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Opto-electrokinetic manipulation for high-performance on-chip bioassays†

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This communication first demonstrates bio-compatibility of a recently developed opto-electrokinetic manipulation technique, using microorganisms. Aggregation, patterning, translation, trapping and size-based separation of microorganisms performed with the technique firmly establishes its usefulness for development of a high-performance on-chip bioassay system.

A cell-based bioassay is an analytical method that assesses concentration and biological activity of chemicals/bio-molecules in a sample using microorganisms such as viruses, bacteria and fungi.1 Bioassays are extensively used in medicine, pharmaceutics and environmental health sciences for various purposes including development of new drugs for disease treatment and exploration of alternative approaches for environmental remediation.2–5 Cell-based bioassays typically have been performed with the support of multiple laboratory procedures such as sampling, separation and extraction in addition to several handling steps needed to prepare a sample for test with indicator microorganisms.6 Continuous advances of microfabrication technologies over past two decades have enabled integration of the microorganism pretreatment procedures on a chip and which in turn motivated many studies for the realization of an ‘on-chip bioassay’ system, together with the numerous advantages such as reduced reagent consumption and detection times.7–14 Most of the studies were concentrated on the development of techniques that can effectively manipulate microorganisms at micro- and nano-scale. Rapid aggregation, transportation, and precise screening of microorganisms on a chip not only facilitates on-chip pretreatment processes but also improves the sensitivity and specificity of a bioassay against target components.15 As electrochemical properties of microorganisms are largely size-dependent, non-homogeneity in cell sizes of microorganisms add to complexity on the cell detection and this necessitates a unique process design for size-based cell separation process.16–18 Ultimately fast and precise on-chip manipulation of microorganisms aids in development of high-performance bioassay systems.

Such substantial advantages led to the creation of various manipulation techniques and their applications to bio-particles: dielectrophoresis,9,11,12 capillary electrophoresis,8,10 optical tweezers3 and magnetophoresis.13,14 However, the existing methods still suffer from the lack of ease, expeditiousness and precision of the manipulation. For example, dielectrophoresis needs design and configuration of a complicated electrode, and its integration into small spaces by microfabrication techniques.12 And the adhesion of bio-particles on the electrode often happens during dielectrophoretic manipulation making continuous progress of the manipulation for long durations difficult.19 Optical tweezers are capable of trapping particles precisely but have the limitation in achieving high-throughput of biochemical analyses, because only a single or few microorganisms can be trapped in the beam waist of a laser.20 Magnetophoresis involves pretreatment of target microorganisms with magnetic beads off-chip for the manipulation, and it inhibits the automated performance of all steps of on-chip bioassay.13 These disadvantages of the existing methods justify the necessity of a simple and versatile alternative technique.

Recently a novel non-contact opto-electrokinetic technique that can manipulate micro- or nano-particles fast and precisely in a microfluidic chip was suggested.21–24 The technique termed rapid electrophoretic manipulation (REP) has been demonstrated to rapidly and dynamically assemble, pattern and translate various artificial colloids of 0.05–3 μm onto an electrode surface by applying a laser illumination to the electrode surface biased with a uniform AC electric field below 200 kHz. Further, sorting three different sized polystyrene particles successively also was shown.24 The manipulation by REP is directly performed by several physical phenomena generated from the simultaneous application of the two driving sources.21,23,24 A uniform AC electric field induces a polarization of particles suspended in a fluid and an electrohydrodynamic flow around the particles and an electrode.21,23,24 A focused laser illumination drives a toroidal-shaped electrothermal vortex with the center at an illuminated site on the electrode through the production of non-uniform temperature distribution in the fluid which interacts with the applied electric field.26 The combination of the three primary phenomena enables REP manipulation by establishing the competition of electrokinetic forces between the particles, and between the particles and the electrode surface.

