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Separation of microorganisms using electromigration techniques

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Abstract

Like other colloidal particles bacteria have a surface charge that originates from the ionization of surface molecules and of the adsorption of ions from solution. Bacterial cell wall and membranes containing numerous proteins, lipid molecules, teichoic acids, lipopolisaccharides which give them characteristic charge. Therefore, bacterial cells undergo electrophoresis in a free solution with their own mobility depending on ionic strength and pH of buffer solution. Various electromigration techniques can be used to separate and determine the intact cells. Successful separation of five species of bacteria was obtained using a trimethylchlorosilane-modified capillary and a divinylbenzene-modified with suppressed EOF over a short distance (8.5 cm). The utilization of coated capillaries prevents adsorption of bacteria to the capillary wall. Another approach is utilization of a dilute dissolved polymer, polyethylene oxide (PEO) in the running buffer as a non-bonded coating for the purpose of altering the EOF. These experiment have proved the possibility of diagnosing a variety of diseases and the ability to separate and identify viable cells.

Keywords: Capillary electrophoresis; Determination of bacteria; Electroosmotic flow; Electrophoretic mobility; Modified capillary

1. Introduction

Detection of microorganisms such as bacteria is very important in bioscience research, medical diagnosis and profiling of some diseases, analysis in food industry and quality control of fermentation processes. Conventional microbiological methods such as a colony counting technique are very time consuming and therefore are not capable of fast diagnosis in case of emergency. Generally, no single test provides a definitive identification of unknown microorganisms.

In recent years, much effort has been made to develop high efficiency and fast instrumentation separation techniques for the determination and detection of biologically macromolecules and living cells. These methods include: polymerase chain reaction (PCR) detection [1], flow cytometry [2,3], differential staining [4], serological methods and recently dielectrophoresis [5–19], field-flow fractionation (FFF) [20,21], a combined dielectrophoretic and field-flow fractionation microsystems [22,23] or microchip-based technology [24] and matrix assisted laser desorption/ionization (MALDI) mass spectrometry [25]. Capillary electrophoresis (CE) [26–46] would be an excellent analytical method for the determination and separation of microorganisms since it is simple and efficient. Earlier, work in bacterial determination using CE was performed in 1987 by Hjerten et al. [26]. They showed that tobacco mosaic virus and *Lactobacillus casei* would migrate through a capillary and provided that the orientation of tobacco mosaic virus affected its electrophoretic mobility. However, there was no separation of the different species of microorganisms because all of bacteria moved together with electroosmotic flow. Ebersole and McCormick were first able to partially separate *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Streptococcus aureus* by CE [28]. *S. pyogenes* and *S. pneumoniae* were resolved in nearly 70 min using 250 cm long, 100 μm i.d. capillaries. Four years later Pfetsch and Welsch [35] applied similar method to separate three of bacteria: *Pseudomonas putida*, *Pseudomonas species* and *Alcaligenes euthropus* but the bandwidths were still broad.

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Armstrong and co-workers applied two electromigration techniques for the separation of several species of microorganisms [33,38]. The first approach involved capillary electrophoresis (CE) of bacteria using a dissolved poly(ethylene oxide) (PEO) solution with UV absorbance detection at 214 nm. They obtained the effective, high efficiency (900,000 plates m^{-1}) and reproducible separation for *Micrococcus luteus*, *E. aerogenes*, *Pseudomonas fluorescens*, and *S. cerevisiae* in <10 min with the capillary dimensions 100 μm i.d. and 27 cm length [33]. Another approach involved capillary isoelectric focusing (CIEF) of three similarly sized bacteria basis on their surface charge with efficiencies 1,600,000 plates m^{-1} [31].

These techniques were used for identification of the causative pathogens of urinary tract infections [31], determination of bacterial viability [36], quantitation of bacteria [44], and determination of living bacterial cells in consumer products [32].

Since bacteria are living organisms, their determination is not easy to perform. Many of microorganisms release biomolecules (enzymes and proteins) and these secretions may produce unwanted peaks. Variations of microbial cell wall composition can result from isolation and preparation procedures, growth conditions or from ages. Moreover, some bacteria may adhere to various surfaces or to other microbes. Microorganisms easy form clusters, because it is their natural behavior and may interact with environment in experimental conditions. The sonication of bacterial suspensions allows obtaining single peaks of different bacteria, but this phenomenon is not always reversed so easily [33,39,47–50].

