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RESEARCH ARTICLE

Cryo-LESA Mass Spectrometry—a Step Towards Truly Native Surface Sampling of Proteins

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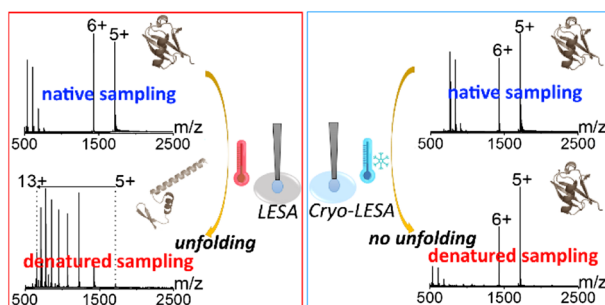
Abstract. Liquid extraction surface analysis (LESA) is a powerful method for measuring proteins from surfaces. In this work, we present development and initial testing of a cryo-platform for LESA mass spectrometry of proteins. We explore the use of native sampling solutions for probing proteins directly from frozen surfaces. Our initial results from analysis of ubiquitin and hemoglobin standards showed that protein and protein complex refolding or unfolding occurs during the liquid solvent extraction stage of routine room temperature LESA. However, by employing the cryo-sampling method, the refolding or unfolding of protein ubiquitin can be dramatically reduced, while for the protein complex of hemoglobin, its native structures can be better preserved compared with room temperature sampling. This indicates that a truly native LESA sampling method of proteins is feasible. We also present detection of proteins directly from a frozen tissue section. Interestingly, slight conformational differences are observed from different regions of the frozen tissue surface. Further development of this strategy should be considered as a method for preserving, maintaining, and studying proteins in their native states, directly from tissue.

Keywords: Liquid extraction surface analysis, Cryo-sampling, Native mass spectrometry, Protein unfolding/refolding, Tissue protein MS

Introduction

Most functionally active proteins, except for those which are intrinsically unstructured, possess characteristic three-dimensional structures, which are the proteins' so-called native structures. Proteins, built from chains of amino acids, are translated from sequences of mRNA by ribosomes. During these translations, which are driven by a series of forces including intramolecular hydrogen bonds and hydrophobic interactions, proteins may begin to fold starting from their N-terminus. Providing this process is not disrupted, the correctly folded structure is achieved following completion of protein synthesis. This correct structure enables the protein to carry out a desired biofunction in cells. However, the folding of a protein does not always follow the biologically preferred pathway. If the cell environment is disturbed by high temperature, extreme pH, abnormal salinity, and intrusion of heavy metal ions, a protein may misfold into a different three-dimensional spatial arrangement other than its native structure. Such misfolding may cause loss of physiological function and induction of disease states. For example, temperature above 37 °C triggers the cell cycle control protein p53 to undergo unfolding and oligomerization, which may lead to tumorigenesis [1]. At intermediate pH (~ 5), prion protein PrP misfolds and aggregates, which is one cause of several neurodegenerative diseases, such as fatal familial insomnia and Creutzfeldt-Jakob disease [2]. Other commonly seen protein-misfolding diseases include Alzheimer's, type II diabetes, and Parkinson's disease [3].

Dozens of biophysical techniques are routinely employed for characterization of protein structures, including X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and

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optical spectroscopy. More than 90% of the known protein structures in the Protein Data Bank have been determined using X-ray studies and NMR spectroscopy. However, X-ray analysis requires proteins to be crystallized which is sometimes challenging, for instance for membrane proteins or highly flexible proteins. In addition, a significant amount (hundreds of micrograms) of purified protein is needed to obtain a successful diffraction pattern. For NMR techniques, a high-concentration solution (more than 10 mg/mL) is also required to study a protein sample. Considering the drawbacks and the potential disruption of sample preparation to protein structures, the aforementioned sophisticated methods cannot be applied to all samples. As one of several complementary tools for structural characterization of biomolecules, mass spectrometry (MS) plays an irreplaceable role in the study of proteins owning to its short analysis time, high sensitivity, and low sample consumption. The emergence of soft ionization methods, such as electrospray ionization (ESI) [4] and matrix-assisted laser desorption/ionization [5], which can bring intact biomolecules into gas phase, has dramatically enhanced the number of applications of MS to study proteins. Coupled with other spectrometric techniques, such as ion mobility spectrometry, collision/electron-induced dissociation, or photo-dissociation, MS has demonstrated extraordinary capability in mapping in detail the structures of proteins together with molecular interactions involved in protein complexes [6–8].

In MS of proteins and protein complexes, care must be taken to maintain molecules' structural features, including folding/unfolding characteristic and non-covalent binding when samples are vaporized. For native MS, proteins are prepared using buffered aqueous solvents to preserve their native quaternary states [9–11]. Compared with typical ESI using acidified organic solvent, native ESI MS has a lower ionization efficiency. However, it is gentler to folded structures and easy-to-break non-covalent interactions. Protonation in native ESI occurs mostly at the surface of protein molecules, leading to a narrow distribution of low charge states ions. Coupled with time-of-flight mass analyzer, native ESI has been applied to interrogate large protein assemblies with molecular masses of more than several hundred kDa [9, 12, 13].

In addition to being applied to solution phase, native MS has been extended to measure proteins from surfaces by several variants of ESI, including desorption electrospray ionization (DESI) [14] and liquid extraction surface analysis (LESA) [15]. Commercially available DESI sources have been mostly utilized to imaging the surface distributions of small molecules [16]. The use of DESI to study native proteins from surfaces had not been described until 2017 when Robinson and her coworkers demonstrated it by using a home-built DESI platform [17]. Later, native DESI imaging of proteins directly from tissue samples was reported [18, 19]. LESA, based on a liquid microjunction surface sampling probe [20], has been proved to be more efficient to transfer native proteins and protein complexes from the surface into mass spectrometer inlets. Cooper and her coworkers have reported native LESA MS studies of proteins from a range of substrates, including dried blood spots, thin tissue sections, and dried droplets of protein solutions [21–24]. Starting from relatively small proteins of 17-kDa holo-myoglobin and 64-kDa

tetrameric hemoglobin [21], they have shown native LESA MS results of protein complexes as large as 800 kDa [23]. In addition, LESA has been used to map the surface distribution of native proteins in tissues [22, 25].

