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Identification of multiply charged proteins and amino acid clusters by liquid nitrogen assisted spray ionization mass spectrometry

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ABSTRACT

The development of liquid nitrogen assisted spray ionization mass spectrometry (LNASI MS) for the analysis of multiply charged proteins (insulin, ubiquitin, cytochrome c, α-lactalbumin, myoglobin and BSA), peptides (glutathione, HW6, angiotensin-II and valinomycin) and amino acid (arginine) clusters is described. The charged droplets are formed by liquid nitrogen assisted sample spray through a stainless steel nebulizer and transported into mass analyzer for the identification of multiply charged protein ions. The effects of acids and modifier volumes for the efficient ionization of the above analytes in LNASI MS were carefully investigated. Multiply charged proteins and amino acid clusters were effectively identified by LNASI MS. The present approach can effectively detect the multiply charged states of cytochrome c at 400 nM. A comparison between LNASI and ESI, CSI, SSI and V-EASI methods on instrumental conditions, applied temperature and observed charge states for the multiply charged proteins, shows that the LNASI method produces the good quality spectra of amino acid clusters at ambient conditions without applied any electric field and heat. To date, we believe that the LNASI method is the most simple, low cost and provided an alternative paradigm for production of multiply charged ions by LNASI MS, just as ESI-like ions yet no need for applying any electrical field and it could be operated at low temperature for generation of highly charged protein/peptide ions.

1. Introduction

Mass spectrometry is a versatile analytical tool for both qualitative and quantitative analysis of various biomolecules including oligonucleotides, proteins and DNA [1–5]. It is uniquely suited for the investigation of biomolecular structures and non-covalent interactions of proteins in modern biology [6]. Therefore, extensive efforts have been directed for the development of new ionization tools. As a result, a variety of desorption/ionization mass spectrometric techniques such as electrospray ionization [1], matrix-assisted laser desorption/ionization [2,3], desorption electrospray ionization [4], electrospray-assisted laser desorption/ionization [5], quantum dot laser desorption/ionization [7] and desorption/ionization on porous silicon [8] have been developed for the identification of various molecules (drugs, peptides and proteins). Among these approaches, electrospray ionization–mass spectrometry (ESI–MS) provides an adequate selectivity for the rapid and accurate identification of amino acid clusters, protein-protein interactions and protein complexes in proteomics. The selectivity, magnitude and detectability of multiply charged protein signals in ESI–MS are influenced by many factors, including denaturing agents, solvent composition [9–11], supercharging reagents [12,13] and chemical derivatization [14], respectively. Meanwhile, multiply charged proteins were also produced by the other ionization methods such as electrosonic spray ionization [15] and extractive electrospray ionization [16], just as in ESI MS. However, in order to produce ESI-like ions without voltage and heat at low temperature, Hirabayashi's group introduced new ionization technique and described that the potentiality of sonic spray-based ionization (SSI) tool for the identification of multiply-protonated organic and biomolecules [17–19]. Apart from this, Yamaguchi's group developed new ionization technique namely cold spray ionization (CSI) that can operate at low temperature [20,21]. The CSI requires a cold desolvation chamber (−80 to 15 °C) for production of nano-sized metal complexes by pouring liquid nitrogen at low temperature (−20 °C). These developments are greatly extended the applications of mass spectrometry for the identification of labile organic species, nano-sized metal complexes, asymmetric catalysts,

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supramolecules and primary biomolecules. Furthermore, charge reduction and charge inversion ion/ion reactions and multiply charged ions were successfully demonstrated by using single-, microchip version-sonic spray ionization and Venturi easy ambient sonic-spray ionization (V-EASI) methods [22–24]. In these approaches, stream of bipolar charged droplets were produced by pouring the sonic stream of nitrogen or air. Very recently, Chen's group successfully illustrated that the use of disposable wooden tips (wooden toothpicks) in ESI MS for the rapid identification of native proteins, protein- and organometallic-complexes [25]. However, the present LNASI MS is entirely different from the above techniques with regard to instrumental set-up, operation conditions, studied parameters and sample selection (Fig. 1 and Tables 1 and 2).

It is well known that the protein structure and number of basic amino acid residues are measured through the identification of multiply charged proteins by ESI MS [26]. In ESI, gas-phase ions are produced from solution by the following steps (i) generation of charged droplets; (ii) shrinkage of charge droplets by sudden desolvation of solvent at high voltage and high temperature which can facilitate to produce very small highly charged droplets and (iii) finally gas-phase ions are generated from these droplets. However, high temperature and high voltage are eliminated in cold-spray or sonic spray ionization methods for the facile, precise and direct solution analysis of various species which might be difficult in ESI due to their (analytes) instability to heat and/or air [17–24]. To take advantages of sonic spray ionization methods, we made an attempt to use liquid nitrogen-assisted spray ionization mass spectrometry for the identification of multiply charged proteins, peptides and amino acid clusters at ambient condition without electric field and high temperature (Fig. 1). This technique is termed as "open probe (without ESI source) liquid nitrogen-assisted spray ionization mass spectrometry". To evaluate LNASI MS performance, a number of parameters such as effect of acid and modifier volume and nebulizer set-up were studied for production of charged ions (proteins, peptides and amino acids).

2. Experimental section

2.1. Chemicals and reagents

Glutathione, arginine, valinomycin, insulin, ubiquitin, cytochrome c, α-lactalbumin, myoglobin, bovine serum albumin (BSA), acids (hydrochloric acid, phosphoric acid, formic acid and trifluoroacetic acid (TFA)) and HPLC grade organic solvents were purchased from Sigma Aldrich, St. Louis, MO, USA and used without further purification. Peptides (HW6, and angiotensin-II (Ang-II)) were obtained from Kelowna International Scientific Inc (Taipei, Taiwan). A Milli-Q ultrapure water system (Millipore, Milford, MA, USA) was used for sample preparations.

2.2. Set-up of LNASI MS

LNASI MS was designed on a Finnigan LCQ ion trap mass spectrometer (Thermoquest Inc., San Jose, CA, USA) by removal of ESI source and replaced with a 'T-shape nebulizer' (it made with stainless steel). The nebulizer was fixed to a stand, in which one end was connected through a Teflon tube (i.d., 0.99 mm) for sample introduction and another tube was inserted at coaxial to sample tube for pouring of liquid nitrogen (99.8%) at flow rate 8 mL min^{-1}. The photograph of LNASI MS set-up was shown in Fig. 1 and schematic instrument set-up was shown in Supporting Information of Fig. S1. Nebulizer tip was exactly fixed at entrance of mass analyzer of ion trap mass spectrometer (the skimmer or the outlet of a heated capillary tube). Logically it can be noticed that automated sample spray takes place by passing liquid nitrogen through nebulizer at coaxial position to sample solution tube (Fig. 1 and Supporting Information of Fig. S1). Ultimately, these can be led to the production of high speed fine charge droplets by sudden desolvation of solvent molecules from solution without electric field and heat. The distance between nebulizer tip and heated capillary tube of mass spectrometer was 1.0 cm (optimal distance).

