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TECHNICAL INNOVATION

Immunosensor towards low-cost, rapid diagnosis of tuberculosis†

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A rapid, accurate tuberculosis diagnostic tool that is compatible with the needs of tuberculosis-endemic settings is a long-sought goal. An immunofluorescence microtip sensor is described that detects *Mycobacterium tuberculosis* complex cells in sputum in 25 minutes. Concentration mechanisms based on flow circulation and electric field are combined at different scales to concentrate target bacteria in 1 mL samples onto the surfaces of microscale tips. Specificity is conferred by genus-specific antibodies on the microtip surface. Immunofluorescence is then used to detect the captured cells on the microtip. The detection limit in sputum is 200 CFU mL^{-1} with a success rate of 96%, which is comparable to PCR.

Introduction

Pulmonary tuberculosis (TB), one of the world's most significant infectious diseases, claims over 1 million lives each year worldwide.[1] The gold-standard diagnostic method is bacteriological cultivation of *M. tuberculosis* (MTB) cells from pulmonary specimens *i.e.* sputum. This method requires extensive laboratory infrastructure and up to 1 month to yield results, which makes it impractical for use in most of the world. The most widely used diagnostic method is smear microscopy, which shows limited sensitivity (approximately 60% relative to culture in some studies).[2] Recently, a semi-automated DNA amplification system, the Cepheid GeneXpert, was shown to diagnose TB in 2 hours with 98% reliability.[3] However, the system is expensive to both set up and run, and has a high electric power requirement. Moreover, 2 hours is too long for "real time" patient care. There is a need for further improvement in TB diagnosis, especially for use in resource-limited settings.

A major technical challenge is in detecting low concentrations of MTB cells in viscous sputum samples. Various microfluidic and electrokinetic approaches[4–7] have been tested for detecting bacteria and biomolecules in samples. However, the methods have not been applied to viscous, complex sputum samples.

In our previous work, microtip or nanotip sensors were used to concentrate target bacteria and DNA by using an alternating current (AC) electric field.[8–10] However, the approach was not specific to target analytes and the small sample volume (<10 μL) limited sensitivity. This paper describes a microtip-based immunosensor that overcomes these limitations. The diagnostic platform specifically detects *Mycobacterium* cells by a combination of electric field, streaming flow, and immuno-affinity binding. The novel concentration mechanism enables rapid detection of target bacteria in sputum samples without requiring bacteriological culture, centrifugation, or nucleic acid amplification. This platform enables fast, inexpensive diagnosis of infectious diseases with low cost equipment.

Device operation

The microtip immunosensor (Fig. 1a) is designed to handle a processed sputum sample of 1 mL, yielding a 'Yes or No' answer. The prototype device (Fig. 1a) is composed of a linear motor to control the dipping and withdrawal of microtips (Fig. 1b) and a 1 mL aluminium well that is vibrated to generate a streaming flow. A function generator (Agilent 33220A, Santa Clara, CA) was used to apply an AC electric field (20 Vpp at 5 MHz). A micromachined chip composed of 5 microtips (Fig. 1b) was coated with polyclonal IgY antibodies raised against *M. tuberculosis* complex (*M. bovis* BCG) cells (ESI†, Fig. S2).

The test was conducted by three simple steps (Fig. 2): (1) sputum was processed to reduce the viscosity in a simple 15 minute procedure; (2) target bacteria in the 1 mL processed sample (containing 0.3 mL of the original sputum sample) were specifically concentrated onto a microtip by a combination of convective flows, AC field, and affinity binding for a total of 3 minutes; and (3) after binding fluorescein-labeled antibodies, the fluorescence signal was processed for 7 minutes. In total, 25

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† Electronic supplementary information (ESI) available: Fabrication procedure of microtips, functionalization method of microtips, streaming flow observation, EHD flow and dielectrophoresis, and ELISA test for anti-BCG IgY. See DOI: 10.1039/c2lc21131a