In comparison with solution phase ESI, LESA involves two additional steps: sample drying and surface sampling. In this work, we conduct experiments at room temperature and under cryo-conditions to explore whether or not protein unfolding or refolding could occur in these stages. Protein standards and the LESA sampling solvent were prepared in either non-denaturing (100-mM ammonium acetate) or denaturing (1:1 water/methanol with 0.1–1% formic acid) conditions in various combinations. Room temperature experiments showed that protein unfolding and refolding may occur depending on the sampling solvent chosen, indicating that the aforementioned hypothesis is possible: that LESA-MS of denatured proteins can result in native-like spectra. In order to solve the protein refolding and unfolding issue, cryo-experiments were tried through minor modifications to the sample recess. Surprisingly, for the standard protein of ubiquitin, the observed spectra are predominantly affected by sample preparation rather than sampling solvent, indicating that the protein refolding and unfolding problem in LESA could be controlled. Analysis of the complex hemoglobin demonstrated that cryo-sampling can be used to generate mass spectra, which far more closely resemble direct infusion native ESI mass spectra. Cryo-sampling experiments were then carried out in frozen tissue. We report detection of proteins directly from frozen tissue sections. Further work is required to optimize this for analysis of frozen sections with native solutions.

Experiment

Standards and Samples

The proteins studied in this work are ubiquitin from bovine erythrocytes and human hemoglobin purchased from Sigma-Aldrich (Gillingham, UK). MS grade salt ammonium acetate was also from Sigma-Aldrich (Gillingham, UK). Optima™ LC/MS grade water, methanol, and formic acid were obtained from Fisher Scientific (Loughborough, UK).

Ubiquitin or hemoglobin with a concentration of 15 μM and LESA sampling solution were prepared using either 100-mM ammonium acetate or 1:1 water/methanol (v:v) with 0.1% formic acid. Ten-microliter aliquots of protein solution were spotted onto pain glass microscope slides. Samples were either left to air dry for at least 1 h or put in − 80- °C freezer for fast freezing prior to LESA or cryo-LESA study, respectively. For air dried samples, the proteins deposited on glass slides after evaporation of the solvent typically show roughly circle marks with diameters of about 5 mm. This would lead to a protein concentration of ~ 0.8 nmol/cm^2 if the deposition is homogeneous across the surface. However, obvious "coffee ring" effects can be observed for both hemoglobin and ubiquitin sample, especially when they are natively prepared. The LESA sampling spot size was observed to be approximately 3 mm in diameter.

For animal tissue LESA MS studies, wild-type mouse brain provided by the Francis Crick Institute, UK was sectioned using the CryoStar™ NX70 Cryostat (Thermo Scientific, Waltham, USA) to thickness of 10 μm. The tissue sections were then thaw mounted onto glass slides. To conduct cryo-LESA experiments, the frozen brain block with a flat surface was analyzed in the sample recess cooled with dry ice. Taking the published protocol suggestions [25], the solvent used was 200-mM AmAc with 5% methanol for native sampling while 1:1 water/methanol with 1% formic acid for denaturing sampling.

LESA-MS Workflow

Both infusion ESI and LESA experiments were performed using the Triversa Nanomate (Advion Biosciences, Inc., Ithaca, USA) coupled to a Synapt G2-Si ion mobility enabled Q-ToF mass spectrometer (Waters, Manchester, UK). For infusion ESI experiments, 5 μL of protein solution was aspirated into a conductive, carbon-impregnated pipette tip and delivered to an ESI chip to form a nano-electrospray. In LESA experiments, the tip held by a robot firstly aspirates 4 μL of extraction solvent from a solvent reservoir. Then the tip was moved to a height of about 0.5 mm above the surface to be analyzed, and 2-μL solvent was dispensed to form a microliquid junction between the tip and the surface. The liquid microjunction was observed to be approximately 3-mm diameter at the surface. After a delay of 5 s, 2.5 μL of the total volume was re-aspirated into the tip, which was translated for direct infusion into the mass spectrometer through the nozzle of a nano-electrospray chip. It is worth noting that the robot's mechanical movement from the surface analyte to the ESI chip takes about 10 s. Typical electrospray voltage and backing pressure employed were 1.4 kV, 0.3 psi for ubiquitin standard while 1.8 kV, 1.0 psi for hemoglobin standard and tissue samples. Data acquisition was performed using the resolution mode of the Synapt MS. The source temperature was set to 80 °C, and the sampling cone voltage was 120 V. The scan time was 1 s, and each spectrum consists of 60–100 scans. At least, triplicate measurements were conducted for each study.

For cryo-LESA experiments, the universal sample adapter plate was replaced with a copper bar, pre-chilled to – 80 °C, and fitted into an insulating nitrile rubber foam, which was used to support the sample. The design allows standard proteins on glass surface or tissue sample to be maintained frozen for more than 30 min. Triplicate measurements were conducted for all studies, and this did incur a variation of 5–10 min from when the copper bar was replaced. No spectral differences were observed, and cryo-temperatures were maintained for all experiments. The total range of temperatures was estimated to be (− 40 to – 5 °C). In future work, constant cooling via Peltier will be ensured. The semi-automated LESA process was controlled by Advion Chipsoft software V8.3.3, and mass spectra were recorded by Waters Masslynx software V4.1.

Results and Discussion

Studies of Ubiquitin

Infusion ESI   The protein ubiquitin has been extensively studied by MS with or without ion mobility separation function [26, 27].
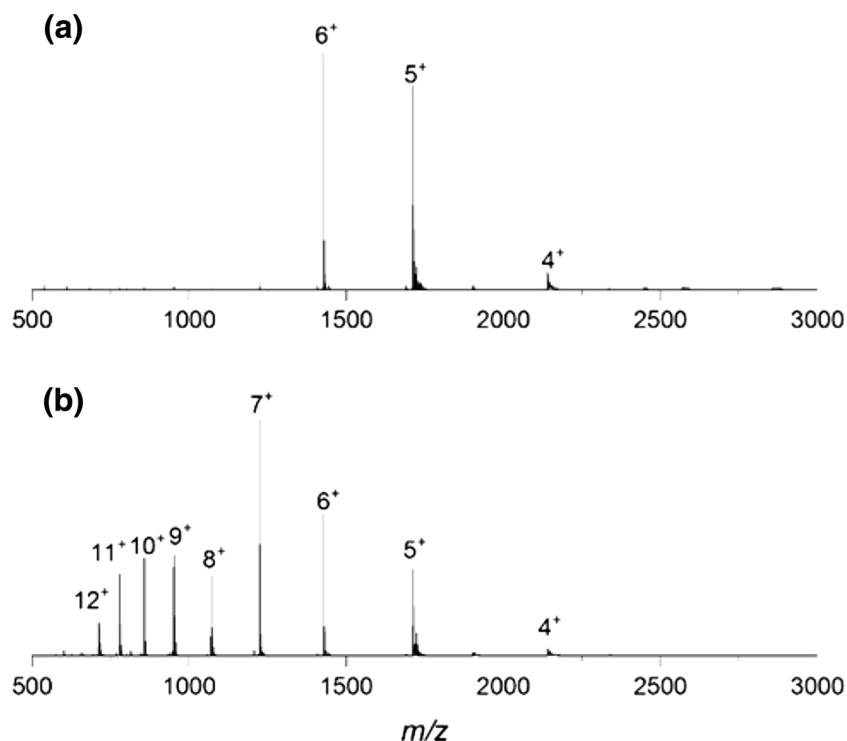

