Integrated Circuits for Rapid Sample Processing and Electrochemical Detection of Biomarkers

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering (IBBME)
University of Toronto

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Abstract

The trade-off between speed and sensitivity of detection is a fundamental challenge in the design of point-of-care diagnostics. As the relevant molecules in many diseases exist natively at extremely low levels, many gold-standard diagnostic tests are designed with high sensitivity at the expense of long incubations needed to amplify the target analytes.

The central aim of this thesis is to design new strategies to detect biologically relevant analytes with both high speed and sensitivity. The response time of a biosensor is limited by the ability of the target analyte to accumulate to detectable levels at the sensor surface. We overcome this limitation by designing a range of integrated devices to optimize the flux of the analyte to the sensor by increasing the effective analyte concentration, shortening the required diffusion distance, and confining the analyte in close proximity to the sensor. We couple these devices with novel ultrasensitive electrochemical transduction strategies to convert rare analytes into a detectable signal.

We showcase the clinical utility of these approaches with several applications including cancer diagnosis, bacterial identification, and antibiotic susceptibility profiling. We design and optimize...
a device to isolate rare cancer cells from the bloodstream with near 100% efficiency and 10 000-fold specificity. We analyse pathogen specific nucleic acids by lysing bacteria in close proximity to an electrochemical sensor and find that this approach has 10-fold higher sensitivity than standard lysis in bulk solution. We design an electronic chip to readout the antibiotic susceptibility profile with an hour-long incubation by concentrating bacteria into nanoliter chambers with integrated electrodes. Finally, we report a strategy for ultrasensitive visual readout of nucleic acids as low as 100 fM within 10 minutes using an amplification cascade. The strategies presented could guide the development of fast, sensitive and low-cost diagnostics for diseases not previously detectable at the point-of-care.
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List of Abbreviations

CE – Coloration efficiency
CTC – Circulating tumour cell
CFU – Colony forming units
CV – Cyclic voltammetry
DNA – Deoxyribonucleic acid
DPV – Differential pulse voltammetry
DCPA – Direct current potential amperometry
ECC – Electrochemical-chemical-chemical
EFD – Electrocatalytic fluid displacement
EpCAM – Epithelial cell adhesion molecule
GFP – Green fluorescent protein
hCG – Human chorionic gonadotropin
mRNA – Messenger ribonucleic acid
NME – Nanostructured microelectrode
OD – Optical density
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PDMS – polydimethylsiloxane
PI – Propidium iodide
PNA – Peptide nucleic acid
RNA – Ribonucleic acid
ssDNA – Single stranded DNA
rpoβ – RNA polymerase β subunit
UPEC – Uropathogenic E. coli
UTI – Urinary tract infection
VVC – Velocity Valley Chip
1 Introduction

1.1 Point-of-care diagnostics

The development of point-of-care diagnostics will revolutionize healthcare by delivering rapid diagnostic results at low-cost.\(^1\) Most existing diagnostic tests must be performed by skilled technicians in centralized laboratories.\(^2\) Samples are taken at the doctor’s office, shipped to the centralized laboratory and the results are reported to the patient during a second follow-up visit several days later. This delays treatment and increases healthcare costs. Moreover, access to expensive, centralized laboratories is often unavailable in low resource settings meaning that many diagnostics tests cannot be applied where they are needed most.

Translating existing diagnostic technologies to the point-of-care is challenging as many tests lack the necessary speed, sensitivity and automation needed to be used during a single doctor’s office visit. As many diseases are manifested by extremely low levels of analytes, gold standard tests such as culture and polymerase chain reaction (PCR) are designed to optimize for high sensitivity. The increased sensitivity often comes at the cost of long test times that are not suitable for a point-of-care setting.

Culture is the gold standard for diagnosing bacterial infections and profiling antibiotic susceptibility. While accurate and inexpensive, culture needs days to amplify the native levels of bacteria present during an infection to visibly detectable levels.\(^3\) In the most serious infections which lead to sepsis, the chances of survival decrease with every hour without treatment with an appropriate antibiotic. Rather than administer an effective targeted antibiotic, doctors must administer broad-spectrum antibiotics which may not cover the etiological organism and will lead to future antibiotic resistance.\(^4\) New technologies to rapidly detect bacterial infections could improve patient survival and bring infectious disease diagnostics to the point-of-care.
Similarly, polymerase chain reaction (PCR) is the gold standard method for detecting specific nucleic acid sequences. The target sequences are drastically amplified through many rounds of enzymatic doublings. While extremely sensitive, PCR is unsuitable in many point-of-care settings as it takes approximately 4 hours and requires a highly skilled operator to prepare the sample. New approaches to rapidly detect nucleic acids could allow point-of-care genetic analysis for a wide variety of diseases.

1.2 Commercially available point-of-care diagnostics

Despite the urgent need, few commercially available point-of-care diagnostics are available. The World Health Organization has stipulated that a point-of-care diagnostic device for a low-resource setting should be ASSURED: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users. Developing such a device is challenging and thus point-of-care diagnostics only exist for a limited number of diseases.

The pregnancy test and the glucose meter are two of the most prevalent examples of commercially available point-of-care diagnostics. The pregnancy test is one of the best examples of a disposable, self-contained test. Sample processing and detection are performed in a hand-held device using passive fluidics. After introducing the sample on a flow pad, the presence of human chorionic gonadotropin (hCG) is rapidly transduced into a visible colour change easily detectable by the end-user.

In the glucose meter, glucose in the blood is introduced onto a disposable test strip which is inserted into a reusable handheld reader. The glucose concentration in the blood is readout electronically by an electrode on the test strip. In one implementation, glucose in the blood is oxidized by glucose oxidase functionalized on the surface of the electrode. This oxidation reaction produces hydrogen peroxide which is readout electrochemically and the results are displayed on screen.
In both cases, the target analytes (hCG and glucose) are present at high levels (micro- to millimolar) which makes both analytes easily detectable. Yet, in many other diseases, the target analytes are present at concentrations as much as 12 orders of magnitude lower. For this reason, the techniques employed in pregnancy and glucose tests cannot be directly applied to detect the relevant analytes in many diseases. There is an urgent need to develop new tools to detect ultra-low levels of analytes rapidly at the point-of-care.

1.3 Biosensors

Recent advances in biosensors have pushed the limits of speed and sensitivity of detection of a broad spectrum of analytes including nucleic acids, proteins, antibodies, cells and metabolites. A diverse array of transduction strategies have been developed which can be grouped broadly into optical, magnetic, mechanical and electronic readout systems.

Optical sensors include those based on reading changes in colour, fluorescence, and surface plasmon resonance. Magnetic sensors typically involve detecting the presence of magnetic micro or nanoparticles that are functionalized with biological recognition elements. Examples of mechanical sensors include devices which detect changes in the resonance of micro-cantilevers or the mechanical properties of cells. A variety of electronic and electrochemical sensors have been reported which detect changes in the surface charge and impedance at electrodes and changes in the redox state of electrochemical reporters and redox active analytes.

Inherent trade-offs arise in the design of a biosensor which include balancing the speed vs. sensitivity and sensitivity vs. specificity of detection. For example, decreasing the turnaround time of a test usually comes at the expense of the test sensitivity. The longer the sample is incubated, the greater the opportunity for the target analyte to accumulate to detectable levels. Similarly, increasing the sensor sensitivity tends to increase false positive results which
decreases specificity. Yet, some important clinical applications call for high speed, sensitivity and specificity. Developing new strategies to manage and minimize these trade-offs will be a central theme of this thesis.

1.4 Electrochemical sensors

Electrochemical sensors are a particularly promising class of sensor as they offer direct electronic and ultrasensitive readout of analytes. As readout is purely electronic, the instrumentation can fit in a handheld or benchtop device. In comparison, optical instruments often require expensive and bulky optical components such as fluorescence microscopes which limit their application at the point-of-care.

As many biological molecules do not have distinct electrochemical signatures, various schemes have been designed to readout both charged and uncharged analytes including DNA, RNA, proteins, cells, and metabolites. Readout methods include detecting covalent reporters, non-cova lent reporters, enzymatic labels, and conformational changes in DNA structures.

1.5 Nanostructured microelectrodes

Nanostructured microelectrodes (NMEs) are electrodeposited noble metal electrodes with controllable surface properties and high sensitivity. The sensors are fabricated by electrodepositing noble metals such as a gold or platinum into small 5 µm apertures defined on a chip. The sensors are functionalized with a thiolated nucleic acid probe molecule complementary to the target sequence of interest. After immersing the sensors in the sample solution, the target analyte hybridizes to the probe molecule and the presence of bound target analyte is readout using an electrocatalytic reporter assay.

By tuning the electrodeposition conditions, sensors grow with varying degrees of nanoscale roughness. The high radius of curvature of the nanoscale features increases the accessibility of
the probe nucleic acids which increases the hybridization efficiency and sensitivity. By tuning the electrodeposition time, sensors grow with sizes ranging from 5-100 µm. Smaller sensors give high signal-to-noise ratios, but have poor sensitivity when challenged with large, slowly diffusing analytes such as RNA. At low concentrations, prohibitively long time scales are required for even a single RNA molecule to interact with sensors with diameters less than 100 µm. Increasing the sensor footprint increases the probability of interaction with the sensor surface and reduces the required hybridization time.

In order to minimize the background current, peptide nucleic acid (PNA), a synthetic, charge-neutral nucleic acid analog, serves as the probe strand. Readout is accomplished using a sensitive electrocatalytic reporter assay. Ruthenium hexamine, a positively charged redox reporter, is electrostatically attracted to the negatively charged backbone of the hybridized target nucleic acid. The presence of bound ruthenium hexamine is readout by applying a voltage at the reduction potential of ruthenium hexamine. The signal is amplified by ferricyanide, which re-oxidizes ruthenium hexamine to allow the transduction of multiple electrons per binding event.

These sensors can have high sensitivity and specificity and can detect the presence of clinically relevant concentrations of RNA, proteins, and small molecules within 30 minutes. A panel of clinical markers can be detected on a single chip by functionalizing each sensor in an array with a different probe sequence.

1.6 Lab-on-a-chip devices

In many applications, the biosensor must be coupled with an upstream sample processing module to prepare the analyte for detection. Examples of common sample processing steps include cell lysis to release the intracellular analytes, and subsequent concentration, isolation and purification of the target molecules. In a point-of-care device, sample processing must be seamlessly integrated with the downstream sensor in an automated, low-cost device.
Recent advances in microfluidics offer the potential to automate high throughput liquid sample processing at low cost. The micron length-scale of microfluidic devices offer a natural way to manipulate and interface with cells and biomolecules. Clinical applications of microfluidics for sample processing include isolation of rare cells from complex matrices, sample concentration, cell lysis, purification, and enzymatic amplification.

1.7 Thesis objectives and overview

The objective of this thesis is to develop new approaches for rapid sample processing and detection of rare analytes to manage the inherent trade-offs that arise in biosensing applications. We will explore methods for overcoming limitations on the speed, sensitivity, and specificity of detection by developing new sensing strategies and coupling these with rapid sample processing using microfluidics. We showcase these new approaches with a range of clinical applications including monitoring cancer progression, diagnosing bacterial infections, and testing antibiotic susceptibility.

The remainder of this thesis will be organized as follows:

Chapter 2: Rapid isolation of rare cells from complex matrices. In Chapter 2, we explore strategies for rapid and efficient isolation of rare cells from complex matrices. We focus on the isolation of circulating tumour cells from the bloodstream as this is the prototypical “needle in a haystack” problem. Highly sensitive and specific isolation techniques are needed as blood cells outnumber circulating tumour cells (CTCs) by a billion-to-one. We design a model to predict the capture efficiency of nanoparticle-labelleled cells flowing in a microfluidic device. We optimize the geometry of the microfluidic device in order to maximize the capture efficiency and minimize non-specific cell adhesion. Finally, we develop a method to sort captured cells by surface marker expression to identify specific sub-populations of rare cells which might have greater prognostic value.
Chapter 3: Rapid electrochemical antibiotic susceptibility testing. In the most serious infections, effective antibiotics must be administered within the first hour. Current antibiotic susceptibility tests are based on standard culture and the results are only available after 1-3 days. In Chapter 3, we study approaches to reduce the time required to perform antibiotic susceptibility tests. We develop a novel electrochemical assay for detecting a redox indicator of bacterial viability and we couple this assay with a strategy to increase the effective concentration of the bacteria by concentrating, isolating and culturing bacteria in nanoliter chambers. Using on-chip electrodes, we electronically readout the susceptibility profile and show that this device can reduce the required incubation period to 1 hour.

Chapter 4: Rapid bacterial lysis and nucleic acid analysis for pathogen identification. In Chapter 4, we ask the question of how to reduce the time required to detect pathogen-specific genetic sequences to identify bacteria rapidly. In particular, we explore strategies to overcome the long incubation periods necessitated by slowly diffusing analytes such as RNA. We increase the probability that released intracellular RNA interacts with the sensor surface by lysing the bacteria in the local environment of the sensor. We find that released intracellular RNA rapidly diffuses across the short distance and accumulates at the sensor surface. Using this method, we show that we can quickly detect clinically relevant concentrations of bacteria.

Chapter 5: Sensitive colorimetric readout of nucleic acids for low-cost pathogen diagnosis. Colorimetric readout is the preferred reporter system for a disposable, point-of-care device as it is inexpensive, easily interpreted, and permanent. In Chapter 5, we explore strategies for rapidly transducing low levels of target analytes into a colour change visible to the naked eye. In order to leverage the sensitivity of nanostructured microelectrodes, we develop an assay to convert the ultra-low currents generated by the sensors into a permanent, visible colour change by introducing an amplification cascade.
1.8 References

2 Rapid isolation of rare cells from complex matrices

In this chapter, we investigate front-end sample processing techniques for rapid and efficient isolation of rare cells from complex matrices. This problem arises in a variety of clinical applications including detecting circulating tumour cells (CTCs) to monitor cancer progression. Circulating tumour cells are cancer cells that have shed from the primary tumour and entered the bloodstream. Detecting CTCs is particularly challenging as it is the prototypical “needle-in-a-haystack” problem. Red blood cells outnumber CTCs by almost a billion-to-one. Here, we explore strategies for increasing the efficiency and purity of rare cell capture from complex matrices. We also investigate methods for sorting subpopulations of tumour cells based on surface marker expression as certain CTCs may have greater metastatic potential and prognostic value.


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2.1 Abstract

The development of strategies for isolating rare cells from complex matrices like blood is important for a wide variety of applications including the analysis of bloodborne cancer cells,
infectious pathogens, and prenatal testing. Due to their high colloidal stability and surface-to-volume ratio, antibody-coated magnetic nanoparticles are excellent labels for cellular surface markers. Unfortunately, capture of nanoparticle-bound cells at practical flow rates is challenging due to the small volume, and thus low magnetic susceptibility, of magnetic nanoparticles. We have developed a means to capture nanoparticle-labeled cells using microstructures which create pockets of locally low linear velocity, termed velocity valleys. Cells that enter a velocity valley slow down momentarily, allowing the magnetic force to overcome the reduced drag force and trap the cells. Here, we describe a model for this mechanism of cell capture and use this model to guide the rational design of a device that efficiently captures rare cells and sorts them according to surface expression in complex matrices with greater than 10 000-fold specificity. By analysing the magnetic and drag forces on a cell, we calculate a threshold linear velocity for capture and relate this to the capture efficiency. We find that the addition of X-shaped microstructures enhances capture efficiency 5-fold compared to circular posts. By tuning the linear velocity, we capture cells with a 100-fold range of surface marker expression with near 100% efficiency and sort these cells into spatially distinct zones. By tuning the flow channel geometry, we reduce non-specific cell adhesion by 5-fold.

2.2 Introduction

Rapid and efficient capture of rare cells from complex matrices is a requirement for a wide variety of applications including the isolation of cancer cells from blood, detecting fetal cells in maternal circulation, and diagnosing viral and bacterial infections. Quantitative monitoring of cancer cells in the bloodstream is a particularly attractive goal, as it would enable non-invasive sampling to track disease progression, potentially using a liquid biopsy rather than one extracted from a tumor. One challenge related to the isolation and analysis of bloodborne cancer cells is that they are present at extremely low concentrations, and large volumes must therefore be processed rapidly to ensure the capture of at least a single cell. Moreover, circulating tumor cells
(CTCs) may be heterogeneous and understanding the distribution of surface marker expression of the component subpopulations would allow greater resolution and precision when monitoring disease progression. For example, recent studies have shown that certain subpopulations of tumour cells have greater metastatic potential.6

Recently, a variety of microfluidic systems for rapid and automated capture of cancer cells have been reported including devices for size-based selection,7–12 immunoaffinity-based capture,5,13–16 fluorescent sorting,17 and magnetic capture of nano- or microparticle labelled cells.18–21 Microfluidic devices offer several advantages over strategies that simply isolate cells in a tube. Microfluidic devices enable the concentration of rare cells into small volumes and facilitate automated processing and analysis of the sample. In many applications, it is advantageous for cells to be released after capture for downstream analysis. Systems in which the device is directly functionalized with a capture agent require an enzymatic step to cleave the cells from the capture antibodies. In comparison, cells captured by magnetic particles can be easily released by removing the permanent magnet or turning off an electromagnet.22 As magnetic capture is non-destructive, the viable cells may be recovered.

Antibody-labeled paramagnetic nanoparticles have several advantages over magnetic microparticles for efficient cell capture, since their high surface-to-volume ratios allow thousands of binding events per cell and rapid binding kinetics.23 Magnetic nanoparticles also possess higher colloidal stability than magnetic microparticles.24 However, due to their small size, magnetic nanoparticles have inherently low magnetic susceptibilities, a consideration which demands a combination of high magnetic field gradients25 and low flow velocities in order to achieve successful trapping of the nanoparticles. Unfortunately, low flow velocities are incompatible with the requirement of rapid processing of large (milliliter) volumes necessary for rare cell capture.
We recently developed a strategy for efficient nanoparticle-mediated capture of subpopulations of cancer cells in spatially distinct zones by manipulating the drag force in a microfluidic device. This approach was validated using samples collected from prostate cancer patients and was shown to be effective at separating CTC subpopulations within these samples. Here we provide a detailed description of the capture mechanism through simulations and we develop a model for cell capture. We optimize a suite of parameters including the device geometry to maximize capture efficiency. The efficient capture of cells with a broad spectrum of EpCAM expression levels is demonstrated by tuning the flow velocity while minimizing non-specific cell adhesion. Using our model and findings, we rationally design a novel strategy for efficient capture and sorting of subpopulations of nanoparticle-bound rare cells directly from complex samples with 5-fold higher purity than the method previously described.
Figure 2.1: The Velocity Valley Cell Capture Chip. (A) Schematic of the cell sorting device with 4 zones of decreasing average linear velocity. By increasing the channel cross section, the drag force drops by a factor of 2 in each sequential zone. Cells with high levels of surface markers are captured in the first zone while cells with low levels are captured in the final zone. The inset shows an optical microscopy image of a capture structure. The capture structures span the full height of the channel in each zone. The scale bar represents 150 μm. (B) Target cells are labelled with anti-EpCAM magnetic nanoparticles and introduced into the device. Target cells are captured by X-shaped capture structures. (C) Spatial distribution of linear velocities at 0.5 mL per hour in the first zone of the device. Velocity valleys are in red. Arrows represent normalized vectors. (D) (i) Magnetic field strength simulated in a cross section of the first zone of the chip. (ii) The strength of the magnetic fields as a function of channel length. (E) (i) Corresponding force on a single nanoparticle in the first zone of the device. As the force is proportional to the field gradient, it is highest at the magnet edges. (ii) The strength of the magnetic force acting on a single nanoparticle as a function of channel length. Scale bar represents 1 mm.
2.3 Results and discussion

In our velocity valley chip (VVC), a flow channel is sandwiched between arrays of magnets that generate a high field gradient inside the chip (Figure 2.1A). Cells are labeled with magnetic nanoparticles coated with the epithelial cell adhesion marker (EpCAM), which is commonly overexpressed in a variety of cancers (Figure 2.1B). Key to this idea is the use of X-shaped capture structures (Figure 2.1C) to generate regions of locally low flow velocity – which we term velocity valleys. Cells in the vicinity of a capture structure slow as they enter the velocity valley. While a cell is in the valley, the magnetic force is sufficient to overcome the lowered drag force. This enables efficient cell capture. Importantly, the structures do not trap non-target cells, and the device effectively captures cells with a wide range of surface marker expression levels.

