Microfluidic Devices for Rapid and Sensitive Identification of Organisms*

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Abstract— **Microfluidic devices for rapid and highly sensitive detection of living organisms were developed for two applications. First, a zebrafish embryo genotyping system was developed and shown to be able to genotype embryos in the first 48 hours of the embryos life without damaging the embryos in any apparent way. Second, a highly sensitive bacteria detection platform has been developed for the rapid detection of pathogens. The system relies on a magnetic bead extraction followed by secondary bead attachment. The secondary beads are barcoded with DNA sequences highly enriched for Gs. The guanine molecules generate an electorchemical response after they are released from the secondary beads and detected at a sensing location downstream from the beads. The amplification with the efficient washing procedures leads to a limit of detection of 3 CFU in 100 mL of water.**

I. Introduction

There is a need to develop highly-integrated and highly parallel microfluidic systems for analysis of nucleic acids and proteins, including biosensor and biodetection platforms. Accordingly, we are working to automate and improve microfluidic-based biomolecule sample preparation modules to maximize the sensitivity and increase the throughput of proposed development and screening platforms. In addition, the sensing modules themselves need to be improved and demonstrated at low limits of detection. In past work, we have integrated high-speed DNA analysis devices performing extraction1,2,3, amplification [4,5], and genotyping [6,7,8] functions. These systems have been demonstrated with a variety of targets including: bacteria, foot and mouth disease virus, and tumor cells. We have developed a number of microfluidic components important for integrated systems including pumps [9,10,11] and sample preparation/separation modules [12,13]. For many of these systems, we have used rapid prototyping methods14,15, which allow faster development of the nanotherapeutics and nanobiosensors needed in society.

In this work, we demonstrate two new systems for rapid identification and molecular analysis of organisms. The first project will focus on genotyping zebrafish embryos within the first 48 hours after fertilization. The second application will focus on a new method for high sensitivity detection of *E. Coli*.

A. Zebrafish Genotyping

Zebrafish is a powerful vertebrate model widely used by the biomedical research community. The strengths of zebrafish (*Danio rerio*) as a small vertebrate model include its rapid development, the transparency of embryos, the molecular tools available to analyze genetic pathways, and its inexpensiveness. The zebrafish body plan and organs are conserved with other vertebrates, and mutations uncovered in zebrafish have had broader applicability to understanding mouse and human development [16,17].Here we introduce a unique method based on microfluidic technology to genotype zebrafish in the first 24-48 hours after fertilization.

The main idea behind the method is to soften the protective chorion surrounding the embryo and then force the embryo through a constricted microchannel. The high forces at microscale shear off the chorion and release the chorionic fluid. Microfluidics is ideally equipped to collect this small amount of chorionic fluid which can be used to genotype the fish using established genotyping techniques. In this way the life/health of the embryo is maintained, which is not possible with current techniques. Furthermore, the approach can facilitate automated high-throughput genotyping of zebrafish embryos by complementing established high-throughput genetic tools.

B. E. Coli Detection

Electrochemical detection has been shown to be very sensitive in the detection of *E. coli*. Han et. al. reported an EC immunosensor for *E. coli* using graphene oxide-Ag nanoparticle composite labels with limits of detection down to 10 CFU/mL [18]. dos Santos recently reported a limit of detection of 2 CFU/mL [19] using an electrochemical impedance spectroscopy-based immunosensor. Note, though that, environmental standards for *E. coli* in water are mostly defined for 100 mL samples. For instance, the U.S Environmental Protection Agency defines protocols for testing *E. coli* limits in the Clean Water Act for 100 mL sampling volumes [20], most likely because 1 mL would not be statistically representative of the volumes involved. Also real world samples generate interference from the sample matrix and background microflora, so isolation and detection of bacterial pathogens is more challenging [21].

Immunoaffinity capture techniques like immunomagnetic separations (IMS) have been applied to isolate and concentrate *E. coli* from water samples. This approach purifies the *E. coli* significantly and reduces the effect of contaminants that might lead to suppression and inhibition of

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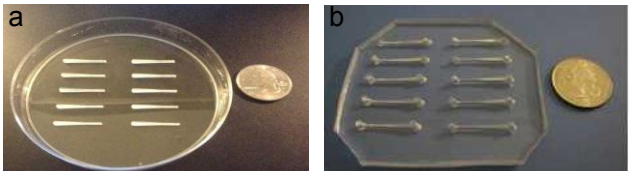

Figure 1. Micromolds and fully fabricated zebrafish genotyping chips alongside a US quarter coin; (a) micromold for chorionic-fluid chip; (b) fully fabricated chorionic-fluid chip with 10 separate chambers for chorionic fluid extraction.

