Henrik Nilsson[1]
Martin Wiklund[2]
Thomas Johansson[1]
Hans M. Hertz[2]
Staffan Nilsson[1]

[1]Technical Analytical Chemistry, Center for Chemistry and Chemical Engineering, Lund University, Sweden
[2]Biomedical and X-ray Physics, Royal Institute of Technology Stockholm, Sweden

Microparticles for selective protein determination in capillary electrophoresis

A system for detection of trace amounts of protein was developed. Two different monoclonal antibodies against human chorionic gonadotropin (hCG) were covalently bound to latex particles. When the latex particles were mixed with a sample containing hCG, a latex-protein-latex complex (immunocomplex) was formed. The complex was separated from the single latex particles using capillary electrophoresis and detected using UV-Vis detection. Limit of detection was 8 amol hCG. The separation was also monitored in real time using laser induced fluorescence – charge coupled device (LIF-CCD) imaging detection. However, a limitation of the method is the restriction to detection of proteins for which monoclonal antibodies are available.

Keywords: Capillary electrophoresis / Particles / Immunoassay / Imaging / Acoustic trapping
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1 Introduction

Medical diagnosis and the search for new drugs is one of the major driving forces for sensitive protein analysis, as methods enabling early diagnosis of disease may facilitate treatment. The absence or presence of specific proteins (the primary target area for drug discovery) is often correlated with disease, thereby obviating the need for specific analysis. A common problem, however, is that proteins of particular interest may be present in extremely low concentrations, especially at the early stages of disease. A system for highly selective and sensitive protein detection was developed. This method is general, and can be applied to analysis of most proteins.

Latex-bound monoclonal antibodies were used to facilitate detection of proteins present in low concentrations using capillary electrophoresis (CE). Two different monoclonal antibodies against spatially separated epitopes of one and the same protein are covalently bound to latex particles. The latex particles are subsequently mixed with the sample and if the protein of interest is present, a latex-protein-latex complex (immunocomplex) is formed (Fig. 1). The sample is then introduced into the CE system and the complex can be separated from the single latex particles. The detection methods used were UV diode array detection or laser-induced fluorescence (LIF) imaging detection [1–3]. Human chorionic gonadotropin (hCG) is a hormone present during pregnancy, and was used as a model system in the present study to experimentally demonstrate the improved limit of detection of this method compared to currently described methods (*e.g.,* ELISA and dipstick immunoassays) [4–6].

Separation of latex particles without covalently bound monoclonal antibodies has previously been performed in CE [7, 8]. Antibody-coated latex particles have been used in agglutination assays using light scattering detection; incubation in 30 min or more gave rise to limit of detection in the lower attomole region in fully optimized systems [9]. Furthermore, fluorescent latex particles have also been used for immunoassays in CE, in which antigens have been identified by the color combination of capture and detection latex particles in an immunocomplex [10]. One goal of the present study is to further improve the detectability by combining the CE-system described above with

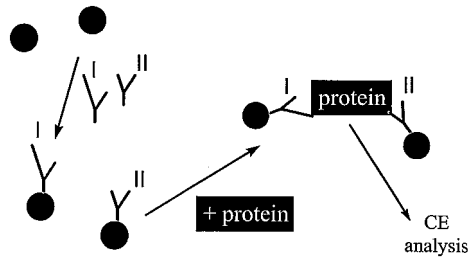

Figure 1. Schematic of the method. Two different monoclonal antibodies specific for different epitopes of the protein are covalently bound to latex particles, only one type of monoclonal antibody on each latex particle. When the latex particles are mixed with a sample containing the protein of interest, a latex-protein-latex complex is formed, which can be separated from the single latex particles using CE.

