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Capillary Electrophoresis (CE) represents a significant tool for the separation of microorganisms as well as the detection and isolation of *Candida albicans* (CA) fungus in human blood. There currently exists few rapid means by which biological pathogens can be tested, at low concentration, in blood, and other organic matrices. Other common chromatographic techniques, such as GC, HPLC, IR and MS do not have the capability of analyzing living microorganisms, nor the ability to separate them with any precision. CE offers the rare opportunity to separate individual cells by the charge to size ratio as well as their isoelectric focusing point (pI). *Candida albicans* fungi was successfully focused and detected in prepared blood samples using surfactant buffer additives to control the cells' surface charge. A second method was developed using capillary isoelectric focusing to separate bacteria species, namely *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Escherichia coli*. The method with which fungus and many other pathogens can be isolated, developed through this study, shows the potential for complete separation cell types within the capillary. These methods will be carried on through further studies of microbial separation using CE and refined.

Introduction

Microbial testing is essential in numerous industries to ensure consumer/patient safety, maintain product quality, and meet regulatory requirements.[1] However, current standard methods for microbial testing are greatly lacking in numerous aspects. The traditional culture method, in which a growth media is inoculated with the sample and allowed to grow, is still the "gold standard" of current sterility tests.[2] Unfortunately, these microbiological tests that rely on the growth of bacteria/fungi fail to meet the demands of modern industry, mainly due to the slow speed of analysis. Furthermore, microorganisms may differ in their requirements for nutrients, cofactors or other environmental conditions, the absence of which can impact their ability to grow in synthetic media. As a result, direct inoculation based methods may be biased – able to only detect only those contaminants capable of growing under the conditions used. Therefore, since no one set of conditions will grow all bacteria or fungi, growth based microbial tests are also fundamentally flawed in the conclusiveness of their results.[3,4]

Because of these factors and the increasing demand on industries to become more streamlined, new methods of microbiological analysis are being developed that lack many of the shortcomings of traditional methods. The presence of viable organisms may be detected by monitoring by numerous methods including bioluminescence, impedance measurements, enzyme immunoassays and immuno-magnetic separations, molecular based techniques (such as PCR), or flow cytometry. While these methods may be used to detect a variety of microorganisms, they still retain some of the basic flaws of plating methods, such as either media specific growth, long analysis times, or low sensitivity (>10^3 cells). [5-10] To overcome these problems, considerable interest has developed in

the microbiology and bioanalytical fields in using capillary electrophoresis (CE) for the analysis of microorganisms.

One of the first published uses of capillary zone electrophoresis (CZE) in the separation of bacteria, by Ebersole and McCormick, occurred in 1993.[11] They were able to successfully separate *Enterococcus faecalis, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus pneumoniae, and Staphylococcus aureus*.[11] Their research, however, was plagued by problems with reproducibility and cell adhesion. It was not until four years later that Pfetch and Welsch were able to separate *Pseudomonas putida*, *Pseudomonas* sp. and *Alcaligenes eutrophus.*[12] Progress was admittedly slow at first as CZE had not yet proven itself as a viable method for separation of bacteria. Separations were made difficult by band broadening and the reproducibility of the electroosmotic flow (EOF). Recently, work has shifted toward using polymeric and surfactant additives to control the surface charge on microorganisms within the applied electric field.[13,14] Isoelectric focusing, which uses a pH gradient to separate analytes by their isoelectric point (pI) has also been explored for the separation of cells and organelles.[15]

Here we demonstrate the ability of capillary electrophoresis as a tool for the detection of microorganisms in "real-world" samples- specifically the detection of *Candida albicans* fungi in blood. *Candida albicans* is a common unicellular fungi and the most common cause of nonsocomial fungal infections in U.S. hospitals. In addition we attempt to physically separate several bacterial species in a mixture using capillary isoelectric focusing. These species all have similar pI values (within 0.2 pH units), demonstrating the ability of CE to separate species with high efficiency.