Li and Harrison used microfluidic system on a glass chip to study mobilization and transportation of living cells under the conditions of electrophoresis [24]. Voltage utilization in different reservoirs could change the direction of transportation of yeast cells.

Buszewski et al. utilized acrylamide-modified capillary to prevent adsorption of bacteria to the capillary wall and to suppress the electroosmotic flow, which allow the bacteria to migrate mainly under the influence of their own electrophoretic mobility. They were able to separate *Eschericha coli*, *Bacillus cereus* and *Proteus vulgaris* using 75 μm coated capillary on 8.5 cm distance [42].

In this contribution, we report our recent achievements and experimental results in separation of microorganisms using capillary electrophoresis (CE) with PEO additive and using modified-capillaries.

2. Experimental

2.1. Chemicals

Phosphoric acid (85%) and dimethylformamide (DMF; 99.8%) were purchased from S. Witko-J.T. Baker (Łódź, Poland), acetic acid (99%), sodium tetraborate, ammonium persulfate, sodium hydroxide, hydrochloric acid, acetone, toluene, hexane, disodium ethylenediaminetetraacetate (EDTA) (all were of analytical grade) were from Polskie Odczynniki Chemiczne (POCh, Gliwice, Poland), Tris (99.5%) was from Merck (Darmstadt, Germany), γ-methacryloxypropyltrimethoxysilane (γ-MAPS) and *N*,*N*,*N*′,*N*′-tetramethylethyldiamine (TEMED), 2,2′-azobisisobutylonitrile (AIBN) and DPPH were from Fluka (Buchs, Switzerland), acrylamide was from Sigma (St. Louis, USA), divinylbenzene (DVB; 80%), trimethylchlorosilane and polyethylene oxide (PEO; M_w = 600,000) was from Aldrich. Deionized water was produced in our laboratory using Mili-Q water purification system (Milipore, Bedford, MA, USA).

Table 1
Capillaries used within study

	i.d. (μm)	L_{tot} (cm)	L_{eff} (cm)	Remarks
1	75	33.5	8.5	Bare fused silica
2	75	33.5	8.5	Trimethylchlorosilane modified
3	75	33.5	8.5	Divinylbenzene modified

i.d., Internal diameter; L_{tot}, total length; L_{eff}, effective length of the capillary.

2.2. Equipment

The separations were performed using $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with diode array detector. Fused silica capillaries were purchased from Composite Metal Services (Worcester, Great Britain). The capillaries that have been used are described in Table 1. Most of the separations have been performed on a short part of the capillary, which is schematically presented in Fig. 1. The spectrophotometric measurements were performed using Helios-α spectrophotometer (Unicam, Cambridge, UK) equipped with quartz cell of 1 cm path length. SEM photography were performed using Leo 1430 VP apparatus (Leo, Electronenmikroskopie, GmbH, Oberkochen, Germany).

2.3. Procedures

The stock buffer solution containing 4.5 mM Tris, 4.5 mM boric acid, and 0.1 mM EDTA (TBE buffer) was prepared by dissolving appropriate amounts of each reagent in deionized water yielding a buffer of pH = 8.53. This solution was then diluted 8:1 with deionized water. Next 0.2 g of PEO was added to 40 mL of this diluted buffer solution to give polymer concentration of 0.5%. This heterogeneous polymer solution was dispersed by placing it in an ultrasonic bath for 4 h at 60 °C. The mixture was removed from the bath and left overnight to dissolve completely. The run buffer solution was prepared by diluting the stock polymer solution with the diluted TBE buffer to a final concentration of 0.0125%. All buffers and polymer solution were prepared fresh daily.

