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Highly Sensitive Method for Detecting and Separating Pathogens using Paramagnetic Particles and a Micro-Fluidic System

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By

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ABSTRACT

Highly Sensitive Method for Detecting and Separating Pathogens using Paramagnetic Particles and a Macro-Fluidic System

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Water is one of the most important compounds on Earth. If a water supply is contaminated with a pathogen, it can have devastating results to an individual or a community. This research is directed at constructing an easily reproducible procedure that could increase the sensitivity of detecting and separating bacteria from the current limits of about 100 cells to 10-20 cells per sample with a ultimate goal of a single cell detection. Here, we report a micro-fluidic system to magnetically capture pathogenic bacteria in water followed by rapid impedance detection using *Escherichia coli* K-12 as a model bacterium. Paramagnetic beads coated with antibodies to target *E. coli* captured the bacteria under a magnet. By controlling the flow rate of the washing solution (*i.e.*, PBS buffer), the excess unbound free magnetic particles, which were also captured by the magnet, can be removed, allowing for separation of the target cells. This is because each bacterium has multiple magnetic beads (>2 beads per cell), so it is more strongly bound to the magnet as compared to the free magnetic particles. The concentrated cells targeted by magnetic particles could yield a much higher signal of impedance, significantly increasing the detection sensitivity. The significant improvement in the detection sensitivity is mainly attributed to the substantial signal amplification by the magnetic particles. The results demonstrate the excellent potential of our system as a rapid, highly effective and sensitive, and economical way to capture and detect pathogens in diluted water samples.

INTRODUCTION

In the most recent worldwide water assessment published in 2012 by the World Health Organization, approximately 780 million people around the world lacked access to "safe drinking water". This is the cause of approximately 1.8 million human deaths each year [1]. Furthermore, if action is not taken to address these people who are incapable of fulfilling their basic needs, it is approximated that as many as 135 million people will have died from waterborne diseases by 2020 [2]. According the United States Centers for Disease Control and Prevention (CDC), there are more than 35 known water-related contaminates. Their adaptation makes filtration and detection a daunting and time consuming task.

Current methods for determining bacterial levels require complex procedures and can have an extensive analysis time. These established methods include polymerase chain reaction (PCR), culture/colony counting, and immunology-based detection [3]. Despite the complexity and time required for these procedures, all current process still fall short of being able to quantify bacterial cells in a heavily diluted sample.

Research has shown that impedance measurement in food requires a less intricate procedure with a rapid analysis time [4]. However, the current limits show that impedance biosensors were only able to detect 1.6×10^2 cells of *Escherichia coli* present in food samples with a detection time 35 minutes [5]. In the last decade there has been an enormous surge of work studying biomagnetism and impedance biosensors. Impedance biosensors measure the electrical impedance of an interface at a particular frequency and measuring the resulting current, with the current-voltage ratio giving the impedance. This approach is known as Electrochemical Impedance Spectroscopy (EIS) [6]. Electrical biosensors rely solely on current or voltage

measurement so that they can perform label-free detection, a desirable property that reduces the need for secondary labeling. Due to the low cost and low power consumption, impedance measurement has high potential to be able to optimize applications where size and cost is important [6]. This impedance technique has been applied to the field of microbiology as a means to detect and quantify pathogenic bacteria.

Microfluidics deal with miniaturizing fluid flow systems, which has the potential to change the way modern biology is performed. This improvement in technology offers the ability to work with smaller volumes, shorter reaction time, and the possibility of running multiple tests simultaneously [7]. Magnetic particles are a potential agent capable of improving impedance signal. These beads have actively investigated for their applications with biosensors and have become a new frontier between biological detection and material science [7].

Magnetic beads have shown promise for pathogen-specific attachment and separation. This technique is possible through the use of biotin-labeled antibodies conjugated with paramagnetic micro-beads that have been functionalized with streptavidin [3]. The stability between these two entities comes from the high binding affinity of the streptavidin-biotin interaction. This has a binding affinity of approximately 1×10^{-15} M^{-1} which is one of the strongest non covalent bonds in nature [8,9]. Utilizing bacterial antibodies is the best practice available because it provides quick and specific attachment between bead and cell. Additionally, it allows for the possibility of multiple paramagnetic beads to attach to each cell.