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However, the technique cannot always manipulate particles stably. The stability of REP-based particle manipulation is governed by a critical frequency, which is the maximum AC frequency at which a stable REP cluster can be obtained.\textsuperscript{21} Phenomenologically explaining, in a particle cluster initiated by REP on an electrode surface, the particles become unstable as applied AC frequency approaches to their critical frequency and, at further increased frequencies, are convected away by a strong electrothermal flow with extinction of the cluster. Therefore, the simultaneous application of a uniform AC electric field within the critical frequency and a focused laser guarantees the concentration, patterning and translation of particles by REP. Meanwhile, the critical frequency of particles bears a complex dependence on their various properties, and hence is not yet completely understood. As partial research achievement, it was demonstrated experimentally that the critical frequency has a close relationship with non-equilibrium electric double layer (EDL) polarization of particles associated with surface charge density ($\zeta$, $\sim \rho_e$).\textsuperscript{21} The dependence is expressed as the inverse of particle diameter squared for the special case of constant surface charges, and it is the basic mechanism of size-based separation of the polystyrene particles mentioned earlier.

The abilities of REP shown in previous studies intuitively are expected to make significant contributions to the achievement of high-speed, high-throughput and high-accuracy of an on-chip bioassay through effective manipulation of indicator microorganisms. However, detailed investigations about bio-compatibility of the technique are yet to be attempted. Manipulation of microorganisms by REP can be often much more complicated as compared to colloidal particles, due to several factors including the motility of microbes. In this paper, we carry out various manipulation experiments using indicator microorganisms, in order to establish the bio-compatibility of REP and report the results. For this purpose, size-based separation of microorganisms also is demonstrated. The capabilities of REP for aggregation and patterning of bio-particles, and translation of the assembly is shown with \textit{Shewanella oneidensis} (\textit{S. oneidensis}) MR-1 which is a gram-negative rod-shaped bacterium of $\sim$1 $\mu$m in diameter and 2–3 $\mu$m in length.\textsuperscript{21} \textit{Saccharomyces cerevisiae} (\textit{S. cerevisiae}) which is a unicellular spherical fungus of $\sim$5 $\mu$m in diameter\textsuperscript{28} and \textit{Staphylococcus aureus} (\textit{S. aureus}), which is a gram-positive spherical bacterium of $\sim$1 $\mu$m in diameter\textsuperscript{29} are employed to demonstrate selective and dynamic size-based separation by the REP technique. These microorganisms are chosen for their easy availability, frequency of usage as an indicator microorganism in bioassay studies, and suitability to each purpose of above experiments.\textsuperscript{29–32} The results shown here demonstrate not only that the manipulation targets of REP can be extended from artificial colloids to biological particles including bacteria and fungi, but also that the technique is a powerful tool for development of a high-performance on-chip bioassay system.

The indicator microorganisms used in this paper, i.e. \textit{S. oneidensis} MR-1, \textit{S. cerevisiae} and \textit{S. aureus} are processed as described below: \textit{S. oneidensis} MR-1 and \textit{S. aureus} are inoculated from their frozen stocks and streaked onto sterile lysogeny broth (LB) agar plates and grown at 30 °C (\textit{S. oneidensis} MR-1) and 37 °C (\textit{S. aureus}) for 24 h. Single colonies from each plate are transferred into sterile culture tubes containing 5 mL of LB broth and grown in an orbital shaker at 120 rpm and appropriate temperatures for 12–16 h. Mid-log phase cells are extracted by centrifugation at 5000 rpm for 5 min, followed by repeated washing with sterile PBS buffer/water three times as required. Media-free cells are resuspended in 0.7% NaCl and incubated with 4 $\mu$L of SYTO-9 green fluorescent dye (Invitrogen Corp.) for 15 min under dark conditions. Finally, the free dye in the medium is removed by centrifugation at 5000 rpm and the cells are suspended in DI water for the experiments. \textit{S. cerevisiae} are grown in YPD broth at 30 °C for 16 h before extracting mid-log phase cells. Cells are washed and labeled as per procedures described above. The above three prepared microorganisms are manually injected into the chip illustrated in Fig. 1 for each experiment and then, are manipulated, or separated along with their size by the simultaneous application of a uniform AC electric field and a focused laser. Detailed specifications of the chip structure and the equipments such as a microscope system, an imaging camera and a laser illumination used in the experiments are available in the previous publications related to REP.\textsuperscript{21,23–26}

To utilize various capabilities of REP shown with artificial colloids in previous studies to manipulation of actual bio-particles, this work shows extensive manipulations of motile \textit{S. oneidensis} MR-1 bacteria with the REP technique. The study began with the measurement of the critical frequency of the bacteria, since the stability of REP manipulation is ensured below the critical frequency (see ESI1\textsuperscript{+}). The critical frequency was determined by observing how the number of MR-1 bacteria in a REP-created assembly changed as the applied AC frequency was changed. As the applied frequency increases gradually from 10 kHz, the number of MR-1 bacteria in the REP-created assembly decreases rapidly at 34 kHz, signaling the arrival of their critical frequency.

