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Ultrasonic-trap-enhanced selectivity in capillary electrophoresis ☆

M. Wiklund [a,*], P. Spégel [b], S. Nilsson [b], H.M. Hertz [a]

[a] Biomedical and X-Ray Physics, Royal Institute of Technology, SE-106 91 Stockholm, Sweden
[b] Technical Analytical Chemistry, Center for Chemistry and Chemical Engineering, P.O. Box 124, SE-221 00 Lund, Sweden

Abstract

We combine ultrasonic trapping and capillary electrophoresis (CE) with the goal to detect ultra-low concentrations of proteins via size-selective separation and enrichment of antibody-coated latex spheres. An 8.5 MHz standing ultrasonic wave is longitudinally coupled into the sub-100-μm diam capillary of the CE system. Competition between acoustic and viscous forces result in in-flow separation of μm-diam spheres according to their size. Experiments separating 2.8- and 2.1-μm-diam fluorescent latex particles, which model a protein-specific immunocomplex/free particle mixture, indicate a potential improvement of the concentration limit of detection of 10^4 compared to current CE systems. Theoretical calculations show room for further improvement.

Keywords: Ultrasonic trapping; Particle separation; Capillary electrophoresis; Concentration limit of detection

1. Introduction

Trace-amount detection of proteins and other biomolecules is of great importance in biomedical analysis. A modern, high-performance tool for this purpose is capillary electrophoresis (CE), providing a sensitive and fast technique for separation of different species in very small sample volumes [1]. We have previously shown that acoustic radiation forces coupled into <100 μm capillaries can be used for in-flow size-selective trapping of μm-sized latex particles [2]. In the present article we combine CE with ultrasonic trapping for improved size selectivity. Furthermore, we evaluate the prospects for improved concentration limit of detection (CLOD) in CE by in-flow highly size-selective long-term sample enrichment via antibody-coated latex spheres.

CE separates analytes according to charge and size with high selectivity and high throughput. CE is very sensitive in terms of low-amount detection, but it is considered to be less sensitive in terms of CLOD as a consequence of the small sample volumes used (typically *pl-nl*). The CLOD using fluorescent analytes is typically 10^{-12}–10^{-14} M [3,4]. Recently, the use of antibody-coated microspheres in CE has been reported as a promising method for trace-amount detection of proteins [2,5]. Here, two different monoclonal antibodies against spatially separated epitopes of one and the same protein are covalently bound to latex spheres. If that protein is present, a latex–protein–latex complex (immunocomplex) can be formed, which may be detected in a conventional CE system. Below we show that ultrasound-trap-enhanced CE with in-flow sample enrichment shows significant potential for improved CLOD of such immunocomplexes.

The theory of acoustic radiation forces in standing-wave fields has been derived by several authors [6,7]. Previous biomedical applications include, e.g., filtering or agglomeration of particles or cells in suspensions with the acoustic field transverse to the flow direction [8,9]. Recently, we have shown that longitudinal ultrasonic standing waves [10] may be used for size-selective trapping and retention of microspheres inside small-diameter capillaries (<100 μm) [2]. Here, the acoustic field and the flow are parallel in a hemispherical geometry to achieve high acoustic radiation forces in the flow direction inside the capillary. The separation principle is based on the competition between acoustic radiation forces and viscous drag forces on the spheres. However, the parabolic flow profile of Ref. [2] caused non-uniform viscous forces, making this system less suitable for highly selective

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* Corresponding author. Fax: +46-8-55378466.
E-mail addresses: martin.wiklund@biox.kth.se (M. Wiklund), hertz@biox.kth.se (H.M. Hertz).

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size separation. In the present article we improve the velocity uniformity by the use of electro-osmotic flow (EOF), i.e., by combining the ultrasonic trap with a CE system. We theoretically evaluate the limits of separation due to Brownian motion and secondary acoustic forces for this plug-flow system. Furthermore, we experimentally evaluate the prospects for improved CLOD and separation capability with an improved laser-induced fluorescence (LIF) detection scheme based on colour identification and single particle counting.

