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Microfluidic Assay for Continuous Bacteria Detection Using Antimicrobial Peptides and Isotachophoresis

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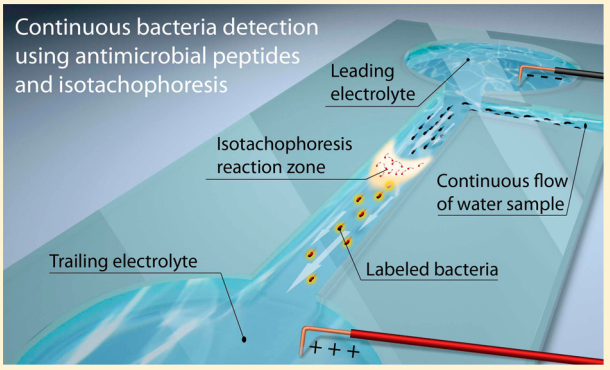
Supporting Information

ABSTRACT: We present a novel microfluidic assay for continuous and quantitative detection of bacteria in water. We leverage isotachophoresis (ITP), an electrophoretic focusing technique, to create a stationary high concentration zone of fluorescently labeled antimicrobial peptides (AMPs) in a microfluidic channel. The tested water sample flows continuously through this high concentration AMPs reaction zone; any bacteria present in the sample is simultaneously labeled by, and separated from, the high concentration AMPs. The labeled bacteria continue into the downstream pure-buffer zone where the fluorescence signal is monitored, providing a direct quantitative measurement of the original bacterial concentration in the sample. We present the principles of the technique, demonstrate its applicability for quantitative detection of *E. coli* as well as its stability over a 1 h monitoring time, and provide a simple model for predicting its performance at different operating conditions. The method could be potentially expanded for use with other types of probes and provide continuous analysis and monitoring of water samples at the point of need.



The quality of drinking water supply remains one of the major causes of mortality worldwide. The World Health Organization (WHO) estimates 3.4 million water-related deaths per year, with 2.2 million caused by diarrheal disease alone.[1] The problem is not limited to developing countries, and outbreaks are often encountered in the developing world. During 2009−2010, in the US alone, 33 drinking water-associated outbreaks were reported, comprising 1040 cases of illness, 85 hospitalizations, and 9 deaths.[2] Yet, it is likely that many outbreaks remain undetected using current methods.[3]

The conventional standard method for microbial examination of water relies on filtration followed by sample cultivation on selective media.[4] This process requires a specialized biosafety laboratory and highly trained personnel and takes between 24 and 72 h to complete. This time lag can be crucial in tracking contamination and preventing waterborne disease outbreaks.

Faster detection techniques are based on genotypic analysis and immunoassays. Genotypic methods target specific nucleic acid sequences, typically rRNA (rRNA) or DNA,[5,6] and utilize amplification methods such as polymerase chain reaction (PCR) which are sensitive, specific, and faster than cultivation methods (typically 2−4 h). However, despite recent advancement in amplification methods, PCR still requires a sterile environment, multiple sample preparation steps for purification of nucleic acids, a dedicated laboratory, and highly trained personnel.[7] Immunoassays rely on binding of species-specific or strain-specific antibodies (Ab) to target bacteria. The traditional and most established immunoassay technique is the enzyme-linked immunosorbent assay (ELISA) and its variants,[8] in which surface-immobilized Ab capture target antigens present in the sample. Modern versions of ELISA make use of immunomagnetic separation (IMS),[9,10] surface plasmon resonance detection (SPR),[11] or immobilization on carbon nanotubes,[12] yet the principle of the technique remains unchanged. An important advantage of Ab-based approaches is that captured bacteria remain viable and can be further studied or analyzed. At the same time, the high costs of associated reagents (primarily antibodies), the relative high complexity in multiple wash steps, and the need for well controlled surface functionalization have largely confined the use of the method to research laboratories, and it has not seen widespread use as a diagnostic tool.

In recent years, there has been significant interest in the use of microfluidic platforms for pathogen detection.[13] However, while continuous monitoring is highly desired in water quality control, the majority of assays are limited by their ability to analyze only a single and finite amount of sample. This is primarily due to their reliance on sample preparation steps,

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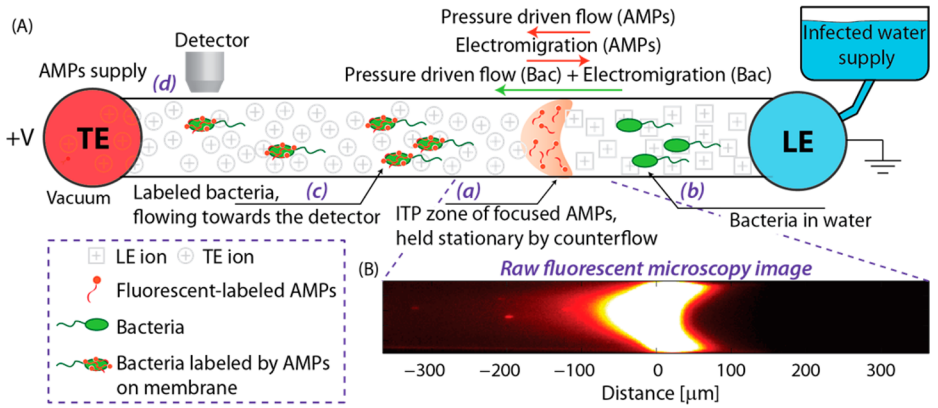

