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Pneumatically assisted electrospray-ion mobility spectrometry for quantitative analysis of intact proteins

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ABSTRACT

In this work, quantitative analysis of intact proteins using ion mobility spectrometry (IMS) is introduced. For this purpose a pneumatically assisted electrospray ionization source with a new design was constructed. Liquid and nebulizer gas flow rates were optimized to achieve the highest response. The pneumatically assisted electrospray-IMS was used for quantitative analysis of insulin, bovine serum albumin (BSA) and human serum albumin (HSA). The analysis of proteins demonstrated that sensitivity of the pneumatically assisted electrospray-IMS is two to three times higher than that of conventional electrospray ionization (ESI) coupled to IMS. The linear dynamic ranges for insulin, BSA and HSA were found to be 1–75, 5–100 and 2–100 $\mu g\ mL^{-1}$ with detection limits of 0.3, 1 and 0.6 $\mu g\ mL^{-1}$, respectively. The relative standard deviations (RSD) for five replicate measurements of insulin, BSA, and HSA at 25 $\mu g\ mL^{-1}$ were recorded as 3, 4 and 1.5%, respectively. The proposed method can be considered as an alternative way for quantitative protein analysis.

1. Introduction

Proteins are complex macromolecules which constitute the building blocks of any living organism and carry out the majority of biological functions, from serving as enzymatic catalysts to protecting the immune system as antibodies. Thus, the quantitative determination of proteins is extremely important in many areas, such as biochemistry, biotechnology and immunodiagnostics. The common quantitative analysis of proteins include the Lowry [1], Bradford [2], biuret reaction [3], bromophenol blue [4] and bromocresol green [5] procedures. These methods generally have some limitations, such as low sensitivity, poor selectivity, instability, narrow linear ranges, slow reactions, and complicated procedures [6]. In recent years, novel methods have been developed for the protein analysis such as spectrophotometry [7], spectrofluorimetry [8], resonance light scattering (RLS) [9], chemiluminescence [10] and electrochemical assays [11]. Nevertheless, these methods require pretreatment and suffer from drawbacks such as high reaction temperature, rigorous acid conditions, relatively long analysis times and lack of selectivity [6].

The IMS is a technique that allows analyte to be detected based on the gas phase mobility, when produced ions travel through a drift tube under the influence of an electric field. The ion mobility depends on the mass, charge and collision cross section (i.e., size and shape) of analyte. The main advantages of this technique are the low detection limit, fast response, simplicity and portability [12]. The separation, detection, and identification of biomolecules with high molecular mass and low volatility such as amino acids, peptides and proteins have been explored using ESI-IMS. The ESI is a soft ionization method, which transfers intact molecules in the liquid phase into gas phase ions. The ESI-IMS was first introduced by Dole and co-workers in 1972 [13]. They reported the spectra of lysozyme with three broad peaks, too broad to be useful. Dole and coworkers [14] showed also the ion mobility spectra of electrosprayed polystyrene oligomers with broad peaks. They reported that low ion mobility resolution in these two works was due to inefficient desolvation of the electrosprayed droplets and concluded that ESI would be useful only for mass spectrometry. Fenn in 1984 [15] demonstrated an attractive and unique characteristic of ESI-mass spectrometry in which macromolecules such as proteins could produce multiply charged ions. Henion and co-workers in 1987 [16] introduced pneumatically assisted ESI for mass spectrometry. In the pneumatically assisted electrospray, a co-axial nebulizing nitrogen gas is utilized to assist the dispersion of a sample solution and to form charged aerosols. Other advantages of pneumatically assisted electrospray are the possibility of using a higher liquid flow rate and the coupling of a high performance liquid chromatograph (HPLC) to a mass spectrometer [17]. The effective use of ESI-IMS was delayed until the late 1980s and 1990s, when an improved

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ESI source for IMS was developed. The modifications included using a counter flow of heated and dry nitrogen drift gas [18] and a cooled electrospray needle [19,20]. The ion mobility spectrum of cytochrome C protein showed an improvement in both sensitivity and resolution. These studies revealed that intact proteins could be electrosprayed under atmospheric conditions. Guevremont and co-workers in 1997 [21] reported the IMS-mass spectrometry (IMS-MS) of insulin and cytochrome C proteins using a new design for ESI. They used a countercurrent stream of nitrogen into the electrospray chamber, allowing only ions and relatively small droplets to introduce into the IMS-MS. The ESI-IMS-MS has been widely used to examine the gas-phase conformations of peptides and proteins [22,23] as well as separation and sequencing of proteins after tryptic digestion to peptides [24,25]. A new design for (ESI-IMS) was introduced by our group in 2007 [26]. In this design the electrospray needle was located outside the cell oven and a desolvation gas was also used to speed up the desolvation process. The advantages of this system were preventing needle clogging, increasing peak intensity and improving resolving power of the apparatus.