The goal of the ultrasonic-enhanced CE system is to size-selectively separate latex–protein–latex complexes (doublets) from single latex particles (singlets), thereby increasing the sensitivity by long-term in-flow sample enrichment [2,5]. In the present article we model the doublets and singlets with big and small latex spheres having a 1.3:1 diameter ratio (typically 2.8 and 2.1 μm), corresponding to the 2:1 volume ratio of doublets and singlets.

2. Theoretical background

In this section we theoretically evaluate limits of in-flow sample enrichment due to Brownian motion and inter-particle forces originating from the scattered acoustic field (Bjerknes forces). The experimental arrangement for the ultrasonic trap is shown in the lower part of Fig. 1 (cf. Section 3). The major competing forces on a particle (cf. insert in Fig. 2) are the acoustic radiation force from the ultrasonic standing wave and the viscous drag force from the EOF. The acoustic force (F_{acoust}) along the capillary axis (z-axis) is [2]

$$F_{\text{acoust}} = C_{\text{a}} I_{\text{a}} r^3 \sin(2k_z z), \tag{1}$$

where I_{a} is the acoustic intensity, r is the particle radius and k_z is the wave number $2\pi/\lambda$. The constant C_{a} depends on the acoustical properties of the system. Eq. (1) shows that the acoustic force on a particle has a strong size-dependence and can be controlled by adjusting the acoustic intensity level. Since the diameter of the acoustic beam in focus is much larger than the capillary, the radial intensity variation over the capillary cross-section area is negligible [2].

The other major force in the capillary is the viscous drag force [2]

$$F_{\text{visc}} = C_{\text{v}} v r, \tag{2}$$

where v is the particle velocity and C_{v} is a constant depending on the viscosity of the liquid. In CE, the driving flow is the EOF characterized by a flat velocity profile, contrary to the parabolic-shaped profile of laminar-flow system used in Ref. [2]. Thus, the system is designed to optimize the uniformity of the radial distribution of both F_{acoust} and F_{visc}.

The longitudinal net force along the capillary axis (z-axis) is the difference between the viscous drag force and the acoustic radiation force ($F_{\text{visc}} - F_{\text{acoust}}$). In Fig. 2, the net force is calculated along the z-axis with parameters chosen as in the experiments. The gray curve is the net force acting on a small sphere (2.0-μm diam) with velocity 1 mm/s and with an acoustic intensity of 100 W/cm^2 and the black curve is the net force acting on a big sphere (2.6-μm diam), same velocity and acoustic intensity. The acoustic intensity level is set as high as possible not to also trap the small sphere. The strength of the ultrasonic trap will then depend on the slope of the net force around the trapping point where the net force on the big sphere equals zero. Long in-flow sample enrichment times requires that the big sphere does not escape via Brownian motion. The escape may be modeled assuming that an isolated sphere moves stochastically in a near-harmonic potential well [10]. The dotted line is a linearization of the net force around the trapping point, with a slope (force constant) $k = 2.2$ pN/μm.

