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Zeta-potential Analyses using Micro Electrical Field Flow Fractionation with Fluorescent Nanoparticles

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Abstract

Increasingly growing application of nanoparticles in biotechnology requires fast and accessible tools for their manipulation and for characterization of their colloidal properties. In this work we determine the zeta-potentials for polystyrene nanoparticles using micro electrical field flow fractionation (μ -EFFF) which is an efficient method for sorting of particles by size. The data obtained by μ -EFFF were compared to zeta potentials determined by standard capillary electrophoresis. For proof of concept, we used polystyrene nanoparticles of two different sizes, impregnated with two different fluorescent dyes. Fluorescent emission spectra were used to evaluate the particle separation in both systems. Using the theory of electrophoresis, we estimated the zeta-potentials as a function of size, dielectric permittivity, viscosity and electrophoretic mobility. The results obtained by the μ -EFFF technique were confirmed by the conventional capillary electrophoresis measurements. These results demonstrate the applicability of the μ -EFFF method not only for particle size separation but also as a simple and inexpensive tool for measurements of nanoparticles zeta potentials.

Introduction

Nanoparticles find application in many areas of biotechnology, including the diagnostics field because they may provide high activity, good selectivity when functionalized with appropriate ligands, a large specific surface area and small size [1]. Widely used nanoparticles for molecular diagnostics[2] are gold nanoparticles[3], quantum dots[4], and magnetic nanoparticles[5]. For the design and optimization of biochemical protocols using nanoparticles, a thorough characterization of their colloidal properties is necessary [6]. The uniformity of size of nanoparticles, within some practical limits, is therefore a precondition to their use. Although some methods for particle synthesis offer good monodispersity, other methods may yield broader size distributions[7]. In the latter case, methods for particle size separation are needed. In yet other cases, a separation based on size may be used to detect binding of large proteins or DNA to a particle.

Electrophoresis of charged particles offers a powerful technology for separations. The electric field flow fractionation (EFFF) method utilizes differences in the ζ -potentials between particles with different sizes to separate them in a flow in a microchannel between two electrodes that are parallel to the flow direction (Fig1.) [8]. When an electric field is applied across the channel, particles with higher ζ -potentials will move closer to the wall of the channel than particles with lower ζ -potentials. The flux of particles towards the accumulation wall will be limited by the Brownian motion which causes a natural diffusion. When particles are located across

the channel, different particle groups move along the direction of the flow toward the outlet port of the channel at different speeds determined by the flow velocity profile.

As a result, the elution time depends on the electrophoretic mobility and the particle size. If one of these two parameters is known independently, the other parameter can be obtained from previously derived theory by measuring the elution time in the EFFF experiment [9-12]. For good particle separation, particles of different size have to possess significantly different electrophoretic mobilities [9,13-16].

Recent trends in microchannel fabrication lead to advances in the miniaturization of chromatography systems. The field strength of a given liquid chromatography system increases as the system dimension is miniaturized [11]. A stronger field is available to drive the separation over a shorter distance which reduces the time of analysis. Typical macro scale EFFF systems have a length of 30-60cm, a height of less than 150 μm , and a breadth of about 2 cm while a number of μ -EFFF channels dimension ranges have heights of 20-30 μm , lengths varying from 4-6 cm, and channel widths from 0.4-8 mm [10].

Several detectors have been used to measure the presence of the particle samples separated from the channel. The most popular detector in previous works is a UV absorbance detector [10,11,14-19]. The UV extinction and light scattering techniques allow for a wide variety of sample types although this method is expensive, complex, and often demands modification of the sample being detected [20]. In addition, the UV detector measures absorbance of all particle sizes and this is an obstacle when analyzing the extent of separation. Alternative detection schemes could be very useful.

Gale et al. [20] reported an electrical conductivity particle detector fabricated by creating low impedance electrodes on the top and bottom surface at the end of a separation channel. Gale et al. also described a new type of electrical impedance spectroscopy particle detector produced by measuring the phase and the magnitude of electrical impedance over a range of frequencies. The impedance detector consisted of 20 μm wide electrodes on the top and bottom surface of the separation channel [21].