In this work, a pneumatically assisted ESI with a new design has been introduced for the IMS. This technique was used for the analysis of intact proteins including insulin and serum albumin. Insulin is a pancreatic hormone protein with low molecular weight (~6 kDa) that is essential for metabolism of carbohydrates and regulation of glucose level in human blood [27]. An inadequate level of insulin in the blood results in the onset of diabetes mellitus. Serum albumin (~67 kDa) is the most abundant protein in blood plasma which functions as a transport protein for numerous endogenous and exogenous substances. It also plays an important role in regulation of colloid osmotic pressure of blood [28].

The pneumatically assisted electrospray uses a nebulizing gas, usually N_2, co-axial to the spraying needle to facilitate droplet formation and evaporation. In pure ESI, droplet formation is caused by instability of the liquid surface due to surface charging, while in pneumatically assisted electrospray, the droplet formation is assisted by the nebulizing gas. This modification allows using higher liquid flow rates, liquids with higher surface tension and solutions with higher conductivity compared to ESI [29]. These advantages result a higher sensitivity and high throughput analysis. Previous works on the analysis of proteins by ESI-IMS have been focused on detection and sequencing of amino acids in proteins. However, the objective of this work is quantitative analysis of intact proteins using pneumatically assisted electrospray-IMS. The performance of pneumatically assisted electrospray-IMS for the analysis of the proteins was compared with that of ESI-IMS.

Table 1
Typical experimental parameters.

Operating parameter	Setting
Mode of operation	Positive
Drift region length (cm)	13
Desolvation region length (cm)	6
Drift field (V cm^{-1})	450
Desolvation field (V cm^{-1})	450
Needle voltage (kV)	10.9
Counter electrode voltage (kV)	8
Shutter grid pulse (ms)	0.4
Drift and desolvation gas	Nitrogen
Drift gas flow rate (ml min^{-1})	500
Desolvation gas flow rate (ml min^{-1})	900
Cell temperature (°C)	165
Solvent	MeoH/H_2O/Ac (50:48:2 v/v)
Liquid flow rate (μL min^{-1})	6
Scan time (ms)	40
Number of point per ion mobility spectrum	500
Number of averaged IMS spectra	100

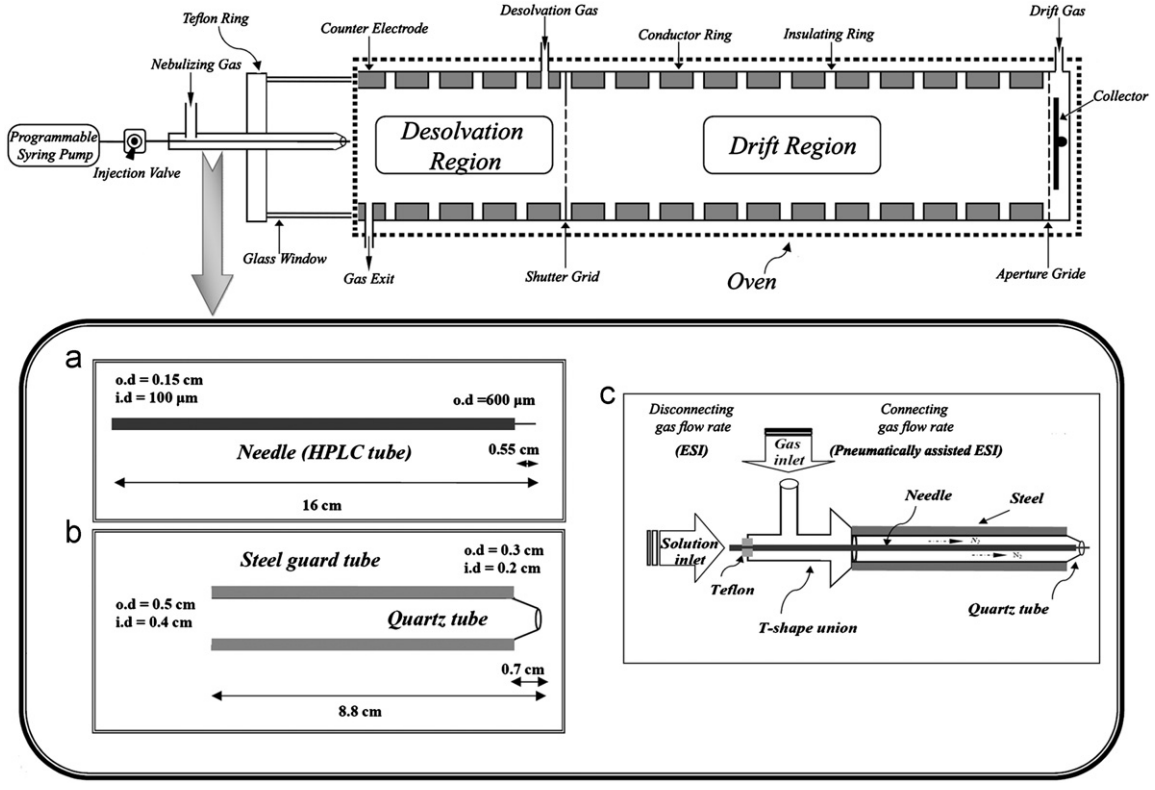

Fig. 1. A schematic diagram of the ion mobility spectrometer apparatus (Top). The pneumatically assisted electrospray and ESI ionization source (Bottom). (a) HPLC tube as a needle, (b) nebulizer and (c) overall schematic diagram.