To spatially sort cells based on surface marker expression, we designed a device with four zones in which the drag force decreases by a factor of 2 in each sequential zone (Figure 2.1A). Cells bound by high numbers of nanoparticles are captured in the first zone with high linear velocities, while cells with lower numbers of bound nanoparticles are captured in the latter zones. The geometry of the zones are optimized to minimize device area which reduces non-specific cell adhesion and the time required to image the captured cells using fluorescence microscopy.

2.3.1 Optimization of capture structures

We sought to evaluate the performance of VVCs by simulating the distribution of linear velocities in devices with a variety of capture structure geometries (Figure 2.2A). These capture structures span the full height of the channel. We calculated the percentage area of the device below a given linear velocity and plotted this as a cumulative distribution function (Figure 2.2B). Given the same average input flow rate of 0.5 mL per hour, the percentage of the chip at low linear velocities is drastically increased when the ‘X’ shaped capture structures are present.
Figure 2.2: Optimization of the capture structures. (A) Spatial distribution of linear velocities in devices with ‘X’, ‘+’, and ‘○’ shaped structures. Velocity valleys are in red. (B) Cumulative distribution of linear velocities in devices with various capture structure geometries compared to a device without structures. The y-axis is the percent area of the chip with linear velocity less than or equal to the corresponding value on the x-axis. X-shaped capture structures markedly increase the percentage area of the chip with low linear velocities. (C) X-shaped capture structures drastically increase the area with linear speed below the threshold for capture when compared to circular posts which do not generate velocity valleys. (D) Capture efficiency as a function of capture structure geometry for VCaP cells. The red lines represent the predicted capture efficiency as a function of structure geometry. Error bars represent standard error.
We hypothesized that these structures could allow for capture of nanoparticle-bound cells while maintaining a high average flow rate by increasing the percentage area of the chip with linear velocities below a threshold for capture \( (v_t) \). We define this threshold linear velocity as the velocity at which the corresponding drag force is lower than the magnetic force acting on the cell. Cells that enter a pocket of low velocity will slow down and experience a reduced drag force and thus, the relative ratio of the magnetic to drag forces will increase. If the cell is slowed below the threshold velocity, the magnetic force can overwhelm the drag force and the cell will be drawn to the chip walls where it will be trapped by a combination of magnetic, frictional, normal and adhesion forces.

Figure 2.2C shows the increase in area of the chip over which the linear velocity lies below the threshold for capture. It compares a chip with ‘X’ shaped capture structures to a device with circular posts which do not generate velocity valleys. The ‘X’ shaped structures provide the greatest enhancement when the threshold velocity for capture is low. This corresponds to the case in which magnetic nanoparticles are employed, since they exhibit low magnetic susceptibility due to their small volume.

To verify the validity of our calculations, we fabricated devices with arrays of ‘X’, ‘+’, and ‘○’ shaped capture structures. We challenged these devices with VCaP cells, a model prostate cancer cell line with a high level of EpCAM expression. VCaP cells were incubated with nanoparticles labelled with an anti-EpCAM antibody for 20 minutes and introduced into the device. Figure 2.2D shows capture efficiency as a function of capture structure geometry. The X-shaped capture structures captured cells with 5× greater efficiency than circular posts. These results agree with the simulations of capture efficiency as outlined below as the posts do not create the low-velocity pockets necessary for effective capture of nanoparticle labeled cells. The capture efficiency of U937 cells, a cell line which does not express EpCAM, was less than 0.1% for all capture structure designs indicating that only cells tagged with magnetic particles will be captured.
2.3.2 Modelling the capture efficiency of a velocity valley chip

As a complement to our numerical simulations of the flow and magnetic fields generated by the magnet array, we analyzed the drag and magnetic forces acting on a cell to develop a predictive model for cell capture using the X-shaped structures. At low Reynolds numbers, drag on a cell is governed by Stokes’ law:

\[ F_d = 6\pi \eta rv \]  

(1)

where \( F_d \) [N] is the drag force, \( r \) [m] is the cell or nanoparticle radius, \( \eta \) [Pa s] is the dynamic viscosity of the medium (0.001 Pa s), and \( v \) [m s\(^{-1}\)] is the relative velocity of the cell compared to the surrounding fluid.

The magnetic force acting on superparamagnetic nanoparticles is:\(^{29}\)

\[ \vec{F}_{m\_\text{bead}} = V_m \frac{\Delta \chi_{\text{bead}}}{\mu_0} (\vec{B} \cdot \nabla)\vec{B} \]  

(2)

where \( V_m \) [m\(^3\)] is the nanoparticle volume, \( \Delta \chi_{\text{bead}} \) [unitless] is the difference between the magnetic susceptibility of the nanoparticle and the medium, \( \mu_0 \) [H m\(^{-1}\)] is the permeability of free space (\( 4\pi \times 10^{-7} \) H m\(^{-1}\)), and \( \vec{B} \) [T] is the applied magnetic field. The magnetic force acting on a cell is given by multiplying the magnetic force on an individual nanoparticle by the average number of nanoparticles per cell (\( N_b \)):

\[ \vec{F}_{m\_\text{cell}} = N_b V_m \frac{\Delta \chi_{\text{bead}}}{\mu_0} (\vec{B} \cdot \nabla)\vec{B} \]  

(3)

Thousands of nanoparticles may bind to a cell\(^{30}\) and the exact value of \( N_b \) depends on the cell type and the affinity constant of the antibodies. For the MACS 50 nm superparamagnetic
nanoparticles, $V_m \Delta \chi_{\text{bead}}$ is $2.5 \times 10^{-16}$ mm$^3$. Since the magnetic fields vary within the channel as a function of distance along the channel and channel height (Figure 7.1.1) we use the maximum magnetic force acting on a cell in our calculations.

Predicting exactly where a cell will be captured in the chip is challenging. We sought to develop a model to analyze various device designs in order to quickly predict performance. Neglecting gravity, the cells will be subjected to several forces as they flow through the microfluidic chip: a drag force in the direction of the flow, a magnetic force in the transverse direction, and a drag force in the transverse direction which opposes the magnetic force (Figure 7.1.2). However, once a cell is captured at one of the boundaries of the chip, friction, adhesion and normal forces are the dominant components keeping the cell fixed in place. In order to determine where the cells will most likely be brought into contact with the chip boundaries, it is useful to compare the relative magnitudes of the drag and magnetic forces acting on a cell. In regions of high flow velocity, the drag force in the direction of the flow will be much greater than the magnetic and transverse drag forces acting on the cell, leading to a resultant cell velocity which is essentially parallel to the flow streamlines. Conversely, in a velocity valley (where the flow velocity is low) the magnitude of the magnetic and transverse drag forces acting on the cell should match or exceed that of the drag force resulting from the flow, yielding a velocity vector which drives the cell towards the boundaries of the chip.

There is a high probability of cell capture if the magnitude of the magnetic force experienced by the cell is much greater than the magnitude of the drag force which opposes capture. Therefore, we define a threshold linear velocity, $v_t$, such that the corresponding drag force is equivalent to the maximum magnetic force acting on a cell inside the channel. Cells in an area of the chip with high local linear velocity (greater than $v_t$) are unlikely to be captured while those in an area of low linear velocity (less than $v_t$) have a higher probability of capture. We approximate that the probability of cell capture at a given structure, $P_{\text{capture}}$, is proportional to $A_{V<v_t}$, the average
percentage of area surrounding a capture structure in which the linear velocity is less than the
threshold. As the time spent in proximity of a capture structure decreases linearly with increasing
flow rate, we also assume the probability of cell capture at a given structure is inversely
proportional to the flow rate.

The total capture efficiency, $E$, can be calculated as:

$$E = 100\% \left( 1 - (1 - P_{\text{capture}})^N \right)$$

where $N$ is the number of capture structures in each cell's path, $P_{\text{capture}}$ is the probability of
capture at a capture structure, and $\alpha$ is an experimentally determined proportionality constant (h mL$^{-1}$) with units set to ensure
that $P_{\text{capture}}$ is unitless. A limitation of this model is that it does not account for the spatial overlay
of the magnetic and drag forces. This model also does not account for the frictional and normal
forces acting on the cell. Additional details of the model are provided in the appendix.

**2.3.3 Capture efficiency as a function of flow rate**

Our initial tests were conducted with VCaP, a cell line with high expression of EpCAM; however, in other cell lines, EpCAM levels may vary over a wide range between $1 \times 10^4$ to $1 \times 10^7$ sites per cell.\(^{32}\) To examine the performance of our device with a cell line with a moderate
level of EpCAM expression, we chose to test SKBR3, a breast cancer cell line which has
approximately 10-fold lower EpCAM expression than VCaP.\(^{26}\) We measured the capture
efficiency of SKBR3 cells as a function of volumetric flow rate (Figure 2.3C). As expected, the
capture efficiency increased with decreasing flow rate.
We used eqn (4) to model the capture efficiency as a function of flow rate for SKBR3 cells. First, we calculated the magnetic force acting on 50 nm nanoparticles using eqn (2) and found the maximum force on a magnetic nanobead is 143 aN (Figure 2.1E). Next, we estimated the number of nanoparticles bound to SKBR3 cells by measuring the deflection of nanoparticle labelled cells flowing in a channel under the influence of a magnetic field (Figure 7.1.3), a method previously established by other authors. The number of nanoparticles per cell can be calculated by measuring the transverse velocity in the channel and using it to calculate the corresponding Stokes drag force. By equating the Stokes drag force to the magnetic force, using eqn (3), it is possible to determine the number of nanoparticles per cell.

We validated the method using magnetic microparticles having known magnetic susceptibilities. Our results indicate that the ratio of the magnetic force to the Stokes drag force is approximately 1, indicating that our chosen measurement technique was valid (Figure 2.3A).

We found an average of $3.3 \times 10^4$ nanoparticles per cell for the SKBR3 cell line (Figure 2.3B). This gives a maximum magnetic force per cell of 5 pN which corresponds to a threshold linear velocity for capture of $25 \mu m$ s$^{-1}$. Using eqn (4), we modeled the capture efficiency as a function of flow rate and fit our model to the capture efficiency data points (Figure 2.3C). We found our model best fit the data when $\alpha$, the experimentally determined proportionality factor, was 0.1. We also calculated the predicted capture efficiency as a function of structure geometry and found good agreement with our measured experimental capture results (Figure 2.2D).

### 2.3.4 Tuning the flow rate to capture a wide range of cell types

We selected a suite of cell lines with a spectrum of EpCAM expression levels to evaluate how the VVC approach performed with varying surface marker levels. The cell lines selected were VCaP, SKBR3 and MDA-MB-231, three cancer lines which have high, moderate, and low levels of EpCAM respectively. VCaP express EpCAM at levels approximately 10-fold higher than
SKBR3\textsuperscript{26} which in turn express EpCAM 10-fold higher than MDA-MB-231.\textsuperscript{21} Using the established method for measuring the number of nanoparticles per cell,\textsuperscript{27} we generated a histogram of the number of beads per cell and found a broad distribution of beads per cell for each cell line. Using the mean numbers of nanoparticles from the histogram, we found an average of $4.3 \times 10^4$, $3.3 \times 10^4$, and $5 \times 10^3$ nanoparticles per cell for VCaP, SKBR3 and MDA-MB-231, respectively (Figure 2.3B). Our calculations are in line with values previously reported in the literature.\textsuperscript{30}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Tuning the linear velocity to capture different cell types. (A) Validation of the method to measure the magnetic force using magnetic microbeads. These results validate the assumption that the Stokes drag force can be equated to the magnetic force. (B) Distribution of the number of nanoparticles per cell for three cell lines. (C) Capture efficiency as a function of flow rate for SKBR3 cells. The black line represents the predicted capture efficiency. (D) Capture efficiency as a function of cell type. By tuning the linear velocity it was possible to capture all three cell lines with greater than 90% efficiency. U937 cells, which do not express EpCAM, are captured with less than 0.1% efficiency. Less than 2% of cells were captured when magnets were removed. The red lines represent the predicted capture efficiency. Error bars represent standard error.}
\end{figure}
To study capture efficiency as a function of EpCAM expression, we challenged the VVC with each of these cell lines and measured the capture efficiency at various flow rates (Figure 2.3D). We found that by tuning the flow rate we could capture all three cell lines with greater than 90% efficiency. As expected, lower flow rates were needed for efficient capture of MDA-MB-231 cells, which have low EpCAM expression, than VCaP cells, which express high levels of EpCAM. These results suggest that by tuning the flow rate, our device is capable of capturing a wide range of cell types.

We challenged the device with 1,000,000 U937 cells to evaluate the non-specific binding that might occur in a blood sample. U937 is a white blood cell line that does not express EpCAM. These cells were tested with a flow rate of 0.5 mL h⁻¹ and the capture efficiency was less than 0.1% (Figure 2.3D). These control experiments suggest the X-shaped structures only are capable of trapping cells when coupled with a magnetic force. We modelled the capture efficiency as a function of the number of bound nanoparticles and flow rate (Figure 2.3D). We found good agreement between our model and the experimental capture efficiency.

2.3.5 Validation in complex matrices

To validate the performance of the VVC in complex matrices, we challenged our device with undiluted, whole blood. We spiked serial dilutions of VCaP cells in blood and incubated the cells with nanoparticles for 20 min. The whole blood was introduced into the device at 0.6 mL h⁻¹. After capture, cancer cells were distinguished from nucleated white blood cells using a series of fluorescent stains for cytokeratin (CK), an epithelial marker, CD-45, a pan-leukocyte marker, and DAPI, which stains the nucleus. Cancer cells were identified as DAPI+/CK+/CD45⁻ and white blood cells were identified as DAPI+/CK⁻/CD45+ (Figure 2.4A). The magnet must be removed prior to imaging which can dislocate the cells from the X-shaped structures (Figure 2.4B).
VCaP cells were captured with high efficiency at all concentrations tested (Figure 2.4C). VCaP cells were not identified in whole blood samples that did not contain spiked cells. In a given device, we would typically observe less than 200 white blood cells, which is less than 0.01% of the WBCs introduced, indicating the cancer cells are recovered with greater than 10 000-fold specificity.

2.3.6 Minimizing non-specific cell adhesion by tuning channel geometry

Motivated by results indicating that the cells with different surface marker expression are efficiently captured at different average linear velocities, we sought to design a device to spatially sort cells based on surface marker expression. We designed a device with multiple zones in which the linear velocity drops stepwise by a factor of 2 in each sequential zone. We hypothesized that cells with high surface marker expression and correspondingly high numbers of bound nanoparticles would be captured in the initial zones of high linear velocity while cells with low EpCAM expression would be captured in the latter zones with low linear velocity.

To study the effect of device geometry on non-specific cell adhesion, we devised two strategies to increase the channel cross-sectional area in successive zones to manipulate the average linear velocity. In the first approach, the channel height is held constant and the channel width doubles in successive zones. In the second approach, we hold the channel width constant while doubling the channel height.

We challenged both devices with 1 mL of undiluted blood at 0.6 mL h⁻¹ and counted the number of non-specific cells bound to the device using fluorescence immuno-staining. Figure 2.4D shows the number of non-specific white blood cells captured in each zone. We found that the numbers of non-specifically bound cells is approximately proportional to the zone width. In comparison, the number of non-specifically bound cells increases much more slowly as a function of zone height. In the latter zones, the number of non-specifically bound cells is 5-fold
higher when successive zones increase in width rather than height. The increase in purity when successive zones increase in height is 1.9-fold, 2.1-fold, 5.3-fold and 5.2-fold in zones 1 through 4 respectively. This is expected as non-specific cell adhesion increases as a function of surface area and thus the propensity for non-specific cell adhesion increases in the wider zones. In comparison, as the channel is much wider than it is tall, the device surface area does not increase appreciably as a function of channel height (Table 7.1.5) which minimizes non-specific cell adhesion.
Figure 2.4: Capture and sorting of rare cells in blood. (A) After capture, cancer cells are stained for DAPI, CK, and CD45. VCaP cells are identified as DAPI+/CK+/CD45− and nucleated white blood cells are identified as DAPI+/CK−/CD45+. (B) VCaP cells trapped near the vicinity of an X-shaped capture structure. The scale bar represents 150 μm. (C) Capture efficiency as a function of the number of VCaP cells spiked in whole, undiluted blood. Error bars represent standard error. (D) The number of non-specifically bound white blood cells (WBCs) across the 4 zones of the device when sequential zones double in height vs. width. (E) Distribution of two cell lines, VCaP and MDA-MB-231, with high and low EpCAM expression respectively across the 4 zones of the device. Whole blood was introduced into the device at 0.6 mL h⁻¹.
2.3.7  Sorting cells by surface marker expression

Motivated by our results, we designed a device to sort cells based on surface marker expression into four spatially distinct zones. This device consists of four zones in which the linear velocity drops by 2 in each successive zone (Figure 2.1A). In the first three zones, the channel width is held constant while the height doubles. Due to limitations in fabricating features greater than 500 μm using photolithography, in the fourth zone, the channel height is held constant and the channel width increases. Although the channel height does increase in the latter zones, the magnetic fields do not decrease appreciably within the channel as the bottom magnet remains at a constant distance below the channel (Figure 7.1.1). We challenged the device with VCaP and MDA-MB-231 cells spiked in blood which have high and low expression of EpCAM respectively and measured the numbers of cells captured in each zone (Figure 2.4E). We found that 92% of VCaP cells were captured in the first zone with highest average linear velocity while 82% of MDA-MB-231 cells were captured in the latter two zones with the lowest linear velocity.