LNASI MS (without ESI source) was effectively produced multiply charged proteins or amino acid clusters without applying any ionization device such as thermal, electrical and laser (eliminated voltage source to ionization). Other instrumental conditions such as heated capillary voltage (in the skimmer) and tube lens offset were set at 40 V and 70 V, respectively. The sample flow rate is 300 μL min^{-1} for LNASI MS and 12.5 μL min^{-1} for ESI MS, respectively. It can be noticed that the system is instinctively sprayed the solution into mass analyzer without electric field-, heat-, corona discharge- and pumping machine. As a result, the charged droplets are produced, ripped apart and then traversed through the heated capillary tube for their separation and identification by MS.

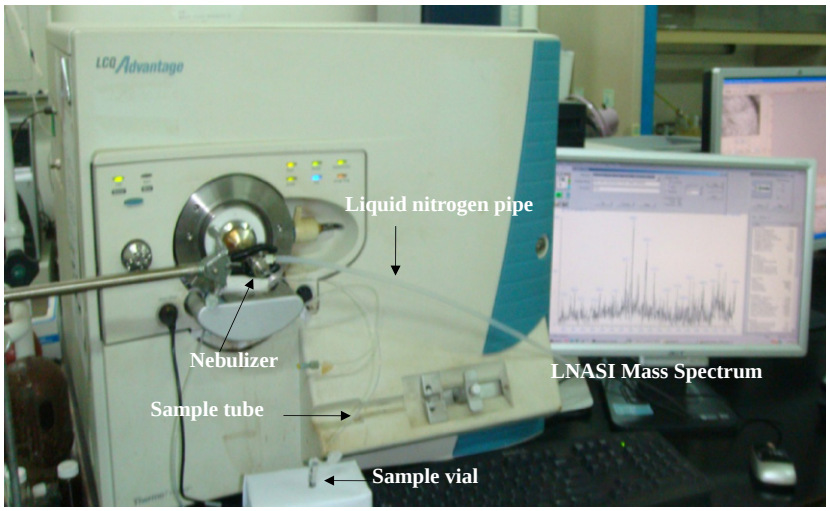

Fig. 1. Photograph of LNASI MS set-up.

Table 1
Comparison of LNASI, ESI and CSI MS parameters.

Instrumental settings	LNASI MS	ESI MS	CSI MS [20]
Sample flow rate (μL/min)	250–300	12.5	8.0
Nebulizer gas flow rate (L/min)	Liquid nitrogen flow–1.5	Aux/Sweep gas flow–0.3	
ISpray voltage (kV)		4.50	0–2.8
Spray current (μA)		−0.10	0.3–0.7
Capillary voltage (V)		9.51	5.0
Capillary temperature (°C)		270	10

Table 2
The optimal solvent compositions and observed charged states of proteins in LNASI MS.

Analytes	Molecular weight (Da)	Number of aminoacids	pI values	Best solution composition in LNASI (*v/v*)	Observed charge states		
					LNASI-MS	SSI-MS [18][a]	V-EASI-MS [24][b]
Insulin	5733.0	51	5.39	H_2O/AcOH, 99/1	+3 to +5 (at 2.0 μM)	NA	NA
Ubiquitin	8565.0	76	6.56	H_2O/ACN/AcOH, 49/50/1	+5 to +11 (at 5.0 μM)	NA	NA
Cytochrome c	12361.0	104	9.59	H_2O/ACN/AcOH, 49/50/1	+7 to +17 (at 5.0 μM)	+13 to +19 (at 1.0 μM)	+9 to +19 (at 80.0 μM)
α-Lactalbumin	14175.0	123	4.50	H_2O/ACN/AcOH, 49/50/1	+8 to +15 (at 5.0 μM)	NA	NA
Myoglobin	16951.0	153	7.36	H_2O/AcOH, 99/1	+9 to +21 (at 5.0 μM)	+18 to +25 (at 1.0 μM)	+11 to +23 (at 58.9 μM)
BSA	66399.0	607	4.70	H_2O/AcOH, 99/1	+34 to +53 (at 5.0 μM)	NA	NA
HW6	905.3		8.23	H_2O/ACN/AcOH, 49/50/1	$[M+H]^+$ (at 5.0 μM)	NA	NA
Ang II	1046.1		6.74		$[M+H]^+$ (at 3.0 μM)	NA	NA
Valinomycin	1111.3				$[M+H]^+$ (at 0.5 μM)	NA	NA
Arginine	174.2		11.15		$[(M)_{11}+H]^+$ (at 2.5 μM)	NA	NA
Glutathione	307.3				$[(M)_6+H]^+$ (at 2.5 μM)	NA	NA

[a] MeOH/H_2O/AcOH (47.5/47.5/5.0, *v/v/v*).
[b] H_2O/MeOH (50/50, *v/v*); NA is Not Applied.

3. Results and discussion

To illustrate LNASI MS capability for biomolecule analysis, an array of proteins with a mass range of 5–60 kDa were investigated. To this, we used T-shape nebulizer, an automated sample spray was achieved by pouring liquid nitrogen through T-shape nebulizer at coaxial position to the sample tube. Since the integration of nebulizer with continuous liquid nitrogen spray produces a suction force to the sample inlet pipe which can cause to automate sample spray with high speed. This spray leads to production of high speed fine charge droplets by sudden desolvation and microscopic fluctuations in ion concentrations [20]. As a result, fine droplet charged ions (gas-phase ions) are formed by the continuous evaporation and fission cycles that can lead to travel fine droplets with high speed by holding charged ions and subsequently transferred them directly into mass analyzer (Fig. S1 of Supporting Information). To obtain good LNASI mass spectra, various experimental parameters such as effect of acid and modifier volume and instrument set-up were studied.

3.1. Effect of acid concentration and solvent composition in LNASI MS

We studied that the effect of acid concentration and modifier volume for the identification of multiply charged proteins by LNASI MS. To this, we selected cytochrome c as a model protein since it is a small and easy ionization nature protein and containing 104 amino-acid residues with molecular weight 12361 Da. To perform protein analysis with LNASI under ambient conditions, first we studied that LNASI mass spectrum of cytochrome c (5.0 μM) by using pure water (without solvent and AcOH) (Fig. 2a). The results indicate that LNASI is able to produce multiply charged ions of cytochrome c directly from aqueous solution without addition of denaturing agents. However, the generated mass spectrum of cytochrome c shows the most abundant charge states from 10+ to 17+ with more background noise. To improve the number of abundant charge states and signal intensities of multiply charged ions of cytochrome c in LANSI, we studied LNASI mass spectra of cytochrome c (5.0 μM) by using H_2O/AcOH (99/1, *v/v*) (Fig. 2b) and H_2O/MeOH/AcOH (49/50/1, *v/v/v*) (Fig. 2c). These results revealed that generated LNASI mass spectra of cytochrome c exhibit more multiply charged ions (from 7+ to 17+) with increased signal intensities by addition of AcOH as denaturing agent. We found that the signal intensities of multiply charged ions of cytochrome c were greatly enhanced by using solution in H_2O/MeOH/ AcOH (49/50/1, *v/v/v*) (Fig. 2c). In this connection, we investigated that LNASI mass spectra of cytochrome c by using various denaturing agents such as formic acid, phosphoric acid, hydrochloric acid and TFA and ammonia at 1% (*v/v*) and obtained mass spectra were shown in Supporting Information of Fig. S2. Among these reagents, AcOH (1%, *v/v*) is acted as the best denaturing agent for effective