Figure 1. Nano-ESI mass spectra of 15-μM ubiquitin in (**a**) 100-mM ammonium acetate and (**b**) 1:1 water/methanol with 0.1% formic acid

As shown in Figure 1, by changing the solvent composition, the nano-ESI mass spectra of ubiquitin differ substantially in ions' charge states and their distribution. The feature of narrow charge state distribution is observed in the mass spectrum obtained of ubiquitin in aqueous salt solvent of 100-mM ammonium acetate (Figure 1a). The ions detected are mainly dominated by 6+ and 5+ charge states. The 4+ state of ubiquitin is also observed. The narrow distribution of low charge states indicates that ubiquitin is mostly in native-like compact structures, which are folded with a limited number of protonation sites exposed at the surface. By altering the solvent to denaturing conditions, 1:1 water/methanol with 0.1% formic acid, a much broader charge state distribution can be seen in the acquired mass spectrum (Figure 1b). The protein is found to be able to adopt from as low as 4 protons to up to 12 protons, of which 7+ ubiquitin is the most abundant. This can be attributed to the protein partially unfolding in the denaturing conditions, in which extended structures with large number of protonation sites are mixed with compact structures. The results from infusion nano-ESI experiments are well reported [26, 27] and used here to compare the charge distributions and conformation of ubiquitin, sampled from a surface, in various conditions.

Room Temperature LESA LESA mass spectra of ubiquitin, sampled from a surface at room temperature, are shown in Figure 2. Using 100-mM ammonium acetate as the LESA solvent to sample, a dried droplet of ubiquitin prepared in the same solvent (see Figure 2a) yielded a very similar ion composition to that observed in the native ESI spectrum (see Figure 1a). The spectrum is dominated by peaks of ubiquitin 6+ and 5+ (6+ the most abundant). The narrow charge state distribution indicates that the ubiquitin is folded, at least after extraction into LESA solvent. However, when the experiments were repeated using denaturing solvents (100-mM ammonium acetate to 1:1 water/methanol with 0.1% formic acid), a distinctly different charge state distribution was observed. The spectra resembled ESI of a denatured protein sample (Figure 1b). These results confirm that use of a denaturing solvent to extract a "native" sample also results in a broad distribution of charge states ranging from 5+ to 13+ of ubiquitin (Figure 2b). The different spectra obtained of ubiquitin acquired using the different sampling solvents indicated that protein unfolding occurs at least in the sampling stage. Hence, whether the deposited protein is in a folded or unfolded state cannot
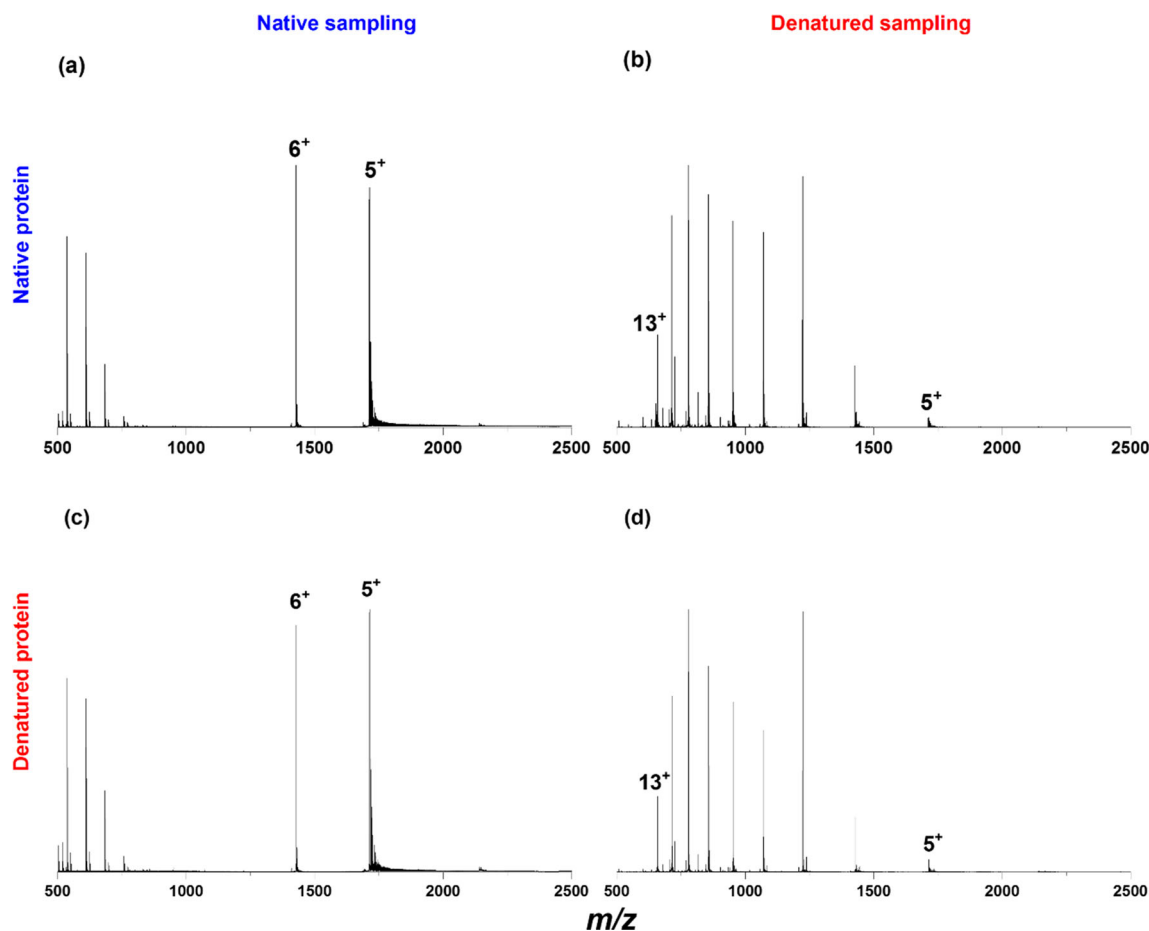

Figure 2. Room temperature LESA mass spectra of ubiquitin prepared in (**a**, **b**) 100-mM ammonium acetate and (**c**, **d**) 1:1 water/methanol with 0.1% formic acid. Sampling solvent used is 100-mM ammonium acetate in (**a**) and (**c**) while 1:1 water/methanol with 0.1% formic acid in (**b**) and (**d**)

necessarily be understood from LESA mass spectra. This is further supported by the following findings when studying protein prepared using denaturing solvent.