These results suggest that this device could be used to spatially sort rare cells as a function of surface marker expression with high purity. Our results indicate that manipulating the linear velocity using a channel height increase can reduce the amount of non-specific binding by minimizing the device surface area. Combinations of increasing both the height and width of the device would expand the dynamic range of linear velocities. Another advantage of this device is that it minimizes the footprint which reduces the time required to image the device using fluorescence microscopy and decreases fabrication costs. By increasing the channel cross-section, it is possible to increase the average flow rate, and thus throughput, while maintaining the same in-channel linear velocity.
2.4 Conclusions

We elaborate the mechanisms, models, and optimization of a microfluidic device for the capture of rare cells in whole blood. This chip enables capture of cells bound by low magnetic susceptibility nanoparticles using capture structures which create velocity valleys, or zones of low-linear velocity. These structures tilt the balance of forces acting on a nanoparticle-bound cell in favour of the action of the magnetic force. Using X-shaped capture structures provides a 5-fold enhancement in capture efficiency over circular posts. By tuning the average flow rate, we efficiently capture cells with a 100-fold difference in EpCAM expression and sort these cells into spatially distinct zones. We optimize the device geometry to reduce non-specific cell adhesion by 5-fold and demonstrate that in whole blood, we successfully recover cancer cells with greater than 10 000-fold specificity. By changing the capture label on the magnetic nanoparticles, this strategy could be used for capture of a wide variety of rare cell types.

2.5 Methods

2.5.1 Simulations

Simulations were carried out using COMSOL Multiphysics. We simulated the spatial distribution of linear velocities of fluid flowing in various device designs. We simulated the spatial distribution of magnetic fields and calculated the corresponding magnetic forces acting on super paramagnetic nanoparticles for our device geometry. Details of the simulations are included in the appendix.

2.5.2 Cell culture

VCaP cells (ATCC CRL-2876) were cultured in DMEM medium (ATCC 30-2002). SK-BR-3 cells (ATCC HTB-30) were cultured in McCoy's Medium Modified (ATCC 30-2007). MDA-MB-231 cells (ATCC HTB-26) were cultured in Leibovitz's L-15 medium (ATCC 30-2008). All media was supplemented with 10% FBS and cells were cultured at 37 °C and 5% CO₂ in T75
flasks. Cells were collected by treating with 0.25% w/v trypsin with 0.53 mM EDTA for 3 minutes.

2.5.3 Chip fabrication
Masters were fabricated on silicon substrates and were patterned in SU-8 3050 (Microchem, MA) using photolithography. PDMS (Dow Chemical, MI) replicas were poured on masters and baked at 67 °C for 45 minutes. PDMS replicas were attached to no. 1 glass coverslips using a 30 s plasma treatment and left to bond overnight. The chip was sandwiched between arrays of N52 NdFeB magnets (K&J Magnetics, PA, 1.5 mm by 8 mm) with alternating polarity. Devices were treated with 1% Pluronic in PBS for 1 hour.

2.5.4 Estimating the number of nanoparticles per cell
We used a previously established method for estimating the number of nanoparticles per cell. Full details are provided in the appendix.

2.5.5 Cell capture in blood
Cells were diluted to the appropriate concentration, spiked in 1 mL of whole blood, mixed with 40 μl of anti-EpCAM Nano-Beads (MACS) and incubated for 20 minutes. The blood was introduced into the device using a syringe pump. Next 200 μl PBS-EDTA at 0.6 ml h⁻¹ was introduced to wash non-specific cells. To measure non-specific cell adhesion as a function of zone geometry, we fabricated two versions of the device in which sequential zones double in width and height. 1 mL of whole blood was incubated with 40 μl of nanoparticles for 20 min and injected into the device at 0.6 mL h⁻¹. 200 μl PBS-EDTA at 0.6 ml h⁻¹ was introduced as a wash step. The numbers of white blood cells were counted as below.
2.5.6 Immunostaining and imaging

Captured cells were counted with the aid of fluorescence microscopy. Before staining, captured cells were fixed inside the chip using 100 μL of 4% formaldehyde, followed by 100 μL 0.2% Triton X-100 (Sigma-Aldrich) for permeabilization. For staining in blood, we used 100 μL of the following reagents: DAPI ProLong Gold reagent (Invitrogen, CA) to stain cell nuclei, CK APC (Genetex GTX80205) and Anti-CD45 AF 488 (Invitrogen MHCD4520) to stain white blood cells. Antibodies were prepared in 100 μl PBS with 1% BSA and chips were stained for 60 minutes at a flow rate of 0.1 ml h\(^{-1}\). For experiments in PBS, we stained cancer cell nuclei using 100 μL of DAPI. After staining, chips were washed twice with 100 μL PBS, dried, and stored at 4 °C. Chips were imaged using a fluorescent microscope (Nikon) with an automated stage controller and CCD (Hamamatsu, Japan) and images were automatically acquired with NES Elements (Nikon). Cells were enumerated by overlaying the bright field, red fluorescent, green fluorescent and blue fluorescent images.

2.6 References


3 Rapid electrochemical antibiotic susceptibility testing

In Chapter 2, we developed a device to concentrate and analyze rare cancer cells rapidly in order to monitor cancer progression. In this chapter, we describe a method to concentrate bacteria in order to readout the antibiotic susceptibility profile of a bacterial infection rapidly.

Culture, which requires days, is the gold standard for antibiotic susceptibility profiling. Yet, in the most serious bacterial infections which lead to sepsis, the chances of survival decrease by almost 8% for every hour without effective treatment. Rather than administer targeted therapy, doctors treat infections with broad spectrum antibiotics while waiting for the results of the susceptibility test. This leads to increased antibiotic resistance and poorer outcomes for patients. In this chapter, we design strategies to reduce the time required to perform antibiotic susceptibility profiling. We describe an assay to electronically detect viable bacteria and couple it with a device to concentrate, isolate and culture bacteria in nanoliter culture chambers. Using this device, the susceptibility profile is readout electronically with a 1 hour incubation.

Disclosure of work within this manuscript; J.D.B, E.H.S. and S.O.K. designed the experiments. J.D.B performed the experiments, and interpreted results with assistance from E.H.S. and S.O.K. J.D.B, E.H.S. and S.O.K. composed and refined the manuscript.

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3.1 Abstract

Rapid phenotyping of bacteria to identify drug-resistant strains is an important capability for the treatment and management of infectious disease. At present, the rapid determination of antibiotic susceptibility is hindered by the requirement that, in existing devices, bacteria must be precultured for 2–3 days to reach detectable levels. Here we report a novel electrochemical approach that achieves rapid readout of the antibiotic susceptibility profile of a bacterial infection within one hour. The electrochemical reduction of a redox-active molecule is monitored that reports on levels of metabolically-active bacteria. Bacteria are captured in miniaturized wells, incubated with antimicrobials and monitored for resistance. This electrochemical phenotyping approach is effective with clinically-relevant levels of bacteria, and provides results comparable to culture-based analysis. Results, however, are delivered on a much faster timescale, with resistance profiles available after a one hour incubation period.

3.2 Introduction

The overuse of antibiotics and the prescription of antibiotics to which a pathogen is not susceptible contribute to rising antibiotic resistance rates – a growing threat to public health worldwide.\textsuperscript{1} Urinary tract infections are among the most prevalent bacterial infections.\textsuperscript{2} Gold-standard antibiotic susceptibility tests for urinary tract infections rely on culture and require 1–3 days in order to allow the bacteria to multiply to detectable levels.\textsuperscript{3} After pre-culture of the bacteria, an additional 18 hours are typically required to perform standard susceptibility tests. Reducing the time needed to determine the susceptibility profile of urinary tract infections could improve clinical outcomes, especially in the case of the most severe infections that lead to urosepsis.\textsuperscript{4} Rapid testing could also contribute to decreased unnecessary antibiotic use,\textsuperscript{5} and could increase the efficiency of centralized diagnostic laboratories.
Tests for antibiotic resistance that rely on enzymatic amplification of antibiotic-resistance genes reduce turnaround times compared to culture. Unfortunately, these assays often require a pre-incubation step to allow the bacteria to multiply, and, further, often require several hours to amplify the genes of interest. Gene-based assays are also limited by the requirement of knowing a priori which genes confer resistance. Dozens of constantly-evolving genes may be implicated in resistance to a given antibiotic, and it is impractical to test for all possible mutations simultaneously.

Assays that monitor bacterial viability in response to antibiotics overcome the limitations of genetic tests. These tests report directly on the question of greatest clinical importance: whether a given antibiotic decreases bacterial survival. New assays for antibiotic resistance include the detection of bacterial motion using AFM cantilevers, electrochemical measurements of bacterial growth, optical detection of bacterial growth, and optical detection of redox reporters of bacterial metabolism. In assays that detect metabolically-active pathogens, the bacteria are incubated with the antibiotic and a redox reporter of metabolism such as resazurin or methylene blue. Metabolically-active bacteria create a reducing environment and either directly or indirectly reduce the compound, and the change in redox state is read out as a change in color or fluorescence. Resistant bacteria continue to multiply and metabolize the compound, while susceptible bacteria do not.

Successful detection using this type of approach hinges on the requirement that a sufficient quantity of the reduced form of the reporter compound accumulates above the detection threshold, a delay that takes at least 12 hours in milliliter-scale culture. Strategies have been proposed that seek to confine bacteria in microliter and nanoliter volumes with the goal of reducing the time of detection by increasing the local concentration of the bacteria. In the most sensitive of these optical techniques, the sample is divided into millions of nanoliter droplets and the signal is readout sequentially from each droplet with a fluorescence
Despite several recent advances in ultrasensitive electrochemical detection of bacteria, few devices have been reported for direct electrochemical detection of antibiotic resistance. Electrochemical readout requires only simple electronics allowing direct electronic detection of antibiotic susceptibility from confined nanoliter droplets without complex optical instrumentation for readout. Here, we describe a new strategy for rapid electrochemical phenotyping that effectively identifies the antibiotic susceptibility profile of bacteria. We describe the development of an assay that uses electrochemical readout to detect metabolically active bacteria. The electrochemical reduction of resazurin is monitored to establish the presence of live bacteria, and analyzed in the presence of antibiotics to determine resistance profiles. We utilize this assay in conjunction with a novel device to concentrate and incubate bacteria in an array of miniaturized culture chambers (Figure 3.1). Captured bacteria are incubated with antibiotics and a redox reporter of bacterial viability. Small changes in the redox state of the dye can be rapidly detected using in-well electrodes (Figure 3.1B). Using this assay, we detect a clinically relevant concentration of bacteria with a 30 minute incubation. Finally, we show that the antibiotic susceptibility profile of a clinically-relevant concentration of bacteria in urine can be determined after an one-hour incubation without any other pre-incubation steps. This is the first approach that provides antibiotic resistance phenotyping on such a short time scale.
Figure 3.1: Overview of the electrochemical phenotypic testing approach. (A) Resazurin (RZ) is reduced to resorufin (RR) by metabolically active bacteria. (B) Schematic of a single well containing a working (WE), counter (CE) and reference electrode (RE). Each well also contains an integrated filter for bacterial capture. (C) Schematic of the antibiotic susceptibility testing device. The bacteria are cultured in miniature culture chambers, each of which contains a filter for bacterial capture and electrodes for readout of bacterial metabolism. (D) i. A urine sample containing bacteria is introduced into the device and bacteria are captured within wells. ii. Resazurin, the culture media, and an antibiotic are introduced. iii. Plugs are formed within wells by introducing an immiscible oil. (E) Representative differential pulse voltammograms (DPVs) illustrating the principle of electrochemical phenotyping. Bacteria are cultured within the wells with resazurin and an antibiotic. Susceptible bacteria do not reduce the compound, while resistant bacteria reduce resazurin to resorufin. The two states of the molecule can be distinguished by in-well electrodes. See Figure 7.2.1 for further electrochemical characterization of resazurin.
3.3 Results and discussion

3.3.1 Electrochemical detection of viable bacteria

Redox dyes that are reduced by metabolically-active bacteria have been used as optical indicators of bacterial viability in the presence of antibiotics, but have not offered significant improvement in the delivery of rapid profiling results. We hypothesized that resazurin, a commonly-employed reporter used to optically assess cell viability, could be used for electrochemical detection of bacterial antibiotic susceptibility (Figure 3.1), and potentially, the sensitivity of this readout method could produce improvements in assay speed. In the presence of an ineffective antibiotic, resistant bacteria will continue to multiply and create a reducing environment which converts resazurin to resorufin. On the other hand, since effective antibiotics hinder bacterial metabolism, they will prevent reduction of the dye. As resazurin and resorufin have different electrochemical signatures, using differential pulse voltammetry we can distinguish between the two states of the dye and thus determine whether the bacteria is susceptible (Figure 3.1E).

We first characterized the electrochemical profile of resazurin to determine if this reporter group would be suitable for monitoring live bacteria (Figure 7.2.1). When resazurin is present in aqueous buffer, the initial irreversible two-electron reduction of the dye to resorufin occurs at \(-0.45\ \text{V vs. Ag/AgCl}.\) An additional reversible process is observed at \(-0.6\ \text{V}\) that represents the two electron reduction of resorufin to dihydroresorufin. In bacterial culture media at 37 °C, the formation of dihydroresorufin occurs at a less negative potential and is visualized as a shoulder on the resazurin reduction peak when differential pulse voltammetry (DPV) is used to monitor the redox reporter. Nonetheless, a significant decrease in the electrochemical signal is observed in the presence of active bacteria (Figure 7.2.2). We studied the effect of dissolved oxygen on the electrochemical signal and found that it did not significantly affect our measurements (Figure 7.2.3).
We tested the limit of detection that could be achieved by monitoring the electrochemical signal of resazurin by incubating serial dilutions of *Escherichia coli* (*E. coli*) with 1 mM resazurin in LB culture media for 5 hours at 37 °C. Figure 3.2A shows representative DPV scans and the average peak currents at −0.35 V as a function of bacterial concentration are plotted in Figure 3.2B. We obtained a detection limit of 100 cfu μL⁻¹, which is clinically relevant and commonly used as a threshold level for the presence of bacteriuria.²,³³ The peak signals decreases with increasing bacterial concentration, as expected given that viable bacteria convert resazurin to resorufin. As there is significant overlap between peaks I and II, a decrease in the height of peak I causes peak II to decrease as well.

![Figure 3.2: Electrochemical detection of bacterial metabolism.](image)

(A) Representative differential pulse voltammograms obtained from culturing serial dilutions of *E. coli* for 5 hours with resazurin. Differential pulse voltammograms of resazurin in LB media exhibit two peaks. Peak I corresponds to the conversion of resazurin to resorufin through an irreversible 2-electron process, while peak II corresponds to the reversible reduction of resorufin to dihydroresorufin. Peak I decreases systematically as metabolically active bacteria metabolize resazurin. (B) Average signal decrease obtained after culturing *E. coli* for 5 hours with resazurin. Data shown represents the average of at least 8 replicates. Error bars represent standard error.
We compared the detection limit of electrochemical and fluorescent detection of bacterial viability using resazurin and found a similar limit of detection of 100 cfu \( \mu \text{L}^{-1} \) indicating that electrochemical detection of resazurin is just as sensitive as fluorescent readout (Figure 7.2.4). The advantage of using electrochemistry is that it does not require complicated or bulky instrumentation for readout and the sensors can be integrated directly into the culture chambers. In the most sensitive fluorescence assays, the assay is performed in a series of nanoliter droplets which require a fluorescence microscope for sequential readout of the droplets. Using electrochemistry, it is possible to integrate the sensors directly into the nanoliter culture chambers, eliminating the need for sophisticated optical equipment for readout. The electronics required for electrochemical readout can be integrated into a small benchtop or handheld device, limiting the cost and footprint of clinical instrumentation that could be developed with this approach.

3.3.2 Design of a microfabricated device for rapid electrochemical analysis of bacteria

With proof-of-concept in hand demonstrating that bacterial viability could be monitored electrochemically, we explored strategies to decrease the detection time to improve the clinical utility of this assay. We hypothesized that by initially concentrating bacteria in a nanoliter well and subsequently conducting the assay within this small volume, we could reduce the assay time to less than one hour. Integrating electrochemical sensors directly into each of the nanoliter incubation chambers allows rapid and direct readout of the antibiotic susceptibility profile in a small volume without requiring bulky optical instrumentation to sequentially readout thousands of nanoliter droplets.

This approach provides two advantages: concentration and confinement. Concentrating the bacteria inside miniaturized wells increases the local effective concentration of the bacteria. For example, 10 bacteria captured in a 1 nL well is equivalent to 10 000 cfu \( \mu \text{L}^{-1} \), while 10 bacteria
captured in 1 μL well gives a concentration of only 10 cfu μL⁻¹. The greater the concentration of bacteria per well, the faster the turnover of resazurin and accumulation of the target redox molecule. As the signal from differential pulse voltammetry is directly proportional to the concentration of the redox molecule, an increase in local concentration of bacteria increases the magnitude of the signal change acquired.

Confinement within a nanoliter volume provides another advantage – as resazurin is reduced, it is prevented from diffusing into bulk solution allowing the reduced form to rapidly accumulate to detectable levels.

A fully integrated device for the concentration of bacteria into an array of independent nanoliter culture chambers was designed with each well equipped with electrodes to electrochemically readout the antibiotic susceptibility profile of the bacteria captured within (Figure 3.1B, C). The culture chambers have dimensions of 100 μm × 50 μm × 550 μm, which is equivalent to a volume of 2.75 nL. Each well also contained a filter for bacterial capture. The well array allows for multiple measurements per sample, thereby increasing the accuracy of the device. Typically, 15 measurements are performed per sample.

To fabricate this device, we patterned gold electrodes on a glass substrate which act as the working, counter and reference electrodes (Figure 7.2.5 and Figure 7.2.6). The substrate was passivated with a 2 μm thick SU-8 layer and openings were defined using photolithography to expose the electrodes beneath. A 50 μm thick SU-8 layer was patterned to form the microchannel and culture wells. This was followed by a 2 μm SU-8 layer patterned to define a barrier at the rear of each well. This barrier is used to immobilize a bed of microbeads which act as an in-well bacterial filter (Figure 3.1B). Figure 7.2.5 shows optical images of the fabricated device. The working electrodes were electroplated with HAuCl₄ to increase the electrode surface area. This increases the magnitude of the acquired signal and thus, the detection sensitivity (Figure 7.2.10A).
To perform the bacterial detection assay within the nanoliter capture device, bacterial cells are introduced into the main channel, which then flow into the wells (Figure 3.1D). The bacteria are prevented by the in-well microbead filters from exiting the wells, while the solution continues to flow. Next, the culture medium, resazurin, and an antibiotic is introduced. An immiscible fluorinated oil, FC-40, is also brought into the device, which displaces the aqueous solution in the main channel. Due to surface tension, a sealed nanoliter plug of culture media remains in each well. The device can then be incubated at 37 °C to allow bacteria to multiply. Antibiotic-susceptible bacteria captured within a well are inhibited from reproducing and do not reduce resazurin while resistant bacteria continue to multiply and reduce the molecule (Figure 3.1E). After incubation, electrochemical measurements are recorded using DPV with the on-chip electrodes.
Figure 3.3: In-well bacterial capture and analysis. (A) Schematic of in-well bacterial capture. Bacteria are trapped within in-well size-based filters fabricated from a bed of polystyrene beads immobilized within each well. (B) Optical image of an additional filter introduced by microbeads immobilized at a pre-fabricated in-well barrier. (C) *E. coli* expressing GFP are trapped within the wells by the microbead filter. Scale bars represent 300 µm. (D) The number of captured bacteria as a function of the concentration of bacteria introduced. 100 µL of sample was introduced.
3.3.3 Validation of in-well bacterial capture

To concentrate bacteria within the wells, a size-based filtration approach was employed. Within each well, we fabricated an SU-8 barrier with a 2 μm tall gap between the surface and roof of the device. This gap size was the smallest feature that could be reliably patterned. Initial experiments indicated that this gap is too large to trap bacteria efficiently (Figure 7.2.7D), thus we contemplated other strategies to enhance the levels of bacterial capture. As high-throughput fabrication of submicron features using standard photolithography is challenging, we explored alternative strategies to generate in-well filters with the submicron pore sizes necessary to trap bacteria. While too large to trap bacteria directly, this in-well 2 μm gap is sufficiently small to trap 5 μm diameter microbeads. Microbeads trapped by the barrier self-assemble into an array that acts as a bacterial filter (Figure 3.3A, B). The submicron pores between beads prevent the passage of bacteria allowing bacteria introduced into the device to concentrate within the wells.

