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# Fast detection of *Candida albicans* and/or bacteria in blood plasma by "sample-self-focusing" using capillary electrophoresis-laser-induced fluorescence

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ABSTRACT

Detection of microbial contamination in blood plasma is critical and necessary in different medical and research fields. Most of the current standard procedures for the detection of bacteria and fungi can be time-consuming, for example, direct inoculation methods of microbial cultures in respective growth media can take a few days to several weeks. A fast analysis method with high sensitivity output such as CE-laser-induced florescence becomes an attractive alternative. Previously, a spacer-injection method with the use of zwitterionic surfactant (SB3-10) as a blocking agent to negate the cells' mobility, induce aggregation and single microbial peak formation in a buffer solution was reported. Here, a fast, simple direct method for microbial detection in blood plasma without using the spacer and blocking agent is reported. To compensate for the natural electrophoretic heterogeneity of microbes, a CTAB additive was used to sweep all microbial cells towards the plasma peak where a single sharp microbial peak is formed and detected. With the use of BacLight™ Green bacterial stain, the microbial peak, generally, can be detected within 10 min in front of the plasma peak using capillary electrophoresis coupled with laser-induced florescence detection. The LOD of microbes detectable were 5 cells per injection. This technique provides a great advantage over traditional, time-consuming microbial inoculation methods.



## 1. Introduction

A rapid detection method for pathogenic microorganisms is an important and necessary component of safety and quality control in many areas of science and technology, including pharmaceutical, food and beverage and medical products [1–3]. Currently, several methods are employed to test for microbial contamination. Among them, the simplest and most widely utilized approach procedure is the direct inoculation method [4]. However, there are drawbacks to this technique including the time required for microbial incubation (over several days or weeks), and great care is needed to prevent any contamination during analysis. Furthermore, this approach does not detect all microorganisms of interest, but only those amenable to the growth media and conditions used. Some molecular based detection methods such as hybridization [5], amplification [6], and immunoassay techniques [7] have been developed to shorten the analysis times. Nevertheless, these techniques can be complex and usually requires extensive training. Also, they are used for the identification of specific microorganisms at the species level. Moreover, reagents and materials required for these types of testing can be expensive. Hence, these approaches are not useful as a general contamination test to determine the presence or completely absence of all microorganisms.

Traditionally, capillary electrophoresis (CE) has been used for separations of molecules based on their mass-to-charge ratio. Recently, this technique has been explored as a method for the analysis and characterization of microorganisms and seems to be very promising [8–14]. Also, due to its unique attributes including rapid, high efficiency analysis and small sample requirements, CE becomes an attractive approach for "biocolloid" analysis. However, the main problem of analysis of intact microbial cells is that separation can be degraded by adhesion of the bacteria to the fused-silica surface of the capillary causing non-reproducible electroosmotic flow (EOF) and decreasing separation efficiency by band broadening [14]. This could happen when cationic components on a bacterial surface interact with anionic silanol groups of the capillary wall. Armstrong and co-worker reported the bacterial migration behavior using a CCD camera coupled with LIF [15]. They showed that under certain experimental conditions, self-focusing process of microbes happened inside the capillary as they migrated in an electric field. Buszewski et al. reported that the aggregation of bacteria can decrease the magnitude of electrophoretic mobilities, leading to poor reproducibility of migration times [16,17]. Recently, we developed a rapid CE method using

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either ultraviolet–visible (UV–vis) or laser-induced fluorescence (LIF) detection to indicate the presence or complete absence of microbes in a solution sample [18,19]. A wide variety of bacteria are compatible using this method and the analysis times, typically, are within 10 min. Subsequently, we adapted this approach to use an ionic liquid supporting electrolyte for the detection of microbial contamination [20] and specifically for *Candida albicans* (*C. albicans*) in samples by using a CE-FISH technique [21]. As yet the determination of microbial contamination in a real biological sample, i.e., whole blood or blood plasma, using CE has not been reported.

