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Simultaneous sizing and electrophoretic mobility measurement of sub-micron particles using Brownian motion

Akilan Palanisami^{1,2} and John H. Miller Jr¹

¹ Department of Physics and Texas Center for Superconductivity, University of Houston, Houston, TX

Abstract

The size and surface chemistry of micron scale particles are of fundamental importance in studies of biology and air particulate pollution. However, typical electrophoretic measurements of these and other sub-micron scale particles (300 nm – 1 μ m) cannot resolve size information within heterogeneous mixtures unambiguously. Using optical microscopy, we monitor electrophoretic motion together with the Brownian velocity fluctuations—using the latter to measure size by either the Green-Kubo relation or by calibration from known size standards. Particle diameters are resolved to $\pm 12\%$ with 95% confidence. Strikingly, the size resolution improves as particle size decreases due to the increased Brownian motion. The sizing ability of the Brownian assessed electrophoresis method described here complements the electrophoretic mobility resolution of traditional capillary electrophoresis.

Keywords

Brownian motion; diffusion; microfluidics; particle electrophoresis; particle sizing

1 Introduction

The electrophoretic mobility is a well-known sensitive probe of particle surface properties and has been used to characterize a variety of biological (e.g. mitochondria) [1] and colloidal [2] particles on the ~ 1 μ m size scale. Increasingly, the electrophoretic mobility is being measured simultaneously with other techniques such as fluorescent intensity [3], light scattering [4] and mass spectrometry [5] to form 2-D analyses. Despite the progress made in these synergistic 2-D studies, extracting the electrophoretic mobility as a function of particle size is still non-trivial; the particles must first be separated by size, and then electrophoretic measurements taken. However, size separation is not an option in sample limited situations. This is particularly relevant in mitochondrial studies, as mitochondrial size has been correlated with aging [6], type 2 diabetes and obesity [7]. Despite this, the correlation of size with the electrophoretic properties of individual mitochondria is relatively unstudied. In this regard, capillary electrophoresis (CE) in concert with laser induced fluorescence (LIF) [3] is a useful technique which can quantify the number of fluorescent molecules associated with an individual particle. However, in addition to being correlated with size, other aspects of the particle may influence fluorescent dye binding. For example, the commonly used fluorescent dye 10-nonyl acridine orange binds to cardiolipin in the inner mitochondrial

²Correspondence to Akilan Palanisami, Texas Center for Superconductivity, 202 Houston Science Center, Houston, Texas 77204-5002, Phone: 713-743-7312, Fax: 713-743-8201, apalanisami@uh.edu.

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membrane [8]. The LIF signal therefore measures the total quantity of cardiolipin per mitochondria, which depends on both mitochondrial size and cardiolipin concentration. Recently, laser scattering has also been used with CE to probe the morphology of individual mitochondria [4]. The degree of laser scattering is affected by size, morphology, internal structure, as well as the molecular composition of the mitochondria. Thus, if the size information could be determined separately, additional information could be extracted from the laser scattering and fluorescence data.

In another example, the size, surface chemistry, and toxicity characteristics of particulate matter air pollution is a serious question due to its correlation with respiratory disease and cardiovascular mortality [9]. Electrophoretic characterization of these particulates could provide unique information on this question. However, separating out airborne particulate by size before electrophoretic analysis is a daunting task due to the small numbers of particulate involved. As air samplers typically filter at only the 2.5 μm and 10 μm size scales, even the size distribution of these particles is poorly characterized. However, there is currently no method for probing the electrophoretic mobility and particle size of individual particles simultaneously.

We address this here by using optical microscopy to observe the Brownian motion of a particle simultaneously with its electrophoretic motion. In traditional particle electrophoresis, a voltage is applied to a suspension containing particles of interest, producing an electrophoretic particle motion. From the resulting drift velocity, the electrophoretic mobility can be extracted. However, more information is contained in the particle's higher frequency random motion (i.e. noise). These fluctuations in particle velocity are due to Brownian fluctuations and correspond to particle size. By measuring this motion component of the noise, the particle size can be estimated with surprising precision. The system described here can be used on any particle in which the Brownian motion can be observed simultaneously with the electrophoretic motion and can thus take advantage of a number of microscope imaging modalities (e.g. epifluorescence, dark field, differential interference contrast, etc.), allowing heightened flexibility in the application of this method. Here, we demonstrate operation and calibration of the instrument by using polystyrene beads of known mean diameter using phase contrast imaging.

