A Multiplexed Approach for the Determination of Intact Protein Mass, Dimension and Oligomerization Using Nanoelectrospray Ion Mobility Spectrometry (GEMMA), LTQ-Orbitrap MS, MALLS and QELS

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OVERVIEW-INTRODUCTION

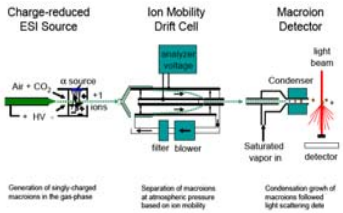
The characterization of proteins and their oligomeric complexes in terms of size, dimensions as well as their relative (*Mr*) or absolute molecular mass, is important in determining protein structure and function. Here we make use of nanoelectrospray ion mobility spectrometry, referred to as gas phase electrophoretic mobility molecule analysis (GEMMA) that has recently shown several potential benefits. GEMMA was evaluated for its capability to determine the size and *Mr* of proteins and protein complexes, and was compared with an established MS technique using an LTQ-Orbitrap. Results were compared with two hydrodynamic approaches quasi elastic (QELS) and multi-angle (MALLS) laser light scattering in terms of sensitivity, accuracy, reproducibility, speed, maintaining intact oligomeric assemblies and other measurement characteristics.

EXPERIMENTAL

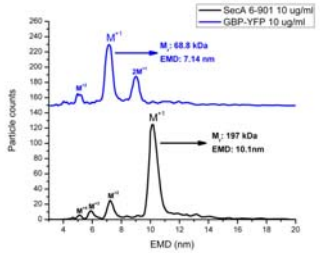
Instrumentation:

- A complete nES-IMS coupled on-line with Condensation Particle Counter (CPC) 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-VPHPLC *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).
- nESI-MS measurements were performed on an LTQ-Orbitrap XL instrument (100,000 resolution at *m/z* 400). Dynamic nanoESI-infusion of 5-40 μg of protein samples with a flow ranging from 400-800 nl min^{-1} was performed using a nano-source (PROXEON) and 30 μm ID stainless steel emitters (PROXEON).

Protein samples: Proteins and protein complexes involved in the Sec and the bacterial Type III secretion pathway of enteropathogenic *E. coli* (EPEC) were isolated and purified using standard biochemical protocols (Karamanou *et al.* 1999; Schenckman *et al.*, 2008). Various shapes and sizes were examined (12,000 - 204,000 Da; 4-11 nm). For the nESI-Orbitrap MS analysis, protein samples were dissolved at various concentrations in ammonium acetate (20 mM) and/or organic solvent.



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 nESI-Orbitrap-MS (high mass accuracy and resolution), and the previously mentioned complementary techniques QELS and 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. Relative Molecular Mass calculated considering that the proteins density is 0.60 g/cm^{-3} [2,3]

Protein Analyzed	Measured	nES-GEMMA	MALLS	QELS	nES-Orbitrap[a]	% Diff.[b]	Predicted M_r[c]	Other studies
SecA 6-901	EMD or D_H (nm)[d]	10.08 ± 0.04	n.a.	11.0 ± 0.5	-	-	-	-
	M_r (kDa)	196.0 ± 2.3	210.5 ± 13.4	n.a.	102433.3667 ± 2.400 204706.7600 ± 19.721	6.9 / **4.3**[g] (6.3)	104.53	-
	Oligomeric State[e]	Dimer	Dimer	-	Monomer/Dimer	-	-	Dimer
	n[f]	5	16	16	1/10	-	-	-
SecB	EMD or D_H (nm)	7.53 ± 0.01	n.a.	8.54 ± 1.14	n.a.	11.8	-	-
	M_r (kDa)	80.9 ± 0.1	80.6 ± 3.5	n.a.	19666.2763 ± 0.156	-0.4 / - (-1.5)	19.93	-
	Oligomeric State	Tetramer	Tetramer	-	Monomer	-	-	Tetramer
	n	3	8	8	5	-	-	-
GBP	EMD or D_H (nm)	7.22 ± 0.13	n.a.	7.6		5.1	-	-
	M_r (kDa)	71.2 ± 4.0	77.4	n.a.	32960.81296 ± 0.279	8.0 / - (-3.1)	34.54	-
	Oligomeric State	Dimer	Dimer		Monomer	-	-	Monomer in reduced form
	n	4	1	1	5	-	-	-
CesAB + EspA	EMD or D_H (nm)	5.72 ± 0.03	n.a.	4.9 ± 1.2	n.a.	-17	-	-
	M_r (kDa)	35.33 ± 0.58	35.4 ± 1.4	n.a.	34358.1076 ± 0.218	0.2 / **-2.8** (-0.7)	35.09	-
	Oligomeric State	1:1 Heterodimer	1:1 Heterodimer	-	1:1 Heterodimer	-	-	1:1 Heterodimer
	n	3	6	-	10	-	-	-
CesAB	EMD or D_H (nm)	5.59 ± 0.32	n.a.	5.6 ± 0.9	-	0.2	-	-
	M_r (kDa)	33.3 ± 5.6	22.7 ± 1.6	n.a.	13439.1153 ± 0.01 26878.3442 ± 0.015	-46.8 / **-23.9** (-18.8)	14.02	-
	Oligomeric State	Dimer	Dimer	-	Monomer/Dimer	-	-	Dimer
	n	6	11	3	1/10	-	-	-
YFP	EMD or D_H (nm)	5.52 ± 0.10	n.a.	6.8	n.a.	18.8	-	-
	M_r (kDa)	31.8 ± 1.8	45.8	n.a.	32052.42304 ± 0.025	30.6 / **0.8** (-9.6)	29.01	-
	Oligomeric State	Monomer	monomer / dimer	-	Monomer	-	-	Monomer
	n	5	1	1	6	-	-	-
PpiA	EMD or D_H (nm)	4.62 ± 0.11	n.a.	4.94 ± 0.24	n.a.	6.09	-	-
	M_r (kDa)	18.7 ± 1.2	19.9 ± 2.0	n.a.	19213.0925 ± 0.006	5.9 / **2.7** (2.7)	19.21	-
	Oligomeric State	Monomer	Monomer	-	Monomer	-	-	Monomer
	n	3	5	4	10	-	-	-