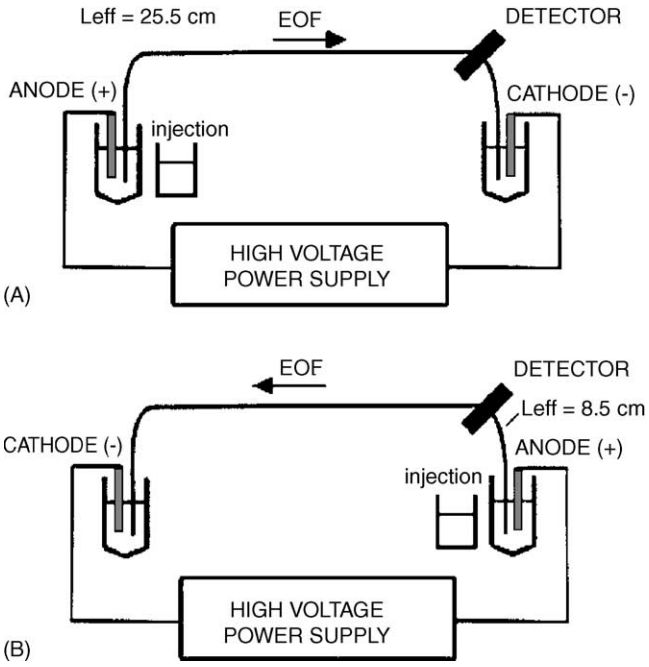

Fig. 1. Scheme of capillary electrophoresis system: (A) classical arrangement; (B) separations performed on a short part of a capillary (polarization reversed, hydrodynamic injection at the short part).

Four species of bacteria were used in this study, namely *E. coli*, *P. vulgaris*, *Bacillus meganterium*, *Micrococcus species* and *Arthorobacter globiformis*. The strains were obtained from the American Type Culture Collection (ATTC). Suspensions of bacteria were prepared the following way. Twenty milliliters of portions of the modified medium A (Lochhead and Chase 1943) with diminished amounts of casamino acid (2 g) were inoculated with 20 μL of bacterial suspension, obtained from 48 h cultures of bacteria grown on slants. The inoculated media (in 50 mL Erlenmeyer flasks) were put on a rotary shaker for 25 h. Afterwards the grown up bacteria were separated from the medium by centrifuged at 4 °C and 10.000 × *g*. Then the bacteria were washed and centrifuged again in deionized water (three times) and finally the density of the culture was designed to have a number of 10^8 cells mL^{-1}. The mixture for separation was obtained by mixing the same volumes of suspensions of each bacteria prior to analysis.

2.4. Capillary pretreatment

Before modification all capillaries were flushed with 1 M NaOH, both ends were sealed and the capillaries were left for 2 h in the oven at 120 °C. Next, the capillaries were flushed with deionized water, 1 M HCl and acetone, and finally dried with nitrogen at 120 °C for 1 h.

2.5. Capillary coating

The DVB-modified capillary was prepared in the following way: the 1:1 mixture of γ-MAPS in DMF containing 0.1% of DPPH was introduced into the capillary, both ends were sealed and the reaction was conducted at 120 °C for 6 h [51]. Finally, the capillary was rinsed with acetone and dried with nitrogen. Next, the capillary was filled with the reaction mixture containing the monomer (DVB) and 1% of the initiator of the polymerization (AIBN) in DMF. After rinsing with this solution for 5 min both ends was sealed and the capillary was left for 24 h in the oven at 65 °C. The remainder of the reaction mixture was flushed out and the capillary was rinsed with toluene for 5 min, dried in air for 2 min and finally rinsed with water for 5 min.

The other capillary after pretreatment was rinsed with trimethylchlorosilane at 25 °C for 24 h and next it was flushed with toluene, methanol and hexane.

The scheme of modifications of capillary wall with DVB and trimethylchlorosilane are presented in Fig. 2A and B, respectively. Fig. 3 demonstrates the SEM micrograph of the capillary wall modified with DVB.

