Nanoelectrospray Differential Ion Mobility Spectrometry for Protein Sizing and Molecular Mass Determination: Method Development and Validation

Efthymios A. Kapellios[1], S. Karamanou[2], M. F. Sardis[2], M. Aivaliotis[3], S. A. Pergantis[1] and A. Economou[2,3]

[1] Dept of Chemistry, University of Crete, Voutes Campus, Heraklion 71003, Greece
[2]Economou Lab and [3]Proteomics Facility, IMBB-FORTH, Vassilika Vouton, Heraklion 71110, Crete, Greece

OVERVIEW-INTRODUCTION

The structural characterization of large biomolecules, including the determination of their size and molecular mass (*Mr*), plays an important role in understanding fundamental aspects of their biochemical behavior. Various techniques are used for this purpose. Even though mass spectrometry has dominated, another similar technique based on nanoelectrospray and **ion mobility spectrometry (IMS)**, referred to as gas phase electrophoretic mobility molecular analysis (GEMMA) has received much less attention, even though it has recently shown several potential benefits. The main objective of the present study was to evaluate GEMMA for its capability to determine the size and *Mr* of proteins and protein complexes, and to compare it with several well established biophysical techniques such as quasi elastic light scattering (**QELS**) and multiangle laser light scattering (**MALLS**) that measure protein dimensions and mass in an aqueous environment in their native state. The various methods were compared in terms of sensitivity, accuracy, reproducibility and other relevant measurement characteristics.

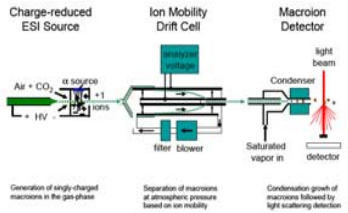
EXPERIMENTAL

Over the past decade considerable progress has been made in the development of analytical techniques for sizing large biomolecules. One of these techniques involves the use of nanoelectrospray (nES) coupled with differential ion mobility spectrometry (IMS) coupled on-line with condensation particle counting (CPC).

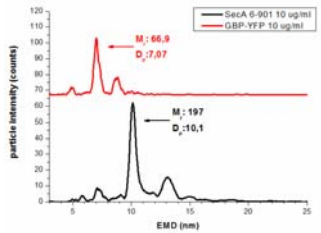
Instrumentation:

- A complete nES-IMS with CPC detection from TSI Inc. (St. Paul, MN) was used in this study. This consisted of a nES source unit (Model 3480C) equipped with a neutralizing chamber (^{210}Po a-source; 5 mCi, model P-2042 Nucleospot local air ionizer; NRD, Grand Island, NY), the differential mobility analyzer which is commercially named macroIMS (Series 3080C), and finally the CPC used was a butanol-based Ultrafine Condensation Particle Counter (Series 3025A)
- *Laser light scattering analyses were carried out using a Shimadzu* LC10A-VP*HPLC system and* size exclusion chromatography (Superdex HR200 10/300 GL prepacked column; GE) coupled to on-line detection with a multi-angle laser light scattering (MALLS) and a Quasi elastic light scattering (QELS) detector (DAWN-EOS; Wyatt) and a refraction index detector (RID-10A; Shimadzu). Data were analyzed and plotted using the Zimm function of the Astra software (Wyatt).

Protein samples: Samples: Several proteins (SecA, GBP, YFP, GBP-YFP, Fluo 5, PPiA) were isolated and purified using established protocols (Karamanou et al.. 1999; Schenckman et al., 2008) and were dissolved at various concentrations in ammonium acetate 20 mM.



Schematic representation of the ion flow path (TSI Inc.)



Typical IMS graph for protein and protein complexes.

RESULTS AND DISCUSSION

nES-IMS was used to determine the size and *Mr* of several proteins. This data was compared with relevant literature data and data obtained by using the previously mentioned complementary techniques (QELS, MALLS). Other analytical figures of merit including amount of protein required for the measurement, amount of protein consumed during the measurement, measurement time and total analysis time are reported on.