*C. albicans* is one of the more common fungal pathogens that exists as a commensal of warm-blooded animals including humans. It colonizes on mucosal surfaces of the vaginal, inside oral cavities as well as in the digestive tract [22]. This dimorphic fungus is responsible for the majority of localized fungal infections in human. Patients with impaired immune system, for example those who have had cancer treatments or AIDS infection, more easily develop *C. albicans* infection called Candidasis [23–25]. About 50–70% of nosocomial blood stream infections are caused by *C. albicans* [26]. The most common detection methods for *C. albicans* in blood include: culturing the infected blood sample on Sabouraud glucose or potato dextrose agar followed by germ tube analysis [27], increasing the DNA of *C. albicans* using the polymerase chain reaction (PCR) [28] and detecting with peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) [29]. There are several drawbacks to these methods such as the time required for the PCR of *C. albicans* DNA and culturing cells before analysis. Alexander et al. reported that the use of PNA-FISH for detection of *C. albicans* can reduce the cost of treatment approximately $ 1,800 per patient [30]. Three drugs are commonly used to treat *C. albicans* infections. They are caspofungin, fluconazole and itraconazole [31–33]. Caspofungin and fluconazole are used as a first-line antifungal agent for the treatment of *C. albicans* infections because of its well-known efficacy and safety profile. However, due to the fact that caspofungin is more expensive than fluconazole, many patients start with caspofungin instead of fluconazole at the beginning of the treatment. If these drugs fail on the treatment, itraconzaole, a wider spectrum antifungal drug, can be used [30]. The length of treatment, depending on the area of infection, is usually from weeks to months [34].

Due to the fact that *C. albicans* has emerged as a significant cause of nosocomial infections, the rapid and direct identification and detection of the presence or complete absence of *C. albicans* and/or other bacteria in blood plasma is necessary.

## 2. Materials and methods

### 2.1. Buffers and stock solutions

Tris(hydroxymethyl)aminomethane (TRIS), citric acid, sodium hydroxide, hydrochloric acid and cetyltrimethylammonium bromide (CTAB), dimethyl sulfoxide (DMSO) were obtained from Aldrich Chemical (Milwaukee, WI). 3-(Decyldimethylammonio)propanesulfonate and caprylyl sulfobetaine (SB3-10) were from Sigma (St. Louis, MO). Yeast and mold (YMB) broth and nutrient broth (NB) were from Difco Laboratories (Franklin Lakes, NJ). Bovine plasma with sodium citrate as anticoagulant was purchased from Innovative Research (Novi, MI). BacLight™ Green bacterial stain (B35000) was purchased from Invitrogen (Carlsbad, CA). Uncoated fused-silica capillaries were with an id of 100 μm and an o.d. of 365 μm were from Polymicro Technologies (Phoenix, AZ).

### 2.2. Bacteria and cell growth

*Brevibacterium taipei* (ATCC no. 13744), *Bacillus cereus* (ATCC no. 10702), *Bacillus subtilis* (ATCC no. 12695), *Candida albicans* (ATCC no. 10231), and *Bacillus megaterium* (ATCC no. 10778) were all purchased from American Type Culture Collection (ATCC, Manassas, VA). *C. albicans* were grown overnight for 20–24 h at 25 °C in YMB. Bacteria were grown overnight for 20–24 h at 30 °C in NB. All microorganisms examined in this study are rated biosafety level one. Standard microbiological practices, therefore, may be employed.

### 2.3. Preparation of blood plasma sample

All bacteria and fungi were grown according to the instructions from the manufacturer (see Section 2.2), which produced a concentration of cells was about $3 \times 10^8$ colony forming units (CFU)/mL (verified by plate counting method). Serial dilutions of microbial solutions were made with working buffer when necessary. The broth containing microbes were centrifuged for 2 min, and the excess broth was then removed to withdraw the microbes. The microbial cells were washed with working TRIS/citric acid buffer, recentrifuged, and finally resuspended in bovine blood plasma. All samples were vortexed for 30 s and sonicated briefly prior to cell staining to prevent cell aggregates.