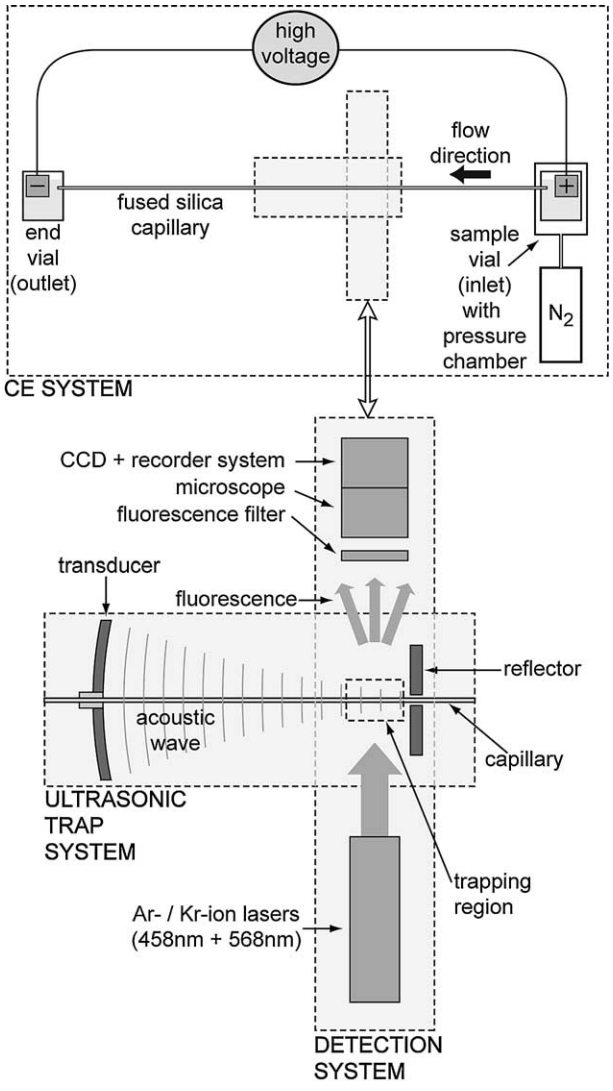

Fig. 1. Experimental arrangement for the ultrasonic-trap/CE system with LIF detection.

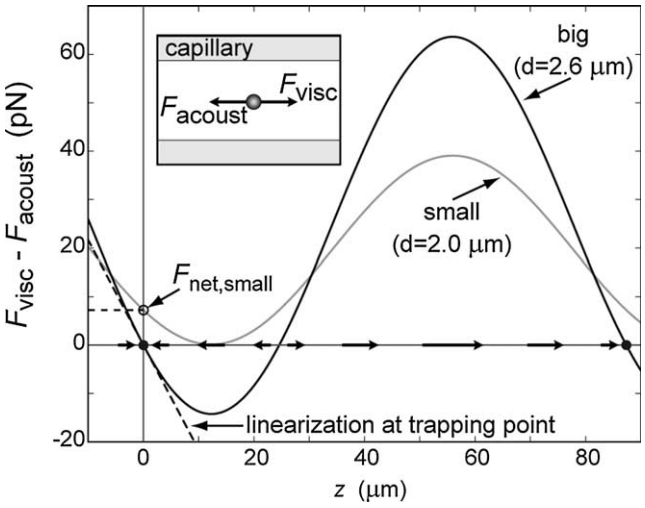

Fig. 2. Calculation of the longitudinal net force ($F_{visc} - F_{acoust}$) on a 2.0-μm-diam particle (gray curve) and a 2.6-μm-diam particle (black curve), simulating a singlet and a doublet, respectively. The acoustic intensity is 100 W/cm^2, the particle velocity is 1 mm/s and the acoustic wavelength in water is 180 μm (i.e., $\lambda/2 = 90$ μm between each trapping point).

The rms stochastical displacement from the trapping point is [11]

$$\sqrt{\langle z^2 \rangle} = \sqrt{kT/M\omega^2}, \tag{3}$$

where k is the Boltzmann constant, T the temperature and $M\omega^2$ the force constant in the harmonic oscillator, resulting in $\sqrt{\langle z^2 \rangle} = 50$ nm for our experimental parameters. Since the probability for a particle to deviate far from the trapping point is very low (e.g., $\sim 10^{-15}$ at 300 nm distance), and the natural oscillator frequency is only 2.5 kHz (from $k = M\omega^2$), a trapped particle should essentially never escape due to Brownian motion. Thus, very long in-flow sample enrichment times are feasible.