Magnetic beads that are on the micro-scale are the most convenient particle to use for this experiment, instead of nano-sized particles. Nano-size magnetic particles can penetrate the cell membrane and accumulate inside the cell [10]. This is problematic because it gives cells that

aren't coated with the specific antigen the same properties as the target cell. When this occurs, specificity is lost and it is difficult for further detection and separation of target cells. Dynabeads MyOne Streptavidin C1 are uniform, superparamagnetic beads of 1.0 μm in diameter with a streptavidin monolayer covalently coupled to the hydrophilic bead surface. This layer is ideal for immunoassays involving small biotinylated antibodies and improves batch consistency and reproducibility of results [11].

The experimental hypothesis is that since *E. coli* cells have multiple receptor sites for antibodies against the cell, multiple magnetic beads will adhere. Since magnetic beads are better conductors than cells, attachment will give off a much stronger signal during impedance measurement. Additionally, these cells with multiple magnetic beads attached will have a stronger attraction for the magnet than the single beads because magnetic force is proportional to particle number, as demonstrated previously [12]. This will allow for the cells to remain attached to the magnet under conditions that would remove single magnetic beads. Since the single magnetic beads will remove from the magnet before the cell bound magnetic beads, the final sample will contain cells targeted by magnetic particles and little or no free magnetic beads. This will yield a substantial decrease in false positive instances by the free magnetic particles remaining in the reaction mixture after cell targeting, enabling us to realize the full promise of impedance-based biosensing.

The objectives of this research are to:

1. Design and fabricate a fluidic testing platform with the modules of magnetic capturing and flow-rate control,
2. Determine bacterial adsorption capacity of paramagnetic micro-beads, and

3. Determine the critical range of flow rates, where the free magnetic particles in excess separate from the cell-targeted magnetic particles.

MATERIALS AND METHODS

Quantifying Cell Adhesion

Preliminary research was conducted in summer 2011 with an REU student. This research was aimed at being able to quantify how many magnetic beads could adhere to a pathogen. *E. coli* K-12 was used as a model microorganism for this study and Dynabeads® MyOne ™ Streptavidin C1 from Invitrogen Life Technologies were used as the means for detection and separation. The primary antibody used was purified IgG from a goat coupled with the N-Hydroxysuccinimide ester of biotin. The secondary antibody used for this experiment was anti-IgG labeled with the Alexa Fluor 488. The Dynabeads are dissolved in Phosphate buffered saline (PBS) with a pH of 7.4. The Dynabeads came in a 2 ml sample containing a concentration of 10 mg/ml, corresponding to approximately $7 – 10 \times 10^9$ Dynabeads per ml.

Beads were washed and prepared using the manufacturer's instructions [11]. A stock solution of magnetic beads was made with 800 µl of PBS and 200 µl of washed bead solution. Both the primary and secondary antibodies were prepared with 600 µl PBS and 200 µl of antibody as a stock solution. For bead and primary antibody conjugation, 300 µl of PBS was added to 100 µl of magnetic bead stock solution and placed in the magnet holder for 5 minutes. After removing the supernatant, 100 µl of primary antibody solution was added to the centrifuge tube and agitated at the slowest speed on the vortex for 10 minutes. The sample was then placed back on the magnetic holder and the supernatant was removed with a pipette. The sample was washed with PBS three times and then labeled S1.

For S1/cell conjugation, 50 µl of S1 was added to four samples of cells, each at a different dilution, and agitated at 800 rpm for 1 hour. Once an hour had passed, the sample was separated and washed again. The secondary fluorescence-tagged antibody was then added to the solution and agitated at 800 rpm for another hour. The conjugated material was then separated from the heterogeneous solution and washed three times with PBS. After washing was complete, the sample was wrapped in foil to prevent light exposure until the sample could be observed using epi-fluorescent and light microscopy. Figure 1 shows images of two samples viewed under light (left) and fluorescent (right) microscopy.