### 2.4. Staining of bacterial cells

Baclight™ Green bacterial stain was used to stain the cells for fluorescence detection using LIF at 516 nm. This dye was dissolved in DMSO to produce 1 mM solution according to the instruction from the manufacture. The cells then were stained by adding 2 μL of dye solution per 1 mL of prepared microbial solution and incubated in the dark for at least 30 min. After incubation, the solution were centrifuged for 2 min and pelleted, then all but the last few microliters of solution was removed. The cells were then washed with working TRIS/citric acid buffer, recentrifuged, pelleted again and all but the last few microliters of the remaining liquid was removed. This washing step was repeated at least 2 times in order to reduce the interference from the plasma peak. The sample was finally resuspended in fresh buffer solution for CE analysis.

### 2.5. Capillary electrophoresis

The CE experiments were performed on a P/ACE MDQ capillary electrophoresis system equipped with photodiode array and 488 nm laser-induced fluorescence detectors (Fullerton, CA). The bare silica capillaries used in this experiment were 30 cm long (20 cm to the detector), with an i.d. of 100 μm and an o.d. of 365 μm. Fluorescence emission from Baclight™ Green bacterial stain cells was detected at 516 nm. New capillaries were initially conditioned with 1 M sodium hydroxide for 5 min, deionized water for 5 min, and running buffer for 5 min. Between each runs, the capillaries were washed with 1 M sodium hydroxide, 1 M deionized water

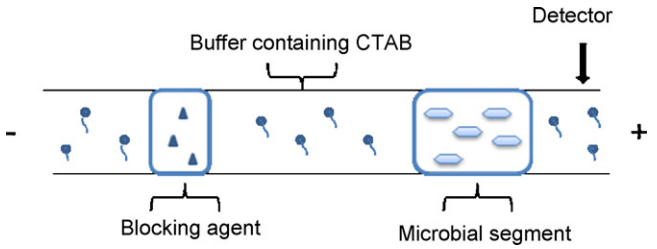


**Fig. 1.** A schematic of the three injection method of microbial detection. Three injections are made as follow: (1) a plug of microbial sample; (2) run buffer as a spacer; (3) blocking agent segment.

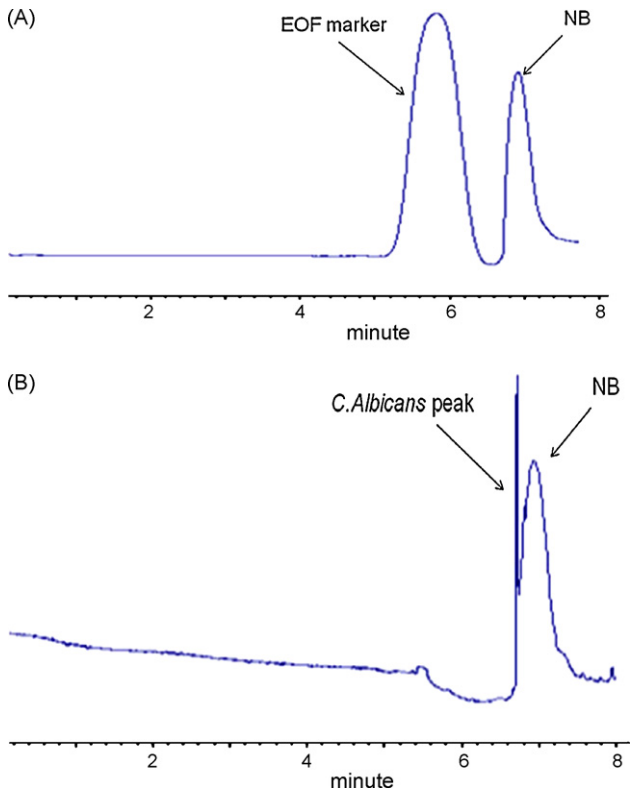


**Fig. 2.** Electropherograms obtained using the three injection method. Sample: (A) EOF marker (DMSO) in run buffer; (B) *C. albicans* in run buffer; other conditions—run buffer: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; blocking agent: 8 g/L nutrient broth (NB) in run buffer; voltage: −3 kV; detection at 214 nm; see Section 2.3 for details.