2 Methods

2.1 Microscope and capillary preparation

The setup (Fig. 1) used an inverted microscope (Olympus IX-71) with a 1.6 $\times$ internal magnification, a 40 $\times$ phase contrast objective and a high speed CCD camera (Imperx IPX-VGA 210L) operating at 98.6 fps with a 10 ms exposure per frame. A halogen lamp provided illumination. The sample chamber was a borosilicate capillary of rectangular cross section (50 μm $\times$ 500 μm $\times$ 5 cm; Vitrocom) placed on a 1'' $\times$ 3'' glass slide. Reservoirs were provided by 0.5'' segments of 0.75'' diameter PTFE tubing epoxied to the glass. PTFE was chosen due to its uniformly hydrophobic surface. Background flow was a confounding factor due to meniscus formation of the fluid in the reservoir [10]. The diameter and hydrophobic character of the reservoir walls minimized this problem. To ensure a fresh glass surface, a 100 mM NaOH solution was slowly flowed for 30 minutes through the capillary. This flow was easily generated by filling one reservoir completely and only partially filling the other (~30 seconds to flow one chamber volume through the capillary with 1 cm height difference between the reservoirs). After the base clean, the reservoirs were washed thoroughly with de-ionized water (DI). Then, by use of a vacuum applied to one reservoir, the capillary was flushed with >150 μL of DI. The capillary was then slowly flushed with DI for 30 minutes before 150 μL of the running buffer was vacuum flushed through the system. The capillary was then equilibrated for > 2 hours under slow flow conditions before

experiments were performed. The running buffer consisted of 130 mM sucrose, 50 mM KCl, 5 mM MgCl_2 , 0.05 mM EDTA, and 50 mM MOPS (pH 7.4) and was chosen to be compatible with biological experiments (in preparation for future studies) as well as minimize the effect of electrode hydrolysis on the pH. The buffer viscosity was measured with a Cannon-Fiske viscometer (0.00110 Pa·s), and the electroosmotic flow (EOF) measured using the current monitoring technique [11].

2.2 Electrophoresis procedure

To load the chamber with sample, both chambers were emptied of buffer, and 50 μl of sample deposited over one end of the capillary. The other reservoir was then subjected to weak vacuum until the capillary had been flushed with several μl of sample. As a test standard, polystyrene beads of 1 μm , 750 nm, and 500 nm mean diameters (manufacturer quoted mean diameters: $1.025 \pm 0.01 \mu\text{m}$, $771 \pm 25 \text{ nm}$, and $456 \pm 15 \text{ nm}$; Polyscience) were diluted to ~10 ppm (w/v) before using. The excess sample in the reservoirs was then removed. 1 ml of buffer was subsequently added to both reservoirs and the Pt electrodes inserted. At this point, beads could be observed with the microscope, allowing removal of any background pressure driven flow by addition of small amounts of buffer to the reservoirs until bead drift motion stopped ($< 200 \text{ nm/sec}$). The microscope was focused in the center of the capillary, to avoid edge effects. Applying 35 volts to the electrodes induced bead movement through the field of view, which was digitally recorded. After measurement (5–20 minutes), the capillary was vacuum flushed with 100 μl of buffer, and a new sample inserted into the capillary.

