

# A multifunctional micro-fluidic system for dielectrophoretic concentration coupled with immuno-capture of low numbers of *Listeria monocytogenes*†

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In this study, we demonstrated a micro-fluidic system with multiple functions, including concentration of bacteria using dielectrophoresis (DEP) and selective capture using antibody recognition, resulting in a high capture efficiency of bacterial cells. The device consisted of an array of oxide covered interdigitated electrodes on a flat silicon substrate and a  $\sim 16 \mu\text{m}$  high and  $\sim 260 \mu\text{m}$  wide micro-channel within a PDMS cover. For selective capture of *Listeria monocytogenes* from the samples, the channel surface was functionalized with a biotinylated BSA–streptavidin–biotinylated monoclonal antibody sandwich structure. Positive DEP (at  $20 V_{pp}$  and 1 MHz) was used to concentrate bacterial cells from the fluid flow. DEP could collect  $\sim 90\%$  of the cells in a continuous flow at a flow rate of  $0.2 \mu\text{l min}^{-1}$  into the micro-channel with concentration factors between  $10^2$ – $10^3$ , in sample volumes of 5–20  $\mu\text{l}$ . A high flow rate of  $0.6 \mu\text{l min}^{-1}$  reduced the DEP capture efficiency to  $\sim 65\%$ . Positive DEP attracts cells to the edges of the electrodes where the field gradient is the highest. Cells concentrated by DEP were captured by the antibodies immobilized on the channel surface with efficiencies of 18 to 27% with bacterial cell numbers ranging from  $10^1$  to  $10^3$  cells. It was found that DEP operation in our experiments did not cause any irreversible damage to bacterial cells in terms of cell viability. In addition, increased antigen expression (antigens to C11E9 monoclonal antibody) on cell membranes was observed following the exposure to DEP.

## Introduction

Foodborne disease has been a serious threat to public health for decades and remains a major public health challenge.<sup>1</sup> *Listeria monocytogenes* has emerged as one of the most hazardous, potentially life-threatening, human foodborne pathogens. The conventional microbiological methods for detection of *L. monocytogenes* based on culture enrichment and plate count techniques are generally time consuming and labor intensive, usually requiring 3–7 days for a presumptive result. As a result, over the past several years, a variety of rapid methods have been investigated for detecting *Listeria*, such as typical or derived immunological assays,<sup>2–4</sup> nucleic acid-based tests,<sup>5–7</sup> and

physicochemical tests based on bacterial growth.<sup>8–10</sup> Many of these rapid methods have made significant progress towards reducing the total assay time. However, when detecting a low number of bacterial cells, pre-enrichment is usually required to obtain a detectable level of bacterial concentration for applications of these methods. The time required for an increase in bacterial concentration solely relying on bacterial growth still causes delay in the overall detection time.

Recent developments have shown the great potential of dielectrophoresis (DEP) as a new technique for microbial concentration and separation. DEP is the electrokinetic motion of dielectrically polarized particles in non-uniform electric fields.<sup>11</sup> Since most biological cells behave as dielectric particles in an external electric field, the generated DEP fields allow the manipulation of the biological cells in a liquid suspension. DEP has been employed for the selective separation of viable and unviable yeast,<sup>12,13</sup> bacterial cells,<sup>14–16</sup> cancer cells,<sup>17,18</sup> viruses,<sup>19</sup> and CD34+ stem cells.<sup>20</sup>

Particularly, recent progress in the development of micro-electrode structures has made DEP a very useful technique for manipulation of biological cells in micro-devices.<sup>21</sup> The ability to fabricate micro- and/or nano-structures with scales and dimensions similar to biological entities has paved the way for new concepts and systems for handling, detecting, and characterizing a few cells or even at a single cell level. Micro-devices are also ideally suited for reducing the time-to-result, to be able to perform ‘point-of-use’ analysis and reducing sample volume. Hu *et al.* developed a cell sorting system using

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DEP in micro-fluidic channels, which can efficiently isolate rare cells from complex mixtures.<sup>22</sup> Our recent studies have demonstrated that DEP is a very valuable technique for manipulation of foodborne pathogenic bacterial cells in micro-fluidic devices. We have successfully used DEP to concentrate live bacterial cells into an extremely small volume of 400 pl in a micro-device, followed by impedance measurements of bacterial metabolic activity.<sup>23</sup> Such a concentration step effectively increases the cell concentration close to the impedance electrodes without increasing the number of cells, thus eliminating the need to amplify the bacterial population by long culture enrichment, and reducing the total assay time. In addition, we have successfully achieved the separation of live and heat-killed *Listeria* bacteria on microfabricated interdigitated electrodes in a static solution,<sup>24</sup> as well as different biological particles in the dynamic flow.<sup>25</sup>

The DEP technique for cell separation requires a distinctive difference in the dielectric properties between target cells and non-target cells. It has been found that using DEP differential affinity alone, it is impractical to separate particles having less than a 50% difference in their crossover frequencies (where DEP force is zero).<sup>26</sup> In many cases the dielectric properties and the sizes of the different cell types are not significantly different, for example several types of bacteria, such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Enterobacter aerogenes*, and *Enterococcus faecalis* CG110. As a result, DEP is not capable of separating these bacteria species, as we observed in our experiments. Therefore, introducing a selective reagent to the DEP separation system is necessary to improve the selectivity between similar bacterial cells for the specific capture of target species.

In this study, we demonstrated a novel micro-fluidic system which utilized DEP as a concentration tool and we coupled the DEP with antibody recognition to realize the selective capture of target bacteria. This system offered advantages inherited from both DEP and antibody recognition. First, this system would concentrate all bacterial cells from a fluid into a small volume channel by a factor of  $\sim 10^3$  or more using DEP. The concentration factor can be much higher and is a function of the volume of starting sample and the time required to flow the sample. Second, the antibodies immobilized on the insulator over the DEP electrode surface would provide the selectivity by capturing only the target bacterial cells in the channel, whereas non-target bacterial cells would be flushed out by the flow when DEP is turned off. This is particularly useful in the cases where dielectric properties of target bacteria are not distinctively different from those of other bacteria in the mixture. Third, greatly increased antibody capture efficiency can be achieved in a continuous flow system, due to the effectively increased cell concentration in the channel, as well as the fact that the DEP force attracts the bacterial cells and allows them to interact and make good contact with immobilized antibodies on the channel surface. This design is particularly suited for micro-fluidic systems involving antibody-antigen reactions to achieve high capture efficiency and good selectivity. In this study, the bacterial cultures of *Listeria monocytogenes*, *Enterobacter aerogenes*, and *Enterococcus faecalis* were used as examples. The micro-fluidic system

consisted of an array of interdigitated microelectrodes on a flat oxidized silicon substrate and a channel right above the array of microelectrodes, which was formed in a polydimethylsiloxane (PDMS) cover. Anti-*Listeria* monoclonal antibody molecules were immobilized onto the surfaces of the interdigitated electrodes to selectively capture *Listeria* cells. Positive DEP was used to collect bacterial cells onto the electrode surfaces and hold these collected cells for a certain time period so that cells had sufficient time to react with antibody molecules. When DEP was turned off, only antibody-specific bacteria would be bound to the immobilized antibody and retained in the channel.

