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Comparison of various nano-differential mobility analysers (nDMAs) applying globular proteins

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The demand for analysis of nanosized particles and assemblies of biologic and inorganic origin has increased in the recent decade together with the growing development of biotechnology and nanotechnology. Recent developments of electrostatic differential mobility analysis (DMA) provide an excellent characterization tool in the nanometer size range. With an increasing number of available nano-DMA (nDMA) systems, the question of data comparability and implementation of possible calibration procedures arise. Here we present analysis of proteins in a range between 3 nm (5.7 kDa) and 15 nm (660 kDa) with five different nDMA systems. Results show differences in the obtained sizes up to 15% between different nDMA systems, which consequently leads to the conclusion that a calibration procedure for each nDMA is necessary when applying such systems for the analysis of nanoparticles with respect to size and molecular mass.

Keywords: Nanoparticles; Proteins; Differential mobility analyser; GEMMA; Electrospray

1. Introduction

Differential mobility analysis is a long established method in aerosol physics [1] designed to characterise ions and charged particles according to their electrostatic mobility, to analyse ambient aerosols and aerosol products of combustion processes and to measure the formation and alteration of atmospheric aerosol particles [1, 2]. The working range of DMA for these purposes is typically in the upper sub-μm to μm range. In the recent decade, mobility analysers were optimized and further developed to

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expand their working range into the single digit nanometer range, or in terms of molecular mass, into the low kDa range [3–5]. This expansion of the working range enabled this technique to be applied in the broad field of chemistry, biochemistry and nanosciences for the analysis of polymers, inorganic nano-sized particles, biopolymers and larger assemblies, such as functional protein complexes, virus-like particles or intact viruses.

The particular advantage of this method compared to mass spectrometry is its high dynamic range in terms of molecular mass (kiloDa to GigaDa) [4] and its operation at atmospheric pressure. Combined with charged particle or molecular ion generation from liquid samples with nano-electrospray sources [6, 7] (n-ES) and subsequent neutralization of the highly charged species [8–10], this method has already been successfully applied in analysing important biochemical analytes, such as viruses and virus fragments [4, 11], bacteriophages, DNA [12], proteins and protein complexes [4, 13], as well as synthetic materials, such as polyethylene glycol polymers [14], polystyrene [15], PAMAM dendrimers [16] and nanometer sized silica particles [17].

With increasing distribution and importance of this method, the need for determination of important analytical parameters, such as accuracy and precision, arises. Until now, nano-DMAs (nDMA) were mainly characterized in a sequential setup [18–22], in which a first mobility analyser produced monodisperse aerosol particles from a polydisperse aerosol source, and a second mobility analyser scanned the size distribution produced by the first one. This setup enabled the comparison of the quality of the mobility analysis in terms of resolution and particle diffusion, but opens the field for interpretation of the collected results, because both DMAs have influence on the characteristics of the resulting size spectrum.

In investigations reported here we produced a monodisperse test aerosol by using various well-defined globular proteins in the size range between 3 and 16 nm. The monodispersivity of the so generated test aerosol is ensured by the inherent uniformity of the protein particles, simplifying the experimental set-up dramatically and allowing an independent characterisation of the sizing performance of various nDMAs. Size spectra obtained with two Vienna-type nDMAs [18, 23], two type-identical commercially available nDMAs (GEMMA, TSI Inc.) and a GEMMA prototype containing a nDMA [4] were analysed according to repeatability, comparability and resolution of the size spectra. Implications for the routine application of nDMAs in the analysis of nanoparticles of biological, inorganic or polymeric nature are discussed.