Figure 1. (A) Schematic illustration of the assay in its simplest format using a single straight channel. (a) Fluorescently labeled AMPs, initially mixed in the trailing electrolyte (TE) reservoir, are focused by cationic ITP and held stationary using vacuum-driven counterflow. (b) The same vacuum line also continuously draws a flow of potentially infected water from the leading electrolyte (LE) reservoir. (c) Any bacteria present in the water travels through the high concentration AMPs zone and is instantaneously labeled due to the locally accelerated reaction. The labeled bacteria continue downstream, while free AMPs remain confined to the stationary ITP zone, thus reducing the downstream background signal. (d) Further downstream, the fluorescent signal is registered by a detector. The signal corresponds to individual bacteria passing through the detector and yields the bacteria concentration in the sample. (B) Fluorescent microscopy image demonstrating the labeling of *E. coli* O:416 with AMPs at the high concentration zone. Initially unlabeled bacteria that flow through the zone are instantaneously labeled and can be clearly seen emerging from the interface. Free AMPs remain contained in the ITP zone.

such as bacterial lysis, labeling, washing, or filtering steps, which are difficult to implement in a continuous manner.

Isotachophoresis (ITP) is an electrophoresis technique that enables separation and preconcentration of ionic molecules based on their effective electrophoretic mobility. A discontinuous buffer system consisting of leading (LE) and trailing (TE) electrolytes, characterized, respectively, by ions having high and low electrophoretic mobility, is used such that, when an electrical field is applied, analytes of interest are focused at a narrow LE-TE interface. Up to a million-fold increase in concentration in 2 min has been demonstrated using this technique.[14] Several recent works make use of ITP for bacteria detection. Bercovici et al. used molecular beacon probes to detect 16 rRNA from bacteria lysates[15] by leveraging accelerated reactions under ITP.[16] Phung et al.[17] and Oukacine et al.[18] took a different approach and used ITP for focusing and detecting whole bacteria, thus avoiding a lysis step. Nevertheless, these methods still require sample preparation steps (off-chip filtering and fluorescence prelabeling) and therefore offer analysis of only finite volume samples. Thus, while existing techniques can indeed provide sensitive and qualitative analysis of water samples, they lack the ability to perform continuous, real-time monitoring. There is an unmet need for continuous detection techniques of infectious disease-causing pathogens, with the potential for simple automation and standardization.

Recent studies used antimicrobial peptides (AMPs) as an alternative molecule for recognition and labeling of bacteria.[19,20] AMPs are relatively short, positively charged peptides that are part of many organisms' innate defense system, contributing to their protection against microbial infections. They target bacteria by nonspecific binding to the negatively charged outer-membrane and then induce microbial killing by several mechanisms, depending on the AMPs type.[21] The AMPs' ability to bind to the bacteria's negatively charged outer-membrane enables their use as bacterial probes. While AMP-based detection cannot be directly compared with high specificity methods such as PCR and ELISA, it nevertheless can serve as an excellent method for initial detection of bacteria presence, guiding further analysis and treatment steps. For instance, the ability to rule out a bacterial infection would be highly advantageous in medical diagnostics, where as much as 85% of blood sample cultures return negative (i.e., show no bacterial growth).[22,23]

We here present a first proof of concept and characterization for a novel microfluidic method capable of continuous monitoring of bacteria in water samples, requiring no sample preparation steps. We use cationic ITP balanced by counterflow to create a stationary zone of highly focused labeled AMPs. The tested water sample flows continuously through the high concentration AMPs reaction zone. Bacteria present in the sample is instantaneously labeled and washed as it transitions through the narrow AMPs zone and into the downstream buffer zone. We demonstrate the use of the method for quantitative detection of both Gram-positive and Gram-negative bacteria and demonstrate its stability over long durations of time. In support of our experimental results, we provide in the theory section a simple yet detailed model for the flow rate of bacteria into the channel and for the binding efficiency of the AMPs probes to the bacteria membrane, which is capable of predicting the sensitivity of the assay at different operation conditions.

THEORY AND PRINCIPLE OF THE ASSAY

Figure 1 illustrates the principle of our assay in its simplest form. Consider a simple straight fluidic microchannel of length L connecting two reservoirs. We fill the channel and East reservoir with LE solution and the West reservoir with TE solution. Applying an electrical field across the channel, we establish cationic ITP and focus a finite sample of AMPs at the LE-TE interface. We balance the AMPs migration by applying negative pressure to the TE reservoir which forms pressure driven flow with an average velocity, $\overline{u}_P$, that matches exactly the electromigration velocity, u_{ITP^+}, such that $\overline{u}_P = -u_{ITP^+}$. We then introduce the sample of interest to the LE reservoir. Any bacteria present in the sample are driven downstream toward the AMPs zone by a combination of advection and electromigration (since bacteria are negatively charged[24]). The LE-TE interface plays two roles: First, since reaction rate is directly

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proportional to reactants' concentrations, the high concentration of AMPs formed at the LE-TE interface serves as a confined reaction zone in which the binding reaction of the AMPs to the bacteria outer membrane is significantly accelerated. Second, the LE-TE interface acts as species-specific filter, in which the negatively charged bacteria are free to pass, but the positive AMPs remain focused and confined in space. Thus, any bacteria present in the sample is sequentially labeled by, and separated from, the high concentration AMPs. The now labeled bacteria continue downstream, where their fluorescence signal can be monitored. From the registered signal, we obtain quantitative measurement of the original bacterial concentration in the sample. A dynamic illustration of the assay and a real-time video of the labeling process are available as Supporting Information.