The quality of the modified capillary wall was evaluated by its wetting properties using the capillary rise method described by Huang and Horváth [52]. The capillaries were immersed 4 cm below the water surface and were left for 1 h to stabilize. All measurements we performed at 25 °C and after each measurement capillaries were dried with nitrogen. The contact angle, θ, was calculated from the capillary rise of water, h_r, using the following equation:

$$\cos\theta = \frac{1}{4}\frac{h_r d(\rho - \rho_v)g}{\gamma},$$

where h_r is the height of the rise (cm), d the internal diameter of the capillary (cm), ρ the density of the liquid used (g mL^{-1}), ρ_v the density of air saturated with vapor of the liquid (g mL^{-1}), g the acceleration of gravity (cm s^{-2}), γ the surface tension of the liquid (dynes cm^{-1}).

The θ angle was calculated using the following data taken from [52]: $\rho = 0.997$ g mL^{-1}, $\rho_v = 1.1845 \times 10^{-3}$ g mL^{-1}, $\gamma = 71.97$ dynes cm^{-1}, and $g = 980.665$ cm s^{-2}.

3. Results and discussion

There have been many attempts to modify conventional microbiological methods in order to determine unknown microorganisms. In our study, we applied capillary electrophoresis to separation of bacteria, but their analysis is not easy to perform. According to the literature data [33,38,44,45] and our experiences [42] we used dissolved polyethylene oxide (PEO) in the running buffer. The purpose of addition of PEO was to alter the electroosmotic flow (EOF) and prevent adsorption of bacteria to the fused silica surface. Without PEO addition the electroosmotic flow was too fast and all species of bacteria migrated with EOF resulting in the broad bandwidths. When the 0.0125% PEO (M_w = 600,000) were used bacteria migrated at the narrow zones and we obtained high-efficiency separation. Of course, when the concentration of polymer in running buffer was