Correspondence: Dr. Staffan Nilsson, Technical Analytical Chemistry, Center for Chemistry and Chemical Engineering, Lund University, P. O. Box 124, SE-221 00 Lund, Sweden
E-mail: staffan.nilsson@teknlk.lth.se
Fax: +46-46-222 45 25

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; **hCG**, human chorionic gonadotropin

standing-wave acoustic trapping. Standing-wave acoustic radiation forces are strongly size-dependent ([11], Wiklund *et al.*, submitted). Thus, size-selective trapping and retention of the immunocomplex is potentially possible. Using an acoustic trap the immunocomplex may be separated from single particles and retained at spatially well-defined positions in the capillary, allowing for improved detection limits based on trace enrichment. To our knowledge, this is a new approach in analytical chemistry, which provides a new type of size selectivity.

2 Materials and methods

2.1 Materials

Affinity-purified monoclonal antibodies 5004 and 5503 (affinity constant $1–9 \times 10^{10}$ L/mol) against hCG were obtained from OY Medix Biochemica AB(Kauniainen, Finland). Clone 5503 was directed against the α-subunit of hCG and clone 5004 was directed against the β-subunit of hCG. Blue-colored latex particles (Estapor K1-080, lot 705, 0.9 μm) and fluorescent latex particles (Estapor F 1 XC-080, lot 440, 0.9 μm) were obtained from Prolabo (Fontenay-sous-Bois, France). 2-(*N*-Morpholino)ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), bovine serum albumin (BSA), and hCG were purchased from Sigma (St. Louis, MO, USA). Tween 20 was purchased from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane (Tris) was purchased from USB (Cleveland, OH, USA). HCl, NaOH, disodiumtetraborate, sodiumdihydrogenphosphate-monohydrate and di-sodiumhydrogenphosphate was purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) was purchased from Fluka. The water used was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Covalent coupling of monoclonal antibodies to latex particles

Blue carboxylated latex particles (25 μL, 10% w/v) were washed by centrifugation (14 000 × *g,* 10 min) in 0.05 M MES buffer, pH 5.5, and resuspended in a final volume of 50 μL. EDC (2 mg) was added to the particles. The mixture was incubated under vortexing for 1 h at room temperature. Antibody solution (300 μL containing 50 μg BSA and 300 μg monoclonal antibody 5004 or 5503; affinity binding $1–10 \times 10^{10}$ L/mol) was then added to the EDC-activated particles. Coupling was allowed to proceed during 5 h with vortexing on ice, followed by incubation at 4°C overnight. Residual activated carboxyl groups were then blocked by adding 1 mL of 0.1 M Tris-HCl, pH 8.0, containing 0.2% BSA and incubated with mixing for 30 min. The particles were then sonicated for 10 s in a Branson sonifier and washed four times by centrifugation (14 000 × *g,* 10 min) with 1 mL of Tris-BSA buffer. The pellet was resuspended in 250 μL Tris-BSA buffer to give a latex concentration of 1% w/v and stored at 4°C until use [6]. For the fluorescent particles 250 μL of fluorescent carboxylated latex particles (1% w/v) were used in each batch, and during the activation a 0.05 M MES buffer, pH 5.5 containing 0.05% w/v Tween 20 was used instead of the 0.05 M MES buffer pH 5.5. The use of Tween 20 prevented particle aggregation.

2.3 Capillary electrophoresis

The CE equipment used was a Hewlett-Packard 3D CE system (Palo Alto, CA, USA) and a Beckman P/ACE System 2050 (Palo Alto, CA, USA). Fused-silica capillaries of 50 μm inner diameter and 375 μm outer diameter were purchased from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 35 cm. Approximately 3 mm of the polyimide coating was removed to create a detection window 28.5 cm from the inlet end. The run buffer was 25 mM sodium borate buffer adjusted to pH 9.2 by addition of 1 M sodium hydroxide. Assays in which 20 mM phosphate buffer, pH 7.1, was used as run buffer were conducted, as well as experiments where 0.05% w/v Tween 20 was added to the sodium borate buffer. All buffers were filtered using Dynagard 0.2 μm filters (Microgon, Laguna Hills, CA, USA) and degassed by sonication. Prior to CE analysis, the latex particles were washed twice in the run buffer by centrifugation (13 000 × *g,* 5 min) and the hCG was dissolved and diluted in run buffer. Samples for CE analysis were made by mixing 40 μL of 0.1% w/v washed latex particles (20 μL each of the two types of particles) with a 20 μL hCG sample (concentrations were varied). Before the first run each day, the capillary was flushed for 1 min with 1 M NaOH, then 1 min with water and finally 3 min with run buffer. This procedure was repeated after the final run each day. Between consecutive runs, the capillary was rinsed with run buffer for 3 min. Separation voltage was set at 15.0 kV with the cathode at the outlet end and injection was done hydrodynamically by applying 50 mbar for 5 s. Detection was made at 195 nm, 280 nm, and 400 nm using a diode array detector (bandwidth 10 nm). The response time was 1.0–1.3 s. The EOF was continuously monitored by the injection of 5% acetone in run buffer.