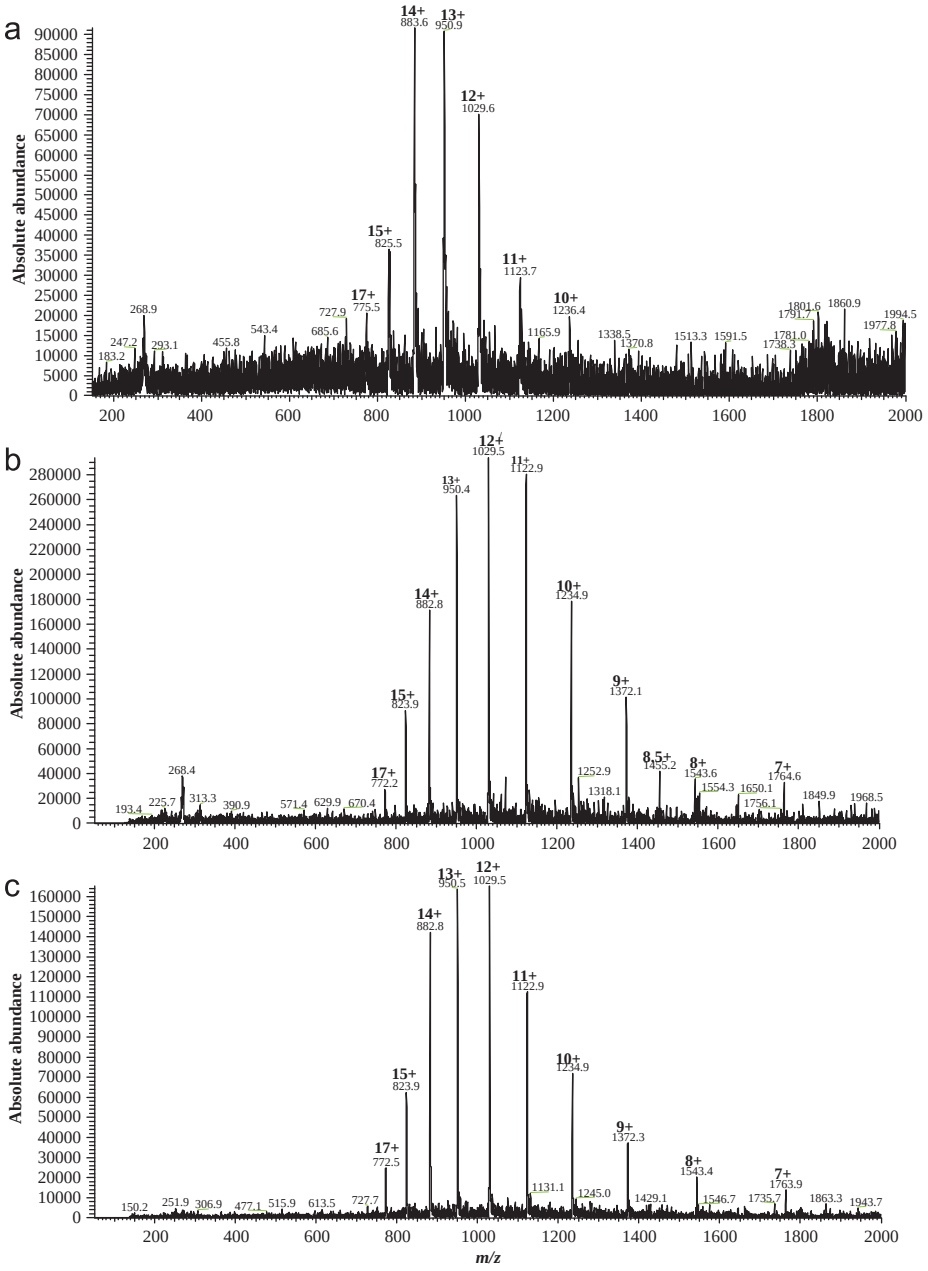

Fig. 2. LNASI mass spectra of cytochrome c (5.0 μM) using solutions (a) pure water, (b) H_2O/AcOH, 99/1, *v*/*v* and (c) H_2O/MeOH/AcOH, 49/50/1, *v*/*v*/*v*.

denaturation and production of maximum multiply charge states of cytochrome c by LNASI MS (Fig. 2b). Since, cytochrome c is a helical globular protein that can be covalently attached with heme group [27]. Cytochrome c exhibits a highly cooperative and acid-induced unfolding transition at pH 2.0–3.0 which is due to its isoelectric point (pI) [28]. Thus, the maximum charge states of cytochrome c were obtained by using solution at 1% (*v*/*v*) AcOH in LNASI MS. Hence, we selected 1% (*v*/*v*) AcOH as denaturing agent for production of multiply charged ions with enhanced signal intensities in LNASI MS.

To understand the effect of acetic acid concentration on the production of multiply charged ions of cytochrome c in LNASI MS, various percentages of acetic acid (0–5%, *v*/*v*) were investigated. Fig. 3a indicates that the concentration of AcOH was greatly influenced on the signal intensities and charge states of cytochrome c. We found that the charge states and signal intensities were increased with increasing amount of acetic acid up to 1% (*v*/*v*). Beyond 1% of AcOH, there was no change in charge states but the signals were decreased. Furthermore, lower change states were observed with decreased signal intensities by increasing concentration of AcOH (to 5%). Since, acetic acid has higher boiling point than that of water, therefore, it is capable to concentrate spray droplets which can enhance the signal intensities of analytes [29]. Hence, the addition of 1% (*v*/*v*) AcOH has significant impact on the formation of multiply charge distribution of cytochrome c by proton-acceptor reactivity in LNASI MS. In ESI, modifier volume plays a critical role on the generation of multiply charge states of proteins through rapid desolvation and deprotonation of solvent molecules [30]. Meanwhile, modifier can increase the rate of proton transfer through gas-phase collision between desolvated protein ions and solvent molecules. Therefore, we studied that LNASI mass spectrum of cytochrome c by using MeOH and ACN as modifiers along with 1% AcOH. Fig. 2c and Fig.4a shows the LNASI mass spectra of cytochrome c by using H_2O/MeOH/AcOH and H_2O/ACN/AcOH at 49/50/1 (*v*/*v*/*v*),