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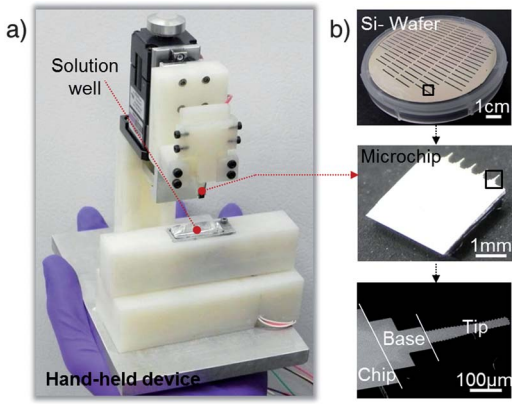

Fig. 1 (a) A prototype device composed of microtips and an aluminium well. (b) A silicon chip consists of 5 microtips that are divided into three regions, namely chip, base, and tip. A total of 340 chips are fabricated on a 100 mm diameter wafer (ESI†, Fig. S1 and S2).

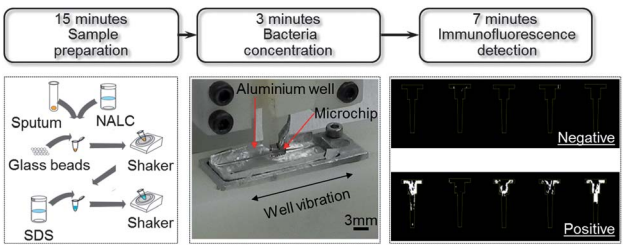

Fig. 2 Detection procedure for 25 minutes. (1) Liquefaction of sputum for 15 minutes, (2) concentration of target bacterial for 3 minutes and (3) binding of fluorescein-labeled antibodies and image processing for 7 minutes.

minutes were required for a single assay, from a sample cup to a numerical result. This timeframe is rapid enough to enable single-visit TB diagnosis in diverse patient care settings.

For rapid concentration of target bacteria, streaming flow, electrohydrodynamic (EHD) flow, and dielectrophoresis were combined (Fig. 3). The bacteria in the 1 mL sample well were transported within 1 mm distance of the microtips by streaming flow. The steady streaming flow was induced by longitudinal vibration of the well with an amplitude of 45 μm at 31.3 Hz. Within this 1 mm distance, the average flow speed was reduced to 100–200 μm s^{-1} based on our observation (ESI†, Fig. S3). The transported bacterial cells were delivered to the vicinity of the microtip surface by EHD flow. The EHD flow could be generated by both electroosmotic- and electrothermal flows (ESI†, Fig. S4). The EHD flow speed was 900 μm s^{-1} at maximum at the microtip end and decreased towards the base- and chip regions of the microtip. The delivered cells were further attracted to the microtip surface by dielectrophoresis. In our observations, the dielectrophoretic force attracted the bacteria onto the microtip surface out of the streamlines of the EHD flow (ESI†, Fig. S5).

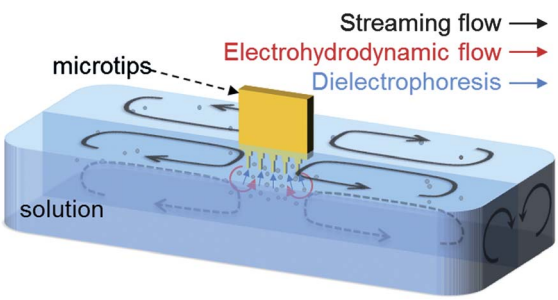

Fig. 3 Concentration mechanism of target bacteria in 1 mL sputum samples using streaming flow, EHD flow, and dielectrophoresis.

Experimental method

For the effective concentration, streaming flow, electric field, and tip withdrawal rate were optimized (ESI†). To study the contribution of streaming flow and an electric field to the concentration of target bacteria, the functionalized microtips were used in combination with *M. bovis* BCG cells at a concentration of 10^7 CFU mL^{-1} in 1× PBS. For counting individual cells, BCG cells were stained with an intercalating dye (SYTO 9® green fluorescent nucleic acid stain; Molecular Probes L7007, Invitrogen), visualized by an epi-fluorescence microscope (Olympus BX-41, Olympus America Inc., Melville, NY), and quantified. A 1 mL sample solution containing stained BCG cells was loaded on the aluminium well. For concentration, the microtips were immersed in the 1 mL sample for 2 minutes. For comparison, four cases involving on–off combinations of vibration and electric field (20 Vpp at 5 MHz) were tested. After 2 minute concentration, the number of captured BCG cells was counted on the microtips surface for each case.