The cross section averaged bacteria velocity at any point along the channel can be expressed as

$$u_{B,i} = \mu_B E_i + \overline{u}_P \tag{1}$$

where the double index $(..)_{B,i}$ denotes a property of the bacteria (B) in the zone i, corresponding to either the LE or TE (i = L or T, respectively). E_i is the electric field in that zone, and μ_B is the effective electrophoretic mobility of the bacteria, which we here assume to be constant across zones. $\overline{u}_P$ is the average pressure driven flow velocity. Accounting for the ITP condition, which dictates $u_{ITP^+} = \mu_i E_i$, and balancing the ITP velocity by counterflow, $\overline{u}_P = -u_{ITP^+}$, we obtain

$$u_{B,i} = u_{ITP^+}\left(\frac{\mu_B}{\mu_i} - 1\right) \tag{2}$$

where μ_L and μ_T are, respectively, the effective mobilities of the leading and trailing ions. The bacterial flux (which is of course equal in the LE and TE zones) can thus be expressed as

$$Q_B = -u_{B,i}c_{B,i}A_i = u_{ITP^+}\left(1 - \frac{\mu_B}{\mu_i}\right)c_{B,i}A_i \tag{3}$$

where $c_{B,i}$ is the concentration of bacteria in the zone i, and A_i is the cross sectional area in that zone. It is important to note that, due to the discontinuity in electric field across the ITP interface, the bacteria concentration in the TE may be different (typically smaller) than the initial bacteria concentration present in the LE. From mass conservation, and accounting for potential cross section area changes between LE and TE,

$$c_{B,T} = c_{B,L}\left(\frac{\mu_T}{\mu_L}\frac{\mu_B - \mu_L}{\mu_B - \mu_T}\right)\frac{A_L}{A_T} \tag{4}$$

The limit of detection (LOD) of the method is dictated by the bacterial flux into the channel; in principle, as the detection is discrete, even a single bacterium could be detected provided that it is labeled by the AMPs. However, at low bacteria concentrations, longer time will be needed for one bacterium to enter the channel. It is most convenient to express the flux in terms of the known LE properties. Denoting the bacteria concentration in the sample, $c_{B,L}$, we obtain

$$Q_B = (\mu_L - \mu_B)A_L E_L c_{B,L} \tag{5}$$

We emphasize that, since all mobilities here are signed quantities, the difference $\mu_L - \mu_B$ is always positive. If the concentration of bacteria in the sample, $c_{B,L}$, is expressed in units of cfu/m^3 (1 cfu/mL = 10^6 cfu/m^3), then the characteristic time scale for detecting the first bacterium is simply $\tau_B = 1/Q_B$. Clearly, higher electric fields, larger cross-section areas, and high LE mobilities contribute to a lower limit of detection, for a fixed detection time.

Another parameter influencing the LOD is the labeling efficiency of the bacteria as it passes through the AMPs zone. Assuming analogy between the binding reaction of AMPs to the bacteria membrane and reaction of biomolecules at a surface (governed by Langmuir kinetics), the concentration of bacteria (surface probes) bound by AMPs (target molecules) follows

$$\frac{db}{dt} = k_{on}c_A(b_m - b) - k_{off}b \tag{6}$$

where c_A is the concentrations of the free AMPs, b_m is the total surface density of receptor probes on the bacteria, b is the density of surface-bound AMPs, and k_{on} and k_{off} are, respectively, the on and off rates of the reaction. These parameters represent properties of the bacteria or probe, so their value varies between different strains and probes and cannot be directly modified. However, they can be partially determined by evaluating the labeling efficiency at different conditions (i.e., measuring b/b_m). The characteristic time scale of the reaction rate is then given by $\tau_R = 1/(k_{on}c_A)$. To achieve sufficient labeling, this time scale must be sufficiently short in comparison with the advection time scale of the bacteria expressed as $\tau_A = l_{AMP}/u_B$, where l_{AMP} is the width of the AMPs zone, and u_B is the velocity of bacteria thorough the zone. This condition could be expressed as $\tau_R < \tau_A$, or

$$u_B < l_{AMP}k_{on}c_A \tag{7}$$

Hence, for a given concentration of AMPs, c_A, and kinetic rate, k_{on}, there is a maximal bacterial velocity for which sufficient labeling will occur. Consequently, this provides an upper bound on the electric field and sets a trade-off on the optimal flux of the system. Since the reaction rate of AMPs is not well characterized, we used an experimental approach to evaluate the assays labeling efficiency, as further described in the Experimental Setup section.