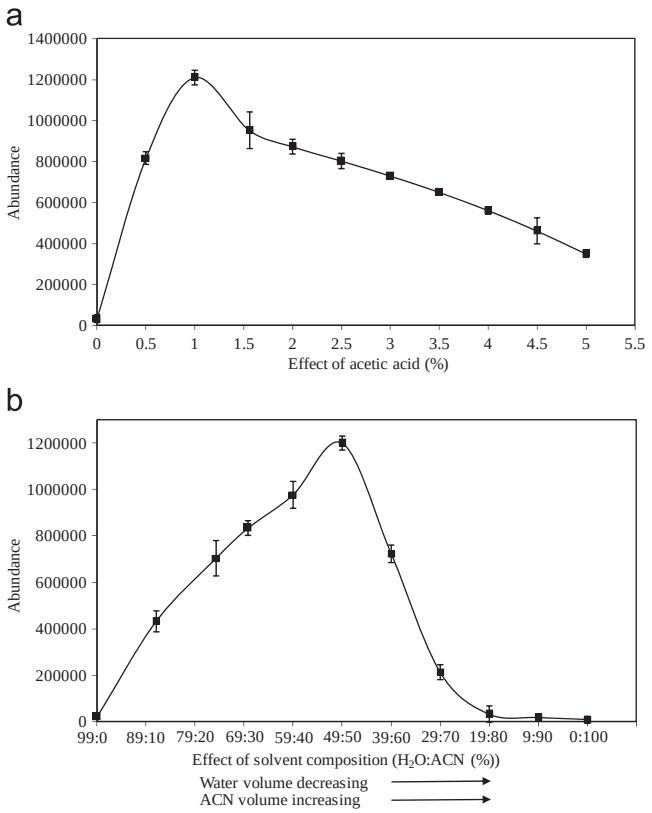

Fig. 3. Maximum abundance and average charged states of cytochrome c as function of (a) acetic acid concentration (%, *v/v*) and (b) effect of modifier volume (H_2O/ACN, *v/v*) along with 1% AcOH.

respectively. The results indicate that similar multiply charged cytochrome c ions were observed by using MeOH and ACN as modifiers at 50% (*v/v*) along with 1% (*v/v*) AcOH. However, 50% ACN solution was provided 7.5 times higher signal intensities than that of MeOH as modifier. Since the droplet size and desolvation process can be strongly depended by the addition of modifiers. Thus, we studied that the effect of ACN percentage (*v/v*) on the production of maximum multiply charged ions of cytochrome c in LNASI MS. Fig. 3b indicates that the effect of ACN percentage (H_2O/AcOH, 99/1, *v/v* to ACN/AcOH, 99/1, *v/v*) on the signal intensities of cytochrome c in LNASI MS. These results illustrated that the signal intensities of multiply charged ions of cytochrome c were increased with increasing volume of ACN up to H_2O/ACN/AcOH, 49/50/1 (*v/v/v*). Above this, signal intensities of multiply charged ions of cytochrome c were drastically decreased. Therefore, we selected H_2O/ACN/AcOH, 49/50/1 (*v/v/v*) as optimal modifier volume for production of multiply charged ions of cytochrome c with increased signal intensities in LNASI MS (Fig. 4a). Importantly, LNASI produced multiply charged ions of cytochrome c (5.0 μM) at ambient temperature that are identical in charge state distribution to ions produced by ESI at H_2O/ACN/AcOH, 49/50/1, *v/v.v* (Fig. 4).

3.2. Effect of nebulizer set-up in LNASI MS

To perform an efficient LNASI, the optimal distance between nebulizer and capillary heated tube (the skimmer of mass analyzer) is very important (Fig. 1 and Supporting Information of Fig. S1). Therefore, we studied that LNASI mass spectra of cytochrome c at 1.0 and 1.5 cm distances between nebulizer and the skimmer of mass analyzer and obtained mass spectra were depicted in Fig. S3 of Supporting Information. These results revealed that the maximum multiply charged ions were observed with good signal intensities by setting-up nebulizer at 1.0 cm distance from the skimmer (Fig. 1). Since, nebulizer distance increases the probability of entering charged ions are greatly decreased, thus decreasing signal intensities by increasing background noise (Fig. S3b of Supporting Information). Table 1 illustrates that operated instrumental conditions for both LNASI and ESI such as sample flow rate, voltage, current, capillary voltage and temperature. We found that LNASI produced multiply charged ions of cytochrome c, just as like ESI and SSI [17].

3.3. Multiply charged proteins by LNASI MS

The capability of LNASI MS was further investigated for the identification of multiply charge states of various proteins including insulin, ubiquitin, α-lactalbumin, myoglobin and BSA, respectively.

3.3.1. Multiply charged ions of insulin

Insulin is a small protein consisting 51 amino acids with molecular weight 5733 Da. We studied that LNASI for production of charged ions of insulin (2.0 μM) by using solutions H_2O/AcOH, 99/1 (*v/v*) (Fig. 5a), H_2O/ACN/AcOH, 49/50/1 (*v/v/v*) (Fig. S4a of Supporting Information) and H_2O/MeOH/AcOH, 49/50/1 (*v/v/v*) (Fig. 4b of Supporting Information). These results revealed that maximum charge states (3+ to 5+) of insulin were observed by using solution H_2O/AcOH, 99/1 (*v/v*), since the maximum charge states of insulin is dependent on the proton affinity of less volatile solvent [31].

3.3.2. Multiply charged ions of ubiquitin

Ubiquitin consists 76 amino acids (12 basic and 11 acidic residues) with molecular weight 8565 Da. Multiply charged ions of ubiquitin (5.0 μM) were studied by using solutions H_2O/ACN/AcOH, 49/50/1, *v/v/v* (Fig. 5b), H_2O/AcOH, 99/1, *v/v* (Fig. S5a of Supporting Information) and H_2O/MeOH/AcOH, 49/50/1, *v/v/v* (Fig. S5b of Supporting Information). These results indicated that the best LNASI mass spectrum of ubiquitin was generated with maximum multiply charged states (5+ to 11+) by using solution H_2O/ACN/AcOH, 49/50/1, *v/v/v* (Fig. 5b).

3.3.3. Multiply charged ions of α-lactalbumin

α-lactalbumin (molecular weight 14175 Da) is an important whey protein and present in cow and many mammalian milks. We also studied that LNASI for production multiply charged ions of α-lactalbumin by using solutions H_2O/ACN/AcOH, 49/50/1, *v/v/v* (Fig. 5c), H_2O/AcOH, 99/1, *v/v* (Fig. S6a of Supporting Information) and H_2O/MeOH/AcOH, 49/50/1, *v/v/v* (Fig. S6b of Supporting Information). These results showed that LNASI was effectively produced multiply charge states of α-lactalbumin from 8+ to 15+ by using solution H_2O/ACN/AcOH, 49/50/1, *v/v/v*. Moreover, the higher charge states of α-lactalbumin (+13 and +15) are disappeared by using solution H_2O/MeOH/AcOH, 49/50/1, *v/v/v* (Fig. S6b of Supporting Information) which is due to the change in solution-phase conditions [32].