To test the sensitivity of microtips, various concentrations of BCG cells (10^1 to 10^6 CFU mL^{-1}) were suspended in PBS buffer. A 1 mL solution of the prepared bacteria sample was loaded in the aluminium well. The microtips were immersed in 1 mL of a bacterial suspension for 2 minutes with vibration and an applied AC field (20 Vpp at 5 MHz). After 2 minutes of immersion, the microtips were withdrawn at a rate of 100 μm s^{-1}. Subsequently, the microtips were immersed in a prepared fluorescein conjugated antibody solution (10 μL, 2 mg mL^{-1}) for 5 min and then rinsed with deionized water (200 μL) for 5 seconds. The rinsed microtips were observed under an epi-fluorescence microscope. The raw fluorescence image was digitized into a white and black image based on the threshold intensity. The threshold value was determined in order to suppress most negative control signals. The experiment was repeated 4 times for each concentration. As a control, 1× PBS buffer (conductivity: $\sigma = 1.58$ S m^{-1}) without bacteria cells was used.

To test the specificity of the microtip sensor, various bacteria were spiked into 1× PBS buffer. Suspensions of BCG (concentration: 10^2 CFU mL^{-1}), *Escherichia coli* (*E. coli*, 10^4 CFU mL^{-1}), and *Staphylococcus epidermidis* (*S. epidermidis*, 10^4 CFU mL^{-1}) were prepared in 1× PBS buffer. The experimental procedure was the same as the sensitivity test using BCG. For verification, 3 experimental runs were conducted for each case.

For a preliminary test of sputum samples spiked with BCG, cell densities of 2×10^2 to 2×10^6 CFU mL^{-1} were used in 10 fold increments. The images of individual microtips were digitized and compared at each concentration. In addition, a total of 11 tests were conducted as 6 negative sputum controls and 5 sputum samples spiked with BCG of 2×10^2 CFU mL^{-1}. Sputum samples from people not known to have TB were purchased from

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BioReclamation, Inc. Sputum spiked with BCG (total 600 μL) was mixed with 4 mg mL^{-1} *N*-acetyl-L-cysteine (NALC; 300 μL, $\sigma = 0.12$ S m^{-1}) and 3 mm glass beads. The sample was vortexed for 5 minutes. 4% sodium dodecyl sulfate (SDS; 300 μL, $\sigma = 0.37$ S m^{-1}) was added to the mixture. Additional vortexing for 5 minutes was conducted for complete liquefaction. Liquefaction of sputum was tested by turbidity (ESI†). As a result of this process, the final concentration of BCG in the processed sputum was half of the initial concentration.

Out of a total volume of 1.2 mL, 1 mL was transferred to an aluminium well without glass beads. Microtips were dipped into the sample with vibration and an applied AC field (20 Vpp at 5 MHz) for a 2 minute concentration step. Subsequently, the microtips were withdrawn at a rate of 100 μm s^{-1}. The microtips were immersed in a fluorescein conjugated antibody solution (10 μL, 2 mg mL^{-1}) for 5 minutes and then rinsed with deionized water (200 μL) for 5 seconds. The fluorescence intensity was measured by an epi-fluorescence microscope, which was digitized for numerical results.

For a dose–response test using MTB (H37Ra) in sputum samples, the concentrations of spiked MTB were 2×10^1, 2×10^2, 2×10^3, and 2×10^4 CFU mL^{-1}. Testing followed the same procedure as the BCG test in sputum. The experiment was repeated 4 times for each concentration. In order to assess the reproducibility of the microtip sensor when challenged with diverse spiked sputum samples, an additional 30 sputum samples (6 negative controls and 24 distinct samples spiked with MTB at 200 CFU mL^{-1}) were tested.

Results and discussion

To study the relative contributions of vibration-induced flow and electric field to concentration of bacteria, four cases involving on–off combinations of vibration and electric field were tested. When both vibration and electric field were activated, the number of captured cells was greatest (Fig. 4a). When either vibration or electric field was applied alone, fewer cells were captured.

When the microtips were tested for capture and detection of various concentrations of BCG cells in a PBS buffer, the sensitivity was 10 CFU mL^{-1} (Fig. 4b). Overall, the fluorescence intensity was enhanced with increasing cell concentration but the signal was not quantitative. In repetitive measurements, the signals usually separated into three regions ranging from the low level of the negative control, to the intermediate level at 10 to 10^4 CFU mL^{-1}, and to the higher level at 10^5 to 10^6 CFU mL^{-1}.

