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Journal of Chromatography B, 721 (1999) 31–37

JOURNAL OF CHROMATOGRAPHY B

Surface characterization and on-line activity measurements of microorganisms by capillary zone electrophoresis

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Received 2 July 1998; received in revised form 20 October 1998; accepted 20 October 1998

Abstract

Capillary zone electrophoresis (CZE) was applied to the electrophoretic characterization for microorganisms. The electrophoretic peaks detected using light scattering phenomena were characteristic of the microorganisms used. The electrophoretic mobility (μ) evaluated by CZE was in good agreement with that obtained by classical electrophoresis of microorganisms. The migration time was reproducible and depended on the ionic strength (I). Analysis of the μ vs. I relationship provided information regarding the charge density and the hardness of the microbial cell surface. The redox enzymatic activity of microorganisms was also evaluated by CZE using a running buffer containing a corresponding substrate and an appropriate exogenous electron acceptor. A decrease in the concentration of the electron acceptor due to microbial activity can be simultaneously monitored during the electrophoretic process without significant modification of the CZE instrument. Effects of some chemical treatments of microbial cells were also studied using this technique.

Keywords: Microorganisms; Surface charge density; Cell hardness; Redox enzymatic reaction

1. Introduction

Establishment of techniques for cell separation and characterization is one of the most important subjects to develop bioscience or biotechnology [1]. The separation and characterization of microbial cells of mixed populations, however, are more complicated and difficult compared to those at the molecular level. The cell surfaces consist of a variety of charged polymers and, in most cases, have net negative charge at physiological pH. The composition of the cell surface polymers is influenced by cell types predominantly and by other several factors, such as growth stages and growth conditions. Thus, classical electrophoresis is utilized for the characterization and separation of cells [2–6].

Capillary zone electrophoresis (CZE) is one of the most powerful tools with advantages of high resolution and small volume in sampling and detection. This technique was applied to the separation of mammalian red-blood cells using fluorinated ethylene–propylene copolymer tubing as a capillary [7]. Ebersole and McCormick [8] have also demonstrated

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0378-4347/99/$ – see front matter
PII: S0378-4347(98)00490-3

the feasibility of the CZE technique to separate and isolate viable bacteria from mixed populations with samples of small quantity. The advantage of CZE in determining the electrophoretic mobility of microorganisms has also also emphasized [9]. Recently, a microchip device was utilized for the separation of various kinds of living cells [10]. However, there is no report dealing with cell surface characterization by means of CZE.

On the other hand, an on-line enzyme assay using CZE has been developed [11]. This technique is recognized as an effective tool for the analysis of minute enzyme quantities and is called electrophoretic-mediated microanalysis [12]. Measurements of a target enzymatic activity, such as that of lactate dehydrogenase in human erythrocytes, were performed by means of CZE after cell lysis within the capillary [13]. Enzymatic activity measurements of baker's yeast cells immobilized on a capillary were also realized by CZE [14]. These concepts of enzymatic analysis by CZE seem to be extended for activity measurements of microbial living whole cells.

In the present study, CZE was utilized for the characterization of the surface charge of microorganisms and simultaneous on-line measurements of redox activities of microorganisms. The surface characterization was performed by analyzing ionic strength dependence of the electrophoretic mobility of microorganisms. The redox enzymatic activity of microorganisms was evaluated from the simultaneous detection of a decrease in the concentration of an appropriate exogenous electron acceptor in a running buffer.

2. Experimental

2.1. Microorganisms and reagents

Escherichia coli K-12 (Institute for Fermentation in Osaka (IFO) 3301) and *Paracoccus denitrificans* (IFO 12442) were cultured in LB media. *Pseudomonas fluorescens* TN-5 [15], *Bifidobacterium longum* 6001 [16], *Acetobacter pasteurianus* NP2503 [quinohemoprotein alcohol dehydrogenase (ADH)-deficient strain] and *A. pasteurianus* NP2503c [ADH-enriched mutant harboring pAA025 (ADH gene)] [17], *Seratia marcescens* (IFO 12648) [18] and *Saccharomyces cerevisiae* [19] were cultured according to the literature. Microorganisms were grown to the late-logarithmic phase at 30°C in a shaking culture medium and harvested by centrifugation at 7000 *g* for 10 min. The cells were washed twice with saline solution (0.85% NaCl) and stored at −80°C. A portion of the stored cells was suspended in a running buffer. The suspension was kept at 5°C and used for electrophoretic experiments within a few days. Cell densities of the suspensions were determined microscopically using a Petroff Hauser counting chamber or spectrophotometrically using calibration curves (for example, for *E. coli*, the unit optical density (OD) at 610 nm was equivalent to 1.55×10^9 cells per ml).

