Rapid Separation of Microorganisms by Quartz Microchip Capillary Electrophoresis

Tomoyoshi SHINTANI,[†] Masaki TORIMURA,^{**} Hiroaki SATO,^{**} Hiroaki TAO,^{**} and Takashi MANABE^{***}

^{*}*Textile Industrial Institute of Ehime Prefecture, 2-5-48 Higashimura-minami Imabari, Ehime 799-1507, Japan*

^{**}*Measurement Technology Research Group, Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology, 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan*

^{***}*Department of Chemistry, Faculty of Science, Ehime University, 2-5 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan*

We developed and optimized a system coupling microchip capillary electrophoresis (MCE) and laser-induced fluorescence (LIF) detection for the analysis of microorganisms. The MCE-LIF system successfully separated pure cultures of lactic acid bacteria and *Saccharomyces cerevisiae* within 200 s. The results indicate that the MCE system can be conveniently used for the rapid and highly sensitive detection of microorganisms. Thus, MCE can provide a cheap and simple method for the on-line detection of microbial contamination.

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Introduction

The inspection of food for pathogenic bacteria has generally been carried out by cultivating samples for several days. Therefore, microbial contamination becomes clear only after product shipment, and this can allow outbreaks of infection and severe economic losses. The development of more rapid, safe, and simple technologies for inspection of microbial contamination is needed not only in the food industry, but also in environmental studies and in medicine.¹ In recent years, advances have been made in the application of capillary electrophoresis (CE) to the separation of biological cells including microorganisms.²⁻⁸ The cell surfaces consist of a variety of charged polymers and, in most cases, have a net negative charge at physiological pH. We had previously developed a rapid and highly sensitive method for detecting bacteria using CE coupled to a laser-induced fluorescence (LIF) detection system.⁹ This technology can be used for the detection of as few as three salmonella cells within as little as 30 min. CE separation technology is now in the process of being transferred to a microchip-based CE system. The microchip capillary electrophoresis (MCE) has several advantages over conventional large-scale counterparts, including high separation efficiency, miniaturization, low sample and reagent consumption, and potential to interface with micro total analysis systems, also known as μ -TAS.^{10,11} MCE systems fabricated by micromachining technologies have been widely used for analyzing biological molecules, such as protein and DNA samples. There have also been some reports that

biological cells can be handled in microfluidic channels by electrokinetic forces.^{12,13} However, there have not been any reports dealing with the injection and separation of microorganisms using MCE, because several technical problems have remained to be solved. For example, if microorganisms were consolidated or aggregated in a loading process, they behaved as a single larger particle rather than many small particles. The key technology for the development of this application is the establishment of techniques for controlling microorganism injection and migration in the capillary.

In this study, we analyzed microorganisms by electrophoresis on a quartz microchip with LIF detection. We focused on improving the loading process and the peak efficiency of microorganisms by adding sodium alginate to a running buffer. The geometry of the injection channels and the buffer composition were optimized to aid in the development of a method for rapidly separating and detecting the microorganisms.

Materials and Methods

Bacterial strains

Streptococcus thermophilus (510) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (B-5b) strains were obtained from the Japan Dairy Technical Association. The bacteria were cultivated for 36 h at 35°C in LB Broth (5 g/L peptonized milk, 5 g/L yeast extract, 20 g/L tryptone 20.0, 2 g/L D-glucose, and 1.5 g/L sodium acetate, pH 4.7 to 4.8). *Saccharomyces cerevisiae* strain K-9 was obtained from the Brewing Society of Japan, and was cultivated for 36 h at 28°C in YM Broth (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L

[†] To whom correspondence should be addressed.
E-mail: shintani@iri.pref.ehime.jp

Fig. 1 MCE2010 quartz microchip. (a) Simple cross-type microchip ($110\ \mu\text{m}$ wide $\times$ $50\ \mu\text{m}$ deep). (b) TwinT-type microchip. The device has separation channels ($110\ \mu\text{m}$ wide $\times$ $50\ \mu\text{m}$ deep) with $500\ \mu\text{m}$ twin-T injectors (offset = $500\ \mu\text{m}$). Bar = $100\ \mu\text{m}$.