2. Experimental

2.1. Chemicals and solutions

Human insulin as a pure powder was purchased from Exirpharma Co. BSA as pure crystals (> 98%) was prepared from Sigma-Aldrich Co. HSA as an injective drug (purity > 96%) was obtained from Biotest Co. Methanol (HPLC grade) and glacial acetic acid (99.5%) were purchased from Merck. Deionized water was used throughout the expriments and prepared by a water purification system (Overseas Equipment & Services).

2.2. Sample preparation

Stock standard 1000 μg mL^{-1} solutions of human insulin, BSA and HSA were prepared in a solvent mixture containing water, methanol and acetic acid in a volume ratio of 48/50/2. Working solutions were prepared by successive dilution of the stock

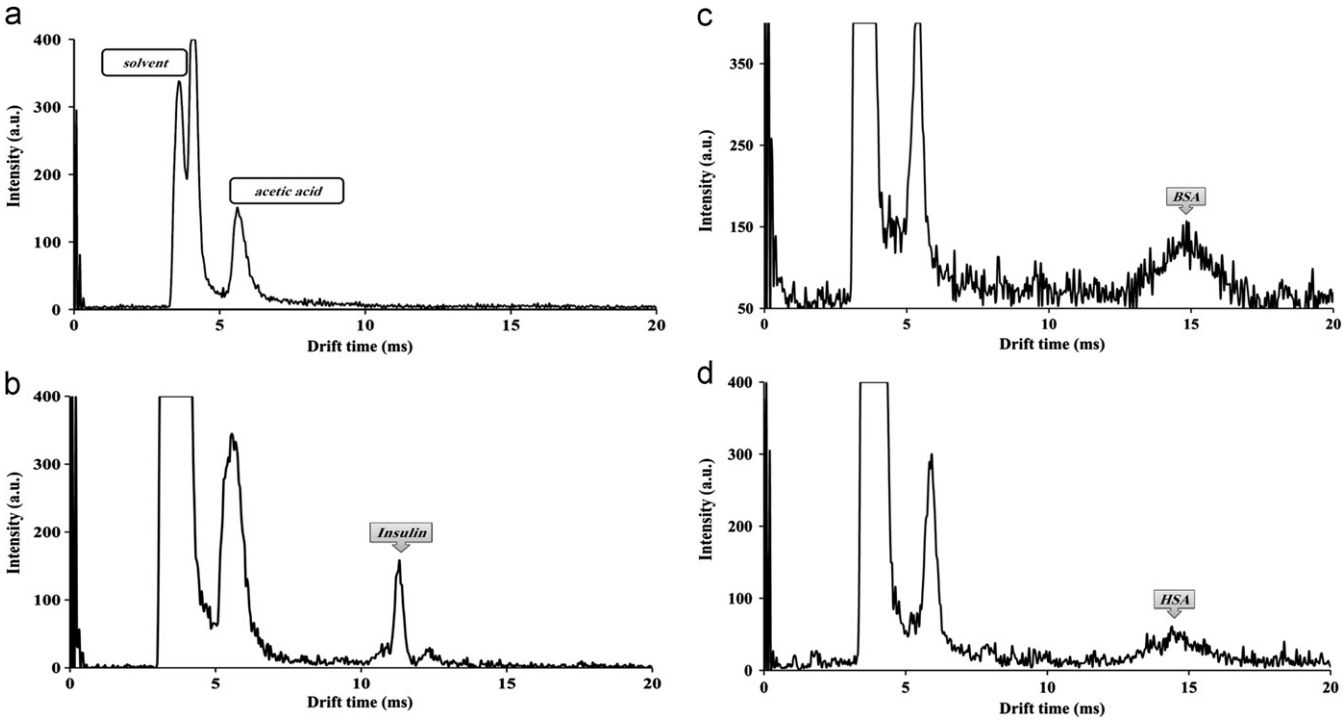

Fig. 2. (a) The IMS spectrum of a mixture of methanol/H_2O/acetic acid (50:48:2 v/v) at the operating conditions (Table 1). Electrospray ion mobility spectra of the proteins (b) Insulin (75 μg mL^{-1}), (c) BSA (100 μg mL^{-1}) and (d) HSA (50 μg mL^{-1}).