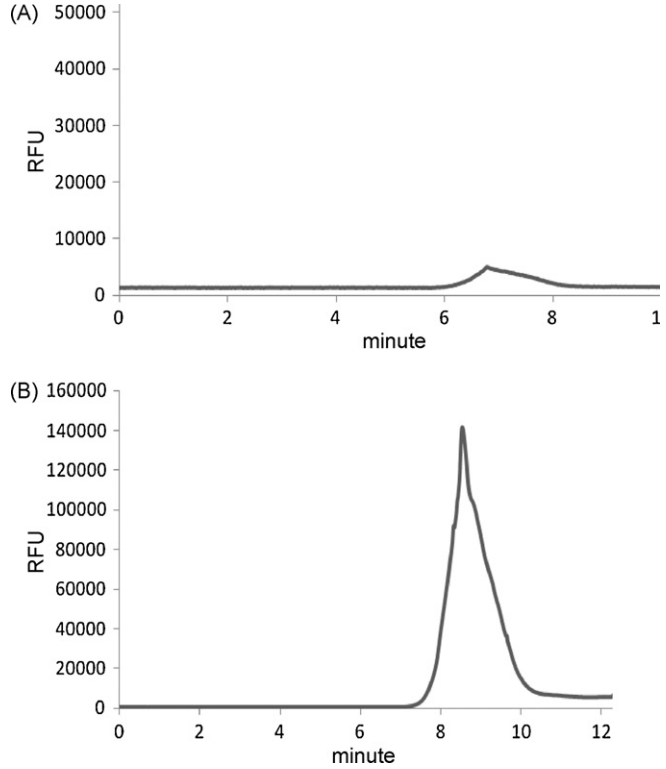


**Fig. 3.** (A) Electropherogram of a blank (blood plasma without microbes) using the three injection method. (B) Electropherogram obtained for Baclight™ Green stained blood plasma. Conditions—run buffer: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; blocking agent: 10 mg/mL SBC-10 in run buffer; voltage: −3 kV; detection at 214 nm; see Section 2.3 for details.

