Developing Genotypic and Phenotypic Systems for Early Analysis of Drug-Resistant Bacteria

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Developing Genotypic and Phenotypic Systems for Early Analysis of Drug-Resistant Bacteria

Yesman Akuoko

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

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Antimicrobial resistance in bacteria is a global health challenge with a projected fallout of 10 million deaths annually and cumulative costs of over 1 trillion dollars by 2050. The currently available tools exploited in the detection of bacteria or their DNA can be expensive, time inefficient, or lack multiplex capabilities among others. The research work highlighted in this dissertation advances techniques employed in the phenotypic or genotypic detection of bacteria and their DNA.

In this dissertation, I present polymethyl methacrylate-pressure sensitive adhesive microfluidic platforms developed using a time-efficient, inexpensive fabrication technique. Microfluidic devices were then equipped with functionalized monoliths and utilized for sequence-specific capture and detection of picomolar concentrations of bacterial plasmid DNA harvested from cultured bacteria. I then showed multiplex detection of multiple bacteria gene targets in these devices with an improved monolith column. Finally, I demonstrated a genotypic approach to studying single bacteria growth in water-in-oil droplets with nanomolar concentrations of a fluorescence reporter, and detection via laser-induced fluorescence after convenient room temperature 2-h incubation conditions. The systems and methods described herein show potential to advance tools needed to address the surging problems and effects of drug-resistant bacteria.

Keywords: microfluidics, droplet microfluidics, DNA analysis, sepsis, laser induced fluorescence, porous polymer monolith, point-of-care, multiplex analysis
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CHAPTER 1. INTRODUCTION

1.1 Bacteria and Antimicrobial Resistance

Bacteria are prokaryotic microorganisms found everywhere from guts of humans and animals, physical surfaces, the atmosphere, water bodies, the earth’s crust to arctic snow. Bacteria are typically µm in length, and exist as single cells, pairs, clusters, or chains. They can be grouped based on their morphology, growth condition requirements, or cell wall composition. Bacteria can be classified into three basic shapes namely, coccus (spherical-shaped), bacillus (rodlike) and spirillum (spiral/twisted). Aerobic bacteria grow in the presence of oxygen, and anaerobic bacteria grow in the absence of oxygen. On the basis of their cell wall composition, bacteria can be grouped either as Gram positive or Gram negative. Gram-positive bacteria have a thick, highly cross-linked peptidoglycan cell wall and no outer membrane, and Gram-negative bacteria have a thinner, partially cross-linked peptidoglycan cell wall and an outer membrane made up of lipoproteins. Gram-positive and Gram-negative bacteria turn blue/purple or pink/red, respectively, after Gram staining.

Bacteria may reproduce using various methods such as binary fission, spore formation or transfer of genetic material. During binary fission, a new cell wall grows down the center of the cell forming two new daughter cells. Additionally, through the transfer of genetic material either by conjugation, transduction or transformation, bacteria can convey resistance genes or genetic advantages to other bacteria. Mutations are usually transferred among bacteria via this means. Bacteria may also produce spores containing their genetic material. This type of reproduction is usually a result of harsh conditions, so the spores produced are resistant to extreme environmental conditions and can remain dormant for centuries until favorable conditions return.
Many bacteria are harmless and/or even essential for human, animal, or plant life, while other forms of bacteria are pathogenic and responsible for infectious or non-infectious diseases. Some “good” bacteria include the following: *Lactobacillus acidophilus* and other *Lactobacilli* help in gut digestion of food, and *Streptococcus thermophilus* is used in the food industry for making yogurt, mozzarella cheese and other fermented dairy products. *Azotobacter*, *Azospirillum*, and *Rhizobium* bacteria fix nitrogen into the soil for plant growth.

Harmful bacteria have been known to cause havoc in living things. Infectious diseases caused by bacteria have been studied and treated with antibiotics for close to a century now. The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928. Since then, research and development of new antibiotics has grown exponentially. Unfortunately, the development of antibiotics has also resulted in the emergence of resistant strains of bacteria. Microbial resistance to the library of antibiotics available has called for new technologies and approaches for the rapid detection of drug-resistant bacteria.

### 1.1.1 Bacteria Detection Methods

Bacteria have unique qualities that differentiate individual species. The various characterization methods available for detecting and identifying specific bacteria can be grouped as phenotypic or genotypic methods. Phenotypic methods utilize information on bacterial cell morphology, growth requirements, Gram staining outcome, or the result of biochemical reactions. Bacterial identification using plate culture is a common method still in use and involves the use of selective and/or differential media. Specialized agar called selective media inhibit the growth of one type of bacteria but encourage the growth of other groups of bacteria. Phenylethyl alcohol agar is a selective media which allows the growth of Gram-
positive bacteria while inhibiting the survival of Gram-negative bacteria.\textsuperscript{16} Differential media on the other hand indicates if target bacteria are present by taking advantage of biochemical interactions between target bacteria and compounds in the agar media to produce distinct changes in the colony or surrounding media.\textsuperscript{14} For example, blood agar is a differential media containing red blood cells (RBCs). This media displays clear colonies in the presence of \textit{Streptococcus pyogenes} since the bacteria lyse RBCs.\textsuperscript{14} Certain agar such as bismuth sulfite agar can function either as a selective or differential media; bismuth sulfite agar is best for identifying Gram-negative \textit{Salmonella typhi} in fecal matter since it prevents the growth of Gram-positive bacteria and most intestinal Gram-negative bacteria.\textsuperscript{17}

Genotypic methods, as the name suggests, are techniques or procedures which utilize nucleic acid sequences of bacteria targets.\textsuperscript{12} Genotypic methods include DNA hybridization,\textsuperscript{12,18,19} whole genome sequencing,\textsuperscript{12} and DNA amplification methods (PCR and RT-PCR).\textsuperscript{12,20} Most of these methods are employed in detecting drug-resistant bacterial strains.

\subsection*{1.1.2 Antimicrobial Resistance Detection Methods}

During bacteria replication, random errors may occur leading to the development of mutations anywhere in the bacterial DNA. These mutations can be harmful or beneficial to the bacteria. A mutation in bacterial DNA can provide an advantage to the bacteria, allowing it to thrive or grow better than other bacteria under the same growth environment.\textsuperscript{4,6} Mutations can also make a bacterial population immune to specific antibiotics, resulting in antimicrobial resistance. Examples of drug-resistant bacteria include the Daptomycin-methicillin-resistant \textit{Staphylococcus aureus} which was identified a year after the antibiotic Daptomycin was released in 2003, and Ceftazidime-avibactam-resistant \textit{Klebsiella pneumoniae carbapenemase} (KPC).
identified the same year the antibiotic Ceftazidime was released in 2015.\textsuperscript{21} The global impact of antimicrobial resistance is estimated to reach 10 million annual deaths and cumulative costs of over 1 trillion dollars by 2050.\textsuperscript{22} Detection of drug-resistant bacteria can be achieved using phenotypic methods like the gold standard broth dilution,\textsuperscript{23} agar dilution and disc diffusion,\textsuperscript{23} flow cytometry,\textsuperscript{24} matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS),\textsuperscript{12} or genotypic methods like PCR,\textsuperscript{12} DNA sensors,\textsuperscript{25} and microarrays.\textsuperscript{12}

Current methods being employed include techniques involving fluorescent in situ hybridization (FISH),\textsuperscript{25} nucleic acid amplification technology (NAAT),\textsuperscript{25} DNA,\textsuperscript{26} and RNA detection or physical properties like impedance or optical density change of multi-well liquid cultures.\textsuperscript{12,27} A major challenge with conventional methods for detecting drug-resistant bacteria is that they are time inefficient, with some methods taking more than 24 h to produce assay results.\textsuperscript{28} Current methods for identifying antimicrobial resistance need to be cost-effective, fast, automatable and point-of-care capable.

\subsection*{1.2 Microfluidics}

Microfluidics involves the manipulation of fluids in 1-1000 µm (in width or height) channel dimensions with the aspiration of allowing complex laboratory bench-top processes to be scaled down into smaller devices.\textsuperscript{29} Potential benefits of microfluidics include low cost, small device footprint, point-of-care capability, small sample size requirement, and the minimal waste generation.\textsuperscript{30} For example, the confinement of sample molecules in micrometer channel dimensions has advantages for sample concentration, kinetics, temperature control, and analysis time.\textsuperscript{30,31} Most microfluidic platforms have the potential for automation, resulting in reduced
A wide range of available, low-cost manufacturing methods can be used for microfluidic devices with the potential for single use or disposable point-of-care devices. Various applications demonstrated with microfluidics include whole genome sequencing, clustered regularly interspaced short palindromic repeat (CRISPR) studies, nanomaterial synthesis, antimicrobial susceptibility testing, identification of disease biomarkers, cell and tissue studies, and drug delivery. Recently, mobile phone operated microfluidic setups or drone mounted microfluidic systems have been developed.

Microfluidics can be grouped based on the method for fabrication, materials utilized and/or applications. In this chapter, I will briefly highlight the fabrication methods, materials and applications pertaining to my research work. Additional details will be provided throughout specific sections of the dissertation where necessary.

1.2.1 Device Fabrication Methods

The intended application of a microfluidic setup dictates the appropriate base material for device manufacturing and fabrication method(s) needed. Some microfluidic devices utilize more than one material and/or fabrication method. Typical fabrication methods for microfluidic devices include computerized numerical control (CNC) micromachining, laser ablation, hot embossing, photolithography, and 3D printing. Here, I provide details on the fabrication methods I worked with or encountered during my research work: photolithography, laser ablation and 3D printing.
1.2.1.1 Photolithography

Photolithography is an integral part of integrated circuit (IC) manufacturing and requires a cleanroom environment. It is a top-down approach for pattern transfer from masks to thin films and requires UV-sensitive resins, a UV radiation source (100-400 nm wavelength), photomasks with patterned design and a mask aligner. A Si wafer or other planar support serves as the substrate for resin deposition and subsequent UV exposure under a mask aligner, as shown in Figure 1.1.

**Figure 1.1** Photolithography process for patterning a substrate.

During the UV exposure step, a pre-designed photomask is aligned with the substrate and used to block off certain sections of the substrate. Post-exposure baking further hardens the leftover resin on the substrate, which can serve as a sacrificial layer for subsequent IC processes or act as a mold for casting microfluidic devices. Positive resist contains molecules that undergo chemical reactions initiated by UV radiation resulting in soluble reaction products which come off during
development in a positive resist developer solution. Negative resist has molecules that react to form insoluble products which do not wash off during resin development in a negative resist developer solution, as demonstrated in Figure 1.1. The minimum feature size obtained is limited by photoresist properties and the wavelength of UV light used during exposure. Feature sizes as low as 5 nm are feasible with the use of extreme UV wavelengths <15 nm.

Photolithography is ideal for creating complex, detailed topography or patterns in a material, which is otherwise difficult if not impossible with other fabrication methods like laser ablation or injection molding.

1.2.1.2 Laser Ablation

Light Amplification by Stimulated Emission of Radiation (Laser) was initially proposed in the 1950s and the first laser was demonstrated in the 1960s. Laser radiation has unique characteristics of being coherent, monochromatic beams that fall within the IR or UV/Vis regions of the electromagnetic spectrum. A typical laser unit consists of an active medium, power supply and optical cavity. The laser beams are either operated in continuous wave or pulsed modes. Continuous wave laser outputs are uninterrupted beams of light. An example of this type of laser is the CO₂ laser which has an active medium of helium and nitrogen with CO₂ as the lasing material. In pulsed lasers, the laser output is emitted as short, high-energy pulses of nanosecond or shorter durations.

Laser ablation utilizes laser energy to break down the structure in a solid substrate, thus producing aerosol products of substrate atoms and/or molecules. Laser ablation is often used for subtractive manufacturing. In subtractive manufacturing the laser ablation process is used to
remove sections of the solid substrate to create unique shapes. The minimum feature size produced is dependent on the laser spot size and other instrument settings like laser power, cutting speed and pulse duration.\textsuperscript{44} In chapter 2 of this dissertation, I used laser ablation in subtractive manufacturing of microfluidic chips.