2. Materials and methods

2.1. *Proteins*

Protein stock solutions of about 1 mg/mL were prepared from commercial protein powders (Sigma-Aldrich) with a 20 mM ammonium acetate buffer (pH 6.0 – 6.7). Because of the negligible salt concentration of the protein samples, it was possible to analyse in buffer diluted solutions without further purification. However, in cases where involatile salt residues are observed and thus could influence the measured size of the protein particles, protein solutions were purified with Microspin (GE Healthcare) or with Microcon Centrifugal Devices (Millipore) until salt concentrations below 1 μg/mL

were reached. The concentration of the different proteins was adjusted to the level that mainly monomer (M) and no significant dimeric (2M) signals were detected. The typical concentration was 20 μg/mL for proteins with a molecular mass > 20 kDa and 5 μg/mL for proteins with a molecular mass < 20 kDa. Insulin (5.7 kDa), ubiquitin (8.6 kDa), cytochrome C (12.3 kDa), myoglobin (17.6 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.6 kDa), avidin (64.0 kDa), bovine serum albumin (66.4 kDa), native enolase dimer (93.4 kDa), fibrinogen (339 kDa), ferritin (483 kDa) and thyroglobulin (660 kDa) solutions were electrosprayed to produce monodisperse aerosol used in this study.

2.2. *Nano-electrospray (N-ES instrument)*

The N-ES source (Mod. 3480, TSI Inc) was used for the aerosolization of the protein samples. For this instrument and above named solvent system, we found that a stable cone-jet mode operation was obtained at 2 kV and 0.1-0.3 L/min CO_2 (99.995%, Air Liquide N) and 1-1.5 L/min compressed air (99.999% synthetic air, Air Liquide) [17]. A pressure difference of 4 psi between sample and spray chamber was applied, which resulted in a sample solution flow of 67 nL/min through the fused silica capillary (25 μm inner diameter and 160 μm outer diameter, uncoated; supplied by TSI Inc). The primary generated droplet size, which is determined by the conductivity of the solvent system and the sample flow through the capillary as well as the inner diameter of the capillary was 150 nm, corresponding to a flow of 2×10^{11} droplets per minute or about 1.7×10^{8} droplets per/cm^3. The N-ES source generates highly positively charged droplets, which are then subsequently neutralised in bipolar ion environment obtained with a Po-210 α-radiation source (TSI Inc). This process results in mainly singly negatively and positively charged airborne molecules and nanoparticles [8, 9]. N-ES settings, buffer concentrations, and solution conductivity were identical for all instruments used in this study.

2.3. *Nano differential mobility analysers*

2.3.1. Gas phase electrophoretic macromolecule mobility analyser (GEMMA and GEMMA Prototype). The commercial GEMMA system, used at the Vienna University of Technology (GEMMA TU Vienna) and University of California Los Angeles (GEMMA UCLA) consists of a nano-electrospray (nES) unit, a nDMA and an ultrafine condensation particle counter (CPC) as detector (all devices from TSI Inc). The operating particle size range of this instrument combination is between 3 nm (limited by the particle detection device, CPC) and 65 nm (the upper scan limit of the nDMA when using maximum sheath flow for maximum nDMA resolution). Both GEMMA systems were operated with negative high voltage at the central nDMA electrode, thus separating the positively charged fraction of the generated singly charged ions/particles. The detection limit of the system (nDMA coupled to the CPC) in terms of particle concentration is in the order of one singly charged particle/cm^3. However, due to the limited charging efficiency in the N-ES source, concentrations of at least 10^4 protein particles/cm^3 are necessary to obtain appropriate particle count statistics across the whole selected sizing range within a reasonable time (120 s/scan).

Ten scans were averaged for each GEMMA spectrum presented in this paper. Both GEMMA systems (TU Vienna and UCLA) were operated under similar settings concerning sheath gas flow, scan time and size range.

Details (operation conditions and construction details) of the GEMMA prototype at the University of Vienna (GEMMA Protoype) are given in table 1 and in detail in a previous published paper [4].