To further characterize the filters, we measured the filter stability (Figure 7.2.8). We found that the filter bed was stable for at least 1 hour after stopping the flow, which is the time required for incubation. Next, we estimated the pore size assuming hexagonal close packing of the spherical microbeads. We calculated a minimum pore size of 0.77 μm, which is sufficiently small to trap a bacterium. Using optical microscopy, we confirmed the validity of these calculations (Figure 7.2.7A–C). These calculations assume perfect hexagonal close packing, but in reality the beads will assemble in a geometry resembling random close packing which causes a distribution in pore sizes, but does not change the diameter of the smallest pores.

We challenged the system with E. coli expressing green fluorescent protein (GFP) and visualized the presence of captured bacteria using fluorescence microscopy (Figure 3.3C). Our initial experiments indicated that bacteria were reproducibly captured within each well. To quantitate the capture efficiency of our device, we introduced serial dilutions of a 100 μL volume of GFP E. coli at a flow rate of 10 μL min⁻¹. After capture, bacteria were introduced onto agar plates and
the *E. coli* colonies were counted after incubating the plates overnight. Figure 3.3D shows the capture efficiency as a function of concentration. Our results indicate this device achieves ~80% capture at concentrations as low as 1 cfu μL⁻¹. As the microbeads assemble randomly, there is a distribution of pore sizes, which allows some bacteria to escape to the filter. As expected, the capture efficiency decreases as a function of flow rate (Figure 7.2.7E). In the case of the 100 cfu μL⁻¹ sample, given that there are 72 wells per device, each well captured on average 120 bacteria. Considering that each well has a volume of 2.5 nL, this represents an effective concentration of approximately 50 000 cfu μL⁻¹. This represents a 500-fold concentration enhancement above the initial concentration of 100 cfu μL⁻¹.

![Figure 3.4 In-well electrochemical measurement of bacterial viability.](image-url) Electrochemical signal generated by resazurin decreases as a function of incubation time for *E. coli* at 100 cfu/μL. Viable *E. coli* are detected within 30 minutes. No positive signal change is observed after a 60 minute incubation with a blank culture media and both purified and unpurified urine.
3.3.4 On-chip detection of viable bacteria

With effective capture demonstrated, we tested the ability of our electrochemical assay to detect viable bacteria captured within the wells. We challenged the device with *E. coli* at 100 cfu μL$^{-1}$, a clinically relevant concentration in urinary tract infections. This concentration corresponds to over 100 bacteria per well. We studied the time dependence of the signal to determine the minimum time necessary to detect a clinically relevant concentration of viable bacteria. As shown in Figure 3.4, we achieved successful detection of metabolically-active bacteria within 30 minutes. In control experiments, we did not observe a statistically significant signal change after 1 hour with a blank culture media sample without spiked bacteria (Figure 3.4). These results represent a greater than 5-fold reduction in incubation time over the previous record of 2.8 h. We observed a small signal increase in the case of blank media which may be due to small chip-to-chip variations. We observe some signal decrease in the case of blank urine and unpurified urine which could be attributed to surface fouling of the electrode as well.
Figure 3.5: Electrochemical phenotypic testing of antibiotic-resistant bacteria. (A) and (B) Determination of the antibiotic resistance profile of *E. coli* and *K. pneumoniae* to ampicillin and ciprofloxacin using a standard microdilution assay with a 24 hour incubation. (C) and (D) Electrochemical determination of the antibiotic susceptibility of *E. coli* and *K. pneumoniae* on-chip after incubating with different levels of antibiotic at 37°C for 1 hour. (E) On-chip electrochemical determination of the antibiotic susceptibility of *E. coli* in unpurified urine after incubating with different levels of antibiotic at 37°C for 1 hour. Currents are normalized to the maximum value. Error bars represent standard error.
3.3.5 Detection of antibiotic susceptibility in complex matrices

After demonstrating detection of viable bacteria, we assessed the suitability of the device to rapidly determine the antibiotic resistance profile of bacteria in undiluted urine. To better simulate a clinical sample, we chose to test uropathogenic strains of *E. coli* (UPEC) and *Klebsiella pneumoniae* (*K. pneumoniae*), two of the most common pathogens implicated in urinary tract infections. The *K. pneumoniae* strain was isolated from the urine of an infected patient and produces extended spectrum β-lactamase enzymes which confer resistance to a wide variety of β-lactam antibiotics. We chose to test for susceptibility to two commonly used antibiotics to treat urinary tract infections – ampicillin, a β-lactam antibiotic, and ciprofloxacin, a fluoroquinolone.

In order to determine a suitable incubation period, we measured the minimum time required for these antibiotics to affect bacterial metabolic activity (Figure 7.2.11). We found that ciprofloxacin inhibited the metabolic activity of *K. pneumoniae* within 30 minutes indicating that a 1 hour incubation period is sufficiently long. We studied the effect of surface fouling induced by incubating the devices with LB media (Figure 7.2.10B) for 1 hour. We noticed only a small change in the acquired signals before and after incubation indicating that fouling could be attributed to approximately a 15% signal change which is acceptable as these devices are not designed to be reused.

*E. coli* (UPEC) and *K. pneumoniae* present at 100 cfu μL⁻¹ in undiluted urine were introduced into the device. After capture, we introduced a culture medium, resazurin, and either ampicillin or ciprofloxacin. Figure 3.5C and D show the signal obtained as a function of antibiotic concentration after a 1 hour incubation. No signal change was observed with a blank sample of urine (Figure 3.4).
For the *E. coli* strain, the signal decrease is low for all ciprofloxacin concentrations, indicating the bacteria are susceptible to the antibiotic at concentrations above 1 μg mL$^{-1}$ (Figure 3.5C). The signal decrease is reduced with ampicillin concentration indicating susceptibility at concentrations between 10 and 100 μg mL$^{-1}$. We confirmed our results using a standard microdilution assay with a 24 hour incubation (Figure 3.5A). We found that the minimum antibiotic concentration that inhibits 90% of bacterial growth (MIC$_{90}$) is 16 μg mL$^{-1}$ for ampicillin and less than 0.1 μg mL$^{-1}$ for ciprofloxacin.

For *K. pneumoniae*, the signal is approximately constant with increasing ampicillin concentration, indicating that bacterial viability is not affected by the ampicillin dose – the hallmark of resistance. As this strain produces a beta lactamase, resistance to ampicillin, a beta-lactam antibiotic is expected. In contrast, we observe a concentration-dependent signal with ciprofloxacin, indicating that bacterial viability is reduced by increasing ciprofloxacin concentration. This indicates that this strain is susceptible to ciprofloxacin and is inhibited at concentrations between 1 and 10 μg mL$^{-1}$ (Figure 3.5D). Using a standard microdilution assay we found the *K. pneumoniae* were indeed resistant to ampicillin but susceptible to ciprofloxacin (Figure 3.5B). The MIC$_{90}$ is 2 μg mL$^{-1}$ for ciprofloxacin and greater than 100 μg mL$^{-1}$ for ampicillin. For both strains, our results on-chip show good agreement with the MIC determined using this gold standard method which required incubation times over 20 times longer than the on-chip assay. We found good correlation between the on-chip susceptibility assay and standard assays with $r^2$ values of 0.81 and 0.82 for *E. coli* and *K. pneumoniae* respectively (Figure 7.2.12). This discrepancy may be attributable to the different detection methodologies and incubation periods used when comparing the two methods.

### 3.3.6 Detection of antibiotic susceptibility in unpurified urine

Next, we performed a series of experiments to determine the antibiotic susceptibility of bacteria in unpurified urine (Figure 3.5E). *E. coli* were spiked directly into undiluted and unpurified urine
at 100 cfu μL⁻¹. The sample was passed through a 10 μm filter that removed large particulates while allowing bacteria to pass (Figure 7.2.13). The filtrate was introduced into the device and tested against ampicillin and ciprofloxacin by incubating at 37 °C for 1 hour. We found that the *E. coli* were susceptible to ciprofloxacin at concentrations 1 μg mL⁻¹ and above and susceptible to ampicillin at a concentration between 10 and 100 μg mL⁻¹. We found no appreciable signal change when using a blank control sample of unpurified urine (Figure 3.4). These results agree with the standard microdilution antibiotic susceptibility assay indicating that the device can be challenged with unpurified urine samples using a simple inline pre-filtration sample processing step. Representative electrochemical scans acquired on-chip are included in Figure 7.2.14.

This device offers the fastest reported detection of antibiotic susceptibility at clinically relevant concentrations directly from unpurified urine. The rapid turnaround time is facilitated by concentrating the bacteria in a nanoliter volume which drastically increases the local effective concentration of bacteria. The turnaround time is further reduced by incubating the bacteria in isolated nanoliter compartments which allows the reduced form of resazurin to rapidly accumulate to detectable levels by confining diffusion. A third advantage of this approach is that it is purely electronic, which facilitates the development of antibiotic susceptibility tests at the point-of-care by eliminating the need for expensive and bulky optical equipment.

In a clinical setting, this device could serve as a rapid alternative to standard susceptibility tests to provide results with a 1 hour incubation after initial culture-based identification of the bacteria. Currently, standard antibiotic susceptibility tests require an additional 18–24 hours after the initial culture step.

The rapid-response device could also be used in conjunction with standard culture-based antibiotic susceptibility tests to provide point-of-care susceptibility results directly from undiluted urine with a 1 hour incubation period. This would thereby permit the rapid
administration of an effective antibiotic in the interim until the results of standard antibiotic susceptibility tests are available 2–3 days later at which point the therapy could be refined. This would allow doctors to administer a targeted antibiotic almost immediately, which would improve patient outcomes and curb the rise of antibiotic resistance by decreasing the use of broad spectrum antibiotics. In infections which lead to urosepsis, the most severe UTIs, this device would have the greatest clinical utility as these infections require immediate administration of effective antibiotics.4

When challenged with a sample containing a single bacterial strain, our device accurately and rapidly determines the susceptibility to various antibiotics. To enable accurate detection in the case of multiple infecting species (polymicrobial infections are present in only 5%–11% of individuals with urosepsis36), the multiple nanoliter chambers employed herein could be devoted to multiplexed combinations of bacteria combined with local metabolic sensing.

3.4 Conclusions

Using an electrochemical approach capable of detecting metabolically active bacteria, we have demonstrated the detection of live bacteria using a short 30 minute incubation period. By concentrating and analysing the bacteria within miniaturized compartments, the time required to detect viable bacteria is drastically reduced. We utilize this assay to monitor bacterial metabolism in response to antibiotics to rapidly readout the antibiotic susceptibility profile. This approach could allow for rapid administration of antibiotics before the results of standard culture-based susceptibility testing are available.
3.5 Methods

3.5.1 Culture device preparation

Gold electrodes were patterned using standard photolithography on glass substrates. A passivating SU-8 2002 (Microchem, Newton, MA) layer was patterned to insulate the electrodes. SU-8 3050 was patterned to create the 50 μm tall channel and wells. A final SU-8 2002 layer was patterned to form the in-well barrier used to trap microbeads. The working electrodes were electroplated by applying −300 mV for 30 s with respect to an Ag/AgCl reference electrode in a solution of 50 mM HAuCl₄ and 0.5 M HCl. Holes were punched in a polydimethylsiloxane (PDMS) lid for the inlet and outlet. The PDMS lid was bonded to the top of the device after an oxygen plasma treatment for 30 s.

To remove bubbles, the device was initially filled with EtOH and flushed with phosphate buffered saline (PBS). 100 μL of microbeads (Sigma Aldrich, St. Louis, MO) with a 5 μm diameter diluted 1 : 100 in PBS were introduced at 10 μL min⁻¹ to form the in-well filters.

3.5.2 Bacterial culture

GFP E. coli (Invitrogen, Carlsbad, CA), E. coli (ATCC 700928) and K. pneumoniae (ATCC 700603) were cultured in an incubating shaker at 33 °C in LB and Nutrient Broth respectively. Concentrations were determined using optical density measurements at 600 nm using a UV-vis spectrometer (Agilent, Santa Clara, CA). These measurements were correlated with the number of colonies on agar plates incubated overnight.

3.5.3 Determination of bacterial capture efficiency

A 100 μL volume of serial dilutions of E. coli were introduced into the capture device at 10 μL min⁻¹. After capture, the device was washed with 100 μL of PBS buffer. Finally the bacteria were
eluted in sterile PBS buffer. The eluted volume was plated on LB agar plates overnight at 37 °C and the colonies were counted.

3.5.4 Electrochemical detection of bacteria

Serial dilutions of *E. coli* were spiked in buffer and introduced into the chip at 20 μL min⁻¹ followed by 200 μL of 1 mM resazurin in LB broth. Air was flushed through the device to form the wells followed by FC-40, a fluorinated oil. The device was incubated in a water bath at 37 °C.

3.5.5 Antibiotic susceptibility microdilution assay

Cultured *E. coli* and *K. pneumoniae* were diluted to 100 cfu μL⁻¹ and incubated at 37 °C in a 96 well plate in Nutrient Broth with serial dilutions of ciprofloxacin and ampicillin. After 24 hours, the absorbance at 600 nm was measured.

3.5.6 Electrochemical detection in urine

Human urine (BioreclamationIVT) was centrifuged at 5000 g for 5 min to remove large particulates. *E. coli* and *K. pneumoniae* were diluted to 100 cfu μL⁻¹ and spiked in the urine. Samples (200 μL) were introduced at 20 μL min⁻¹. Next, 200 μL of either ampicillin or ciprofloxacin in 1 mM resazurin and LB media were introduced at 20 μL min⁻¹. Air was flushed through the device to form the wells followed by FC-40 (200 μL) (Sigma Aldrich, St. Louis, MO). Thus the total volume of all solutions introduced is 600 μL which requires 30 min to process at 20 μL min⁻¹. The device was incubated in a water bath at 37 °C for 1 hour. 10 minutes were required to scan the leads. Thus the total time for the assay from sample introduction to readout was 1 hour and 40 minutes.
3.5.7 Electrochemical detection in unpurified urine

*E. coli* were diluted to 100 cfu μL⁻¹ and spiked in the unpurified human urine (BioreclamationIVT). The spiked urine (200 μL) was passed through a 10 μm filter to remove large particulates and directly introduced at 20 μL min⁻¹ into the chip. Next, 200 μL of either ampicillin or ciprofloxacin in 1 mM resazurin and LB media were introduced at 20 μL min⁻¹. Air was flushed through the device to form the wells followed by FC-40 (Sigma Aldrich, St. Louis, MO). The device was incubated in a water bath at 37 °C.

3.5.8 Electrochemical measurements

Electrochemical measurements were performed using a potentiostat (BASi, West Lafayette, IN) and a three electrode set-up. For off-chip electrochemical experiments, we used a Ag/AgCl reference electrode and a Pt counter electrode. For on-chip experiments, we used the in-well Au reference and counter electrodes. Electrodes were scanned using differential pulse voltammetry.

3.6 References

4 Rapid bacterial lysis and nucleic acid analysis for pathogen identification

In Chapter 3, we investigated sample processing strategies to readout the susceptibility profile of a bacterial infection quickly. In this chapter, we develop a method to identify bacteria rapidly through genetic analysis, an important first step in choosing an effective antibiotic to treat the infection.

Detecting species-specific genetic markers could allow rapid diagnosis of bacterial infections. To detect intracellular genetic sequences, the pathogens must first be lysed to release the intracellular contents. Typically, lysis and sensing are performed in distinct modules. The sample is lysed in one chamber and sent to a second for genetic analysis. The slow diffusion of large analytes like RNA limits the response time of this approach when detecting clinically relevant concentrations of bacteria. Prohibitively long incubation periods are required for even a single molecule to reach the surface of nano- or micro-sensors. In this chapter, we explore strategies for overcoming the limitations imposed by diffusion by performing lysis in close proximity to the sensor in order to allow the released nucleic acids to rapidly diffuse across the short distance and hybridize with the capture probe.

Disclosure of work within this manuscript; J.D.B, J.D., E.H.S. and S.O.K. designed the experiments. J.D.B and J.D. performed the experiments, and interpreted results with assistance from E.H.S. and S.O.K. J.D.B, J.D., E.H.S. and S.O.K. composed and refined the manuscript.


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4.1 Abstract

Rapid and direct genetic analysis of low numbers of bacteria using chip-based sensors is limited by the slow diffusion of mRNA molecules. Long incubation times are required in dilute solutions in order to collect a sufficient number of molecules at the sensor surface to generate a detectable signal. To overcome this barrier here we present an integrated device that leverages electrochemistry-driven lysis less than 50 μm away from electrochemical nucleic acid sensors to overcome this barrier. Released intracellular mRNA can diffuse the short distance to the sensors within minutes, enabling rapid and sensitive detection. We validate this strategy through direct lysis and detection of *E. coli* mRNA at concentrations as low as 0.4 CFU/μL in 2 min, a clinically relevant combination of speed and sensitivity for a sample-to-answer molecular analysis approach.

4.2 Introduction

New strategies for rapidly detecting low levels of bacteria are urgently needed to control and manage infectious disease.\(^1\) No existing method simultaneously satisfies the needed speed and sensitivity requirements to detect sufficiently low levels of bacteria in a clinically-relevant time period.\(^2\) Culture, the gold standard for diagnosis for most types of bacterial infection diagnosis, requires hours to days to amplify the bacteria to visibly detectable levels. Enzymatic amplification methods such as the polymerase chain reaction (PCR) are complex to automate and often require sample purification that slows analysis.\(^3,4\)

A wide variety of molecular sensors have been developed to address the limitations of culture and PCR.\(^5-8\) The direct detection of nucleic acid sequences using chip-based sensors has been pursued for some time as an attractive solution.\(^9-14\) The use of mRNA sequences such as that corresponding to the RNA polymerase β (rpoβ) subunit can provide species-level identification of bacteria,\(^15\) and many copies of mRNA may exist in a single cell which provides an inherent
signal enhancement. However, using this type of biomarker to rapidly detect bacteria at clinically relevant levels remains a challenge; the long diffusion times of large mRNA molecules impose an inherent trade-off between speed and sensitivity of detection.\textsuperscript{16,17} In a typical sample-to-answer detection scheme, bacteria are lysed in a lysis chamber and the homogeneous lysate is then transported to a detection chamber (Figure 4.1A).\textsuperscript{18} As each bacterium may harbor multiple copies of the target mRNA, prior to lysis, the molecules are present at a locally high concentration inside each bacterium. However, after lysis, intracellular mRNA is released into bulk solution, and thus the overall concentration of mRNA is very low. When sensing low concentrations of mRNA, long incubation times are required in order to accumulate enough target molecules at the sensor surface to generate a detectable signal.