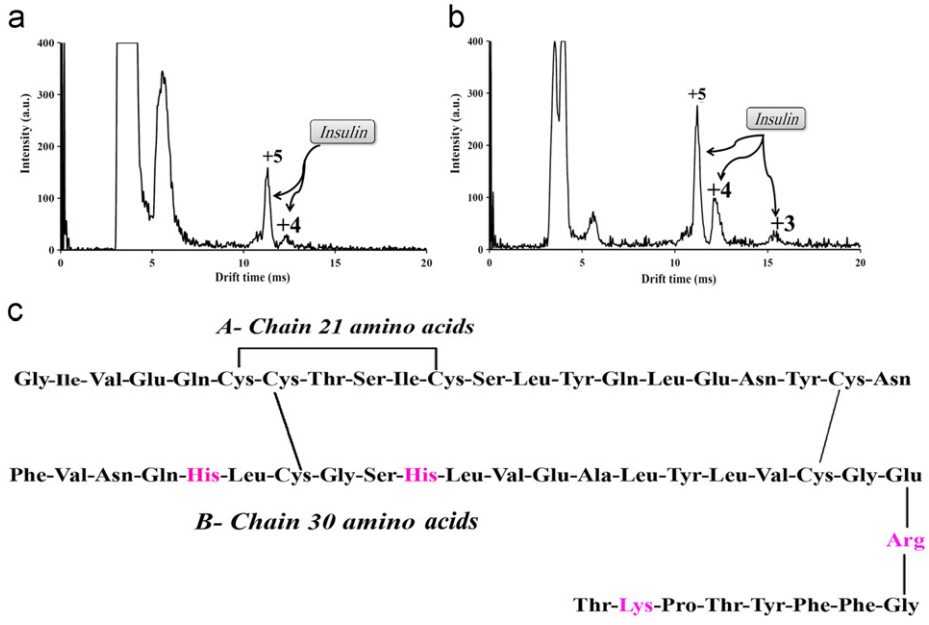

Fig. 3. The ESI-IMS spectra of insulin at two different concentrations: (a) 75 μg mL^{-1}, showing almost two individual ion peaks and (b) 100 μg mL^{-1}, for which a new peak appears. (c) Amino acid sequence of insulin illustrating the presence of basic amino acids (Lysine, Histidine, Arginine) corresponding to multiply charge states in positive mode of ESI-IMS.

solutions. Since the purpose of this work was the analysis of the proteins in the positive IMS mode, the small amount of acetic acid in the solvent mixture was sufficient to lower the pH below the isoelectric point of the proteins. All the sample solutions were filtered through a 0.22 μm PTFE syringe filter (Jet Biofil Co.), stored at4 °C and protected from light prior to use.

2.3. Instrumentation

The IMS apparatus was constructed in our group at Isfahan University of Technology. The major components of this instrument are an ionization source, an atmospheric pressure ion mobility tube, two high voltage power supplies, a pulse generator and a data acquisition system. The ion mobility tube was constructed from stacked aluminum rings alternating with PTFE insulating rings. The conducting and the insulating rings were 1 and 0.16 cm wide, respectively. The inner and outer diameters of the rings were 3.5 and 8 cm, respectively. The conducting rings were connected in series by a chain of 5.6 MΩ resistors to form a uniform electric field in the ion mobility tube and to guide ions towards the collector plate at the end of the tube. The tube consists of two sections: a desolvation and a drift region separated by a Bradbury-Nielsen ion gate that made of two series of parallel wires biased to a potential for blocking ion passage to the drift tube. The grid potential is removed for a short period of time by the pulse generator at a frequency of 25 Hz, allowing ion packets to enter into the cell. An aperture grid ring (stainless steel screen, 37-mesh) and a Faraday plate detector (positioned ~1 mm behind the aperture grid) were located at the end of the drift tube. Nitrogen after passing through a 13X molecular sieve (Fluka) served as the desolvation and drift gases at flow rates of 900 and 500 mL min^{-1}, respectively. Temperature of the IMS drift tube was maintained at 165 °C by an oven. Electronic parts of this instrument included two high-voltage power supplies for the spray needle and the drift field (isolated ±5 kV and non-isolated ±10 kV, respectively); a current to voltage preamplifier with a gain of 10^9 V A^{-1} to amplify the ion current produced by the Faraday plate; a personal computer with a high-speed A/D module (12-bit dynamic range), and data acquisition software to collect ion mobility spectra of the analytes.

Fig. 1 shows a schematic diagram of the pneumatically assisted electrospray and ES-IMS.

The spray needle was placed completely outside the heating oven [26]. In this design, the spray needle was made from a 16 cm long stainless steel tube (HPLC type) with 0.1 mm i.d. and 1.5 mm o.d. The outer diameter of the tube tip (5.5 mm) was reduced to 0.6 mm in order to produce a stable spray (Fig. 1a). The spray needle was inserted into an 8.8 cm long quartz tube

Table 2
Multiply charge state for insulin in ESI.