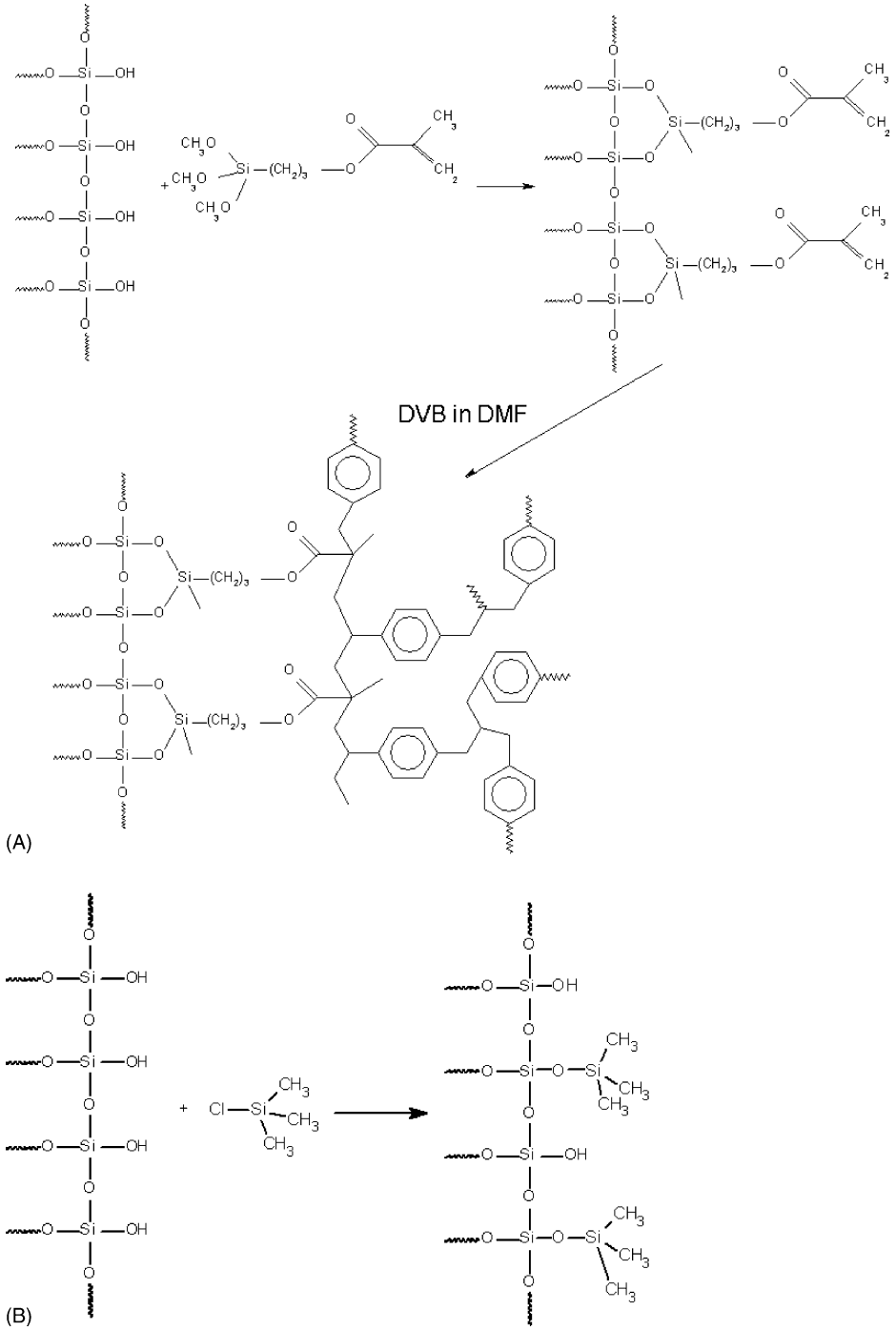

Fig. 2. Scheme of modification of capillary wall with: (A) DVB; (B) trimethylchlorosilane.

changed the migration times were different but the bacteria were still focused into sharp peaks. We observed a decrease of EOF with increasing concentration of polymer in running buffer (Fig. 4).

During our experiments we observed that sample preparation is a very important step in order to obtain good separation. Microorganisms easy form multicellular associations because it is their natural behavior in the environment. These interactions vary from weak associations to strong electrostatic or covalent bonds [46]. To prevent self-aggregation the suspension of bacteria was shaken before injection or the microbes were dispersed in an ultrasound bath. We observed that cell aggregation is not always reversed so easily, because some microorganisms associate more strongly than others. Moreover, solution of microbes can change with time, with different growth stages and storage conditions. Fig. 5 demonstrates two electropherograms of *E. coli* obtained with the same sample without and with dispersion of bacterial cells.

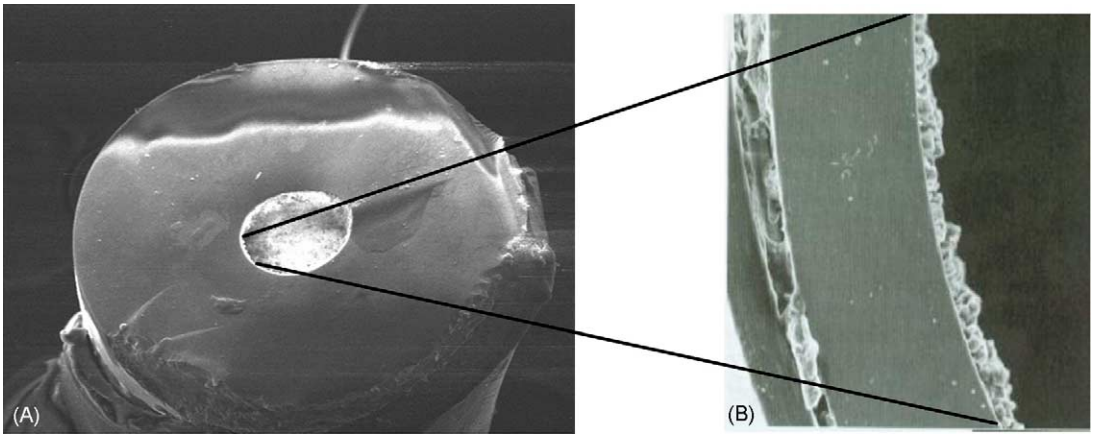

Fig. 3. SEM micrographs of capillary modified with DVB.

Microorganisms easy adhere to many surfaces. Self-aggregation of bacteria is sometimes described as a special case of microbial adhesion [46], in which the substrata are also another microbe of the same or different species. We observed that without PEO additive in the running buffer bacteria strongly adhered to the fused silica capillaries and bacteria migrated with the electroosmotic flow. Fig. 6 demonstrates separation of five bacteria without and with PEO additive.