The mass spectrum acquired by denaturing LESA sampling of denatured protein is presented in Figure 2d. The charge states of ions of ubiquitin show a wide distribution, ranging from 5+ to 13+, indicating unfolded protein ions. However, surprisingly, when shifting the sampling solvent from denaturing to native conditions, a native-like spectrum is obtained for LESA of denatured protein, as shown in Figure 2c. As for native LESA sampling of natively prepared sample (see Figure 2a), only two charge states, 5+ and 6+, were found to be dominant, providing evidence of structure refolding in 'native LESA' of a denatured protein. No notable spectral differences were observed by varying the delay time between surface sampling and ionization (a minimum time of 10 s is needed for the mechanical motion of the LESA platform). This could be attributed to the millisecond timescale of ubiquitin refolding [28]. In addition, no obvious difference between drift times of ions of the refolded protein and that of the initially folded protein was recorded (Figure S1). Again, this indicates that in LESA, it is the choice of extraction solvent rather than the sample preparation, which determines the spectra reflecting protein structures. The only difference noted is that the intensity of 5+ ion is a

little higher than that of 6+ ion in the spectrum of refolded protein, while the opposite was observed for the protein prepared in native conditions. This might be caused by the fact that the refolded protein prefers a relatively more compact structure with five charges to that with six charges.

It is possible that the protein sample in all cases above, dried at room temperature, is denatured. In this scenario the results obtained using native sampling solvent conditions would be indicative of refolding. We developed a cryo-sampling strategy to try to eliminate this possibility and to re-evaluate native surface sampling of ubiquitin.

Cryo-LESA The mass spectrum obtained by "cryo-LESA" using 100-mM ammonium acetate to extract a frozen native ubiquitin solution is presented in Figure 3a. A 5% methanol was added to the sampling solvent to prevent freezing of the liquid microjunction with the cooled surface. The charge state distribution was again dominated by 5+ and 6+, indicating that proteins detected are predominantly seen in a folded state after extraction from the frozen substrate. In the room temperature experiments, 6+ ubiquitin was found to be more abundant. From a frozen sample, the 5+ ubiquitin ions were the most abundant.
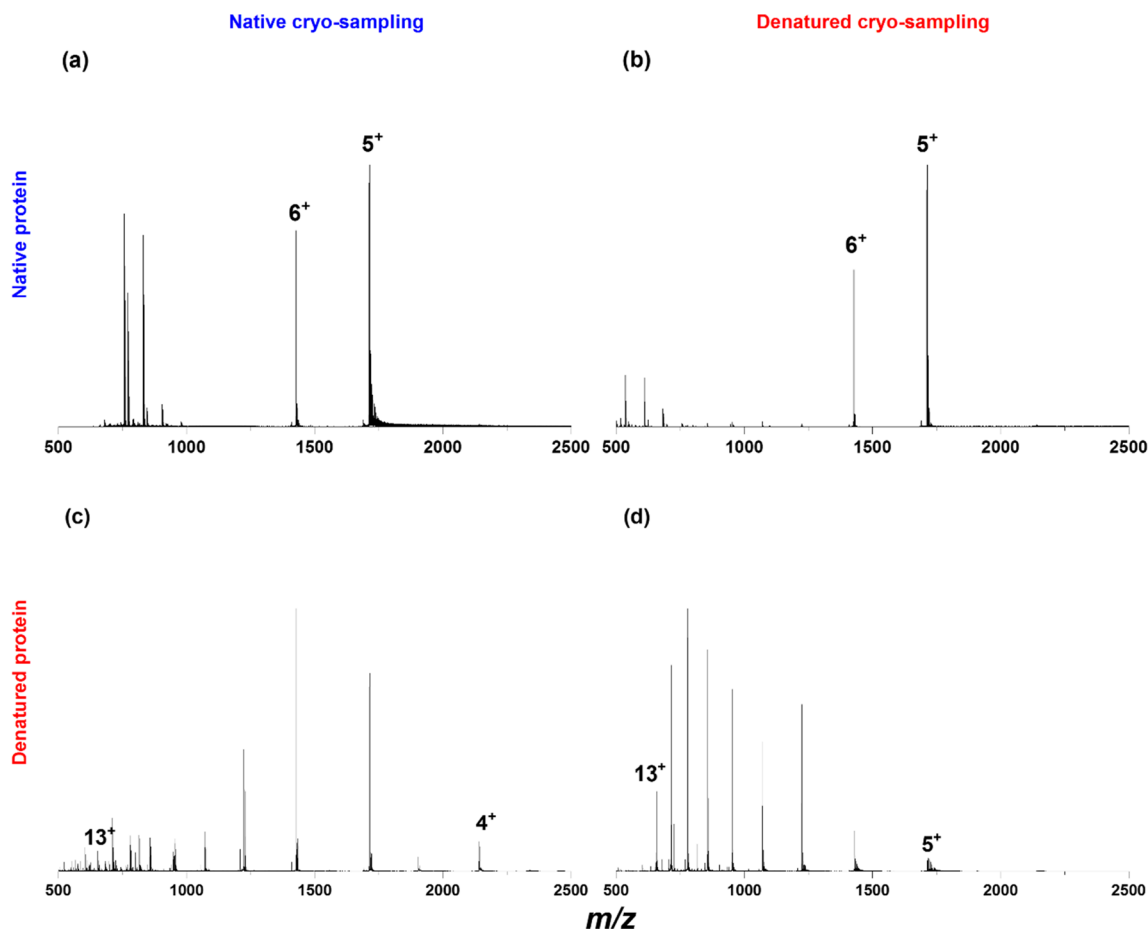