1.2.1.3 3D Printing

Another fabrication technique currently gaining prominence in microfluidics is 3D printing.\textsuperscript{32} 3D printing is a bottom-up fabrication method which builds 3D shapes layer by layer from the base up. Depending on the type of 3D printer, the designed object can be printed either by polyjet, stereolithography, or fused deposition modeling.\textsuperscript{32} A 3D printer can cost a few hundred to a few hundred thousand dollars. Several resources are available in the literature highlighting the various 3D printing methods mentioned.\textsuperscript{32,45,46} Some advantages associated with 3D printing are the ease of revising designs, the lack of a cleanroom environment requirement, and the automated nature of the printing process that limits the need for skilled personnel. Complex device structures are possible due to the bottom-up fabricating style of 3D printing. Microfluidic platforms with 3D printed components have been used for bacteria preconcentration and DNA purification,\textsuperscript{47} antimicrobial susceptibility testing (AST),\textsuperscript{48} detection of pathogenic bacteria,\textsuperscript{49} and label-free counting of bacterial cells.\textsuperscript{50}
1.2.2 Materials

Various materials suitable for fabricating microfluidic devices are available. Microfluidic devices fabricated from glass, thermoplastics, paper, ceramics, metals, silicon, quartz, and 3D printing resins have been demonstrated in the literature. Herein, I focus on the go-to materials utilized in the fabrication of microfluidic devices used for bacterial analysis.

1.2.2.1 Thermoplastics

Thermoplastics melt at accessible temperatures and can be shaped using embossing, injection molding or extrusion. The chemical composition is made up of covalently bonded polymer chain molecules with or without side branches. The ordered nature of the polymer chains distinguishes crystalline and amorphous thermoplastics from each other, with crystalline thermoplastics having ordered polymer chains. Thermoplastics are available naturally from plants, synthesized from petroleum products or engineered in the laboratory and sold under various brand names at cost-effective prices. Commonly used thermoplastics for microfluidic devices include polyurethane, cyclic olefin copolymer, polycarbonate, polymethyl methacrylate (PMMA), and polypropylene.

PMMA is a laboratory engineered thermoplastic originally developed in the 1920s. It is formed from the polymerization of methyl methacrylate and sold globally under numerous brand names such as Plexiglas, Acrylite, Perspex, and Lucite. PMMA is low density (1.17–1.20 g/cm³), durable, transmits >90% visible light and can be made into microfluidic devices using a wide range of fabrication methods like hot embossing, laser ablation and micromilling. Thermal bonding is the most common technique for bonding PMMA to other PMMA surfaces,
thermoplastics, or pressure sensitive adhesives (PSAs).\textsuperscript{26,54} The bonding process involves heating PMMA sheets to near the glass transition temperature ($T_g$ between 430 to 460 K),\textsuperscript{54} then holding the PMMA sheet and substrate together using pressure during the cooling stage for effective bonding, as shown in Figure 1.2. Other bonding techniques involve ultrasonic welding, solvent bonding or surface modification.\textsuperscript{54}

![Figure 1.2](image)

**Figure 1.2** Hot embossing and thermal bonding for PMMA sheets.

### 1.2.2.2 Polydimethylsiloxane

Polydimethylsiloxane (PDMS) is an elastomeric material that is heavily used for microfluidic research. PDMS is chemically inert, optically transparent, moderately thermally conductive, and gas permeable. This makes it a suitable substrate for biological studies and other applications requiring inert environments. The PDMS silicone encapsulants are provided as two-part liquid components, silicone elastomer base and curing agent, which are typically mixed in a 10:1 ratio, degassed, poured over a mold, and cured at room temperature or heat cured for pattern transfer.\textsuperscript{55}

The patterned PDMS is bonded to other solid substrates like glass, PDMS or silicon by plasma bonding.\textsuperscript{56} Air/O$_2$ plasma activates the surfaces of the substrate leaving silanol (Si-OH) groups, as shown in Figure 1.3. After plasma treatment, the two surfaces are quickly placed in contact with each other to form an irreversible Si-O-Si covalent bond at the interface.\textsuperscript{56}
Figure 1.3 Plasma bonding for PDMS microchips.

PDMS swells in the presence of organic solvents and absorbs small molecules and proteins.\textsuperscript{55}
Some applications also require pretreatment or coating of PDMS microchannels to minimize nonspecific adsorption or alter the hydrophobicity of channel walls.\textsuperscript{55}

1.3 Microfluidic Processes and Components for Bacterial Analysis

As discussed earlier, bacterial analysis can be accomplished through genotypic and phenotypic assays. Microfluidics provides unique approaches and techniques for studying bacteria and their molecular components. Microfluidic systems utilizing streptavidin magnetic beads,\textsuperscript{57} dielectrophoresis,\textsuperscript{58} nanoporous membranes,\textsuperscript{59} or immunoaffinity microbead columns\textsuperscript{60} have been developed for detecting sepsis-related bacteria, separation of bacteria from blood, bacteria capture, or enrichment respectively.\textsuperscript{60} Microfluidic systems for antimicrobial susceptibility testing have been developed.\textsuperscript{28,31,61} One such system was equipped with a droplet generating setup, multiple droplet docking stations each with > 8000 droplet docking sites capable of trapping droplets containing 1–4 bacteria cells with antibiotics, and the bacteria
response was monitored over 2 h duration. Herein, I focus on the process of droplet microfluidics and on polymer monolith components that are used in this dissertation.

1.3.1 Droplet Microfluidics

As the name suggests, droplet microfluidics involves the generation and manipulation of discrete droplets in microfluidic channels. Droplet microfluidics exploits the immiscible nature of organic and aqueous solvents to generate monodisperse water-in-oil (W/O) or oil-in-water (O/W) droplets. Each droplet can act as a microreactor unit and can be filled with reagents for chemical and/or biological reactions. Droplets can be a few hundred to millions in number, have nanoliter-to-picoliter volumes and can be mixed, undergo fusion or fission, or be sorted to different sections of a droplet microfluidic device. These physical manipulations of droplet(s) are achieved through the incorporation of unique geometries within the channel designs. A key advantage of droplet microfluidics is the high number of data sets available for analysis. Droplet microfluidics are therefore high-throughput, and multiple bacteria studies can be carried out in parallel experiments. The confinement of reagents to nanoliter or picoliter volumes encourages diffusion and heat transfer, resulting in faster processes.

Single-cell studies using droplet microfluidics are being explored by numerous labs globally for single cell analysis and study of bacterial resistance. Droplet microfluidics at the single cell level has been used to screen for bacterial heteroresistance, a phenomenon in which susceptible bacteria populations harbor resistant subpopulations. The screening approach focused on the antibiotic concentration per bacterium and not the absolute antibiotic concentration. This same lab monitored the growth profile of both Gram-positive and Gram-negative bacterial strains in single-cell experiments, and utilized a label-free detection method.
relying on the intensity of scattered light from each droplet when hit with a laser beam. In single-cell experiments detecting *E. coli* cells in milk samples, a team of researchers demonstrated that droplets could retain their size to within >90% when immobilized during the duration of incubation with the use of a thermosetting liquid as the continuous phase. A microfluidic “SlipChip” device capable of direct extraction and preconcentration of bacteria from whole blood prior to droplet encapsulation for ASTs has been developed.

Through single-cell bacterial analysis, chemical or biochemical information from individual bacteria cells can be monitored and studied, thus eliminating limitations associated with ensemble-averaged data from multiple cells. The early diagnosis of bacteria and their resistant strains becomes probable since individual bacteria cells are separated from their neighbors, and abnormalities in the growth profile of droplet-encapsulated single bacteria from possible mutations can be separated and investigated through droplet sorting techniques.

1.3.2 Polymer Monoliths

Monoliths are porous cross-linked organic polymers. Monoliths are commonly prepared using various percentage compositions of one or more monomers, crosslinker, initiator and porogenic solvents. Premade monoliths can be introduced into microchannels directly through a complex process, or prepared via the common in-situ process which involves introducing the monolith mixture into the microchannel and irradiating the device for monolith polymerization. During the polymerization process, the initiator begins a radical polymerization reaction involving the monomer(s) and crosslinker molecules. The porogenic solvents occupy spaces within the polymer and are flushed out post-polymerization using vacuum pressure or solvents. This creates free volume within the monolith and is responsible for
the porosity of the monoliths. The porous nature of column monoliths provides high surface area, and the reduced back pressure permits the use of simple fluid flow systems compared to packed columns in chromatography. Structural characterization of monoliths can be achieved using the scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Electron microscopy images can also provide information on the pore and nodule sizes of the monolith.

Monoliths have been exploited for a wide range of applications including analyte separation, preconcentration or detection. Porous silica monoliths within microfluidic devices for isolation of bacteria from whole blood have been demonstrated. The strong monolith adhesion to microchannel walls provide large cross-sectional area for high-throughput analysis. Other labs have demonstrated the use of porous organic monoliths in microfluidic systems for monitoring bacterial cell-to-cell communication.

Microfluidics has been used to screen for drug-resistant bacteria and their genes. However, several challenges persist which I address in this dissertation. In genotypic attempts to detect bacterial genes, earlier monolith microfluidic systems utilized materials that required expensive, time-inefficient fabrication methods such as hot embossing. Furthermore, droplet microfluidic methods developed for phenotypic antimicrobial resistance testing either lose droplet traceability, require high levels of fluorescence reporter molecules, lack single cell studies, or require offline incubation and monitoring. The work presented in this dissertation advances knowledge for bacteria detection via DNA hybridization using polymer monoliths, multiplex detection of bacterial DNA, and droplet microfluidic analysis of bacteria.
1.4 Dissertation Overview

Microfluidics provides time-efficient, cost effective and point-of-care solutions to dealing with many challenges relying on benchtop solutions. Microfluidic techniques for studying antimicrobial resistance are still being explored. This dissertation focuses on microfluidic devices and techniques for genotypic and phenotypic diagnosis of antimicrobial resistance.

In Chapter 2, I describe the development of simple, robust microdevices fabricated using a time-efficient, inexpensive technique. These microfluidic devices were used to demonstrate sequence-specific capture and detection of picomolar concentrations of bacterial plasmid DNA harvested from cultured bacteria.

In Chapter 3, I present an improved PMMA-PSA microfluidic devices with monolithic columns modified with two different DNA capture sequences, and used to demonstrate selective capture, fluorescent labeling, and multiplex detection of multiple bacteria gene targets.

In Chapter 4, I develop a microfluidic setup capable of studying single bacteria growth in W/O droplets. Droplet-encapsulated *E. coli* were incubated with nanomolar concentrations of a fluorescence reporter and detected via laser-induced fluorescence after convenient room temperature 2-h incubation conditions.

In Chapter 5, I summarize the outcomes from the earlier chapters and provide guidance on future projects worth pursuing in relation to the work described in this dissertation.
1.5 References


47. Abafogi AT, Kim J, Lee J, Mohammed MO, van Noort D, Park S. 3D-Printed Modular Microfluidic Device Enabling Preconcentrating Bacteria and Purifying Bacterial DNA in Blood
for Improving the Sensitivity of Molecular Diagnostics. Sensors. 2020;20:1202.
doi:10.3390/s20041202

doi:10.1007/s42242-021-00173-0

microfluidic chip biosensor detection of foodborne pathogenic bacteria: a review. Anal Bioanal

50. Duarte LC, Figueredo F, Ribeiro LEB, Cortón E, Coltro WKT. Label-free counting of
Escherichia coli cells in nanoliter droplets using 3D printed microfluidic devices with integrated
doi:10.1016/j.aca.2019.04.045

51. Niculescu A-G, Chircov C, Bîrcă AC, Grumezescu AM. Fabrication and Applications of

52. Asim M, Jawaid M, Saba N, Ramengmawii, Nasir M, Sultan MTH. Processing of hybrid
polymer composites—a review. In: Hybrid Polymer Composite Materials. Woodhead

53. Zhou W, Dou M, Timilsina SS, Xu F, Li X. Recent innovations in cost-effective polymer and

54. Sivakumar R, Lee NY. Microfluidic device fabrication mediated by surface chemical


58. Yoon T, Moon HS, Song JW, Hyun KA, Jung HI. Automatically Controlled Microfluidic System for Continuous Separation of Rare Bacteria from Blood. Cytometry A. 2019;95:1135-44. doi:10.1002/cyto.a.23909


69. Han JY, Wiederoder M, DeVoe DL. Isolation of intact bacteria from blood by selective cell lysis in a microfluidic porous silica monolith. Microsyst Nanoeng. 2019;5:30. doi:10.1038/s41378-019-0063-4

70. Nischang I, Causon TJ. Porous polymer monoliths: From their fundamental structure to analytical engineering applications. TrAC. 2016;75:108-117. doi:10.1016/j.trac.2015.05.013
CHAPTER 2. RAPID AND SIMPLE PRESSURE-SENSITIVE ADHESIVE MICRODEVICE FABRICATION FOR SEQUENCE-SPECIFIC CAPTURE AND FLUORESCENCE DETECTION OF SEPSIS-RELATED BACTERIAL PLASMID GENE SEQUENCES*

2.1 Introduction

Microbial resistance to antibiotics is a major threat to global healthcare\textsuperscript{1,2} with an estimated 10 million deaths and economic impact of over 1 trillion US dollars by the year 2050.\textsuperscript{3} Effective treatment of these infections requires knowledge of what antibiotics the bacteria are susceptible to, through ASTs. Genotypic and phenotypic ASTs are currently used in detecting drug-resistant bacteria. Phenotypic ASTs provide specific resistance data but require hours to several days for bacterial growth, identification, and susceptibility testing, delaying effective antimicrobial therapy.\textsuperscript{4,5} Genotypic ASTs can be faster but require advance knowledge of the gene, utilize expensive equipment, and often need bacterial culture prior to testing, all of which hinder effective treatment.\textsuperscript{2,6,7} Here, I focus on genotypic testing and address some of its limitations. ASTs that are inexpensive, simple, rapid, and effective should help to improve treatment of bacterial infections.