2,6-Dichlorophenolindophenol (DCIP) was purchased from Dojin Chemical (Kumamoto, Japan). Colistin sodium methanesulfate (colistin) was purchased from Wako Chemical (Osaka, Japan) and ofloxacin (9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2, 3-dihydro-7*H*-pyrido-(1,2,3-de)1, 4-benzoxazine-6-carboxylic acid) was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). All other chemicals used were of analytical reagent grade and were used without further purification.

2.2. Capillary zone electrophoresis

The CZE system used here consisted of a Glassman EH high voltage power supply (Whitehouse Station, NJ, USA), a Shimadzu SPD-M10AVP photodiode array detector (Kyoto, Japan) and a Takara Riken digital multimeter TR6843 (Tokyo, Japan) for current monitoring. Occasionally, an Olympus microscope BX50 (Tokyo, Japan) equipped with an Olympus high-sensitive CCD video camera HCC-1950A was used as a detector in place of the photodiode array detector. Fused-silica capillaries (Chromtech, The Netherlands) with a 50-μm I.D. and a 200-μm O.D. were used at a total length of 800 mm and an effective length of 500 mm. A 5-mm section of the polyimide coating on the capillary was removed for the detection, and the capillary was attached to the detector in a house design.

Just before each run, the separation capillary tube was rinsed with distilled water for 10 min using an aspirator. Running solutions used in the present

study were 10 m*M* phosphate buffers of pH 7.0 and 7.8, the ionic strength (*I*) of which was adjusted with NaCl in the range from 0.019 to 0.227 *M*. Buffer solutions were prepared with distilled water. Linearity between the electric current (10–60 μA at 5 kV) and *I* was confirmed for each running buffer.

Microbial suspensions were introduced into the capillary by siphoning at a height of 22 cm for 5 s (gravity injection). The injected volume of microbial suspensions was roughly estimated according to an equation for the gravity injection [20]. During the separation, the capillary surface was cooled with an electric fan. All experiments were performed at room temperature (25±3°C).

2.3. On-line activity measurements

On-line enzyme assays of microorganisms were performed using a running buffer containing a corresponding substrate of the microorganisms and an appropriate exogenous electron acceptor. The reduction of the electron acceptor due to the whole cell redox catalytic activity was monitored using the photodiode array detector. Before and during CZE measurements, the running buffer was deaerated with high-purity argon gas in order to prevent respiratory electron flow into dioxygen and the autoxidation of the reduced form of the electron acceptor.

Colistin, KCN, or ofloxacin were used for the chemical treatment of *E. coli* cells. The chemicals were added into the cell suspension (OD=5.3) at a concentration of 0.2 m*M* at room temperature. The suspension was subjected to the CZE analysis ≅30 min after the addition of the chemicals, unless otherwise noted.

3. Results and discussion

3.1. Electrophoretic behavior of microorganisms

Fig. 1 shows typical electropherograms of four kinds of microorganisms, monitored spectrophotometrically at 210 nm. Each kind of microorganism gave one main broad peak and occasionally several spike-like peaks. The migration times and shapes of the main peaks were reproducible and characteristic of the microorganisms, while the appearance of the spike-like peaks depended on the kind of microorganisms used and the migration time was not reproducible. The absorption spectra of both kinds of peaks exhibited typical characteristics of light scattering, indicating that both of the peaks can be ascribed to the migration of the microorganisms. The microscopic device was also available in place of the photodiode array for the detection of the cells. Microscopic detection revealed the existence of cell aggregates as well as well-dispersed cells. The spike-like peaks were attributed to cell aggregation, while the well-dispersed cells gave the broad main peak. The broadening of the main peak is most probably due to the heterogeneous characteristics of each microorganism.

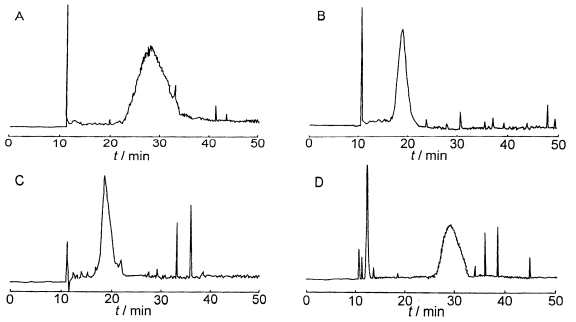

Fig. 1. Typical electropherograms of (A) *E. coli* K-12, (B) *P. fluorescens* TN-5, (C) *B. longum* and (D) *P. denitrificans* by CZE. Conditions: electrolyte, 10 m*M* phosphate buffer (pH 7.0, *I*=0.019), applied voltage, 10 kV; detection, 210 nm.