Fig. 1 Microfluidic setup used for manipulation and size-based separation of microorganisms by REP technique. The chip consists of microfluidic chambers sandwiched between two parallel-plate electrodes. The top and bottom electrodes are made from an indium tin oxide (ITO) coated glass substrate and cover slip, and are transparent for illumination and microorganism observation. During experimentiation, an AC electric field is supplied by a function generator and an optical illumination is provided from a Nd : YVO$_4$ laser (1064 nm wavelength). Then the laser is focused on the bottom electrode by an objective lens. Liquid is introduced through the ports on the top electrode.
Therefore all manipulation experiments of the *S. oneidensis* MR-1 bacteria by REP were performed by simultaneously applying a uniform AC electric field within the identified frequency range and a focused laser. Fig. 2 shows various manipulations that can be performed on MR-1 bacteria by the REP technique (also see the ESI†). A large number of the bacterial cells under the effect of an AC electric field in Fig. 2(a) were rapidly assembled by REP onto the electrode surface as in Fig. 2(b). The electric signals applied to the chip are 18.69 kHz and 17.8 V$_{pp}$ and the intensity of the provided laser illumination is 20 mW. The aggregation procedure of the bacteria is described as follows: when the suspension containing *S. oneidensis* MR-1 bacteria is injected into the chip, the bacteria swim freely in the channel using their flagella. With the application of a uniform AC electric field, they are transported toward an electrode surface by an electrohydrodynamic flow, experiencing a reorientation caused by the AC frequency-dependent torque because of the geometric shape of their body (prolate ellipsoids).33 Then the torque aligns the principal axis of the bacterial body to the applied electric field lines. On the electrode surface, the MR-1 bacteria show an irregular configuration in the competition of repulsive forces between their induced dipole moments, and attractive forces by the electrohydrodynamic flow (Fig. 2(a)). The additional application of a laser illumination induces an electrothermal vortex by generating the temperature-dependent gradients of permittivity and conductivity in the medium which interact with the applied electric field.$^{21,33}$ The toroidal microvortex transports the upright MR-1 bacteria continuously towards the illumination location on the electrode, forming their assembly at a broad, non-uniform temperature region produced by the laser (Fig. 2(b)).$^{21,26}$ The rapidity of REP manipulation observed in the aggregation process is demonstrated with an exponential fitting curve in Fig. 2(c). By the application of the technique, the number of MR-1 bacteria in the cluster increased with time and as a result, about 700 MR-1 cells were aggregated on the electrode surface in 0.73 s. The process was saturated when all of the bacteria within the electrothermal vortex had been collected. The assembly of the bacteria by REP can be made anywhere on the electrode surface biased with a uniform AC electric field along with location of the focused laser, and the experimental observation led to the attempt of REP-based patterning using multiple laser spots (Fig. 2(d)). When two laser spots were supplied on the electrode surface, MR-1 bacteria formed their separated assemblies at and around each of the illuminated regions. The applied frequency, electric potential and laser intensity is 17.32 kHz, 16.9 V$_{pp}$ and 30 mW, respectively. If a laser illumination of a periodic lattice type is applied to the chip, multiple isolations of the bacteria can be formed on the electrode. REP also achieved successful translation of MR-1 bacteria in the channel. It was performed by moving a focused laser across an electrode surface, and the experiment result is shown in Fig. 2(e)–(f). REP-based manipulations of *S. oneidensis* MR-1 first attempted in this paper revealed new capabilities of REP.