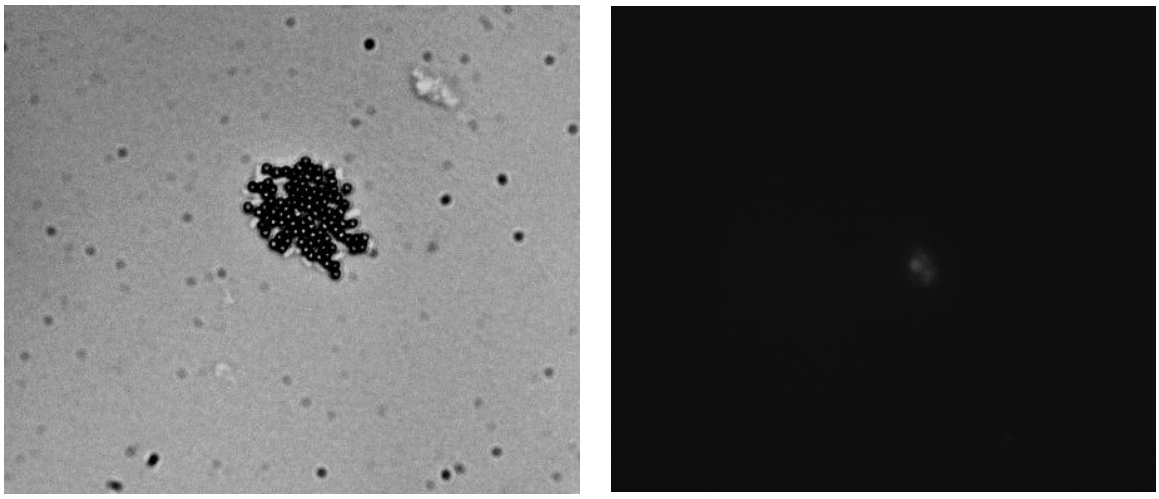

Figure 1. The picture on the left shows a magnified image using light microscopy of a static conjugation of magnetic beads and *E. coli*. Cell bound magnetic beads are present in this image, as well as a large amount of single magnetic beads. The picture on the right shows an image taken by epi-phosphorescent microscopy confirming the presence of cell bound magnetic beads.

Separation Technique

The goal was to find the range of flow rates to remove single magnetic beads yet allow for multiple magnetic beads that are attached to the cell to remain within the system. Figure 2 shows a fluidic testing platform, which was used in this study, with the modules of magnetic capturing and flow-rate control. A KD Scientific KDS-200 Dual-Syringe pump was used to vary the flow rates for the system. This pump had software that could accurately produce the same flow rate from various syringes, given their diameter. Masterflex® Tygon® lab tubing with a 1.6 mm inside diameter was used for the system. Magnetic capturing was conducted by using a strong Neodymiumblock (1" × 1" × 1") rare-earth magnet (surface magnetic field strength = 0.684 Tesla; K&J Magnetics).

Experiments consisted of two control groups (*i.e., E. coli* only and magnetic particles only) and one experimental group (a mixture of *E. coli* and antibody bound magnetic particles, which contains magnetic particle targeted *E. coli* and free magnetic particles in excess). Each group contained sixteen 1 ml samples. The first control group comprised of flushing 100× diluted *E. coli* cells that had been suspended in PBS buffer through the system. *E. coli* cells had been diluted in 1.5 ml centrifuge tubes using the serial dilution method. Before the sample was flushed, 300 µl of the solution was taken to the spectrophotometer and optical density was measured at 650 nm wavelength. The 3 ml syringe with the *E. coli* sample was attached to the tubing and then secured onto the pump device. The diameter of the 3 ml syringe was 8.66 mm. The initial flow rate was set to 0.05 ml/min, as to ensure that any paramagnetic particles present in the solution would bind to the magnet.