Figure 3. LESA mass spectra of frozen sample of ubiquitin prepared in (**a**, **b**) 100-mM ammonium acetate and (**c**, **d**) 1:1 water/methanol with 0.1% formic acid. Sampling solvent used is 100-mM ammonium acetate with 5% methanol in (**a**) and (**c**) while 1:1 water/methanol with 0.1% formic acid in (**b**) and (**d**)
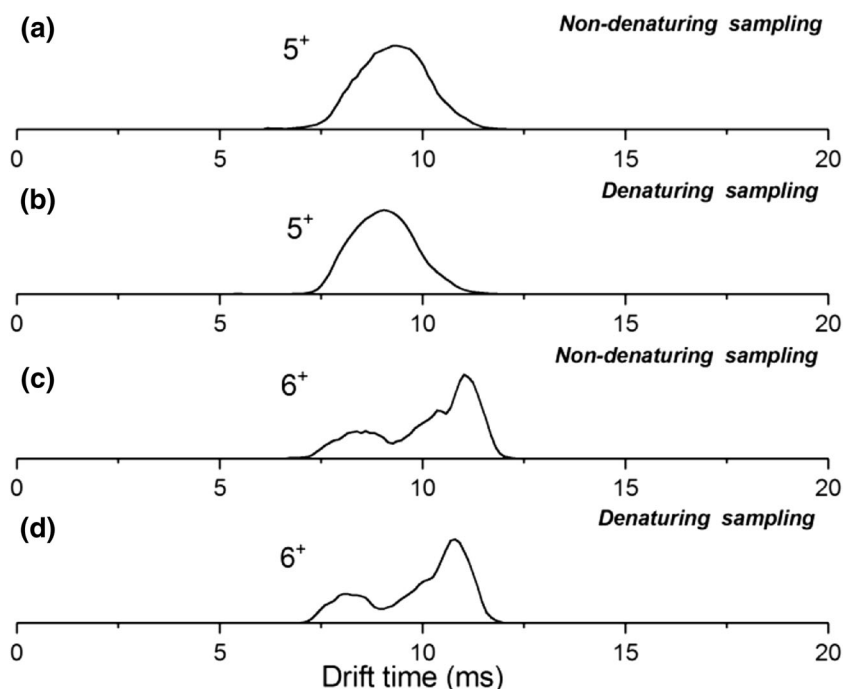
Figure 4. Drift time curves of 5+ (**a**, **b**) and 6+ (**c**, **d**) ubiquitin ion acquired in LESA studies of frozen protein samples prepared using 100-mM ammonium acetate. Both 100-mM ammonium acetate with 5% methanol and 1:1 water/methanol with 0.1% formic acid are used to perform non-denaturing (**a**, **c**) and denaturing sampling (**b**, **d**), respectively

This may be because the lower temperature favors a higher percentage of more compact structures, leading to a higher number of lower charge state of ubiquitin ions. Nevertheless, for the same ions detected in different temperatures, there is no apparent mismatching observed in the comparison of their drift times (see Figure S2), indicating that proteins are folded in a similar way.

Repeating the studies which were conducted at room temperature, a denaturing solvent of 1:1 water/methanol with 0.1% formic acid was also used to sample a protein prepared in native conditions. Surprising, the spectrum (presented in Figure 3b shows that under these conditions, the protein appears unaffected by the denaturing LESA solvent. Denaturing solvent sampling of frozen native protein also results in narrow charge state distribution of ions. The relative ratio between two dominant charge states and drift time of same ions (presented in Figure 4b, d) appears

similar to that observed using native solvent sampling of the same frozen sample (see in Figure 4a, c). Protein unfolding does not occur under cryo-sampling conditions. We repeated this experiment and consistently found that compact folded protein structures were detected from frozen samples, even using denaturing conditions.

We also surveyed frozen proteins prepared using denaturing solvent. Here, a broad charge state distribution of ions (Figure 3d) was detected when the LESA sampling solvent was also denaturing. Interestingly, if non-denaturing solvent was used for sampling frozen denatured protein (Figure 4c), the charge state distribution was broad. However, the average charge state is observed to be lower. The relative intensities of low charge states ($z < 7$) are dramatically enhanced, while that of high charge states ($z > 7$) show opposite trend. It is

Table 1. Lists of Detected Proteins Together with Their Parent Ion Charge States Distribution Using Different LESA Sampling Methods to Study Standard Protein Sample (Rows 2–5) and Mouse Brain Tissue Sample (Rows 6–13)

	Protein IDs	Native LESA	Denaturing LESA	Native cryo-LESA	Denaturing cryo-LESA
Standard protein sample	Native ubiquitin	5–6	5–13	5–6	5–6
	Denatured ubiquitin	5–6	5–13	5–13	4–13
	Native hemoglobin	Tetramer, dimer, and monomer	Monomer	Tetramer, dimer, and monomer	Monomer
	Denatured hemoglobin	Dimer and monomer	Monomer	Dimer and monomer	Monomer
tissue sample	β-thymosin 10	4–5	5–7	N. D.	5–7
	β-thymosin 4	4–5	5–7	N. D.	5–7
	PEP 19	5–8	7–9	N. D.	7–9
	Ubiquitin	5	7–11	N. D.	8–11
	Acyl Co-A binding protein	6	8–13	N.D.	9–11
	α-globin	11–19	11–20	9–18	10–20
	β-globin	12–18	11–18	N.D.	10–19
	Myelin basic protein	9–20	17–22	N.D.	N.D.

possible that the extended structures of the frozen protein refolded in the LESA sampling stage. Compared with the thorough refolding observed at room temperature, the refolding induced by native solvent sampling occurs to a less extent under cryo-conditions. These results demonstrate the potential of this novel sampling strategy for retaining the conformation of the protein from substrate to gas phase. The effects of protein preparation and LESA solvent on features of observed mass spectra are summarized and presented in Table 1.