In order to increase speed, selectivity, sensitivity of bacterial analysis we investigated modification of the internal capillary surface. Such approach should result in changed electroosmotic flow and prevent the adsorption to the capillary wall. In our previous study, we performed the separation on an acrylamide-modified capillary [42,44], but this modification was not stable in our experimental conditions and peak shapes were not satisfactory.

When the trimethylchlorosilane-modified capillary was used bacteria migrate at the narrow zones and the peak shapes were very sharp. When the sample was injected into the shorter part of the capillary (changing the polarization) we were able to separate five species of bacteria on 8.5 cm distance in a very short time (8 min; Fig. 7) and with high efficiency ($N \sim 4{,}000{,}000$ plates m^{-1}). On the longer part of the capillary we still obtained high efficiency separation, but microorganisms were resolved in nearly 30 min.

Modification of capillary wall with divinylbenzene gave similar results. A successful separation of four species of bacteria on an 8.5 cm distance was obtained in 5 min (Fig. 8). All of the microbes migrated in the narrow zones, and the electrophoretic mobilities of the separated bacteria are presented in Table 2.

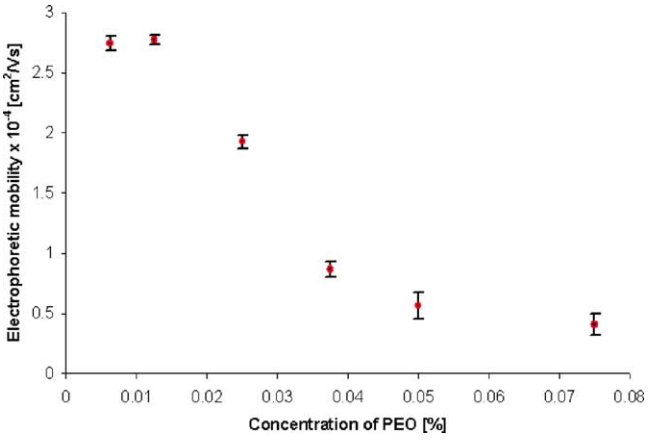

Fig. 4. Decrease of EOF with increasing concentration of PEO. Buffer TBE (pH = 8.53). Capillary: bare fused silica (L_{tot} = 33.5, L_{eff} = 8.5 cm), detection at 210 nm, $V = -15$ kV, injection 100 mbar s. Each point is the average of four measurements.

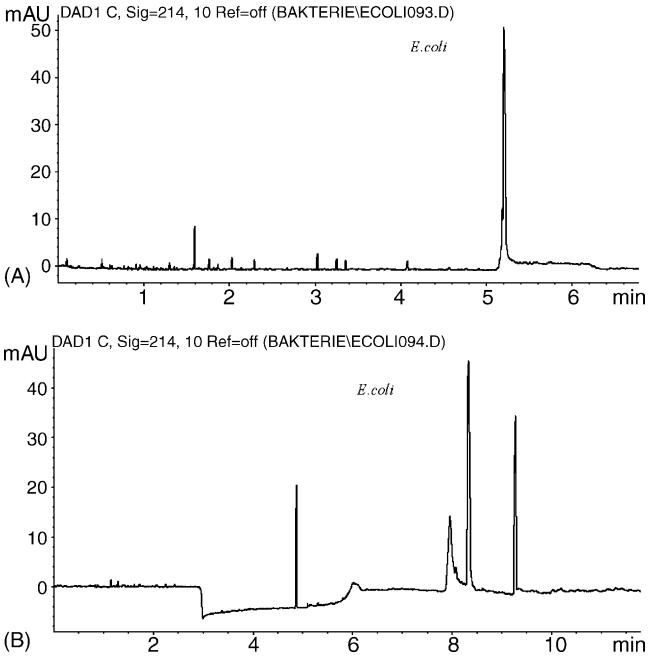

Fig. 5. Effect of sample pretreatment: (A) an electropherogram after the cells dispersion in an ultrasound bath; (B) an electropherogram showing clusters of bacteria. Capillary: bare fused silica (L_{tot} = 33.5 cm, L_{eff} = 8.5 cm). The buffer was 0.0125% PEO dissolved in TBE buffer (pH = 8.53), detection at 210 nm, $V = -15$ kV, injection 100 mbar s.