There is also a possibility that a small sphere passes an already trapped big sphere/doublet at very close distance. Then, radiation forces from the scattered acoustic field (Bjerknes forces) [7] can be relevant in comparison to the primary forces F_{acoust} and F_{visc} The Bjerknes forces can be both attractive or repulsive depending on the angle between the centerline of the spheres and the direction of the acoustic beam, and to the position in the standing wave. The worst possible case is when a small and a big sphere are oriented at right angles to the acoustic beam. Comparing the Bjerknes force in this case with the longitudinal net force on the small sphere at the trapping point for the big sphere ($F_{net,small} = 8$ pN in Fig. 2), the Bjerknes force is maximum 15% of $F_{net,small}$ in the case of particles in direct contact. Furthermore, electrostatic forces between the particles due to their surface charge will counteract the Bjerknes forces, and since the Bjerknes forces decrease rapidly with distance ($R^{-2} - R^{-6}$) they will be of even lesser importance. Finally, we should mention that the Bjerknes force on a small sphere will increase if several big trapped spheres are agglomerated at the same point. However, this situation can be avoided by continously releasing the trapped spheres after they are counted.

Thus, we may conclude that Brownian motion or Bjerknes forces are very small and should not limit the capabilities of an ultrasonic-trap-enhanced CE system. The achievable sample enrichment and separation capability will thus depend primarily on experimental factors such as the uniformity of the acoustic field coupled into the capillary.

3. Experimental arrangement

The experimental arrangement is depicted in Fig. 1 and consists of three units, the CE system, the ultrasonic trap system and the LIF detection system. The in-house built CE system consists of a ~40 cm fused silica capillary (inner diameter 40–75 μm/wall thickness 20–25 μm), two 600 μl sample/buffer vials at each end and a 0–30 kV DC supply with positive polarity. At the inlet end of the capillary, the vial is enclosed in a pressure chamber used for fast hydrodynamic sample or buffer injection, or for rinsing the capillary.

The ultrasonic trap system is integrated in the CE system, placed in the middle of the capillary. A detailed description of the ultrasonic trap is given in Ref. [2]. Basically, it consists of a 8.5 MHz focusing transducer (radius of curvature of 50 mm) and a plane high-impedance molybdenum reflector placed in focus, creating a high-intensity standing wave parallel with the capillary in a hemispherical geometry. The ultrasonic trap is submerged in degassed water to minimize acoustic reflection losses and the capillary is guided through narrow-bore holes in both the transducer and the reflector. Thus, when the trap is activated, the acoustic intensity is evanescent-wave-coupled into the capillary via the very thin capillary wall (thickness <5% of the acoustic wavelength in quartz).

The detection is performed with a LIF imaging system, consisting of laser-excitation (with a 458 nm Ar-ion laser and a 568 nm Kr-ion laser), a multi-bandpass fluorescence filter, a 63× stereo microscope and a colour-CCD camera with a video recorder system. Thus, particles can be identified on basis of their colour and the system is designed to operate with the commonly used fluorophores fluorescein-5-isothiocyanate and rhodamine with fluorescence in the green and red wavelength regions, respectively.

In all experiments, the particles used were red fluorescent carboxylate-functionalized 2.1-μm-diam latex spheres (Molecular Probes, OR, USA) and green fluorescent carboxylate-functionalized 2.8-μm-diam latex spheres (Polyscience, PA, USA). These small and big

spheres are used to simulate singlets and doublets, respectively. Since the surface charge properties (i.e., the number of carboxylate groups) differed for the two vendors, bovine serum albumin (BSA) was coupled to the latex particles to equalize their charge properties. The surface charge density will then be almost equal on the two types of spheres since the BSA molecule is large in comparison to the distance between the carboxyle groups. In this way, the uniformity of the particle velocities during CE could be optimized which is important for highly selective separation.

Coupling of BSA to the carboxylated latex particles was achieved using a carbodiimide coupling procedure [12]. The carboxyl groups of the latex particles were activated, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, to promote condensation between primary amines on the BSA and free carboxyl groups on the particles to form peptide links by acid catalysed removal of water. Thus, covalent bonds are generated that link the protein to the particle. Before injection, the particles are diluted in a run buffer (borat/hydrochloric acid buffer, pH 8.0) with 0.02% (w/v) free BSA added to prevent particle agglomeration. To achieve a high reproducible EOF, the capillary is hydrodynamically flushed at high pressure with NaOH (0.1 M), then with water and finally with run buffer. All experiments are initiated with this procedure.