Microfluidics are an emerging platform for DNA analysis,\textsuperscript{8–11} allowing sample processing, fluid handling, and analysis to be automated and simplified.\textsuperscript{12} Microfluidic sequence-specific DNA capture can be valuable when coupled with downstream processes such as PCR,\textsuperscript{13} single-molecule counting,\textsuperscript{14} or next-generation sequencing.\textsuperscript{15} Noviana et al.\textsuperscript{16} described an assay

\*This chapter is adapted with permission from: Akuoko, Y.; Hanson, R. L.; Harris, D. H.; Nielsen, J. B. Lazalde, E.; Woolley A. T. Rapid and simple pressure sensitive adhesive microdevice fabrication for sequence-specific capture and fluorescence detection of sepsis-related bacterial plasmid gene sequences; \textit{Analytical and Bioanalytical Chemistry}. \textbf{2021};413:1017-25.
for sequence specific DNA capture in a microcentrifuge tube and utilized a paper-based lateral flow assay for colorimetric detection. However, the long device fabrication time and the use of a detection method known to produce false positives in field experiments due to humidity changes limit potential widespread use. Shokoufi et al.\textsuperscript{17} reported DNA hybridization using unmodified gold nanoparticles in a glass microchip. However, they utilized brittle materials and equipment intensive microfabrication techniques for channel formation and enclosure, and devices resulted in the detection of modest, nanomolar concentrations of synthetic target DNA. Wang et al.\textsuperscript{18} demonstrated sequence-specific capture, isolation, and separation of target ssDNA from non-targets across two reservoir wells connected by a tapering microchannel in a PDMS microchip. Although the microfluidic format was desirable, significant nonspecific ionic interactions and adsorption, likely due to the device material, were reported.

In prior studies on DNA capture performed in my lab,\textsuperscript{19} porous polymer monoliths formed in a channel in polypropylene microdevices allowed extraction of KPC DNA from bacterial lysate purified from spiked whole blood. Although that work demonstrated proof of concept, the hot embossing and thermal bonding of polypropylene posed challenges in terms of fabrication time and complexity, as well as device yields.

Simpler and faster DNA extraction microdevice fabrication could be desirable for timely identification of drug-resistant bacteria, leading to treatment. The ease and versatility of laser micromachining have led to its use in the fabrication of microdevices.\textsuperscript{20,21} The most common method for enclosing laser-cut polymer microchannels is thermal bonding to various materials,\textsuperscript{22–25} requiring long bonding processes to achieve adhesion, with the additional tendency to undergo distortion or breakage.\textsuperscript{24,25} An alternative channel enclosure approach uses PSAs, which are coated onto one or both sides of a flexible polymeric material. PSAs have been used to enclose
microchannels because of simplicity, versatility, and rapid processing. Bazaz et al. fabricated an inertial microfluidic device with 3D printed microchannels bonded to PMMA sheets using double-sided adhesive tape and successfully demonstrated particle/cell separation and focusing. Kinahan et al. developed an integrated liver assay using a lab-on-a-disk fabricated from PMMA sheets and PSAs. Serra et al. investigated how factors such as adhesive tape structure, adhesive thickness, and bond time affect PSA bond quality and strength on various polymeric substrates including PMMA, polycarbonate, and cyclic olefin copolymer. Some downsides with the PSAs utilized are their poor optical quality, lack of robustness, limited bond strength, and chemical incompatibility.

Herein, I utilized simple, robust microdevices fabricated using a time-efficient, inexpensive fabrication technique to demonstrate sequence-specific capture and detection of picomolar (pM) concentrations of bacterial plasmid DNA harvested from cultured bacteria. I created microdevices from laser-micromachined PMMA microchannels enclosed using a PCR compatible optical adhesive tape PSA. I further demonstrated the formation of monoliths with capture oligonucleotides in these devices. I performed sequence specific DNA capture, fluorescent labeling, and laser induced fluorescence (LIF) detection of antibiotic resistance gene targets in these devices. The simplicity of the fabrication method and performance of these microdevices highlight the general applicability of this approach, particularly in front-end sample preparation for single-particle counting or other assays.
2.2 Materials and Methods

2.2.1 Chemicals and Materials

The following reagents were used in the preparation of 20 mM Tris-HCl hybridization buffer (pH 8): tris(hydrochloride), tris(hydroxymethyl)aminomethane, sodium chloride, and magnesium chloride. To prepare monoliths, I used benzoin methyl ether (BME), ethylene dimethacrylate (EDMA), poly(ethylene glycol)diacrylate (PEGDA, MW 575), 1-dodecanol, and 2-propanol. All reagents used for buffer and monolith preparation were obtained from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All chemicals were analytical grade purity or higher. Hybridization buffer and deionized water (18.3 MΩ) were autoclaved and sonicated before use.

PMMA sheets, optical adhesive films (PSAs), and microfluidic connectors used for devices were obtained from Plaskolite (Columbus, OH), Applied Biosystems (Foster City, CA), and Idex Health & Science (Bristol, CT), respectively. DNA oligonucleotides were purchased from Eurofins (Louisville, KY), and bacterial plasmid vectors were obtained from Twist Bioscience (San Francisco, CA). Temperature control was achieved using a thermoelectric module (VT-31-1.0-1.3, TE Technology, Traverse City, MI) operated by a TE Technology controller (TC-48-20) and control thermistors (MP-2444).

2.2.2 Microfluidic Device Design

The microdevice channels were designed (see Fig. 2.1) using AutoCAD (Autodesk, San Rafael, CA) and formed into 4.0 cm× 9.0 cm× 0.3 cm PMMA blocks using a CO2 laser cutter (VersaLaser VLS3.50, Universal Laser Systems, Scottsdale, AZ) with the following settings: 70% laser power, 10% cutting speed, and 1000 pulses per inch. The microchannel was 0.2 mm
wide at the top and 1.6 mm deep. For channel enclosure, the PMMA was heated at 100 °C for 2 min on a benchtop laboratory press (Model 4120, Carver, Wabash, IN), after which an optical adhesive film (4360954, Applied Biosystems) was laminated onto the channel side of the PMMA at 2 bar for 2 min. The microdevice was left to cool to ambient temperature over ~6 min to avoid thermally induced stress.

![Microdevices and monoliths.](image)

**Figure 2.1** Microdevices and monoliths. (a) Schematic view of a PMMA—PSA microfluidic device. (b) Top-view photograph of a PMMA—PSA microfluidic device with monolith. (c) Low-magnification cross-sectional SEM image of a PMMA—PSA channel with a monolith. (d) High magnification cross-sectional SEM image of a monolith showing its porous nature.

Total cost per device was ~1 US dollar, with the optical adhesive film comprising most of the price; laser cutters can be obtained for a few thousand US dollars. Microfluidic connectors were then attached to the inlet and outlets of the PMMA—PSA microdevice using Solarez UV resin (Vista, CA) and cured under UV light for 2 min.
2.2.3 Monolith Preparation

The monolith was prepared using a single-step photopolymerization method outlined in previous work,\textsuperscript{19} with minor modifications. In brief, the microdevice was filled with a polymerization mixture comprised of (all w/w): 3% BME, 18.5% PEGDA, 18.5% EDMA, 14% 2-propanol, 41.5% 1-dodecanol, and 4.5% 500 μM acrd-NDM (in 1:1 2-propanol:water, see Table 2.1). The entire microdevice surface was masked with black tape except for a 0.25-cm opening in the straight section of the Y-shaped channel (Fig. 2.1a). The device was placed under a SunRay 600 UV lamp (Uvitron International, West Springfield, MA) at 100 mW/cm\textsuperscript{2} for 5 min, which formed the monolith in the microchannel. The monolith-modified microdevice was then connected via polytetrafluoroethylene tubing to a Fluigent pressure-driven pumping and valving system (Lowell, MA) and solvent reservoirs. After formation, the functionalized monolith was rinsed with 2-propanol for 10 min and deionized water for 5 min. Cross-sectional SEM images of the functionalized monoliths were obtained using an Apreo scanning electron microscope (Thermo Fisher, Waltham, MA).

2.2.4 DNA Design

For extraction and detection of antibiotic resistance genes, the New Delhi metallo-beta lactamase (NDM) and KPC genes were selected. The NDM sequences in Table 2.1 were subjected to NCBI nucleotide database search using a BLAST tool.
Table 2.1 DNA oligonucleotide sequences.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrd-NDM</td>
<td>Acrd-ACCCAGGTTTAGACTCACTTGAGCAGTCGGTCGCAGA GGTCGGAGAC[sp18]GCAGCACACTTCCTATTCGACAT</td>
</tr>
<tr>
<td>NDM</td>
<td>CATCCCTGACGATCAAAACCGTTGGAAGCGACTGCCCAGAA ACCCGGCATGTCGAGATAGGAAGTGTGCTGC</td>
</tr>
<tr>
<td>KPC</td>
<td>TATCGCCGTCTAGTTCTGCTCTTGCTCTTCATGGCGCTG GCTGCTTTCTGCCACCGCGCTGACCAACCTCGTCCGGA ACCATTCGCTAAACTCGAACAGGACTTTG</td>
</tr>
<tr>
<td>NDM hybridization probe</td>
<td>Fl-GCTCGACAGTCCCTTCCAACGGTTTGATCGTCAGGGATG TCGAGC-Fl</td>
</tr>
</tbody>
</table>

Abbreviations: Acrd = acrydite, [sp18] = 18 carbon atom spacer, Fl = fluorescein

The capture sequence for the NDM gene (see Table 2.1) is adapted from previous work,\(^\text{19}\) the acrd-NDM DNA is attached to the monolithic column during polymerization via the acrydite functional group. A 50-base sequence, located at the 5’ end of the capture oligonucleotide, and an 18-carbon spacer keep the NDM capture oligonucleotide away from the solid support for DNA hybridization. The synthetic NDM target (71 bp) is 47 bp longer than the NDM hybridization sequence (24 bp), such that a section of the target oligonucleotide hybridizes to the capture DNA leaving the unpaired bases available for hybridization with the complementary hybridization probe (see Table 2.1). Melting data for the NDM-related sequences (Table 2.1) was obtained by using a PCR instrument to analyze the melting temperatures of both NDM capture-target and NDM target-probe duplexes.

The NDM sequence was synthesized and inserted into a proprietary cloning vector (pTwist Amp High Copy, Twist Bioscience), which was provided as plasmid DNA. For plasmid control experiments, I used KPC as the insertion sequence. Full plasmid insert sequences for NDM and KPC are given in Table 2.1. I then introduced the plasmid DNA into \textit{E. coli} cells
using the New England Biolabs (Ipswich, MA) standard transformation protocol. The *E. coli* cells containing the plasmid DNA with the NDM gene were stored in a screw cap microcentrifuge tube at −77 °C until usage. Similar processes were followed in obtaining plasmid DNA with the KPC gene and subsequent insertion into *E. coli* cells.