## Materials and methods

### Micro-fluidic device design, fabrication and assembly

The schematic design of the micro-fluidic biochip used in this study is presented in Fig. 1. The device consisted of an array of interdigitated microelectrodes on a flat oxidized silicon substrate and a channel right above the array of microelectrodes which was formed in a PDMS cover. The array of interdigitated electrodes (Ti/Pt of 200/800Å) was constructed using sputtering and lift-off processing. The electrodes were 23  $\mu\text{m}$  wide with a spacing of 17  $\mu\text{m}$ . The whole electrode array (including the electrode, edge and space areas) was covered with a silicon oxide layer ( $\sim 0.3 \mu\text{m}$ ) using plasma-enhanced chemical-vapor deposition (PECVD), to prevent electroosmotic currents at the electrodes.<sup>24</sup>

The mold for the PDMS cover was made by patterning 16  $\mu\text{m}$  SU-8 25 photoresist (MicroChem Corporation, Newton, MA, USA) on a silicon wafer. PDMS solution, a 10 : 1 (w/w) ratio of elastomer monomer/curing agent (Sylgard 184 Silicone Elastomer, Dow Corning Corporation, Midland, MI, USA) was thoroughly mixed, allowed to cure for 1 h at room temperature, poured into a petri dish with the mold on the bottom, and finally cured at 75°C overnight. PDMS layers were then removed from their mold, and two holes were punched at the two ends of each channel with a needle. The channel on the PDMS was  $\sim 260 \mu\text{m}$  wide and  $\sim 16 \mu\text{m}$  deep. Considering its effective length of  $\sim 3000 \mu\text{m}$ , the volume of the channel was  $\sim 12.5 \text{ nl}$ .

Both the silicon chip and the PDMS cover were treated with oxygen plasma ( $p\text{O}_2 = 0.2 \text{ Torr}$ ) at 200 W for 10 s. They were aligned immediately and allowed to cure for 2 h at room temperature. Microbore tubes (OD of 0.016", ID of 0.004", Cole-Parmer Instrument, Vernon Hills, IL, USA) were inserted into the two holes on the PDMS cover for injection and to drain the liquids through the channel.

### Antibody immobilization in the DEP channel

Biotinylated bovine serum albumin (BSA), with 8 mol of biotin for each mole of BSA, and streptavidin were purchased from Pierce (Rockford, IL, USA). Phosphate-buffered saline (PBS) buffer (pH 7.4) was obtained from a reagent package purchased from Sigma-Aldrich (St. Louis, MO, USA), and the pH value was adjusted to be 4–5 by adding 1 M HCl when needed. Anti-*L. monocytogenes* monoclonal antibody (MAb) C11E9 (which belongs to the IgG2b subclass and binds to a

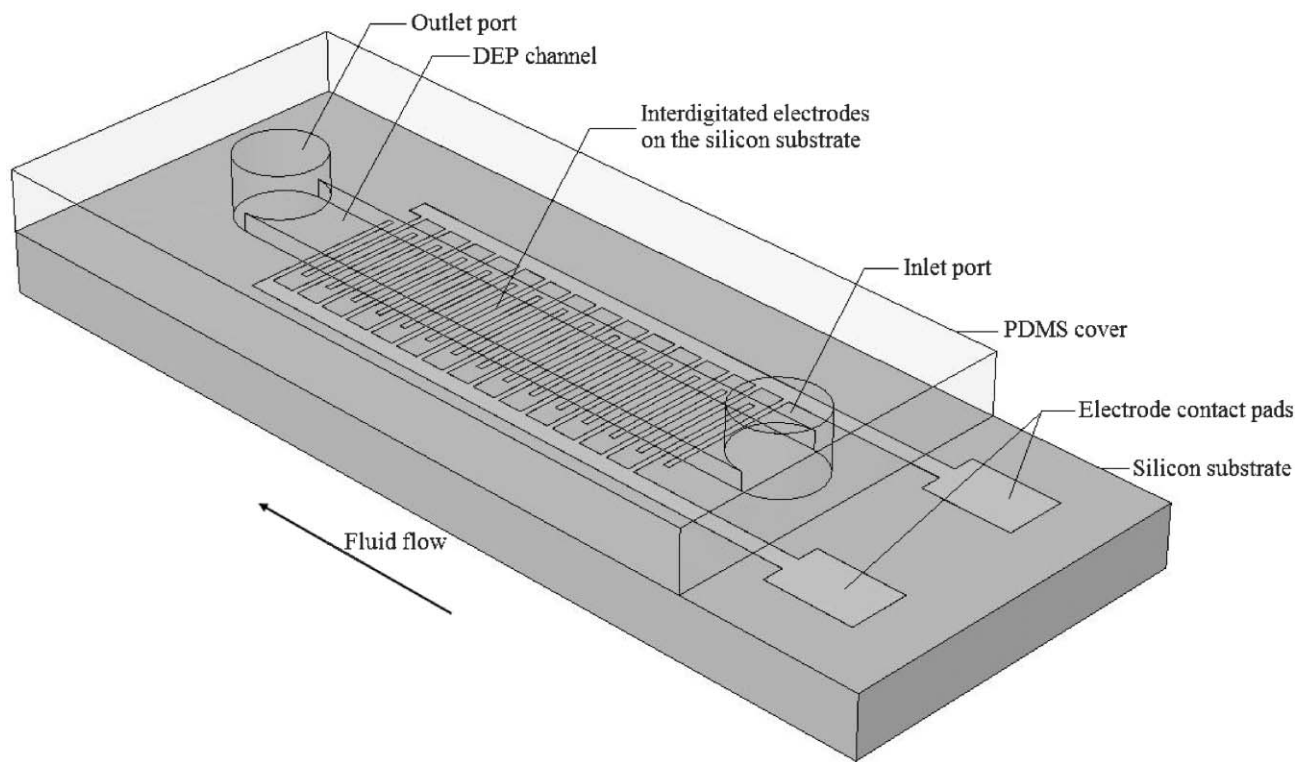


Fig. 1 Schematic of the micro-fluidic biochip used in this study.