Although fluorescent detectors are generally considered the most sensitive technique [21], they have not been used to analyze the sample fractions in previous studies of EFFF. Using fluorescence detection the size of particles can be identified precisely by their fluorescence emission.

In this work we employ the μ -EFFF method for separation of fluorescent nanoparticles with different sizes and corresponding fluorescent spectra. We use the fluorescent detection to demonstrate the use of a μ -EFFF system for measurement of the ζ potential of the particles using the well-developed theory of μ -EFFF.

Micro EFFF theoretical background

A brief description of the relevant theory is required to understand the analysis of the experimental results. A primary variable in this theory is the electrophoretic mobility μ that refers to the velocity of a particle during electrophoresis [11,22].

$$\mu = \frac{U}{E} \quad (1)$$

where U is the velocity of a particle and E is the electric field. Due to the complexity caused by the accompanying counter-ions packed around the particle surface and molecules around the particle, electrophoretic mobility is usually classified in terms of the product $\kappa\epsilon$ [23,24]

$$\kappa \cdot r < 1, \quad \mu = \frac{2\zeta\epsilon}{3\eta} \quad (\text{Hückel}) \quad (2)$$

$$\kappa \cdot r > 100, \quad \mu = \frac{\zeta\epsilon}{\eta} \quad (\text{Smoluchowski}) \quad (3)$$

where κ is the electrical double layer thickness, ζ is the zeta-potential, η is a background electrolyte viscosity, ϵ is the dielectric permittivity of the medium, and r is the radius of a particle.

The cross-channel driving forces arise from the electric field and diffusion. This concept can be quantified theoretically by using several equations [10-12]. The modified Einstein equation can be used to describe the dispersive effect represented by D , the diffusion coefficient.

$$D = \frac{kT}{3\pi\eta d} \quad (4)$$

where k , T , η and d are the Boltzmann constant, an absolute temperature of a solution, a background electrolyte viscosity, and the particle diameter, respectively. The dimensionless parameter λ is a ratio of the particle cloud thickness (D/U) to the height of the channel w .

$$\lambda = \frac{D}{Uw} \quad (5)$$

This parameter λ can be related to the retention ratio, R , defined as a measure of the average particle velocity along the channel compared to the average carrier velocity.

$$R = 6\lambda \left(\coth\left(\frac{1}{2\lambda}\right) - 2\lambda \right) \quad (6)$$

The retention ratio can be found from the ratio of void time and elution time. The void time is the time required for filling the solution into the channel completely and the elution time is a time required to get the particles of interest to the channel exit. So the retention ratio can be derived experimentally as

$$R = \frac{V_o}{V_e} = \frac{t_o}{t_e} \quad (7)$$

where V_o is a void volume, V_e is an elution volume, t_o is a void time, and t_e is an elution time. This equation is valid when the flow is a constant fluid flow with time. From equations (4) - (7) the theoretical estimate of the retention time can be very different from that of experiment result when t_e is derived by measuring t_o and λ found from D and U . The electrophoretic mobility is related not only to the zeta-potential of the particle but also the applied electric field. Typically, the theoretical retention time is of the order of 2-3 days when naively assuming that the electric field in the channel is equal to the applied field. However, the experimental retention time, usually 10-30 minutes, suggests that the actual field inside the channel is much less than the applied field. This behavior is due to the electric double layer between the electrode and the carrier fluid. Counter-ions and molecules close to top and bottom walls tend to shield the electrodes inside the channel and the actual electric field strength, which affects the particle motion in the medium, is reduced considerably. Thus, the actual effective field for retention in the channel is around 1% of the applied field [10,14]. The actual field strength is proportional to the effective voltage across the solution and it is given as

$$V_{eff} = E_{eff} w \quad (8)$$

where V_{eff} and E_{eff} are the effective voltage and the effective field, respectively. Using equations (1) and (8),

$$U = \mu E_{eff} = \frac{\mu V_{eff}}{w} \quad (9)$$

and λ is found from

$$\lambda = \frac{D}{\mu V_{eff}} \quad (10)$$

Experimental

Carriers

Various buffer solutions with different pH values and purified de-ionized water were used in the experiments. The solutions were 10mM Citrate-Acetate buffer with pH 4.5, 10 mM MES (Morpholinoethanesulfonic) buffer with pH 5.3, 10 mM Citrate buffer with pH 6, 10 mM Citrate Phosphate buffer with pH 7, and 10 mM Carbonate buffer with pH 8.6 and pH 9.24. The buffers altered the zeta potentials of the particles.

