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Establishing SI-Traceability of Nanoparticle Enumeration Techniques: A Case Study on Electrospray Differential Mobility Analysis

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Nanostructured materials and their specific physical and chemical properties have been widely used over these past decades for a large range of applications, from electronics to energy, catalysis or medicine. However, for process optimization in the context of industrial production, air quality survey, biomedical applications and almost all areas where nanoparticles are involved, thorough and accurate characterization of these materials is needed [1-3]. According to the European recommendation 2011/696/EU, a nanomaterial is "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate, and where for 50% or more of the particles in the number size distribution, one or more external dimension is in the size range 1 nm-100 nm" [4]. As evidenced by this definition, two major parameters are to be measured in order to characterize a nanostructured material and to determine whether it is considered nano or not: the particle size and the corresponding particle number concentration. However, the classification of a material should be independent of the method(s) chosen for its characterization, which implies that methods must provide comparable results.

The privileged way to ensure method comparability is to establish the traceability of measurement results to the international system of units (SI) through standardization. This concept refers to an ideal condition in which results of a measurement procedure are traceable to a higher order reference procedure, that is typically calibrated with an appropriate higher order reference material which number concentration is traceable to the SI units via a primary reference procedure [5]. Consequently, to standardize analytical procedures for nanoparticle enumeration, two requirements can be identified: produce reference materials, in liquid and/or solid phase, and value-assign them using a primary reference procedure for number concentration measurements. As defined in the guide of the International Vocabulary of Metrology (VIM), a primary reference procedure is *"used to obtain a measurement result without relation to a measurement standard for a quantity of the same kind"* [6]. This implies that all parameters involved in the calculation of the particle number concentration be calibrated with standards of different nature than the analyte, meaning that a procedure involving calibration with a certified reference material constituted of the same particles intended to be quantified cannot be considered as primary. However, to date, no standards and no primary reference procedures are available for particle enumeration. In their review, Shang and Gao notably evidence a lack of accurate methodology for nanoparticle enumeration and the associated need for validated procedures to accurately measure it [7]. Similarly, Hofmann-Amtenbrink et al. highlight the current need for standardization of analytical procedures intended for nanoparticle characterization and especially for industrial and biological applications [1].

In this context, numerous analytical procedures have been developed for nanostructured materials characterization in the frame of European research projects. As an example, the NanoDefine project aims to develop measurement strategies for nanostructured materials characterization in accordance with the European definition [4]. This project lead to development of a tool called the NanoDefiner e-Tool dedicated to selecting the most appropriate measurement methods to identify whether a material can be considered as "nano" or not. An associated tiered approach is used and consists in choosing *tier-1* methods for pre-characterization depending on the material's nature (powder or colloidal suspension), and *tier-2* method for confirmative measurements [8]. Another example is the European Joint Research Project EMPIR InNanoPart that is dedicated to the development of traceable measurement and calibration protocols to measure particle number concentrations in liquid suspension with a target relative uncertainty inferior to 10%, for spherical particles in the 1 to 1000 nm size range. Small Angle X-Ray Scattering (SAXS) and single particle Inductively Coupled Plasma Mass Spectrometry (SP-ICPMS) have notably been optimized as candidate reference methods for the measurement of absolute particle number concentrations [9].

Both projects additionally identified the Electrospray Differential Mobility Analysis (ES-DMA) method as a promising technique for nanoparticle enumeration and number size distribution measurements of nanoparticles in colloidal suspensions [8]. This system is typically composed of a charge reduced electrospray generator (ES), i.e., an electrospray generator directly coupled to a photoionization source such a soft X-Ray source, or to an α-emitting source such as ^{210}Po, to nebulize colloidal suspensions and to apply a known charge distribution to the generated aerosols. This generator is then connected to a Mobility Particle Size Spectrometer (MPSS), a coupling between a Differential Mobility Analyzer (DMA) and a Condensation Nucleus Counter (CNC), that selects and counts particles as a function of their electrical mobility diameter [10-12]. ES-DMA presents many advantages for particle enumeration as it allows measuring both the size distribution and the particle number concentration, which makes it a versatile system. Analyses are fast, and the system is easy to set-up and to operate, although it requires some technical skills.