3.3.4. Multiply charged ions of myoglobin

Myoglobin (molecular weight 16951 Da) is a globular all-helical protein, consisting 153 amino acids. Its native form can be characterized by a tightly folded conformation and a heme group which is non-covalently bound with a hydrophobic pocket [33]. The substantial unfolding of polypeptide chain and the disruption of noncovalent heme-protein interaction can be observed by acid-induced denaturation of proteins. The charge states of myoglobin are mainly due to the denaturation and the reconstitution of acid-denatured proteins [34,35]. Fig. 6a and

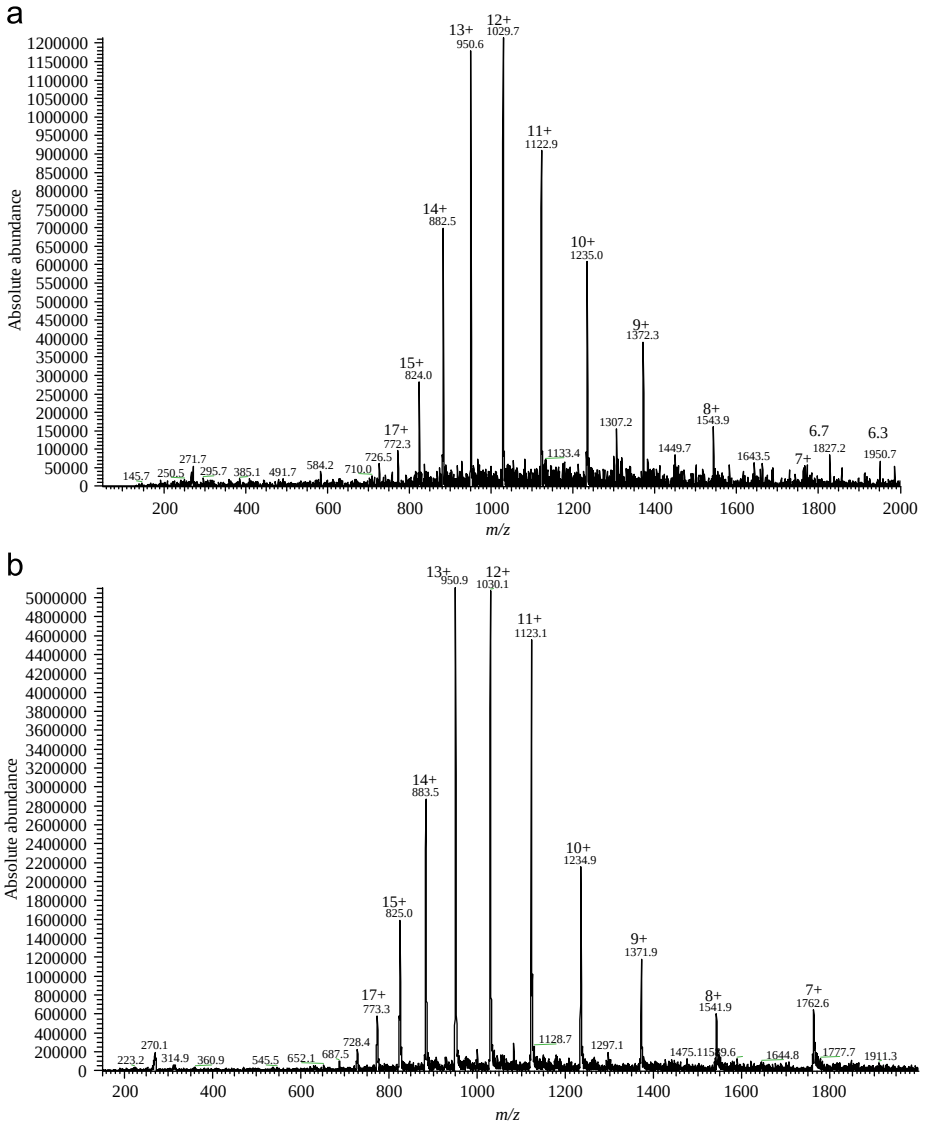

Fig. 4. (a) LNASI mass spectrum of cytochrome c (5.0 μM) using solution H_2O/ACN/AcOH, 49/50/1, *v/v/v*. (b) ESI mass spectrum of cytochrome c (5.0 μM) using solution H_2O/ACN/AcOH, 49/50/1, *v/v/v*.

Fig. S7 of Supporting Information display LNASI mass spectra of myoglobin by using solutions H_2O/AcOH, 99/1, *v/v*; H_2O/ACN/AcOH, 49/50/1, *v/v/v* and H_2O/MeOH/AcOH, 49/50/1, *v/v/v*, respectively. These results showed that multiply charged ions of myoglobin (charge states +9 to +21) were effectively generated by using solution H_2O/AcOH, 99/1, *v/v* (Fig. 6a). It can be noticed that multiply charged ions were produced with poor signal intensities by using solutions H_2O/ACN/AcOH, 49/50/1, *v/v/v* and H_2O/MeOH/AcOH, 49/50/1, *v/v/v* (Fig. S7 of Supporting Information). Furthermore, the highly charged myoglobin ions (10+ to 29+) were produced by using 1% (*v/v*) AcOH [36] and TFA vapor [9] in ESI.

3.3.5. *Multiply charged ions of BSA and cytochrome c*

BSA is a serum albumin protein consisting 607 amino acid residues with molecular weight 66399 Da. Fig. 6b and Fig. S8 of Supporting Information indicate that LNASI mass spectra of BSA by using solution H_2O/AcOH, 99/1, *v/v*; H_2O/ACN/AcOH, 49/50/1, *v/v/v* and H_2O/MeOH/AcOH, 49/50/1, *v/v/v*, respectively. The results indicate that the highly charged BSA ions (34+ to 53+) were effectively produced by using solution H_2O/AcOH, 99/1, *v/v* (Fig. 6b). On the other hand, multiply charged BSA ions were not observed by using solutions H_2O/ACN/AcOH, 49/50/1, *v/v/v* and H_2O/MeOH/AcOH, 49/50/1, *v/v/v* (Fig. S8 of Supporting Information). Table 2 represents the molecular weights, number of amino acid residues, optimal volume of modifier and observed multiply charge ions of various proteins in LNASI MS. These results provided evidence that multiply charge states of proteins can be depended on the modifier and the presence of disulfide bridges that can lead to various ionization sites or different conformations around protonated sites [37]. In fact, multiply charged proteins can also be easily produced from the solid matrix without electric field or laser source [38]. Furthermore, LNASI produced multiply charged ions are very similar to our ESI produced spectra of proteins (cytochrome c ESI mass spectrum is shown in Fig. 4b, the other spectra are not shown) and reported ESI methods [9,10,12,36]. After tuning the best conditions for LNASI and ESI, we compared absolute intensities of cytochrome c in both LNASI and ESI instruments (Fig. 4). From these results, the signal intensities of LNASI mass spectrum of cytochrome c is 2-times lower than ESI MS, however, LNASI MS produced ESI-like multiply charged