Multiply charge state	Reduced mobility (K_o)	
	Klopsch report	This work
+5	0.73	0.786
+4	0.69	0.716
+3	0.54	0.580

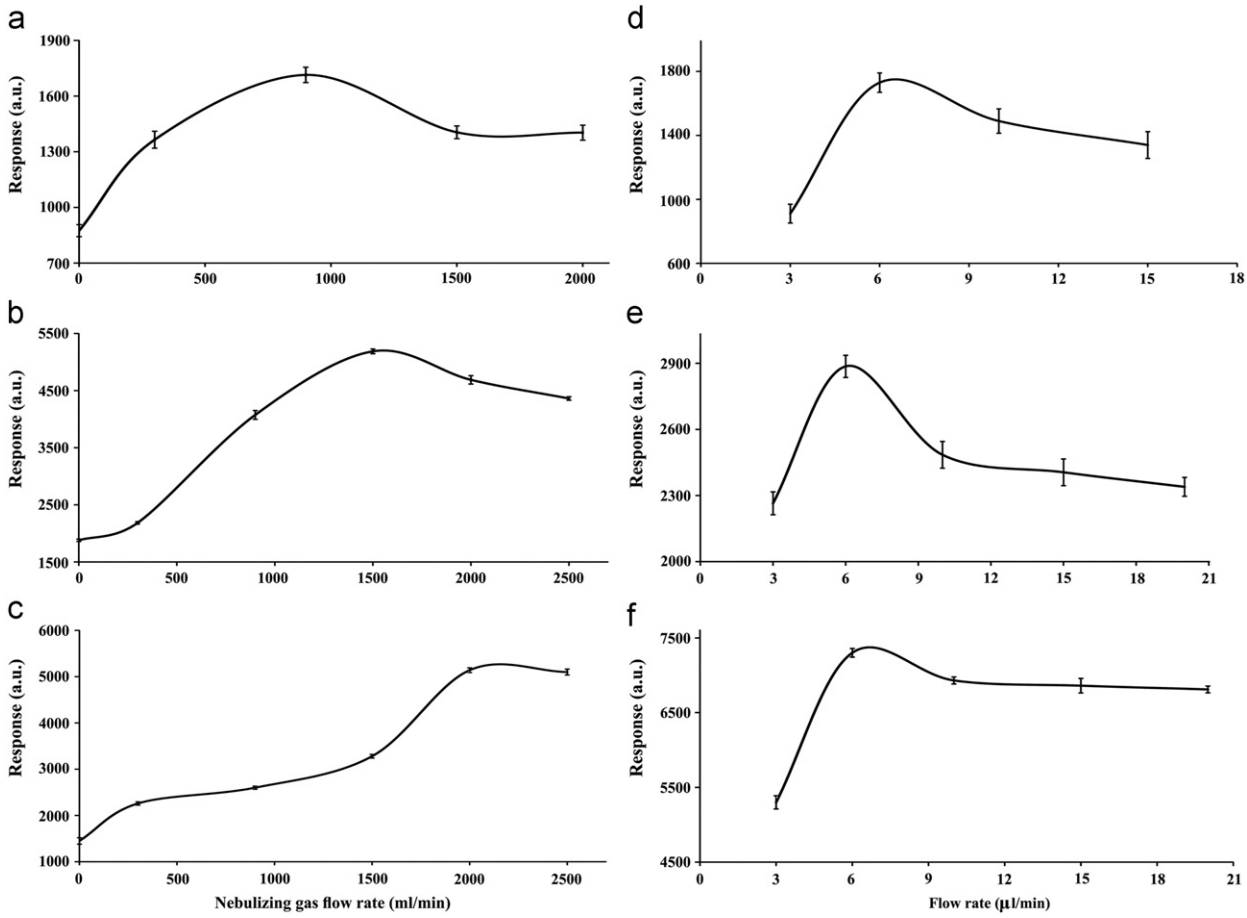

Fig. 4. The Effect of nebulizing gas flow rate and liquid flow rate on the IMS response for the three proteins at the operating conditions given in Table 1. (a,d) insulin, (b,e) BSA and (c,f) HSA.

(5 mm o.d. and 4 mm i.d.). The diameter of the quartz tip was gradually reduced to 2 mm i.d. The spray needle tip was protruded by about 3 mm from the quartz tube. A metal tube was used to protect the quartz tube except at the tip (Fig. 1b). The end of the tubes was connected to the nebulizing gas and syringe pump through a T-shape union (Fig. 1c). Typical operating conditions for obtaining ion mobility spectra of the compounds are listed in Table 1.

3. Results and discussion

3.1. ESI-IMS analysis of intact proteins

Fig. 2a illustrates the ion mobility spectrum of the solvent (water, methanol and acetic acid in a volume ratio of 48/50/2) at the experimental conditions listed in Table 1. Also the ESI-IMS spectra of human insulin (75 μg mL^{-1}), BSA (100 μg mL^{-1}) and HSA (50 μg mL^{-1}) are shown in Fig. 2(b, c and d, respectively).