**Fig. 2** Manipulation of *S. Oneidensis* MR-1 by REP technique (also see the ESI†). Red-dots in the figure represent the locations of a focused laser beam on the bottom ITO electrode surface, and their size corresponds to the actual diameter of the laser beam (1.3–1.5 μm in diameter). (a) A random distribution of MR-1 bacteria on the electrode when only a uniform AC electric field is applied to the chip. (b) Aggregation of *S. Oneidensis* MR-1 by REP technique. The applied electrical signal is 17.8 V$_{pp}$ at 18.69 kHz and the laser power is 20 mW. (c) The change of the number of MR-1 bacteria in the assembly along with a time after opto-electrokinetic technique is applied to the chip. (d) Patterning of the *S. Oneidensis* MR-1 using two laser illuminations under an electric field. The applied electrical frequency and voltage is 17.32 kHz and 16.9 V$_{pp}$ and the laser power is 30 mW. (e)–(f) Translation of the *S. Oneidensis* MR-1 assembly by the movement of a laser beam. The provided frequency, voltage and laser power is 18.69 kHz, 13.8 V$_{pp}$ and 20 mW respectively. In Fig. 2(f), blue-dot reflects the location and actual size of the beam before the movement and the arrow represents the direction of the laser movement. All of above images were taken from the bottom view.
technique, such as the ability to trap motile bacteria and localize them into clusters at an arbitrary location on an electrode surface. Moreover, death of the bacteria also was not observed during the manipulation as they could move freely again after the termination of REP operation. The ability of REP offers many benefits to studying bacterial chemotaxis response against particular contaminants present in highly contaminated areas, such as superfund sites.\(^3^4\) The above experiment results clearly demonstrate biocompatibility of REP technique.

REP can also be used to achieve size-based separation of microorganisms. The idea for the cell separation lies in the existence of a critical frequency of particles which is associated with their surface charge density.\(^2^1\) The critical frequency that is characterized as the inverse of particle diameter squared for the special case of constant surface charges makes possible the application of REP to size- or length-based cell separation. For instance, while individual cells in a pure culture show significant size variations along with growth phases of cells, their surface charges are almost constant.\(^3^5^–^3^7\) This indicates that size-based separation of live cells in different phases of the cell cycle can be achieved by REP. Also, the dependence of a critical frequency is useful in isolating a particular species of bacteria from a cocktail based on size differences, as long as they have like surface charges. In this paper, the size-based separation by REP was performed with \(S.\) cerevisiae (fungus) and \(S.\) aureus (bacterium) which are both spherical and have similar zeta potential (surface charge) but have different cellular diameters, and the experiment results are presented in Fig. 3 (also see ESI2†). When two driving sources of 17.5 kHz, 10.07 V\(_{pp}\) and 20 mW were applied to a chip, the two microorganisms formed a single combined cluster at the bottom electrode surface (Fig. 3(a)). As the frequency was increased from 17.5 to 38.9 kHz, \(S.\) aureus remained at the bottom electrode while all \(S.\) cerevisiae were swept away towards the top electrode by an electrothermal flow (Fig. 3(b) and (c)). During this process, some of \(S.\) aureus cells also were removed from the original cluster possibly due to collision with in-flowing \(S.\) cerevisiae. However, most of the \(S.\) aureus population remained in the cluster located at the bottom electrode surface even in the presence of a strong electrothermal vortex of the suspending medium. This selective manipulation ability of REP was also utilized to dynamically trap and separate only \(S.\) aureus in the fluid where both the microorganisms were suspended, and the result is presented in ESI3†. Regarding with the mortality of \(S.\) aureus and \(S.\) cerevisiae during the size-based separation and dynamic trapping, the two microorganisms settled onto the bottom electrode surface after the deactivation of REP technique, and this indicates that REP does not threaten their viability. Dead micro-organisms would float towards the top electrode. Therefore, size-based separation and trapping of the two microorganisms by REP not only more strengthens its position as a bio-manipulation technique, but also further clearly shows its utility for an on-chip bioassay.

Conclusion

In summary, in this work we have demonstrated the biocompatibility of REP for the first time. A large number of the microorganisms could be rapidly concentrated and patterned as well as dynamically traveled on an electrode surface. Moreover, the precise size-based separation and dynamic/selective trapping of two different microorganisms also was achieved. These abilities of REP can make critical contributions to the realization of a high performance on-chip bioassay system. The development of continuous flow based bio-separator/sorter using REP is currently in progress, and it is expected to enhance the manipulation capabilities of the technique.

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