Here, we propose a novel approach to rapid genetic analysis that uses an electrochemical approach to lyse bacteria in close proximity to a microelectrode where their mRNA can be analyzed. Proximal electrochemical lysis shortens the required distance over which the target mRNA molecules must diffuse. This approach provides a 10-fold improvement in performance and permits rapid analysis of bacteria at clinically relevant levels.
Figure 4.1: Schematic of lysis and electrochemical readout. (A) (i) Typically, bacteria are lysed in bulk solution, and the homogeneous lysate is transferred to the sensor. (ii) In an alternative proximal lysis approach, bacteria are lysed in the vicinity of the sensor, and released mRNA can rapidly diffuse the short distance to the sensor. (B) Diffusional flux of mRNA released from a single bacterium harboring 1400 transcripts. The flux from lysis at varying distances from the sensor is compared to the flux from homogeneous lysate from 4 CFU/μL. (C) Molecules accumulated at the sensor over time after local lysis (50 μm away) of a single bacterium compared to homogeneous lysate at varying concentrations. Detecting less than ~1 CFU/μL in 5 min is not possible in a homogeneous lysate. In contrast, intracellular RNA from just a single bacterium lysed locally accumulates within minutes at the sensor surface. The dashed black line represents a typical threshold for detection of 10 molecules. (D) Each well contains two lysis electrodes and an NME sensor. Bacteria are lysed electrochemically by an applied potential. Released intracellular mRNA rapidly hybridizes to the complementary PNA probe molecules functionalized on the NME surface. (E) The amount of hybridized mRNA is read using an electrocatalytic reporter pair and DPV to measure the peak current before and after hybridization.
4.3 Results and discussion

4.3.1 Summary of Approach

Detection of mRNA using chip-based sensors is limited by the rate of molecular diffusion to the sensor. Molecules of bacterial mRNA, which can be up to thousands of base pairs long, have low diffusion coefficients compared to short synthetic oligomers, and long time scales are therefore required to collect enough molecules to generate a robust response. To study the diffusional flux of analytes at the sensor after lysis, we created a model of a single \textit{E. coli} inside a well that contains a 20 μm hemispherical sensor. We assumed each \textit{E. coli} contained multiple copies of RNA polymerase β subunit (rpoβ), a 4000 bp transcript. We assumed a copy number of 1400 as calculated previously. Figure 4.1B compares the diffusional flux of the analytes at the sensor as a function of time for the homogeneous and local lysis approaches. In the homogeneous lysis case, the flux is low and nearly constant. In the local lysis approach, the flux rapidly increases in the first minutes as the analytes reach the sensor and slowly decays as the molecules diffuse away. Figure 4.1C shows the number of analyte molecules captured at the sensor surface over time for both lysis scenarios. Rapidly (<30 min) detecting less than 1 CFU/μL is not possible with a homogeneous lysis approach as no analyte molecules are captured after 30 min. On the other hand, if just a single bacterium is lysed 50 μm from the sensor, many target analytes will accumulate on the sensor surface within minutes.

In light of the findings from our diffusional model, we designed a device that would minimize the distance between the site of cell lysis and mRNA detection. The device featured electrodes for electrochemical lysis that surrounded a detection sensor (Figure 4.1D). Previous studies have shown that hydroxide ions can be produced locally to initiate cellular lysis, but this approach has primarily been used in bulk solution as a means to prepare samples for analysis using PCR or other detection approaches. Here, lysis electrodes were placed within 50 μm of a microelectrode sensor, which allows large mRNA molecules to diffuse to the sensor within 10
The reaction that initiates cell lysis is based on the production of hydroxide ions from water at a cathode at a potential of 20 V. Hydroxide ions can break down bacterial membranes, causing the contents of the bacteria, including the nucleic acids, to be released into solution.

The placement of a chip-based sensor in close proximity to the lysis electrodes provides the potential for very sensitive analysis of bacterial cells because limited diffusion is required of the molecules that provide information on cellular identity. The functionalization of a nearby sensor with a thiolated probe molecule that is complementary to a unique portion of a bacterial mRNA allows capture of the marker if bacteria are present within a sample undergoing analysis. The presence of bound target mRNA can be read using an electrochemical reporter system that senses the change in electrostatics at the sensor surface (Figure 4.1E).\textsuperscript{23} Ru(NH\textsubscript{3})\textsubscript{6}\textsuperscript{3+} ions accumulate at the sensor surface and serve as electron acceptors. Fe(CN)\textsubscript{6}\textsuperscript{3–} ions serve to reoxidize Ru(II) as it is made electrochemically and regenerate the electron acceptor to make the reaction electrocatalytic. Differential pulse voltammetry provides an effective sampling method that can be used to investigate whether current levels rise above a threshold that indicates that a sample is positive for a particular pathogen.
Figure 4.2: Device design. Simulations of the electric fields induced by lysis electrodes which span the sensor (A) and wrap around the sensor (B). (C) Optical microscopy image of NMEs electrodeposited into the well array. (D) Optical microscopy and scanning electron microscopy images of an electrodeposited NME. Scale bars represent 100 μm.
4.3.2 Design and Validation of Lysis Electrodes for Electrochemical Hydroxide Generation

Given that large applied potentials could disrupt the bond between a probe sequence and the sensor, thereby compromising the integrity of the surface assembled monolayer, we simulated the electric fields generated by different lysis electrode geometries (Figure 4.2A,B). Lysis electrodes which sandwich the sensor induce a high potential at the sensing electrode which could reduce probe coverage by dissociating the gold–thiol bond (Figure 4.2A). Figure 4.2B shows an alternative layout in which both lysis electrodes wrap around the sensing electrode. The electrodes act as an on-chip faraday cage to shield the sensor from the electric fields. We chose this layout to minimize the electric fields experienced by the sensor.

Another feature of the device we fabricated is patterned wells that contain the lysis and sensing electrodes to enable the contents of the bacterial cells to remain close to the sensor. The wells introduced on the surface of the chip hold 1 nL of liquid and were generated using SU-8 and patterned using photolithography. Nanostructured microelectrode (NME) sensors were plated within the wells and were shown to exhibit similar morphologies to what has been demonstrated previously (Figure 4.2C,D).17,18,24

As an initial assessment of lysis, we measured _E. coli_ growth on agar plates after they were subjected to on-chip electrochemical lysis (Figure 4.3A). We applied pulse voltages from 0 to 20 V at 1 Hz for 1 min to _E. coli_ and allowed the lysate to incubate on agar plates overnight. _E. coli_ viability dropped after applying 5 V, and no growth was observed after applying 20 V. While this approach is an indirect measure of whether lysis is occurring, it provides a means to identify an interesting potential range to look for membrane permeability.

To study whether lysis made the _E. coli_ membrane permeable, we measured cellular uptake of propidium iodide (PI), a fluorescent dye which intercalates with DNA (Figure 4.3B). Cellular uptake of PI is used as an indicator of lysis as PI cannot cross intact cell membranes. Using flow
cytometry, we measured propidium iodide uptake as a function of pulse voltage. Increasing voltage caused greater uptake of propidium iodide, with the largest uptake at 20 V.

Using fluorescent microscopy, we visualized PI uptake in real time (Figure 4.3C). Voltage pulses (20 V) were applied to E. coli expressing green fluorescent protein (GFP) in the presence of PI. We observed greater PI uptake as increasing number of voltage pulses were applied. E. coli lyse first at the positive outer electrode and then near the grounded inner electrode.
Figure 4.3: Validation of electrochemical lysis. (A) Effect of applied potentials on *E. coli* viability after incubating on agar plates. IPA was used as a positive control. (B) Uptake of PI measured by flow cytometry as a function of voltage. IPA is used as a positive control. (C) Uptake of PI measured with optical microscopy as a function of the number of pulses applied. *E. coli* expressing GFP are green, and *E. coli* that uptake PI are red. Scale bars represent 100 μm.
4.3.3 Identification of Compatible Solution Conditions for Electrochemical Lysis and Detection

Initiating lysis close to a chip-based sensor has the potential to improve detection limits but may also introduce issues that would interfere with the function of the sensors. As discussed above, the presence of a strong field could interfere with the attachment of the probe to the sensor. In addition, the ions generated electrochemically could degrade the probe molecules.\(^{25}\)

We tested different lysis conditions and investigated whether probe molecules were dissociating from the sensor surface. An assay based on the blocking of the surface in the presence of probe was used, where signals generated by Fe(CN)\(_6^{4-}\) were analyzed. The iron reporter group is repelled by the anionic probe, and if stripping occurred, the signal it generates would increase.

As shown in Figure 4.4, differing levels of probe stripping were observed when buffer conditions were varied. Lysis performed in water with short (30 \(\mu\)s) or long (10 ms) pulses did not cause probe dissociation. However, lysis in 1× phosphate-buffered saline did cause damage with large signal changes occurring for both pulse times. Diluting the PBS or the use of phosphate buffer attenuated this effect. In PBS, the presence of chloride ions likely contributes to the production of hypochlorite ions after the production of Cl\(_2\) at the anode. Hypochlorite is highly reactive and persists in buffered solutions and is expected to cause significant damage both to immobilized probes and the molecules being liberated within a sample. It is therefore desirable to avoid generating this species.

Lysis in phosphate buffer, especially with short pulses, resulted in minimal probe loss and provides buffering capacity to ensure that samples only transiently experience elevated hydroxide levels and provides ionic strength to promote hybridization on the sensor surface. This buffer system was therefore identified as the best set of solution conditions that was compatible with both electrochemical lysis and detection.
Figure 4.4: Effect of lysis on probe integrity. Percent change in magnitude of peak oxidation currents of Fe(CN)$_6^{3-}$ measured before and after on-chip lysis. The percent increase in current measured after applying 30 μs and 10 ms pulses in various buffers. High percentage changes indicate that the probe is removed from the surface. Error bars represent standard error.
4.3.4 Bacterial Detection with Proximal Lysis

With effective lysis demonstrated, the detection of bacteria using the devices was tested. A series of studies were conducted that focused on the effect of lysis pulse length on detection of *E. coli* (Figure 4.5A). Increasing the pulse time from 30 to 300 μs increased the amount of current change generated with a solution of 400 CFU/μL *E. coli* cells per microliter, indicating greater lysis efficiency with the longer pulses. The optimal current at 300 μs is likely due to the competing effects of hydroxide generation. High hydroxide concentrations lead to efficient lysis but also to degradation of the target molecules. Below 300 μs, the lysis is not as efficient, while above 300 μs, the target likely degrades due to excessive hydroxide concentration and the probe is removed from the sensor which causes a lower current change.

To evaluate the detection limit of the sensors with proximal lysis, *E. coli* were serially diluted from 400 to 0.4 CFU/μL (Figure 4.5B). *E. coli* were lysed on-chip by applying the optimal 300 μs pulses for 1 min, and the lysate was allowed to incubate at 37 °C for 30 min. The 5000 CFU/μL *S. aureus* was used as a negative control. *E. coli* were detected with a limit of detection of 0.4 CFU/μL and high specificity as no signal was observed from *S. aureus*.

The sensitivity enhancement provided by the combination lysis/detection device was tested by challenging the sensors with serial dilutions of *E. coli* lysed off-chip. The limit of detection of the device when challenged with *E. coli* lysate prepared off-chip was only 4 CFU/μL, which is 10 times higher than when *E. coli* were lysed on-chip. This indicates that lysis in close proximity to the sensors provided a 10-fold sensitivity advantage.

In addition to providing enhanced detection limits, proximal lysis should also speed the progress of hybridization and produce fast results. To determine the time dependence of the detection approach, we challenged the sensor with *E. coli* at 0.4 CFU/μL using 2 and 5 min hybridization times (Figure 4.5C). We observed a positive signal from 0.4 CFU/μL after both 2 and 5 min
hybridization periods, indicating that the sensor has a rapid response time. This is a record-breaking level of speed and sensitivity combined in a single sensor system. The previous sensitivity record for direct electrochemical detection of mRNA in crude lysate is 1 CFU/μL after a 20 min incubation time. Here we show detection of 0.4 CFU/μL within 2 min.

Real sample matrices have a wide range of pH and salt concentrations which need to be controlled in proximal electrochemical lysis. To use this device with complex matrices, the sample could be prediluted in the appropriate buffer. Alternatively, this device could be coupled to a preconcentration step to separate the bacteria of interest from the sample and allow for buffer exchange.
Figure 4.5: Lysis and electrochemical detection of *E. coli*. (A) Effect of electrical lysis pulse length on the electrochemical signal change. (B) Concentration-dependent signal change of *E. coli* lysed on-chip as compared to *E. coli* lysed off-chip. The data were normalized to the signal from 400 CFU/μL. (C) Hybridization time dependence of the signal from *E. coli* lysed on-chip. The dashed line corresponds to the average signal change in the absence of *E. coli*. Error bars represent standard error.
4.4 Conclusions

We developed an integrated device capable of electrochemical lysis and detection of *E. coli* at concentrations as low as 0.4 CFU/μL in 2 min. Lysing in the vicinity of the sensors allowed high concentrations of released intracellular mRNA to reach the sensor rapidly. Our experiments highlight the importance of optimizing the buffer and electrode geometry when lysing nearby surface-modified electrodes in order to maintain the integrity of the probe monolayer and provide a powerful sample-to-answer approach for bacterial detection.

4.5 Methods

4.5.1 Simulations

Simulations of electric fields for various lysis electrode designs were conducted using COMSOL Multiphysics. The applied voltages were set to keep the average field constant across both geometries.

Simulations of the flux of analyte molecules at the sensor surface were calculated using a COMSOL model. These calculations are described in Appendix A.

4.5.2 Device Fabrication

Devices were fabricated by the Canadian Photonics Fabrication Centre (Ottawa, ON) on 300 μm thick 6 in. silicon wafers coated with 450 nm of thermally grown SiO₂. To pattern the electrodes, 300 nm Au was deposited on a 25 nm Ti adhesion layer. After patterning the electrodes using standard photolithography and wet etching, the electrodes were passivated with 500 nm SiO₂ using plasma-enhanced chemical vapor deposition. The lysis electrodes, contacts, and apertures were exposed using reactive ion etching. Wells were patterned using SU-8 30-25 (Microchem, Newton, MA) using photolithography. The devices were diced and fixed to the bottom of a custom-designed PMMA reservoir (QuickCUTCNC, Atlanta, GA).
4.5.3 NME Electrodeposition

Nanostructured microelectrodes (NMEs) were electrodeposited using a two-step process using a three-electrode setup with a Pt counter electrode and a Ag/AgCl reference electrode. To grow a sensor with a large footprint, we applied 0 mV with respect to the Ag/AgCl reference electrode for 20 s in a solution of 50 mM HAuCl₄ and 0.5 M HCl. Sensors were decorated with nanostructured features by applying −250 mV in a solution of 5 mM PdCl₂ and 0.5 M HClO₄ for 5 s.

4.5.4 Preparation of Bacterial Samples

*E. coli* (Invitrogen, Carlsbad, CA) and *S. aureus* (ATCC) were cultured in an incubating shaker at 33 °C in LB Miller and tryptic soy agar broth, respectively. Concentrations were measured using optical density measurements at 600 nm with a UV–vis spectrometer (Agilent, Santa Clara, CA) and by counting the number of colonies on agar plates incubated overnight at 37 °C. Using centrifugation, the growth medium was replaced with the appropriate buffer before lysis. To assess cell viability, 100 μL of lysate was spread on LB agar plates and incubated overnight at 37 °C. *E. coli* were lysed off-chip using OmniLyse (Claremont Bio, Upland, CA).

4.5.5 Flow Cytometry

*E. coli* were diluted to 1 × 10⁷ CFU/mL in various buffers. Potentials from 0 to 20 V were applied to lysis electrodes using a 1 Hz repetition rate for 60 s. After lysis, samples were incubated with 2 μg/mL propidium iodide for 30 min in the dark. Measurements were made using a BD FACS Canto flow cytometer and plotted as histograms of fluorescence intensity.

4.5.6 Fluorescent Microscopy

*E. coli* expressing GFP were diluted to 1 × 10⁷ CFU/mL in the appropriate buffer and lysed with varying numbers of 20 V pulses (30 μs pulse time). Samples were incubated with 2 μg/mL
propidium iodide, and red and green fluorescent images were acquired with a Nikon Eclipse LV150 microscope.

4.5.7 Synthesis and Purification of PNA Probes

Probes complementary to *E. coli* rpoβ mRNA (4029 bp) were designed with the following sequence: \( \text{NH}_2\text{-Cys-Gly-Asp-ATC TGC TCT GTG GTG TAG TT-Asp-CONH}_2 \). Probes were synthesized using a protein Technologies Prelude peptide synthesizer and purified using reverse-phase high-performance liquid chromatography. Probe concentration was calculated using the extinction coefficient and the absorbance at 260 nm.

4.5.8 Sensor Functionalization, Lysis, And Hybridization

Sensors were functionalized with 1 μM PNA probe and 9 μM mercaptohexanol for 30 min at room temperature. Chips were washed twice for 5 min with 1× PBS buffer after probe deposition. *E. coli* and *S. aureus* were diluted to the appropriate concentration in 50 mM phosphate buffer, and 50 μL was added to the chip. After washing, potentials were applied to lysis electrodes using 1 Hz pulses with varying voltages and pulse times. The chip was incubated in a humidity chamber for 2 to 30 min at 37 °C followed by washing twice with PBS for 5 min. After washing, DPV measurements were performed following probe deposition and sample hybridization in the electrocatalytic solution.

4.5.9 Electrochemical Measurements

For all electrochemical measurements, we used a three-electrode setup with a Ag/AgCl reference electrode and Pt counter electrode connected to a potentiostat (BASi, West Lafayette, IN). To measure the effect of lysis on probe detachment, NMEs modified with a PNA probe were scanned from 0 to 0.5 V using differential pulse voltammetry in a solution of 2.5 mM Fe(CN)\(_6\)\(^{4-}\) and 0.1× PBS. Oxidation currents were measured before and after applying 30 μs and 10 ms 20 V pulses to the lysis electrodes at 1 Hz for 60 s.
To measure the amount of hybridized nucleic acid, electrochemical signals were measured in 0.1× PBS with 10 μM [Ru(NH$_3$)$_6$]Cl$_3$ and 4 mM K$_3$[Fe(CN)$_6$]. DPV signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and a pulse period of 100 ms. Signal changes that corresponded to target hybridization were calculated with background-subtracted currents: $\Delta I = (I_{\text{after}} - I_{\text{before}})$ (where $I_{\text{after}}$ = current after target hybridization and $I_{\text{before}}$ = current before target hybridization, i.e., current with only probe).