Experimental

Materials

Supplies: Phosphate salts, sodium hydroxide, acetic acid, hydrochloric acid, cetyltrimethylammonium bromide (CTAB), and caprylyl sulfobetaine (SB3-10), and ampholyte (pH 2-4) were all purchased from Aldrich (Milwaukee, WI). Ethanol, 10% formalin, Triton X-100, and bovine blood were purchased from Sigma (St. Louis, MO). Uncoated capillaries were obtained from Polymicro Technologies (Phoenix, AZ). YM broth was from Difco Laboratories (Detroit, MI). All cultures were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Microbial Detection in Blood Methods

Sample Preparations

Fungi cultures were grown overnight (20–22 h) at 24ºC in YM broth. 1 mL portions of fungi culture were pelleted for 5 min at 2000 rpm using a microcentrifuge. The supernatant was discarded and the cell pellet was resuspended in PBS wash. The solution was then centrifuged again, supernatant discarded, and resuspended in 1 mL of 60% formalin-40% EtOH and allowed to fix for 1 hour. The fixed cells were then centrifuged and resuspended in 60% PBS-40% EtOH and kept refrigerated until analysis. Prior to CE runs, the sample was pelleted and suspended in running buffer. For the spiked blood samples, the pre-fixed cells were suspended in 1 mL of bovine blood, spun down, and resuspended in 1 mL 0.1% Triton X-100 to lyse red blood cells. This solution was then fixed and prepared as described above. Thanks for Dr. Brehm-Stecher and the Iowa State University Department of Food Science for providing these samples.

CE Conditions

All separations were conducted using a Beckman Coulter P/ACE 5100 CE with a 37 cm capillary, 30 cm to the detector. The injection order was as follows: fungi sample for 5 seconds (0.5 psi), spacer plug of buffer for 4 seconds (0.5 psi) and finally the SB3-10 blocking agent (BA) for 2 seconds (0.5 psi).

Throughout all analysis, the voltage at which CE runs were conducted was 5 kV. Initially the analysis was run at 2.5 kV, which produce a low EOF and did not separate the cells present in the capillary well. When the voltage was increased to 5 kV, the efficiency of the separation increased and the analysis time remained short (approximately 8 minutes per analysis).

Isoelectric Focusing Methods

Sample Preparations

Cells were grown overnight from cultures in nutrient broth at 37ºC. Cells were harvested, centrifuged into pellets, washed and resuspended in ampholyte solution several times. For the separation of bacteria, a high resolution ampholyte solution of pH 2-4 was used at a concentration of 1% by volume in water. All biological samples present in the study are of minimal risk to humans through direct contact. All fungi samples have been rendered impotent by the EtOH/Formalin solution in which they were suspended. The strains of *B. subtilus*, *E. coli* and *P. fluroscens* studied are BSL 1 and non pathogenic toward humans.

CE Conditions

All separations were conducted using a Beckman/Coulter MDQ with a 60 cm hydroxy propyl methyl cellulose (HPMC) coated capillary, 50 cm to the detector. The injection order was as follows: water rinse for 30 seconds, ampholyte rinse for 30 seconds, sample injection for 30 seconds.

With the capillary filled with the ampholyte/bacteria mixture, a 20 kV current was applied across the capillary for a period of 30-40 minutes allowing for the formation of the pH gradient and migration time of cells. During this focusing period, the current across the capillary fell, eventually plateauing, indicating that the gradient had formed and the cells had migrated to their isoelectric focusing point (pI) and become neutral. At this point, with the voltage still applied, a pressure of 0.1 psi was applied for approximately 30 minutes to the capillary, pushing the focused sample toward the detector.

Results and Discussion

Microbial Detection in Blood

In the process of CE, a running buffer exists between the inlet and outlet vials of the CE instrument. The majority of the research conducted in the present study involved the optimization of the running buffer, cetyltrimethylammonium bromide (CTAB). The purpose of the CTAB running buffer is to “sweep” all of the CA cells through the capillary by coating the surface of the cells in a net-positive charge. CTAB is, however, a cation surfactant used in topical antiseptics due to its ability to lyse common bacteria. Using an exceedingly concentrated CTAB solution poses the risk of lysing the rather robust *albicans* cells in the sample. To prevent destroying the analyte, the concentration of CTAB used to sweep the cells was qualitatively increased until the minimum concentration of CTAB required to homogonously sweep all CA cells into a single wall of analytes to be bound into a plug by the blocking

agent (BA) caprylyl sulfobetaine (SB3-10). The BA, SB3-10, is a zwitter-ionic surfactant that acts to trap all fungi cells in a plug that will pass the detector simultaneously (Figure 1).