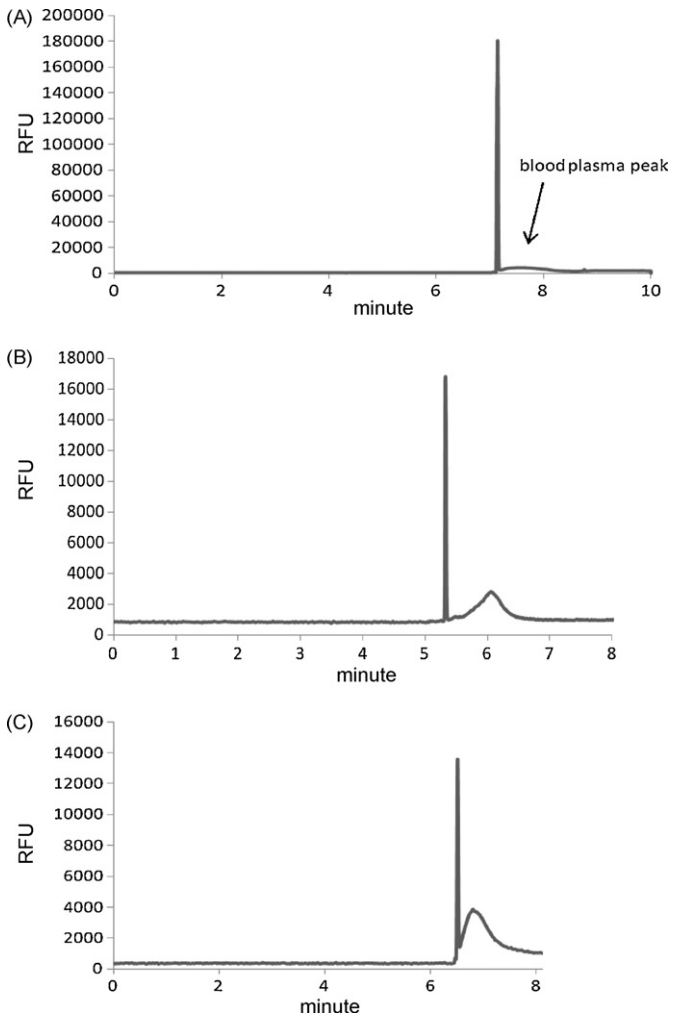


**Fig. 4.** The electropherograms obtained with the three injection method for *C. albicans* in blood plasma. Samples contain: (A) $3 \times 10^8$ CFU/mL *C. albicans*, approximate 48,000 cells/injection; (B) $3 \times 10^4$ CFU/mL *C. albicans*, approximately 5 cells/injection. Prior to CE analysis, washing and dilutions of dye interference plasma with working buffer was performed (see Section 2.3 for details). Experimental conditions are the same as listed in Fig. 3. (C) The electropherogram of *C. albicans* in blood plasma (concentration: $3 \times 10^4$ CFU/mL) using the self-focusing method without spacer segment and blocking agent plug. Experimental conditions: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; sample buffer: 1 mM TRIS/0.33 mM citric acid at pH 7. See Section 2.3 for details.

for 1 min each and running buffer for 3 min. The working buffer of 1 mM TRIS, 0.33 mM citric acid was prepared from 10× dilution of 10 mM TRIS, 3.3 mM Citric acid. pH was adjusted to 7 using 1 M sodium hydroxide or 1 M hydrochloric acid. CTAB was added freshly into the working buffer to obtain actual running buffer. All run buffers and vials used in the study were autoclaved prior to the experiment. Prior to the separation, the capillary was filled with running buffer. All separations were performed in 3 kV in reverse polarity due to reversal of the electroosmotic flow (EOF) by CTAB. All experiments were repeated for at least 3 times to ensure reproducibility of the results. Data were analyzed with Beckman System Gold software.

## 3. Results and discussion

### 3.1. Three plug injection method in buffer samples

The goal of this study was to develop a rapid and simple method capable of determining whether any microbial contamination is