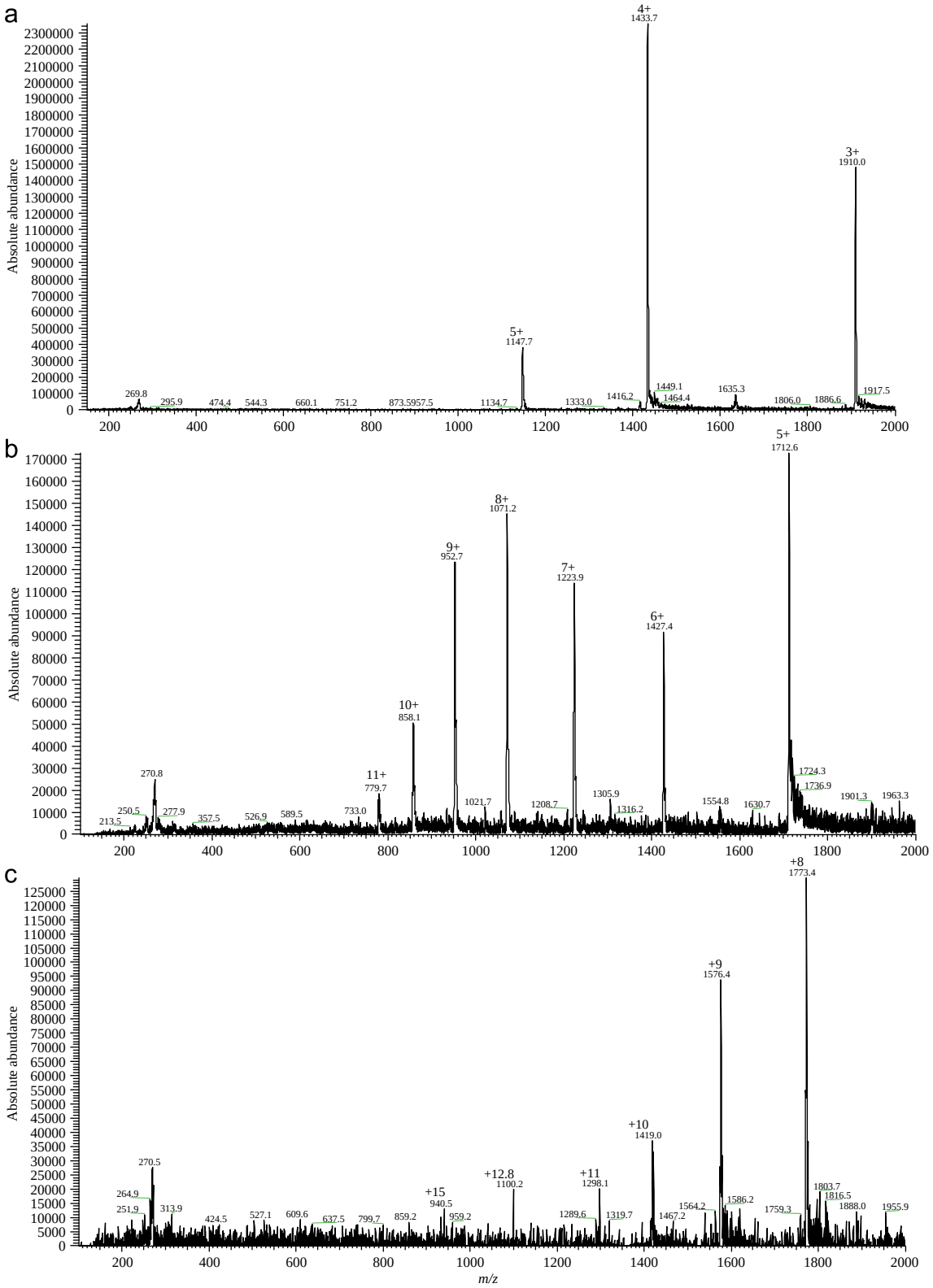

Fig. 5. LNASI mass spectra of (a) insulin (2.0 μM) using solution at H_2O/AcOH, 99/1, *v/v*, (b) ubiquitin (5.0 μM) at H_2O/ACN/AcOH, 49/50/1, *v/v/v* and (c) α-lactalbumin (5.0 μM) at H_2O/ACN/AcOH, 49/50/1, *v/v/v*.

proteins. Moreover, LNASI MS was effectively identified multiply charged ions of cytochrome c at 400 nM, and obtained spectrum was shown in Fig. 6c. These results revealed that LNASI MS was successfully produced multiply charged proteins at low concentration without heat or electric field.

3.4. Peptide mixture by LNASI

To demonstrate the feasibility of LNASI MS for peptide mixture analysis, we examined that the ability of LNASI MS for the analysis of peptide mixture (HW6, Ang II and valinomycin) using