66 kDa surface protein on *L. monocytogenes*), produced in the laboratory of one of the coauthors (A. Bhunia) in the Food Science Department at Purdue University, were used for the experiments.<sup>27</sup> MAb C11E9 was conjugated with biotin using biotin labeling reagents following the procedure described by the supplier (product# 21336, Pierce Biotechnology, Inc., Rockford, IL, USA). Based on the instructions of the product, NHS-activated biotins react with primary amine groups ( $-NH_2$ ) to form amide bonds which links the biotin to the antibody.

Immobilization of antibody on the  $SiO_2$  surface of the DEP channel was carried out by using biotin and streptavidin chemistry.<sup>28</sup> In the immobilization procedure, the DEP channel was first cleaned with deionized (DI) water at a flow rate of  $0.5 \mu\text{l min}^{-1}$  for 20 min. Biotinylated BSA solution ( $0.5 \text{ mg ml}^{-1}$  in PBS, pH 5) was then injected into the channel at a flow rate of  $0.05 \mu\text{l min}^{-1}$  for 30 min, and the solution was held in the channel for another 2 h. Biotinylated BSA was physically adsorbed directly onto the silicon oxide surface.<sup>28</sup> After washing with PBS (pH 5) at  $0.5 \mu\text{l min}^{-1}$  for 15 min, streptavidin ( $250 \mu\text{g ml}^{-1}$  in PBS, pH 5) was injected into the channel at  $0.05 \mu\text{l min}^{-1}$  for 30 min and incubated for 1 h, allowing streptavidin to react with the biotinylated BSA. Excess streptavidin was washed out with PBS (pH 5). Biotinylated anti-*Listeria monocytogenes* monoclonal antibody (C11E9) solution ( $250 \mu\text{g ml}^{-1}$ ) was injected into the channel at  $0.05 \mu\text{l min}^{-1}$  for 30 min and incubated overnight at room temperature. The binding sites for biotin on streptavidin molecules allowed biotinylated antibodies to anchor. The micro-chip was ready for use after being washed with PBS and DI water.

#### Bacteria cultures and media

*Listeria monocytogenes* V7, *Enterobacter aerogenes* DUP14591, and *Enterococcus faecalis* CG110 cultures were grown in brain heart infusion (BHI) broth at  $37^\circ\text{C}$  for 16–18 h. The cells were pelleted by centrifuging (Eppendorf, Westbury, NY, USA) at  $6,000 \times g$  for 5 min and resuspended in sterilized deionized water. The cell numbers of these cultures were determined by surface plating onto brain heart infusion (BHI) agar (Difco, Sparks, MD, USA). Colonies were counted after incubation of the plates at  $37^\circ\text{C}$  for 24 h. The number of cells usually reached  $10^9$  colony forming units per milliliter ( $\text{cfu ml}^{-1}$ ). *L. monocytogenes* cells were stained with 3,3'-dihexyloxycarbocyanine iodide ( $\text{DiOC}_6(3)$  dye (green), Molecular Probes, Eugene, OR, USA), and *E. aerogenes* and *E. faecalis* cells were stained with BacLight<sup>™</sup> red bacterial stain (Molecular Probes, Eugene, OR, USA). All stained bacteria suspensions were centrifuged and washed with DI water 4–5 times to remove excess dye molecules. Serial dilutions were prepared in DI water for further applications.

#### DEP and immuno-capture of *Listeria* cells in the micro-device

Bacterial cultures were injected into the DEP channels by using a WPI SP200i syringe pump (World Precision Instruments, Inc., Sarasota, FL, USA) and a gas-tight luer-lock syringe (ILS100TLL, World Precision Instruments, Inc.) at a flow rate of  $0.2 \mu\text{l min}^{-1}$ . An Agilent 33120A arbitrary waveform generator (15 MHz) (Agilent Technologies, Inc., Palo Alto, CA, USA) was used as the a.c. signal source to provide a sinusoidal signal with a frequency of 1 MHz and amplitude of 20 V (peak to peak) to the interdigitated electrodes. For

examining the efficiency of DEP collection, each 20  $\mu\text{l}$  of bacterial samples with different concentrations were injected into the DEP channel at the flow rate of 0.2  $\mu\text{l min}^{-1}$  when a 20  $V_{\text{pp}}$  potential at a frequency of 1 MHz was applied to the interdigitated electrodes. The cell concentrations in the samples before injection into the channel and in the elution at the outlet were determined by plating appropriate dilutions onto Oxford plates. The efficiency of DEP collection was calculated using eqn 1.

$$\text{DEP capture efficiency (\%)} = \frac{\text{Cell concentration at inlet} - \text{Cell concentration at outlet}}{\text{Cell concentration at inlet}} \times 100\% \quad (1)$$

In the experiments with DEP coupling with immuno-capture of *Listeria*, each 5  $\mu\text{l}$  of bacterial samples with different cell concentrations were injected into the DEP channel at a flow rate of 0.2  $\mu\text{l min}^{-1}$  when DEP was in operation (20  $V_{\text{pp}}$  at 1 MHz). DEP effectively collected *Listeria* cells into the channel. After the flow was stopped, DEP remained in operation for another 30 min to hold the collected cells in contact with the channel surface. Then DEP was turned off and the flow started again. By this procedure, *Listeria* cells bound to the antibodies were retained in the channel, while unbound cells were washed out by the flow. Cells collected by DEP in the channel and cells bound with antibodies when DEP was off were analyzed by fluorescence imaging.

### Fluorescence imaging

The fluorescence images were taken on a Nikon ECLIPSE E600FN fluorescence microscope (Japan) using the filters FITC (Fluorescein isothiocyanate), TRITC (tetramethylrhodamine isothiocyanate), and a FITC–TRITC–DAPI (4',6-diamidino-2-phenylindole dihydrochloride) triple filter (so both green and red cells can be observed simultaneously) by a CCD camera (Pixera, Los Gatos, CA, USA). The numbers of *L. monocytogenes* cells were estimated by image analysis performed with shareware software ImageJ (Wayne Rasband, National Institute of Health, USA). The capture efficiencies of antibody to *Listeria* cells were estimated by the number of cells collected in the channel when DEP was on and the number of bound cells when DEP was off, using eqn 2.

$$\text{Antibody capture efficiency (\%)} = \frac{\text{Cell number at DEP off}}{\text{Cell number at DEP on}} \times 100\% \quad (2)$$

### Field emission scanning electron microscopy (FESEM)

*Listeria* cells collected in the micro-fluidic chip by DEP were dried in air overnight at room temperature. After removing the PDMS cover, the chip was directly imaged using a Hitachi S 4800 FESEM microscope (Tokyo, Japan) without coating. Acceleration voltage was kept constant at 2.0 kV. Images were acquired digitally using Quartz PCI v.7 software (Hitachi High-Technologies Canada, Inc., Rexdale, Ontario, Canada).