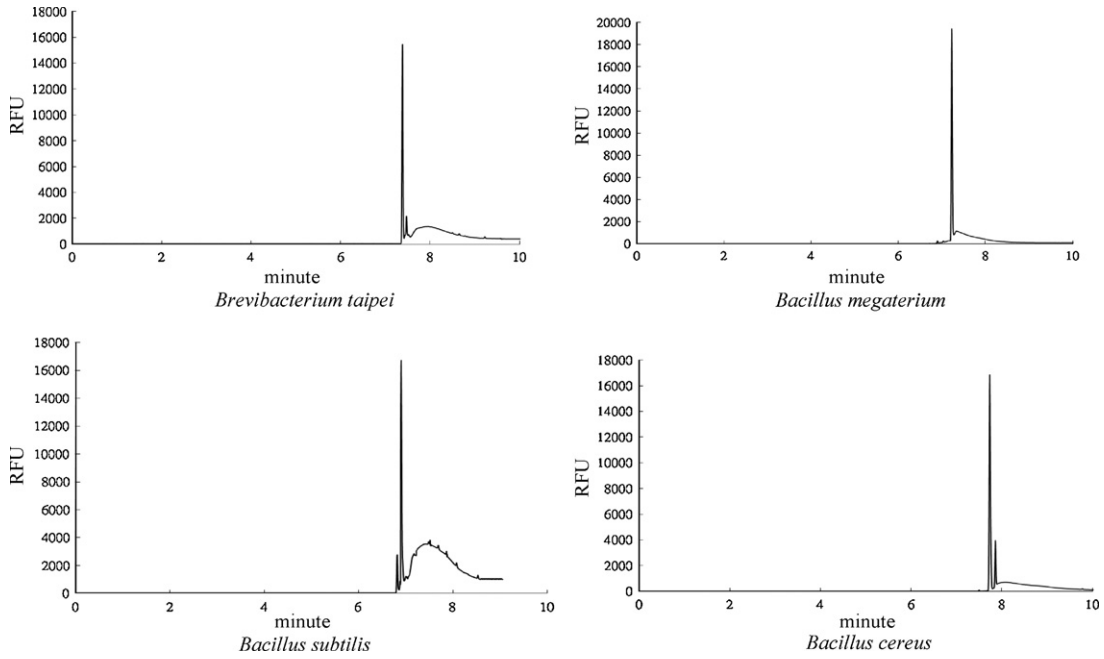


**Fig. 5.** Four different bacteria in blood plasma using the self-focusing method without spacer segment and blocking agent plug. Conditions for all electropherograms are the same those listed in Fig. 4C except that the CTAB concenration was 1 mg/mL. See Section 2.3 for details.

present or completely absent in a blood plasma sample. As reported previously, a single peak of microbes, regardless of the individual species and their electrophoretic heterogeneity, was achieved using CTAB as a run buffer additive with a three plug injection method consisting of the microbial sample, running buffer spacer, and blocking agent [18,19]. Fig. 1 shows the schematic of this three injection method. Briefly, the capillary was initially filled with running buffer containing CTAB. The sample of bacteria without CTAB was then injected followed by an injection of a spacer containing CTAB. Finally, a segment of SB3-10 which serves as a blocking agent and does not contain CTAB, was injected into the capillary. The run buffer additive, i.e., CTAB, residing in the front of the microbes (on the anode side) migrates towards the cathode while the microbes move towards the anode. As the CTAB passes through the microbial sample zone, it carries the bacteria with it. As the microbes travel through the spacer, they are removed from any contaminants in the sample plug region. Upon reaching in the front of the blocking agent, microbial aggregation occurs and a large macroparticle is formed, at which point the electrophoretic mobility of microbes is lost and the plug then migrates at the same speed and direction as the EOF. The EOF direction, under these conditions, is reversed as it flows towards the anode, as does the flow of the microbial sample plug and the blocking agent. Fig. 2 shows the electropherograms obtained using this three injection method. In Fig. 2A only DMSO was dissolved in sample solution while in Fig. 2B only the *C. albicans* were present in sample. It is clear that the *C. albicans* form a sharp peak in the front of blocking agent zone, which is away from the sample plug zone. The microbes can therefore be removed from sample plug that might contain neutral contaminants (e.g. DMSO in Fig. 2A).

### 3.2. Application to blood plasma sample

A similar experiment using the three injection method was performed with a real blood plasma sample spiked with the fungi *C. albicans* used instead of the microbial buffer sample plug of Fig. 1. Fig. 3A was a control run with a blank sample, i.e., blood plasma sample without *C. albicans*. The electropherogram shows that a small plasma interference peak was detectable at 6.5 min. The effect of adding Baclight™ Green bacterial stain to the blood plasma is shown in Fig. 3B. Exactly, 2 μL of Baclight™ Green bacterial stain dye was added to 1 mL of the blank blood plasma sample prior to the separation. The migration time of the interference peak remained the same. However, the peak width was substantial and the peak area was greatly enhanced (at least 50 times). This indicates that Baclight™ Green bacterial stain could interact with components of the blood plasma. Fig. 4A and B shows the electropherograms of the three injection method using blood plasma spiked with different *C. albicans* concentrations ($3 \times 10^8$ and $3 \times 10^4$ CFU/mL, respectively). Results show that the plasma-dye interference peak was greatly reduced compared to Fig. 3B and a single *C. albicans* peak was obtained in front of the interference peak on both electropherograms. These indicate that washing and dilution of blood plasma-dye microbial sample with working buffer in the experimental procedures is able to reduce the effect of interference peak prior to CE analysis (see Section 2.3).