D-glucose, pH 6.8 to 7.0). One milliliter from each culture was harvested by centrifugation (Model Himac CF15D2, Hitachi, Japan) at $600 \times g$ for 2 min. The supernatant was removed, and the sedimented cells were washed with 0.85% NaCl to remove the culture media. The microorganisms were suspended in a TBE (0.1 M Tris, 0.1 M boric acid, and 2 mM EDTA, pH 8.4) at a density of $[6\text{ to }8] \times 10^8$ cells/mL.

Running buffer and sample solutions for CE of microorganisms

Sodium alginate (500 to 600 cP, Wako Pure Chemicals, Japan) was added to TBE at a final concentration of 1.0% (w/v). This mixture was stirred overnight at room temperature to completely dissolve the alginate. In addition, a 10% (w/v) NaCl solution was prepared in TBE. The MCE running buffer was prepared by mixing appropriate amounts of TBE, 1.0% sodium alginate, and 10% NaCl. Unless otherwise indicated, for injection into the MCE system, the microorganisms were suspended in TBE at approximately 6×10^6 cells/mL.

Fluorescent labeling of microorganisms

SYTO 9 nucleic acid stain solution was obtained from Molecular Probes (Eugene, OR, USA). A 0.5 mL sample of microbial suspension was adjusted to $5\ \mu\text{M}$ SYTO 9 and incubated at room temperature in the dark for 15 min, as recommended by the manufacturer. The free SYTO 9 dye was removed from the microorganism suspension by centrifugation at $600 \times g$ for 2 min. The sedimented, stained microorganisms were resuspended in a running buffer and then applied to the MCE-LIF system.

MCE

The MCE separations were performed on a MCE-2010-LIF system (Shimadzu, Japan) equipped with MCE-2010 (version 1.3) software. Type D ($110 \times 50\ \mu\text{m}$) and Type TwinT ($110 \times 50\ \mu\text{m}$) microchips were manufactured by Shimadzu. A schematic diagram of the chip from above and the shape of the channels in cross section are shown in Fig. 1. The channels are $50\ \mu\text{m}$ deep (at the center) and $100\ \mu\text{m}$ wide (across the top), and the reservoirs are 2 mm in diameter. As shown in Fig. 1b, the plug length of the TwinT-type microchip, which is the distance between center to center in the vertical sample loading channel, is 0.5 mm. The sample reservoir was HV2, the sample

Fig. 2 Electropherograms for the MCE-LIF of *S. thermophilus* 510 (approximately 400 injected cells) in TBE (pH 8.3) containing 0.05% sodium alginate and 0.1% NaCl.

waste reservoir was HV1, and the buffer focusing reservoirs were HV3 and HV4. The microchip was washed with $100\ \mu\text{L}$ of both 1 M NaOH and 0.1 M NaOH, followed by $100\ \mu\text{L}$ of distilled water. The microchannels were filled with a running buffer solution from reservoir HV4 using a syringe. Using a pipette, reservoirs HV1, HV3, and HV4 were filled with a running buffer and reservoir HV2 was filled with a sample. The microchannel in simple cross-type microchips were filled with samples by a 5 s application of 420 V at reservoir HV2 and 300 V at reservoirs HV3 and HV4, while HV1 was maintained in a grounded state. Sample separation for this type of MCE was performed using a 320 s application of 860 V at reservoir HV3 and 260 V at reservoirs HV1 and HV2, while HV4 was maintained in a grounded state. The microchannels in TwinT-type microchips were filled with a 5 s application of 800 V at reservoir HV2, 500 V at reservoir HV3, and 600 V at reservoir HV4 while HV1 was maintained in a grounded state. Sample separation for this type of MCE was performed using a 320 s application of 860 V at reservoir HV3, 200 V at reservoir HV1, and 400 V at reservoir HV2 while maintaining HV4 in a grounded state. These conditions were roughly optimized with a fluorescein (FITC) sample solution under the conditions used for microorganism experiments. The separation was maintained at a temperature of 30°C using a thermostat control, and the fluorescently labeled microorganisms were detected with a LIF detector (488 nm semiconductor laser module) equipped with a 520 nm bandpass filter. A fluorescence microscope (COOLPIX micro system VI, Nikon, Japan) was used to count the cell numbers in the suspension and to observe the cell behavior in the MCE channels.