EXPERIMENTAL SETUP

Chemicals and AMPs. *ITP Buffers.* We performed cationic ITP using LE buffer composed of 100 mM NaOH and 200 mM HEPES and TE buffer composed of 10 mM pyridine and 20 mM HEPES. To suppress electroosmotic flow (EOF), we added ∼1% 1 MDa poly(vinylpyrrolidone) (PVP) to both buffers. All buffer components were purchased from Sigma-Aldrich, (St. Louis, MO) and prepared in purified UltraPure DNase/RNase free distilled water (Milli-Q water purification, Millipore Corp., Billerica, MA, USA).

AMPs. We used in this study TAMRA labeled Indolicidin, 5-TAMRA-ILPWKWPWWPWRR, synthesized by Biomatik (Wilmington, Delaware, USA). Indolicidin is present in nature in bovine neutrophils and demonstrates broad spectrum of activity against Gram-negative and Gram-positive bacteria, fungi, and protozoa.[25] A stock solution was prepared by solubilizing the AMPs in 1 to 8 acetonitrile in deionized water and stored at −20 °C. Further dilutions were freshly prepared in acetonitrile before each set of experiments from a stock solution concentration of 100 μM which was kept refrigerated.

We estimated the peptide properties using the PepCalc.com online calculator. The pI of the peptide is at pH 12.41, and at a neutral pH, the overall charge is +2. Thus, it can likely be considered as a fully ionized cationic species in all experiments.

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We estimated the AMPs mobility to be around 5×10^{-9} $m^2/(V\ s)$ by observing the focusing of sample in a set of ITP experiments with various TE compositions having different effective mobilities. We noticed a tendency of the AMPs to attract and adhere to the negatively charged channel walls, likely due to electrostatic interaction. Thus, stringent cleaning of the channels between experiments was necessary. In addition, at very high concentrations (above 10 μM), we noticed aggregation and precipitation of AMPs, which can result in precipitants flowing toward the detector causing false positives. To avoid this, we worked with smaller concentrations of AMPs (as indicated in the Results and Discussion section) and introduced to the channel only a finite amount of AMPs which remains at a soluble concentration when focused.

Bacteria Growth Conditions and Sample Preparation. *Bacteria Growth Conditions.* We used *E. coli* O:416 as a model strain in this study. We prepared stock cultures by incubating *E. coli* in Luria broth (LB) at 37 °C to an OD600 of 0.3, corresponding to approximately 3×10^8 cfu/mL, as measured by standard plating. We then transferred 1 mL of cell suspensions to sterilized 1.5 mL vials, centrifuged at 14 650 rpm for 2 min, and discarded the supernatant. We repeated the centrifugation process twice to remove any medium remnants and stored the bacteria pellets at −20 °C.

Sample Preparation. For experiments performed with unlabeled bacteria, pellets were resuspended in 1 mL of tap water and serially diluted in tap water to the final concentrations. For labeling efficiency experiments performed with prelabeled bacteria, we incubated the pellets in 200 μL of tap water and 4 μL of 34 μM SYTO9 *BacLight* (LIVE/DEAD BacLight Bacterial Viability Kit, Life technologies) for 10 min. To discard remaining free fluorophores, we centrifuged at 14 650 rpm for 2 min, then removed supernatant, and resuspended the pellets in 1 mL of tap water, for a final concentration of 3×10^8 cfu/mL.

Experimental and Imaging Settings. All experiments were performed on a NS-12A microchip made of isotropically etched soda lime glass (PerkinElmer, Waltham, Massachusetts, USA). An overview of the chip geometry is provided in Figure 2. The chip consists of two intersecting channels, each having maximum width and depth of 90 and 20 μm, respectively. Images were obtained using an inverted epifluorescent microscope (Ti−U, Nikon, Tokyo, Japan) equipped with a metal halide light source (Intensilight, Nikon Japan), 20× objective (Plan Fluor, NA = 0.75, WD = 0.66 mm, Nikon, Tokyo, Japan), TAMRA compatible filter-cube (model 49004, 545/25 nm excitation, 605/70 nm emission, and 565 nm dichroic mirror, Chroma, Bellows Falls, VT, USA), SYTO9 compatible filter-cube (480/15 nm excitation, 535/20 nm emission, and 505 nm dichroic mirror, Nikon, Tokyo, Japan), and motorized stage (MS-2000, Applied Scientific Instrumentation, Eugene, Oregon). Images were captured using a 14 bit, 512 × 512 pixels CCD camera (Clara, Andor, Belfast, Ireland). We controlled the camera and stage using NIS Elements software (v.4.11, Nikon, Tokyo, Japan) and processed the images with MATLAB (R2011b, Mathworks, Natick, MA). Constant voltage or current were applied using a sourcemeter (model 2410, Keithley Instruments, Cleveland, OH).

Assay Procedure. Before each experiment, we cleaned the channel by sequentially flowing bleach and DI water, for 2 min each. In each experiment, we first establish a stationary ITP zone containing focused AMPs and then introduce the sample to the channel.