The electrophoretic mobility (μ) of the microorganisms was evaluated from the migration time at the top of the main peak as well as that of a neutral marker (mesityl oxide). The μ values of several microorganisms are summarized in Table 1. All of the microorganisms used here gave negative values of μ, indicating that the cell surfaces are negatively charged, probably due to dissociated carboxyl groups of (lipo)polysaccharides, sialic acids and/or proteins. The μ value of *E. coli* is in good agreement with the reported value obtained by classical electrophoresis [6]. This method can be utilized for the separation of microorganisms, although relatively large differences in μ are required for complete separation because of peak broadening. It is interesting that the ADH-enriched *A. pasteurianus* cells have a slightly less negative μ than the ADH-deficient cells. This might mean that such genetic modification (or the expres-

Table 1
Electrophoretic mobility (μ) of microorganisms[a]

Microorganisms	μ ($m^2\ s^{-1}\ V^{-1}$)$\times 10^{-8}$	[RSD (%)[b]]
E. coli K-12	−3.44	[1.7]
P. fluorescens TN-5	−2.30	[2.4]
B. longum	−2.10	[1.2]
P. denitrificans	−3.26	[1.5]
S. marcescens	−0.21	[3.5]
A. aceti	−0.23	[3.0]
A. pasteurianus NP.2503 (ADH-deficient)	−0.40	[3.9]
A. pasteurianus NP.2503c (ADH-enriched)	−0.19	[3.5]
S. cerevisiae	−0.23	[2.8]

[a] CZE conditions: 10 m*M* phosphate buffer (pH 7.0, $I=0.019$ *M*).
[b] Relative standard deviation at $n=3$.

sion of ADH) affects the structure and the composition of the cell surface (or cell wall) in part.

3.2. Surface characteristics of microorganisms

As shown in Fig. 2, the μ values of *E. coli* and *P. fluorescens* cells decreased parabolically with an increase in I and reached certain values. The limiting values were obviously below zero. Such behavior is known for colloidal particles covered with an ion-penetrable surface-charged layer [6,21]. The μ value of such colloidal particles with a uniformly charged layer composed of a z-valence charged group at a concentration of N in a 1:1 electrolyte solution is given as a function of I as follows [21]:

$$\mu = \frac{\varepsilon(\Psi_0\lambda + \Psi_{DON}\kappa_m)}{\eta(\lambda + \kappa_m)} + \frac{1000zFN}{\eta\lambda^2} \quad (1)$$

with

$$\Psi_{DON} = \frac{RT}{F}\ln\left[\frac{zN}{2I} + \sqrt{\left(\frac{zN}{2I}\right)^2 + 1}\right] \quad (2)$$

$$\Psi_0 = \Psi_{DON} + \frac{2I}{zN}\left[1 - \sqrt{\left(\frac{zN}{2I}\right)^2 + 1}\right] \quad (3)$$

$$\kappa_m = \sqrt{\frac{2000F^2I}{\varepsilon RT}}\sqrt{\left(\frac{zN}{2I}\right)^2 + 1} \quad (4)$$

where ϵ and η are the permittivity and viscosity of the medium, respectively, Ψ_{DON} is the Donnan potential, Ψ_0 is the potential at the boundary between the medium and the surface region, κ_m is the Debye–Hückel parameter of surface region, λ is a parameter characterizing the resistance to liquid flow in the surface region, R is the gas constant and F is the Faraday constant. Eq. (1) coupled with Eqs. (2)–(4) was fitted to the experimental μ vs. I plots using N and λ as adjustable parameters by means of a non-linear least squares method, where z is assumed to be −1 (for dissociated carboxyl groups). The regression curves are depicted in Fig. 2 by solid lines. The resultant parameters are summarized in Table 2. The values for *E. coli* are in acceptable agreement with the reported values obtained with a classical electrophoretic technique [6]. Judging from Table 1, the surface charge density of *E. coli* cells is higher than that of *P. fluorescens*. Considering that $1/\lambda$ is a