Nanoparticle Samples

Commercially available, quasi-monodisperse, fluorescent polymer nanospheres (Duke Scientific Corporation) were used. Particles with diameters of 28 nm, impregnated with green dye, and 98nm particles with red dye, were used throughout this study. The spheres were made of polystyrene, which has a density of 1.05 g-cm³ and a refractive index of 1.59 at 589 nm. The aqueous suspensions are packaged as 1% solids in a multicomponent diluent which prevents clumping and aids in dispersion. The Coefficient of Variation of the diameter (standard deviation as a percent of the mean) ranged from 10% to 20%. All samples were appropriately diluted in the carrier prior to injection into the channel. The 28 nm particles were diluted from 10 to 0.016 mg-mL⁻¹ and 98 nm particles were diluted from 10 to 0.25 mg-mL⁻¹ to improve the separation efficiency and to conserve expensive reagents.

EEFF Device fabrication

The EEFF device shown in Figure 1 was made of ITO-one side coated glass slides with dimensions of 25 × 75 × 0.7 mm and a sheet resistance R_s of $6.2 \pm 2 \Omega$ (Delta Technologies, MN 55082-1234). A 60 μ m thick spacer made of epoxy (Devcon) was used to form the side walls of the channel. Inlet and outlet ports with a diameter of 1.6 mm for external fluid connections were drilled in the lower glass plate. The channel has a ribbon-like structure with trapezoidal ends (60 μ m height, 8 mm breadth and 62.5 mm length).

μ EEFF Instrumentation

The system consisted of the EEFF device, a DC power supply (GW, model GPS-3030D), two alligator clamps, a polyethylene tubing with I.D. 1.14 mm and O.D. 1.57 mm (Becton Dickinson and Company, Sparks, MD 21152-0370), a Monoject aluminum hub blunt needle (Tyco Healthcare Group LP, Mansfield, MA 02048), a Fluke 83 III multimeter (John Fluke MFG. Co., Inc., Everett, Washington), 1 mL syringe needle (Allometrics, Inc., Baton Rouge, LA 70815), Teflon tubing, a 12 cc Monoject syringe with regular tip (Sherwood Davis & Geck, St. Louis, MO 63103), 50 μ L syringe (Hamilton Co., Reno, Nevada), a rubber stopper, a syringe pump (Cole Parmer, Vernon Hills, Illinois 60061), 96 well plates (Nunc, Kamstrupvej 90, DK-4000 Roskilde, Denmark, model 96F Untreated Black Microwell SH), and a microplate

reader (Molecular Devices Corporation, Orleans Drive, Sunnyvale, CA 94089, model SpectraMax M2).

A T-shaped connector was attached on the drilled inlet hole for both injecting the particle samples and carrying the fluid into the EFFF channel. One opening in of the connector was linked to the Teflon tubing to inject the 3 μL sample volume ($0.133 \text{ mg}\cdot\text{mL}^{-1}$) of the nanoparticles and the other opening was connected to the syringe pump via the polyethylene tubing to transport the carrier into the channel. The sample and the carrier exited via the 1 mL syringe needle glued to the drilled outlet hole. The DC power supply was connected to the top and the bottom ITO layers via two alligator clamps.

Capillary Electrophoresis System

A conventional capillary electrophoresis device was used for comparison with the μEFFF device. The system consisted of a standard glass rectangular cell with section of $10\times 1 \text{ mm}$ (Mk II, Rank Brothers Ltd.), platinum wire (0.5 mm diameter, Alfa Aesar), DC power supply (GW, model GPS-3030D), Opolette tunable optical parametric oscillator laser (Opotek Inc.), PTG Programmable Timing Generator (Princeton Instruments), PI-MAX Camera (Princeton Instruments), and ST-133 Controller (Princeton Instruments). The rectangular U-shaped capillary tube was attached to a translation stage. To avoid errors due to electroosmotic flow, the detector was focused at stationary level positions following the instructions of the Mk II user manual[25]. Two electrodes made of platinum wire were added to the ends of the tube. The electrodes were connected to the DC power supply. An OPO (Optical Parametric Oscillator) tunable laser system and spectrophotometer system were used to excite the fluorescent particles and to detect the fluorescence.