4. Experiments

To investigate the uniformity of the particle velocities, experiments were performed with equal proportions of big and small spheres. Fig. 3 shows a 4-s-clip from a video recording with sphere migration in an electric field of 15 kV/m (no acoustic field). Here, two big spheres (green) and three small spheres (red) illustrate the uniformity of the velocity distribution, where the spheres have a velocity of ~160 μm/s ± 5%. Thus, by coating

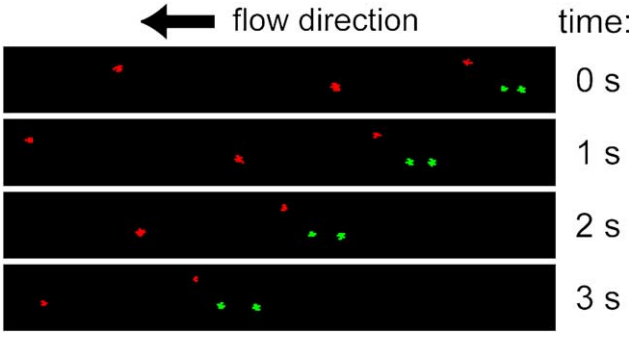

Fig. 3. Contrast-enhanced LIF images from the experiments showing the uniformity of the particle velocity distribution. Here, the green particles (2.8-μm-diam BSA-coated latex spheres) and the red particles (2.1-μm-diam BSA-coated latex spheres) move in an EOF.

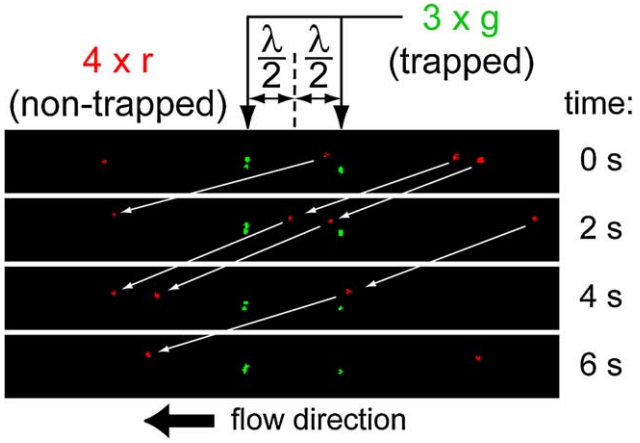

Fig. 4. Contrast-enhanced LIF images from a separation experiment. Here, three 2.8-μm-diam particles are trapped while four 2.1-μm-diam particles enter and escape from the trap, illustrating the separation and detection principle.

the particles with BSA, the variation in electrophoretic mobility due to differences in the number of carboxylate groups on the non-coated particles is efficiently minimized, resulting in a very uniform particle velocity distribution, equal for both big and small spheres.

The idea of the latex-particle ultrasonic-trap-enhanced CE method is to do long-term in-flow sample enrichment and detection of immunocomplexes (doublets), by size-selectively trap and retain very few doublets among a large number of singlets. In the experiments, this is simulated with samples having particle concentrations of $\sim 10^3$ spheres/μl, and with the ratio 10:1 between the number of small and big spheres. The samples were continuously run during 3 min with an electric field strength of 10–40 kV/m, corresponding to particle velocities of 100–400 μm/s. The acoustic intensity was gradually increased until no big spheres could pass the trap. The number of small spheres passing the trap at this acoustic intensity level was documented, as well as the number of trapped small spheres. In Fig. 4, a typical view is shown, here with three trapped big spheres and four passing small spheres. During the 3 min of separation in each experiment, ~200 particles (of which ~10% are big spheres) entered the trap. The number of unwanted trapped small spheres (“false doublets”) is usually 5–10% when trapping all of the big spheres (“true doublets”). The best result obtained was nine unwanted trapped small spheres among 189 (4.8%).