2.3.2. Parallel nano-differential mobility analysing system (PDMA). The PDMA system is an advanced *in-house* development (Faculty of Physics, University of Vienna) combined with the above mentioned N-ES source and with an electrical ion/particle detection device working on the Faraday cup (FC) principle. Parallel to the scanning nDMA an identical separation nDMA is simultaneously operated for sampling of the selected nanoparticles, hence the acronym PDMA [24]. Recent development extended the lower sizing limit of the PDMA system down to 0.7 nm. The applied detection method needs a certain minimum number of charged species (concentration) in order to achieve measurable electrical signal. The detection limit of this nDMA/FC system is currently in the order of 10^3 charged particles/cm^3 to ensure good counting statistics.

High voltage of positive polarity was applied to the central electrode, thus analyzing the negatively charged particle fraction. The PDMA was operated under scanning conditions to yield a particle size spectrum from 1 to 20 nm within 120 s. Ten spectra were averaged for the presented data of each protein sample.

2.3.3. Long nano-differential mobility analyser (L-nDMA). The L-nDMA is also an in-house (Faculty of Physics, University of Vienna) constructed nDMA coupled with the above described N-ES source. The difference to the PDMA is a ten-fold increased separation length, namely 150 mm (see figure 1), which results in a different sizing range, starting from 2 nm up to 200 nm. The detection system of the L-nDMA is also based on the FC principle. This system was used with identical scanning rate (2 s/channel) as the PDMA and all GEMMA systems. Ten spectra of each protein were averaged for the final data analysis.

Properties and operation parameters which are important for the subsequent comparison and discussion of the results, are summarized in table 1 for all here described nDMAs.

2.4. *Performance and resolution of n-DMAs*

The principle and features of a DMA has been described extensively in literature [1, 3, 5, 23], so only key aspects of this technique are briefly summarized here, which are relevant for this investigation. Electrical ion mobility is defined by equation (1) and is the ratio between electrostatic forces and friction that leads to constant particle movement when facing a certain electrical field. The electrical mobility can also be expressed in terms of design and operation parameters of the DMA (figure 1) and leads as a consequence to only one particular size fraction of ions/particles that is analysed by the DMA at certain sheath gas and voltage settings (equation 2). Because the number of

Table 1. Properties and operating parameters of the evaluated nDMAs.

Operating Conditions	GEMMA (TU Vienna/UCLA)	L-nDMA	PDMA	GEMMA prototype
Sheath gas flow through nano-DMA (L/m)	15/20	14	20	18
Aerosol flow to sheath gas flow ratio (%)	8.7/7.5	9.3	6.5	7.2
Polarity of the central electrode	Negative	Positive	Positive	Negative
Feasible working range in terms of size (nm)	3–65	2–200	0.7–50	3–65
Scan range applied (nm)	3–25	2–20	1–20	3–65
Number of channels across scan range	60	47	68	90
Voltage scan direction	Up	Down	Down	Up
Scan rate (sec/channel)	2	2	2	2
Applied detector	CPC	FC	FC	CPC

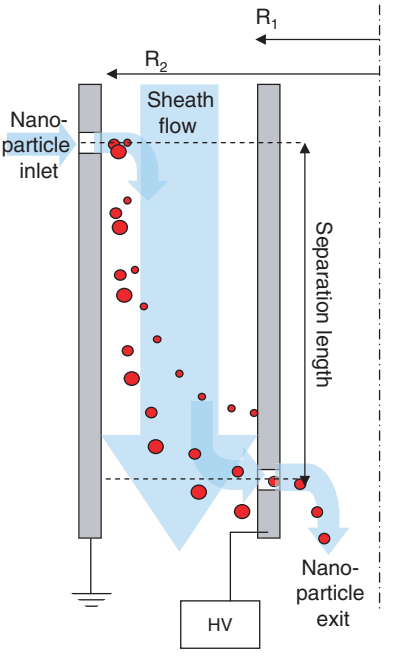

Figure 1. Operating scheme of a nDMA. HV – High Voltage–scanned from 0 to 10 kv during mobility analysis, R_1 – Inner Radius of the central electrode, R_2 – Outer Radius of the central electrode.