### 2.2.5 Bacterial Growth and Plasmid Sample Preparation

*E. coli* cells containing plasmid DNA were inoculated into 50 mL lysogeny broth containing 100 μg/mL ampicillin using a 10-μL inoculating loop (Fisher Scientific), all in a 50-mL conical centrifuge tube. The centrifuge tube was loosely capped and left overnight in an incubator-shaker (Benchmark Scientific, Edison, NJ) at 37 °C and 200 rpm for aerobic growth of *E. coli* cells. After incubation, the plasmid DNA in the *E. coli* cells was extracted into a microcentrifuge tube using a QIAprep Spin Miniprep Kit (Hilden, Germany) with minor protocol modifications. In the final extraction step using Buffer EB (10 mM Tris-Cl, pH 8.5), instead of a single, 50-μL extraction, I performed multiple extractions in parallel with 10 μL of Buffer EB on each QIAprep 2.0 spin column, then pooled the eluents together. This adaptation allowed me to extract more plasmid DNA from the *E. coli* cells than by the single extraction approach. Plasmid DNA was then stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Plasmid DNA was digested with EcoR1-HF (20 U/ml, R3101S, New England Biolabs, Ipswich, MA) by incubating at 37 °C for 30 min in an incubator-shaker at 200 rpm. Linearization of plasmid DNA occurred because there is only one EcoR1 restriction site in the circular plasmid. After incubation, the microcentrifuge tube containing the enzyme-digested plasmid DNA was heated in a water bath at 65 °C for 30 min to stop further enzyme activity. The linearized plasmid DNA
samples were then stored at 4 °C. The plasmid DNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher).

2.2.6 Operation of the Microfluidic Setup and Fluorescence Measurement

The setup and procedure for carrying out DNA hybridization and LIF measurements in the microdevice were adapted from prior work. In brief, the fluid control and detection for the microdevice consisted of a Fluigent pump system, electronics for temperature control, and an optical system for LIF measurements. The optical system consisted of a solid-state 488 nm laser (CrystaLaser, Reno, NV), optical filters, and a photomultiplier tube (PMT). The PMT output was filtered and amplified before being recorded and displayed with a LabVIEW (National Instruments, Austin, TX) program. I did a 9-point boxcar average on the LIF data to reduce noise. Calculation of elution peak areas was performed using a custom MATLAB code (see Table 2.2).

The fluid reservoir connected to the microfluidic device was initially filled with hybridization buffer that was flowed through the microdevice, thus saturating the monolith with pH 8 hybridization buffer. Solution was initially loaded into the microdevice through the sample inlet and out through outlet 1 (see Fig. 2.1a). I then closed outlet 1 and opened outlet 2 to flow the solution through the monolith. The monolith was kept at 45 °C by placing a thermoelectric heater directly on top of the monolith section of the microdevice (see Fig. 2.1a). The average (± standard deviation, n = 20) flow rate through devices was 7 ± 1 μL/min at a pressure of 200 mbar.
Table 2.2 MATLAB code for calculating fluorescence signal peak area.

<table>
<thead>
<tr>
<th>MATLAB Code for Baseline Subtraction</th>
<th>MATLAB Code for Determining Peak Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>function [m b sm sb sy] = fitline(x,y)</td>
<td>%data to change</td>
</tr>
<tr>
<td>%written by Marie Killian May 2012</td>
<td>data=csvread('filename.csv');</td>
</tr>
<tr>
<td>%edited June 5, 2012</td>
<td>t=insert; %time increment between points</td>
</tr>
<tr>
<td>%Accepts vectors x and y and fits a line to the data</td>
<td>signal=data(:,insert excel column number with the</td>
</tr>
<tr>
<td>with slope m,</td>
<td>signal data);</td>
</tr>
<tr>
<td>%intercept b, and standard deviations of the fit sm,</td>
<td>%don't change this part</td>
</tr>
<tr>
<td>sb, and sy.</td>
<td>time=data(:,insert excel column number with the</td>
</tr>
<tr>
<td>if size(x,1) == 1</td>
<td>time data);</td>
</tr>
<tr>
<td>x = x';</td>
<td>plot(time,signal);</td>
</tr>
<tr>
<td>end</td>
<td>f=ginput(4);</td>
</tr>
<tr>
<td>if size(y,1) == 1</td>
<td>close</td>
</tr>
<tr>
<td>y = y';</td>
<td>tdiff=abs(f(1,1)-time);</td>
</tr>
<tr>
<td>end</td>
<td>[~,I]=min(tdiff);</td>
</tr>
<tr>
<td>if size(x,2) ~= 1</td>
<td></td>
</tr>
<tr>
<td>error('x and y must be vectors')</td>
<td>[~,I2]=min(tdiff2);</td>
</tr>
<tr>
<td>end</td>
<td>tdiff3=abs(f(3,1)-time);</td>
</tr>
<tr>
<td>X = [x , ones(length(x),1)];</td>
<td>[~,I3]=min(tdiff3);</td>
</tr>
<tr>
<td>[fit bint r] = regress(y,X);</td>
<td>tdiff4=abs(f(4,1)-time);</td>
</tr>
<tr>
<td>m = fit(1);</td>
<td>[~,I4]=min(tdiff4);</td>
</tr>
<tr>
<td>b = fit(2);</td>
<td>xfit=cat(1,time(I:I2),time(I3:I4));</td>
</tr>
<tr>
<td>n = length(y);</td>
<td>yfit=cat(1,signal(I:I2),signal(I3:I4));</td>
</tr>
<tr>
<td>sy = sqrt(sum(r.^2)/(n-2));</td>
<td>[m,b]=fitline(xfit,yfit);</td>
</tr>
<tr>
<td>sm = sy<em>sqrt(n/(n</em>sum(x.^2)-sum(x)^2));</td>
<td>bsignal=signal-(m*time+b);</td>
</tr>
<tr>
<td>sb = sy<em>sqrt(sum(x.^2)/(n</em>sum(x.^2)-sum(x)^2));</td>
<td>plot(time,bsignal);</td>
</tr>
<tr>
<td>end</td>
<td>g=ginput(2);</td>
</tr>
<tr>
<td></td>
<td>btdiff=abs(g(1,1)-time);</td>
</tr>
<tr>
<td></td>
<td>[~,I5]=min(btdiff);</td>
</tr>
<tr>
<td></td>
<td>btdiff2=abs(g(2,1)-time);</td>
</tr>
<tr>
<td></td>
<td>[~,I6]=min(btdiff2);</td>
</tr>
<tr>
<td></td>
<td>peak=bsignal(I5:I6);</td>
</tr>
<tr>
<td></td>
<td>area=trapz(peak)*t</td>
</tr>
</tbody>
</table>

2.2.7 DNA Oligonucleotides

In hybridization experiments involving DNA oligonucleotides, 100 μL of synthetic NDM target DNA (100, 300, or 500 pM) was flowed through the functionalized monolith, followed by 100 μL of 5 nM NDM hybridization probe (see Table 2.1 for sequences). The monolith was then
rinsed of excess probe with hybridization buffer. Thereafter, the flow of buffer was paused, during which the monolith was heated to 65 °C for 3 min for DNA de-hybridization. The buffer flow was resumed, and the eluted DNA was detected using the LIF optical setup.

2.2.8 Bacterial Plasmid DNA

For extraction and detection of antibiotic resistance genes, the NDM and KPC genes were selected. The NDM sequences in Table 2.1 were subjected to NCBI nucleotide database search using a BLAST tool. Both the capture and hybridization probe sequences (Table 2.1) were run on NCBI’s Standard Nucleotide Blast Tool. Based on the BLAST hits, the capture sequence is specific to the NDM gene with 100.0% Identity. In the case of the hybridization probe, 64% of the BLAST results reported 100.0% Identity while 36% of the BLAST results reported 97.2% Identity for the NDM gene. The capture sequence for the NDM gene (see Table 2.1) is adapted from previous work; the acrd-NDM DNA is attached to the monolithic column during polymerization. Plasmid DNA samples were prepared from stock solutions and 20 mM Tris-HCl hybridization buffer. For hybridization experiments involving NDM bacterial plasmids, 100 μL of the plasmid DNA sample (100, 300, or 500 pM) was heated for denaturation at 94 °C for 4 min, and immediately quenched in an ice bath. The fast cooling causes misalignment of denatured DNA strands, thus preventing full renaturation. The plasmid DNA sample was subsequently flowed through the functionalized monolith, followed by 100 μL of 5 nM NDM hybridization probe. Excess hybridization probe was rinsed off and the monolith heated to 65 °C for 3 min before elution of the captured plasmid DNA.
2.2.9  **Negative Control**

To confirm sequence-specific capture of DNA on functionalized monoliths, 100 μL of synthetic or plasmid KPC target (500 pM) was loaded on the monolith as above. The hybridization probe and subsequent rinsing, heating, and elution steps were then performed along with LIF detection as in earlier experiments.

2.3  **Results and Discussion**

2.3.1  **Microfluidic Device and Monolith Preparation**

A photograph of a completed device is shown in Fig. 2.1b. The laser ablation of PMMA formed microchannels with an approximate “V” shape and steep edges that converged at a depth of ~ 1.6 mm, as seen in Fig. 2.1c and Fig. 2.2.

![Figure 2.2](image)

**Figure 2.2** Additional high magnification cross-sectional SEM images of monoliths in PMMA—PSA microdevices.

During fabrication of PMMA–PSA microdevices, I heated at 100 °C for 2 min for strong adhesion between the PSA and PMMA. Multiple different types of PSA films were evaluated and several provided comparable performance, such that this approach is not dependent on a
single film supplier. The heating step also annealed any cracks formed in PMMA during the laser ablation process and protected the PMMA against solvent during monolith preparation. The total time spent in laser micromachining PMMA (2 min), heating (2 min), laminating with PSA (2 min), cooling (6 min), attaching microfluidic connectors (1 min), and UV curing (2 min) was \(~15\) min. The Y-shaped microchannel design made it possible to easily switch solutions being flowed and avoided introducing air bubbles into the monolithic column during these solution changes. The function of the monolith is to provide a porous support and anchor for the capture DNA sequence for hybridization. In a previous study,\textsuperscript{19} a polymerization time of 12 min for a 5-mm monolithic column was utilized in a polypropylene device. However, I found that a 12-min polymerization time resulted in delamination at the PMMA–PSA interface directly above the monolith, likely due to the difference in materials from the previously used polypropylene. I therefore studied shorter polymerization times to decrease delamination without compromising adhesion of the monolith to the channel walls. I found that a polymerization time of 5 min provided the best outcome. I used a 2.5-mm monolithic column to decrease the back pressure relative to prior studies,\textsuperscript{19} thus enabling multiple experiments to be performed on a single microdevice without delamination. The adhesion of the monolith to the PMMA and PSA and its porous nature (see Fig. 2.1c, d) ensured that fluid flowed through for hybridization. I measured the mean (\(\pm\) standard deviation, \(n = 60\)) pore and nodule sizes in the monoliths to be \(3.7 \pm 1.7\) and \(1.1 \pm 0.1\) \(\mu m\), respectively.

2.3.2 On-Chip Hybridization Experiments for Oligonucleotides and Plasmid DNA

I used the NDM gene as a model system to demonstrate sequence-specific capture and elution of nucleic acid material from a complementary capture oligonucleotide immobilized in a
monolith in a PMMA–PSA microdevice. Figure 2.3 shows fluorescence detected after the monolith (see Fig. 2.1a) during all analysis steps from sample loading through elution, as well as the set point temperature for the monolith heater. The initial flow of 100 μL of the NDM target through the functionalized monolith produced no increase in fluorescence, because the target had no fluorescent label.

![Figure 2.3](image)

**Figure 2.3** Time profile illustrating fluorescent signal and temperature for on-chip capture of a 300 pM synthetic NDM target, labeling with 5 nM NDM hybridization probe, rinsing and elution.

The background fluorescence decreased slightly over time, likely due to minor photobleaching of the device material as previously reported in some microfluidic devices. The sharp dips in signal (created by transiently blocking light to the PMT) at ~12 and 34 min (after loading target DNA and rinsing hybridization probe, respectively) served as time stamps, indicating a transition between steps. The labeling step of flowing 100 μL of the NDM probe through the functionalized monolith produced a gradual increase in fluorescence signal which plateaued at ~ 0.8 relative fluorescence unit (RFU). The subsequent rinse step removed excess, unhybridized labeling probe, which resulted in a gradual decrease in fluorescent signal to
background by ~33 min. At this point, I paused the flow of hybridization buffer and heated the monolith for 3 min to 65 °C. When flow restarted, it eluted the fluorescently labeled nucleic acid, detected as the peak at ~36 min.

Figure 2.4 Melting curve data for the DNA sequences in Table 2.1. (a) Melting curve for the capture-target DNA duplex. (b) Derivative plot of the melting curve in (a) (Tm = 79 °C). (c) Melting curve for the target-probe DNA duplex. (d) Derivative plot of the melting curve in (c) (Tm = 77°C).

Melt curve analyses of the NDM sequences in Table 2.1 (see Fig. 2.4) indicated similar melting temperatures for the capture-target and target-probe DNA duplexes, such that both duplexes may be denatured during the heating step prior to elution. Experimentally, appreciable signal from DNA denaturation was observed (e.g., Fig. 2.3) for elution heater settings that were lower than the measured melting temperatures, which could be attributed to buffer solution differences or a lower effective melting temperature for the capture sequence when immobilized on the monolithic column.
Figure 2.5 Fluorescence-elution time profile for various concentrations of synthetic NDM target and an off-target sequence as a negative control.