Studies of Hemoglobin

From the aforementioned LESA studies of ubiquitin, part of the initial hypothesis has been verified: that denatured protein refolds when being analyzed with native sampling solvent during LESA. This opens the question about how to achieve a truly native surface sampling of proteins using LESA. Surprisingly, we have shown that by employing the cryo-LESA method, the structures of ubiquitin, whether native or denatured, can be preserved from sample solution to gas phase through cryo-state sampling. Even though, it is still ambiguous as to whether natively prepared protein becomes denatured once air dried at room temperature. Therefore, to further explore the differences between LESA and cryo-LESA in preserving protein complexes' structures, in this paper, the same studies have also been performed on holo-hemoglobin, which consists of four subunit proteins (two α-chains and two β-chains) non-covalently bound. Using LESA platform on Orbitrap or Q-TOF instruments, extensive studies have been performed on natively prepared hemoglobin deposited either on plain glass slide or polyvinylidene fluoride membranes [21]. The results showed that the intact complex could be detected by a combination of LESA sampling from glass substrate and Q-TOF mass analyzer with a 30-°C inlet temperature. Here, we compared direct infusion ESI, with LESA at room temperature and cryo-LESA of hemoglobin.
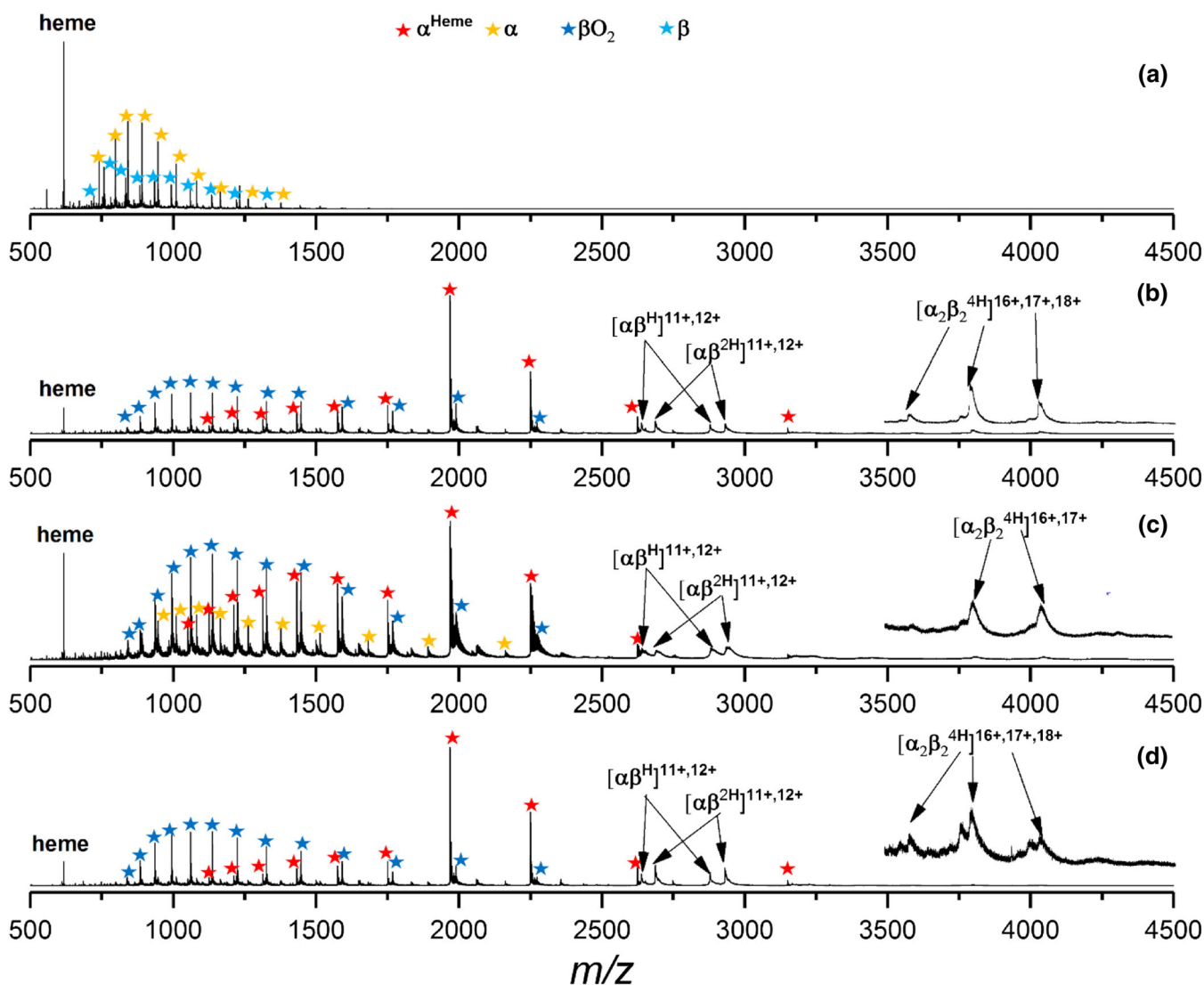

Figure 5. Mass spectra of hemoglobin prepared in (a) 1:1 water/methanol with 0.1% formic acid (b–d) 100-mM ammonium acetate analyzed by direct infusion ESI (a, b), room temperature LESA (c), and cryo-LESA (d)

For natively prepared hemoglobin, the mass spectra collected from direct infusion ESI (Figure 5b) show peaks relating to ions of isolated subunits, i.e., α^H (α-globin with heme group) from 5+ to 14+ and βO_2 (oxidized heme-free β-globin) from 7+ to 19+. Peaks from complex ions, which include 11+ and 12+ ions of dimers in the form of $\alpha\beta^H$ and $\alpha\beta^{2H}$ and ions of the intact form of the molecule, i.e., 16+ to 18+ tetramers $\alpha_2\beta_2^{4H-}$, are also evident. In contrast, for hemoglobin prepared in denaturing conditions, the ESI mass spectrum presented in Figure 5a only show ion peaks relating to ions of the respective subunits, i.e., 10+ to 22+ α-globin and 11+ to 19+ β-globin, both in heme-loss states. In addition, the relative intensity of peaks relating to the heme ion indicates a very high extent of denaturation of the protein.

The liquid droplet of natively prepared hemoglobin was then deposited onto the plain glass slide, air dried, and analyzed by room temperature LESA. A buffered aqueous neutral solution was used as the LESA sampling solvent, and the resulting ESI mass spectrum was presented in Figure 5c. As was observed from direct infusion of native ESI-MS of hemoglobin, ion of the subunits and the intact molecules were detected. However, in the direct infusion ESI experiment, α-globin is detected only in holo-form (labeled as α^{Heme} in the figure); some apo-α-globin (labeled as α in the figure) has also been observed at room temperature LESA. The relative abundance of ions of heme was found to be higher in LESA spectra. The

different mass spectra obtained from infusion vs LESA indicate that a structural change may have occurred during air drying, again pointing to the need of a method, which could better preserve the structure of proteins during LESA sampling.

The cryo-LESA mass spectrum of natively prepared hemoglobin can be seen as Figure 5d. No obvious discrepancy has been observed in the spectra between cryo-LESA and direct infusion ESI, suggesting that protein structures can be better maintained using this platform. Furthermore, it can be seen that the signal-to-noise ratio obtained under cryo-conditions was approximately three-fold that of room temperature.