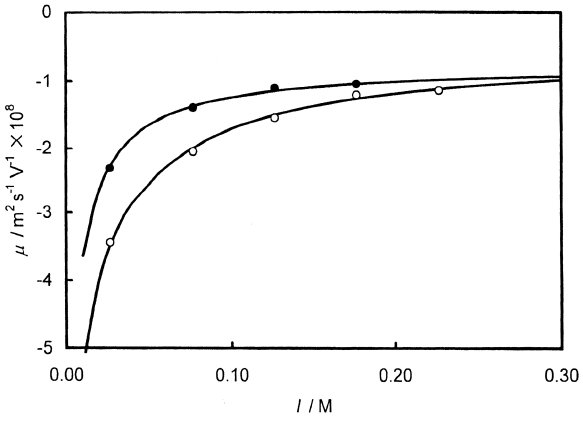

Fig. 2. Ionic strength dependence of the electrophoretic mobility of (●) *P. fluorescens* TN-5 and (○) *E. coli* K-12 cells. Solid lines represent the regression curves obtained based on Eqs. (1)–(4). Conditions: electrolyte, 10 m*M* phosphate buffer (pH 7.8) with various ionic strength values, adjusted with NaCl; applied voltage, 5 kV; other conditions were the same as those in Fig. 1.

Table 2
Estimated values of N and $1/\lambda$ for *E. coli* and *P. fluorescens*[a]

Bacteria	N (M)$\times 10^{-3}$	$1/\lambda$ (m)$\times 10^{-11}$
E. coli K-12	184.9±0.2	51.9±0.1
P. fluorescens TN-5	65.0±0.4	103.5±0.2

[a] Standard deviations were obtained as the goodness of the fitting.

parameter representing the softness of the charged surface [6,21], it can be concluded that the charged surface (or wall) of *E. coli* cells is harder than that of *P. fluorescens*.

3.3. Activity measurements during separation in the capillary

It is well known that microbial whole cells exhibit the reducing activity of several exogenous electron acceptors in the presence of corresponding substrates [22,23]. *E. coli* metabolizes glucose in their first metabolic pathway and the electron is transferred finally to dioxygen. DCIP is often used as an electron acceptor in several redox-enzyme reaction systems and it can serve as an exogenous electron acceptor for glucose oxidation of *E. coli*. Most probably, glucose dehydrogenase bound to the membrane is responsible for the reduction of DCIP [24].

Fig. 3 shows typical electropherograms of native *E. coli* cells in the presence of an excess amount of glucose and 0.5 m*M* DCIP. The migration of *E. coli* cells and the change of the DCIP concentration were simultaneously detected at 210 and 605 nm, respectively. The migration of *E. coli* cells was practically independent of the presence of glucose and DCIP. When monitored at 605 nm, a flat plateau region with decreased absorbance was observed between the negative peak at 14 min and the *E. coli*-migration peak at 20 min (detected at 210 nm). Such a plateau was not observed in the absence of glucose in the running buffer. The negative peak (at 14 min) originated from the sample injection, which causes a topical decrease in the DCIP concentration in the running buffer. These results indicate that *E. coli* catalyzes the DCIP reduction using glucose as a substrate during the electrophoretic process. The migration rate of the DCIP is larger than that of *E. coli* cells and the reduction of DCIP proceeds at a steady state. Thus, the plateau region with a decreased absorbance is generated between the injection peak and the peak of *E. coli* cells.

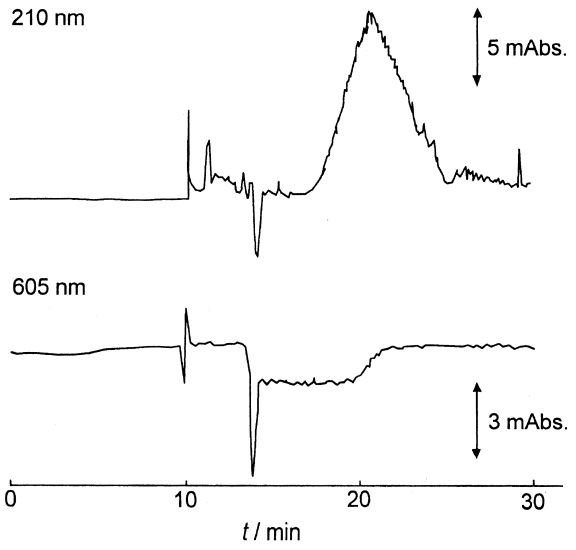

Fig. 3. Typical electropherograms of *E. coli* cells in the presence of 10 m*M* glucose and 0.5 m*M* DCIP, monitored at 210 and 605 nm. Conditions: electrolyte, 10 m*M* phosphate buffer (pH 7.0); applied voltage, 10 kV; injection amount, ca. 6×10^4 cells.

The decreased absorbance of the plateau at 605 nm was well correlated to the number of *E. coli* cells injected in the range from 4×10^4 to 4×10^5 cells. The correlation coefficient was 0.990. The detection limit of the enzymatic activity measurements for *E. coli* cells was about 1×10^4 cells at a signal-to-noise ratio of three. The number of *E. coli* cells can also be evaluated from the height of the main peak top at 210 nm. The correlation coefficient of the analysis was 0.990 in the range described above.