Figure 2.5 shows fluorescence detected during elution in four different experiments after loading three different concentrations of synthetic NDM target DNA and one concentration of an off-target synthetic sample. The elution peak height increased with loaded target DNA concentration. Some peak tailing was also seen, indicative of stronger retention or possible nonspecific adsorption on the monolith or channel. As a control experiment, synthetic KPC target DNA (500 pM) was flowed through the monolith containing NDM capture oligonucleotides. A small signal resulted after labeling and elution, indicating good specificity. This off-target signal could be due to minor nonspecific adsorption of DNA target or labeling probe to the microdevice surface that was reversed during the heat denaturation step.

After each experiment, the fluorescence system was calibrated by flowing various hybridization probe concentrations through the microdevice and recording fluorescence signal. From this calibration curve ($s_y = 0.029$) and the fluorescence signal during elution, I calculated the instantaneous concentration of eluted DNA and its corresponding standard deviation.
Then, using the volume of the eluted DNA determined from the elution times and flow rates recorded by the Fluigent pump system, I calculated the moles of DNA eluted, standard deviation, and percent recovery given the moles of target DNA loaded.

**Table 2.3** Experimental results for elution of various concentrations of synthetic NDM target and a negative control off-target sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA loaded (fmol)</th>
<th>DNA eluted ± std. dev. (fmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pM</td>
<td>10</td>
<td>5.66 ± 0.57</td>
<td>57</td>
</tr>
<tr>
<td>300 pM</td>
<td>30</td>
<td>8.59 ± 0.55</td>
<td>29</td>
</tr>
<tr>
<td>500 pM</td>
<td>50</td>
<td>16.85 ± 0.89</td>
<td>34</td>
</tr>
<tr>
<td>500 pM off-target</td>
<td>50</td>
<td>0.47 ± 0.44</td>
<td>0.94</td>
</tr>
</tbody>
</table>

The percent recovery was lower for 300 and 500 pM, than for 100 pM loaded target DNA (see Table 2.3), consistent with similar observations in an earlier study. The percent recovery for DNA hybridization could potentially be improved by lowering loaded DNA concentrations (which is consistent with levels found in bacterial infections), using slower fluid flow rates, lengthening the monolithic column, or increasing the density of the capture oligonucleotides on the monolith.
**Table 2.4** Elution peak areas for various concentrations of both synthetic and plasmid target DNA.

<table>
<thead>
<tr>
<th>Sample concentration</th>
<th>Synthetic DNA</th>
<th>peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pM</td>
<td></td>
<td>0.150</td>
</tr>
<tr>
<td>300 pM</td>
<td></td>
<td>0.216</td>
</tr>
<tr>
<td>500 pM</td>
<td></td>
<td>0.299</td>
</tr>
<tr>
<td>500 pM off-target sequence</td>
<td></td>
<td>0.035</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample concentration</th>
<th>Plasmid DNA</th>
<th>peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pM</td>
<td></td>
<td>0.107</td>
</tr>
<tr>
<td>300 pM</td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>500 pM</td>
<td></td>
<td>0.151</td>
</tr>
<tr>
<td>500 pM off-target plasmid</td>
<td></td>
<td>0.028</td>
</tr>
</tbody>
</table>

To demonstrate specific capture and detection of bacterial DNA, I performed similar DNA hybridization experiments on plasmids isolated from grown bacteria as a step towards real sample analysis. Figure 2.6 shows fluorescence detected during elution in four different experiments after loading three different concentrations of NDM plasmid target DNA and one concentration of an off-target plasmid sample.

**Figure 2.6** Fluorescence-elution time profile for various concentrations of NDM plasmid and an off-target plasmid sample as a negative control.
I loaded relatively large numbers of plasmid (6.0 × 10^9 copies or 6.0 × 10^{10} CFU/mL for the 100 pM plasmid target DNA sample, of which 2.3 × 10^9 copies were detected) on the monolith. Although I characterized these results with fluorescence detection, the devices are designed for use in front-end sample preparation applications. Similar to the observed trend in Fig. 2.5, the elution peak height increased with higher loaded target DNA concentrations, but the fluorescence signals of the elution peaks for plasmid DNA were comparatively lower than those of synthetic target DNA for a given loaded target concentration (compare Figs. 2.5 and 2.6). More peak tailing was also observed for the plasmid DNA than for synthetic DNA, likely because of greater nonspecific adsorption of the longer plasmid DNA. The elution peak areas for various concentrations of both synthetic and plasmid target DNA are provided in Table 2.4. The percentage of plasmid DNA recovered (Table 2.5) was determined, similarly to how it was done for Table 2.3, using a calibration curve (s_y = 0.066), the observed fluorescent signal, and flow rates. Plasmid DNA percent recovery was less than that of synthetic DNA for the same molar concentrations of loaded target (compare Tables 2.3 and 2.5).

**Table 2.5** Experimental results for elution of various concentrations of NDM plasmid and a negative control off-target plasmid sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA loaded (fmol)</th>
<th>DNA eluted ± std. (fmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pM</td>
<td>10</td>
<td>3.8 ± 2.0</td>
<td>38</td>
</tr>
<tr>
<td>300 pM</td>
<td>30</td>
<td>4.6 ± 1.8</td>
<td>15</td>
</tr>
<tr>
<td>500 pM</td>
<td>50</td>
<td>10.3 ± 2.2</td>
<td>21</td>
</tr>
<tr>
<td>500 pM off-target plasmid</td>
<td>50</td>
<td>0.3 ± 1.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>
The much larger size of the plasmid DNA compared to the synthetic DNA may partially account for the lower percent recovery, as the capture sequence could be sterically hindered and thus less available for capture in the monolith compared to the shorter synthetic oligonucleotide. Additionally, the plasmid is double-stranded and needs to be denatured prior to capture, which could also reduce the percent recovery relative to synthetic DNA. The limit of detection for the fluorescence system in these experiments was determined to be ~30 pM from the sample peak heights and the standard deviation of the blank in Fig. 2.6.

As a control experiment, 500 pM of KPC plasmid DNA was loaded into the monolith. The off-target plasmid peak was much smaller, with ~3 % capture relative to the same concentration of target (see Table 2.5). This small off-target signal might result from minor nonspecific ionic interactions or adsorption of DNA to the channel wall. Importantly, these results demonstrate good sequence-specific capture, fluorescent labeling, and detection of sepsis-related bacterial plasmid DNA using easily and rapidly fabricated microdevices.

### 2.4 Conclusions

In summary, I have demonstrated a simple and fast method for fabricating microfluidic devices for sequence-specific DNA capture, labeling, and fluorescence detection of sepsis related antibiotic resistance genes. I fabricated microdevices from widely available polymeric materials (PMMA sheets and optical adhesive tapes) using a simple, time-efficient technique involving laser micromachining and heat sealing, which did not require a clean room environment. I formed porous functionalized monoliths in the microdevices and demonstrated sequence-specific capture, fluorescent labeling, and detection of bacterial plasmid DNA with performance comparable to traditional devices.\(^{19}\) The overall process, including device fabrication, DNA
capture, labeling, and detection, was performed in less than 90 min. In a related recent work, the total time spent from bacteria extraction in a blood sample to target analysis in an optofluidic single-particle counting system was ~1 h. The devices described herein could be incorporated into that detection setup to provide comparable analysis times with simpler device fabrication. The simple, time-efficient process for achieving effective sequence-specific capture of bacterial plasmid DNA demonstrated herein can be integrated with other processes for analysis of bacteria, including capture, DNA labeling, and detection.

2.5 References


CHAPTER 3. MULTIPLEX DETECTION OF SEPSIS-RELATED BACTERIAL PLASMID DNA USING MONOLITH-MODIFIED RAPIDLY PROTOTYPED MICROFLUIDIC DEVICES*

3.1 Introduction

Sepsis remains a global threat with an estimated cumulative financial impact of over 1 trillion dollars by 2050.¹ The survival rate of patients with sepsis-related bacterial infections drops by more than 7.6% per hour of delayed treatment.²,³ Currently available tools for detecting sepsis-related bacteria either take >24 h,⁴ or lack the ability to detect different types of bacterial DNA simultaneously.⁵⁻⁸ Tools which can efficiently detect multiple bacterial types, or their DNA can improve the early diagnosis of sepsis-related bacteria, and limit inaccurate diagnosis, along with misuse of antibiotics.

Microfluidic systems can provide unique and convenient approaches to capturing and detecting bacterial DNA. Boissinot et al.⁹ developed a microfluidic cartridge with a bead bed capable of capturing bacteria DNA for real-time monitoring. The system was able to identify bacterial DNA in test samples in <15 min, but lacked multiplex capability. The glass microchip developed by Shokoufi et al.¹⁰ for DNA hybridization using unmodified gold nanoparticles lacked the ability for multiplex detection of different bacterial DNA simultaneously. Improving existing tools capable of multiplex detection will encourage the early detection of bacteria DNA associated with bacteria resistance.

In Chapter 2 of this dissertation, I demonstrated the selective capture, labeling and detection of picomolar concentrations of sepsis-related bacterial DNA. In this chapter, I present the use of PMMA-PSA-PMMA microdevices for multiplex detection of two different bacterial DNA sequences. Microfluidic devices were made from laser micromachined PMMA sheets and double-sided PSAs. The microfluidic devices were modified with polymer monoliths containing multiple DNA capture sequences and used to demonstrate sequence-specific capture, fluorescent labeling and LIF detection of corresponding DNA targets. The efficacy of the sequence-specific DNA capture and label hybridization process was verified using different laser excitation wavelength sources. The ability of this setup to selectively trap bacterial plasmid DNA for detection can be adapted for front-end sample preparation techniques like single-particle counting.

3.2 Materials and Methods

Materials. The chemical reagents utilized in this work are presented in Chapter 2.2.1.

Microfluidic device design. Microchannel features on a custom CNC machined aluminum master were hot embossed into 2.8 cm $\times$ 6.4 cm $\times$ 0.3 cm sized PMMA blocks at 130 °C on a benchtop laboratory press (Model 4120, Carver, Wabash, IN). The PMMA microchannels were 500 µm deep and 500 µm wide at the top. Holes for microfluidic connectors were then drilled at the inlets and outlets of the microchannel using a CO$_2$ laser cutter (see Figure 3.1B). A 1-inch-wide double-sided PSA was laminated onto the microchannel side of the PMMA block and enclosed with an unpatterned 2.8 cm x 6.4 cm x 1 cm PMMA block (Figure 3.1A). The final PMMA-PSA-PMMA bulk device was held together with a benchtop laboratory press at
100 °C for 2 min and allowed to cool to room temperature. Microfluidic connectors were then attached to the devices using Solarez UV resin (Vista, CA) and cured under UV light for 2 min.

![Image](image1.png)

**Figure 3.1** Microdevices and monoliths. (A) Device cross section with two layers of PMMA attached by double-sided PSA. (B) Schematic view of a PMMA—PSA—PMMA microfluidic device with monolith. (C) High magnification cross-sectional SEM image of channel with porous polymer.

Monoliths were prepared and introduced into the PMMA microdevices using the method outlined in Chapter 2.2.3. The monolith polymerization mixture was composed of (all w/w): 3% BME, 18.5% PEGDA, 18.5% EDMA, 14% 2-propanol, 41.5% 1-dodecanol, 2.25% 500 μM acrd-NDM and 2.25% 500 μM acrd-KPC (see Chapter 2.2.3 and Table 3.1).

**Bacterial growth and plasmid sample preparation.** *E. coli* cells containing plasmid DNA were grown using the procedure outlined in Chapter 2.2.5. After incubation, the plasmid was extracted and purified from the *E. coli* cells using the standard Zymo Midiprep kit protocol (Irvine, CA). Plasmid DNA was then digested with EcoR1-HF (20 U/ml, R3101S, New England Biolabs, Ipswich, MA) and Cutsmart buffer (New England Biolabs) by heating to 37 °C for 15
min, and quenching the reaction at 65 °C for 20 min using a VWR heat block. Digested samples were then stored at 4 °C.