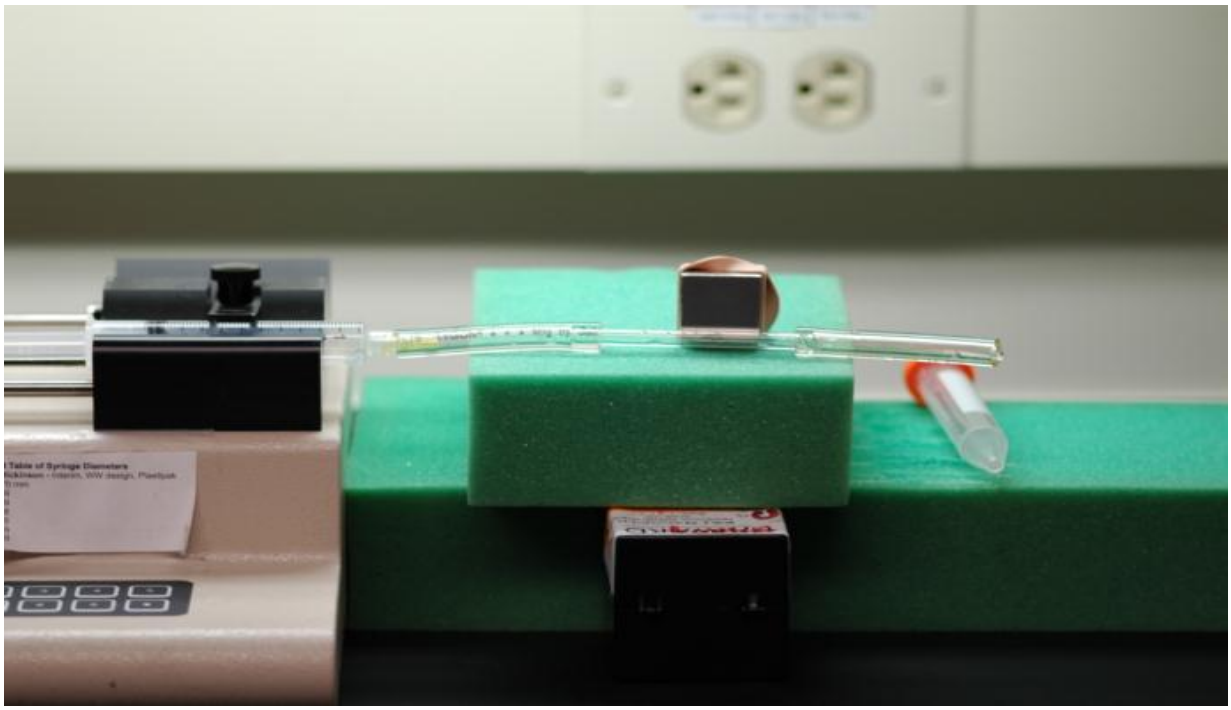

Figure 2. This picture depicts the system used in the experiment. The syringe pump is on the left, in the middle of the tubing is where the magnetic beads were separated from the solution via the magnet; the sample was collected with centrifuge tubes on the far right end.

After a sample was injected to the system at the low flow rate, the 3 ml syringe was replaced by a 60 ml syringe (diameter = 26.7 mm) filled with PBS. The outside diameter of the syringe head was roughly equal to the inside diameter of the tubing which prevented any leakage from occurring once they were connected. Air pockets carry an incredible amount of force and if they are present in the system they will wash away the magnetic particles regardless of the flow rate. Air pockets were removed from the syringe by blocking the tip and inverting. The syringe was then pushed until a convex meniscus forms at the opening and there is no gap between the flush and the original sample.

Then, 2 ml of PBS was flushed at a given flow rate and the outflow was collected for further analyses. This process was repeated at 1 ml/min increments, until a flow rate of 15 ml/min was reached. The 2 ml sample, collected at each flow rate, was concentrated to 300 µl by centrifugation to make it same as the original volume of the magnetic particle sample. The concentrated samples were used for spectrometric (at $\lambda = 650$ nm) as well as microscopic analyses.

The second control group consisted of 1 ml samples containing 100× diluted Dynabeads® MyOne ™ in PBS buffer with no antibodies attached. The experimental group was comprised of a cell and magnetic bead conjugated solution, which was combined as described above. Each sample contained 150 µl of 100× diluted cells, 250 µl of S1 solution, and 600 µl of PBS buffer. The samples were transported through the system and the collected samples were analyzed in the same manner as the first control group.

RESULTS/DISCUSSION

Conjugation of Magnetic Beads to Cells

According to the light and epi-fluorescent microscopy (Figure 1), multiple magnetic beads are shown to attach to each cell as hypothesized. The number of beads per cell was estimated to be 2.4.