### Indirect ELISA for *Listeria* surface antigen expression

Aliquots of 50  $\mu\text{l}$  ( $10^9$  cfu  $\text{ml}^{-1}$ ) of DEP treated and normal *Listeria* cells were dispensed into the wells of a flat-bottomed 96-well plate (1B immulon, ThermoLabsystems, Milford, MA, USA). Incubated overnight at 4°C and the wells were washed with PBS containing 0.5% Tween 20 (PBST, pH 7.4) to remove unbound cells. Anti-*Listeria* monoclonal antibody C11E9 (0.02 mg  $\text{ml}^{-1}$ ) was added into the wells and incubated for 1 h and washed three times with PBST to remove unbound antibody. The wells were reacted with 100  $\mu\text{l}$  of 1 : 5000 dilution of horse radish peroxidase (HRP)-conjugated anti-mouse antibody (Jackson Immuno Research Laboratories, Westgrove, PA, USA), and developed with a substrate solution containing hydrogen peroxide and O-phenylene diamine (OPD) (Sigma, St. Louis, MO, USA). The reaction was stopped after 10–15 min by adding 100  $\mu\text{l}$  0.1 M HCl. The absorbance was measured at 490 nm using an ELISA reader (Bio-rad, Hercules, CA, USA).

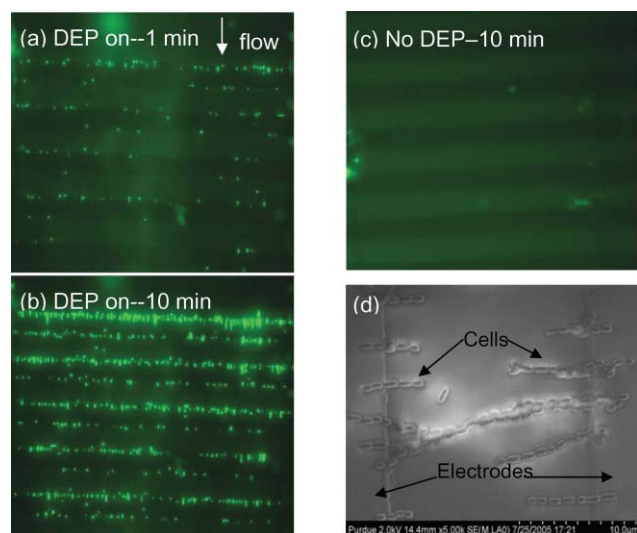
### Transmission electron microscopy (TEM)

For TEM samples, individual TEM grids were exposed to  $\sim 10^9$  cfu  $\text{ml}^{-1}$  of DEP treated and normal *Listeria* cells and were incubated at room temperature for 10–15 min, and the grids were washed with PBST buffer to remove unbound cells. Anti-*Listeria* monoclonal antibody C11E9 (0.02 mg  $\text{ml}^{-1}$ ) was added and incubated for 15 min and washed ten times with PBST to remove unbound antibody. Grids were incubated with 10 nm gold particles (Ted Pella, Inc., Redding, CA, USA) (1 : 50 dilution) for 10–15 min at room temperature. After washing with PBST, the grids were negatively stained with 0.25% uranyl acetate and imaged using a Philips CM-100 TEM (Philips Electron Optics, Eindhoven, The Netherlands) operating at 100 kV, and were captured on Kodak SO-163 film.

## Results and discussion

### DEP concentration of *L. monocytogenes* in the micro-channel

In our experiments, *Listeria* cells were suspended in DI water. The conductivity of the interior of the cell can be as high as 1  $\text{S m}^{-1}$ , whereas the conductivity of the DI water range from a low of about 1–2  $\mu\text{S cm}^{-1}$ , up to about 10–15  $\mu\text{S cm}^{-1}$ . When the sample containing *Listeria* cells was injected into the DEP channel, bacteria cells experienced positive DEP forces and were collected at the electrode edges where the electric field was the strongest. Fig. 2 presents the fluorescence images, taken at (a) 1 min and (b) 10 min, of the sample flow (0.2  $\mu\text{l min}^{-1}$ ) at a concentration of  $10^6$  cfu  $\text{ml}^{-1}$  into the DEP channel with 20  $V_{\text{pp}}$  applied at 1 MHz to the interdigitated electrodes. It can be seen that increasing numbers of *Listeria* cells were trapped at the edges of the interdigitated electrodes with increasing time when DEP was in operation, whereas no cells were visible on the microelectrodes when DEP was not applied to the electrodes, as shown in Fig. 2(c). Comparison of Fig. 2(b) and Fig. 2(c) indicates that collection of *Listeria* cells from a continuous-flow sample stream was successfully achieved by DEP using this microelectrode design, while capture of cells in the fluidic channel by immobilized antibody alone was much less efficient. Fig. 2(d)



**Fig. 2** Fluorescence images taken at, (a) 1 min and (b) 10 min, of the sample flow ( $0.2 \mu\text{l min}^{-1}$ ) at a concentration of  $10^6 \text{ cfu ml}^{-1}$  into the DEP channel with 20 V (peak to peak) applied at 1 MHz to the interdigitated electrodes; (c) no cells were visible on the microelectrodes when DEP was not applied to the electrodes; (d) SEM picture of the DEP collected *Listeria* cells in the micro-fluidic channel.

presents the SEM picture of the DEP collected *Listeria* cells in the micro-fluidic chip, clearly showing that bacterial cells are collected at the edges of the interdigitated electrodes. It also can be seen that the maximum value of the polarization occurs at the two ends of the rod shaped cells and parallel to the electric field. The magnitude of the induced polarization charge is equivalent to  $\sim 0.1\%$  of the net surface charge of a cell and can be generated within one microsecond.<sup>29</sup> If a large number of cells are collected, cells tend to align in rows, usually referred to as 'pearl chains', which bridge the electrodes as shown in Fig. 2(d). This phenomenon has been used for bacterial detection based on the admittance of the microelectrode changes as the number of trapped cells increase and bridge the electrodes.<sup>30</sup>

The collection of cells by DEP can be dramatically rapid, but it is not always highly efficient in practice. It is reported that in microelectrodes, efficiency can be as low as 1–3%, but high efficiency (over 50%) can be achieved depending on the design of the system.<sup>31,32</sup> Theoretically, the DEP collection efficiency is affected by the combination of numerous factors, including dielectric properties of the cell, the medium, operation parameters of the flow and DEP, the design of the electrode configuration, and dimensions of the channel above the electrodes. Detailed analytical expressions for the DEP force, at height greater than the electrode width, was reported by Huang *et al.*<sup>33</sup> and Morgan *et al.*<sup>34</sup> Our recent study

reported the characterization and modeling of the DEP forces in terms of different particle size, horizontal distance, vertical distance using polystyrene beads and biological cells, which provided useful information in designing and operating DEP systems for collection of biological cells.<sup>25,35</sup>