charges of the analysed ions/particles is determined by the neutralization step via the Po-210 source [8, 9], a size spectrum can be obtained by scanning through all size fractions by varying the voltage of the central electrode and by incorporating the charging probability of the analysed size fractions.

$$Z = \frac{i^* e_o}{3\pi\eta} * \frac{C_{(D_P)}}{D_P} \tag{1}$$

$$\frac{i^* e_0}{3^*\pi\eta} * \frac{C_{(D_P)}}{D_p} = Q_{sh} * \frac{\ln(R_2/R_1)}{2\pi * L} * \frac{1}{V} \tag{2}$$

i Number of charges
Z Electrical mobility diameter
R_1, R_2 Inner and outer radius of the DMA electrode
D_P Particle/ion diameter, exiting the DMA for a given voltage
Q_{sh} Sheath gas flow
L Separation length of the DMA
$C(D_P)$ Cunningham slip correction factor (see equation (3))

$$C_{(D_P)} = 1.0 + 2.492 \cdot \left(\frac{\lambda}{D_P}\right) + 0.84 \cdot \left(\frac{\lambda}{D_P}\right) \cdot \exp\left(-0.43 \cdot \left(\frac{D_P}{\lambda}\right)\right) \tag{3}$$

λ Mean free path of the surrounding gas molecules: 66 nm at 20°C and 101 kPa

The resolution of a DMA is influenced by the DMA geometry, particle/ion diffusion and physical properties of the sheath gas. Equations (4) to (6) describe the relationship between the resolution of a DMA and conditions in the separation path and particle/ion properties. A detailed discussion about the DMA transfer function can be found in literature [5, 23, 25, 26].

$$\left(\frac{\Delta V}{Vp}\right)^2 = 16^* \ln 2^* \frac{(b + b^{-1}) * G}{Pe} \approx \left(\frac{\Delta Dp}{Dp}\right)^2 \tag{4}$$

ΔV Half width of the in voltage space normalized peak of a monodisperse particle/ion
Vp Voltage setting of the peak center of a monodisperse particle/ion
G Constant factor from theory [5] which depends on geometry, usually very close to unity
Pe Peclet number (5)
b Device parameter (6)

$$Pe = \frac{Q_{sh}}{\pi^* D(R_1 + R_2)} \tag{5}$$

D Diffusion coefficient of the particle/ion in question

$$b = \frac{L * G}{R_2 - R_1} \tag{6}$$

As can be seen from equations (4) to (6), several parameters contribute to a DMA performance. Due to particle/ion diffusion, which decreases the resolution and leads to particle losses, transit time through the DMA has to be minimized, which is especially important for particles or ions below a size of 30 nm. This leads to the conclusion that a DMA should be operated with highest possible sheath gas flow (see equation 5). To decrease the separation path, the separation length L of the DMA (inlet into the DMA and exit point in the central electrode) has to be minimized. This leads as

a consequence to a narrower annular width (R_2 minus R_1) within the DMA minimizing the term $b + b^{-1}$ in equation (5), resulting in higher voltages which have to be applied to a DMA (figure 1). Chen and Pai [27] has found from simulations that the geometry of particle/ion inlet and outlet slits has also a substantial influence on establishing a homogenous electrical field and a turbulence free zone between the inlet and outlet slits. A comparative study of the of the broadening of the transfer function of three practically identically constructed DMAs [28] showed, that even minor differences in the inlet section for sheath gas flow lead to a different performance of the DMAs concerning resolution. The effectiveness of electrophoretic mobility analysis of nanoparticles/ions paved the way to further DMA prototypes in which attempts are made to optimize the transfer function of the DMA in producing turbulence free flows even with extremely high sheath flow rates [29], or as alternative for example, to scan the sheath flow instead of the voltage of the central electrode or to apply a DMA with variable separation length [30, 31].