Sampling a Frozen Tissue Section

At room temperature, using native sampling solvents to analyze sectioned and thaw-mounted tissue resulted in mass spectra mainly dominated by the signal of myelin basic protein detected in charge states ranging from 9+ to 20+ (Figure 6a). When using ion mobility separation, the α-chain (11+ to 19+) and β-chain (12+ to 18+) of hemoglobin were also detected (shown in Figure S3). Ions relating to several other proteins (tentatively assigned by mass) including β-thymosin 10 (4+), β-thymosin 4 (4+ and 5+), PEP19 (5+ to 8+), ubiquitin (5+), and Acyl Co-A binding protein (6+) were all observed in the ion mobility resolved mass spectra. To date our, results show successful detection of only relatively small (molecular weight
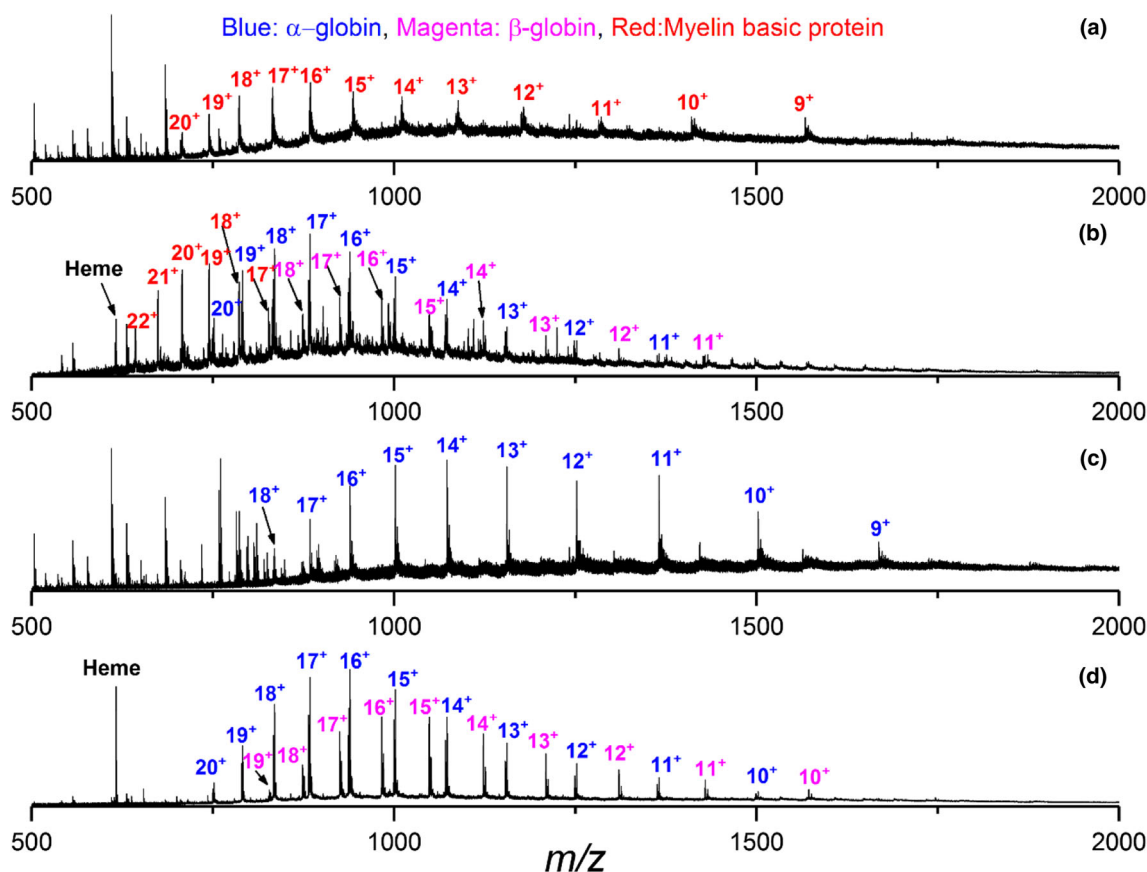

Figure 6. LESA mass spectra of mouse brain tissue samples at room temperature (**a, b**) and cryo-condition (**c, d**). The sampling solvent used was 200-mM AmAc with 5% methanol in (**a**) and (**c**) while 1:1 water/methanol with 1% formic acid in (**b and d**)

less than 10 kDa) protein ions exhibiting narrow charge state distributions using native solvents for LESA MS of animal tissue samples. The Cooper group at the University of Birmingham has presented detection of an intact hemoglobin tetramer directly from liver tissue using native LESA solvents [22]. Our above protein standard experiments also show that intact hemoglobin complex can be extracted from surface to LESA solvent. However, we did not detect the intact tetramer or the dimer here from the tissue. Instead, only ions of subunits α-chain and β-chain with broad charge state distributions were observed. This could be partially explained in terms of sample concentration as we were not sampling any vascular features. It is also possible that the protein hemoglobin was denatured and unfolded before LESA sampling. The myelin basic protein was also detected across a broad charge state. Once again, these results indicate just how important the preservation of the sample is in direct analysis and imaging experiments.

When using a denaturing LESA sampling solvent, ions from three protein species were detected: 17+ to 22+ myelin basic protein, hemoglobin 11+ to 20+ α-chain, and 11+ to 18+ β-chain (Figure 6b). Considering the connection between proteins' solubility and their isoelectric pH (lowest solubility at the isoelectric pH), this may help explain why acidified denaturing LESA sampling conditions seem to result in extraction of more proteins with near neutral isoelectric pH (for instance 6.9–7.4 for hemoglobin; 8.12 and 7.08 for its α-chain and β-chain, respectively, [29]) than under native sampling conditions.

Furthermore, low pH values favor protonation of molecules, which as a result lead to relatively higher signal of hemoglobin subunits. Apart from the three dominant species, ions with lower intensities of some other proteins were also detected: β-thymosin 10 (5+ to 7+), β-thymosin 4 (5+ to 7+), PEP19 (7+ to 9+), ubiquitin (7+ to 11+), and Acyl Co-A binding protein (8+ to 13+). Compared with native sampling, except for hemoglobin α-chain and β-chain, the charge states of other proteins are either higher or broader in distribution, indicating that different protein structures are extracted under the two sampling conditions. These results indicate that at room temperature, during LESA sampling and analysis of tissues, protein unfolding/refolding may occur. To eliminate (or minimize) the LESA solvent effect on acquired mass spectra of animal tissues, the cryo-sampling method was also employed.