The anti-BCG polyclonal IgY was used for both detection as well as capture. Its specificity was evaluated by using an ELISA test. Compared with the response for *S. epidermidis* and *E. coli*, the antibodies bound more avidly to BCG (ESI†, Fig. S6). In a specificity test using the immunofluorescence microtip sensor, the BCG signal significantly exceeded the signals of *E. coli*, and *S. epidermidis* whose concentrations were greater by two orders of magnitude than that of BCG (Fig. 4c).

In a preliminary test using human sputum spiked with BCG, the white spots indicated captured *M. tuberculosis* complex cells on the microtips (Fig. 5a). The bacteria were observed in greater numbers at the base regions of the microtips. Interestingly, the cells were detected at the terminal end of the microtip only when the initial concentration was greater than 10^5 CFU mL^{-1}. In this test, the signal was discernible down to 200 CFU mL^{-1}. At this concentration, a total of 11 tests were conducted. The signals generated by spiked samples were clearly differentiated from the negative controls (Fig. 5b).

In the dose–response test using MTB H37Ra spiked into sputum, the detection limit was 200 CFU mL^{-1} (Fig. 5c). In order to assess the reproducibility of the microtip sensor when challenged with diverse spiked sputum samples, an additional 30 samples (6 negative controls and 24 distinct samples spiked with H37Ra at 200 CFU mL^{-1}) were tested. Relative to the 6 negative controls, 23 out of 24 positive samples showed positive results, corresponding to a 96% success rate. The samples in Fig. 5d varied in terms of viscosity, opacity, and color, suggesting that the method is robust and can be applied to diverse sputum samples (ESI†). The detection limit of 200 CFU mL^{-1} is equivalent to PCR amplification-based methods applied to sputum samples following similar protocols.[11] Given that sputum samples are replete with human cells, bacteria, and other microorganisms, these results also demonstrate the specificity of the microtip sensor.

The specificity of this method is conferred in part by the polyclonal IgY antibody. The antibody has not yet been tested for cross-reactivity to non-tuberculous *Mycobacterium* (NTM) species that frequently occur in respiratory samples from symptomatic patients. Given the close antigen relatedness between MTB and NTM, cross-reactivity is likely. However, in the resource-limited countries for which the microtip technology is designed, NTM-related disease remains relatively rare. For

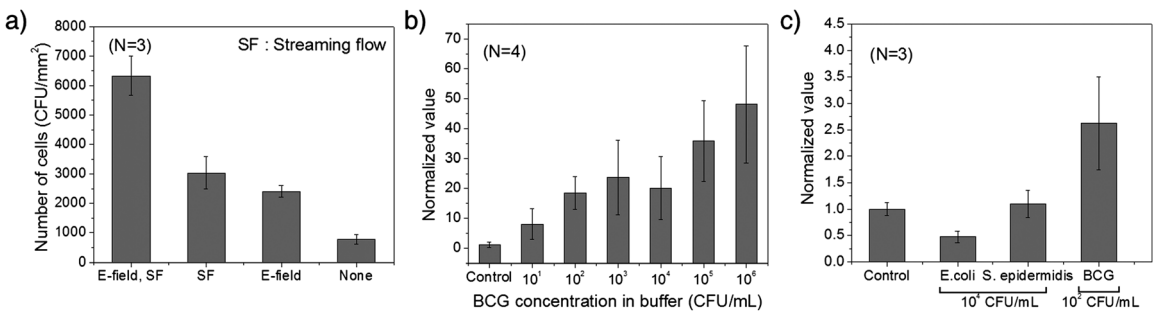

Fig. 4 (a) Effect of each concentration mechanism. Values are number of captured cells per microtip. (b) Sensitivity test for BCG cells in PBS buffer by using the immunofluorescence microtip sensor. (c) Specificity test for *E. coli*, *S. epidermidis*, and *M. bovis* (BCG) solutions. The concentration of *E. coli* and *S. epidermidis* is 10^4 CFU mL^{-1} while that of BCG is 10^2 CFU mL^{-1}.