μEFFF Operation

The channel was filled with the various buffer solutions via the polyethylene tubing while the stopper was opened; the opening of the rubber stopper was also filled with the solutions to prevent air bubble formations from the sample injection. After this step, the sample injection opening was closed by using the stopper. The pump flow was stopped before the sample plug entered the device. A 3 μL sample volume ($0.133 \text{ mg}\cdot\text{mL}^{-1}$) was injected into the channel by inserting the 50 μL syringe into the Teflon tubing. The electric field was applied at this point. No flow was applied in order to provide the relaxation time, about 60 seconds, needed to obtain colloidal stability of the particles. While continuing to apply the chosen voltage, the syringe pump was restarted with a constant carrier flow to start the separation process. Samples of 8 droplets each were collected from the 1 mL syringe needle in the outlet port; the samples were transferred to individual wells of a 96 well plate. The volume of each droplet was 5 μL ; the 40 μL volume of the combined sample was the minimum quantity required to get a measurable fluorescent response. The fluorescent spectra of the collected samples were measured on a microplate reader.

Electrophoresis System Operation

The capillary tube was filled with 7.96 mL of 10 mM carbonate buffer with pH of either 8.6 or 9.24. A potential of 70 V DC was applied across the electrodes. Forty μL of single size particle suspension were injected into the inlet. The OPO laser and the spectrophotometer system were used to detect the particle cloud at the start point. The laser was operated at 430 nm to excite 28 nm particles and at 490 nm for 98 nm particles. After measuring the fluorescence intensities at the inlet, the capillary tube system was translated so that the focused beam was directed to a location near the outlet. The time needed for the particle cloud to reach the end point was noted when the fluorescence signal appeared at the second location.

Results and Discussion

Current / Voltage Characteristics

The current-voltage relationship for the channel is important as the voltage should be applied within a safe working-range for the channel. This dependence is shown in Fig. 2. These two figures reveal that different kinds of buffer solution exhibit different relationships of voltage and current. At low voltages, the current across the channel is very small; at a “turn-on” voltage, current rises. In the low voltage plateau region, the particles do not have enough surface charge to move downward to the bottom of the glass slide – particle separation was not observed in this region. At a voltage particular to each solution pH, the current rises quickly in an approximately linear fashion. The voltage-current relationship was also affected by the flow rate. The turn on voltage was relatively low with a fast flow rate. The currents inside the channel in Fig. 2 were measured at a flow speed of $2.8 \text{ mL}\cdot\text{h}^{-1}$.

These data are helpful in determining a proper range of voltages. Low voltages will not yield sufficient effective electric field for a separation, while high voltages exceeding the turn-on voltage can cause electrolysis inside the channel and also damage the ITO layer coated on the glass slides. In Fig. 2, the turn-on voltages are 1.4 V for the 10 mM Carbonate buffer with pH 8.6.

Particle separation by size

For particle size separation experiment, a $30 \mu\text{L}$ solution was prepared with $0.016 \text{ mg}\cdot\text{mL}^{-1}$ 28 nm particle concentration and $0.25 \text{ mg}\cdot\text{mL}^{-1}$ 98 nm particle concentration giving a total concentration of $0.133 \text{ mg}\cdot\text{mL}^{-1}$, in 10 mM carbonate buffer pH 8.6. Voltage of 1.7 V was applied across the channel generating a current of $29.8 \mu\text{A}$. Stop flow time of 1 min was used. Flow rate was set at $2.8 \text{ mL}\cdot\text{h}^{-1}$. Droplets were collected from the outlet for 60 s intervals in separate wells of a microplate. The volume collected in each well was $40 \mu\text{L}$. The fluorescent spectra of the collected samples were recorded under excitation at 260 nm and are presented in Fig. 3. The first and the second samples show the presence of the 98 nm red dye particles and therefore their elution during the first 120 s. The spectra of the ninth, the tenth, and the eleventh samples indicate the elution of the 28 nm green dye particles in the time interval from 600 to 720 s. Particles were clearly separated from each other.