4.6 References

5 Sensitive colorimetric readout of nucleic acids for low-cost pathogen diagnosis

In Chapter 4, we developed a method to readout disease-related nucleic acids quickly. To readout the signal, a benchtop instrument called a potentiostat records the current generated by the sensors. While a benchtop instrument is suitable for a hospital laboratory or doctor’s office, a disposable test with simple, colorimetric readout is needed in low-resource settings. Existing disposable diagnostics are available only for analytes present natively at high levels. Converting ultra-low levels of biomarkers, such as nucleic acids, into permanent, visible colour changes is not possible using existing methods as they lack the necessary sensitivity. In this chapter, we investigate methods to transduce ultra-low levels of nucleic acids into a visible colour change by introducing a cascade of amplification steps. This work lays the foundation for the development of low-cost, point-of-care sensors for pathogen-specific nucleic acids.

Disclosure of work within this manuscript; J.D.B, J.D, I.B.B., W.L., E.H.S. and S.O.K. designed the experiments. J.D.B, J.D, I.B.B. and W.L. performed the experiments and interpreted results with assistance from E.H.S. and S.O.K.; J.D.B., J.D., I.B.B., W.L., E.H.S. and S.O.K. composed and refined the manuscript.


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5.1 Abstract

Diagnosis of disease outside of sophisticated laboratories urgently requires low-cost, user-friendly devices. Disposable, instrument-free testing devices are used for home and physician office testing, but are limited in applicability to a small class of highly abundant analytes. Direct, unambiguous visual read-out is an ideal way to deliver a result on a disposable device; however, existing strategies that deliver appropriate sensitivity produce only subtle colour changes. Here we report a new approach, which we term electrocatalytic fluid displacement, where a molecular binding event is transduced into an electrochemical current, which drives the electrodeposition of a metal catalyst. The catalyst promotes bubble formation that displaces a fluid to reveal a high contrast change. We couple the read-out system to a nanostructured microelectrode and demonstrate direct visual detection of 100 fM DNA in 10 min. This represents the lowest limit of detection of nucleic acids reported using high contrast visual read-out.

5.2 Introduction

Low-cost, user-friendly diagnostics have the potential to expand the ubiquity of molecular testing in clinical medicine.\textsuperscript{1-11} Disposable, instrument-free devices are used today, but have so far only achieved the detection of certain analytes that happen to be highly abundant. A key feature of these devices is the use of an easy-to-interpret visual read-out strategy. Existing read-out approaches require the accumulation of a high level of an analyte, and therefore only abundant analytes have been detected visually. Developing ways to link a visible, unambiguous colour change to rare biological molecules remains an unmet need. Recently, a variety of direct, colorimetric read-out strategies have been reported: these include approaches based on nanoparticles,\textsuperscript{12,13} plasmonic nanomaterials,\textsuperscript{14} 2D materials,\textsuperscript{15} and enzymatic reactions.\textsuperscript{7} Unfortunately, these approaches require interpretation of subtle colour changes. This can make analyses operator-dependent, or, in other cases, diminishes the benefits of a test being instrument-free by requiring a scanner device.
Developing new, easy-to-interpret interfaces that convey diagnostic results obtained with low-abundance analytes would enable the development of low-cost diagnostics for a spectrum of new diseases. Motivating this work are rapid recent advances in biosensors that produce nanoampere electrical current changes as a function of specific biomarkers present in a sample.\textsuperscript{16,17,18} New strategies to transduce extremely small electrochemical currents into easily perceived, high-contrast visual changes would allow the visual detection of low abundance analytes using electrochemical biosensors. In addition, low-cost current-to-colour conversion is of broad interest in displays and in sensors for non-medical applications.

Strategies for direct colorimetric read-out of electric currents include paper-based electrochromism,\textsuperscript{19} electrochromic polymers,\textsuperscript{20} metal oxides\textsuperscript{21} and fluorescent dyes.\textsuperscript{22} Electrochromic polymers and dyes allow for rapid and reversible colour switching in response to electrical currents, but the currents required to switch areas detectable to the naked eye are above the threshold necessary for sensitive electrochemical detection. Inducing visible colour changes using currents below 1 $\mu$A is a fundamental challenge, for such currents fail to supply enough electrons to electrochemically reduce a visibly perceptible quantity of electrochromic material. Directly translating such low currents into visible changes has yet to be achieved without the aid of costly, power-consumptive active electronics such as amplifiers.

We here develop an approach to amplify the changes to optical density triggered by the levels of electrochemical current generated at a nucleic acid sensor. We term our new approach electrocatalytic fluid displacement (EFD). An electrochemical current drives the deposition of a catalyst, which promotes the growth of a bubble that actuates a fluid. Specifically, the electrochemical current drives the electrodeposition of a metal catalyst for hydrogen peroxide decomposition. On the introduction of hydrogen peroxide liquid, a bubble catalytically forms, and this displaces a fluid. The bubble displaces a dye, or, in the alternative, modifies the index of refraction to reveal a structural colour change. We begin by providing a conceptual basis for our
approach, and we benchmark it against other colorimetric read-out strategies. After optimizing the device parameters and geometry, we determine the minimum current necessary for successful colorimetric read-out. To showcase this approach, we demonstrate sensitive colorimetric detection of ssDNA by coupling the read-out to a nanostructured microelectrode (NME) and a novel electrocatalytic assay.

5.3 Results and discussion

5.3.1 Overview of electrocatalytic fluid displacement

The electrocatalytic fluid displacement (EFD) approach is based on the electrodeposition of platinum, which catalyses the evolution of a bubble that actuates a fluid (Figure 5.1). An electrochemical sensor is connected to a read-out chamber by a metallic bridge (Figure 5.2A). On the introduction of the sample, the target analyte hybridizes to the complementary probe functionalized on the surface of the sensor (Figure 5.2B). After hybridization, the electrocatalytic solution is introduced into the sensing chamber (Figure 5.2B). On the application of a potential at 250 mV for 10 s, ruthenium is oxidized at the sensing electrode and platinum is simultaneously electrodeposited at a mesh electrode in the read-out chamber. The current is further amplified by two additional reducing agents in the electrocatalytic solution. After the application of the potential, hydrogen peroxide is introduced into the read-out chamber (Figure 5.2C). The deposited platinum catalyses the decomposition of hydrogen peroxide into water and oxygen, which forms a bubble. The growing bubble displaces a fluid to reveal a colour change. In the case of read-out based on dye displacement, the opaque dye is displaced and the read-out window becomes transparent revealing a blue spot underneath the device (Figure 5.2D). In the case of read-out based on a structural colour change, the growing bubble causes an index mismatch that unveils the diffraction grating patterned in the underside of the PDMS chamber lid, which causes light to diffract into its component colours (Figure 5.2E).
Figure 5.1: Schematic of an integrated device for electrocatalytic fluid displacement. An electrochemical current from a nanostructured microelectrode is converted into a visible change through the deposition of a catalyst that catalyses bubble formation. As the bubble grows, the white dye is displaced to reveal a blue colour.
Figure 5.2: Colorimetric detection of DNA using electrocatalytic fluid displacement. (a) Target hybridization. The analyte hybridizes to a complementary PNA probe. Ru(NH₃)₆³⁺ is electrostatically attracted to the negatively charged backbone of the target nucleic acid. (b) Signal transduction. A potential is applied to the NME which oxidizes Ru(NH₃)₆³⁺. The current is amplified using an electrochemical-chemical-chemical (ECC) reporter system. Ru(NH₃)₆³⁺ is regenerated by MPA, which is in turn regenerated by cysteamine. The electrochemical current drives the deposition of platinum, a catalyst for hydrogen peroxide decomposition, on a mesh electrode immersed in platinum solution. (c) Colorimetric read-out. After the introduction of peroxide, a bubble forms as the platinum catalyses the decomposition of peroxide. The growing bubble is transduced into a colour change either through an optical density change or a structural colour change. In the optical density approach, the bubble displaces a white dye to reveal the blue spot. To induce a structural colour change, the bubble displaces peroxide that causes an index mismatch at a diffraction grating patterned in the underside of the chamber lid. Incident white light is diffracted into its component colours. (d) Optical density change. A cross-section of the electrocatalytic fluid displacement approach with read-out based on dye displacement. (e) Structural colour change. A cross-section of the electrocatalytic fluid displacement approach with read-out based on a structural colour change. In the case of electrocatalytic fluid displacement based on a structural colour change, the underside of the PDMS lid of the read-out chamber is patterned with a diffraction grating.
To sense nucleic acids, the EFD read-out system is connected to a nanostructured microelectrode (NME), which acts as an ultrasensitive electrochemical biosensor (Figure 5.2A). The NME sensors were fabricated on silicon substrates using a two-step electrodeposition process as previously described. The gold microstructures protrude from the surface and reach into solution, which increases the probability of interaction with the target molecules. The sensors are decorated with a second layer of finely nanostructured gold. The nanoscale roughness maximizes sensitivity by enhancing the hybridization efficiency of the probe and target. These sensors have been used previously to detect a variety of chemical and biomolecular targets.

We use a multi-pronged strategy to minimize the current in the absence of target nucleic acid. We functionalize the sensors using a charge-neutral probe, and we read the current using a novel electrochemical assay. Specifically, the sensors are functionalized with thiolated peptide nucleic acid (PNA) probes complementary to the target sequence. PNA is a synthetic nucleic acid analogue that has a neutral charge. This neutral charge minimizes the background current and increases the signal-to-noise ratio.

After target hybridization and washing (Figure 5.2A), we read-out the electrochemical signals using an electrochemical-chemical-chemical (ECC) redox cycle reporter system, which radically amplifies the current. It is worth noting that this is the first reported use of ECC for the detection of nucleic acids. To simplify the electronics in a disposable device, we use a DC potential for read-out as opposed to voltammetry, which requires a potential sweep and thus more complicated electronics. Using a DC potential, it is not possible to resolve the contribution from unwanted redox reactions occurring at nearby potentials to the overall signal. In the past we have used an electrocatalytic redox reporter system consisting of ruthenium hexamine and ferricyanide for nucleic acid detection using differential pulse voltammetry, but this gave high background currents using DC potential amperometry. At the reduction potential of ruthenium hexamine,
ferricyanide is reduced as well, which contributes to the overall current even in the absence of bound-target nucleic acids. Therefore, we designed the ECC redox reporter system such that there are no interfering redox reactions near the potential of interest.

Our new assay employs Ru(NH$_3$)$_6^{3+}$, mercaptopropionic acid (MPA) and cysteamine. Ru(NH$_3$)$_6^{3+}$ is electrostatically attracted to the negatively charged phosphate backbone of the bound target nucleic acids. On the application of a potential at 250 mV, Ru(NH$_3$)$_6^{3+}$ is oxidized to Ru(NH$_3$)$_6^{4+}$ (ref. 31). The MPA present in solution chemically reduces Ru(NH$_3$)$_6^{4+}$ back to Ru(NH$_3$)$_6^{3+}$, allowing for multiple turnovers of Ru(NH$_3$)$_6^{3+}$, which generates a high electrocatalytic current. This signal is further amplified by cysteamine, another reducing agent, which is chemically oxidized to cystamine by reducing the oxidized form of MPA (R-S-S-R) back to its reduced form (R-SH).

This electrical current from the sensor is coupled to the EFD electrode immersed in a platinum solution to drive the deposition of the catalyst. When the NME is challenged with the target analyte, the current drives the electrodeposition of platinum on the EFD electrode, which catalytically forms a bubble that displaces the dye to reveal the blue spot. When the target sequence is not present, the current is too low to deposit a sufficient amount of platinum to catalyse bubble growth and no colour change occurs.

5.3.2 A comparative model of colour change

Catalytic electrochromic transduction methods offer significant signal amplification needed for transducing the ultra-low currents generated by the ECC assay compared with direct electrochromic reduction. To study the prospective performance of this approach, we calculated the predicted time required to induce a visible colour change using a variety of transduction strategies.
We illustrate the challenge of directly inducing a colour change by considering the example of electrodepositing an optically discernible quantity of metal. A 1 nA current applied for 10 s supplies $6 \times 10^{10}$ electrons, which can turnover a maximum of $6 \times 10^{10}$ molecules. Even under the generous assumption that a single molecular layer is visible, given an atomic radius of 1 Å, this yields a spot of only $50 \mu m \times 50 \mu m$. This is too small to be easily visible to the naked eye as the spatial resolution of human eyesight is ~100–200 μm (ref. 32).

We hypothesized that we could instead develop a means to amplify, by orders of magnitude, the colour change per charge. We would electrodeposit a catalyst, such as platinum, to turn on the colorimetric reaction. By depositing a catalyst, each electron effectively converts multiple molecules, amplifying the colour transformation. However, as Figure 5.3 shows, even the catalytic reduction of an electrochromic compound in bulk solution requires exceedingly long times to induce a visible change. Assuming a 50-μm tall chamber with a 200 μm diameter window filled with enough pigment, with the absorbance of malachite green, to give an OD of 1, it would take over 4 h to turnover the compound using the platinum deposited from a 1 nA current.
Thus, instead of catalytic reduction of a solution-based pigment, we considered catalytic evolution of gas as an equivalent molar amount of gas occupies a much larger volume than a liquid. At STP, the volume of one mole of gas is 22.4 l, which is three orders of magnitude larger than a mole of liquid H₂O (18 ml). Platinum is an excellent catalyst for the decomposition of hydrogen peroxide to form oxygen and water. As shown in Figure 5.3, the catalytic production

Figure 5.3: Calculations of the time to visual appearance. We calculated the time to visual appearance using electrocatalytic fluid displacement and reduction of an electrochromic compound. We assume the read-out window is a 200 µm × 200 µm × 50 µm chamber and the current is applied for 10 s. The onset of bubble formation occurs as the solution is saturated with oxygen. A bubble is defined as visible once it reaches the volume of the chamber. We assumed the electrochromic dye had the absorbance of malachite green and a visible change corresponds to a ΔOD of 1.
of a visible bubble that fills the same window requires under 3 min, over 80 times faster than catalytic reduction of an electrochromic dye in solution.

We hypothesized that electrocatalytic bubble formation could be converted into a colorimetric change by actuating a fluid to modulate the optical density (OD) of the read-out window. This is a central step in EFD.

5.3.3 Optimization of device geometry

Motivated by our calculations, we sought to experimentally validate the electrocatalytic fluidic displacement approach. We patterned a rectangular gold electrode on a glass substrate, which sits at the bottom of a 50-μm tall by 1.5-mm wide circular chamber. After depositing platinum for 10 s at 1 nA, we introduced hydrogen peroxide and measured the rate of bubble growth using a microscope (Figure 5.4A). Although we did not observe rapid bubble growth, we noticed that bubbles formed preferentially at the electrode edges.
Figure 5.4: Electrocatalytic fluid dye displacement. (a) Bubble evolution as a function of time for various electrode geometries. Platinum was deposited using a 1 nA current for 10 s. Bubble growth increases with the ratio of edges to surface area. (b) Average bubble area after 20 min as a function of applied current using the electrodes with the highest mesh density. The bubble is confined to a 50-μm tall channel. Error bars represent s.e.; all measurements represent n>5 trials. (c) Bubble growth as a function of time for various deposition currents using electrodes with the highest mesh density. Bubbles do not form when no current is applied. (d) Images of bubble growth as a function of dye concentration acquired using an optical microscope. (e) Images of colorimetric read-out as a function of deposition current and time. One-nA currents are detectable in 5 min. Scale bar, 1 mm. (f) Transmission spectrum of the read-out window before and after bubble growth.
To test the enhancement provided by edges, we designed mesh-shaped electrodes with increased ratios of edges to surface area. We applied 1 nA for 10 s to deposit platinum and recorded the rate of bubble growth (Figure 5.4A). We found that the rate of bubble evolution increased with increasing numbers of edges. The highest density mesh, with $3.4 \times$ the edge to surface area ratio of the rectangular electrode, provided the fastest bubble growth. No bubbles formed when no current was applied as no platinum was electrodeposited. Bubble growth was not observed after immersing the device in platinum solution for 25 min, indicating that platinum is not deposited via electroless deposition (Figure 7.4.1).

Using the high-density mesh electrodes, we measured the average growth of the bubble for various applied currents. Figure 5.4B shows the average bubble area measured after 20 min as a function of electrodeposition current while Figure 5.4C shows the bubble growth over time. After 20 min, a 1 nA current applied for 10 s yields a bubble with an area of 0.25 mm$^2$, which is visibly detectable.

### 5.3.4 Electrocatalytic fluidic dye displacement

To induce a visible colour change that is easily interpretable by the end-user, we utilize the bubble to displace an opaque dye that obscures a blue spot beneath the read-out window. As the chamber fills with oxygen, the blue spot is revealed.

Increasing the dye concentration increases the opacity of the dye, but also increases its viscosity. We found that at higher viscosities, bubble formation was inhibited (Figure 5.4D). We optimized the dye concentration and found that using a concentration of 25 μg ml$^{-1}$ allowed for sufficient optical density to conceal the blue spot while promoting bubble growth (Figure 5.4D).

To determine the minimum visibly detectable current, we deposited platinum at various rates for 10 s and measured the exposed area of the blue spot (Figure 5.4E). Using a 1 nA deposition current, the spot area grows to 0.09 mm$^2$ in 5 min. The exposed area expands to 0.24 mm$^2$ by
20 min. No bubble growth is observed when platinum is not electrodeposited. As the spatial resolution of human eyesight is about 200 μm (ref. 32) the smallest visible area ~200 μm × 200 μm or 0.04 mm². Thus, the spot area of 0.09 mm² obtained from a 1 nA current after 5 min is visible to the naked eye.

To quantify the performance of our device we calculated the colouration efficiency, a metric that quantifies the efficiency of converting an electrical current into a colorimetric change. Colouration efficiency, CE, is given by:

\[
\text{CE} = \frac{\Delta \text{OD} \cdot A}{Q}
\]  \hspace{1cm} (1)

Where \(\Delta \text{OD}\) is the change in optical density, \(Q\) is the charge required for switching [C] and \(A\) is the spot area [cm²]. We measured the optical density before and after switching and found a \(\Delta \text{OD}\) of 0.27 (Figure 5.4F). Given a switchable area of 0.24 mm² after 20 min using a 1 nA current applied for 10 s, this device has a colouration efficiency of \(6.48 \times 10^4\) cm² C⁻¹. Figure 5.5A compares the switchable area as a function of charge for devices with the highest reported colouration efficiencies for a range of read-out strategies. Given the previous records of \(2.6 \times 10^4\) cm² C⁻¹ for fluorescent polymers²² and \(9.3 \times 10^2\) cm² C⁻¹ for non-fluorescent electrochromic compounds,²⁰ a colouration efficiency of \(6.48 \times 10^4\) cm² C⁻¹ is, to our knowledge, the highest reported value in the literature for an electrochromic device.
Figure 5.5: Electrocatalytic fluid displacement reveals a structural colour change. (a) Comparison of the charge required to induce a visible colour of a certain area and optical density change for a variety of read-out strategies. The dashed red line represents the calculated exposed area of a bubble generated using electrocatalytic fluid displacement. We assume the bubble is confined to a 50-μm tall chamber, the reaction proceeds for 10 min, and the ΔOD is 1. The dashed blue line represents the area of a monoatomic layer of platinum directly reducible by the current. We assume the ΔOD is 1 and thus this represents an upper bound using this strategy. (b) Spot size as a function of time for various deposition currents using electrodes with the highest mesh density. Bubbles do not form when no current is applied. Error bars represent s.e. (n=3). (c) Images of colorimetric read-out as a function of deposition current and time using a diffraction grating. The window turns from optically transparent (which appears as black due to a black background) to cyan as light at that wavelength is diffracted towards the camera. One-nA currents are detectable in 1 min. Scale bar, 1 mm.
5.3.5 Induction of a structural colour change

As optical absorbance increases with path length, the read-out window must be sufficiently tall for the dye to obscure the coloured spot beneath. This limits the response time of a colorimetric device based on dye displacement, as the bubble must grow large enough to reach the chamber ceiling.