Insulin at low concentrations shows two peaks, as shown at Fig. 3a; but at higher concentrations (up to 75 μg mL^{-1}) a new peak appears and a total of three peaks are observed (Fig. 3b). Guevremont and co-workers [21] also observed three relatively broad peaks in the IMS spectrum of bovine insulin, which were recognized by time of flight as $[M+3H]^{3+}$, $[M+4H]^{4+}$ and $[M+5H]^{5+}$ ions. Klopsch [30] reported two dimensional IMS-MS spectra for bovine insulin with three charge states (+5, +4, and +3) and reduced mobility of the ions. The reduced mobility of the multiply charge ions produced by insulin in our study and the values reported by Klopsch are listed in Table 2. In this work, nicotinamide was used as a reference standard for the instrument (k_0=1.85 $cm^2\ V^{-1}\ s^{-1}$ [31]) and the reduced mobility values of all the insulin ions with unknown mobility (K_{0u}) were determined according to the following equation:

$$K_{0_u} = K_{0_{ref}} \times \frac{t_{d_{ref}}}{t_{d_u}} \quad (1)$$

where K_{0u} is the reduced mobility of the unknown. $t_{d\ ref}$ and $t_{d\ u}$ are the drift time of the reference standard and an unknown compound, respectively. Since the structures of human and bovine insulin are very similar and reduced mobility of the produced ions are very close, it might be concluded that the three observed peaks in our insulin spectrum (Fig.3b) also created from the multiply charge states of +5, +4 and +3.

In practice, the number of charge states in positive ESI of proteins depends on the number of basic amino acids (arginine, lysine and histidine) and also on the N-terminus in proteins. The sequence of the human insulin is shown in Fig. 3c. Insulin can get maximum of 5 protons corresponding to the number of sites with significant basicity in solution (two histidine, one arginine and one lysine residues together with the N-terminal primary amine group). Rauschenbach and co-workers [32] represented a distribution of molecular ion charge states from +25 to +70 in the ion mobility spectrum of BSA by mass spectrometry. Therefore, presumably the broad peak of BSA in our work is due to presence of these multiply charge state ions.

3.2. Pneumatically assisted electrospray-IMS analysis of intact proteins

3.2.1. Optimization of instrumental parameters

As discussed above, the pneumatically assisted ESI uses the nebulizing gas to enhance the nebulization efficiency and this modification allows using higher liquid flow rates relative to the conventional ESI. The effect of nebulizing gas flow rate on peak area of the samples was investigated and the results are shown in Fig. 4. The results for insulin (Fig. 4a) show that the IMS response increases by increasing the nebulizing gas flow rate from 0 to 900 mL min^{-1} and then decreases, possibly due to instability of the spray. Therefore, 900 mL min^{-1} was selected as the optimized flow rate for the analysis of insulin. Fig. 4b (for BSA) and Fig. 4c (for HSA) show similar trends, but with the optimum values of 1500 and 2000 mL min^{-1}, respectively. The reason for the higher optimum flow rates of the nebulizing gas for BSA and HSA relative to insulin might be attributed to higher molecular weights of these compounds.

Fig. 4 shows also the effect of liquid flow rate on the IMS response for the compounds at their corresponding optimized nebulizing gas flow rates. Fig. 4d shows that the IMS response for insulin increases by increasing the liquid flow rate from 1 to 6 μL min^{-1} and beyond that no significant increase is noticed, in spite of observing stable sprays. Fig. 4e for BSA and Fig. 4f for HSA show the same results as insulin. Therefore the flow rate of 6 μL min^{-1} was selected as the optimum for subsequent measurements. Hill et al. [20] have also investigated the effect of liquid flow rate on IMS response using electrospray as the ionization source.

3.2.2. Pneumatically assisted electrospray versus ESI

The pneumatically assisted electrospray- and ESI-IMS spectra of insulin, BSA and HSA were obtained and compared. Fig. 5