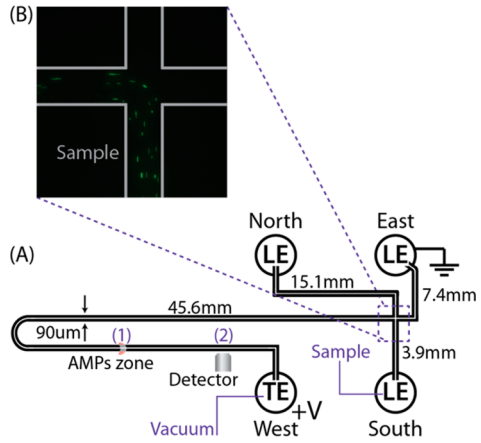

Figure 2. Schematic of the microfluidic chip layout and experiment setup. (A) The chip is a commercially available design (NS-12A, PerkinElmer) made of isotropically etched soda lime glass with dimensions of 90 μm (width) × 20 μm (depth). Also shown are the length dimensions of each intersected channel. A finite amount of AMPs is injected through the West reservoir, focused by cationic ITP, and remains confined and stationary at point (1) by negative pressure applied at the West reservoir. Electric field is applied on the channel by setting a constant voltage or current between East and West reservoirs, oriented for cationic ITP propagation from the West to the East. Detection of the fluorescent signal is obtained by a camera located at point (2), 4 mm downstream from the labeling zone. (B) Raw fluorescence image of the channel intersection showing an *E. coli* sample prelabeled with SYTO9, initially mixed in the South reservoir, flowing into the main channel, toward the labeling site.

Initial Formation of Labeling Zone. For chip loading, we filled the North, East, and South reservoirs with 20 μL of LE and applied vacuum at the West reservoir for 1 min to fill the channel. Next, we rinsed the West reservoir with DI water and filled it with 18 μL of TE and 2 μL of 1 μM AMPs. We applied constant voltage between the West and East reservoirs. When the focused AMPs plug traveled a distance of 6 mm along the channel, we stopped the voltage, rinsed the West reservoir, and filled it with 20 μL of pure TE. We then reapplied the voltage (or current) to regain focusing of the injected AMPs. This resulted in a finite and well controlled amount of focused AMPs. To then hold the AMPs plug stationary, we applied negative pressure to the West reservoir using a water column, resulting in pressure driven flow countering electromigration. We controlled the flow rate by changing the height of the water column according to visual monitoring of the ITP interface progression and feedback on the current reading, which is a good indication for the location of the interface in the channel. The AMPs plug, i.e., the labeling reaction zone, was positioned stationary 12 mm from the West reservoir (marked as station (1) in Figure 2).

Detection Procedure. We introduced 10 μL of the sample of interest to the South reservoir and actuated the pipet several times to homogenize the solution. For time dependent detection, we positioned the objective 4 mm downstream from the labeling reaction zone (marked as station (2) in Figure 2) and triggered the camera at 5 Hz for 2 min. For labeling efficiency experiments, the same procedure was used, but the spiked samples were also prelabeled with SYTO9 (labeling procedure described above). As further detailed in the Supporting Information, after turning off the electric field (and counter flow), we captured images at 10 stations downstream

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from the interface zone using both FITC and TRITC filters (total set of 20 images).

RESULTS AND DISCUSSION

Proof of Concept for Quantitative Bacteria Detection. Figures 3 and 4 demonstrate the ability of the method to achieve continuous and quantitative bacteria detection in water samples. Figure 3 presents the measured bacteria flux versus the known bacteria concentration introduced in the reservoir. Here, the bacteria in the sample are originally unlabeled and obtain their fluorescent labeling as they pass through the high concentration AMPs region. Images are recorded at a distance of 4 mm downstream from the ITP interface, and the number of bacteria in each frame is counted, as described in the Supporting Information (S1). After binding to the outer-membrane of the target bacteria, the AMPs may lyse and disrupt it as part of their killing mechanism. However, as this process typically takes several minutes[26] and our detection takes place only a few seconds after the binding, we do not expect a significant effect on the detection.

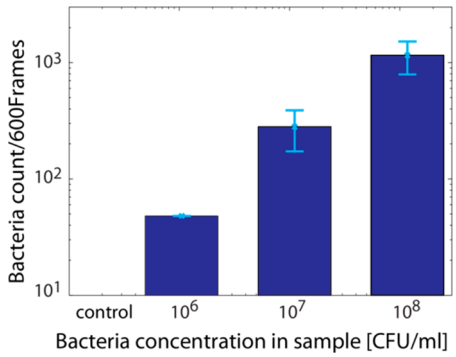

Figure 3. Experimental results demonstrating quantitative bacterial detection. The detected bacterial flux (which is defined as the number of detected bacteria per frame) correlates well with the original bacterial concentration in the sample. Thus, quantitative assessment of the original bacterial concentration can be obtained. No signal was obtained for tap water used as the control case. The signal was acquired at 5 Hz frame rate for 2 min. The height of each bar represents the average of at least 5 realizations, with the range of the bars representing 95% confidence of the mean. Constant voltage of 400 V was applied on the channel, resulting in a current of approximately 2 μA.

