A New Role of Biological Macromolecules as Reference Materials for the Sub-10 nm Calibration for the Real-time Electrophoretic ES-SMPS

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Abstract

Electrospray scanning mobility particle sizer (ES-SMPS) is an electrophoretic method for size characterization of nanoparticles (NPs) and its population in biological samples such as macromolecules and virus is growing. To ensure the measurement precision and accuracy, especially for the sub-10 nm measurement, size calibration of ES-SMPS system is necessary. Gold nanoparticles (AuNPs) are commonly chosen as the reference materials (RMs) while the surfactants preventing NP aggregate coat among NP surface lead to the measurement uncertainty in ES-SMPS. In this paper, both AuNPs and self-disperse biological macromolecules, bio-NPs, were selected for the evaluation of their suitability as RMs for ES-SMPS. Two methods including centrifugation and heating were used for the surfactant removal from AuNPs suspension while the results showed little influence of the centrifugal and thermal treatments on the accuracy improvement of AuNPs, where more than 22% difference from the nominal diameter remained in AuNPs. In the case of bio-NPs, bovine serum albumin (BSA), ubiquitin, low-density lipoprotein (LDL) and high-density lipoprotein(HDL) were suspended in electrolyte. BSA and ubiquitin presented good disperse and repeatable size compared to literature, high stability at time intervals, and consequently were suggested as ideal sub-10 nm RM for ES-SMPS calibration.

1 Introduction

Electrospray scanning mobility particle sizer (ES-SMPS) or electrospray ionization-gas-phase electrophoretic mobility molecular analysis (ESI-GEMMA) is a common method for size characterization in the field of aerosols and nanoparticles (NPs) science [1-5]. The size differentiation of NPs is based on the size-dependent, single-charged electrophoretic mobility of NPs so that NPs with various diameter will be separated under a series of voltages in a differential mobility analyzer (DMA). DMA works in a size range from 1 nm to 500 nm and the downstream condensed particle counter (CPC) quantifies the number of NPs screened by DMA. Consequently, both size and amount of NPs could be obtained with a scan time of few minutes.

Except for the applicability in NP measurement, ES-SMPS has demonstrated its performance in size measurement of bio-nanoparticls (bio-NPs) including proteins varies of sizes [6-8], viruses and virus-like particles [9-11], liposome [12], and etc. The correlation between mobility size and molecular weight of biomolecules has been reported. [6,13], and the aggregate of proteins can accordingly characterized [14,15]. Another advantage of the nanoscale ES-SMPS is the real time scanning of sample, which make the technique fascinating for biomanufacturing process, nanomedicine and pharmaceutical development. Taking the drug progress for Alzheimer's disease for instance, the fast monitoring of ES-SMPS verified the oligomerization of the 42-residue amyloid-β peptide and provided the knowledge of the aggregation mechanism [16]. For the quantification of absolute number concentration for bio-NPs, Li et al. proposed the method by evaluating the droplet size, measuring the number concentration of NP formed as different oligomers for the total sample

number summarization [17]; the internal calibration for the electrospray transmission efficiency proposed by Clouet-Foraison et al. [18].

To obtain accurate and reliable size measurements via ES-SMPS, the absolute calibration with proper reference materials (RMs) is needed. Commercially available RMs including polystyrene latex, metallic nanoparticles, and metal oxides in the solution are frequently selected for calibration. However, the presence of additives such as surfactant hinders the size calibration of ES-SMPS for NPs having size < 20 nm due to the formation of non-volatile residue coated on the pristine NPs (**Figure 1**). Alternatively, bio-NPs possess highly repeatable size and shape and long-term stability, and have been considered as candidate RMs for ES-SMPS recently. You et al. systematically evaluated five different bio-NPs illustrated their capability of offering superior calibration of ES-SMPS over conventional NP standards with high stability over several weeks [19]. Even though the consideration of bio-NPs as candidate RMs, research focusing on the selection of bio-NPs for calibration.is hardly found.