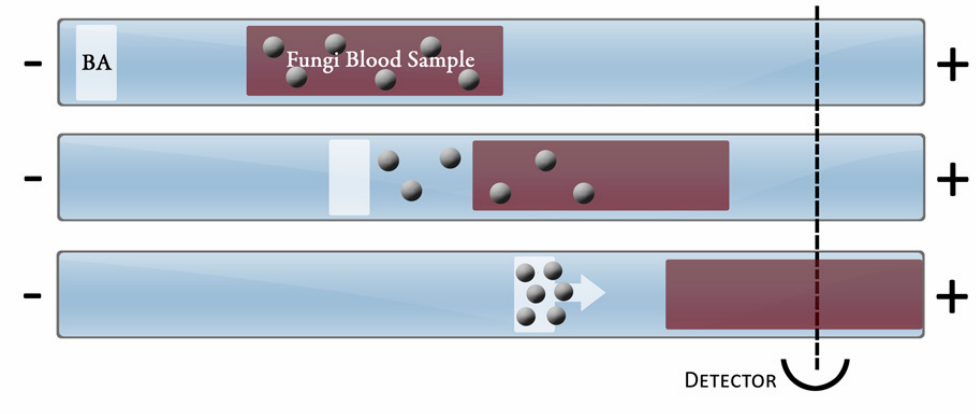

Figure 1. CE Based Sterility Test Diagram

The process of determining the correct concentration of CTAB and SB3-10 was a concerted effort. Both needed to be present to observe the behavior of each. This presented a difficulty in that changing one concentration led to unexpected results and behavior of the other. A process of ramping up concentrations until it was apparent that both the CTAB and BA were working in conjunction began, followed by a slow process of scaling back the concentrations of each to observe the result.

Initially, the CTAB buffer was produced by adding 0.036 grams of CTAB to 25.00 mL of 1.00 mM phosphate buffer, giving an initial concentration of 4 mM CTAB. The SB3-10 BA was produced in the same manner, by adding 0.006 grams of SB-310 to 25.00 mL of 1 mM phosphate buffer giving an initial concentration of 2 mM SB3-10. These early concentrations proved to be far too low and produced undefined, spread peaks rather than one well defined peak. The CTAB was not properly coating the surface of the *albicans*, and the SB3-10 was not forming a well defined cell plug. To solve this problem, the concentrations of both the CTAB and SB3-10 were increased to 20 mM and 15 mM respectively. The result was a well defined peak, where in all *albicans* were swept toward the blocking plug in a uniform manner. However, the chromatograph showed several peaks after where the blocking plug was expected to be.

To determine the position of the BA in the chromatograph, an EOF marker was added to the SB3-10. The EOF marker works by producing an exceedingly strong peak at 214 nm, showing, without question, the position of the BA. This allows a similar run to be performed afterwards using the same BA without any EOF marker to determine the elution time of the *albicans* cells. The EOF marker showed that the focusing that was observed was indeed the border of the BA, and that the peaks after the initial peak wall were *albicans* cells that managed to pass through the BA.

In the next experimental run, the concentration of *albicans* in the sample was reduced by a factor of approximately 4. The concentrations of CTAB and SB3-10 were kept at 20 mM and 15 mM respectively and when the experiment was re-run, only a single peak appeared. Since it was likely that the concentrations of CTAB and SB3-10 had been increased too much to compensate for sample overloading, which was no longer a problem, the concentrations of CTAB and SB3-10 were scaled back until the ideal balance of ~5 mM CTAB and ~15 mM SB-310 were attained (see Figure 2).