2.5 *Sizing accuracy of nDMAs*

The obtained raw size data were first normalized to the maximum height of the analyte peak and then the full width at half maximum (FWHM) was determined (see figure 2). The count mean diameter (CMD) of the monomer peak was calculated for all size spectra according to equation (7). Subsequently all CMDs corresponding to one protein sample analysed with one nDMA instrument were averaged to give one data point (used in figure 3). The standard deviation of each CMD was calculated to obtain a value that reflects the repeatability of the nDMA analysis on each instrument.

$$C(nm) = \frac{\sum_{i=b}^{e} I_i * D_i}{\sum_{i=b}^{e} I_i} \tag{7}$$

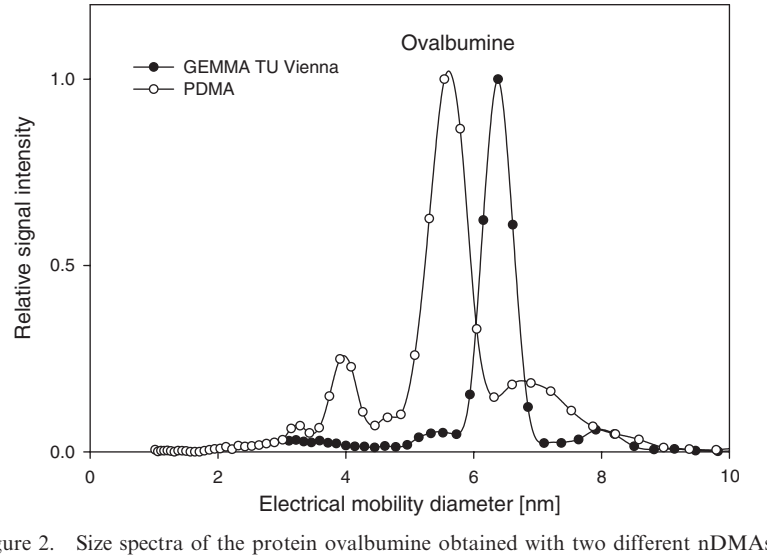

Figure 2. Size spectra of the protein ovalbumine obtained with two different nDMAs.

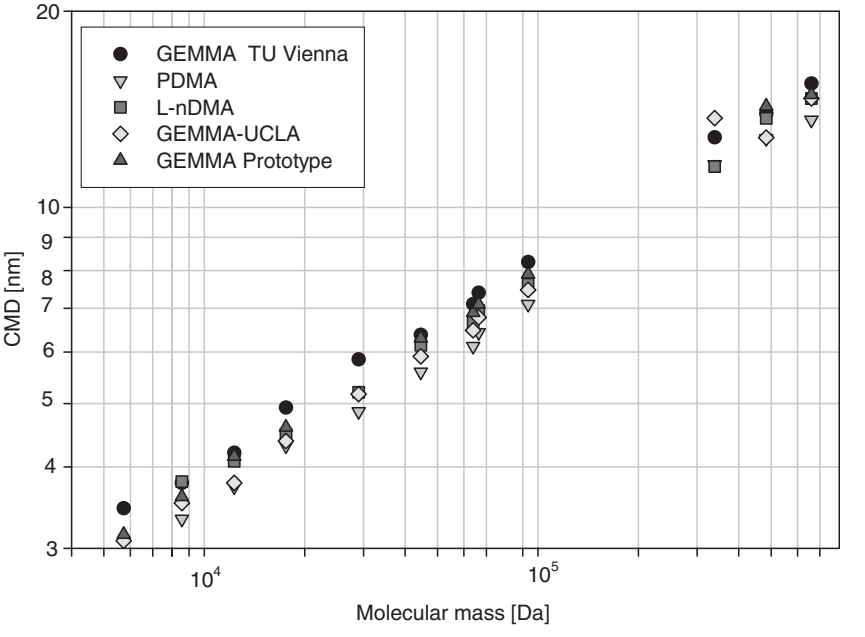

Figure 3. Count mean diameter (CMD) for the selected standard proteins obtained with the five different nDMAs plotted as a function of molecular mass of the proteins.