Figure 1 also indicates that there is an excessive amount of free magnetic beads present from the static magnetic separation method. Because of these extra magnetic particles, it is difficult to accurately quantify how many cells are present, for example using impedance measurement. The presence of non-cell-bound free magnetic beads would yield a false positive result.

Magnetic Capturing and Separation

When the *E. coli* only control samples (Control 1) were administered through the system beginning at 0.05 ml/min, the cells washed through the system and no adhesion was observed (Figure 3). The results are not surprising since *E. coli* has no paramagnetic properties.

When the magnetic bead only control samples (Control 2) were administered through the system beginning at various flow rates, 100% of free magnetic beads were detached from the magnet at the flow rate $\geq$ 11 ml/min (blue line, Figure 4).

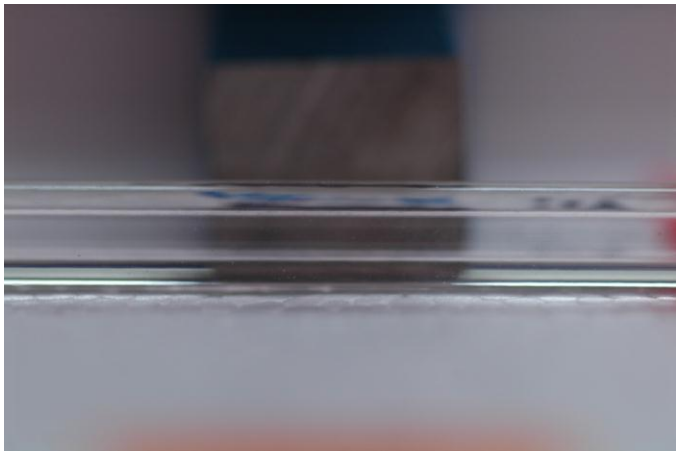

Figure 3. The *E. coli* control samples were administered through the system beginning at 0.05 ml/min. The cells washed through the system and no adhesion was observed.

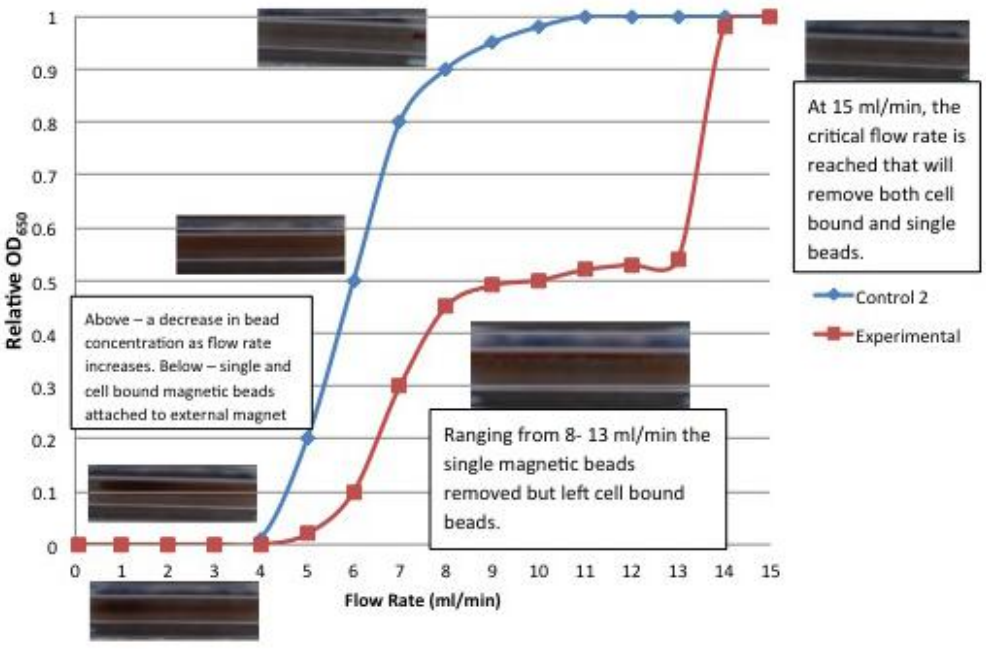

Figure 4. Relative optical density of control 2 and experimental samples with respect to flow rate (mL/min). They were tested against each other to determine the range of optimal flow rates at which the free magnetic particles can be separated from the magnetic particle bound cells. A flow rate between 11 – 13 ml/min allows the separation.