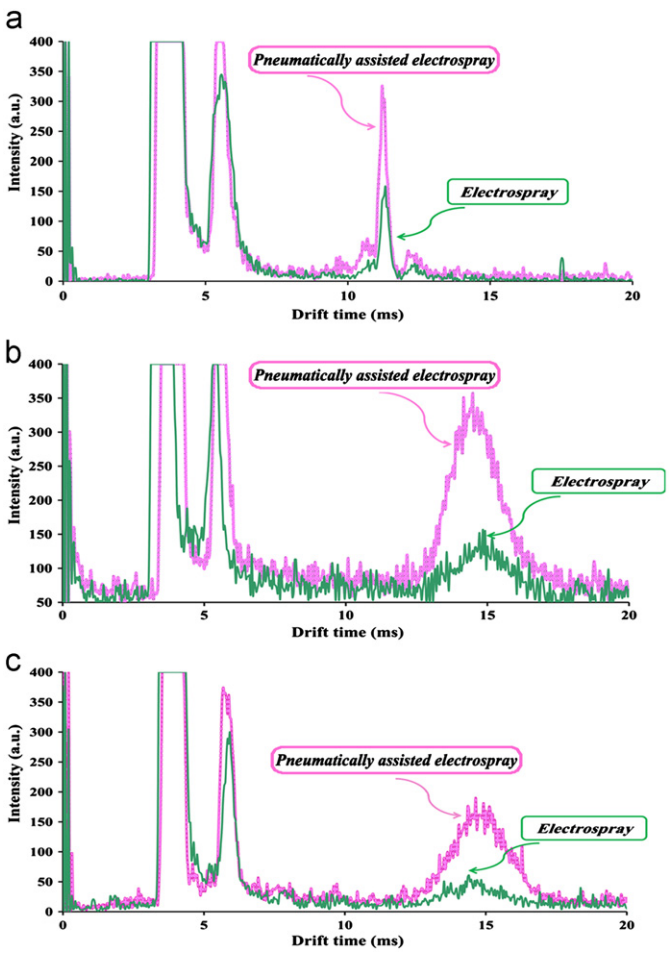

Fig. 5. Comparison of ion mobility spectra obtained from the three protein solutions (a: insulin, b: BSA and c: HSA) with EIS and pneumatically assisted electrospray ionization sources, indicating a more stable spray and higher sensitivity when pneumatically assisted electrospray is used as the ionization source to analyze intact proteins.

shows that, a significant improvement in the response can be observed when pneumatically assisted electrospray is used as the ionization source. The advantage of pneumatically assisted electrospray over ESI is more pronounced for HSA and BSA, because these compounds are more influenced by the nebulizing gas than insulin.

3.3. Quantitative analysis of intact proteins

The quantitative analysis of insulin, BSA and HSA were performed at the optimized conditions, by injecting 20 μL of each protein solution into the system and recording the spectra from first appearance until they completely disappeared. The average signal for each compound was calculated by averaging the recorded spectra and used as the response corresponding to the injected concentration. Fig. 6 shows the IMS and the average spectra.

The calibration plots for insulin, BSA and HSA are shown in Fig. 7. The analytical parameters were calculated and listed in Table 3. It can be noticed that the linear ranges for insulin, BSA and HSA are 1–75, 5–100 and 2–100 μg mL^{-1} with detection limits of 0.3, 1 and 0.6, respectively. The RSDs for five replicate measurements of insulin, BSA, and HSA at 25 μg mL^{-1} were found to be 3, 4 and 1.5%, respectively. The calibration plots with two orders of magnitude linear range together with low detection limits reveal the capability of pneumatically assisted electrospray-IMS for quantitative analysis of intact proteins.

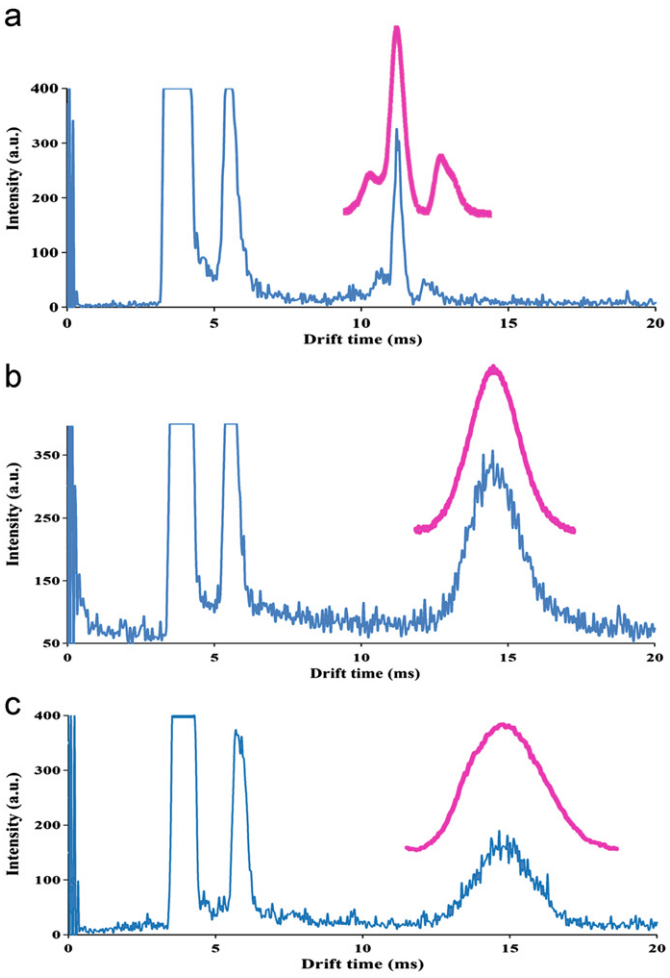

Fig. 6. The pneumatically assisted electrospray-IMS spectra taken at time with maximum peak intensity (top) and average spectra obtained by Matlab software (bottom) for one concentration of (a) insulin (b) BSA and (c) HSA.