a: nES-Orbi determined Mr;
Mr of different oligomeric state detected is presented seperately
b: % difference between nES-GEMMA and MALLS/QELS / nES-Orbitrap results;
in parenthesis % difference between predicted M_r and nES-GEMMA determined Mr.
c: Predicted M_r value obtained from the primary sequence of the protein monomer
d: EMD and D_H refered to GEMMA and QELS measurments, respectively
e: Main oligomeric state detected by each technique
f: Number of replicate analyses
g: % difference between nES-GEMMA and nES-Orbitrap determined Mr
n.a.: not applicable

Protein EMD, D_H and M_r values determined using nES-GEMMA, nES-Orbitrap, MALLS and QELS and their comparison

CONCLUSIONS

1. nES-IMS (or GEMMA) and nESI-MS (Orbitrap) 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 a proteomics intact protein analysis flow (if coupled on-line to a high resolution mass spectrometer Orbitrap).
3. Quaternary non-covalent protein structures "survive" gas-phase traveling in GEMMA (e.g. SecA is a dimer) whereas in Orbitrap only a minor fraction of them is finally detected.
4. GEMMA "shrinks" protein diameter by approximately 10%, compared MALLS and QELS. The same is most probably true for the other gas-phase analysis, namely nES-MS.
5. Size and Molecular Mass measurements are similar between the compared methods.
6. Disadvantage of GEMMA and nESI-MS (Orbitrap) is that it can not be used to measure high concentration levels because artifact oligomers tend to form.
7. nESI-Orbitrap MS determines with high accuracy the protein mass while is able to identify possible Post-translational modifications, truncations and mutations which can not be obtain/predicted by the theoretical proteome of any given organism/cell. Therefore it is a necessary complementary technique to GEMMA .

Comparison of general characteristics of the techniques used in this study for protein sizing and M_r determination.

Technique Characteristics	nES-GEMMA	nES-Orbitrap	MALLS	QELS
Sizing range (nm)	3 - 150	n.a.	n.a.	1 - 1000
Size measurement precision (nm)	0,1	n.a.	n.a.	n.d.
Relative molecular mass range (kDa)	8 - 80000	HR ≤75KDa[a] LR ≤130KDa	10 - 5000	n.a.
Mass measurement accuracy (%)	1 - 10	0.0001-0.0010	0.1 - 1	n.a.
Measurement accuracy limitation	Theoretical upper limit is 150 nm or 80 MDa	HR ≤75KDa LR ≤130KDa	Larger particles scatter more than smaller ones. SEC separation is critical.	
Measurement bias	Assumes proteins to be spherical	Proteins with high charge capacity	Nonbiased, independent of markers	
Measurement environment	Gas phase		Aqueous phase	
Capability to measure non-covalent complexes	Yes	Limited	Yes	
Buffer Compatibility / Main buffer	nES compatible buffers / AmAc pH 7-8		No restrictions / Tris pH 8.0	
Typical concentration used for analysis	1 - 10 μg mL^{-1}		1 mg mL^{-1}	10 mg mL^{-1}
Amount required for analysis	0.025 - 0.25 μg		> 0.2 mg	> 1 mg
Amount Consumed during analysis	~1.6 ng		non-destructive	
Single measurement time (min)	1 - 4	5-10	20 - 40 (for SEC separation)	
Data Analysis time (min)	< 1	5-10	20 - 40	
Sample Collection after analysis while maintaining functionality	Potentially yes	No	Yes	

a: HR refer to High Resolution
LR refer to Low Resolution

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