The acquired signal (i.e., bacterial flux) is proportional to the original bacterial concentration in the sample, illustrating that quantitative detection of *E. coli* can be obtained. We use the two sample *t* test method[27] to determine whether the difference between any two measured mean values is significant. We define a *p*-value of $p = 0.05$ (corresponding to 95% confidence on a statistical difference between results). In all cases, the calculated *t*-values were significantly higher than the required threshold (for example, the calculated *t*-value for the difference between the signal at 10^6 and 10^7 cfu/mL is 5.26, whereas the threshold based on the number of repeats is 2.57), indicating clear statistical significance. As discussed in the theory section, the limit of detection is determined by the flow rate of sample into the channel. Here, for channel cross section of 90 μm × 20 μm, ITP velocity of $u_{ITP} = 100$ μm/s, and approximated bacteria mobility of $\mu_B = -30 \times 10^{-9} m^2/(V\,s)$, the bacterial flux for the 10^8 cfu/mL sample as obtained using eq 3 is 1542 cfu/min. This is in good agreement with the order of magnitude of measured experimental values. Under these conditions, the lowest concentration we detected in 2 min was 10^6 cfu/mL; in this time period, approximately 40 bacteria pass through the ITP interface, get sufficiency labeled, and are detected. The extrapolated limit of detection is thus 10^5 cfu/mL (yielding 4 bacteria in 2 min).

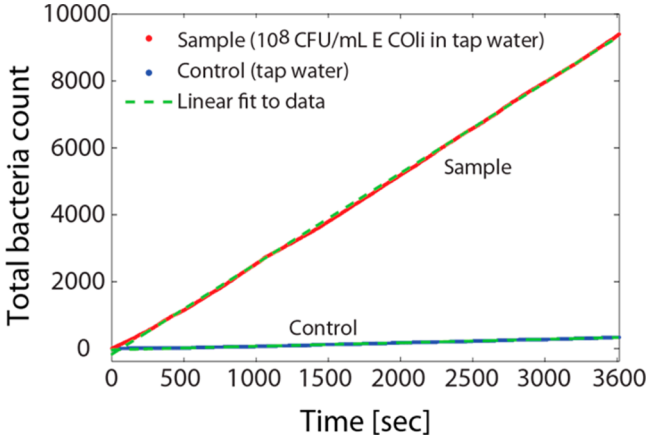

Figure 4. Experimental measurements of bacteria counts vs time, demonstrating continuous bacterial detection during a 1 h period. The obtained signal exhibits stable behavior and increases linearly with time, suggesting no significant deterioration of the finite AMPs sample focused at the interface. The control sample contained only tap water and shows only a moderate increase, after long times, likely due to autofluorescence of contaminants or precipataion of AMPs. The signal was acquired at a 1 Hz frame rate for 1 h. Constant voltage of 400 V was applied on the channel.

Using the same cross-section geometry, better limits of detection could be obtained with longer monitoring times. Figure 4 presents bacteria count versus time and demonstrates continuous operation of the assay for over an hour. At a concentration of 10^8 cfu/mL, we obtain approximately 9000 detections per hour (or 150 per minute). Importantly, the number of detected bacteria grows at a constant rate, substantiating and validating the assay stability and possible use as a continuous water monitoring platform. To further support the claim of the assay stability, we present a trace of the monitored current during the whole procedure in the Supporting Information (Figure S3). Furthermore, no bacteria aggregation or channel clogging was observed during this 1 h time frame. As measured by Harden and Harris[28] many other bacterial species are also negatively charged over a wide range of pH values, enabling their detection in using our assay. Figure S4 in the Supporting Information presents the applicability of the assay to other (Gram-negative and Gram-positive) bacteria species.

Labeling Efficiency. The sensitivity of the assay is directly affected by the flow rate, as well as the labeling efficiency of bacteria as it passes through the AMPs confined at the LE-TE interface. To characterize the latter, we performed the assay on tap water samples spiked with bacteria which were prelabeled with SYTO9. After stabilizing the assay, we simultaneously stopped the counterflow and voltage, such that all bacteria remain stationary. We then imaged the channel at 10 stations downstream of the ITP interface zone. At each station, we took images using two filters: 480/535 for detecting the bacteria prelabeled with SYTO9 and 545/605 for detecting the bacteria which were labeled on-chip by AMPs. SYTO9 emits at a

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wavelength of 500 nm, which is sufficiently shifted from the 579 nm emission of the TAMRA labeled AMPs, and thus, it is possible to measure the number of bacteria which were successfully labeled and compare it with the total number of bacteria that passed through the labeling zone. We wish to clarify that the use of SYTO9 dye was not to monitor the viability of the bacteria but only to be able to count the total number of bacteria in frame. We define the labeling efficiency as the ratio between the number of detected AMPs labeled bacteria and the total bacteria present in all the frames. To obtain additional statistics, we repeated the process several times by reapplying the current and counterflow to fill the channel with a new set of labeled bacteria. Imaging after stopping the flow enables one to perform this colocalization test of the signal, which is not possible during the standard assay operation. Figure S2 in the Supporting Information illustrates in more details this detection process. We examined the influence of both the applied current (affecting the electromigration speed of the bacteria) and the initial AMPs concentration (affecting the peak concentration at the ITP interface) on the binding reaction of AMPs to the bacteria membrane.