In this study, the suitable RM for the sub-10 nm size calibration for ES-SMPS was evaluated. AuNPs with diameter of 10 nm and 5 nm and four biomolecules involving bovine serum albumin (BSA), ubiquitin, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were selected for the size measurement. The pre-treatments of AuNPs by centrifugation and heating were applied for the removal of additives. Those treatment methods presented little effect on the accurate improvement of detected diameter and the difference was up to 112 % in Au-5 compared to the information from electron microscopy. On the contrary, BSA and ubiquitin performed consistent diameters with 6.9 nm and 3.6 nm, respectively, and showed comparable size distribution that referred to good stability as a function of time after dissolving in electrolyte. LDL and HDL contained multiple peaks in size distribution and were similar with that observed in previous study [20]. The results of this research revealed the advantages of using the self-disperse proteins and suggested the suitability of BSA and ubiquitin as the reference nanoparticles for sub-10 nm calibration for ES-SMPS.

2 Materials and methods

2.1 AuNP suspension preparation

The commercially available AuNPs with diameter of 10 nm (NIST-8011) and 5 nm (nanoComposix) were combined with electrolyte, which was 50 mM ammonium acetate (AmAc) in deionized water, at a volume ratio of 3:2 and had the final AmAc concentration of 20 mM.

Centrifugal sedimentation was applied on AuNP solution to reduce the amount of surfactant in original solution. 400 µl of the original AuNP solution was centrifuged at 14,000 rpm for 15 minutes and AuNPs were separated from the solvent by protein concentrator. 400 µl deionized water were then added and pipetted for AuNP resuspension. To perform two or more centrifugal cycles, AuNP suspensions were experienced another centrifugal process and solvent replacement. The centrifugal treated AuNP (in 400 µl deionized water) were mixed with 266 µl 50 mM AmAc solution and the final AuNP suspensions with 20 mM AmAc were prepared for ES-SMPS size scanning.

The thermal treatment was performed using a tube furnace installed between aerosol generator and ES-SMPS for the burnout of the surfactant in AuNP aerosols. 300 µl of the original AuNP solution was mixed with 200 µl 50 mM AmAc solution to form the AuNP suspensions with 20 mM AmAc. Four temperatures 20 @C, 200 @C, 500 @C, 800 @C were performed in gas condition.

2.2 Protein suspension preparation

Four biological macromolecules, bovine serum albumins (BSA; Sigma-Aldrich), ubiquitin (Sigma-Aldrich), low-density lipoproteins (LDL; Sigma-Aldrich), and high-density lipoproteins (HDL; Sigma-Albumin), were selected for testing. 10 mg proteins were suspended in 10 ml 20 mM NH4CH3COO solution in an ultrasonic both for 15 minutes for the good dispersion of proteins. Protein suspension samples with different concentrations were prepared by serial dilutions. For the stability monitoring, protein suspensions were stored at 4 @C and sonicated for 15 minutes at 20 @C.

2.3 ES-SMPS size characterization

The ES-SMPS system consisted of an aerosol generator (electrospray aerosol generator, ES; TSI 3480), a scanning mobility particle sizer (SMPS; TSI 3080) with a nano- differential mobility analyzer (DMA) column (TSI 3085), a condensed particle counter (CPC, TSI 3776). To generate droplets by the electrospray aerosol generator, the electrolyte with suitable conductivity is required and AmAc with a concentration of 20 mM has been recorded to produce droplets with the smallest size and the best performance. Therefore, AuNPs and proteins were suspended in 20 mM AmAc in this work. For NPs differentiation, the sheath flowrate was set as 20 L/min and aerosol flowrate was 1.5 L/min.

2.4 Data analysis

Size distributions of the AuNPs were measured three times and the detected diameters were showed as the mean with standard deviation. Biological macromolecules were measured five times and the mean with standard deviation were presented.