2.3 Image analysis

A typical 5 minute experiment produced ~10 gigabytes of data. This large volume of data created a tradeoff between the detail of analysis and speed. The protocol described here allowed analysis of 10 GB of data in 20 minutes with an ordinary desktop processor (Intel Core2 Duo E6750). Images were analyzed using LabVIEW (National Instruments) software. Because of the phase contrast objective, 500 nm beads appeared as dark regions and the larger beads appeared as bright regions. Beads were identified by applying a pixel intensity threshold, where all clusters of pixels below (or above) a user-defined intensity were identified as potential beads. The algorithm then created a binary image of the clusters, with the pixel intensity information discarded. Dark (or bright) clusters smaller than 10 pixels were discarded. The center of each remaining dark (or bright) cluster was then estimated using centroid analysis. To link the particle positions into a particle trajectory, the algorithm outlined in Sbalzarini et al. [12] was implemented in ImageJ (written in Java). Briefly, a particle in frame n was paired to a particle in frame $n+1$. This pairing was shuffled between particles to produce the optimum set of connections, where the optimum is defined by the minimum of a cost functional; defined as the sum of quadratic distances between all paired particles. This linking procedure was relatively fast, as opposed to particle identification, which compromised the bulk of the analysis time. Tracks less than 3 seconds in duration as well as particles that came within 4 μm of each other were discarded. By discarding beads which are within 4 μm of each other, the Coulomb surface charge interaction between beads is negligible, as the Debye length (distance at which charge interactions become screened) of the buffer is ~1 nm. For the purpose of benchmarking the instrument, bead aggregates were identified using the type factor (see Supporting Information), which measures the circularity of the particle from 1 (most circular) to 0, and clusters below the 0.95 level were rejected.

3 Results and discussion

3.1 Velocity fluctuations within a mixed bead sample

To study the feasibility of characterizing the diameter distribution of an inhomogeneous particle mixture, 1.025 μm , 771 nm, and 456 nm beads were mixed in a 1:1.5:2.6 number concentration ratio and injected into the capillary. An applied electric field (7 V/cm in the x direction) led to bead motion against the EOF. A typical bead path is shown in Fig. 2a. The instantaneous velocity in the x direction (v_x) was calculated by subtracting the x positions of the bead between consecutive frames and multiplying by the frame rate (Fig. 2b). The instantaneous velocity in the y direction (v_y) was similarly calculated (Fig. 2c). The fluctuations in v_x and v_y were due to Brownian motion and were expected to be size dependent. To quantify the fluctuation, the velocity variance (σ_v^2) for each bead was measured:

$$\sigma_v^2 = \sigma_{v,x}^2 + \sigma_{v,y}^2 \quad (1)$$

$$\sigma_{v,x}^2 = \langle (v_x(t) - \langle v_x(t) \rangle)^2 \rangle \quad (2)$$

$$\sigma_{v,y}^2 = \langle (v_y(t) - \langle v_y(t) \rangle)^2 \rangle \quad (3)$$

where $\langle \rangle$ is the time average over the observation of the bead. The velocity variance is sensitive to only the fluctuations in velocity (the mean velocity is subtracted out). Therefore constant electrophoretic motion will not influence this quantity. However, a fluctuating electrophoretic mobility (perhaps due to the surface charge properties of the particle) can induce velocity fluctuations. For moderately spherical particles, these mobility related velocity fluctuations will only occur along the direction of electrophoretic motion (x direction), resulting in $\sigma_{v,x}^2 > \sigma_{v,y}^2$ (for Brownian motion $\sigma_{v,x}^2$ equals $\sigma_{v,y}^2$). For all measurements reported here, $\sigma_{v,x}^2$ equals $\sigma_{v,y}^2$ (within statistical error)—indicating the bead electrophoretic mobility (i.e., surface charge behavior) was not fluctuating on the measurement timescale. In the mixed bead sample, 471 tracks were characterized, with three distinct peaks apparent in the σ_v^2 histogram (Fig. 3a). Each peak corresponds to a particular size bead. This was confirmed by measuring the σ_v^2 histograms of each bead type separately (Figs. 3b–d). In the case of the 1.025 μm beads, the beads themselves displayed considerable size variation when anchored to a substrate and optically inspected (see Supporting Information). This size variation is reflected in Table 1 as well as Fig 3. Focusing our attention on the more homogeneous 771 nm and 456 nm beads, we find the σ_v^2 relative standard deviation (RSD) ~6% (Table 1). Thus, a single track provides enough information to measure the bead diameter to within $\pm 12\%$ with 95% confidence. Strikingly, because diffusion is greater for smaller particles, the resolution of this method improves with decreasing particle size (see Supporting Information), in contrast to other measurement techniques.