Table 2
Electrophoretic mobilities ($\mu_{EP} = \mu_{OBS} - \mu_{EOF}$) of microbes separated in capillaries with different modification ($n = 4$)

Bacteria	PEO		CH_3SiCl		DVB	
	$\mu_{EP} \times 10^{-4}$ ($cm^2(V\,s)^{-1}$)	R.S.D. (%)	$\mu_{EP} \times 10^{-4}$ ($cm^2(V\,s)^{-1}$)	R.S.D. (%)	$\mu_{EP} \times 10^{-4}$ ($cm^2(V\,s)^{-1}$)	R.S.D. (%)
E. coli	−0.16	8.01	−1.36	5.76	−1.25	1.46
B. meganterium	−0.72	7.87	−3.00	8.03	−2.38	0.31
P. vulgaris	−1.25	3.80	−3.43	1.89	−2.49	0.86
M. species	−1.35	1.94	−3.49	1.74	−3.19	0.59
A. globiformis	−1.44	1.80	−3.60	1.82	–	–

Divinylbenzene-modified capillary gave more reproducible separations resulting in low R.S.D. values (0.59–1.46%). All the bacteria exhibited negative electrophoretic mobilities which reflected their surface charge. It can be noticed in Table 2 that the mobilities of the microbes in the TBE/PEO buffer are significantly lower than in modified capillaries. This is because of higher viscosity of this separation medium in comparison to TBE. We observed that electroosmotic mobilities in both modified capillaries were almost the same:

$\mu_{EOF,\,trimethylchlorosilane} = 3.90 \times 10^{-4}$ and $\mu_{EOF,\,DVB} = 3.81 \times 10^{-4}\,cm^2(V\,s)^{-1}$. As the bacteria were separated in the same buffer (TBE) in modified capillaries (trimethylchlorosilane and DVB) it is likely that weak adhesion to the capillary wall may have some effect on the separation, which resulted in slightly greater values of mobilities in DVB capillary. These observations can be confirmed by the results of capillary rise measurements, which were presented in Table 3. In the DVB-modified capillary no rise was observed, which is the proof of high hydrophobicity of the inner capillary wall. The effect of weak interactions of water (and, of course, polar species) with DVB layer is known from gas chromatography PLOT columns, where water is very quickly eluted, before organic compounds [53]. However, despite the hydrophobicity of modified capillaries the electroosmotic mobilities are quite high, which may result from adsorption of organic constituents of the running buffer on the wall and creation of the double layer.

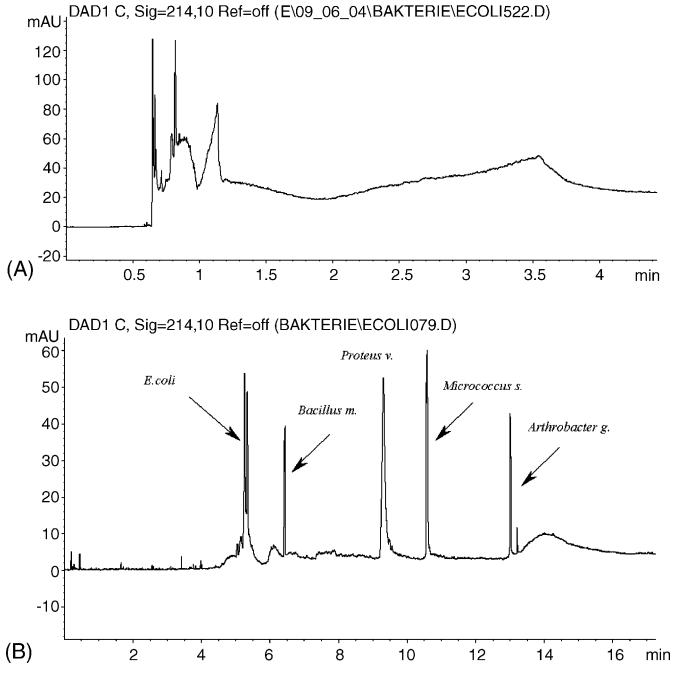

Fig. 6. Separation of five bacteria species on 8.5 cm distance (L_{tot} = 33.5 cm). (A) TBE buffer (pH = 8.53), detection at 210 nm, $V = -15$ kV, injection 100 mbar s. (B) The buffer 0.0125% PEO dissolved in TBE buffer (pH = 8.53), detection at 210 nm, $V = -15$ kV, injection 100 mbar s.