2.4 Fluorescence imaging detection of immunocomplexes in CE

The in-house built CE-system used a stabilized power supply (0–30 kV; Mark II Zeta Elektronik, Höör, Sweden). The capillary was mounted onto a holder and the outer

protective polyimide coating was removed using fuming nitric acid and wiped off with a soft tissue, thus creating a detection window of approximately 10 cm. The inlet and outlet electrode vessels were 1.5 mL microfuge tubes. Buffers and fluorescent latex bound antibodies were prepared as described previously. The injection was made hydrodynamically by inserting the inlet end of the capillary into the sample vessel and elevating the sample vessel 10 cm for 5–30 s and the separation voltage was 15 kV. The excitation source was an argon ion laser Spectra Physics SP-171 at 488 nm, 150 mW. The laser light was focused on the detection window of the capillary. LIF was monitored using an 1100 X 3300 pixel CCD camera. The CCD was cooled to –40°C and was connected to a PC to display and store the fluorescence profiles of the capillary. A timer was connected to the CCD and one image was stored every 2 s. The exposure time was 200 ms. The software WinSpec (TEA/CCD-1100-PB; Princeton Instruments, Trenton, NJ, USA) was used to record and store the fluorescence data. Data processing was performed according to [14].

3 Results and discussion

3.1 Covalent coupling of monoclonal antibodies to latex particles

In order to verify the success of the covalent coupling of antibodies to latex particles, CE was used. The two types of coated latex particles, with covalently bound monoclonal antibody 5004 and 5503, respectively, were introduced into the CE system and analyzed. The electropherogram showed baseline separation of the two different antibody-coated latex particles in the absence of hCG, originating from the difference in p*I* of the two antibodies (Figs. 2, 3). The electrophoretic mobilities of the two groups of particles is also dependent on the degree of coupling of each monoclonal antibody. In case of a low degree of antibody coupling, the p*I* of the blocking protein (BSA) will be of importance. The electrophoretic mobility of the latex particles with covalently bound monoclonal antibodies is determined by the charge of the antibody at the pH of the run buffer. Since the number of monoclonal antibody molecules that bind to each latex particle during the coupling procedure may vary, a Gaussian distribution of electrophoretic mobilities is achieved, which results in the observed peak shapes (Figs. 2, 3). In order to further prove the success of the coupling procedure, noncoated latex particles were analyzed using CE, under the same conditions as the coated latex particles. The electropherogram obtained from this assay differs significantly from those obtained for antibody-coated latex particles (Fig. 4), thus indicating that the procedure of binding the monoclonal antibodies covalently to the latex particles has been successful. For the noncoated latex particles the electropherogram shows a large number of peaks, probably due to polydispersity in mass-to-charge ratio.