E. coli detection signal during subsequent detection assays. They method also reduces false positive and false negative test results by removing virtually all inhibiting materials that could be incorrectly detected. Zhu et. al. applied IMS coupled with fluorescent detection (using a spectrofluorometer) for detection of *E. coli* 157:H7 with a limit of detection of 10 CFU/mL. However fluorescent detection requires related optical detection equipment, which is often not miniaturized making them less amenable to point-of-use.

In this paper, we report the use of immunomagnetic capture coupled with amplification and indirect EC detection of *E. coli* O157:H7 on an electrochemically reduced graphene oxide glassy carbon electrode (RGO-GCE). *E. coli* O157:H7 specific antibodies coated magnetic beads were used to capture *E. coli* O157:H7 strains from water samples. To enable multiplexing and amplification we use synthetic polyguanine oligonucleotides (PolyG) as an EC barcode and amplification system. The bacteria collected using magnetic beads is attached to another set of *E. coli* O157:H7 antibody functionalized nonmagnetic agarose beads. These nonmagnetic beads have the EC barcode, PolyGs and can be correlated to the *E. coli* O157:H7 concentration in the sample. After washing steps, we transfer this complex (magnetic beads, bacteria and nonmagnetic beads) to the RGO-GCE electrode. These PolyGs are hybridized with complementary probes on the electrode surface and upon an EC scan generate a guanine oxidation signal that is correlated to *E. coli* O157:H7 concentration in the sample. Using the protocol we demonstrate detection of *E. coli* O157:H7 in phosphate buffer solution (PBS) and waste water samples.

II. Methods

A. Zebrafish Genotyping

There are three steps for this procedure. First, zebrafish embryos at 24 hpf (hours post-fertilization) are exposed to pronase solution for about 5-6 minutes at room temperature and pressure. Pronase is a proteolytic enzyme that softens the chorion surrounding the embryo [22]. Pronase solution is prepared by mixing 30 mg/ml of commercial pronase in 1 ml of E3 buffer. E3 buffer is used by zebrafish researchers to breed the fish.

Second, the embryos are washed with E3 as pronase over-exposure is harmful for embryos. This is done by exchanging the pronase solution containing the embryos with fresh E3 buffer.

In the final step the embryos are collected individually by suction in a tube (~ 5 cm long) connected to a disposable 3-ml syringe. Only one embryo is collected in each tube. Caution is taken that during suction the embryo (along with the E3 buffer around it) does not enter or touch the syringe-tip. Then the free end of the tubing is connected to the inlet of a microchannel formed in Polydimethylsiloxane (PDMS) by replica molding (shown in Figure 1). The microchannel is designed so that its width tapers from 2 mm at the inlet to 0.75 mm at the outlet; the height remaining constant at 1.5 mm. A typical size of a zebrafish embryo is approximately 600 µm (without chorion) or 1200 µm (with chorion) [23]. Hence the channel design allows shear forces to act on the chorion, but not on the embryo when it separates from the chorion, thereby keeping the embryos safe from harm.

Once the tubing is connected to the microchannel inlet, pulsating pressure is applied on the syringe's plunger. This generates a pulsating flow inside the microchannel making the embryo travel across the microchannel in a series of small movements. The pulsating flow provides time to the embryo to adjust its body with the varying microchannel dimensions and avoid any harm to the embryo. Eventually the chorion of the embryo tears and releases the chorionic fluid. When the embryo surfaces out of the microchannel at the outlet, the fluid (~60 µl) around the live embryo is collected manually using a pipette. The live embryo is separately collected by another pipette. The tubing is then discarded and a new tube is used for the next embryo which undergoes the same process described earlier, but in a new/unused microchannel. This reduces the chance of DNA contamination between different embryos.

The collected fluids (a mixture of chorionic fluid and E3) from processed embryos are then analyzed by standard genotyping techniques (DNA extraction, Polymerase Chain Reaction (PCR) and High-Resolution Melting Analysis (HRMA)) and associated commercial hardware to genotype the embryos of interest.

B. E. Coli Detection

The mechanism of indirect sensing of *E. coli* O157:H7 consists of three steps which are: IMS to selectively capture *E. coli* O157:H7, analyte amplification consisting of a barcode or electrochemical PolyG tag attached to secondary beads, and EC detection of the PolyG tags.

In the IMS step, the bacteria sample is concentrated from 100 mL sample by filtration and isolated using *E. coli* O157:H7 specific antibody coated magnetic beads. To enable amplification synthetic PolyG oligos are used as a barcode and amplification system. The bacteria collected using magnetic beads is attached to another set of secondary beads containing barcode PolyG oligos and can be correlated to the *E. coli* O157:H7 concentration in the sample. The sample is then washed to remove any unbound secondary beads. The magnetic bead/*E. coli*/secondary bead complexes are transferred to the EC detector and the PolyGs on the secondary beads are hybridized with complementary probes on the electrode surface. A DPV scan generates a signal corresponding to the PolyGs on the secondary beads that is indirectly correlated to *E. coli* O157:H7 concentration in the sample. The probes on the electrode surface are specific to the PolyGs on the secondary beads to ensure selectivity.