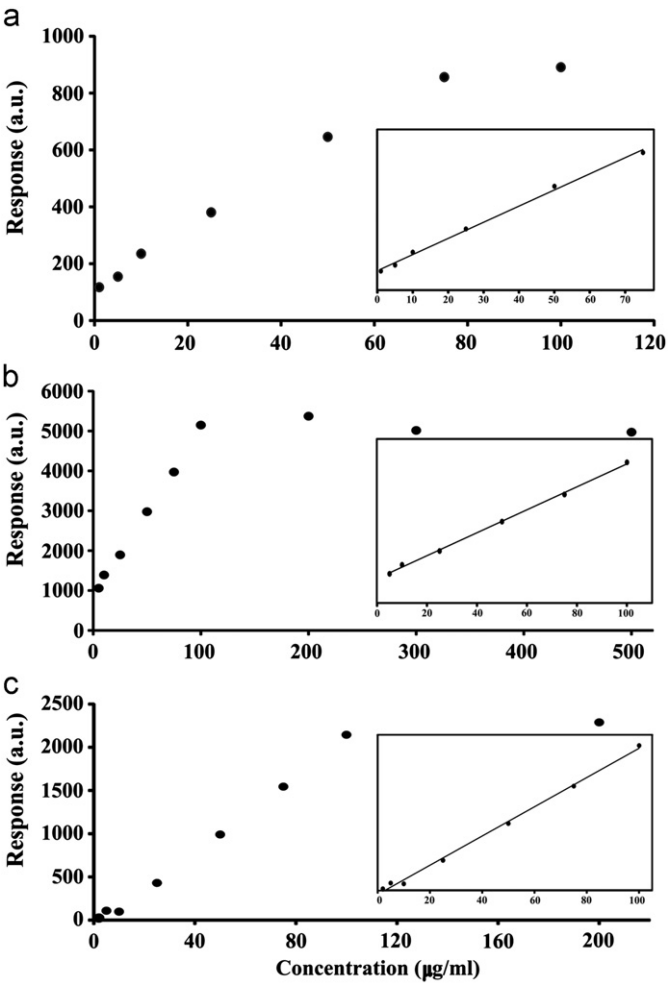

Fig. 7. The response of pneumatically assisted electrospray-IMS at the optimum conditions against the concentration of insulin (a), BSA (b) and HSA (c). The inset figure represents the linear sections of the calibration curves.

3.4. Comparison with other methods

Table 4 summarizes the detection limits and linear response ranges obtained for HSA by this study and previously reported methods. The results show that the detection limit of the proposed method is lower than or comparable with some other methods such as linear sweep voltammetry [33], LC-MS [34] and some of the light scattering methods [35]. However, a number of light scattering methods [36–38], fluorescence [39], chemiluminescence [40] and a few electrochemical methods [41], offer lower detection limits. The results of Table 4 also show that the calibration linear range of the proposed method (two orders of magnitude) is comparable or better than the other methods. Furthermore, the IMS is generally considered as a simple and fast technique [12].

The proposed method with its low detection limit and wide dynamic range can be utilized for analysis of HSA in urine samples of patient. Persistent albumin in the range of 30–300 mg per day (~20–200 mg L^{-1}) in urine, called microalbuminuria, is an early marker for diabetic nephropathy and cardiovascular events [42,43], could be analyzed provided that albumin is separated from urine samples.

Table 3
Analytical characteristics for the determination of proteins by pneumatically assisted electrospray-IMS.

Protein	Linear range (μg mL^{-1})	Linear regression equation (c, μg mL^{-1})	Correlation coefficient, R2	Detection limit (μg mL^{-1})	RSD % ($n=5$)
Insulin	1–75	$Y=10.078C+119.62$	0.996	0.3	3
BSA	5–100	$Y=42.107C+881.45$	0.999	1.0	4
HSA	2–100	$Y=21.663C-60.869$	0.997	0.6	1.5

Table 4
Comparison of analytical parameters of the proposed method with other methods in determination of HAS.

Method	Linear dynamic rang (μg ml^{-1})	Detection limit (μg ml^{-1})	Reference
Linear sweep voltammetry	3–15	0.53	[33]
LC-MS		11.3	[34]
Resonance Rayleigh scattering (RRS)	26.5–3188.5	6.643	[35]
Second-order scattering (SOS)	26.5–3188.5	16.61	[35]
Resonance Rayleigh scattering (RRS)	0.2–4	0.075	[36]
Resonance light scattering (RLS)	0–9	6.9×10^{-5}	[37]
Flow injection analysis-RLS (FIA-RLS)		0.11	[38]
Fluorescence	0–9.2	0.064	
	9.2–34.5	0.115	[39]
FI-Chemiluminescence (FI-CL)	0.05–24	0.03	[40]
Anodic stripping voltammetry	0.03–0.67	0.01	[41]
Proposed method (pneumatically assisted electrospray-IMS)	2–100	0.6	

4. Conclusions

The IMS with ESI and newly designed pneumatically assisted electrospray as the ionization sources was utilized for quantitative analysis of intact proteins including insulin, BSA and HSA. The results showed that the sensitivity of pneumatically assisted electrospray-IMS was two to three times higher than that of ESI-IMS. The effect of nebulizing gas flow rate on sensitivity of the measurements was investigated and it was observed that the enhancing effect is more pronounced for the compounds with higher molecular weights. The results of quantitative analysis of insulin, BSA and HSA demonstrated that the method could be considered as an alternative way for protein analysis.

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