In order to separate the microbial single peak and the plasma interference peak, varying the injection length of the spacer plug was performed. However, similar results were obtained where a single peak was always detectable in front of a small plasma interference peak with similar migration times. Based on these results, we hypothesized that blood plasma containing different kinds of proteins, albumins and peptides that could potentially be a surface active species was responsible for cellular aggregation. Therefore, another experiment without the use of a spacer plug and blocking agent was performed. Results show that a similar electropherogram was obtained where a single microbial peak was also obtained at about 7 min followed by a small interference peak. Apparently, when the voltage was applied in this situation, the microbial cells migrate towards the anode out of the blood plasma plug while the surfactant, CTAB, residing in the front of the microbe-containing plasma plug migrates towards the cathode. When the microbes encountered the cationic surfactants, they were dynamically coated by the surfactant. The microbes then reverse their migrating direction towards the cathode. Upon reaching the blood plasma segment, the microbes aggregated, lost their electrophoretic mobility and formed a large macroparticle in front

of the blood plasma peak. The large macroparticle then migrated towards the anode with the EOF.

It is important to note that the actual concentrations of the fungi and bacteria in the original sample after 20–24 h incubation were about $3 \times 10^8$ CFU/mL. This concentration was higher than that in real infected blood plasma sample. Wain et al. and Werner et al. showed that concentration of bacteria in blood during bacteremia rarely exceeds $10^3$ CFU/mL [35,36]. As a result, the LOD for this method must be evaluated. Fig. 4C shows the electropherogram of $3 \times 10^4$ CFU/mL (10,000× dilution from the original concentration) of *C. albicans* concentration. With the physical limitation of our CE instrument, it requires a minimal sample volume of 0.5 μL for proper injection. Also, based on our previous studies, the optimal injection of sample (158 nL using 5 s at 5 psi) was used [19]. Therefore, approximately five cells were injected per each separation.

If this method is to provide an alternative means for determining the presence or complete absence of microbial contamination in blood plasma, this method should be applicable to virtually any microorganism. However, Rodriguez et al. showed that CTAB may lyse bacterial cells when the concentration exceeds 2 mg/mL, resulting in lower peak heights causing inaccurate results [37]. Conversely, higher CTAB concentration has no influence on fungi such as *C. albicans* probably due to protection by its cell wall [20]. Therefore, four different kinds of Gram positive bacteria (*Brevibacterium taipei*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus megaterium*) were examined with lower CTAB concentrations (1 mg/mL) (see Fig. 5). All bacterial peaks could also be obtained within 10 min using 1 mg/mL of CTAB. These results indicate that this method is not only applicable to the detection of fungi but also on the detection of bacteria in blood plasma with the use of low concentration of CTAB. In order to specifically identify *C. albicans* from a blood sample, we, currently, are evaluating the herein described method with the use of capillary electrophoresis-fluorescence *in situ* hybridization (CE-FISH) to determine and quantify the *C. albicans* from a mixed-microbial blood sample.

## 4. Concluding remarks

A rapid detection method for determining the presence or complete absence of microorganisms in a real biological sample is needed. In this study, a simple, fast detection approach without the use of spacer and blocking agent was examined to provide a quick answer for presence/complete absence of microbes in blood plasma sample within 10 min. Results show that blood plasma containing various kinds of blood plasma proteins and peptides is capable of aggregating microbial cells to form a single sharp peak in front of the plasma interference peak in CE experimental conditions. In order to prevent lysing of cells, CTAB concentration as low as 1 mg/mL was also able to sweep all bacterial cells, while fungi cell required at least 5 mg/mL, to form a single peak in front of the blood plasma peak. The LOD of approximate 5 cells per injection was able to be detected using this approach.

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