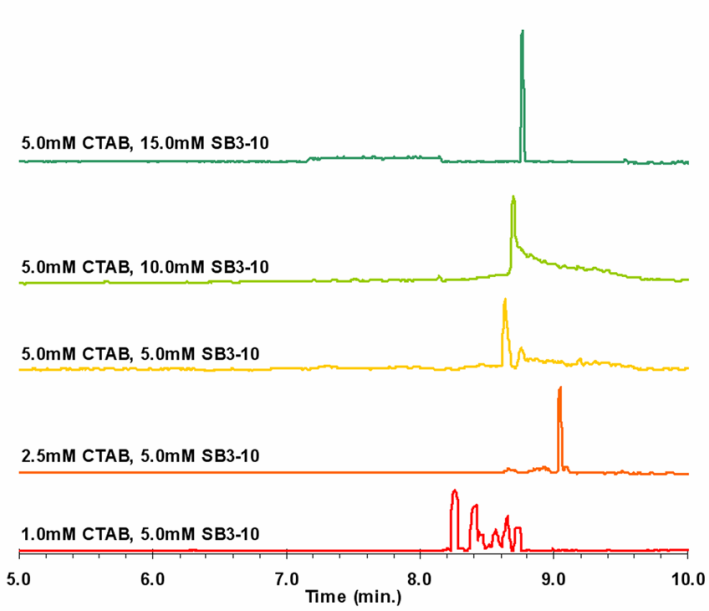

Figure 2: Buffer Additive Concentration Optimization

Once the ideal concentrations of CTAB and SB3-10 were determined for the *Candida albicans*, it became necessary to determine whether the analysis could be conducted using lysed bovine blood spiked with CA. An issue that had still not been addressed was whether other cell materials left in the blood sample after lysing would be swept into the sample plug, giving a potential false positive for CA. A sample of lysed bovine blood was analyzed under the same conditions as the spiked blood and produced no peak when analyzed at 310 nm (the optimum wavelength of analysis). The results are shown in Figure 3.

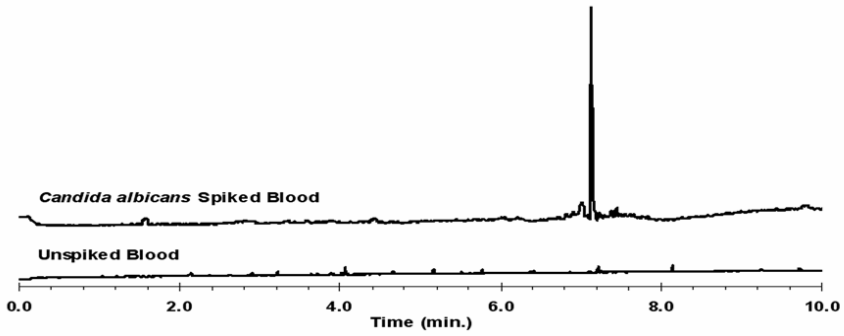

Figure 3: Blood Matrix Response

The ideal concentration of CTAB and SB-310 needed to produce a single, strong peak did, however change. The additional components of the blood matrix increased the necessary concentration of CTAB for an effective separation to approximately 10-12 mM.

The detector itself is a UV/Vis spectrophotometer that measures the absorbance of the running buffer and any particles in the capillary as the exit into the output vial. The wavelength, at which the detector was set, needed to be adjusted to 310nm so that only the desirable species absorbed, and not the buffer or other background components.

The voltage at which the CE was run needed to be set to an appropriate level. If the voltage was too low, the analysis would be slow due to decreased EOF. Conversely, if the voltage was too high, the cells may potentially lyse or die, preventing further analysis or false negatives.

The current research produced viable results and a process that can be used to isolate and detect *Candida albicans* in blood samples. The actual concentration of CTAB and SB-310 required for the analysis of any sample varies based on the concentration of the sample and the components that occupy the blood matrix. As a result, most separations must be initially conducted with a guideline CTAB and SB-310 concentration of 10 mM and 15 mM respectively, and then adjusted qualitatively based on the individual conditions of the analysis. The research currently being undertaken is meant to act as a springboard to more specialized analysis. It was observed, midway through the experiment, that often times a lower cell concentration can be advantageous in the separation of CA. This is beneficial, since most fungal infections exist in low concentration, and early detection methods do not exist.