**Operation of the microfluidic setup and fluorescence measurement.** The setup and procedure for carrying out DNA hybridization and LIF measurements in the microdevice are outlined in Chapter 2.2.6. Solid-state 488 nm and 532 nm lasers (CrystaLaser, Reno, NV) were utilized as fluorescence excitation sources in this work. The 488 nm laser primarily excites the fluorescein labeled target and the 532 nm laser primarily excites Atto-532 and TAMRA. Atto-532 fluorescent label has a higher emission signal than TAMRA. The monolith was kept at 40 °C while loading devices with DNA targets or labels. The monolith temperature was then raised to 70 °C for 2 min prior to eluting labeled DNA targets. The flowrate was maintained at 5–15 µL/min using pressures from 200–800 mbar. The LIF Data were averaged over 10-points, elution peaks were baseline corrected, and peak areas were calculated using the custom MATLAB code in Table 2.2.

**Multiplex detection of NDM and KPC plasmid targets.** Plasmid DNA was initially heated for denaturation at 94 °C for 4 min, and immediately quenched in an ice bath. The fast cooling causes misalignment of denatured DNA strands, thus preventing full renaturation. A 100 µL mixture of 2.5 nM NDM and 2.5 nM KPC DNA was loaded onto the device containing monolith modified with both NDM and KPC capture sequences. A 120 µL hybridization probe solution containing 100 nM each of Atto 532-NDM and FL-KPC probe (see Table 3.1) was then flowed through the monolith, followed by a rinse step with hybridization buffer until the fluorescence signal returned to baseline. The monolith temperature was then raised to 70 °C for 2 min and labeled DNA targets were eluted by resuming the flow of buffer through the monolith.
Table 3.1 DNA sequences for capture probes, targets and fluorescent labels.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence 5' to 3'</th>
<th>Peak Excitation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrd NDM</td>
<td>Acrd-ACCCAGGTTTGGAGCAGTCGGTCGCAGAGG TCGGAGAC[sp18]GCAGCACACTTCTATCTCGACAT</td>
<td></td>
</tr>
<tr>
<td>Acrd KPC</td>
<td>Acrd-ACCCAGGTTTGGAGCAGTCGGTCGCAGAGG TCGGAGAC[sp18]TATCGCCGTCTAGTTCTGCTGTCTTG</td>
<td></td>
</tr>
<tr>
<td>KPC</td>
<td>CATTCAAGGGCATCTTTCCGAGATGGGTGACCACGGAACCCAG CGGATGCCCATGCCCTATCAGTCAAGACAGCAGAAGTGAAGTGGCTGC</td>
<td></td>
</tr>
<tr>
<td>NDM</td>
<td>ACCACCAGCAGCGCCGCATCCCCTGACGATCAAACCGTTG GAAGCGACCGCCATGCCCTATCAGTCAAGACAGCAGAAGTGGCTGC</td>
<td></td>
</tr>
<tr>
<td>FL–NDM</td>
<td>[FL]ACCACCAGCAGCGCCGCATCCCCTGACGATCAAACCGTTG GAAGCGACCGCCATGCCCTATCAGTCAAGACAGCAGAAGTGGCTGC</td>
<td></td>
</tr>
<tr>
<td>TAM–KPC</td>
<td>[AminoC6+TMR]ATTCAAGGGCATCTTTCCGAGATGGGTGACCA CGGAACCGACGGATGCCCATGCCCTATCAGTCAAGACAGCAGAAGTGGCTGC</td>
<td></td>
</tr>
<tr>
<td>FL-NDM Probe</td>
<td>[FL]GCTCGACAGTCGCTTTCAACCGGCTTTGATCGTCAGGGATGT CGAG[FL]C</td>
<td></td>
</tr>
<tr>
<td>FL-KPC Probe</td>
<td>[FL]TATCCGCTGGTTCGCGGATCCCTCGGAAAGATC[FL]C</td>
<td></td>
</tr>
<tr>
<td>Atto 532-NDM Probe</td>
<td>[Atto 532]GCTCGACAGTCGCTTTCAACCGGCTTTGATCGTCAGGGATGT CGAG[Atto 532]C</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Acrd = acrydite, sp18 = 18 carbon atom spacer, FL = fluorescein, TAM = TAMRA, AminoC6 = Amino Modifier C6

The device was heated to 70 °C for 2 min and labeled DNA targets were eluted and detected with either the 488 nm or 532 nm laser. For control experiments, KPC and NDM synthetic DNA were used as targets, with FL-KPC and Atto 532-NDM as fluorescence labels, and the 488 nm laser was used for detection.
3.3 Results and Discussion

The earlier device fabrication method employed in making PMMA-PSA microfluidic devices used a single PMMA block, and the microchannels were enclosed with a single-sided PSA (see Figure 2.1). The devices for the multiplex experiments were made using two PMMA blocks held over each other with a double-sided PSA. The new devices showed more robustness and could withstand pressures >2.5 bar, compared to pressures <500 mbar for devices described in Chapter 2. The monolithic column was constrained by the two PMMA blocks, creating a tight seal around the monolith for complete fluid flow through monolith. The modification of the monolith composition with two different acrydite-functionalized NDM and KPC capture sequences allowed two different gene targets to be caught simultaneously. Based on the success of this method, the procedure could be modified with monoliths containing >2 capture sequences for applications which require entrapment of several gene targets.
Figure 3.2 Fluorescence-elution time profile for separate synthetic DNA targets and mixed target samples on a multiplexed monolith column. The green, amber, and blue-shaded elution peaks were obtained when the monolith was loaded with 5 nM KPC target, 5 nM NDM target, or a mixture of KPC and NDM targets respectively. A 100 nM FL-NDM, FL-KPC or both FL-NDM and FL-KPC hybridization probe was used for the KPC target, NDM target, or mixed KPC and NDM target sample run, respectively. All detection was done using the 488 nm laser.

Figure 3.2 demonstrates the ability of the monolith to capture multiple targets and shows elution peaks from fluorescence detected under three different experimental conditions after loading monolith with synthetic DNA targets, labeling with fluorescein probes, and detecting with the 488 nm laser.
Table 3.2 Baseline-subtracted elution relative peak areas.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Laser</th>
<th>Condition</th>
<th>Elution Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>Blue</td>
<td>5 nM KPC</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 nM NDM</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 nM KPC + 5 nM NDM</td>
<td>1.00</td>
</tr>
<tr>
<td>3.3A</td>
<td>Blue</td>
<td>10 nM FL-NDM + 10 nM TAM-KPC</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM Fl-NDM</td>
<td>0.84</td>
</tr>
<tr>
<td>3.3B</td>
<td>Green</td>
<td>10 nM TAM-KPC + 10 nM FL-NDM</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM TAM-KPC</td>
<td>1.18</td>
</tr>
<tr>
<td>3.4</td>
<td>Blue</td>
<td>2.5 nM KPC + 2.5 nM NDM</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 nM KPC</td>
<td>0.68</td>
</tr>
<tr>
<td>3.5</td>
<td>Blue</td>
<td>10 nM KPC + 10 nM NDM</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM NDM</td>
<td>0.09</td>
</tr>
</tbody>
</table>

As expected, the elution peaks from the individual KPC or NDM target runs had similar signal intensities and peak areas (see Table 3.2). Comparing the elution peaks in Figure 3.2, the elution peak from the mixed KPC and NDM DNA target run was ~96% of the combined peak areas of the individual KPC and NDM target elution peaks (Table 3.2). This demonstrates the ability of the monolith to selectively capture both DNA targets, with potential for quantitation.

To illustrate multiplex detection on monolith columns using different excitation wavelength sources, individual fluorescently labeled synthetic DNA targets as well as mixtures of prelabeled targets were flowed through the monolith in separate runs and detected with either the 488 nm or 532 nm laser. Figure 3.3 shows elution peaks from detecting labeled NDM or KPC targets with the 488 nm or 532 nm laser. The NDM and KPC targets were labeled with TAMRA and fluorescein, respectively. The two elution peaks in Figure 3.3A have similar signal intensities and areas (see Table 3.2), signifying that the elution peak was mainly the result of fluorescence from the FL-NDM by the 488 nm laser in both the individual and mixed target DNA samples.
Figure 3.3 Fluorescence elution signal from fluorescently labeled synthetic DNA targets. (A) FL-NDM target (shown in red), and mixture of FL-NDM and TAM-KPC target (shown in blue) detected with the 488 nm laser. (B) TAM-KPC target (shown in red), and mixture of FL-NDM and TAM-KPC target (shown in blue) detected with the 532 nm laser.

In Figure 3.3B, the 532 nm laser was used for detection of two separate runs, one of which utilized prelabeled TAM-KPC targets, and the other a mixture of FL-NDM and TAM-KPC targets. In Figure 3.3B, the elution peak area from the TAM-KPC target was slightly larger than the elution peak from the FL-NDM and TAM-KPC target mixture (see Table 3.2). The elution peaks in both runs are from the fluorescence of TAM-KPC by the 532 nm laser. The difference in peak areas might also be attributed to the degrading of the monolith quality after multiple runs or fluctuations in monolith porosity with time.
Figure 3.4 Fluorescence signal from elution of KPC plasmid target (shown in red), and mixed KPC and NDM plasmid targets (shown in blue) on a multiplexed monolith column. The red-shaded elution peak was obtained when the monolith was loaded with 2.5 nM KPC target, and 100 nM each of FL-KPC and Atto-532 NDM probe. The blue-shaded elution peak was obtained when the monolith was loaded with a mixture of 2.5 nM each of KPC and NDM targets, and labeled with 100 nM each of FL-KPC and Atto-532 NDM probe. All detected with the 488 nm laser.

The multiplex analysis was extended to plasmid DNA. Figure 3.4 shows elution peaks for an individual KPC target run, and a mixed KPC and NDM target sample run. The elution peak from the mixed KPC and NDM target sample was 47% greater in peak area (see Table 3.2) than the elution peak from the individual KPC target. The extra signal intensity and peak area observed for the combined KPC and NDM target sample run might be due to excitation of the Atto-532 labeled NDM by the 488 nm laser. The fluorescence from the Atto-532 adds to the overall fluorescence recorded in the elution peak for the mixed KPC and NDM target sample run. The elution peaks for the plasmid DNA were also smaller than those of the synthetic DNA, similar to what was observed between Figures 2.5 and 2.6. This is due to the much larger size of the plasmid DNA compared to the synthetic DNA, resulting in more steric hinderance with the
capture sequences, and thus less capture in the monolith compared to the shorter synthetic oligonucleotide.7

**Figure 3.5** Fluorescence signal from elution of single NDM DNA target, and mixed KPC and NDM targets on a multiplexed monolith column in control experiments. The monolith was loaded with 100 nM NDM target (red) or 100 nM each of NDM and KPC target mixture (blue), labeled with 100 nM each of Atto-532 NDM and FL-KPC probe, and detected with the 488 nm laser.

Figure 3.5 presents data for control experiments which demonstrate sequence-specific capture, labeling of DNA targets on the multiplexed monolith column and LIF detection. For the elution peak obtained from the individual NDM sample (red), the capture-target NDM duplex was labeled with Atto-532 NDM which is poorly excited by the 488 nm laser; hence the small elution peak intensity and area observed (see Figure 3.5 and Table 3.2). The signal observed might be a result of minor nonspecific ionic adsorption of DNA to the device wall.7 For the elution peak from the mixed KPC and NDM sample (blue), the NDM and KPC targets captured on the multiplexed monolith column were labeled with Atto-532 NDM and FL-KPC, respectively. The larger elution peak signal and area (see Figure 3.5 and Table 3.2) observed is
primarily from fluorescence of the fluorescein labeled KPC target, since fluorescein is effectively excited by the 488 nm laser. Importantly, the results demonstrate good multiplex detection of sepsis-related bacterial DNA using a monolith functionalized with multiple capture sequences.

3.4 Conclusion

The above results demonstrate the ability to use the monolith setup highlighted herein for the multiplex detection of bacterial plasmid DNA. Microfluidic devices were fabricated using two PMMA blocks and double-sided PSAs. These microdevices supported porous polymer monolith columns under fluid pressures >2.5 bar, and microdevices could be used for multiple experimental runs. The monolith was modified with two different DNA capture sequences and used to demonstrate selective capture, fluorescent labeling, and multiplex detection of KPC and NDM gene targets. The method developed herein could be integrated with other bacteria detection processes which rely on capturing multiple gene targets, or single-bacteria counting.

3.5 References


of effective antimicrobial therapy is the critical determinant of survival in human septic shock.


CHAPTER 4. IMPROVING DROPLET MICROFLUIDIC SYSTEMS FOR STUDYING SINGLE BACTERIA GROWTH*

4.1 Introduction

Antimicrobial resistance remains a global threat with ~ 5 million deaths in 2019 alone and 10 million deaths projected every year by 2050. In the USA, the Centers for Disease Control and Prevention reported ~ 2.8 million antimicrobial resistant infections and 35,000 deaths occurring annually. Current tools employed in the diagnosis of antimicrobial resistance can be time inefficient leading to delayed diagnosis and treatment. New techniques for improved study of bacteria will reduce mortality rate, antimicrobial resistance, and medical costs resulting from delayed treatment.