With the practical design of this DEP system, the efficiency of DEP collection of *Listeria* cell from samples was examined. Table 1 shows the results of efficiency of DEP collection for samples with different concentrations and at different flow rates. It can be seen that the efficiency of DEP collection of *Listeria* cells was about 87–92% for samples with cell concentration varying from  $10^5$  to  $10^7 \text{ cfu ml}^{-1}$  at flow rate of  $0.2 \mu\text{l min}^{-1}$  when 20  $V_{pp}$  was applied to the interdigitated electrode at a frequency of 1 MHz, indicating that the collection efficiency is quite stable regardless of the cell concentration in the sample. It was also noticed that there is approximately 5–10% variation in the collection efficiency by using different micro-fluidics chips even at the same operation conditions. The collection efficiency decreased to  $\sim 65\%$  when the flow rate increased from  $0.2 \mu\text{l min}^{-1}$  to  $0.6 \mu\text{l min}^{-1}$  for the sample containing  $2.6 \pm 0.3 \times 10^6 \text{ cfu ml}^{-1}$  of *Listeria* cells. Assuming that the fluid follows a parabolic laminar flow profile, the average velocity of the flow in the micro-channel (the flow rate divided by the cross-sectional area of the channel) is  $800 \mu\text{m s}^{-1}$  at a flow rate of  $0.2 \mu\text{l min}^{-1}$ , and  $2400 \mu\text{m s}^{-1}$  at a flow rate of  $0.6 \mu\text{l min}^{-1}$ . Considering the significant increase in the flow velocity, the bacterial cells experienced higher hydrodynamic drag forces at a flow rate of  $0.6 \mu\text{l min}^{-1}$  than at a flow rate of  $0.2 \mu\text{l min}^{-1}$ , which possibly accounts for the lower collection efficiency at higher flow rate. Theoretically, higher collection efficiency is achievable under an optimally designed system and optimal operation conditions. The collection efficiency of  $\sim 90\%$  at  $0.2 \mu\text{l min}^{-1}$  with the variations in different systems was acceptable with our tests. Therefore, any further tests were all performed at 20  $V_{pp}$ , 1 MHz, and a flow rate of  $0.2 \mu\text{l min}^{-1}$ . At this flow rate, it would take 25 min and 100 min for a sample volume of 5  $\mu\text{l}$  and 20  $\mu\text{l}$ , respectively. The concentration factor, which is the ratio of the original sample volume (5–20  $\mu\text{l}$ ) to the micro-channel volume (12.5 nl) times the DEP collection efficiency ( $\sim 90\%$ ), is between  $10^2$  to  $10^3$ . The concentration factor can easily be orders of magnitude higher if a larger starting volume is used, which requires a longer flow time.

#### DEP coupling with antibody capture of *L. monocytogenes* in the micro-fluidic chip

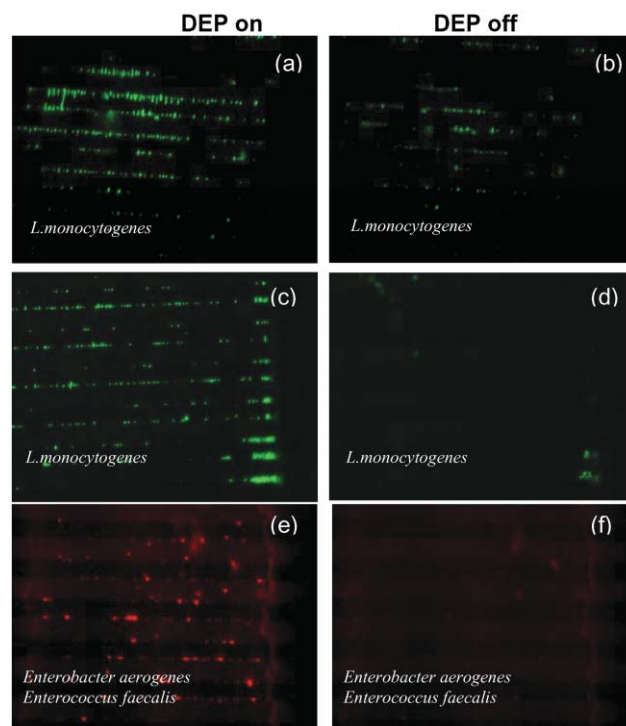
Dielectrophoresis is probably the most broadly applied method for cell separation among all chip-based cell separation technologies. In typical separations, cells are brought into

**Table 1** The efficiency of DEP collection of *Listeria monocytogenes* cells in the tested micro-fluidic system for samples with different concentrations of cells and at different flow rate. DEP: 1 MHz, 20  $V_{pp}$ . Cell concentrations were calculated based on colony counts on 3–5 Oxford plates

Flow rate/ $\mu\text{l min}^{-1}$	Cell concentration at inlet/cfu $\text{ml}^{-1}$	Cell concentration at outlet/cfu $\text{ml}^{-1}$	Capture efficiency
0.2	$2.2 \pm 0.4 \times 10^5$	$2.6 \pm 0.3 \times 10^4$	$\sim 88\%$
0.2	$1.8 \pm 0.8 \times 10^6$	$2.3 \pm 0.6 \times 10^5$	$\sim 87\%$
0.2	$3.0 \pm 1.0 \times 10^7$	$2.5 \pm 0.7 \times 10^6$	$\sim 92\%$
0.2	$2.6 \pm 0.3 \times 10^6$	$3.3 \pm 0.5 \times 10^5$	$\sim 87\%$
0.6	$2.6 \pm 0.3 \times 10^6$	$9.0 \pm 1.4 \times 10^5$	$\sim 65\%$

the channel by fluid flow and the cells are collected on the electrodes by DEP force. By adjusting the conditions in the channel, including the frequency of the applied field, the conductivity of the suspending medium, or the flow rate, separation can be achieved by the selective release of certain types of cells while other types of cells are retained. By this means, DEP has been employed for the selective separation of viable and unviable yeast,<sup>12,13</sup> bacterial cells,<sup>14,15</sup> cancer cells,<sup>17,18</sup> viruses,<sup>19</sup> and CD34+ stem cells.<sup>20</sup> Suehiro *et al.*<sup>16</sup> reported a selective detection method, in which positive DEP was used to exclusively collect viable bacterial cells from a suspension also containing heat-treated nonviable cells, thus selective detection of viable bacterial cells was achieved. A recent study in our group has demonstrated an effective separation of live and heat-treated *Listeria* cells by DEP with interdigitated electrodes.<sup>24</sup> By choosing a critical frequency of the applied field, separation was achieved by collecting live cells at the edges of the electrodes using positive DEP, and heat-treated cells were collected at the centers of the electrodes using negative DEP.<sup>24</sup> In all these cases, the separation of cells is making use of the distinctive difference in dielectric properties between the target cells and other cells in the mixture. For example, the separation of live and dead cells is generally based on the difference in conductivity of the cell membrane between live cells and dead cells. The cell membrane of a live cell consisting of a lipid bilayer is highly insulating, with a conductivity of around  $10^{-7}$  S m<sup>-1</sup>. On death, the cell membrane becomes permeable and its conductivity increases by a factor of about  $10^4$ .<sup>21</sup> Separation of different types of cells is based on the consideration of the chemical differences between cells, particularly at their surfaces, which provide characteristic polarization properties with resulting distinctive dielectrophoretic responses. However, in many cases, the chemical difference between target cells and non-target cells are not sufficient, thus separation of similar cells using DEP cannot be achieved.