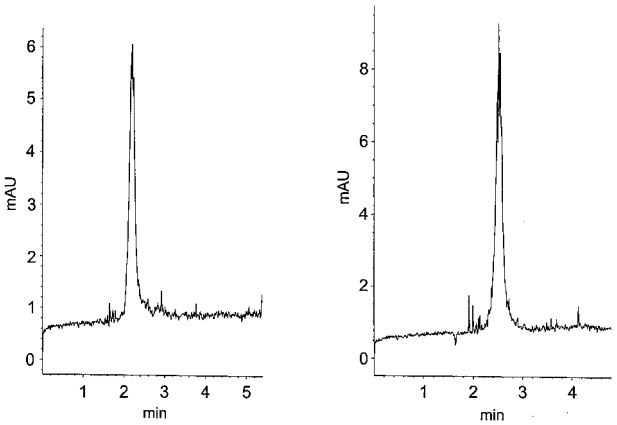

Figure 2. Electropherograms from a CE separation of latex particles coated with two different monoclonal antibodies against hCG (left: coupling with monoclonal antibody clone 5004, right: coupling with monoclonal antibody clone 5503). Capillaries: fused silica, 50 μm inner diameter and 375 μm outer diameter, 35 cm total length. The run buffer was 25 mM sodium borate buffer adjusted to pH 9.2 by addition of 1 M sodium hydroxide. Separation voltage was set at 15.0 kV with the cathode at the outlet end and injection was done hydrodynamically by applying 50 mbar for 5 s. Detection at 195 nm, 280 nm (shown) and 400 nm using a diode array detector.

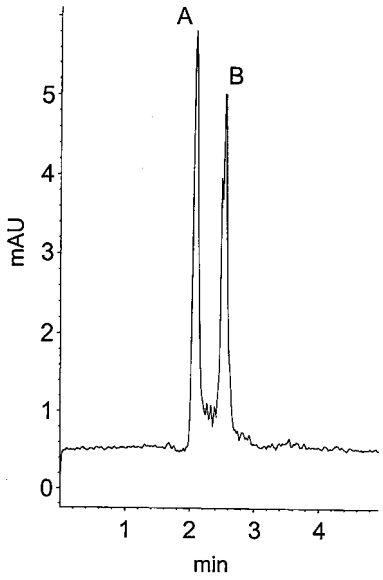

Figure 3. Electropherogram from a CE separation of a mixture of latex particles coated with two different monoclonal antibodies against hCG. (A) Latex particles with covalently bound monoclonal antibody 5004; (B) latex particles with covalently bound monoclonal antibody 5503. No hCG was present in this assay. Conditions as in Fig. 2.

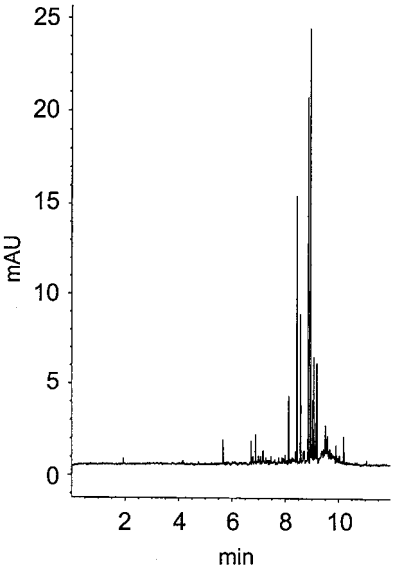

Figure 4. Electropherogram from a CE separation of non-coated latex particles. Conditions as in Fig. 2.

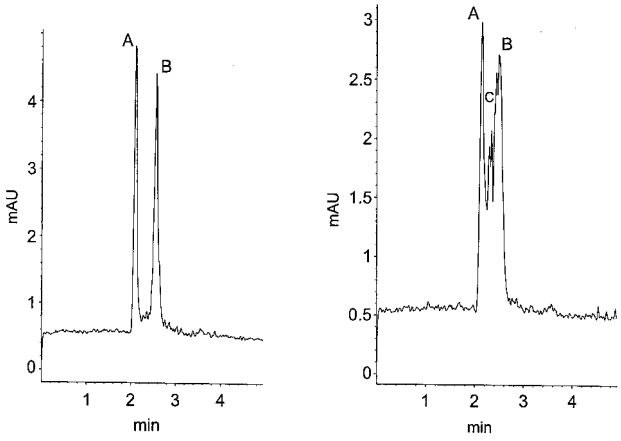

Figure 5. Electropherograms from a CE separation of latex particles coated with two different monoclonal antibodies against hCG in the presence of 33 μg/mL of hCG. Left: 0 min incubation time. Right: 25 min incubation time. (A) Latex particles with covalently bound monoclonal antibody 5004; (B) latex particles with covalently bound monoclonal antibody 5503; (C) immunocomplex (latex-hCG-latex complex). Conditions as in Fig. 2.