Microfluidics as a platform for novel analytical tools has been explored for some time. Microfluidic applications include DNA analysis, bacterial analysis, and single-cell genomics to name a few. Droplet microfluidic systems use immiscible phases to confine contents in discrete droplets, which can be advantageous for controlling diffusional losses, high throughput biological experiments or automated control of multiple microreactors. Bacteria can be grown within discrete droplets, and when enclosed with appropriate reagents, online analysis can be performed. This type of single cell analysis helps overcome limitations associated with ensemble-averaged data from multiple cells.

Various droplet microfluidic setups or techniques for examining bacteria growth have been reported. Boedicker et al. presented a method for performing susceptibility testing of

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*This chapter is adapted with permission from: Akuoko, Y.; Nagliati, H. F.; Millward, C. J.; Woolley A. T. Improving droplet microfluidic systems for studying single bacteria growth. Analytical and Bioanalytical Chemistry. 2023; 415:695-701.
bacteria which involved trapping bacteria cells in nanoliter-sized plugs for incubation, imaging, and fluorescence detection. Plugs enclosing *S. aureus* bacteria were collected in Teflon tubing and analyzed for > 7 h in a microscope incubator; however, the setup employed off-chip incubation and long assay times. Keays et al. also utilized a tubing system for droplet generation, incubation, and monitoring of bacterial cell growth. The setup utilized a plate reader to record changes in optical density of droplets to determine the growth rate of encapsulated bacteria over 10 h. A droplet microfluidic system with an array of docking sites capable of trapping individual droplets was developed by Kang et al. The microfluidic platform comprised four units each with > 8000 droplet docking sites, and each docking station could trap droplets containing 1–4 bacteria cells and be monitored over 2 h duration. Kaushik et al. developed droplet-based microfluidic chips for studying the growth of single bacteria in pL-sized droplets in a shorter time. The confinement of bacteria in small droplet incubation chambers coupled with resazurin-based fluorescence detection provided assay results in ~ 1 h.

The above-mentioned works demonstrate capability for droplet microfluidics of bacteria; however, these techniques either lose droplet traceability, require high levels of fluorescence reporter molecules, lack single cell studies, or require offline incubation and monitoring. Notably, droplet destabilization and unwanted merging can occur during the transfer of droplets with offline systems, particularly limiting droplet traceability. Herein, I develop a microfluidic setup capable of studying single bacteria growth in droplets over 2 h. I use PDMS blocks as microfluidic tubing connectors to simplify interfacing with flow systems. My devices allow for online incubation, droplet monitoring, and detection, offering an integrated setup with potential for future automation and rapid availability of assay results. I performed bacteria counts in droplets with microscopic imaging and demonstrated the encapsulation of single bacteria with
the droplet microfluidic platform. I studied droplet-encapsulated *E. coli* in the devices and evaluated room temperature growth over multiple hours. Even with a 100 × lower concentration of fluorescent probe compared to earlier studies, I was able to confirm bacteria viability. I use this platform to monitor the growth of single *E. coli* cells over 2 h in room temperature incubation conditions. The setup’s ability to avoid droplet overlap or desequencing will enable droplet tracking in future studies.

4.2 Materials and Methods

4.2.1 Chemicals and Materials

The following materials were used in making the PDMS—glass microfluidic devices: Sylgard 184 silicone elastomer base and curing agent (H047LAC000, Midland, MI), glass slides (1 mm thick, 12-550C, Thermo Fisher Scientific, Waltham, MA), cover glass (0.13–0.16 mm thick, 5075-1D, Thermo Fisher Scientific), and Aquapel (47112, Cranberry, PA). The SU-8 2075 photoresist used in photolithography was obtained from Kayaku Advanced Materials (Westborough, MA). The continuous phase for the W/O droplets was composed of Novec 7500 engineered fluid and polytetrafluoroethylene-polyethylene glycol-polytetrafluoroethylene (PTFE-PEG-PTFE) surfactant purchased from 3M (St. Paul, MN) and Creative PEGWorks (PPP-224, Chapel Hill, NC), respectively. The bacteria gram staining kit, lysogeny broth, and agar media (Difco brand) were obtained from Thermo Fisher Scientific. All broth solutions and deionized water (18.3 MΩ) were autoclaved before use. The *Escherichia coli* (ATCC 29522) bacterial strain and resazurin sodium salt (AAB2118706) were procured from ATCC and Thermo Fisher Scientific, respectively.
4.2.2 Device Design and Fabrication

Microfluidic channels of dimensions 200 × 75 μm² (width by height) were designed using CleWin software, and PDMS microfluidic devices were fabricated from molds created using standard photolithography. In brief, the chrome mask design was patterned onto a 4 in. Si wafer coated with SU-8 2075 photoresist. When the Si-wafer molds were ready, PDMS mixture from combining Sylgard 184 base to curing agent at a 10:1 ratio was poured onto the Si wafer and baked at 70 °C for ~ 20 min. The PDMS replicas were then bonded to glass slides via oxygen plasma treatment in a plasma cleaner (PDC-32G, Harrick Plasma). Cube-shaped PDMS blocks (0.5 × 0.5 × 0.5 cm³) were used as microfluidic connectors for attachment of PTFE tubing to the microfluidic devices. The connectors were perforated with a 1.5 mm biopsy punch (Integra Life Sciences, Mansfield, MA) and attached to the PDMS-glass devices using freshly degassed PDMS. The microfluidic devices were then left to bake in an oven at ~ 80 °C for 30 min, and the microchannels were later treated with Aquapel, flushed with nitrogen, and baked at ~ 80 °C for 30 min.

4.2.3 Device and Experimental Operation

Droplet generation and fluorescence measurements were achieved using the fluid control and detection system described in prior work with slight modifications [5]. The Fluigent pump system (Le Kremlin-Bicêtre, France) was used for fluid control and generation of W/O droplets. A pump system pressure of 500 mbar was used for both oil and sample aqueous phases. The optical setup for fluorescence measurements consisted of a solid-state 532 nm laser (CrystaLaser, Reno, NV), optical filters, a photomultiplier, and a data display LabVIEW (National Instruments, Austin, TX) program as described previously.²⁴ For W/O droplet generation, the oil phase was
mixed with 0.1% (w/w) PTFE-PEG-PTFE surfactant, and the sample aqueous phase consisted of LB media, 500 nM resazurin, and $2 \times 10^8$ CFU/mL *E. coli*. I used 0.1% (w/w) surfactant in oil to avoid micelle formation and material transport between droplets and to minimize droplet shrinkage. After droplets were generated in-chip, the initial fluorescence signal was measured in the optical setup, and the droplet-encapsulated bacteria were incubated in-chip at room temperature for ~2 h, after which the fluorescence signal from incubated droplets was measured again.

### 4.2.4 Bacteria Staining, Imaging, and Cell Counting

To demonstrate the ability of the setup to encapsulate single bacteria in W/O droplets, $2 \times 10^8$ CFU/mL of *E. coli* was initially centrifuged, and the solid residue mixed with both crystal violet and Gram’s iodine before decolorization with acetone and then counterstained with safranin dye. Each staining step was performed in between rinse, centrifugation, and decanting steps. The final solution was centrifuged, and the solid residue made up of labeled *E. coli* cells was diluted with LB media and used as the aqueous phase in generating W/O droplets. Droplets were generated in microfluidic channels with dimensions of $100 \times 10 \mu\text{m}^2$ (width by height) and visualized with a $100 \times$ Nikon Plan Fluorite oil immersion objective (Melville, NY) and a CCD camera (C14440-C0UP, Hamamatsu, Japan). For bacteria imaging, thinner (0.13–0.16 mm) cover glass slides were used in fabricating the PDMS-glass microfluidic devices. These measurements on dead bacteria inferred the number of live bacteria in droplets in all other experiments.
4.3 Results and Discussion

4.3.1 Device Setup and Operation

Figure 4.1a shows the microchannel design with oil and aqueous inlets for droplet generation, a mixing zone for complete blending of droplet components, and an incubation zone for storing of droplets for ~2 h before fluorescence detection. The detection point is located near the outlet of the microchannel, although the device setup and operation also allow detection to be accomplished anywhere in the incubation zone.

![Figure 4.1a](image1.png)  
![Figure 4.1b](image2.png)  
![Figure 4.1c](image3.png)

**Figure 4.1** Microdevices for droplet microfluidics. (a) Schematic view of PDMS microdevice. (b) Top-view photograph of PDMS microdevice. (c) W/O droplet generation in microchannel.

Figure 4.1b displays a top view photograph of a completed PDMS microfluidic device filled with red food dye solution for easy visualization of the microchannel, and Fig. 4.1c shows uniform-sized W/O droplets generated in a microchannel, enroute to the incubation zone. PDMS microfluidic devices often are created as thick layers to allow microfluidic connector tubing to be held in place by the PDMS device material. As an alternative, I utilized cube shaped PDMS
blocks for attachment of PTFE tubing to the microfluidic devices, so the final microfluidic devices were more compact and required less PDMS (see Fig. 4.2a).

I studied various microchannel depths ranging from 10 to 200 μm and widths from 10 to 100 μm. W/O droplets generated in shallower or narrower microchannels lost volume during incubation. Channel depths below 75 μm produced rod-shaped droplets, which fully contacted the PDMS microchannel walls, potentially leading to analyte or solvent transport to the bulk PDMS.\textsuperscript{27} On the basis of these data, I used microchannel dimensions of 200 × 75 μm\textsuperscript{2} (width by height). I utilized a Fluigent flow control system (see Fig 4.2b), which provided constant pressure-driven flow rates and reproducible W/O droplet volumes. The final setup provided steady generation of 40–70 droplets/min with uniform sizes as seen in Fig. 4.1c. The mean (± standard deviation) droplet diameter was 155 ± 15 μm, corresponding to a calculated volume of 1.7 ± 0.4 nL.\textsuperscript{28}

Figure 4.2 Device and pump system setup. (a) Side view of PDMS microdevice showing fluid connectors made from PDMS. (b) Fluigent pump system connection to a PDMS microdevice.
4.3.2 Bacteria Cell Count and Fluorescence Measurements

In Fig. 4.3, I show the ability of the setup to generate droplets that contain single bacteria. Figure 4.3a–b shows droplets containing single bacteria (circled in red), while Fig. 4.3c–d shows droplets containing two bacteria each. Controlling the droplet size via aqueous and oil phase flow rates and selecting the concentration of bacteria solution allowed droplets with desired numbers of bacteria to be made.

![Figure 4.3 Images of W/O droplets containing stained *E. coli*. (a–b) Single bacteria in a droplet and (c–d) two bacteria in a droplet.](image)

Figure 4.4 shows a histogram of the number of droplets containing zero, one, or two bacteria. About 60% of the droplets imaged under these conditions had no bacteria, while ~ 33% had single bacteria and ~ 7% had two bacteria, which is consistent with Poisson distribution statistics. 29
E. coli is a robust microorganism capable of replicating over a wide range of temperatures and growth conditions. E. coli replicates approximately every 20 min under optimal aerobic conditions in LB broth at pH 7.0 and 37 °C. I performed these experiments in the same solution but at room temperature. Growing and dividing E. coli produce NADH, which reduces non-fluorescent resazurin to fluorescent resorufin, resulting in an increase in fluorescent signal. I evaluated E. coli droplet incubation times ranging from 1 to 5 h in the detection setup. Before/after fluorescence signal differences were difficult to discern after 1-h incubation. The fluorescence signal often exceeded the dynamic range of the detection setup after incubation times > 3 h. I thus selected 2-h incubation times for experiments as a compromise between signal and experimental time. Other droplet microfluidic studies reported in the literature on bacteria with resazurin utilized > 50 μM resazurin concentrations.\textsuperscript{9,20} Utilizing μM resazurin concentrations potentially causes any slight amounts of resorufin produced from bacteria growth to be masked by the bulk resazurin, further increasing incubation times. I utilized a 100 × lower
concentration of resazurin for these experiments, which reduced background and allowed slight changes in resorufin concentration during incubation to be picked up in fluorescence detection.