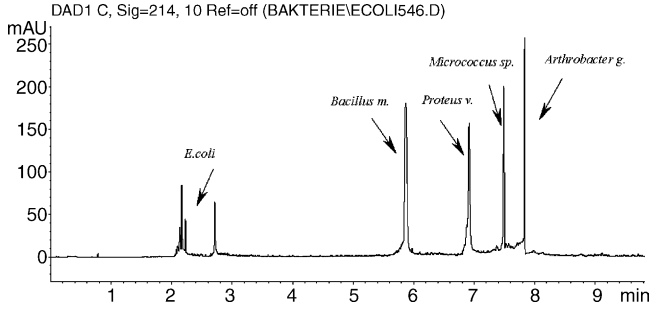

Fig. 7. Separation of five species of bacteria using trimethylchlorosilane modified capillary on 8.5 cm distance. Buffer TBE (pH = 8.53), detection at 210 nm, $V = -15$ kV, injection 100 mbar s.

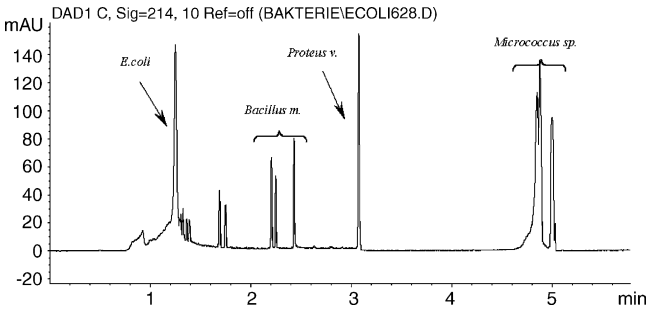

Fig. 8. Separation of four species of bacteria using divinylbenzene modified capillary on 8.5 cm distance. Conditions the same as in Fig. 7.

Table 3
Results of capillary rise measurements

Capillary modification	Height of the rise (cm)	Wetting angle θ (°)
Trimethylchlorosilane	3.4	85.4
Divinylbenzene	–	–

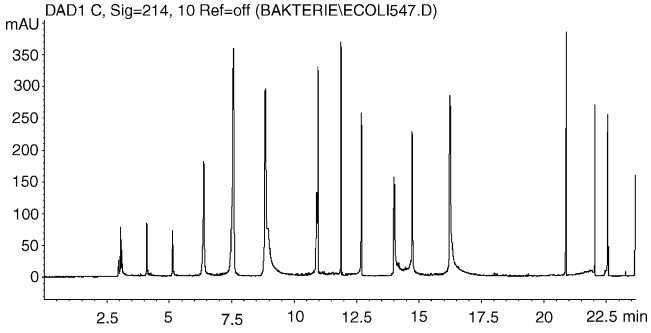

Fig. 9. Clusters formation between five bacterial species. Conditions the same as in Fig. 7.

In our experiments, we also observed formation of clusters between different species of bacteria but all of bacteria and their aggregates still migrated at the zones narrow (Fig. 9).

4. Conclusions

The ability to quickly identify and characterize of microorganisms is very important in many branches. Capillary electrophoresis of the cells is very convenient and significant analytical methods in these achievements. Many problems are associated with analysis of living cells such as: adsorption to the capillary wall, self-aggregation, stability and reproducible results. We were able to separate and identify five species of bacteria using two different approaches. Polymer additive to the running buffer could resolved microorganisms via size and shape. Capillaries modification gave high efficiency separation results on very short distance.

General methods based on capillary electrophoresis (CE) or microfluidic device seem to be very promising and the impact on this high efficiency separation on modern science will continue to grow.

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