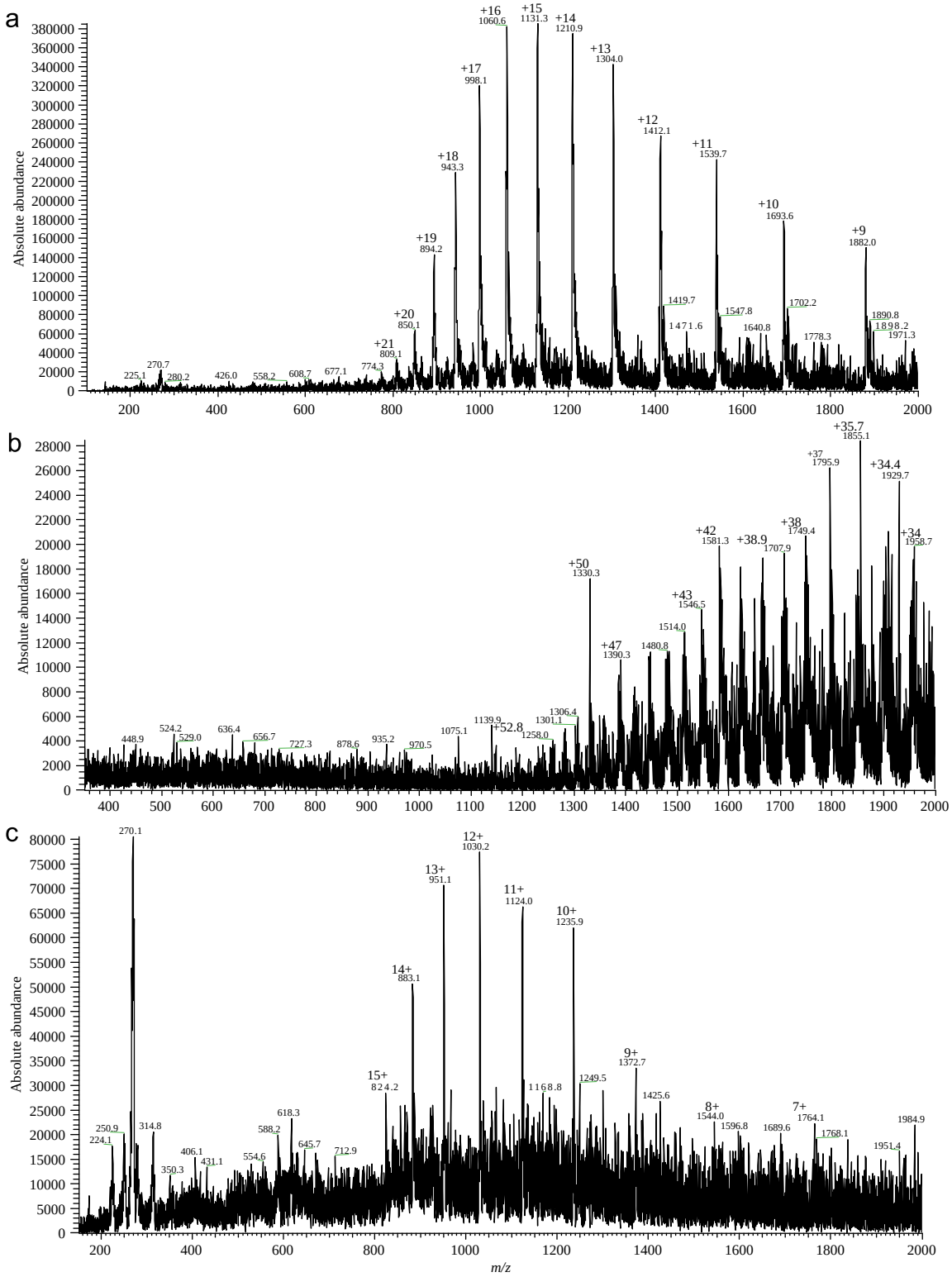

Fig. 6. LNASI mass spectra of (a) myoglobin (5.0 μM) using solution at H_2O/AcOH, 99/1, *v/v*, (b) BSA (5.0 μM) at H_2O/AcOH, 99/1, *v/v* and (c) cytochrome c (400 nM) at H_2O/ACN/AcOH, 49/50/1, *v/v/v*.

solution H_2O/ACN/AcOH, 49/50/1, *v/v/v* (Fig. 7a). This result showed that LNASI MS has well ability to detect peptides and generated ions at *m/z* 905, 1045, 1128 and 1810 which corresponded to $[HW6+H]^+$, $[Ang\ II+H]^+$, $[Val+H]^+$ and $[2HW6+H]^+$, respectively. These results confirmed that LNASI MS is effectively identified peptide ions from the peptide mixtures.

3.5. Arginine and glutathione clusters by LNASI MS

Cluster ions of amino acids can be produced in ESI MS [39]. Thus, the applicability of LNASI MS was also examined for the identification of amino acid clusters. Fig. 7b and c and Fig. 8 display LNASI and ESI mass spectra of arginine and glutathione by using solution H_2O/ACN/AcOH, 49/50/1, *v/v/v*. Fig. 7b and c shows

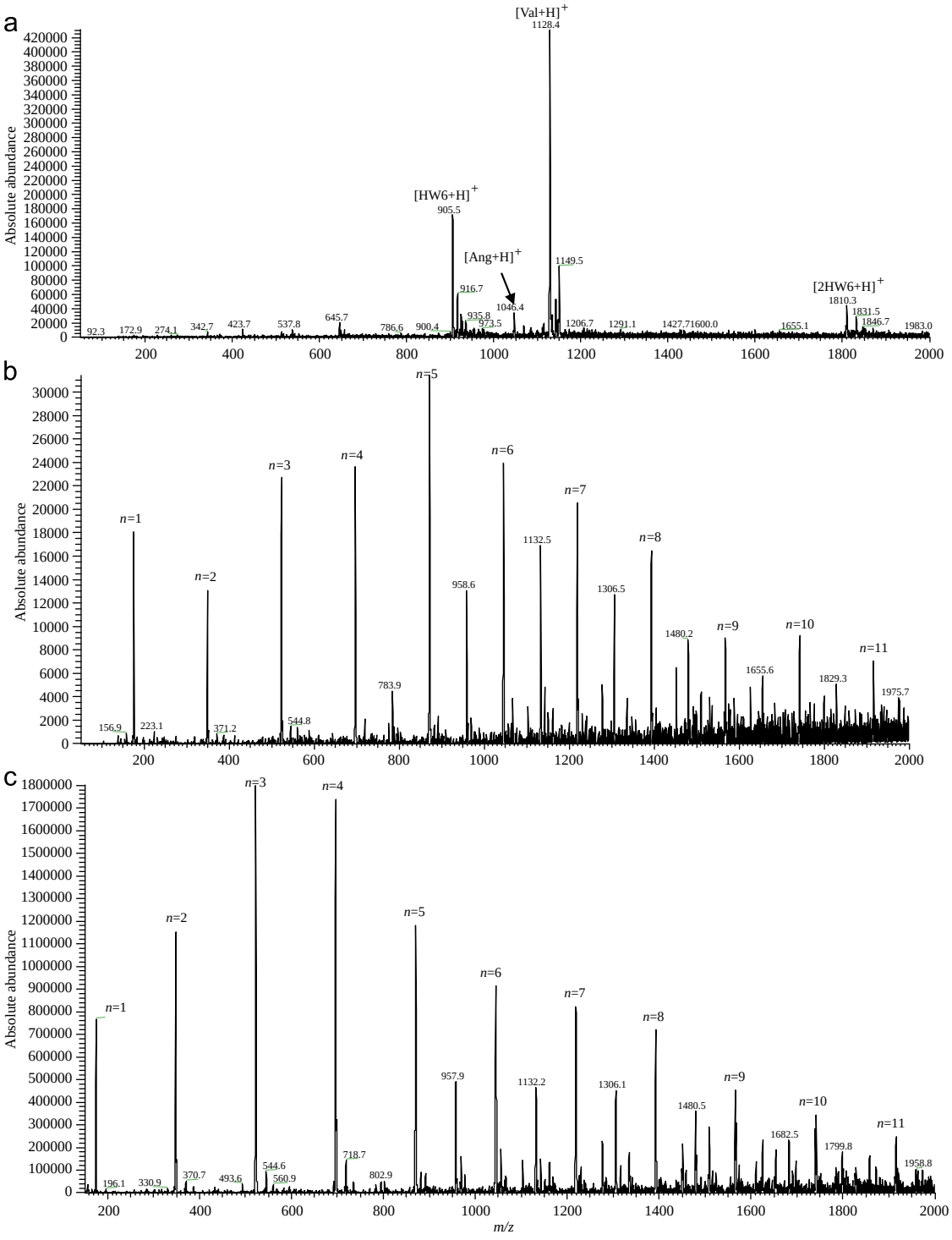

Fig. 7. LNASI mass spectra of (a) peptide mixture (HW6 5.0 μM; Ang II 3.0 μM and valinomycin 0.5 μM) at H_2O/ACN/AcOH, 49/50/1, *v/v/v*, (b) arginine (2.5 μM) using solution at H_2O/ACN/AcOH, 49/50/1, *v/v/v*. (c) ESI mass spectrum of arginine (2.5 μM) using solution at H_2O/ACN/AcOH, 49/50/1, *v/v/v*.

the LNASI- and ESI-mass spectra of arginine clusters $[(Arg)_n+H]^+$ ranging from $n=1$ (175 Da) to $n=11$ (1916 Da). It can be noticed that ESI mass spectrum of arginine shows the maximum signal intensity of arginine cluster at $n=3$ $[(Arg)_3+H]^+$ (523 Da) (Fig. 7c). Interestingly, LNASI mass spectrum of arginine shows the maximum signal intensity of arginine cluster at $n=5$, $[(Arg)_5+H]^+$ (870 Da) (Fig. 7b). Fig. 8 shows LNASI- and ESI-mass spectra of glutathione. These results indicated that LNASI and ESI were produced same glutathione clusters $[(Glu)_n+H]^+$, ranging from $n=1$ (308 Da) to $n=6$ (1844 Da). Importantly, it can be noticed that LNASI MS shows the maximum signal intensity of glutathione cluster at $n=6$ $[(Glu)_6+H]^+$ (*m/z* 1844) (Fig. 8a). Meanwhile, ESI MS shows the maximum signal intensity of glutathione cluster at $n=1$ ($[Glu+H]^+$, *m/z* 308) (Fig. 8b). These results clearly indicated that LNASI was effectively produced amino acid clusters and showed superior signal intensities for large clusters of arginine ($n=5$) and glutathione ($n=6$) than that of ESI.