In this study, anti-*Listeria* antibodies pre-coated on the SiO<sub>2</sub> layer on top of the electrode array were able to selectively capture *Listeria* cells. When the sample containing *Listeria* cells or a mixture of *Listeria* and other cells was injected into the channel, positive DEP was used to collect all bacteria cells at the electrode edges against the flow stream and retain them for a certain time period. When DEP was turned off, the fluid flow flushed the unbound *Listeria* cells and other non-target bacteria cells by the fluidic drag force; only target bacterial cells were selectively captured by the immobilized antibodies and stayed on the channel surface. Fig. 3 shows the results of cell capture in the DEP channel immobilized with anti-*Listeria* antibodies (C11E9) and control experiments. Fig. 3(a) presents the fluorescence images of the *L. monocytogenes* cells collected in the channel when DEP was applied. Fig. 3(b) shows the *L. monocytogenes* cells left in the channel after DEP was turned off and the channel was washed with DI water for 5 min. As a control experiment, Fig. 3(c) and (d) show the fluorescence images of *L. monocytogenes* in the DEP channel that was modified with 1% BSA when DEP was on and off, respectively. It can be seen that *L. monocytogenes* cells collected by DEP force on the electrode surface could not stay on the electrode surface in this BSA modified chamber



**Fig. 3** Fluorescence images of *L. monocytogenes* collected in the DEP channel (a) when DEP was applied, (b) when DEP was turned off and the channel was flushed for 5 min, in the DEP channel that was immobilized with anti-*Listeria* antibodies C11E9; (c) when DEP was on and (d) when DEP was off in the control channel that was modified with BSA; (e) fluorescence images of *Enterobacter aerogenes* and *Enterococcus faecalis* cells collected in the DEP channel immobilized with *Listeria* antibodies C11E9 after DEP was applied, and (f) after DEP was turned off and washing process. DEP: 1 MHz, 20 V<sub>pp</sub>.

after DEP was turned off (Fig. 3(d)), while some *L. monocytogenes* cells were retained on the channel surface that was modified with *Listeria* antibodies when DEP was off (Fig. 3(b)). Fig. 3(e) shows the fluorescence images of *Enterobacter aerogenes* and *Enterococcus faecalis* cells collected in the channel when DEP was applied, but they did not stay in the channel when DEP was turned off as shown in Fig. 3(f). These results demonstrated that *L. monocytogenes* cells could be captured by anti-*Listeria* antibodies immobilized on the channel surface after DEP was off, and they could not be retained in the DEP channel without the help of antibody capture after DEP was turned off, whereas non-target bacteria *Enterobacter aerogenes* and *Enterococcus faecalis* were not captured by anti-*Listeria* antibody.

#### Antibody capture efficiency

In general, capture efficiencies of antibodies immobilized on solid surfaces to bacterial cells are very low. For example, the binding efficiency of anti-*Salmonella* antibodies on a roughened glassy carbon electrode surface is less than 0.5% by placing a droplet (containing  $\sim 10^5$  cells) directly on the electrode surface, and it is less than 0.01% by immersing the electrode into 1 ml solution containing  $10^7$  cells.<sup>36</sup>

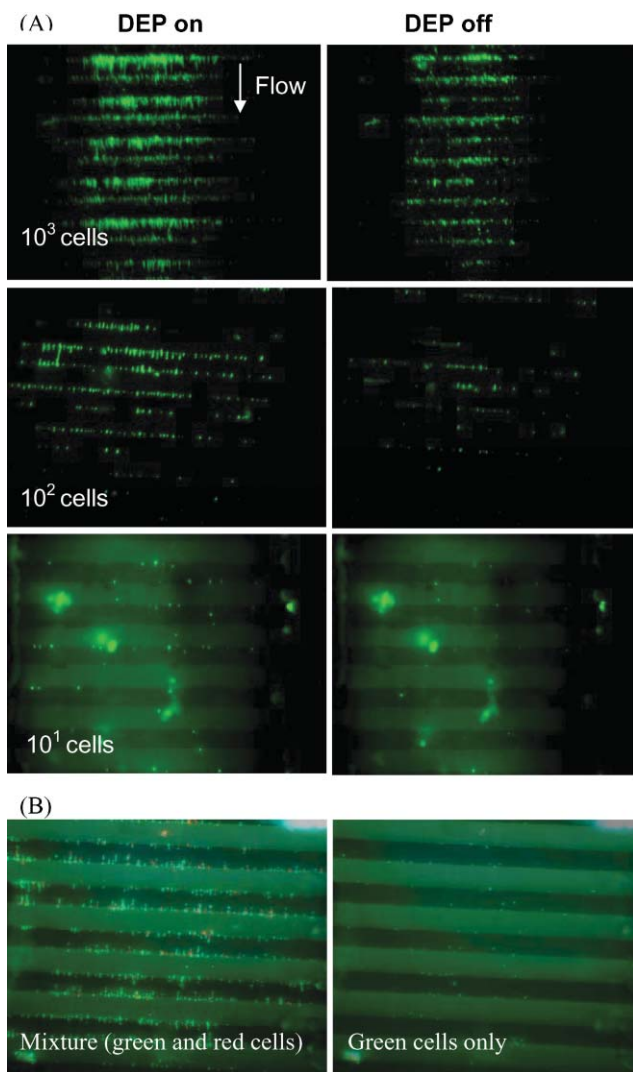
In the case of micro-fluidic devices, the bacteria cells travel in a micro-channel at velocities in the range of several hundred

microns per second at normal sample injection conditions, therefore, very few bacteria have the possibility to contact the channel surface, interact with the immobilized antibodies, and be captured on the channel surface. Therefore, capture efficiency of immobilized antibodies in the flow-based micro-channel would be lower than that of immobilized antibodies on general solid surfaces. With the micro-fluidic device described here, DEP electrodes collected bacterial cells from the flow onto the electrode surface, which provided the right conditions for bacterial cells to physically interact with the antibodies immobilized on the channel surface. Hence, one can

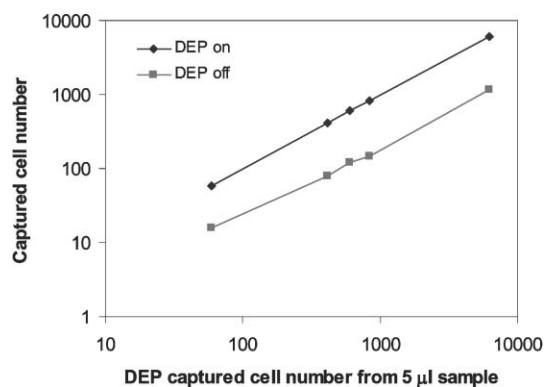
expect a greatly increased capture efficiency of these antibodies for capturing target bacterial cells.