Figure 5A presents the labeling efficiency as a function of the applied current to the channel. The average labeling efficiency varies between 65% and 85% for all the current values. We again use the two sample t test method[27] to determine whether the difference between any two measured mean values is significant. Using a p-value of $p = 0.05$, the calculated t-values were significantly lower than the threshold required statistical significance (for example, the calculated t-value for the difference between the labeling efficiencies at 8 and 10 μA is 1.65, whereas the threshold based on the number of repeats is 2.78). Hence, we conclude that there is no significant advantage in the labeling efficiency of one current over another. This is despite the fact that higher current results in higher electromigration velocity and, consequently, shorter reaction time. This indicates that, at these electric current values, the labeling process is reaction limited and the advection time of the bacteria through the labeling zone is significantly higher than the time required for binding. In other words, k_{on} is sufficiently high such that eq 7, $u_B < l_{AMP}k_{on}c_A$, is well satisfied. We hypothesize that at sufficiently high currents there would be a decrease in labeling efficiency as the advection time decreases. However, the highest current presented corresponds to the maximum voltage (2200 V) possible in our experimental setup, and we were unable to experimentally observe this decrease. Within the range of currents tested, the highest current is thus optimal, as it provides the highest flow rate, without reducing efficiency. Figure 5B presents experimental results of labeling efficiency as a function of the AMPs concentration in the well, for a fixed current of 3 μA. Consistent with theory, labeling efficiency increases with AMPs concentration. We conclude that the optimal concentration is 0.1 μM, as beyond this value significant precipitation of AMPs was observed, with no significant gain in signal (applying a t test analysis, the calculated t-value between the labeling efficiency at 0.1 and 1 μM is 1.12, whereas the critical t-value for 95% confidence is 2.31), suggesting no significant difference in labeling efficiency.

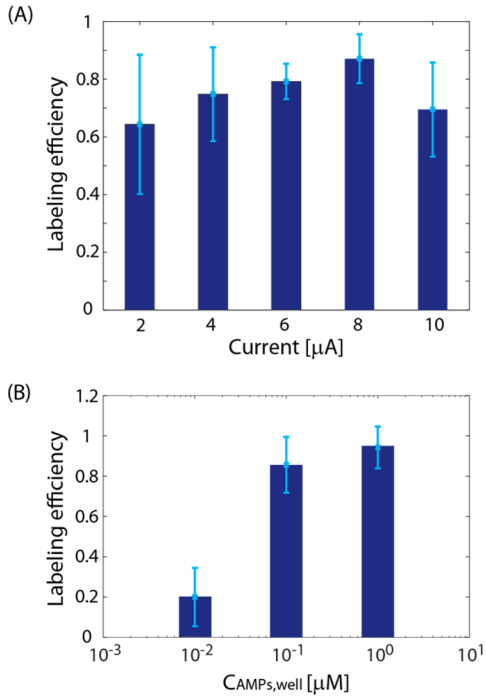

Figure 5. Experimental results for characterization of labeling efficiency. (A) Labeling efficiency as a function of applied current. Each measurement corresponds to analysis of pairs of FITC and TRITC images, acquired in 10 predetermined stations (total set of 20 images). Labeling efficiency was defined as the ratio between the number of detected bacteria in the TRITC images and the FITC images. For bacteria concentration in the sample, c_B, equal to 10^8 cfu/mL, the number of total detected bacteria in 10 stations ranged between 5 and 86 bacteria. The height of each bar represents the average of at least 3 realizations (5 repeats for 2.4 μA; 6 repeats for 6 μA; 3 repeats for 8 μA; 3 repeats for 10 μA), with the range of the bars representing 95% confidence of the mean. The mean labeling efficiency of the assay is ~75% regardless of the applied current value as was supported by t test statistical analysis which showed no significant difference (at 95% confidence) between the labeling efficiency at different electric current values. (B) Dependence of bacteria labeling on AMPs concentration. The results are shown as a function of the initial AMPs concentration in the reservoir. Constant current of 3 μA was applied on the channel. The height of each bar represents the average of 5 realizations for each concentration, with the range of the bars representing 95% confidence of the mean. Higher concentration results in better labeling, with no significant improvement beyond 0.1 μM as was demonstrated by t test statistical analysis.

CONCLUSIONS AND FUTURE WORK

We developed a novel microfluidic assay for continuous, real-time, and quantitative detection of bacteria in water. Our assay leverages cationic ITP to focus antimicrobial peptide probes in a confined region of the channel, through which bacteria can flow freely. This enables continuous labeling, separation, and detection of bacteria in a simple microchannel, without requiring any human intervention between steps.