3.2 CE analysis of immunocomplex

CE was utilized to determine whether or not an immunocomplex could be formed as previously suggested (Fig. 1) and be separated from the single latex particles and detected. In the present method, hCG was added to a mixture of latex particles coated with the two different monoclonal antibodies (monoclonal antibody 5004 and 5503, respectively). The electropherograms obtained before and after addition of the analyte protein were compared to identify the immunocomplex peak. The EOF was constant during this study, as determined by injection of acetone in run buffer. hCG *per se* could not be detected in the present study due to the low concentration of the protein.

Initially the particles were mixed with a high concentration of hCG (33 μg/mL). CE analyses were made immediately after mixing and after incubating for 15–90 min. After 25 min incubation without agitation, immunocomplexes could be detected with a migration time intermediate between those of the two single latex particles (Fig. 5). Incubation for 25 min appears to be required for the formation of the immunocomplex, since no immunocomplexes could be detected with shorter incubation times. However, the ultrasonic equipment could also be used to obtain in-capillary acoustic mixing and facilitated immunocomplex formation [12], which most likely would lead to significantly shortened incubation times. Furthermore, in-capillary incubation has previously been shown to be useful in CEC separations [13]. This type of incubation could also be used in the present method.

The electrophoretic mobility of the immunocomplex can be expected to be intermediate between that of the two latex particles (with covalently bound monoclonal antibody 5004 and 5503, respectively) since the charge-to-size ratio of the complex will be intermediate between that of the two single latex particles. In a worst case, *i.e.,* using a high concentration of latex particles, a high concentration of sample and a high degree of antibody bound to the particles, multimeric immunocomplexes could be formed. Multimeric complexes can be expected to have the same electrophoretic mobilities as the latex-protein-latex complex, since the charge/size ratio of a large complex will be equal to that of a smaller complex, provided that the number of latex particles carrying each of the two different monoclonal antibodies is equal. However, multimeric complexes would only be expected at high concentrations of hCG, whereas lower concentrations of hCG would yield complexes comprising only two latex particles. Therefore, diluted samples and particle solutions are to be used.

The lowest concentration at which detection of the protein could be made was 0.33 μg/mL hCG, which corresponds to the UV detection of approximately 200 amol (Fig. 6). Assuming a signal-to-noise ratio of 2 as limit of detection, the limit of detection was 8 amol hCG (Fig. 6). The concentration limit of detection was 20 nM. Subsequently, fluorescent latex particles with covalently bound monoclonal antibodies were analyzed using the same method. The samples were prepared in the same way as previously described, and the hCG concentration was

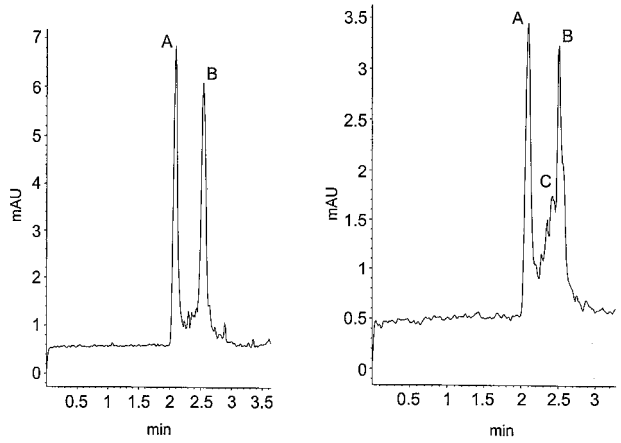

Figure 6. Electropherograms from a CE separation of latex particles coated with two different monoclonal antibodies against hCG in the presence of 0.33 μg/mL of hCG. Left: 0 min incubation time. Right: 75 min incubation time. (A) Latex particles with covalently bound monoclonal antibody 5004; (B) latex particles with covalently bound monoclonal antibody 5503; (C) immunocomplex (latex-hCG-latex complex). Conditions as in Fig. 2.