By patterning substrates with feature sizes on the order of the wavelength of light, it is possible to produce vibrant structural colours.\(^{34}\) Examples of this include diffraction gratings and iridescence. The colour of the substrate can be modified by matching the index refraction between a second medium and the substrate.\(^{35}\)

We hypothesized that we could exploit a structural colour change to decrease the read-out turnaround time. As structural colour changes rely on the index matching at an interface, the colour change is largely independent of the path length through the index-matching medium. Thus, we could expect a vibrant colour change using a device with a much smaller channel height than required when using dye displacement. As the substrate provides the colour, there is no need to increase the opacity of the peroxide by introducing additional compounds, which might interfere with the reaction.

To prove out this approach, we patterned a diffraction grating into the underside of the PDMS lid affixed to the top of the device with a 7-μm tall channel. As the index of refraction of peroxide (n=1.35) is similar to that of PDMS (n=1.4), the diffraction grating is invisible to incoming light when the device is initially loaded with peroxide. As the bubble forms, the peroxide is replaced with O\(_2\), which has an index of refraction of 1. This index mismatch between the bubble and PDMS unveils the diffraction grating. The incident white light is diffracted into its component colours to reveal the circular spot.
Figure 5.5B shows the growth of the coloured spot using the diffraction grating approach while Figure 5.5C shows the corresponding images of the spot over time. As the bubble grows, white light begins to diffract into its component colours. The window turns from optically transparent (which appears as black due to a black background) to cyan as light at that wavelength is diffracted towards the camera. Using a 1 nA deposition current, the spot size is 0.06 mm$^2$ after 1 min, which is visible by eye. This spot grows to 0.36 mm$^2$ and 1.1 mm$^2$ by 5 and 15 min, respectively. Given a spot size of only 0.1 mm$^2$ after 5 min using electrocatalytic fluidic displacement of a dye, the structural colour spot of 0.36 mm$^2$ is over three times larger in the same time frame. No spot forms when no current is applied Figure 5.5C.

### 5.3.6 Colorimetric read-out of ssDNA

To test the capability of the EFD device to detect biomarkers, we connected the NME sensors in serial (Figure 7.4.2) to the EFD read-out chip and challenged the NMEs with ssDNA. As an initial characterization of the ECC assay, we challenged the sensors with serial dilutions ssDNA. We then measured the corresponding currents after applying 250 mV (Figure 5.6A). The average peak current decreases with decreasing target ssDNA concentration giving a detection limit of 1 fM (Figure 5.6B). The current generated from 100 nM non-complementary ssDNA is less than 2 nA, which is similar to the background current, indicating this read-out method is specific.
Figure 5.6: Colorimetric detection of DNA. (a) Electrochemical current as a function of time for various analyte concentrations after applying 250 mV w.r.t. to a Ag/AgCl reference electrode for 3 s. (b) Average peak electrochemical current as a function of analyte concentration. Negative control is non-complementary (NC) DNA. (c) Spot size as a function of target DNA concentration after 10 min using dye displacement. One-pM ssDNA is detectable by eye. The visible threshold is defined as an area of 200 mm × 200 mm. (d) Images of the EFD device showing growth of the bubble over time as a function of ssDNA concentration using dye displacement. (e) Spot size as a function of target DNA concentration after 10 min using a structural colour change. (f) Images of the EFD device showing growth of the bubble over time as a function of ssDNA concentration using a structural colour change. Scale bar, 1 mm. Error bars represent s.e.; all measurements represent n > 5 trials.
To demonstrate colorimetric read-out of biomarkers, we coupled the assay to our read-out device and challenged the sensors with serial dilutions of ssDNA. To connect the sensors to our EFD device, we immersed the NME sensors in the ECC solution and the EFD read-out device in the platinum electrodeposition solution. To bridge electronically the sensor and read-out device, a platinum wire electrode immersed in the ECC solution is connected to a second platinum electrode in the electrodeposition bath. The EFD read-out device acts as the counter electrode for the entire system (Figure 5.2). After applying 250 mV for 10 s to the NME, we introduced peroxide into the EFD chip and measured the rate of colour formation (Figure 5.6C). We found a detection limit of 1 pM after 10 min with an average spot size of 0.068 mm². To our knowledge, a detection limit of 1 pM is the lowest reported limit of detection for colorimetric detection of ssDNA using an electrochemical sensor. No visible spot was observed when the sensors were challenged with 100 nM of non-complementary ssDNA indicating a specificity discrimination ratio of $1 \times 10^5$ (Figure 5.6D).

We studied the performance of the diffraction grating approach for colorimetric ssDNA detection. First, we optimized the peroxide concentration to minimize bubble formation from currents at the background level. We found that bubble growth at low currents could be suppressed using 10% peroxide (Figure 7.4.3). We challenged the devices with ssDNA and measured the growth of the diffracting area (Figure 5.6E). Figure 5.6F shows the corresponding images of the growth of the visible spot over time. Using 1 pM complementary ssDNA, the spot size was 0.15 mm² after 10 min. In that same time frame, the spot using dye displacement was 0.068 mm², which is about two times smaller. This is expected as the chamber height using the diffraction grating approach was five times smaller than the chamber in the dye displacement device, and thus the bubble can rapidly grow laterally. Using this method, 100 fM of ssDNA was also detectable by eye with an average spot size of 0.085 mm² (Figure 5.6E). No spot was visible with 100 nM non-complementary ssDNA (Figure 5.6F).
This colorimetric read-out approach is an inexpensive, disposable and low-power alternative to using electronics for read-out of electrochemical currents. We use three stages of amplification to transform ultra-low currents into colorimetric changes. The ECC redox assay uses two chemical catalysis steps to amplify the electrodeposition current. Thus, each bound nucleic acid is converted into multiple deposited platinum atoms. Next, by electrodepositing a catalyst, the colorimetric reaction continues long after the initial application of the electrochemical current, obviating the need for high currents to induce colorimetric changes. Last, colorimetric read-out is accelerated by exploiting the fact that a gas occupies a much larger volume than an equivalent molar amount of liquid. A catalytic reaction can evolve a large volume of gas much faster than it can turnover a visible amount of dye of the same volume.

Even though spot sizes as small as 100–200 μm are visible under perfect conditions to those with 20/20 vision, spot sizes <1 mm² may be difficult to see for some. Small spot sizes could be easily magnified using inexpensive lenses fabricated from elastomers such as PDMS.

A fully integrated device would require the timed introduction of reagents with automated flow. This could be integrated onto an instrument-free device using passive fluidic systems such as paper microfluidics, capillary pumps or on-chip vacuum pumps. As this strategy only requires the application of a DC potential, the potential could be applied using a DC power source such as a battery as opposed to a potentiostat.

5.4 Conclusions

In summary, we introduce a strategy for rapid and sensitive colorimetric read-out of electrochemical currents based on electrocatalytic fluid displacement. This approach relies on the electrochemical mediated deposition of platinum which catalyses the growth of a fluid displacing bubble. We present two strategies for converting this fluidic displacement into a visible colour change using a dye and a structural colour change. We demonstrate successful colorimetric
detection of a 1 nA current in 1 min and calculate a colouration efficiency of $6.48 \times 10^4$ C cm$^{-2}$, which to our knowledge is the highest value reported in the literature. We showcase this approach by coupling our device to a novel electrocatalytic assay and nanostructured microelectrode sensor to demonstrate successful and specific colorimetric detection of 100 fM of ssDNA in 10 min with a discrimination ratio of over $1 \times 10^5$.

5.5 Methods

5.5.1 Calculation of time required for visible bubble formation

To calculate the rate of colour change using direct electrochromic colorimetric read-out, we assume a channel 50-μm tall by 200-μm wide filled with enough electrochromic dye to give an OD of 1. We assumed a high molar absorptivity of $1 \times 10^7$ M$^{-1}$ m$^{-1}$, which is similar to that of malachite green. Using the catalysis rate of platinum, we calculate the time needed to turn over the dye in the channel. To calculate the rate of colour change using electrocatalytic fluidics, we assume a channel a chamber that is 50-μm tall with a 200-μm width. Using the catalysis rate of platinum, we calculated the rate of oxygen formation. The onset of bubble formation occurs as peroxide in the chamber is saturated with oxygen. We assume the bubble is visible once it grows to the volume of the chamber. Parameters used in the calculations are listed in Table 7.4.1.

5.5.2 Device fabrication

The device was fabricated using standard photolithographic methods. In brief, electrodes were patterned on a glass substrate. The device was passivated using SU-8 2002 (Microchem, Newton, MA) and apertures were patterned to expose the electrodes below. The channel was fabricated by patterning SU-8 3050 (Microchem, Newton, MA).
5.5.3 Platinum electrodeposition

The electrode was immersed in K$_2$PtCl$_4$ (Sigma-Aldrich, MO) and connected to an Epsilon potentiostat (BASi West Lafayette, IN) using a three-electrode set-up with a Ag/AgCl reference electrode and a Pt counter electrode. Using chronopotentiometry, various currents were applied for 10 s. After electrodeposition, the device was washed thoroughly with H$_2$O and covered with a PDMS (Dow Chemical, MI) lid.

5.5.4 Colorimetric read-out using a dye

Hundred microlitres of white dye (Liquitex Titanium White Ink) was centrifuged for 5 min at 15 000 g. The supernatant was removed and replaced with 400 μl of 30% H$_2$O$_2$ (Sigma-Aldrich, MO). The dye (25 μg ml$^{-1}$) was introduced into the channel and the amount of bubble generation was measured over time using a camera (Canon).

5.5.5 Colorimetric read-out using structural colour

A diffraction grating was patterned in PDMS by curing PDMS on a DVD-R. The PDMS diffraction grating lid was removed and attached to the device with a 7-μm tall channel patterned using SU-8 2010 (Microchem, Newton, MA). Twenty-seven percent H$_2$O$_2$ with 1% pluronic (Sigma-Aldrich, MO) was introduced into the device, and colour changes were measured over time using a camera (Canon).

5.5.6 Sensor chip fabrication

Six-inch silicon wafers (University Wafer, MA) were passivated using a thick layer of thermally grown silicon dioxide and coated with a 25 nm Ti adhesion layer. A 350-nm gold layer was deposited on the chip using electron-beam-assisted gold evaporation, which was again coated with 5 nm of Ti. The electrodes were patterned in the metal layers using standard photolithography and a lift-off process. A 500 nm layer of insulating Si$_3$N$_4$ was deposited using
chemical vapour deposition. The 5-μm apertures were etched at the tips of the metal leads using standard photolithography. Contact pads (0.4 mm × 2 mm contact) were patterned using wet etching as well.

5.5.7 Fabrication of sensors

Chips were cleaned by sonication in acetone for 5 min, rinsed with isopropyl alcohol and DI water, and dried with nitrogen. Electrodeposition was performed at room temperature. The 5-μm apertures on the fabricated electrodes were used as the working electrodes and were contacted using the exposed contact pads. Nanostructured microelectrode sensors were electrodeposited in a solution of 50 mM HAuCl₄ (Sigma-Aldrich, MO) and 0.5 M HCl (Sigma-Aldrich, MO) using DC potential amperometry at 0 mV for 100 s. After washing with DI water and drying, the sensors were coated again with a thin layer of Au to form nanostructures by plating at −450 mV for 10 s.

5.5.8 Functionalization of sensors

An aqueous solution containing 1 μM of probe (5′-GGT CAG ATC GTT GGT GGA GT-3′) (PNA Bio, CA) was mixed with 10 μM of aqueous Tris(2-carboxyethyl)phosphine hydrochloride solution (Sigma-Aldrich, MO) and then the mixture was left for overnight to cleave disulphide bonds. After mixing 100 nM of 6-mercaptohexanol (MCH) (Sigma-Aldrich, MO) to this probe solution mixture, 20 μl was pipetted onto the chips and incubated for 3 h in a dark humidity chamber at room temperature for probe immobilization. The chips were then washed thrice for 5 min with 0.1 × PBS (Life Technologies, CA) at room temperature. The chips were then treated with 1 mM MCH for an hour at room temperature for back filling. After washing, the chips were challenged with different concentration of targets for 30 min at room temperature. After hybridization, the chips were washed thrice for 5 min with 0.1 × PBS at room temperature and the electrochemical scans were acquired.
5.5.9 Electrochemical detection of ssDNA

All electrochemical experiments were carried out using a Bioanalytical Systems Epsilon potentiostat with a three-electrode system featuring a Ag/AgCl reference electrode and a platinum wire auxiliary electrode. Electrochemical signals were measured in a Tris buffer solution (50 mM, pH 9) containing 10 μM [Ru(NH₃)₆]Cl₃ (Sigma-Aldrich, MO), 0.5 mM 3-mercaptopropionic acid (MPA) (Sigma-Aldrich, MO) and 0.5 mM cysteamine (Cys) (Sigma-Aldrich, MO). DC potential amperometry (DPCPA) signals were obtained at +250 mV for 10 s. Signal changes, ΔI, were calculated with ΔI=Iₖ−I₀ (where Iₖ is the current at a given concentration and I₀ is the current without analyte).

5.6 References


6 Conclusions and future outlook

6.1 Thesis findings

In this thesis, we report the development of a variety of techniques for rapid sample processing and detection of disease-related analytes.

Rapid isolation of rare cells from complex matrices could enable early diagnosis and improved monitoring of cancer progression. In Chapter 2, we developed a strategy to isolate rare cells from the bloodstream both rapidly and efficiently. We showed that x-shaped structures introduced into the flow stream increase the capture efficiency of magnetic nanoparticle-labelled cells by 5-fold over using circular posts. By analysing the magnetic and drag forces acting on a nanoparticle-labelled cell, we optimized the capture structure geometry to maximize capture efficiency and minimize non-specific cell adhesion in order to recover cancer cells with over 10 000-fold specificity. Finally, we designed and tested a device to spatially sort cancer cells based on surface marker expression. We found that we can efficiently sort rare cell subpopulations directly from the bloodstream into four spatially distinct zones.

In Chapter 3, we studied strategies to reduce the time required to perform antibiotic susceptibility tests. First, we designed a novel electrochemical assay to readout live bacteria by electrochemically detecting the reduction of resazurin by metabolically active bacteria. We used this novel assay, in conjunction with a device to concentrate and culture bacteria in nanoliter wells, to readout the antibiotic susceptibility profile of bacteria rapidly. Bacteria are concentrated on-chip into isolated nanoliter culture chambers with integrated electrodes. The susceptibility profile is readout electronically after incubating the bacteria with the culture media, the antibiotic and resazurin. By concentrating and confining the bacteria in nanoliter chambers, the effective concentration of bacteria is drastically increased and the reduced form of the redox indicator can rapidly accumulate to detectable levels. We showed that using a 30 minute incubation period, we
can detect clinically relevant concentrations of bacteria at 100 cfu/µL. We could readout the antibiotic susceptibility profile with a 1 hour incubation period. We compared our approach to standard susceptibility tests with an overnight incubation and found similar susceptibility profiles using a 20-fold shorter incubation period.

In Chapter 4, we developed a strategy to detect pathogen-specific RNA molecules rapidly for sensitive bacterial detection. We overcame the limitations imposed by slowly diffusing RNA molecules by performing lysis in close proximity to the sensor. Released intracellular RNA can rapidly diffuse the short distance to the sensor and accumulate on the sensor surface. We optimized the lysis conditions to maximize the integrity of the probe monolayer and found that short pulse times and low salt buffers minimize damage to the probe. We showed that the proximal lysis approach is 10 times more sensitive than standard homogenous lysis for *E. coli* RNA detection.

Rapid and sensitive colorimetric readout of biomarkers is an important step in the development of disposable, point-of-care diagnostics. In Chapter 5, we developed an ultrasensitive colorimetric readout strategy to detect clinically relevant concentrations of nucleic acids. We showed that by introducing a cascade of amplification steps, we can rapidly convert the nanoamp currents generated by sensitive electrochemical sensors into a permanent visible colour change. The current generated by the sensors drives the electrodeposition of a small amount of platinum, a metal catalyst that induces the decomposition of hydrogen peroxide. The breakdown of peroxide forms a bubble which displaces the solution. We developed two methods to convert this fluid displacement into a colour change by displacing a dye and by inducing a structural colour change. We connected the readout device with a nanostructured microelectrode sensor and found that we could detect 100 fM of ssDNA, a clinically relevant level of nucleic acids, in 10 min.
6.2 Future outlook

This work lays the foundation for the development of several tools to process and analyze biological samples rapidly in order to detect disease-related biomarkers. The emphasis of this thesis is on the design of proof-of-concept devices to demonstrate new sample processing and sensing techniques. In order to develop automated and operator-independent devices, the necessary instrumentation, such as fluidics, pumps, and readout electronics, must be integrated into a single device. Further validation of all of these devices with clinical samples is needed before these approaches could be translated into the clinic.

In Chapter 2, we designed and optimized a device for rare cell isolation using both theory and experiments. The model described in this chapter does not consider the three-dimensional flow profile, the probability of interacting with the capture structures or the likelihood that a cell crosses a streamline due to the magnetic force. This model does not account for cell-cell interactions, turbulence, wall effects, frictional forces or adhesion forces. This model also does not account for variations in the spatial overlay of the magnets with the cell capture array. The model could be enhanced by including many of these factors in future work. In order to further analyse the isolated cells, it will be important to integrate downstream analysis such as gene expression profiling and drug susceptibility testing. Validating this device with patient samples will also be critical before it can be translated to the clinic.

In Chapter 3, we described a strategy for rapid electrochemical antibiotic susceptibility testing. As this device was only validated with two different gram negative species, additional efforts to test the device efficacy against a broad spectrum of gram negative and positive bacteria are needed. As antibiotics have various modes of action, this device should be tested against a wide range of antibiotics with both bactericidal and bacteriostatic activity. Clinical applications call for multiplexed analysis of at least 10 different antibiotics at multiple concentrations. This could be accomplished by using microfluidics to load the antibiotics in an on-chip well array. A range
of antibiotic concentrations could be generated on-chip using a branched network of microfluidic channels.