3 Results and discussion

3.1 Enlarging diameter of AuNPs in ES-SMPS

After mixing the original AuNP solutions and AmAc solution and having the final AmAc concentration of 20 mM, Au-10 and Au-5 suspensions were loaded to ES-SMPS system for the size scanning. Multiple peaks among the size distributions of both AuNP samples were found, as displayed in**Figure 2(A)** & **(D)** , and were considered as the influence of the surfactant or other impurity existing in the original AuNP solution. The centrifugal process replaced the most of the solvent in the original AuNP solution with deionized water/AmAc solution, and**Figure 2(B)** & **(E)** exhibited the reduction in surfactant/impurity in the AuNP suspensions as the dramatically decrease of NP numbers and disappearance of the peaks positioning at smaller diameters. In according with the size distribution of AuNP samples with centrifugal treatments, the diameters of Au-10 and Au-5 from the original solution were determined as 12.6 nm and 10.6 nm, which presented a respective difference of 26 % and 112 % from the nominal diameter.

3.2 Treatment for removing surfactant/impurity from AuNP solution

Figure 2(B) & **(E)** illustrated the decrease in both AuNP diameter and number corresponding to the reduction of surfactant/impurity by centrifugal treatment. A decreasing trend of diameter referring to more centrifugal cycles could be seen and 2-cycle of centrifugation (C2) was examined with the greatest reduction of AuNP diameters, where Au-10 and Au-5 were 12.2 nm and 8.2 nm and the difference from nominal diameter were 22 % and 64 % (**Table S1**). No apparent change in detected diameter between C2 and C4 while C4 displayed $>$ 10 % loss in AuNP number compared to C2 (**Figure S1**). The similar result of C2 and C4 processes were thought as the equilibrium of surfactant in solution, which was the basic amount for the nanoparticle dispersion.

To completely remove the surfactant, tube furnace was installed between the aerosol generator and ES-SMPS to burn out the surfactant/impurity from the original AuNP solution. The thermal treatment showed the decrease AuNP size and number compared to the original AuNP at 20 @ C (**Figure 2 (C)** & **(F)**). The diameter of Au-10 was measured as 12.2 nm at 200 @ C and remained constant at 500 @ C. In the case of Au-5, its diameter was 10.1 nm and 9.0 nm under temperature of 200 @ C and 500 @ C, respectively. At 800 @C both Au-10 and Au-5 showed a reverse growth, where Au-10 had a diameter of 13.6 nm and Au-5 was 9.3 nm (**Table S1**). The other method applying higher temperature to burnout the surfactant in the aerosol of gold nanoparticle was found to remove the organic components of surfactant, which resulted in the size reduction, but the inorganic surfactant remained on the nanoparticles. The expansion in AuNPs might be a result of reshape or recharge of AuNPs under high-temperature treatment and the phenomenon was not discussed in this paper.

3.3 Characteristic size of biological macromolecules

Dissolving in 20 mM AmAc, serially-diluted samples were prepared and the correlation between the protein concentration and size distribution was investigated. At 10^{-1} mg/ml scale of the protein concentration in electrolyte, only the BSA exhibited clear and separate peaks among size distribution, as shown in **Figure 3(A)** . Based on the electrophoretic mobility of NP, the primary peak, the representative size, was 6.9 nm, and the dimer (8.8 nm), and trimer (10.2 nm) of BSA could be clearly recognized [17]. On the other hand, the ubiquitin, LDL, and HDL possessed wider range of size distribution and the positions of monomer, dimer, and etc., were obscure (**Figure 3 (B) -(D)**). At the concentration of 10^{-3} mg/ml, the representative sizes of ubiquitin revealed and were 3.6 nm and the dimer was 4.6 nm for ubiquitin. Although a primary peak at 10.2 nm and 20.9 nm extinguished in HDL and LDL sample at 10^{-3} mg/ml protein concentration, the emergence of multiple peaks with inconsistent relation between size and number could not be explained by the theory of NP charging efficiency [20]. Consequently, HDL and LDL that possessed unknown electrophoretic behavior was not considered as the RM in this article.