An issue that arose throughout the study, was the degradation and hydrolysis of the CTAB and SB-310 solutions when left for several days or, at low concentrations, several hours. New solutions of CTAB and SB-310 should be continually produced and immediately used to ensure that the data being obtained was valid. In a real-world application the concentration of the CTAB and SB-310 needs to be adjusted qualitatively, and therefore would require less time and precision in preparation of the solutions.

Isoelectric Focusing of Bacteria

Isoelectric focusing allows not only for the detection of bacteria, but also physical separation of the cells. Briefly, an ampholyte solution containing the mixture of cells is injected into the capillary. Upon application of the voltage, a pH gradient is formed within the capillary due to the migration of ampholyte components. The cells will also migrate based on their surface charges until they reach the pH which equals their pI, at which point their mobility is lost and they focus into a sharp zone. A mobilization step is then used to force the cells past the detector for analysis (Figure 4).

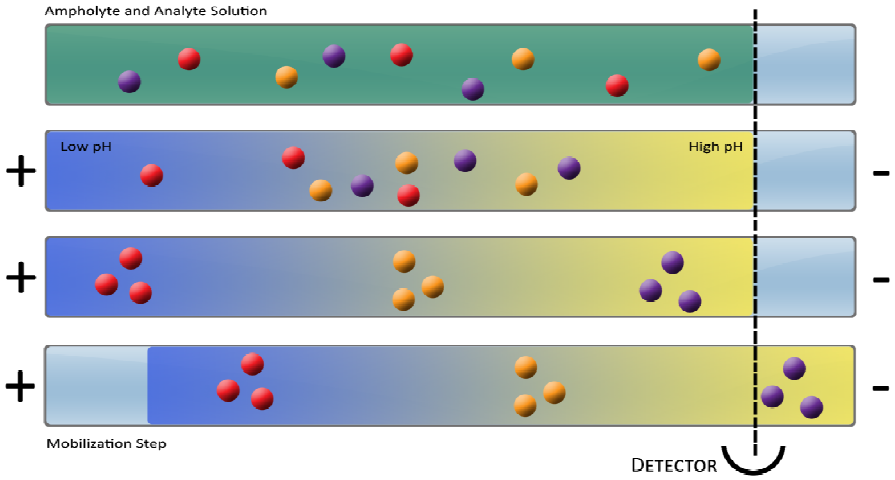

Figure 4. Diagram of Capillary Isoelectric Focusing

As shown in Figure 5, bacteria cells were successfully separated using the above isoelectric focusing methodology. The peaks were highly efficient allowing these cells, whose pI values are very similar, to be completely isolated. Bacteria species that were analyzed were *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Escherichia coli*.

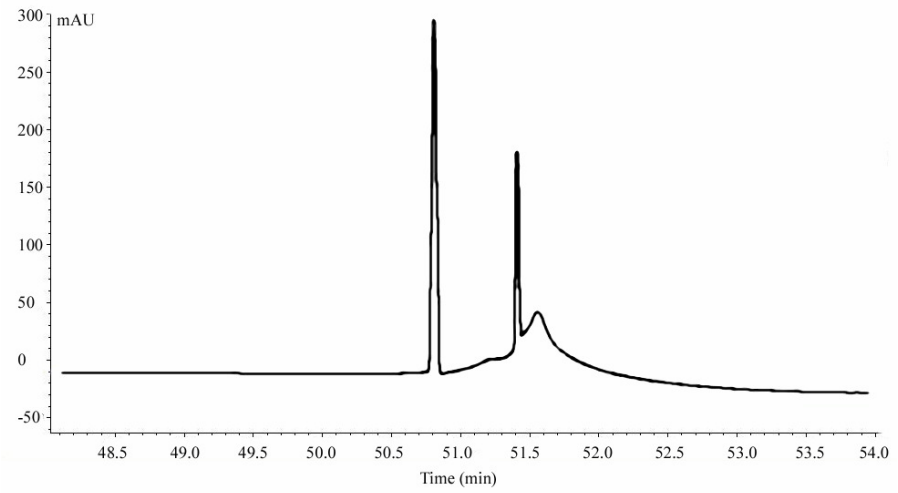

Figure 5. Isoelectric Focusing of *E. coli* (Peak 1) and *B. subtilis* (Peak 2).