5. Discussion

A typical CLOD in CE with the UV absorbance detection is in the 10^{-5}–10^{-6} M range [13], while fluorescence detection results in 10^{-12}–10^{-14} M [3,4]. The ultrasonic-trap-enhanced CE method has significant

potential for improved CLOD by in-flow sample enrichment of immunocomplexes (doublets). If one doublet is trapped and detected during a 3-min enrichment time (cf. Section 4) with an EOF velocity of 500 μm/s (8 μl/h), the CLOD is on the order of 10^{-18} M. With increased enrichment times, deemed feasible from the theoretical discussion in Section 2, the number may be even lower. Thus, the ultrasonic-trap/CE method has potential to increase the CLOD several orders of magnitude in comparison to current CE methods. The detection error is currently estimated to be in the range of 5–10%, corresponding to the amount of singlets (''false doublets'') trapped.

There are several reasons for the non-100% separation capability in the experiments. One problem is that the positioning of the capillary in the center of the reflector hole is inaccurate. Thus, the centerline of the reflected acoustic wave may be radially displaced ≈20–30 μm, corresponding to the difference between the capillary (outer) diameter and the reflector hole diameter. This can result in a uniformity-disturbance of the standing wave coupled into the capillary. Furthermore, at the acoustic intensity levels used in the experiments (10–100 W/cm^2), problems with temperature and acoustic streaming can disturb the flow properties inside the capillary, especially if the acoustic field is non-uniform. The main reason for the uniformity problems is that the experimental setup is designed for a removable capillary through the ultrasonic trap. By rigidly attaching the capillary to the acoustic reflector, a much higher uniformity would be achieved.

6. Summary

We have combined CE with ultrasonic trapping for size-selective in-flow sample enrichment. The idea is to use the system for trace-amount protein detection via latex-bound monoclonal antibodies. As a model system for immunocomplexes (latex–protein–latex complexes) and single latex particles, 2.8- and 2.1-μm-diam BSA-coated latex spheres have been used to investigate the separation capability of the ultrasonic-trap-enhanced CE system. We show that the method has potential to increase the sensitivity in terms of CLOD by a factor 10^4, to the attomolar-range, compared to current CE methods. The main advantage with the method is the possibility to enrich the sample during the separation and to use much larger sample volumes and longer detection times than is possible in ordinary CE. Calculations of the separation capability including particle interaction forces and Brownian motion predict good marginal to size-selectively trap immunocomplexes from single particles, but experimental results indicate that a small part (5–10%) of the free particles can also be trapped, probably due to disturbances in the uniformity of the acoustic standing wave inside the capillary.

Acknowledgement

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Reference

[1] H.J. Issaq, Electrophoresis 21 (2000) 1921.
[2] M. Wiklund, S. Nilsson, H.M. Hertz, J. Appl. Phys. 90 (2001) 421.
[3] T. Johansson, M. Petersson, J. Johansson, S. Nilsson, Anal. Chem. 71 (1999) 4190.
[4] A.T. Timperman, K. Khatib, J.V. Sweedler, Anal. Chem. 67 (1995) 139.
[5] H. Nilsson, M. Wiklund, T. Johansson, H.M. Hertz, S. Nilsson, Electrophoresis 22 (2001) 2384.
[6] L.A. Crum, J. Acoust. Soc. Am. 50 (1971) 157.
[7] M. Gröschl, Acustica 84 (1998) 432.
[8] R.Y. Wang, J.A. Jarratt, P.J. Keay, J.J. Hawkes, W.T. Coakley, Talanta 52 (2000) 129.
[9] N.E. Thomas, M.A. Sobanski, W.T. Coakley, Ultrasound Med. Biol. 25 (1999) 443.
[10] H.M. Hertz, J. Appl. Phys. 78 (1995) 4845.
[11] S. Chandrasekhar, Rev. Mod. Phys. 15 (1943) 1.
[12] S. Nilsson, C. Lager, T. Laurell, S. Birnbaum, Anal. Chem. 67 (1995) 3051.
[13] M. Petersson, J. Nilsson, L. Wallman, T. Laurell, J. Johansson, S. Nilsson, J. Chromatogr. B 714 (1998) 39.