3.4 Protein stability in electrolyte at sequential time frame

The major concern of selecting the bio-NPs as RM was its degradation and deactivation. To learn the stability of bio-NPs, the candidate proteins selected as the sub-10 nm RM, BSA and ubiquitin, which exhibited consistent results with literature [6,19,21], were suspended in 20 mM AmAc with the respectively protein concentrations of 0.1 mg/ml and 0.5 mg/ml. The size distribution and representative diameter of BSA and ubiquitin were monitored as a function of time to confirm the dispersive and structural stabilities of these proteins. In **Table S2** , the diameters of BSA and ubiquitin measured on sequential days were recorded and the size variance of their representative diameters remained < 10 % difference from literature.

On the 63^{th} day of BSA suspension, the detected diameter declined to 6.6 nm and had a 4.3 % difference from the value obtained on the 1^{st} day. A new BSA solution at the sample protein concentration was prepared and tested on the same day to approve the change in size, which contributed to the structural alternation of BSA in electrolyte. **Figure S3** indicated the similar size distributions and the same measured representative diameter of BSA generated from both the 63^{th}-day BSA solution and the fresh BSA solution measured on the same day. In association with the diameter of the 1^{st}-day BSA solution, the shift in size distribution was evident, from which not only the change in monomer but also dimer. On the 120^{th} day of BSA suspension, the representative diameter of BSA was again measured as 6.9 nm, which suggested the slight change in diameter was the floating of the ES-SMPS system. As a result, the consistent diameters of BSA and ubiquitin obtained in this experiment and reported in literature illustrated the repeatability of BSA and ubiquitin and presented their suitability as the sub-10 nm and sub-5 nm RM.

4 Conclusion

The conventional NPs such as AuNPs have been known to show the measurement uncertainty in ES-SMPS system because of the addition of surfactant, and the surfactant-depended uncertainty is more serious especially for the NPs with sub-10 nm diameter. In this experiment, two treatments including centrifugation and heating were used to remove the surfactant while both were found to present little effect on the detected diameter. Instead of working on the size improvement of AuNPs, the self-disperse biological macromolecules were considered for the sub-10 nm RM. Bio-NPs of four proteins were examined and the BSA and ubiquitin were characterized to have the similar charging efficacy with the conventional NPs, where the monomer, dimer and trimer could be seen among their size distributions. The representative diameter of BSA was 6.9 nm and ubiquitin was 3.6 nm. The suspense stability of these bio-NPs was monitored and the consistence in the detected size was approved. In conclusion, the BSA and ubiquitin were suggested as the sub-10 nm RM for the ES-SMPS calibration.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

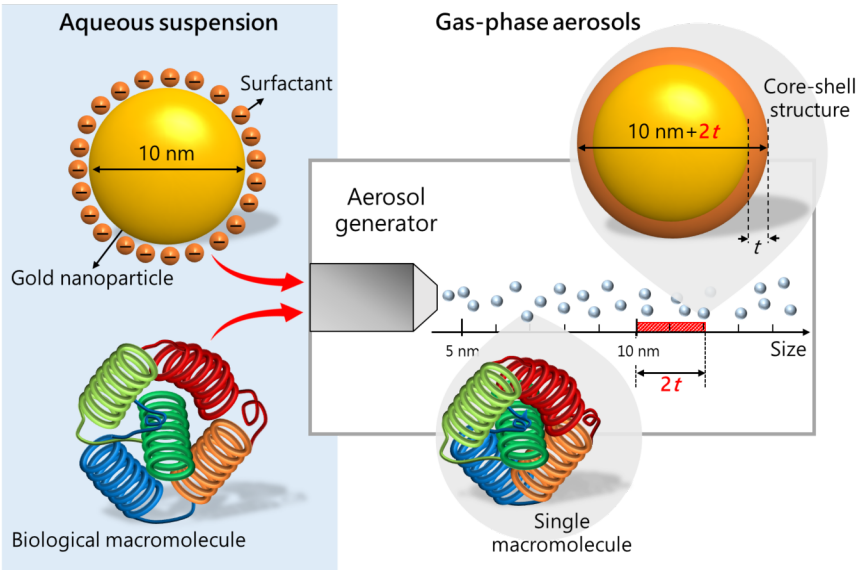

Figure 1. Scheme for the generation of gas-phase aerosols and the advantage of using bio-NPs as RM for ES-SMPS system. Formation of core-shell structure in AuNPs contributed to the surfactant, which prevents the aggregate of NPs. Biological macromolecules consist of different functional groups and natively separate from each at proper dissolving concentration.