To determine the elution time of most of the particles from a given size class, the individual spectra of the 98 nm particles for 1, 2 and 3 min are compared in Fig. 4 at different times. The intensities decayed as time increased, showing that the 98 nm particles were separated from the mixture of the green and red dye particles when the flow was restarted after a one minute stop time with the electric field applied to the channel. The larger particles eluted first and no more large particles were detected with the plate reader after the first three minutes. On the other hand, the 28 nm particles persisted in the channel longer and started to be eluted from the outlet port at 8 minutes; most of these particles were eluted between 9 and 10 minutes as shown in Figure 5. The channel voltage was 1.7 V in these experiments, a voltage higher than the turn-on voltage of 1.4 V at pH 8.6. It was found that effective particle separation could not be obtained at lower voltages. However, experiments using a higher voltage than 1.7 V caused electrolysis and formed bubbles inside the channel. Similar measurements were carried out at pH 9.24.

Particle Group Transit Time Measurements

The velocity of a group of particles of a single size was measured in the rectangular glass capillary tube under the conditions described in the previous section, not only to confirm the values of the electrophoretic mobilities of the fluorescent nanoparticles but also to compare these values with numbers estimated from the μ -EFFF separation in next section. The applied

field strength between the electrodes was about $318.2 \text{ V}\cdot\text{m}^{-1}$. The time period needed for the injected $40 \text{ }\mu\text{L}$ of particle suspension to move between the ends of the rectangular cell in the capillary tube was measured repeatedly with the laser/spectrometer set up to obtain an average time for particle movement. The time periods are shown in Fig. 6. The time standard deviation for each measurement was approximately 49 seconds. The distance between the two interrogation regions of the capillary was 130 mm. The particle velocities could be obtained based on the mean transit times. These results suggest that the 98 nm particles have almost zero charges on the surfaces in the carbonate buffer comparing with 28 nm particles and support the observation that the 98 nm particles were eluted from the micro-channel ahead of the 28 nm particles.

Zeta-potential Analyses

The carbonate buffers with pH 8.6 and pH 9.24 were prepared using Na_2CO_3 and NaHCO_3 mixed with double distilled deionized water. For these particular solutions, the Debye-Hückel parameter κ has a value of 0.64 nm^{-1} and the $\kappa\cdot r$ products of 28 nm particles and 98 nm particles are $8\cdot 10^{-9}$ and $31\cdot 10^{-9}$, respectively [26]. These particles can therefore be supposed to follow the Hückel equation and the zeta-potential of each particle size group can be determined using equation (2).

The ζ -potentials of the particles can be estimated from results of the capillary electrophoresis experiment. Using particles velocities based on the particles' transit times in each pH buffer, the ζ -potentials are derived as shown in Table I. The ζ -potentials of the fluorescent nanoparticles can also be obtained from the particle separation results in the microchannel device. This is, in a sense, an inverse problem and uses the theoretical framework that was described earlier. The void time is defined as

$$t_o = \frac{w \times b \times L}{R_v} \quad (11)$$

where w is the height of the channel, b is the breadth of the channel, L is the length of the channel, and R_v is a volume flow rate of the buffer. The retention ratio, R can be obtained from (7) by using the calculated void time and the measured elution time. In addition, the dimensionless parameter, λ can be found by solving eq. (6). The electrophoretic mobility μ can be obtained from equation (9) by inserting the drift velocity caused by the electric field, U from equation (5), followed by calculation of the diffusion coefficient, D from equation (4). The effective voltage was considered to be an average 0.6% of the applied voltage in this case – the effective voltage across a channel is known to be in the range of 0.25% to 1% of the applied voltage, depending on the composition of the buffer [10,11,14]. Values of the parameters used were: temperature, $T = 298.15 \text{ K}$, and background electrolyte (carbonate buffer) viscosity, $\eta = 8.903\cdot 10^{-4} \text{ kg}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$.

Table 1 compares the particle parameters for the conventional electrophoresis system and the μ EFFF system. The two different approaches generated similar electrophoretic mobilities and zeta-potentials, although the effective applied voltage in the micro-channel is not known precisely.

