa cytochrome c and 64.4 kDa bovine serum albumin, using 200 mM ammonium acetate in 1:1 water/methanol as DESI spray solvent, narrow charge state distribution of protein ions were observed in the recorded mass spectra, indicating that folded compact structures were detected. Using the same solvent

for analysis of noncovalent ligand bound protein complexes including 17.5 kDa size monomeric holo-myoglobin and 64.4 kDa size tetrameric hemoglobin shows that intact complexes are detected. This was especially clear for myoglobin, with the resulting spectrum dominated by heme group attached protein ion peaks. Importantly, the observation of hemoglobin tetramer proves that the truly native protein complex structures can be preserved in DESI studies.

Further studies reveal that protein refolding and protein complex reassembling occur during native DESI analysis of denatured samples. Rather than commonly detected broad charge state distribution of protein ions or/and ligand-free apo-protein peaks in typical denaturing MS studies, we observed a narrow CSD in native DESI analysis of denatured proteins and peaks of holo-proteins in that of denatured protein complexes. The refolding was most likely to occur during initial spray wetting process. These findings, remind us that extra care should be taken in native DESI-MS data interpretation, especially for unknown non-standard samples such as tissues etc. Employing a much faster moving speed of the DESI stage may help inhibit protein refolding but raise additional requirement of improving DESI sensitivity.

Our next study plans will investigate methods to improve the detection sensitivity of native DESI analysis and explore strategies to overcome the protein refolding phenomenon therein. The solutions for the latter may include 1) narrowing the spray coverage area on surface or employing faster DESI stage moving speed to reduce the protein analytes extraction time into solvent before they are ejected 2) employing a cryo sampling strategy like we used in our native LESA studies[29] to frozen the structures of surface proteins to be analyzed.

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ACKNOWLEDGMENTS

We thank Dr. Emrys Jones from Waters company for providing us the prototype DESI sprayer and its general operation guidelines. Dr. Alex Dexter is appreciated for the internal review of this work and Amy Burton is acknowledged for maintaining and troubleshooting the prototype setup. We also acknowledge the funding support from the National Measurement System project 'AIMS HIGHER' and CRUK Cancer Grand Challenges program.

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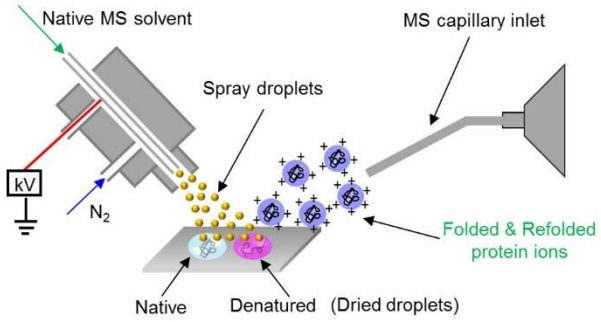
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