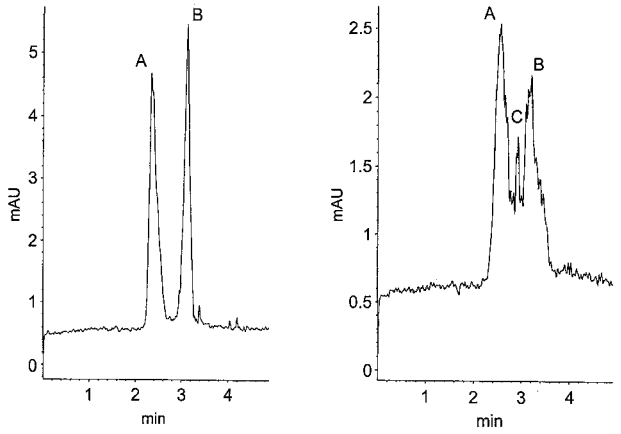

Figure 7. Electropherograms from a CE separation of fluorescent latex particles coated with two different monoclonal antibodies against hCG in the presence of 33 μg/mL of hCG. Left: 0 min incubation time. Right: 115 min incubation time. (A) Fluorescent latex particles with covalently bound monoclonal antibody 5004; (B) fluorescent latex particles with covalently bound monoclonal antibody 5503; (C) immunocomplex (latex-hCG-latex complex). Conditions as in Fig. 2.

33 μg/mL (Fig. 7). As expected, the same types of immunocomplexes were obtained as when using blue latex particles. The purpose of the use of fluorescent particles was to follow the CE separation in real time using imaging detection, as discussed further below. The results indicate that the method can be used as a system for detection of trace amounts of protein. The results also show that the stability of the immunocomplex is sufficient for separation using CE, as indicated in repeated runs. The binding strengths of the antibody-protein binding (affinity constant 1–9 $\times$ 10^{10} L/mol) are thus sufficient for the application.

3.3 CE analysis of latex-protein-latex complex using imaging detection

To monitor in real time the separation of the latex-protein-latex complexes from the single latex particles, fluorescence imaging detection was used. Imaging detection provides a unique opportunity to follow a CE separation process. Previous studies [14] have shown that imaging detection can give valuable and in some case unexpected information regarding separation processes. Thus, in-depth understanding of an entire separation process is obtained by the use of this detection method. In contrast to previous studies on CE separations [14], however, the present separation process did not show any unexpected abnormalities. CE separations of mixtures of latex particles coated with the two different monoclonal antibodies in the absence as well as in the presence of hCG were performed using fluorescence imaging detection. In these experiments, one monoclonal antibody was bound to fluorescent latex particles while the other monoclonal antibody was bound to blue, nonfluorescent particles. When analyzing the mixture of latex particles in the absence of hCG, one Gaussian peak was expected. When analyzing the mixture of latex particles in the presence of hCG, however, a split peak would be expected due to the formation of an immunocomplex with a different electrophoretic mobility from the single latex particles. The results obtained showed that the expected Gaussian peak was obtained from the analysis of the mixture of latex particles (with covalently bound monoclonal antibody 5004 and 5503, respectively). As expected, a split peak could be seen in the analyses of the mixture of latex particles in the presence of hCG (Fig. 8). The results obtained using LIF-CCD imaging detection and fluorescent latex particles show that the immunocomplex can be detected and the CE separation followed in real time. These results also indicate that fluorescence imaging detection could be useful in monitoring trapping of immunocomplexes using the acoustic trap, possibly used in conjunction with confocal microscopy.