Fig. 4(A) shows the representative results of antibody capture of *L. monocytogenes* after DEP collection for different numbers of *L. monocytogenes*, (a)  $10^4$  cells, (b)  $10^3$  cells, and (c)  $10^2$  cells from 5  $\mu\text{l}$  of the sample volume with cell concentrations ranging from  $10^4$  to  $10^6 \text{cfu ml}^{-1}$ . It is clear that a significant portion of *Listeria* cells were attached to channel surface, which indicate that target cells can be capture by the immobilized antibodies on the channel surface. As shown in the images, the lowest number cells captured by DEP from a 5  $\mu\text{l}$  of the sample in this study was about 60 cells, when DEP was turned off, about 16 cells were captured by the antibodies on the channel surface. This result indicates that this device is capable of capturing very few bacterial cells, which is very useful for detection of foodborne pathogenic bacteria. Fig. 4(B) shows the fluorescence images of the sample containing the mixture of *L. monocytogenes* (green), and other non-target bacteria *Enterobacter aerogenes* and *Enterococcus faecalis* (both are labeled red). As seen in the images, both green and red cells are present in the image when DEP was on (left), indicating DEP could collect all species of the bacteria in the flow. When DEP was turned off, only some green cells were retained in the channel (right), indicating that non-target bacteria, *Enterobacter aerogenes* and *Enterococcus faecalis* (red) were not captured by anti-*Listeria* antibody.

Analysis of these images by using ImageJ software enabled captured cell numbers to be estimated, based on the total fluorescence particles occupied area divided by the area of a single bacterial cell. Fig. 5 shows the cell numbers when DEP was on and off obtained by analyzing fluorescence images of five samples with the cell number ranging from  $10^1$  to  $10^3$  cells. The antibody capture efficiency of this system, calculated based on the cell numbers obtained from the image analysis (the cell number when DEP was off to that of when DEP was on), varied from 18% to 27% for the five samples, with an average capture efficiency of 20.7% and a standard deviation of  $\pm 3.7\%$ . The capture efficiency achieved with this DEP electrode in the micro-fluidic device is much higher than those of traditional electrodes by immersing the electrodes into a



**Fig. 4** (A) Fluorescence images of *L. monocytogenes* cells collected in the micro-channel by DEP (left) and by antibody when DEP was off (right) for different concentrations of *L. monocytogenes* of  $10^3$  cells,  $10^2$  cells, and  $10^1$  cells. Bacterial sample: 5  $\mu\text{l}$  with concentrations of about  $1-3 \times 10^4$  to  $1-3 \times 10^6 \text{cfu ml}^{-1}$ . (B) Fluorescence images of the mixture of *L. monocytogenes* (green), and other non-target bacteria *Enterobacter aerogenes* and *Enterococcus faecalis* cells (both are red) collected in the micro-channel by DEP and antibody (left) and only *Listeria* cells (green) were captured when DEP was off (right). Bacterial sample: 5  $\mu\text{l}$  with concentration of  $10^6 \text{cfu ml}^{-1}$  (1 : 1 : 1). Flow rate:  $0.2 \mu\text{l min}^{-1}$ , DEP:  $20 V_{pp}$  at frequency of 1 MHz. Big green spots in the images are impurities and contaminants.



**Fig. 5** Captured cell numbers in fluorescence images obtained by analyzing images using ImageJ software, based on the total fluorescence particles occupied area divided by the area of a single bacterial cell. Three of the five data points are from analysis of images in Fig. 4(A).

solution or by placing solution onto the electrode surfaces.<sup>36</sup> It is also about twice the capture efficiency of around 8–10% achieved in our previous protocol.<sup>37</sup>

Besides the concentration effect, DEP forces hold these collected bacterial cells in good contact with the immobilized antibody molecules on the channel surface, which greatly increased the opportunity for bacterial cells to be captured by the antibodies. Our results demonstrated the great advantage in dramatically increasing the capture efficiency of the antibodies in the flow-based micro-devices. It also implied that integration of DEP technique to traditional antibody-based assays such as ELISA is possibly an effective way to improve the detection limits for those methods. More attractively, the protocol could provide selective capture of target bacterial cells from a mixture of other biological cells with similar dielectric properties, in which separation of these cells is impractical by using DEP technique alone. It also allows the use of much reduced sample volumes when compared to traditional methods of cell analysis such as flow cytometry.<sup>38</sup>

#### Effects of the electric field on cell viability and antibody expression

As described above, in the DEP collection, the cells are exposed to the non-uniform electric field and become electrically polarized. Cell viability was examined by checking the viable cell numbers in the samples before and after DEP treatment at 2 MHz, 20 V<sub>pp</sub> for 2 h, using the plating method. Two levels of bacterial cell concentration were tested for the viability of *Listeria* cell upon DEP treatment. The viable cell numbers in the samples were  $2.2 \pm 1.2 \times 10^8$  cfu ml<sup>-1</sup> and  $1.8 \pm 0.4 \times 10^6$  cfu ml<sup>-1</sup> before DEP treatment, and  $2.2 \pm 0.8 \times 10^8$  and  $1.3 \pm 2.4 \times 10^6$  cfu ml<sup>-1</sup> after DEP treatment. It can be seen that the viable cell numbers in both samples did not change significantly upon the DEP treatment, indicating that DEP did not cause any irreversible damage to the cells in terms of cell viability. This observation is consistent with the results of other studies on a variety of cell types. For example, the viabilities of erythrocytes,<sup>17</sup> CD34+ cells,<sup>20</sup> yeast cells,<sup>12</sup> and a number of Gram-positive and Gram-negative bacteria,<sup>14</sup> following exposure to DEP forces were all confirmed using cell stain and/or plate counting methods.