Results and Discussion

Compared to DNA, peptides, or low molecular weight compounds, it is difficult to analyze whole cells using microchannel or capillary tubes in the electrokinetic mode. In the current studies, MCE for cells was first tested using SYTO 9-stained *Streptococcus thermophilus* strain 510 cells in a simple cross-type chip. Similar to the results of CE experiments,⁹ the electrophoretic migration profiles of SYTO 9-stained *S. thermophilus* 510 in TBE running were not reproducible using the MCE-LIF; in fact, in some cases, no apparent main peaks were observed (data not shown). In addition, a tendency for a gradual flow of *S. thermophilus* 510 cells into reservoir HV4 during the separation process was frequently observed, although this was not found with FITC samples.

Fig. 3 CCD pictures showing the electrophoretic movement of *S. cerevisiae* K-9 and *S. thermophilus* 510 in the TwinT-type microchip.

To solve these problems, we added polymers to the running buffer because they had been reported to improve the peak shape during the separation of microorganisms by CE. For example, Armstrong and co-workers⁶⁻⁸ have achieved the rapid and highly efficient separation of mixtures containing several species of bacteria and fungi by adding polyethylene oxide (PEO) to the CE running buffer. PEO was effective in this case because it promotes microorganism aggregation during electrophoretic migration.^{14,15} We therefore examined the separation of *S. thermophilus* 510 by MCE-LIF using a simple cross-type chip and TBE containing 0.025% PEO. We found that the addition of 0.025% PEO caused the microorganisms to aggregate in the loading process, so that they behaved as a flocculate with random sizes rather than as many small particles in the cross section to be injected. Thus, it was not surprising that the number of injected cells and the migration time was not reproducible under this condition. In contrast, the peak shape for *S. thermophilus* 510 was dramatically improved when the TBE running buffer contained 0.05% sodium alginate and 0.1% NaCl (data not shown). This carbohydrate polymer is essential in the optimized running buffer to sharpen the peak,⁹ because no such improvement was observed when the same amount of NaCl was independently added to the running buffer. However, the reproducibility of the injected cell number was not as good (coefficient of variation of peak area was > 10%; $n = 3$).

To improve the reproducibility of the sample plug volumes and to better control the fluid flow within the channels, a TwinT-type (double-T) microchip format was introduced.^{16,17} Therefore, we attempted to separate microbes using a TwinT-type microchip. When the *S. thermophilus* 510 sample was subjected to MCE-LIF analysis using this system, a reproducible single sharp peak was easily obtained (Fig. 2). SYTO 9-stained lactic acid bacteria (*S. thermophilus* 510, *L. delbrueckii* B-5b) and yeast (*S. cerevisiae* K-9) were applied to the MCE-LIF system under the optimized running buffer conditions. Figure 3 shows the fluorescent microscopic observations of SYTO 9-stained microorganisms in a channel of a TwinT-type microchip. Judging from the image captured at

Fig. 4 Electropherograms for the MCE-LIF of *S. thermophilus* (510), *L. delbrueckii* (B-5b), and *S. cerevisiae* (K-9) in TBE (pH 8.3) containing 0.05% sodium alginate and 0.1% NaCl. Approximately ten cells were injected for total of microorganism.

1.0 s (Fig. 3), an almost complete separation of the lactic acid bacteria and yeast was achieved within 1.0 s. Electropherograms (Fig. 4) show that the lactic acid bacteria co-migrated at approximately 140 s, whereas yeast co-migrated at approximately 100 s. The migration times for lactic acid bacteria and yeast alone, or in a mixed solution, were identical, and each peak was completely separated with good resolution. The results show that the device can efficiently separate and detect SYTO 9-labeled microorganisms under the optimized conditions. However, using these buffer conditions, we could not separate two types of lactic acid bacteria from each other by MCE or standard CE.

In this report, we have described the rapid separation of microorganisms using a MCE device coupled with LIF detection. The MCE-LIF system successfully separated pure cultures of microorganisms within 200 s. The MCE-LIF system can be conveniently used for the rapid detection of microorganisms when it is compared with the standard CE system. The balance between dispersive forces and intercellular

adhesive forces affects the observed bandwidths. Understanding and controlling the aggregation and dispersion of cells in microfluidic processes is essential for the further development of MCE methods for microorganism analysis.

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