Once the particles are separated, the properties of nanoparticles in any buffer solution can be estimated by knowing the retention ratio, the dimensionless parameter, the diffusion coefficient, and the particle drift velocity inside the microchannel (Table 1). This analysis is a powerful tool to estimate size-dependent properties of particles. The system offers significant improvement in terms of size and economical efficiency in comparison with the typical zetameters.

The zeta potential estimates can also explain why big particles were eluted earlier than small particles in the μ -EFFF system. In this paper, the separation results were typical for the steric mode. Unlike previously reported data [10,14,15,20,21], the 98 nm particles did not seem to possess higher surface charge when compared to the 28 nm particles. The values of the calculated dimensionless parameter λ were close to zero. As a result, the drift velocity caused by the electric field inside the channel was significantly greater than the transport due to diffusion and the predominant force was the electrostatic force. In both cases of pH 8.6 and pH 9.24, the drift velocity of 28 nm particles was up to 65.5 times higher than that of 98 nm particles shown in Table 1. Consequently, the 28 nm particles were concentrated closer to the bottom plate of the channel than the 98 nm particles. They were retained in a zone with lower flow velocity and were eluted from the microchannel after the 98 nm particles.

Conclusions

Fluorescence emission spectra offer a useful diagnostic tool for studying and evaluating the next generation of precise μ -EFFF separation systems. In comparison to UV light absorption detection, fluorescent emission is more sensitive and better resolved, resulting in an improved detection sensitivity for size discrimination of separated fluorescent polymer nanoparticles from a micro-channel. Fluorescent particle mixtures of different electrophoretic mobility or particle size were successfully separated in an ITO-coated microchannel. Particle separation was governed by a steric mode of electrophoresis. Important particle properties such as the electrophoretic mobility and the zeta-potential were successfully compared with the experimental results from a conventional capillary electrophoresis system that also made use of fluorescence emission for detection of the nanoparticles. The proposed analysis should permit rapid determination of nanoparticle properties in specialized buffers when the particles are separated by a μ -EFFF system.

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Figure 1. Schematic representation of the EFFF channel. Epoxy glue is used as a spacer to form side walls of the channel.

Figure 2.
Measured current values of 10 mM carbonate buffer with pH 8.6 and pH 9.24.

Figure 3.
Separation of 28 nm particles from 98 nm particles at pH 8.6

Figure 4.
Separated 98 nm red dye particles emission spectra in pH 8.6.

Figure 5.
Separated 28 nm green dye particles emission spectra in pH 8.6.

Figure 6.
Particle transit times in capillary electrophoresis

Table 1
Comparison of Particle Properties of μ -EFFF system with Capillary Electrophoresis System

Parameter		28nm particle groups in pH 8.6	98nm particle in pH 8.6	28nm particle in pH 9.24	98nm particle in pH 9.24
Electro-phoresis System	U [m / s]	$(4.276 \pm 0.738) \times 10^{-5}$	$(9.640 \pm 0.035) \times 10^{-7}$	$(5.058 \pm 0.327) \times 10^{-5}$	$(7.763 \pm 0.017) \times 10^{-7}$
	μ [m^2/Vs]	$(1.344 \pm 0.232) \times 10^{-7}$	$(3.029 \pm 0.011) \times 10^{-9}$	$(1.590 \pm 0.102) \times 10^{-7}$	$(2.440 \pm 0.056) \times 10^{-9}$
	ζ [V]	$(2.582 \pm 0.045) \times 10^{-1}$	$(5.821 \pm 0.021) \times 10^{-3}$	$(3.054 \pm 0.020) \times 10^{-1}$	$(4.688 \pm 0.010) \times 10^{-3}$
μ -EFFF system	R	0.071	0.638	0.060	0.714
	λ	0.012	0.153	0.010	0.190
	D [m^2/s]	1.752×10^{-11}	5.006×10^{-12}	1.752×10^{-11}	5.006×10^{-12}
	U [m / s]	2.411×10^{-5}	5.450×10^{-7}	2.883×10^{-5}	4.403×10^{-7}
	μ [m^2/Vs]	1.418×10^{-7}	3.206×10^{-9}	1.696×10^{-7}	2.590×10^{-9}
	ζ [V]	2.725×10^{-1}	6.159×10^{-3}	3.258×10^{-1}	4.976×10^{-3}