4 Concluding remarks

The results obtained in the present study show that sensitive protein detection can be made using latex-bound monoclonal antibodies in a CE system. Fluorescent latex

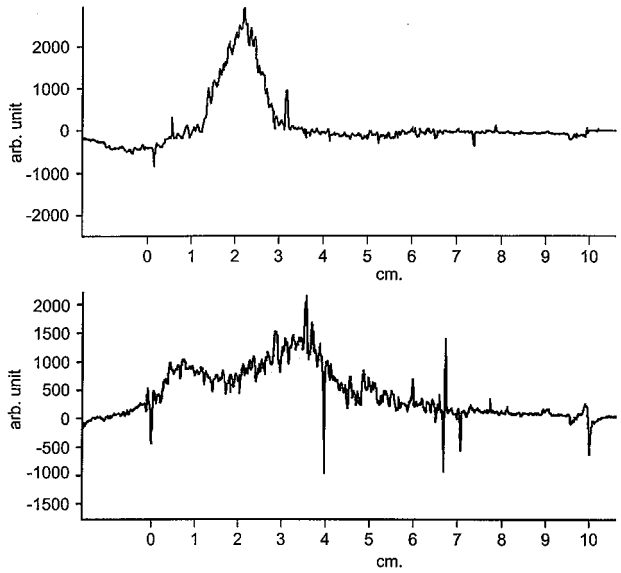

Figure 8. Images from a CE separation of a mixture of latex particles (fluorescent latex particles coated with monoclonal antibody 5503 and blue latex particles coated with monoclonal antibody 5004) in the absence of hCG (upper) and in the presence of hCG (lower). Detection was performed using LIF imaging. Capillaries: fused silica, 50 μm inner diameter and 375 μm outer diameter, 100 mm total length. The run buffer was 25 mM sodium borate buffer adjusted to pH 9.2 by addition of 1 M sodium hydroxide. The injection was made hydrodynamically by inserting the inlet end of the capillary into the sample vessel and elevating the sample vessel 10 cm for 5–30 s. The separation voltage was 15 kV.

particles can also be used, which can be expected to reduce the limit of detection significantly with LIF point detection, as well as allowing the use of LIF-CCD imaging detection. The method developed in the present study can thus be used for detection of trace amounts of protein. As pointed out in the introduction, combining the latex-particle CE method described above with acoustic trapping provides promise for further improvement of the detectability in protein determination. The goal is to produce a size-selective trap in the capillary by which the larger particles (*i.e.,* the immunocomplexes) are selectively separated and retained from the smaller single latex spheres. In order to demonstrate the concept, a proof-of-principle system has been developed (Wiklund *et al.,* submitted). With this capillary acoustic trap system, size-selective separation of latex spheres with different diameters was recently demonstrated. The size selectivity is due to the competition between the acoustic radiation force (F_a) and the viscous drag force (F_v). F_a is proportional to the volume of the particle and to the acoustic intensity, while F_v is proportional to the particle radius and to the particle velocity. Thus, at a certain flow velocity, particles will be trapped at different acoustic intensity levels depending on the particle sizes, allowing trapping and separation of larger particles from smaller particles. Compared to conventional CE, a capillary ultrasonic trap would not only allow separation, but also in-flow sample enrichment at spatially defined positions inside the capillary. This will provide trace enrichment and long detection times, thus potentially improving the limit of detection to the single-molecule level. Furthermore, the acoustic trap provides the introduction of a new type of size selectivity in CE separations.

In-column incubation can be achieved by the injection of the latex-coupled antibodies prior to the sample [13]. This procedure could be improved by in-capillary mixing obtained through the introduction of sound waves (acoustically enhanced mixing) [12] and allow the construction of a highly automated system for high-throughput analysis. One potential application of the presented method is for detection of prion proteins in biological samples. Prion proteins may cause transmissible neurodegenerative disorders such as BSE (bovine spongiform encephalopathy, the “mad cow disease”) and scrapie in animals, as well as Creutzfeldt-Jakob’s disease in humans [15, 16]. The sensitivity and selectivity of the presented method could provide improvements in this field. However, it is obvious that the method can only be applied to the specific analysis of any protein against which two different monoclonal antibodies can be obtained. Even higher sensitivity and selectivity can be expected when the ultrasonic trap is used in conjunction with the presented method.

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