EC deposition and differential pulse voltammetry (DPV) were carried out using a Gamry Reference 600 potentiostat (Gamry Instruments, Warminster, PA). A conventional three-

electrode system, which consisted of a bare or modified glassy carbon electrode (GCE) as a working electrode, an Ag/AgCl electrode as a reference electrode and a platinum mesh as an counter electrode, was employed for the DPV and EC deposition.

Graphene oxide for EC deposition was purchased from Graphene Supermarket (Calverton, NY, USA). *E. coli* O157: H7 nonpathogenic strain (Catalog no. 700728) was obtained from ATCC. The *E. coli* O157:H7 antibody coated magnetic beads for pathogen extraction was obtained from Invitrogen (Dynabeads MAX kit, Invitrogen, Carlsbad, CA, USA). The streptavidin coated polystyrene (secondary) beads were purchased from Bangs Laboratories (Catalog no. CP01N, Bangs Laboratories, Inc., Fishers, IN)). Sulfo-NHS (N-hydroxysulfo-succinimide) and EDC (1-ethyl-3(3-dimethly aminopropyl) carbodiimide hydrochloride) were obtained from Pierce/Thermo Fisher Scientific (Rockford, IL, USA). Sodium hydroxide was ordered from Macron Fine Chemicals (Center Valley, PA, USA). Tris(2,2'-bipyridyl)ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2$) was purchased from Sigma-Aldrich (Catalog no. 224758-1G, St. Louis, MO, USA). The oligonucleotides were obtained from DNA/Peptide synthesis core facility, University of Utah (Salt Lake City, UT, USA).

All reagents were of analytical grade and were used as received without further purification. Ultra-pure water prepared by Purelab System (ELGA Purelab, UK) was used throughout the experiment.

III. Results and Discussion

A. Zebrafish Genotyping

Based on the described methods an experiment was performed to determine the following important aspects of the work:

1. The ability of the method to collect chorionic fluid;
2. The presence of enough DNA in chorionic fluid for genotyping embryos;
3. The source of the DNA (maternal or embryonic).

For the experiment we used a cross of wild-type male fish, to females heterozygous for a Gal4-transgene. The Gal4-transgene is on a Tol2-transposon carrying a transgenesis marker of GFP (Green Fluorescent Protein) expressed in the heart. Embryos are either wild-type (GFP-negative), or Gal4+ (GFP-positive). We found that all of the wild-type embryos did not show any PCR amplification, demonstrating that no maternal cells or DNA was contained in the chorionic fluid (shown in Figure 2). Conversely, all transgenic embryos' DNA was successfully amplified. Furthermore, the embryos maintained normal heart rhythms, showed sensitivity to touch with normal swimming behaviors, and had normal morphological development through 6 days following chorion removal. Thus, the approach and methods were successful.

B. E. Coli Detection

Three runs of *E. coli* O157:H7 extraction from 100 mL samples using vacuum filtration yielded an average percentage recovery of 47%. The efficiency of capture of *E. coli* using IMS after vacuum pre-filtration from different concentrations (500, 50 and 5 bacteria/mL) of *E. coli*

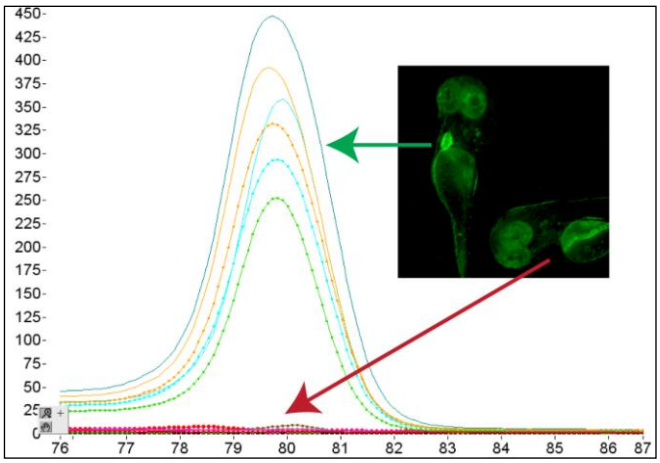

Figure 2. HRMA PCR melt-curve analysis demonstrates that chorionic fluid carries embryonic cells/DNA and can be amplified from chorionic fluid collected in the microfluidics device. Gal4-transgenic embryos, visualized because of cardiac GFP expression, amplify the Gal4 PCR product (green arrow), but non-transgenic embryos which have a transgenic mother do not have PCR product (red arrow).