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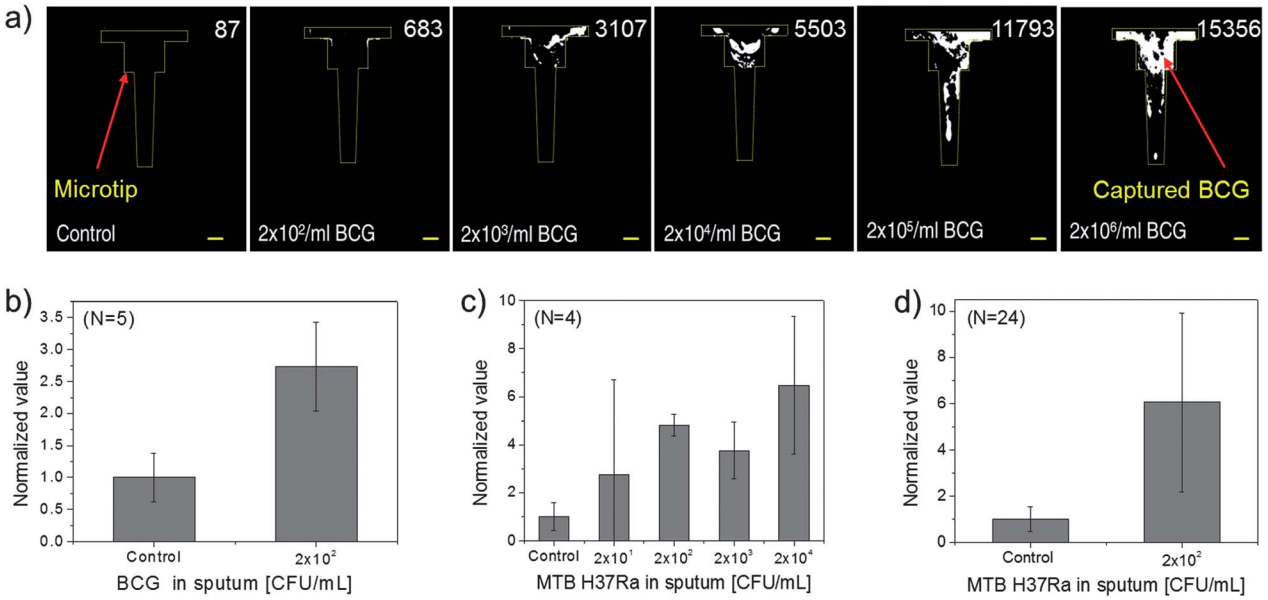

Fig. 5 Test results for detection limit of the microtip immunosensor for sputum. (a) Digitized images from sputum samples spiked with BCG. The concentrations of BCG and the average numbers of white pixels are shown at the bottom and top in the images, respectively. The scale bar is 50 μm. (b) Immunofluorescence detection of BCG at 200 CFU mL^{-1}. (c) Dose–response for MTB spiked in sputum samples. At an MTB concentration of 20 CFU mL^{-1}, 3 positive signals out of 4 samples are higher than the negative controls. (d) Reproducibility test at an MTB concentration of 200 CFU mL^{-1} in sputum. Compared with 6 negative controls, 23 out of 24 samples show positive signals. The total success rate is 96%.

example, in a recent large study conducted on 1730 suspected TB cases in Peru, Azerbaijan, South Africa, and India, fewer than 3% of confirmed *Mycobacterium*-positive cases had NTM disease.[3]

Conclusions

In summary, the immunofluorescence microtip sensor specifically detected *Mycobacterium* cells by the combination of electric field, streaming flow, and immuno-affinity binding. The detection limit was 200 CFU mL^{-1} in human sputum, which was comparable to PCR but without requiring bacteriological culture, centrifugation, or nucleic acid amplification. In spite of the complex nature of physical, chemical, and biological mechanisms, the simple operation of "dipping and withdrawal" of tips will allow for screening by minimally trained personnel within 25 minutes. In addition, the minimal power requirement (5 W) combined with low assay cost is ideal for point-of-care (POC) screening in limited resource settings. With rapid advancement of low-cost optical microscope technology,[12] and with further development of high-throughput manufacturing, stabilization, and quality control protocols, this technology should enable fast, inexpensive diagnosis of infectious diseases with low cost (<$1000) equipment.

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