3.2 Theoretical considerations and calibration

The Brownian motion can be considered using the Green-Kubo relation [13],

$$D = \frac{1}{2} \int_0^\infty (\langle v_x(t) \cdot v_x(0) \rangle + \langle v_y(t) \cdot v_y(0) \rangle) dt \quad (4)$$

where D is the diffusion constant. At these timescales, the bead velocities from Brownian motion at different times are uncorrelated. This lack of correlation along with the Einstein relation (Eq. 5) and the Stokes drag for spherical particles (Eq. 6),

$$D = \frac{k_B T}{\zeta} \quad (5)$$

$$\zeta = 6\pi\eta r \quad (6)$$

where ζ is the particle drag, k_B the Boltzmann's constant, T the temperature, F the camera frame rate, r the particle radius, and η the buffer viscosity, allows calculation of r via the velocity fluctuations:

$$r = \frac{k_B T F}{3\pi\eta\sigma_v^2} \quad (7)$$

For all 3 bead types studied here, the measured mean σ_v^2 was roughly 25% larger than the theoretical prediction (Table 1). Deviation from the prediction is due to other sources of noise in addition to the Brownian motion of the particle. These sources include microscope stage vibration, camera dark count noise, the background spatial pixel intensity variation, and noise due to pixilation of the image in combination with the centroid position algorithm. To account for these and other sources of noise, the simplest protocol is to calibrate the system with beads of known diameter.

To calibrate using commercial beads, it is important to note that even though the mean bead diameter quoted by the manufacturer may be quite accurate (often < 0.01 RSD), the standard deviation in bead size within a given lot may be substantial, e.g., in the $1.025 \mu\text{m}$ beads used here, a diameter RSD of 0.08 was found (see Supporting Information). When calibrating, it is important to analyze a sufficient number of beads such that that bead size fluctuations can be averaged out. For instance, if 100 of the aforementioned $1.025 \mu\text{m}$ beads are used for calibration, the standard deviation in bead diameter drops from ~ 80 nm (for a single bead) to ~ 8 nm.

When using beads for calibration, there is a risk that particles of interest may image differently than the calibration beads, and this difference may influence σ_v^2 . The principle concern here is the edge of the particle, as pixel intensity noise inside the particle is removed by the binary image analysis. To probe this concern, the particle determination threshold was varied by 12% full scale. This had a negligible ($< 1.2\%$) effect on the mean of the bead σ_v^2 distributions. We also examined the average area of each particle image and compared it with its σ_v^2 , under the notion that smaller particles may have a larger σ_v^2 due to the proportionally larger number of noisy edge pixels as compared to the interior pixels. Again, a negligible correlation (Pearson product-moment correlation of $+0.004$) was found between the particle area and the σ_v^2 (Fig. 4). These tests suggest that calibrating σ_v^2 against bead size standards is a viable protocol, at least under the conditions used here.

3.3 Time of observation vs. size resolution

As discussed in the previous section, the bead diameter RSD will contribute to the σ_v^2 RSD; however the length of the bead time track also contributes to the σ_v^2 RSD. This is because the Brownian velocity fluctuations are random in time, resulting in sampling noise due to the finite time measurement. This noise shows up as a contribution in the σ_v^2 RSD. For pure Gaussian velocity fluctuations of identically sized beads, the RSD of the σ_v^2 histogram will equal $M^{-0.5}$, where M is the number of position measurements [14]. As beads were typically observed for 3–6 seconds (300–600 position measurements), the theoretical σ_v^2 RSD is roughly 0.05, very close to what was measured for the 771 nm and 456 nm beads (Table 1). The excess σ_v^2 RSD was largely due to the beads of a particular quoted size not being perfectly uniform. This is clearly apparent in the 1.025 μm beads which have a diameter RSD of 0.08 (see Supporting Information). Including this contribution increases the theoretical σ_v^2 to 0.10, which is very close to the measured σ_v^2 RSD of 0.108 for the 1.025 μm beads.