C Count mean diameter of the monomer spectrum
b, e Integration borders of the Peak
I_i Signal intensity at the size channel i
D_i Mobility diameter of the size channel i

3. Results and discussion

The size spectra of the protein ovalbumin (same sample solution) obtained with two different nDMAs are shown in figure 2. A shift of about 1 nm between the spectra can be observed, although all parameters necessary to calculate a size spectrum from the raw data, such as gas properties, charging probability of the particles, geometrical parameters, sheath flow rate, are precisely known and implemented in the analysers software, [3, 18, 23]. The observed differences in the size for identical protein particles has been found for al five nDMA systems (see figure 3). As can be seen, the obtained CMDs are within a certain size interval, with a spread of up to 15% related to average particle size obtained by all instruments for all measured proteins. This difference between the investigated nDMAs becomes even more significant, as the repeatability of the position of the CMD for each protein particle/ion was just of the order of $\pm 0.1\%$ within a series of ten subsequent gathered size spectra obtained with one randomly chosen nDMA. This is about 100 times smaller than the spread of measured CMD values.

It is noticeable that the relationship between molecular mass and the CMD of the analysed globular proteins exhibits an excellent correlation coefficient ($R^2 \geq 0.98$) for each instrument.

The relative (normalised to the CMD value) FWHM of measured peaks are similar for all investigated instruments and typically below 6% for proteins with a molecular mass >30 kDa except for the L-nDMA showing the relative FWHM in this molecular mass range of about 8%. This is a result of the longer separation distance connected with increased peak broadening by diffusion [23]. This effect becomes dominating for measurements of species with a molecular mass <30 kDa, which corresponds to 5 nm diameter. For such small particles, the diffusion-driven broadening leads to relative FWHM of the order of 10% for smallest investigated proteins for all DMAs but the L-nDMA, where values of about 15% were found.

The observed size differences between the investigated instruments may result from various sources such as differences in particle/ion detection method, as well as the voltage scanning algorithm which recovers the actual size distribution from raw data [18]. The L-nDMA and PDMA systems use for particle/ion detection a FC device, thus they measure directly and for practical reasons instantaneously the presence of a charged nanoparticle or ion. The three GEMMA systems use a CPC for the charged particle/ion detection. This method is known to deliver somewhat varying peak shape depending on the nDMA scan procedure and the CPC detection event [32]. Whatever the reasons for the differences in the determined size values found in this study are, it is obvious that the use of calibrants of known size, molecular mass and high structural homogeneity (monodisperse) are absolutely necessary for each individual instrument even in case of identically constructed devices. The use of calibration compounds such as proteins, polymers or inorganic particles, are indispensable for the precise functioning of any nDMA system, especially in case of the analytical task of molecular mass determination (the nDMA system is used as 'mass spectrometer' at ambient pressure [4,15]) or in which conclusions about size, changes of size caused by agglomeration, denaturation, degradation or the formation of complexes of certain analytes have to be drawn.

4. Conclusions

The repeatability ($\pm 0.1\%$) of the measured size values in one series of ten subsequent collected size spectra with each nDMA shows evidently the ability of these instruments for the characterisation and analysis of nano-sized biological, inorganic and polymeric particles. But, the fact that all nDMA systems even though operated under optimal and nearly identical working conditions consistently show a sizing disagreement indicates that not all parameters which influence the size analysis can be controlled to a level at which sizing would agree to 5% or better. This leads to the final conclusion that for analysis of nanoparticles, macromolecules, proteins and polymers, a calibration of each individual nDMA system with well-defined size or molecular mass standards is inevitable.

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