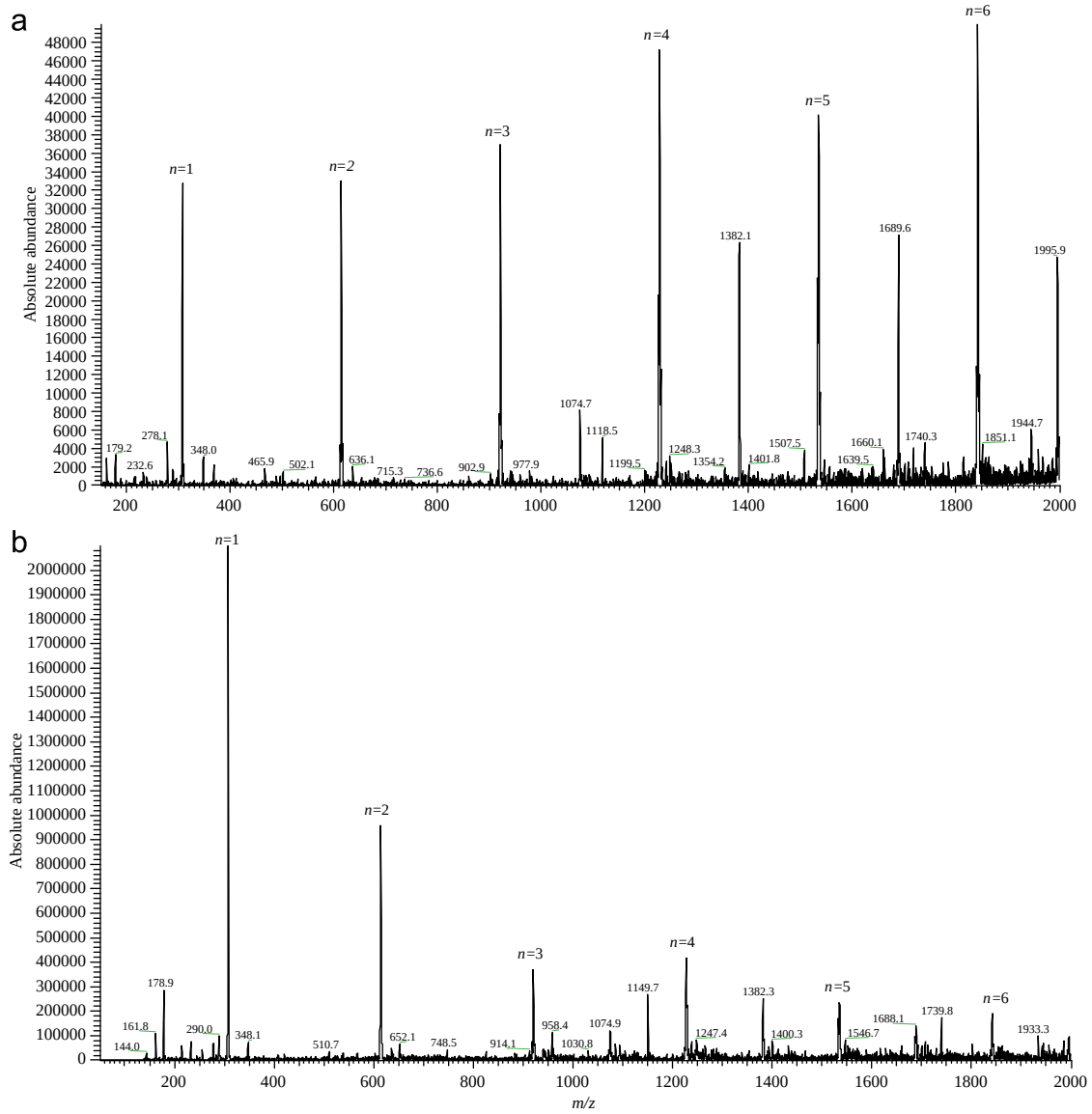

Fig. 8. (a) LNASI mass spectrum of glutathione (2.5 μM) using solution at H_2O/ACN/AcOH, 49/50/1, *v/v/v*. (b) ESI mass spectrum of glutathione (2.5 μM) using solution at H_2O/ACN/AcOH, 49/50/1, *v/v/v*.

3.6. Comparison of LNASI with ESI and CSI

The developed LNASI MS method is compared with ESI and CSI methods relating to instrumental conditions and molecule analysis (Tables 1 and 2). It can be noticed that LNASI was achieved by using a simple nebulizer for automate sample spray through liquid nitrogen flow without electric voltage and temperature control. Meanwhile, ESI was achieved by applying high electric voltage (3–5 kV) through heated capillary tube. In CSI, charged ions are produced by electrospray or ion spray ionization probe using desolvation chamber at low temperature. Typically, CSI requires two sophisticated apparatus such as cold-spray ion sources and special cooling devices for the precise temperature control from −80 to +15 °C [20]. Therefore, CSI was produced fine droplet charged ions with increased polarizability at low temperature. As shown in Fig. 1, LNASI requires only a simple nebulizer for the sample spray through liquid nitrogen. Importantly, it can be noticed that LNASI sample flow (250–300 μL/min) is larger than that of CSI, SSI and V-EASI methods [17,20,24]. Since, SSI [17], CSI [20] and V-EASI [24] methods have sample flow at 8.0, 30.0 and 5–20 μL/min by using very small diameter needles (i.d., 0.25 mm–SSI; i.d., 0.1 mm–V-ESI) when compared with LNASI (nebulizer needle–I.d., 0.45 mm and Teflon tube–I.d., 0.99 mm). Table 2 shows that the comparison of LNASI, SSI and V-EASI methods with regard to multiply charged proteins (cytochrome c and myoglobin), modifier volume and used analytes in sonic spray ionization methods [18,24]. Based on the above results, LNASI MS is effectively identified multiply charged proteins, peptides and amino acid clusters without voltage and heat at ambient conditions.

4. Conclusions

This paper demonstrates that an alternative paradigm for production of multiply charged ions by open probe liquid nitrogen assisted spray ionization mass spectrometry. This system was effectively produced high speed fine droplets from the sample solution by passing liquid nitrogen without heat or voltage and pumping machine. The highly charged droplets are directly passed into mass analyzer to produce ESI-like ions. Another key attractive feature of LNASI is simple and successfully detected

multiply charged proteins, peptide mixture and amino acid cluster ions at ambient temperature without voltage, heat and sample pumping machine, just as like ESI. Thus, this study confirms that LNASI MS is a new ionization method for identification of multiply charged ions of biomolecules (proteins, peptides and amino acids) without heat or voltage at ambient condition.

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Appendix A. supplimentry information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.011.

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