Comparison of nES-IMS vs MALLS vs QELS

Feature	nES-IMS (or GEMMA)	MALLS	QELS
What is measured	Electrophoretic Mobility Diameter (EMD: defined as the diameter of a sphere that has the measured electrophoretic mobility) Relative molecular mass (determined indirectly from electrophoretic diameter assuming a protein density of 0.62 g.cm^{-3})	Mass, Radius of gyration	Stokes (hydrodynamic) radius
Principle	Measurement of electrophoretic mobility (Z_p) Calculation of EMD (D_p) $D_p = \frac{neC}{3\pi\mu Z_p}$ Calculation of relative molecular mass (M_r) $M_r = \rho * D_p^{\,x}$ $x = 3, \rho = 0.60 g/ml$	Solution scattered laser light	Solution scattered laser light, processed auto-correlation function
Ionization	NanoElectroSpray (nES) plus Polonium charge neutralizer to generate singly-charged migrating species	none	none
No of charges per protein	Single positive charge	-	-
Time of actual Measurement	Each molecule spends approx. 0.1-0.4 sec in gas phase under atm. pressure	Multiple Millisecond measurements in buffer	Multiple Millisecond measurements in buffer
Time of experimental run per final Measurement	2-4 min per run Sample in capillary 112 sec Sample in Ion Mobility Drift Cell 0.4 sec Sample in Detector 3.27 sec, <1 min per software analysis	40 min column equilibration, 20-40 min per run, 20-40 min per software analysis	40 min column equilibration, 20-40min per run, 20-40 min per software analysis
Measurement bias	Relative molecular mass determination is biased by the assumption that the protein measured is approximately spherical	Unbiased, independent of markers (calibrants)	Unbiased, independent of markers (calibrants)
Measurement accuracy limitation	No commercial marker proteins exist above ~1MDa Theoretically the instrument can go up to 80 MDa or 150 nm (tested in the present study up to 1 MDa) good IMS separation is important in the case of mixtures	Higher order aggregates scatter more than smaller proteins-good SEC separation is important in the case of mixtures	Higher order aggregates scatter more than smaller proteins-good SEC separation is important in the case of mixtures
Amount of protein	1-10 μg/ml solutions analyzed; 0.25 μg required; 0.14 ng consumed	>200 μg in 1mg/ml solution	>1 mg in 10 mg/ml solution
Protein environment during measurement	Gas phase atmospheric pressure	Aqueous	Aqueous
Operating temperature	21 ºC laboratory temperature	4-37 ºC	4-37 ºC
Buffer	AcONH$_4$ pH 7.8 but others possible providing they are compatible with nanoES	Tris pH 8.0 but others possible	Tris pH 8.0 but others possible
Protein dimension	2.5 nm-150 nm	10nm (for Rg)	1 nm-micrometers
Protein mass	10^6 – 80 x 10^6 Da	10^6 – 5 x 10^6 Da	-
Accuracy of measurement	5-10% Mass value, EMD: +/-0.5 nm	0.1-1%	1-10%
Non-covalent protein complexes	Yes	Yes	Yes

Protein size and molecular weight comparison:

A set of different proteins were used for the validation of the accuracy of the technique. Comparison of the protein size and molecular weight, with relevant literature data and data obtained by using the previously mentioned complimentary techniques (QELS and MALLS), is provided in the table below. Relative Molecular Mass calculated considering that the proteins density is 0.60 g/cm^{-3} [2,3]

DIAMETER (nm)/ MOLECULAR WEIGHT (kDa)						
METHOD		GEMMA	MALLS	QELS	% Difference	Theoritical Mol. Mass
PROTEIN	Complex					
SecA 6-901 *Dp* (nm) Rel. Mol. mass	Dimer	(n=5) 10.08 ± 0.05 196 ± 2.3	(n=17) 207.8 ± 8.1	11.4 ± 0.5	-11,6 -5.7	204.0
SecA 6-834 *Dp* (nm) Rel. Mol. mass	Dimer	(n=4) 9.87 ± 0.01 181.00 ± 0.5				
GBP *Dp* (nm) Rel. Mol. mass	Dimer	(n=4) 7.21 ± 0.13 71.2 ± 3.9	77.4	7.6	-5.1 -8.0	71.4
YFP *Dp* (nm) Rel. Mol. mass	Monomer	(n=3) 5.52 ± 0.11 31.8 ± 1.8	45.8	6.8	-18.8 -30.8	27.1
GBP-YFP *Dp* (nm) Rel. Mol. mass	Monomer	(n=4) 7.01 ± 0.05 65.2 ± 1.4	67.0	8.2	-14.5 -2.7	60.0
Fluo 5 *Dp* (nm) Rel. Mol. mass	Monomer	(n=5) 7.96 ± 0.08 95.8 ± 2.9	91.4	8.8	-9.5 4.8	87.9
PPiA *Dp* (nm) Rel. Mol. mass	Monomer	(n=3) 4.62 ± 0.11 18.7 ± 1.25	22.2			19.2

Molecular weight and electrophoretic mobility diameters of proteins studied by different techniques (IMS, QELS, MALLS).

CONCLUSIONS

1. nES-IMS (or GEMMA) requires considerably less amount of material and is faster per measured sample (by a factor of ~15 when the time for SEC column elution and regeneration is taken into account)
2. Because of short analysis times GEMMA is better suited for large sample set screening and could make an excellent front-end to an proteomics intact protein analysis flow if coupled on line to a high resolution mass spectrometer
3. quartenary non covalent protein structures "survive" gas-phase traveling (e.g. SecA is a dimer)
4. GEMMA "shrinks" protein diameter by approximately 10%, compared MALLS and QELS.
5. Size and Mole. Mass measurements are similar between the compared methods.
6. Disadvantage of GEMMA is that it can not be used to measure high concentration levels because artifact oligomers tend to form.

REFERENCES

[1] Loo, J. A.; Berhane, B.; Kaddis, C. S.; Wooding, K. M.; Xie, Y.; Kaufman, S. L.; Chernushevich, I.V. J. Am. Soc. Mass Spectrom. 2005, 16, 998–1008.
[2] Kaddis, C. S.; Lomeli, S. H.; Yin, S.; Berhane, B.; Apostol, M. I.; Kickhoefer, V. A.; Rome, L. H.; Loo, J. A. J. Am. Soc. Mass Spectrom. 2007, 18, 1206-1216.
[3]. Papanikou, E., Karamanou,S. and Economou, A. (2007) Bacterial protein secretion through the translocase nanomachine.Nature Reviews in Microbiology 5, 839-851.

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