To demonstrate successful bacteria incubation within droplets, I studied droplets generated from a sample solution containing $2 \times 10^8$ CFU/mL *E. coli*, 500 nM resazurin and LB broth. The use of low concentrations of fluorescent label reduces costs and waste, limits perturbation to bacteria, and allows low resorufin concentrations produced post incubation to be effectively detected, potentially limiting false positive results. High concentrations of resazurin have further been reported to limit cell survivability over extended incubation times, so my approach is well-suited for applications requiring extended incubation of cells with resazurin.

![Figure 4.5 LIF of droplets containing $2 \times 10^8$ CFU/mL *E. coli* and LB broth. (a) Before incubation and (b) after ~2-h incubation. (c) Zoomed view of data in (a). (d) Zoomed view of data in (b).](image)

Figure 4.5 shows the fluorescence vs. time profile from streams of droplet-encapsulated bacteria before and after incubation. I observe RFU signals with similar peak heights from droplets before incubation as seen in Fig. 4.5a. In contrast, in Fig. 4.5b, I observe RFU signals with varying peak heights after incubation. I show an expanded view of the data from Fig. 4.5a.
and b in Fig. 4.5c and d. The RFU signal from each droplet before incubation was $2.7 \pm 0.02$ RFU ($n = 243$) in Fig. 4.5a. After 2-h incubation, peaks in Fig. 4.5b range from 1.5 to 6.5 RFU. The increase in RFU in some droplets is supportive of the presence of one or more bacteria and their growth in the droplets during the incubation period. Droplets with static or decreased fluorescence after 2-h incubation likely lack encapsulated bacteria; a decrease in RFU for some droplets after incubation could be from loss of analyte from droplet(s) during that time. Changes in droplet spacing in the fluorescence data before and after incubation (Fig. 4.5) occur when droplets undergo relative motion, creating clusters with reduced space in between. Slight backpressure during flow of droplets through the incubation zone may also lead to minor compression in the droplet spacing observed.

**Figure 4.6** Control experiment: LIF of droplets containing 500 nM resazurin, LB broth and no *E. coli*. (a) Before incubation and (b) after ~2 h incubation. (c) Zoomed view of data in (a). (d) Zoomed view of data in (b).

To confirm that the fluorescence increase in Fig. 4.5 is due to droplet-encapsulated bacteria, I performed control experiments involving droplets without bacteria. The fluorescence-
time profile in Fig. 4.6a–b clearly shows that the droplet fluorescence before and after incubation is largely unchanged. These data confirm a lack of resorufin production during incubation due to the absence of bacteria in droplets. Small changes in fluorescence signal are attributed to minor droplet shrinkage or generation of small amounts of resorufin from resazurin reduction under the incubation conditions, and similar outcomes were obtained in replicate experiments. Changes in spacing between peaks before and after incubation (see Fig. 4.5b and Fig. 4.6b) could be attributed to relative motion between droplets during incubation (see Fig. 4.7), also resulting in altered spacing and closer proximity between some adjacent droplets producing fluorescence signals which are not baseline resolved in Fig. 4.5 and Fig. 4.6.

Figure 4.7 Uniform sized W/O droplets in a microchannel. (a-c) Droplets in different sections of the incubation microchannel.

To further demonstrate the changes in fluorescence in bacteria-encapsulated droplets after on-chip incubation, I generated histograms of droplet fluorescence. In Fig. 4.8a and b, I provide histogram distributions showing the fluorescence signal of droplets for a control experiment. Figure 4.8a shows a single peak (1.25 ± 0.03 RFU, n = 213) before incubation, with no change after incubation in Fig. 4.8b (1.25 ± 0.03 RFU, n = 196). This is further supported by the
expanded view images where I observe no change in peak heights for RFU signals before incubation (Fig. 4.6c) and after incubation (Fig. 4.6d).

**Figure 4.8** Histograms of RFU signals. Bacteria-free droplets (a) before incubation and (b) after 2-h incubation. Droplets containing $2 \times 10^8$ CFU/mL *E. coli* (c) before incubation and (d) after 2-h incubation. Bin width is 0.5 RFU.

In contrast, the histograms in Fig. 4.8c and d show the RFU signal of *E. coli* containing droplets before and after incubation. Figure 4.8c shows a single peak (2.7 ± 0.02 RFU, n = 243) before incubation and multiple peaks after incubation (Fig. 4.8d) demonstrating the presence and growth of *E. coli* in some droplets, with some droplets likely enclosing different numbers of bacteria. The higher pre-incubation droplet fluorescence signal in Fig. 4.5c relative to Fig. 4.6c is likely due to between-day variations in optical system alignment. The incubation channel is designed to retain droplet ordering, which could enable droplet tracking before and after incubation in future studies. As further evidence of this possibility, I demonstrated droplet tracking within the devices by generating different sequences of colored dye (red and green).
W/O droplets and tracked them from the incubation region to the outlet/detection region. Figure 4.9 shows that droplets maintain their sequence and avoid mixing/merging between incubation and detection, further demonstrating potential for tracking.

![Figure 4.9](image)

**Figure 4.9** Different colored W/O droplets in microchannels. (a, c) Droplets of varied sizes and colors (red and green) in the incubation section. (b, d) Images near the detection/outlet section of the microchannel show how droplets maintain their position and sequence, allowing for traceability.

### 4.4 Conclusion

In summary, I demonstrated an approach to making compact PDMS chips with straightforward tubing connections to fluid pump systems for droplet generation, in-chip incubation, and detection of *E. coli* bacteria. I utilized soft lithography and plasma bonding in fabricating my microfluidic devices and prepared connectors using PDMS blocks for interfacing of the devices to a flow control system. I produced uniform droplets enclosing zero, one, or
two bacteria within the devices, incubated droplet-encapsulated bacteria with nanomolar concentrations of a fluorescence reporter, and performed detection via laser-induced fluorescence after convenient room temperature 2-h incubation conditions. These devices allow for online droplet incubation, monitoring, detection, and traceability all within a single microfluidic device. This platform should allow the testing of various concentrations of antibiotic(s) on single bacteria and could be adapted to work at 37 °C in future experiments. Developing microfluidic chips with sample droplet generation and fluorescence detection would advance commercialization and potential field deployment of droplet microfluidic devices for bacteria studies.

4.5 References


16. Tan SJ, Phan H, Gerry BM, Kuhn A, Hong LZ, Ong Y, Poon PSY, Unger MA, Jones RC, Quake SR, Burkholder WF. A microfluidic device for preparing next generation DNA sequencing libraries and for automating other laboratory protocols that require one or more column chromatography steps. PLoS One. 2013;8:e64084. https://doi.org/10.1371/journal.pone.0064084


5. CONCLUSIONS AND FUTURE WORK

5.1 Conclusion

Microfluidics is a promising technique for the development of tools and systems needed for the detection of drug-resistant bacteria and their DNA. However, various limitations persist which hinder the widespread adaptation and mass deployment of conventional tools for detecting drug-resistant bacteria or their DNA. In this dissertation, I present different tools for genotypic detection of sepsis-related bacterial DNA, and I describe a phenotypic approach for studying drug-resistant bacteria. First, I developed microfluidic devices for sequence-specific DNA detection of sepsis-related antibiotic resistance genes in Chapter 2. I fabricated microfluidic devices using polymeric materials (PMMA sheets and optical adhesive tapes) and laser ablation, and integrated porous functionalized monoliths in the channels of my microdevices. I utilized the microfluidic platform to demonstrate sequence-specific capture, fluorescent labeling, and detection of picomolar concentrations of bacterial plasmid DNA with performance comparable to traditional devices. In Chapter 3, I discussed improvements made in the monolith-functionalized PMMA-PSA microdevices and presented experimental work on the multiplex detection of different sepsis-related bacterial DNA. Finally in Chapter 4, I described a droplet microfluidic approach, detailing the fabrication of compact PDMS chips with straightforward tubing connections to fluid pump systems. Devices were used to produce uniform droplets enclosing zero, one, or two bacteria with nanomolar concentrations of a fluorescence reporter, and probed after convenient room temperature 2-h incubation conditions.

The overall work presented in this dissertation advances knowledge for bacteria detection via DNA hybridization using polymer monoliths, multiplex detection of bacterial DNA, and
droplet microfluidic analysis of bacteria. Further scientific improvements are envisioned and outlined below.

5.2 Future Work

5.2.1 Sequence-Specific Detection of Bacterial DNA on Functionalized Monoliths

My work described in Chapter 2 could be improved through various processes. For example, reduction of microchannel surface roughness from laser ablation could be addressed by treatment with a solvent. However the microchannel roughness may be important to monolith attachment to channel walls. Moreover, improving the percent recovery of plasmid DNA could be achieved through modifications in experimental operation, such as lowering fluid flow rates, increasing the monolith length, or raising the density of capture oligonucleotides on the monolithic column. The future integration of this simple, inexpensive microdevice with an optofluidic device capable of detecting single fluorescently labeled nucleic acid molecules from bacteria could lead to rapid clinical diagnosis of bacterial antibiotic resistance compared to currently available methods that take 24 or more hours.

5.2.2 Multiplex Detection of Bacterial DNA on Functionalized Monoliths

The selective capture of multiple DNA targets simultaneously with a multiplexed monolith in PMMA-PSA microdevices increases the number of antibiotic resistance genes probed. Future experiments could be conducted to study the binding capacity of the monolith column and how many capture sequences can be integrated in the monolith without compromising factors like limit of detection, signal intensity and/or elution peak areas. The monolith length can be increased for more DNA capture in future experiments since
microdevices could withstand fluid pressures >2.5 bar. Also, the use of multiple lasers and fluorescent probes with minimal spectral overlap would be ideal for future experiments.

5.2.3 Dosing Droplet-Encapsulated Bacteria with Antibiotics

The droplet microfluidic platform provided assay results after 2 h incubation at room temperature. The incubation period could be shortened by performing the incubation at 37 °C, the optimum temperature for bacteria growth. This could be achieved by introducing a thermoelectric heater at 37 °C to the device platform. Using this approach might increase dehydration in the PDMS and encourage size shrinkage in aqueous droplets. An alternate approach would involve building a compact water bath on the device platform; this may improve the humidity within the bulk PDMS while providing the needed 37 °C temperature for incubation.

![Figure 5.1](image)

**Figure 5.1** Possible appearance of AST data generated from small numbers of ciprofloxacin-dosed single *E. coli* cells encapsulated in W/O droplets. The position of the MIC for the hypothetical data is also indicated.
Introducing antibiotics to the droplet-encapsulated bacteria will provide information on the minimum inhibitory concentration (MIC) of antibiotics for single bacteria in droplets (see Figure 5.1). To achieve this, the current device design shown in Figure 4.1a will be adapted to include channels for introduction of different antibiotic concentrations (see Figure 5.2).

**Figure 5.2** Microfluidic approaches for creating W/O droplets with bacteria and different concentrations of antibiotics. (a) Using valves and multiple channels with different solutions; additional branches and valves could readily be added. (b) Droplet merging; groups of droplets with different antibiotic concentrations are created by dilution before encapsulation and then merged with W/O droplets containing bacteria.

The incorporation of multiple channels as shown in Figure 5.2a for introducing varying antibiotic concentrations will allow for rapid analysis of MIC values for different antibiotic types. Resazurin fluorescent label, bacteria and antibiotic solutions can be combined prior to droplet generation at the interface between the aqueous and oil phases, or a narrow constriction can be built as shown in Figure 5.2b to encourage the merging of antibiotic and bacteria-containing droplets.

Dosing droplet-encapsulated bacteria with more than one antibiotic in a multiplex study could be performed with the droplet microfluidic setup described above. By studying
encapsulated single bacteria under the influence of multiple antibiotics, the efficacy of treating bacteria with multiple antibiotics simultaneously can be ascertained. Also, the potential synergy between multiple antibiotics towards bacteria could lead to low MIC values that encourage low dosages of antibiotics for bacteria treatment. Figure 5.3 shows a hypothetical heat map of average fluorescence for single cells for given antibiotic concentration sets, with the corresponding MIC curve.

**Figure 5.3** Multiplexed AST plots with hypothetical data showing a heat map of average fluorescence for single cells at each antibiotic concentration set, with MIC curve.

Since the discovery of penicillin about a century ago, the need to develop improved tools for detecting bacterial infections is now more important than ever. Laboratory research focused on detecting antimicrobial resistance is making its way towards commercialization at a greater rate.\(^5,6\) The work I describe herein adds to scientific knowledge related to the early detection of antimicrobial-resistant bacteria. This approach will allow treatment of infection with appropriate
antibiotics which addresses the problem of emerging antibiotic resistance. The methods developed herein could also be adapted and/or integrated in both current and future tools for front-end analysis of drug-resistant bacterial DNA or antimicrobial susceptibility testing.

5.3 References