A possible explanation for this could be that the cell membrane, which consists of a lipid bilayer containing many proteins, acts as an effective capacitor that shields the interior of cells from the applied field. As a result, the membrane sustains the full electric potential applied to the cell.<sup>29</sup> It is reported that if the induced membrane potential exceeds ~1 V, cells would be lysed by the effects of electroporation and electrofusion.<sup>39</sup> For cells with a radius of around 2.5–5 μm, an applied field lower than about  $1-3 \times 10^5$  V m<sup>-1</sup> would not produce such effects.<sup>21</sup> In our experiments, the maximum electric field was ~ $10^4$  V cm<sup>-1</sup> (20 V/17 μm), which is believed not to be detrimental to bacteria cells.<sup>25,40</sup>

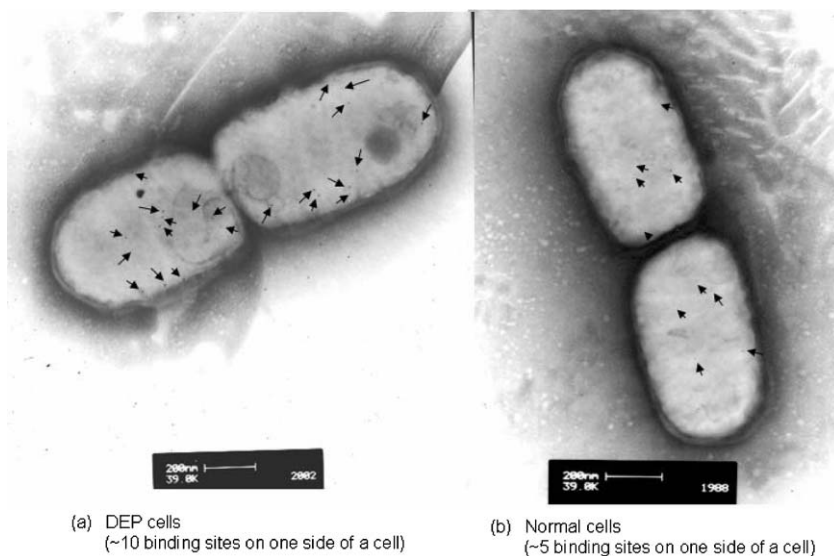
However, as the cell membrane sustains the electric field applied to the cell, the electric field possibly causes changes in protein expressions on the cell membrane following exposure to DEP. Since we mainly used C11E9 monoclonal antibody to

capture *Listeria* cells in our tests, we examined the expression of the antigens to C11E9 monoclonal antibody on the *Listeria* cells upon the treatment of DEP by using the enzyme-linked immunosorbance assay (ELISA) and scanning electron microscopy (SEM). Monoclonal antibody (MAb) C11E9 belongs to the IgG2b subclass and binds to the surface proteins of *L. monocytogenes* (it reacts with 5 different surface antigens with a major reactive antigen being a 66 kDa protein n-acetylmuramidase). In ELISA tests, 50 μl of normal cells or DEP treated cells ( $10^9$  cfu ml<sup>-1</sup>) was introduced over the ELISA well overnight at 4 °C. Unbound cells were washed away by PBS buffer. Bound cells were reacted with anti-*Listeria* monoclonal antibody C11E9 and further reacted with HRP-conjugated anti-mouse antibody. Absorbance was measured at 490 nm after the plate was developed with the substrate solution containing hydrogen peroxide and *o*-phenylene diamine (OPD). It turned out that DEP treated cells produced a higher signal (OD<sub>490</sub> =  $0.87 \pm 0.058$ ) compared with normal cells (OD<sub>490</sub> =  $0.48 \pm 0.019$ ), indicating that more C11E9 antibody molecules could bind to the DEP treated *Listeria* cells. Fig. 6 shows the TEM pictures of the DEP treated and normal *Listeria* cells with 10 nm gold particles as the labels for binding sites of anti-*Listeria* monoclonal antibody C11E9 on cell surfaces. The pictures confirm that the number of binding sites for C11E9 on a DEP treated *Listeria* cell (~10 binding sites) are about twice those on a normal cell (~5 binding sites). This result suggested that the induced cell membrane potential upon the DEP treatment might cause some proteins in the membrane to get more exposed to the surface, and thus makes them easier for the antibody to access. Differences in other protein expression might also occur upon DEP treatment. However, a detailed mechanism accounting for the difference in protein expression is not clear and needs further detailed investigation.

#### Conclusions

In this study, we demonstrated a micro-fluidic device which used the combination of DEP and antibody-antigen recognition to achieve high capture efficiency for *L. monocytogenes*. This device afforded multiple functions in manipulation of bacterial cells, including concentration, selective capture, and a resulting high efficiency of antibody capture for bacteria cells. DEP could collect ~90% of the cells in a continuous flow of 0.2 μl min<sup>-1</sup> in the micro-channel with concentration factors between 10<sup>2</sup> and 10<sup>3</sup> with a sample volume of 5 to 20 μl. This device achieved antibody capture efficiencies of about 18–27% when bacterial cell number ranged from 10<sup>1</sup> to 10<sup>3</sup> cells per 5 μl in the samples. The results in this study clearly indicated that dielectrophoresis is an effective way to trap a vast majority of the bacteria and bring the target species to interact with antibodies immobilized on the surface of the DEP channel, which greatly increases the capture efficiency as compared to systems without DEP or other traditional electrodes. On the other hand, introduction of antibody recognition into the DEP system could provide the selectivity of DEP separation of biological cells, especially in the case of separating cells with similar dielectric properties and sizes. This DEP and antibody-mediated capture system has the potential to enable selective





**Fig. 6** SEM pictures of *Listeria monocytogenes* cells with the binding sites to anti-*Listeria monocytogenes* monoclonal antibody C11E9 labeled with 10 nm gold particles in (A) DEP treated cells and (B) normal cells. DEP treatment: 20 V<sub>pp</sub> at 5 MHz for 2 h. The binding sites (tiny black dots) are indicated by arrows.

capture of *L. monocytogenes* from the mixture of *Listeria* and other cells. It is expected to provide a useful way to isolate low numbers of target cells from hundreds of millions of competent cells.

It was found that DEP operation in our experiments did not cause any irreversible damage to bacterial cells in terms of cell viability. Unexpectedly an increase in antigen expression (antigens to C11E9 monoclonal antibody) on cell membranes was observed following the exposure to DEP.

In addition, this system could be coupled with several detection methods for subsequent detection of captured cells, such as cell lysis and analysis of target molecules or impedance measurement for monitoring bacterial metabolism, which would provide sensitive methods for the detection of biological cells. It is believed that the principle of this system could be applied to capture of other biological cells by immobilizing desirable antibodies onto the DEP channel surface.

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