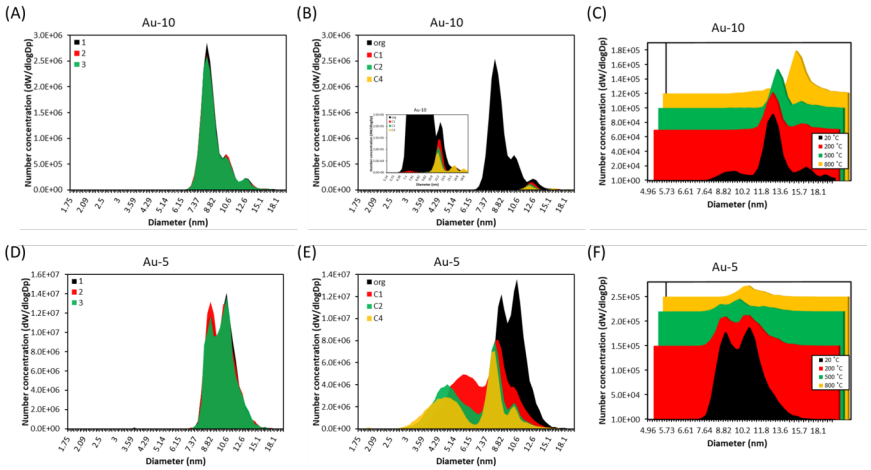

Figure 2. Size distributions of AuNPs. The original AuNP solution (org) of (A) Au-10 and the size alternation by treatments including (B) different centrifugal cycle, and (C) heating at various temperature.

(D)-(F) were the same situation for Au-5.

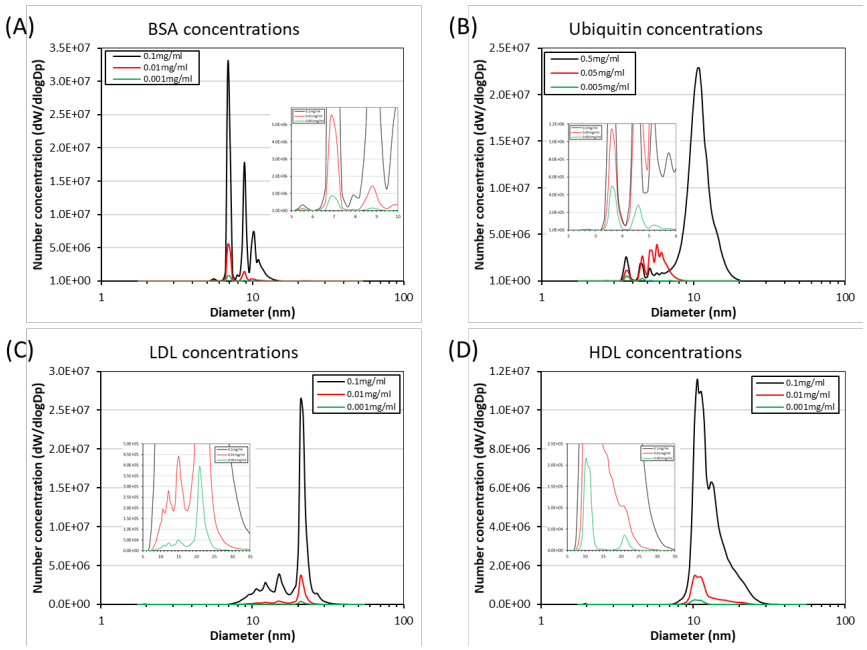

Figure 3. Size distributions of bio-NPs at different concentrations in electrolyte. (A) BSA ranging from 0.1 mg/ml to 0.001 mg/ml, (B) ubiquitin from 0.5 mg/ml to 0.005 mg/ml, (C) LDL at a range of 0.1 mg – 0.001 mg/ml, and (D) HDL with the same range as LDL.