3.4 Electrophoretic mobility

The velocity fluctuation measurements were all done simultaneously with the application of an electric field driving electrophoretic motion. The electrophoretic mobility was measured by fitting the position vs. time data of each bead with a linear fit and subtracting out the EOF contribution. Thus every tracked bead has an estimated electrophoretic mobility and σ_v^2 . These data for the mixed bead sample (the same data as used in Fig. 3a) are displayed as a scatter plot in Fig. 5. The three bead groups are clearly resolved in the figure, validating this technique's utility as a method to simultaneously measure size and electrophoretic properties of individual $\sim 1 \mu\text{m}$ particles. The spread in the electrophoretic mobility measurements of a cluster are mostly due to the Brownian motion and are discussed in more detail later. We note the electrophoretic measurements alone give a smeared distribution between the three clusters—the σ_v^2 measurements are critical in distinguishing the clusters. The slight dependence of bead size with the electrophoretic mobility is consistent with a previous study [3].

3.5 Count efficiency

When interpreting the number of counts between different clusters, it is important to note that different classes of particles will be counted with different efficiency. For instance, larger particles are more likely to lie within the focal plane of the microscope, making them more likely to be counted. In addition, smaller particles are more likely to drift in and out of the focal plane (due to their larger D). Finally, particles with a faster net velocity will be oversampled relative to slower objects. Interpretation of the cluster count number is feasible, but the correction is sample dependent. These effects are seen when the original bead number concentration ratio for the 1.025 μm /771 nm/456 nm mixed bead sample (1:1.5:2.6), is compared with the ratio of the areas under the peaks of Fig. 3a (1.3:1.2:1). The qualitative observation of clusters is unaffected by these effects.

3.6 Electrophoretic mobility resolution

In this method, an inherent tradeoff exists between the resolution of the electrophoretic mobility and σ_v^2 , as increasing the measured Brownian “noise” improves the estimate of σ_v^2 but smears out the electrophoretic mobility distribution. The theoretical plate number of the electrophoretic measurement (N) is

$$N = \frac{L^2}{2Dt} \approx 400 \quad (8)$$

; where L is the length of the particle track (~50 μm) and t is the period of particle observation (~5 sec). For Brownian motion limited measurements, the electrophoretic mobility RSD is equal to $N^{-0.5}$, in this case giving ~5%, very similar to the measurement (Table 2). This degree of uncertainty, while not ideal, is sufficient to provide useful information on particles with heterogeneous electrophoretic mobilities, such as mitochondria [15]. If necessary, the plate number can be improved by increasing the applied voltage and camera frame rate. For example, increasing both the applied voltage to 875 V (185 V/cm) and camera frame rate to 2500 fps (attainable with a CMOS camera) would increase the N to a more respectable 10,000.

4 Concluding remarks

Noise is often a limiting factor in measurements, but here the particle velocity noise is put to good use by extracting particle size information simultaneously with the electrophoretic mobility. While other techniques such as electron beam microscopy or optical microscopy can have superior resolution (nm or less), they require the particles to be fixed to a surface and are not suitable for online CE detection. An unusual feature of this fluctuation sizing technique is the improvement of size resolution with decreasing particle size (until the particle can no longer be imaged). Furthermore, the resolution can be improved even further from what was shown here by simply increasing the frame rate of acquisition. In addition, particles are viewed directly, allowing removal of aggregates or other types of classification. By imaging only particles in the center of the capillary, sidewall sticking problems are also obviated (a serious consideration in some samples). These singular characteristics complement the high electrophoretic mobility resolution of traditional CE systems, and, as both CE and the Brownian-assessed electrophoresis technique described here require only μl amounts of sample, these two types of systems can be used together to obtain a more complete particle characterization than could be obtained by either system individually, particularly of sample limited inhomogeneous systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kostal V, Arriaga EA. Electrophoresis. 2008; 29:2578–2586. [PubMed: 18576409]
2. Rodriguez MA, Armstrong DW. J Chromatogr B. 2004; 800:7–25.
3. Duffy CF, McEathron AA, Arriaga EA. Electrophoresis. 2002; 23:2040–2047. [PubMed: 12210257]
4. Andreyev D, Arriaga EA. Anal Chem. 2007; 79:5474–5478. [PubMed: 17555300]
5. Klampfl CW. Electrophoresis. 2006; 27:3–34. [PubMed: 16315165]
6. Brunk UT, Terman A. Eur J Biochem. 2002; 269:1996–2002. [PubMed: 11985575]