O157:H7 1 mL samples was 95%, yielding an overall efficiency of 46%.

Three runs of IMS using *E. coli* O157:H7 specific magnetic beads in non-specific pathogen samples (3000 CFUs Salmonella) yielded an average zero CFU Salmonella captured on the beads signifying that the IMS is highly specific to E. coli O157:H7.

Graphene oxide was deposited on the GCE electrodes in preparation for bacteria detection. An increase in the peak currents with successive potential scans from cycle 1 to 18 indicated the deposition of reduced graphene oxide on the bare GCE. The graphene electrodeposition happens on conducting surfaces only, and the resultant graphene coating is very stable due to its poor insolubility in common solvents.

Electrochemical DPV was used to quantitatively measure the amount of hybridized PolyG tags on the electrodes and hence indirectly measure the amount of captured *E. coli* O157:H7. Figure. 3 shows the change in absolute DPV signals (S1) with an order of magnitude change in CFUs from 300 to 3 CFUs. Figure 4 shows the relative DPV signals (S1-S5) corresponding to varying concentrations of *E. coli* O157:H7 (0 to 300 CFUs enumerated by plate counting) in

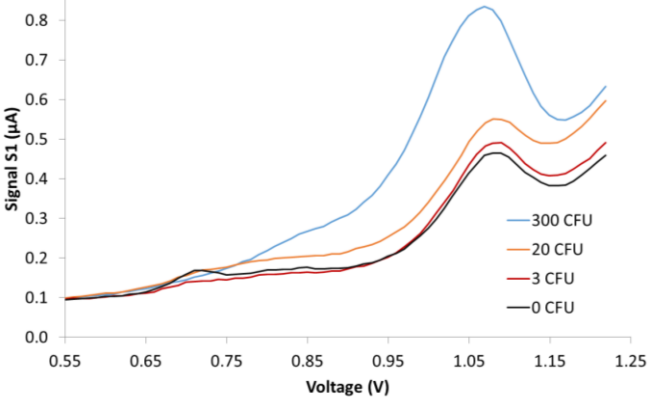

Figure 3. Absolute DPV signals (S1) corresponding to an order of magnitude change in concentration of E. coli O157:H7 from 3 to 300 CFUs.

the initial seeded 100 mL PBS buffer samples.

The relative oxidation signal due to guanine increased from 0 to 300 CFUs. The standard deviation was found to be 56.5% for three successive 300 CFU measurements. From Fig. 3, the calibration curve is linear in the range from 3-300 CFUs, with a regression equation of y=79.74+0.34x with R^2=0.9. The detection limit was 3 CFU/100 mL with a signal-to-noise ratio of 3 (the noise being the probe only signal). The 0 CFU does give a signal of 15 nA which corresponds to the base signal due to ruthenium in the electrolyte. The average probe only signal (RGO-GCE with functionalized probes) was higher than the signal corresponding to 0 CFU. This is because there is a drop in signal during DPV cycles due to passivation by acetate buffer in the electrolyte. Since the probe scans were initially run for all the electrodes before hybridized target scans was performed, there is a drop in signal for 0 CFU compared to probe only signal.

Our assay was able to detect *E. coli* O157:H7 in waste water plant effluent. The amount of *E. coli* O157:H7 in waste water effluent samples was unknown. The initial test yielded a 65 nA signal. The waste water effluent was then seeded with 300 CFU *E. coli* O157:H7 and tested. The negative control (DI water) gave a signal of 20 nA which corresponds to signal range for 0 CFUs in buffer solution. Post autoclaving the waste water sample gave a detection signal, indicating that dead bacteria are also detected. One possible solution to fix this would be to run an additional scan after a prescribed time (about 1 hour) to gauge the amount of live bacteria that are generating new bacteria.

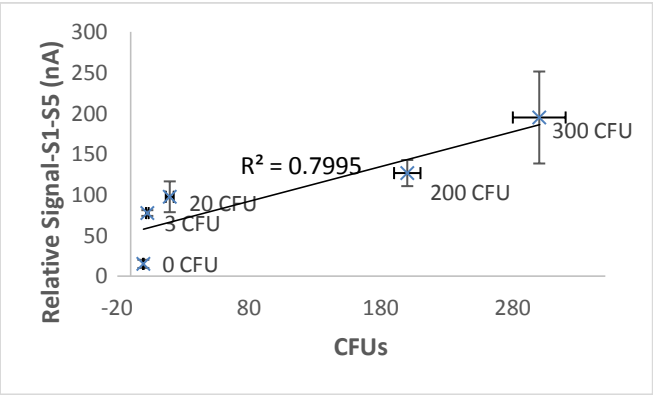

Figure 4. Relative DPV signals (S1-S5) corresponding to varying concentrations of E. coli O157:H7 in seeded 100 mL PBS buffer samples

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