Previous methods which have applied ITP for whole bacteria detection used it as a mechanism to focus and concentrate the bacteria to facilitate highly sensitive detection. Oukacine et al.[29] used simultaneous electokinetic and hydrodynamic injection with UV detection and thus required no labeling. They have demonstrated the method both on tap water and river water samples. Prior to injection, sample was filtered and isolated from the original water matrix and then resuspended in a low conductivity electrolyte which was then used in the analysis. This method provided a limit of detection of 2×10^4 cfu/mL. Another approach was explored by Phung et al.[17] in order to improve the sensitivity of detection. Their assay involves a

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prelabeling step in which the sample was incubated with SYTO9 dye (a cell permeable nucleic acid stain) for approximately 30 min. This was followed by ITP focusing of bacteria from the sample and fluorescence detection of the formed peak. The assay was performed in a standard capillary electrophoresis apparatus and achieved an excellent limit of detection of 135 cfu/mL. The authors have also demonstrated the detection of bacteria at a concentration of $\sim 10^4$ cfu/mL from contaminated river water samples, after filtering the sample to remove particulates.

The main advantage of these techniques is in their ability to provide relevant limits of detection, in a relatively short time, and using existing commercial equipment. However, both methods cannot perform continuous analysis and require sample preparation to obtain these limits of detction. The method we presented here serves to demonstrate a different working point, in which sample is analyzed continuously, an important capability for water quality monitoring. Within the scope of this work we have demonstrated, using standard commercially available microfluidic chips, the assay's ability to provide quantitative measurements, and its stability over 1 h of continuous monitoring.

While Phung et al.[17] and Oukacine et al.[29] analyzed river water, we tested only tap water (spiked with bacteria) which is likely cleaner. However, we did not perform any sample processing whatsoever and used the raw samples directly on chip. Further improvements to the sensitivity of our assay are clearly required in order to achieve the desired sensitivity (10^2–10^4 cfu/mL[30]) at a reasonable time. Since in our assay sensitivity is governed by the incoming flow rate of the sample (as discussed in the theory section), improved sensitivity can be achieved by working in larger channel dimensions. Our microfluidic channel (which is a standard commercially available design) was 90 μm wide and 20 μm deep and provided a LOD of approximately 10^4 cfu/mL over a 60 min detection window. A set of 100 such parallel channels would have a total width of only 1 cm and would enable a LOD of approximately 10^2 cfu/mL over a 1 h window. Furthermore, increased flow rate can be achieved by maximizing the applied electric field on the channel and thus the flow velocity. Our labeling efficiency experiments revealed that at least a 10-fold increase in the applied current is possible without affecting the labeling efficiency and impairing detection. Hence, designing a dedicated microfluidic chip, using the same principles presented here, may allow one to detect a concentration as low as 10^2 cfu/mL in minutes.

Higher throughput could also be achieved by using a larger diameter channel or capillary, but as temperature due to joule heat scales with the diameter,[31] this would lead to excessive heating and require specialized cooling. In contrast, the use of multiple parallel channels in a planar format maintains the depth of the channel, and thus, temperature is expected to remain essentially unchanged. Higher throughput and longer analysis time would also require scaling the size of the reservoirs to avoid pH changes due to hydrolysis. As detailed by Persat et al.,[32] operating the assay at 200 μA with a 100 mM LE, without exceeding a pH change of 0.2 in the reservoirs, would require a reservoir volume of 1.2 mL for 10 h of operation. This is a sufficiently small volume to be easily integrated with a microfluidic system.

In this work, we demonstrated the assay using AMPs, which are relevant for detecting bacteria but do not provide information on the bacterial strain or species. Nonspecific detection of bacteria is useful in water monitoring as an early alert step and a first warning sign but still cannot be compared to the advantages of specific detection. Therefore, in the future, we intend to adapt the technique to work with specific antibodies as probes. Many bacteria species are negatively charged over a wide range of pH.[28] Thus, to achieve this, one would need to design conditions in which the antibodies are positively charged and remain active, and their effective mobility is bracketed between those of the leading and trailing electrolytes. Most antibodies produced in mammals have pI values between 6.1 and 8.5.[33] Thus, by designing a cationic ITP buffer system at a pH < 6, it may be possible to focus the majority of antibodies and apply our assay for detection of specific bacteria strains or species.

The method demonstrated here also has an inherent capability to operate with the use of only very small volumes of reagents due to the ITP focusing. The typical reagent amounts used in other AMP-based methods for bacteria detection ranges between 450[19] and 2500 ng.[34] In contrast, in our assay, we were able to use only 4.6 ng of AMPs in the reservoir. This amount can be even further reduced by allowing more focusing of the AMPs at the ITP interface during the formation of the labeling zone.

To conclude, the assay we developed and demonstrated has the potential to enable continuous monitoring of water at the point-of-need (e.g., water source, water treatment facilities, municipal networks, and even consumers), relieving the dependence on clinically trained personnel and eliminating the need to transport samples to a centralized lab. The use of a microfluidic platform, as well as the significant focusing of labeling probes by ITP, results in a significant reduction in the amount of expensive reagents required for detection and enables online continuous monitoring which is not possible in other applications. We believe that the assay may also be applicable for pathogen detection in food safety and medical diagnostics applications, where rapid pathogen detection is also crucial.

■ ASSOCIATED CONTENT

Supporting Information

Further details on the image analysis process; the electric current trace of a 1 h operation of the assay, for a complete picture of the conditions during the assay's operation; the applicability of the assay to a variety (Gram-negative and Gram-positive) of bacteria species; a movie demonstrating the principle of the assay and real-time capturing of its operation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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