7. Kelley DE, He J, Menshikova EV, Ritov VB. Diabetes. 2002; 51:2944–2950. [PubMed: 12351431]
8. Fuller KM, Duffy CF, Arriaga EA. Electrophoresis. 2002; 23:1571–1576. [PubMed: 12179973]
9. Pope C III, Dockery D. J Air Waste Manage Assoc. 2006; 56:709–742.
10. Crabtree HJ, Cheong ECS, Tilroe DA, Backhouse CJ. Anal Chem. 2001; 73:4079–4086. [PubMed: 11569795]
11. Huang X, Gordon MJ, Zare RN. Anal Chem. 1988; 60:1837–1838.
12. Sbalzarini I, Koumoutsakos P. J Struct Biol. 2005; 151:182–195. [PubMed: 16043363]
13. Kubo R. Rep Prog Phys. 1966; 29:255–284.
14. Riley, KF.; Hobson, MP.; Bence, SJ. Mathematical Methods of Physics and Engineering. Cambridge University Press; 2002.
15. Duffy CF, Fuller KM, Malvey MW, O’Kennedy R, Arriaga EA. Anal Chem. 2002; 74:171–176. [PubMed: 11795787]

Figure 1.
Electrophoresis schematic.

Figure 2.

Motion of a 456 nm bead in a 7 V/cm field. **a)** Bead motion is to the right. The vertical fluctuations are due to Brownian motion. Note x and y scales are not equal. **b)** Instantaneous velocity in the x direction vs. time. The average value is non-zero due to the electrophoretic motion. **c)** Instantaneous velocity in the y direction vs. time.

Figure 3. σ_v^2 histograms of beads undergoing electrophoresis. **a)** was taken on a mixed bead sample. The remaining histograms are of pure **b)** 1.025 μm , **c)** 771 nm and **d)** 456 nm diameter bead samples (106, 134, and 93 total counts respectively). Gray arrows confirm that the peaks seen in **a)** correspond to particular bead sizes.

Figure 4. Particle area vs. σ_v^2 for 771 nm beads. The x-axis is the mean area of a tracked particle over its entire track.

Figure 5. Electrophoretic mobility vs. σ_v^2 for 1.025 μm , 771 nm, and 456 nm beads. Fig. 3a is a histogram of this data along the x-axis.

Table 1**Bead Velocity Noise Properties**

Diameter (μm)	Velocity Variance ($10^{-10} \text{ cm}^2\text{s}^{-2}$)			Excess Noise
	$\sigma_{v,x}^2$ Mean $\pm$ SD	$\sigma_{v,y}^2$ Mean $\pm$ SD(RSD)	σ_v^2 Mean $\pm$ SD	$\frac{\langle \sigma_v^2 \rangle}{\left(\frac{k_B T F}{2\pi\eta r} \right)} - 1$
0.456	111 $\pm$ 8	111 $\pm$ 7	222 $\pm$ 12 (5.4%)	0.27
0.771	64 $\pm$ 5	65 $\pm$ 5	129 $\pm$ 8 (6.2%)	0.25
1.025	48 $\pm$ 6	49 $\pm$ 6	97 $\pm$ 11 (10.8%)	0.24

Data corresponds to Fig 3b–d. The “Excess Noise” is the fraction of the measured σ_v^2 that is in excess of the theoretical σ_v^2 expected from Brownian motion (Eq. 7).

Table 2

Bead Electrophoretic Mobility

Diameter (μm)	<u>Electrophoretic Mobility $\times 10^{-4}$ ($\text{cm}^2\text{V}^{-1}\text{s}^{-1}$)</u>	
	Mean $\pm$ SD	RSD
0.456	-3.07 ± 0.21	6.8%
0.771	-3.19 ± 0.16	5.0%
1.025	-3.48 ± 0.13	3.8%

Data corresponds to Fig. 5.