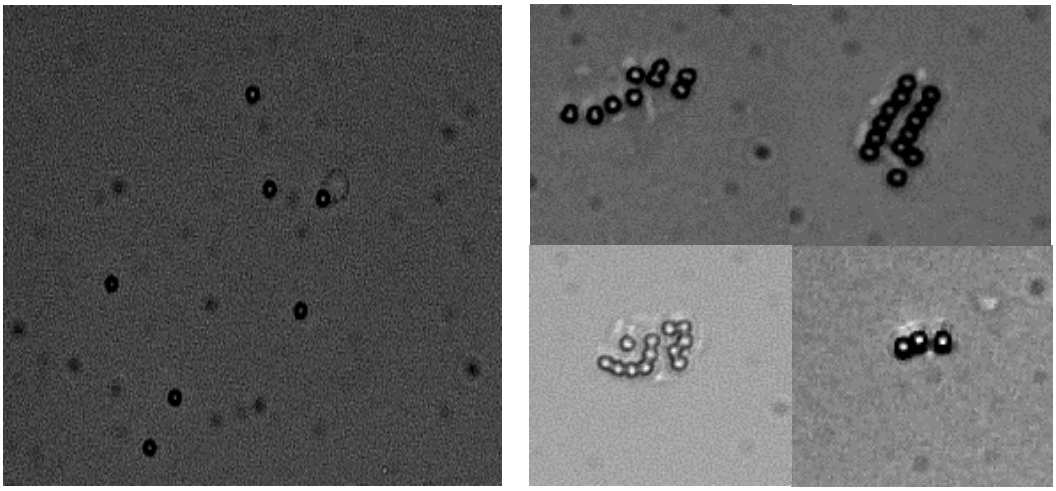

Figure 5. The picture on the left is a sample taken at 11 ml/min. Only individual magnetic beads are visible. It is also important to notice that no cell bound magnetic beads are present, meaning they are still attached to the magnet. The images on the right were from the flush (at 15ml/min) administered to remove the cell bound magnetic beads. Mostly magnetic bead bound cells yet little free magnetic beads are visible.

When repeated the experiments with a mixture of *E. coli* and antibody bound magnetic particles (Experimental Sample), which contains magnetic particle targeted *E. coli* and free magnetic particles in excess, the optical density increased up to 9 – 10 ml/min, but no increase between 11 – 13 ml/min with subsequent increases at flow rates ≥ 14 ml/min (red line, Figure 4). This implies that the free magnetic particles, which present in the sample, first detached from the magnet and their removal was completed at the flow rate of ~11 ml/min as similarly as the result with Control 2. The plateau between 11 – 13 ml/min shows that the magnetic particle bound cells are still bound to the magnet at the range of flow rates. The increased signals at flow rates of over 14 ml/min imply the removal of the magnetic particle bound cells. According to the microscopic analyses, only individual magnetic beads are visible and no cell bound magnetic

beads are present for the samples at the flow rate of 11 ml/min (left, Figure 5). This indicates that the cells with magnetic beads are still attached to the magnet. However, at the subsequent increase of the flow rate, *i.e.*, 15 ml/min, the magnetic bead bound cells are visible with little free magnetic beads (right, Figure 5). The results clearly indicate that the flow rates of 11 – 13 ml/min are the optimal range of flow rates, at which the free magnetic particles are removed yet the magnetic particle bound cells retained. The results prove the hypothesis of the research, *i.e.*, cells with multiple magnetic beads attached have a stronger attraction for the magnet than the single beads because magnetic force is proportional to particle number.

Our findings should serve as a key index to develop rapid, highly effective and sensitive biosensing systems using magnetic particles as separation agents as well as detection signal amplifiers, such as those applied to the impedance based pathogen detection systems [3-5]. To realize the overall goal of this project, the next step would be to scale down the system volume by utilizing micro-fluidic systems.

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