Denaturing cryo-sampling conditions (Figure 6d) resulted in mass spectra dominated by hemoglobin α-chain (10+ to 20+) and β-chain (10+ to 19+). In addition, just as was observed at in room temperature LESA, other proteins detected at low intensities were also observed (listed in Table 1). Compared with room temperature study, the cryo-spectra reveal a lower background and fewer peaks in general. This is probably due to decreased solubility at lower temperature, which leads to a reduced number of species extracted from surface into solvent.

For these reasons, native LESA cryo-sampling did not provide rich protein MS data. This might be caused by weak surface extraction ability at low temperature and fewer proton
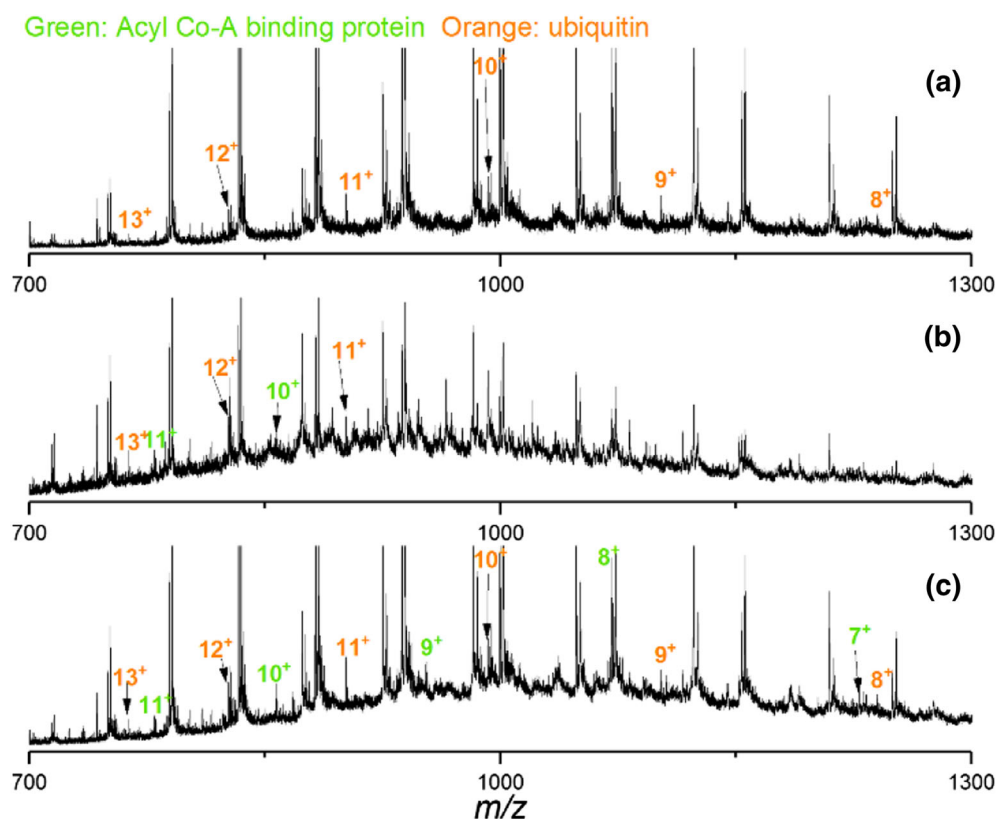

Figure 7. Zoomed-in LESA mass spectra of mouse brain tissue samples analyzed in different regions at cryo-condition. The sampling solvent used was 1:1 water/methanol with 1% formic acid

donors by native solvents. In fact, in these first experiments, the only protein detected was hemoglobin α-chain (Figure 6c), with a broad charge distribution.

In addition, studies were also conducted in different regions (midbrain, cerebral cortex, and cerebellum, respectively) of the frozen brain tissue sample. As very limited peaks were detected using native sampling solvent conditions from a frozen section (see above), the different regions were sampled using denaturing solvents only. The acquired mass spectra at different surface locations (see Figure S4) were very similar and resemble those presented in Figure 6d. The most abundant protein ions were those of α-chain and β-chain of hemoglobin, with a broad charge state distribution. No evidence of intact tetramer or dimer was observed. In addition, ubiquitin and Acyl Co-A binding protein were also detected at very low amount (see Figure 7). It can be seen that ubiquitin was detected in all three regions. However, the charge state distribution was slightly different. A broad distribution from 8+ to 13+ of ubiquitin was seen in Figure 7a, c, while relatively narrow distribution from 11+ to 13+ was shown in Figure 7b. The Acyl Co-A binding protein was observed only in Figure 7b, c, with the charge distribution from 7+ to 11+ in Figure 7c while only 10+ and 11+ in Figure 7b. From the different charge state distributions observed for the two small proteins, it is possible that cryo-sampling has allowed us to sample different conformers of the same molecule from different regions. Further work in model systems of known concentration would be needed to confirm this possibility. A number of exciting surface-sampling/native protein experiments could be conducted using a cryo-LESA stage in the future.

Conclusion

Protein unfolding or refolding was found to occur in room temperature LESA MS analysis of standard protein samples and also for proteins sampled from the surface of a mouse brain tissue section. For protein standards, the resulting detected charge states and ion conformation were found to be governed mainly by the sampling solvent. Use of a non-denaturing LESA solvent results in more native like mass spectra and vice versa. Hence, the charge state distributions in the acquired mass spectra can not necessarily be used to confirm a truly native protein conformation.

By employing a new cryo-sampling strategy and surveying frozen protein standards, we were able to better preserve a protein in native state from the sample to the gas phase. Native-like mass spectra are observed for LESA of natively prepared ubiquitin, whether the sampling condition is native or not. Also, the protein refolding issue observed during LESA sampling can be reduced. In the studies of hemoglobin, the spectral differences observed in LESA vs direct infusion ESI in terms of both varieties of detected species and their relative ratios have disappeared.

The results we obtained from directly sampling frozen tissue sections were relatively hard to interpret. The heterogeneous nature of the tissue sections firstly made it hard to directly compare the proteins, which were extracted under each of the conditions. It is clear that fewer proteins are extracted and/or detected when sampling a frozen section. While this is perhaps not a promising result, successful detection of proteins directly from a frozen section is exciting, especially that conformation differences of same proteins are detected. Coupled with the evidence presented from standard proteins in frozen vs room temperature conditions, further development of this strategy should be considered as a method for preserving, maintaining, and studying proteins in their native states directly from tissue.

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