In the case of nano-bio-objects, and more specifically nano-bioparticles, ES-DMA has already proven its added value and has

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been used to characterize a wide variety of nano-bioparticles: proteins [12,13], protein aggregates [14,15], viruses [16,17], supramolecular complexes [18] or lipoproteins [19] for example. Li et al. and You et al. notably demonstrated that accurate absolute quantification of proteins, and protein aggregates, was possible with this system [12,13]. Similarly, Caulfield et al. reported the use of ES-DMA as a tool to directly measure lipoprotein particle number concentrations in biological samples using direct calculations [20]. In a recent study, we thus evaluated the potential of ES-DMA as a primary reference procedure for bio-nanoparticle enumeration in biological samples, and more specifically investigated the case of lipoproteins, large globular supramolecular assemblies which diameter typically range from 10 to 60 nm [21]. These particles are in charge of lipid transportation in blood and have notably been causally related to cardiovascular diseases [22]. In this study, we presented a metrologically improved procedure to measure lipoprotein number concentrations in liquid biological matrices, based on number size distribution measurements in aerosol phase with ES-DMA [23]. We however evidenced some limitations making ES-DMA unsuitable as a primary reference procedure in the current state-of-the-art. We demonstrated that method robustness and associated uncertainties did not meet the requirements of a primary reference procedure. In particular, the electrospray generator was identified as a key limitation for an accurate and precise measurement of lipoprotein number concentration.

During the optimization process, the major parameters involved in the aerosolization by ES were optimized; liquid injection flow-rate and gas flow-rates in the nebulization chamber were regulated using calibrated mass-flow regulators with SI traceability. The position of the photoionization source and sample preparation steps were also optimized. Nevertheless, some parameters appeared hardly stable over time. For instance, the sample liquid flow-rate, which is usually 100 to 300 nL/min, may be disrupted by the presence of microbubbles in the capillary connections, which induce instabilities of the Taylor cone and generate a high variability of the number size distribution measurements. Analyses being performed at ambient pressure and temperature, small variations of these parameters may also induce small variations of the gas viscosity, then impacting flow-rates in the system and again, number size distribution measurements [17]. However, we demonstrated that the major parameter contributing to the uncertainty budget associated to absolute lipoprotein quantification by ES-DMA was the ES transmission efficiency *E*, i.e., the percentage of aerosolized particles that are actually detected and counted by the system compared to the initial particle number concentration in the colloidal suspension [23,24].

The transmission efficiency *E* is the key limitation to making ES-DMA a candidate primary reference procedure as it impacts both measurement traceability to the SI, and method robustness and accuracy. To characterize *E* without losing the SI-traceability and primary aspect of the method, it is necessary to dispose of a higher order reference material with a certified particle number concentration consisting of particles of different nature than lipoproteins (VIM definition). However, as mentioned before, such material is not commercially available. Our study additionally highlighted the fact that *E* probably depends on the physico-chemical characteristics of the particles and on the conductivity of the analyzed samples [23] Either way, it appears that *E* is specific for a given sample and that its value may not be transferable to any sample regardless of sample composition and of the entity being measured. These observations thus indicate that using a reference material made of inorganic monodisperse particles with certified number concentration may not be suitable to measure *E* for lipoproteins in biological samples due to the physico-chemical differences of biological and inorganic matrices. This implies that ES-DMA necessarily needs to be calibrated using lipoprotein material which prevents it from being considered a potential primary reference method.

Nevertheless, it could be envisioned that, with the production of a certified reference material for particle number concentration, this issue could be addressed. Indeed, a way to overcome this difficulty could be inspired from isotopic dilution mass spectrometry that uses internal standards to account for the variability of the ES ionization efficiency from one injection to another. Spiking samples with a solution of inorganic nanoparticles of different electrical mobility diameter and known particle number concentration could indeed allow to normalize sample matrices and conductivity and thus to neglect the impact of *E* on quantification accuracy. A first prerequisite is that the spiking process does not affect the sample matrix. This could be potentially achieved through sufficient sample dilution. Another prerequisite would be that spiked particles with different chemical composition and electrical mobility diameter are nebulized like particles intended to be measured. This hypothesis still needs to be verified in order to ensure that this methodology does not introduce a bias for quantification. This approach, which represents a huge challenge, would moreover allow making lipoprotein concentration measurements traceable to the SI units as ES-DMA would then be a primary reference procedure for such particles.

This case study of lipoprotein quantification by ES-DMA thus illustrates the challenges associated with establishing measurements traceability to the SI and with developing a primary reference procedure for nanoparticle enumeration. Indeed, not only should the method be robust and precise, it should also provide SI-traceable measurements. In the current state-of-the-art, limitations were identified for ES-DMA to meet these requirements, although the development of a higher order non-lipoprotein reference material for particle number concentration could help overcome these difficulties.

Finally, despite these different initiatives, standardizing particle concentration measurements and enumeration techniques appears to be a huge challenge. A reference procedure for enumeration would need to be versatile and applicable to any kind of nano-objects on a large size range [7]. High accuracy, low measurement uncertainties and robustness of the method should additionally be sufficient for the method to serve as an anchor to a whole traceability chain, requirements that prove to be technically challenging in the current state-of-the-art. Given the fast-growing use of nanostructured materials in our modern societies, further work appears highly needed in the field.

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