A similar experiment was performed for *P. fluorescens* using nicotinic acid in place of glucose, nicotinic acid being one of the specific substrates of *P. fluorescens* [23,25]. A decrease in the absorbance at 605 nm, due to the reduction of DCIP (and the oxidation of nicotinic acid) catalyzed by *P. fluorescens*, was detected. However, a clear plateau region could not be observed because of the close migration rate of DCIP and *P. fluorescens* under the present conditions. Some appropriate baseline corrections or selection of another suitable electron acceptor would be required to obtain quantitative

information. It would be important for better mediators to have an electrophoretic mobility that was sufficiently different from that of target microorganisms.

Since the metabolic activity of cells is one of the indicators that they are alive, on-line activity measurements were applied to chemically treated *E. coli* cells. Colistin is one of the antibiotics specific to gram-negative bacteria and is known to cause cell membrane injury [26]. The electropherogram of colistin-treated *E. coli* cells is depicted in Fig. 4A. The profile of the electropherogram monitored at 210 nm was almost identical to that of the untreated one (Fig. 3). However, complete suppression of the DCIP reducing activity was observed, as judged from the electropherogram monitored at 605 nm. It may be assumed that colistin treatment causes the inhibition of glucose dehydrogenase (and/or related enzymes) in the membrane of *E. coli* without significant changing the characteristics of the cell surface (or cell wall).

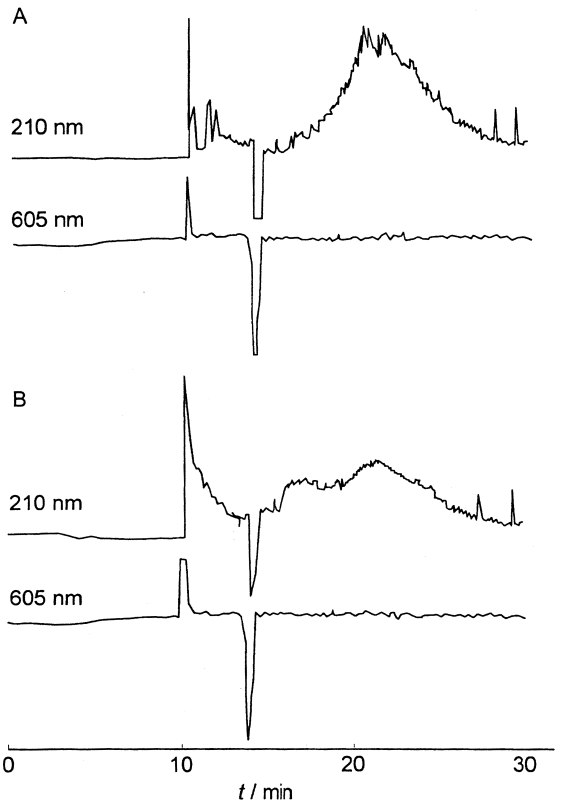

Fig. 4. CZE electropherograms of *E. coli* cells pretreated with (A) colistin and (B) ofloxacin for 30 min before injection. Other conditions were given in Fig. 3.

Similar behavior was observed for *E. coli* treated with KCN, an inhibitor of the respiratory chain. However, a pretreatment period of about 2 h was required to attain the complete inhibition of the DCIP reducing activity. This suggests that KCN could not inhibit directly the DCIP reducing activity and that the inhibition of the respiratory chain may cause destruction of the membrane and then the inhibition of membrane-bound enzyme activities.

On the other hand, ofloxacin is a synthetic pharmaceutical for bacteriolysis [27]. Fig. 4B shows electropherograms of *E. coli* pretreated with ofloxacin. In contrast to Fig. 4A, remarkable changes were observed in the electropherograms monitored at 210 and 605 nm after ofloxacin treatment. This may imply a significant change in the *E. coli* cell surface, most probably due to bacteriolysis induced by ofloxacin. The inhibition of the DCIP reducing activity might have resulted from cell lysis.

4. Conclusion

In this paper, we have demonstrated that CZE can be used for cell surface characterization and activity measurements of microorganisms. This method can serve as a new tool for various studies of cell biology, such as cell cycle interaction with chemicals. Since CZE is a powerful tool for the separation of microorganisms in complicated cell populations, simultaneous measurements of the activity will enable one not only to assess viability but also to realize selective detection of target microorganisms during the separation. This method could also be utilized for the analysis and monitoring of mixed cultures of microorganisms.

Acknowledgements

This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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