Isoelectric focusing proved to be more sensitive at lower concentrations of bacteria than the sterility test and had fewer stray cells due to the increased focusing time. Isoelectric focusing did however require additional separation time and had a higher incidence of aborted runs due to equipment malfunctions. Additionally, coating the wall of the capillary proved difficult and time consuming. Therefore, in situations where the presence of any bacteria is unacceptable, the basic sterility test would be ideal. In a situation where the identity of the bacteria is in question, isoelectric focusing is necessary.

Proper coating of the capillary wall was of significant importance. The coating needed to be homogeneous as well as resistant to repeated separations. A 0.5% solution of HPMC was prepared by dissolving 0.15 grams of HPMC in 30 mL of de-ionized water, using a magnetic stirrer, overnight. The resulting solution was highly viscous and difficult to inject into the capillary. A syringe, along with an HPLC rubber seal were used to inject the HPMC into the capillary. Once filled with HPMC, the capillary was then placed into a GC and attached to the nitrogen outlet. A stream of nitrogen was applied to the capillary at 5 psi to push out any excess HPMC and prevent blockages. Proper nitrogen flow was checked by submerging one end of the capillary in a beaker of de-ionized water and waiting for bubbles to emerge. Once flow was ensured, the capillary was placed in the GC oven under a temperature gradient that ramped from 60°C to 140°C over a period of 20 minutes, once at 140°C the capillary was allowed to bake for 40 minutes. Upon completion of the oven cycle, the capillary was allowed to cool to 60°C, at which point, the detector window was burned into the capillary with nitrogen still flowing. The capillary was then removed and inserted into the capillary cartridge.

The resolution of the buffer has proven to be the biggest drawback to CIEF. Most of the buffers commercially available have a range of approximately two pH units. Most of the cells being separated have a pI difference of approximately 0.2 pH units. As a result, unless the pI of the cells in question are significantly different, differentiation of peaks on the electropherogram becomes increasingly difficult.

Future Research

The next step in this research will be to fluorescently tag the CA in blood samples using fluorescence in-situ hybridized (FISH) probes to positively identify CA as the pathogen present in a blood sample. The method developed so far proves that fungi in blood can be focused and detected, however identification the species of fungi is where the FISH probes are needed.

The Fish probes bind to the unique complementary strands of rRNA in ribosomes of the CA, producing a strong fluorescent signal only for the target cells. This allows not only for the ability to determine whether a fungal infection exists or not, but also, what type of fungus is present. This will allow medical professionals the opportunity to identify and treat a wide variety of fungal infections with increased certainty.

Also, during experimentation with bacterial samples, it was observed that the pI of the samples appeared to decrease with time. This may be due to changes in the proteins expressed on the surface of the cells as the bacteria age, resulting in a shift in the surface pI of the cells. Additional studies must be conducted to confirm this hypothesis, but if true, the age of the bacteria from the time of inoculation could be determined through isoelectric focusing. Additionally, the separation of bacteria and fungus has not been attempted. Whether the resolution of a buffer with the pI range needed to capture both is high enough is unknown.

Conclusions

The present study has proven the feasibility and viability of the method in question. Though the methods outlined above work in the given conditions, field conditions will vary, and thus the method may require further optimization. Evaluation of the methods' robustness is still needed. This research is the genesis of capillary electrophoretic techniques for detecting biological pathogens in blood. Further research will need to be conducted to determine the timeline of detection and infection as well as field methods that do not require a high theoretical knowledge of the process. The results though, are promising, and with further research, it is likely more applications of CE in the separation of biological materials will arise.

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