In Chapter 4, we described a strategy for proximal lysis and detection of bacterial nucleic acids. Further efforts are needed to fully characterize the lysis method described in this chapter including determining the lysis efficiency and the effect of lysis on the integrity of released mRNA. As the lysis is currently performed in a controlled buffer, additional work is needed to develop a device to handle pathogens in complex matrices. This could be accomplished using a microfluidic device to isolate the pathogens from the matrix using immuno-magnetic capture, at which point the bacteria could be transferred to the desired lysis buffer. Testing a wide range of both gram negative and positive bacteria in complex matrices is critical before translating this approach to a clinical setting.

The findings in Chapter 5 point toward the development of integrated point-of-care diagnostics for infectious diseases. In Chapter 5, we outlined a strategy for rapid and sensitive colorimetric readout of nucleic acids. This readout platform could serve as the display on a disposable diagnostic for infectious diseases such as HCV and malaria, two diseases in which treatment is dependent on the pathogen genotype. Before translating this device to a clinical setting, it will be important to investigate the specificity and sensitivity of this approach with analytes in complex matrices such as blood. This will likely call for additional sample purification steps that need to be integrated onto a disposable device using passive fluidics. All necessary electronics should be integrated onto the device as well. To validate the efficacy of this strategy for point-of-care diagnosis, this device will need to be tested against a panel of clinical samples in low-resource settings.

The findings from this work could guide the development of diagnostic devices for previously intractable clinical applications such as sepsis, a bloodstream infection. Rapidly determining the susceptibility profile of a bloodstream infection is critical as the chance of survival drops by
almost 8% for every hour without administration of effective therapy. No existing method can provide results fast enough as sepsis may be manifested by extremely low levels of bacteria at 1-100 CFU/mL. The approaches outlined in Chapters 2, 3 and 4 could have particular relevance to sepsis diagnosis. In Chapter 2, we described an approach for rapid isolation of rare cells using magnetic nanoparticles. By tuning the capture antibody on the nanoparticles, the approach outlined for capturing rare cancer cells could be modified to isolate and concentrate rare blood-borne pathogens. In Chapters 3 and 4 we described tools for rapid detection and susceptibility profiling of bacteria. After isolating and concentrating the bacteria from the bloodstream, the approaches described in Chapters 3 and 4 could be used to determine the infecting bacterial species and readout the susceptibility profile electronically. Combined, these methods may allow rapid diagnosis and susceptibility profiling of low levels of blood-borne pathogens such as bacteria, fungi, and malaria.

6.3 Summary

The response time of surface-based biosensors is limited by how quickly the target molecules can reach the sensor surface. In this thesis, we presented three approaches to increase biosensor sensitivity by increasing the flux of the target analyte at the sensor surface: [1] increase the effective analyte concentration, [2] shorten the required diffusion distance, and [3] prevent diffusion into bulk solution.

The simplest way to increase the flux of the target analyte is to increase the analyte concentration. Directly amplifying the target analyte however is time-consuming and only possible for a limited class of analytes such as nucleic acids and bacteria. Thus, we outlined strategies to increase the effective analyte concentration. As concentration is a function of both the amount of the analyte and the solution volume, we sought strategies to both increase the analyte abundance and decrease the solution volume. For example, to decrease the time needed to perform a susceptibility test, we increased the abundance of analyte using a filter to trap the
bacteria in a microfluidic chip with a volume on the order of microliters. The chamber volume is then further reduced by introducing an immiscible oil phase to confine the trapped bacteria to the nanoliter culture chambers. Together, these methods increase the effective concentration of the bacteria 1000-fold.

Decreasing the required diffusion distance to the sensor surface can also increase biosensor speed and sensitivity. For example, to reduce the time needed to detect bacterial nucleic acids, we lysed bacteria in close proximity to the sensor. As bacteria contain multiple copies of the target sequence, the analyte is at a locally high concentration within the bacteria. After lysis, multiple target nucleic acids are released close to the sensor surface and thus, in the vicinity of the sensor, the analyte is present at a locally high concentration. These molecules can diffuse the short distance to the sensor more quickly than if evenly distributed throughout the bulk solution.

A third strategy to increase flux is to constrain the analyte within a small spatial volume around the sensor. In the antibiotic susceptibility chip, both culture and readout are performed within an isolated nanoliter compartment. Thus, as the bacteria turnover resazurin, the redox indicator of bacterial viability, the reduced compound, resorufin, is confined to this nanoliter compartment, prevented from diffusing to bulk solution and can quickly accumulate to detectable levels.

To further improve sensor performance, we also explored strategies to increase the sensitivity of the electrochemical transduction method. This is especially relevant for low-cost, point-of-care diagnostics as the instrumentation must be integrated onto a disposable device. We realized that no single transduction method is sufficiently sensitive for direct visual of ultra-low levels of nucleic acids. Thus, in the electrocatalytic fluidic displacement approach we developed, three amplification strategies are connected in serial. Firstly, the electrochemical-chemical-chemical amplification assay converts a single binding event into many electrons. Secondly, the deposition of a catalyst allows the colorimetric reaction to progress long after the initial application of the potential. Thirdly, the product generated is in the gas phase which occupies a 1000-fold greater
volume than an equivalent molar amount of liquid at STP. Only by combining all three amplification strategies was it possible to visually readout the nucleic acids with high sensitivity. This electrocatalytic fluid displacement strategy is not limited to nucleic acid detection and could be used to detect a wide range of biological analytes.

Ultimately, to be useful in a clinical setting, the approaches outlined above must be integrated into an automated benchtop or handheld device. Throughout this thesis, we placed special emphasis on designing electronic detection strategies which could be translated to a commercial device. While optical detection methods are sensitive, they often rely on expensive fluorescent microscopes, microplate readers and other bulky optical components, limiting their application in low-resource or point-of-care settings. On the other hand, electronic components are routinely integrated into benchtop, handheld or even disposable devices. Integrating sample processing techniques is equally important, and thus we designed microfluidic devices with on-chip sample manipulation, filtration, and lysis.

We showcased these advances with a variety of proof-of-concept clinical applications ranging from cancer detection to antibiotic susceptibility testing. The methods presented here are general prescriptions for increasing biosensor performance and could be applied to the detection of variety of biological analytes across a range of clinical applications. Ultimately, the work outlined here could aid the development of more effective point-of-care diagnostic technologies to guide physicians in choosing the most effective treatment for a given disease.
7 Appendix A – Supporting Information

7.1 Supporting Information for Chapter 2

Magnetic field simulations. For ease of calculation, a simplified 2D magnetic geometry was used for the simulations. The magnetic field was calculated using COMSOL Multiphysics with four alternating NdFeB magnets (1/4” x 1/16””) with magnetization of 0.75 T (K&J Magnetics).

Figure 7.1.1: Simulations of the magnetic fields. (A) and magnetic forces (B) on a single nanoparticle in Zones 1 and 4. Close up simulations of the magnetic fields (C) and magnetic forces (D) with overlaid normalized field vectors. (E) The magnetic field inside the channel as a function of height above the channel center (y=0) and distance along the channel.
Fluid flow simulations. Using COMSOL Multiphysics, we simulated the distribution of linear velocities inside the chip for the five different trapping structure designs using an average linear speed of 600 µm/s which corresponds to a 1 mL/hour flow rate for a 50 µm channel height.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \eta )</td>
<td>Dynamic viscosity of the medium</td>
<td>0.001 Pa x s</td>
</tr>
<tr>
<td>( V_m \Delta \chi_{\text{bead}} )</td>
<td>Magnetic nanobead parameter (( V_m ) is the nanobead volume and ( \Delta \chi_{\text{bead}} ) is the difference of the magnetic susceptibility of the beads and the surrounding medium)</td>
<td>2.5 ( \times ) 10(^{-16} ) mm(^3)</td>
</tr>
<tr>
<td>( \mu_0 )</td>
<td>Permeability of free space</td>
<td>4( \pi \times ) 10(^{-7} ) H/m</td>
</tr>
<tr>
<td>( r )</td>
<td>Cell radius</td>
<td>10 µm</td>
</tr>
</tbody>
</table>

Table 7.1.1: Simulation parameters.

Model of capture efficiency. In each chip there are 17 rows of capture structures (Figure 2.1C). On a path from the inlet to the outlet, each cell will pass by one structure, per row, for each of the 17 rows. Thus, there are 17 opportunities for a cell to be captured. The probability of escaping all 17 rows is \((1 - p_{\text{capture}})^{17}\). Therefore the capture efficiency, \( E \), can be calculated as:

\[
E = 100\% \left(1 - \left(1 - p_{\text{capture}}\right)^{17}\right)
\]
where \( N \), the number of structures in each cell’s path, is 17, \( P_{\text{capture}} = \alpha \frac{A_{\nu < \nu_t}}{Q} \), \( Q \) is the flow rate (mL/hr), \( A_{\nu < \nu_t} \) is the average percentage of area surrounding a capture structure in which the linear velocity is less than the threshold, and \( \alpha \) is an experimentally determined proportionality constant with units set to ensure \( P_{\text{capture}} \) is unitless (units are hr/mL).

Figure 7.1.2: Forces acting on a cell in the channel under various flow conditions.

**Calculation of \( \nu_t \).** First we measured the average number of nanoparticles per cell using the method described below. Using the magnetic simulations, we calculated the maximum magnetic force acting on a cell in the channel. The threshold velocity, \( \nu_t \), is defined as the required linear velocity such that the drag force acting on the cell is equal to the maximum magnetic force.

**Calculation of \( A_{\nu < \nu_t} \).** Using the fluid flow simulations, \( A_{\nu < \nu_t} \), the average percentage of area surrounding a capture structure in which the linear velocity is less than the threshold, was calculated for each structure design. We simulated the spatial distributions of linear velocity and
used COMSOL to calculate the percentage area of the chip in which the linear velocity was less than the threshold linear velocity for capture.

**Fitting the model to the experimental data ($\alpha$).** The data was fit to the SKBR3 capture efficiency data, and we found the model best fit the data using a proportionality constant of 0.1. For VCaP we found the model best fit the data using a proportionality constant of 0.4.

<table>
<thead>
<tr>
<th>Chip geometry</th>
<th>$A_{v&lt;v_t}$</th>
<th>N</th>
<th>$\alpha$</th>
<th>$E_{\text{model}}$</th>
<th>$E_{\text{experimental}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘x’</td>
<td>0.17</td>
<td>17</td>
<td>0.4</td>
<td>91.5</td>
<td>100</td>
</tr>
<tr>
<td>‘+’</td>
<td>0.078</td>
<td>17</td>
<td>0.4</td>
<td>66.8</td>
<td>59</td>
</tr>
<tr>
<td>‘o’</td>
<td>0.018</td>
<td>17</td>
<td>0.4</td>
<td>21.8</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 7.1.2: Simulation parameters for different chip geometries using VCaP cells.
<table>
<thead>
<tr>
<th>Flow rate [mL/hr]</th>
<th>$A_{v&lt;\eta}$</th>
<th>N</th>
<th>$\alpha$</th>
<th>$E_{model}$</th>
<th>$E_{experimental}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.19</td>
<td>17</td>
<td>0.1</td>
<td>74.7</td>
<td>81</td>
</tr>
<tr>
<td>1</td>
<td>0.13</td>
<td>17</td>
<td>0.1</td>
<td>20.3</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>0.063</td>
<td>17</td>
<td>0.1</td>
<td>2.76</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 7.1.3: Simulation parameters used to validate the model as a function of flow rate using SKBR3 cells.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Q</th>
<th>$A_{v&lt;\eta}$</th>
<th>N</th>
<th>$\alpha$</th>
<th>$E_{\text{model}}$</th>
<th>$E_{\text{experimental}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCaP</td>
<td>0.25</td>
<td>0.19</td>
<td>17</td>
<td>0.4</td>
<td>99.8</td>
<td>90</td>
</tr>
<tr>
<td>SKBR3</td>
<td>0.125</td>
<td>0.22</td>
<td>17</td>
<td>0.1</td>
<td>96.1</td>
<td>93</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.0625</td>
<td>0.15</td>
<td>17</td>
<td>0.1</td>
<td>99.1</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 7.1.4: Simulation parameters used to validate capture efficiency as a function of cell line.

**Measurement of nanoparticles per cell.** To estimate the number of magnetic nanobeads bound to a cell, we used a previously described method. Cells labelled with magnetic nanobeads were flowed in a straight channel adjacent to a magnet (Figure 7.1.3). In the presence of the magnetic force, labelled cells flow on a diagonal towards the magnet.

The magnetic force was calculated according to Eq. 2. Neglecting wall effects, the transverse drag force acting on a cell (modelled as a spherical particle) at low Reynolds numbers is given by Stokes’ law:
\[ \vec{F}_{Dy} = 6\pi \eta r \vec{v}_y \]  

(2)

where \( \eta [\text{Pa}\cdot\text{s}] \) is the fluid viscosity, \( \vec{v}_y [\text{m/s}] \) is the transverse component of cell velocity, and \( r [\text{m}] \) is the cell radius. Neglecting inertia and assuming no other forces are acting on the cells, the magnetic and drag forces acting on the cells should be equal and opposite:

\[ F_{Dy} = -F_m \]  

(3)

Substituting in for the drag and magnetic forces and rearranging, we arrive at the following equation for the number of beads per cell:

\[ N_b = \frac{6\pi \eta r v_y}{\mu_0 m_{\text{max bead}} B_f B} \]  

(4)

**Validation of Stokes’ Drag.** In order to confirm that the drag force on the cells was accurately described by Stokes’ law, a series of validation experiments were performed using fluorescently labelled paramagnetic microbeads. Microbeads having diameters of 8 \( \mu \text{m} \) (COMPEL, Bangs Laboratories) and 30 \( \mu \text{m} \) (PLA-M-greenF, micromod) were tested. A NdFeB permanent magnet provided the magnetic field, and the magnetic flux density & magnetic field strength were calculated via computer simulations carried out in COMSOL Multiphysics. Magnetization curves provided by the microbead suppliers were used to calculate the magnetic susceptibility of the paramagnetic beads, which was a function of the local magnetic field strength.

The 8 \( \mu \text{m} \) and 30 \( \mu \text{m} \) microbeads had densities of 1100 kg/m\(^3\) and 1400 kg/m\(^3\), respectively. Initial experiments were carried out in PBS, however the high density of the beads relative to PBS led to a significant portion of the microbeads settling out of the solution. Particle settling velocity, known as Stokes’ settling velocity, is governed by: \(^3\)
where \( v_s \) [m/s] is the particle settling velocity, \( D \) [m] is the particle diameter, \( \eta \) [Pa s] is the fluid viscosity, \( \rho_p \) [kg/m\(^3\)] & \( \rho \) [kg/m\(^3\)] are the particle and fluid densities, respectively, and \( g \) [m/s\(^2\)] is the gravitational acceleration. Clearly, the particle settling velocity is minimized when the difference between the particle and fluid densities is minimized and when the fluid viscosity is maximized. The viscosity and density of the test solutions were increased by adding glycerol, which is much more viscous and dense (\( \eta_{\text{glycerol}} = 1.412 \) Pa s, \( \rho_{\text{glycerol}} = 1261 \) kg/m\(^3\)) than PBS (\( \eta_{\text{PBS}} = 0.001 \) Pa s, \( \rho_{\text{PBS}} = 1000 \) kg/m\(^3\)). Experiments were carried out with 75% and 85% glycerol solutions. The viscosity of the test solutions was characterized at laboratory temperature (22°C) using a shear rheometer fitted with a 40 mm, 0.5° cone (TA Instruments, AR2000).
Figure 7.1.3: Schematic of the experimental setup used for measuring the number of nanoparticles per cell.

For each experiment, a small amount of the microbeads was added to the test solution, which was then pumped at a constant flow rate ranging from 100 µl/hr to 1000 µl/hr in a straight walled channel having a width of 1000 µm and a height of 105 µm (Figure 7.1.3). A sequence of images was captured using fluorescent microscopy at a frame rate of 100 FPS. After each experiment, the positions and velocities of the particles in each image were calculated using a suite of MATLAB functions developed at Yale University.\(^4\)

Figure 2.3A shows that the ratio of the magnetic force to the measured Stokes drag force for both the 8 µm and 30 µm microbeads was consistent with the expected value of unity, confirming the validity of Stokes’ law for this experimental setup.
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<td>400</td>
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</tr>
</tbody>
</table>

Table 7.1.5: Device surface area across four zones of the device.

7.1.1 References

7.2 Supporting Information for Chapter 3

Figure 7.2.1: Electrochemistry of resazurin. A) (i) Cyclic voltammograms and (ii) differential pulse voltammograms of 10 mM resazurin in PBS + 20% ACN. B) (i) Cyclic voltammograms and (ii) differential pulse voltammograms of 10 mM resazurin in LB media. Scans were acquired using an Au macroelectrode and a Ag/AgCl reference electrode.
Figure 7.2.2: Electrochemical reduction of resazurin by metabolically active bacteria. A) Cyclic voltammograms of *E. coli* at 1x10^6 CFU/mL before (red) and after (blue) incubating with 1 mM resazurin for 6 hrs at 37°C in LB media. B) Corresponding differential pulse voltammograms. Scans were acquired using an Au macroelectrode and a Ag/AgCl reference electrode.

Figure 7.2.3: Effect of dissolved oxygen on the electrochemistry of resazurin. (A) CV and (B) DPV acquired using a gold macroelectrode with 1 mM resazurin in LB media before and after purging with N2 for 20 min. We observed a 13% decrease in the DPV peak current after purging with N2.
Figure 7.2.4: Fluorescent detection of metabolically active *E. coli*. Serial dilutions of *E. coli* were incubated for 5 hours at 37°C with 1 mM resazurin in LB media. The fluorescence signal was measured using a microplate reader at 585 nm with an excitation wavelength of 570 nm. Metabolically active bacteria convert resazurin to resorufin which increases the fluorescence signal. 100 CFU/µL were detectable using fluorescence which corresponds with the detection limit achieved using electrochemistry using the same 5 hr incubation period. The dashed line represents the signal from the blank sample.

Figure 7.2.5: Optical images of the device. (A) An optical image of the well array. (B) (i) An optical image of a single well with a working, counter, and reference electrode before introducing the microbeads. (ii) Microbeads are trapped by a barrier at the rear of each well. (iii) After bacteria are captured, a nanoliter plug is formed by introducing an immiscible organic phase. Scale bars represent 100 µm.
Figure 7.2.6: Steps in the fabrication of the device. (1) First a 100 nm gold layer is patterned using standard photolithography. Cr is used as an adhesion layer. (2) Next the patterned electrodes were passivated with 2 µm of SU-8 2002 using photolithography. (3) A 50 µm well layer was patterned using SU-8 3050. (4) A second layer of SU-8 2002 is patterned as a thin 2 µm spacer small enough to trap the 5 µm microbeads necessary to fabricate the in-well filters. (5) To increase the surface area of the electrodes, gold is electrodeposited on the working electrodes by applying -300 mV for 30 s with respect to an Ag/AgCl reference electrode in a solution of 50 mM HAuCl4 and...
0.5 M HCl. (6) The device is capped with a PDMS lid and 5 µm microbeads are injected into the wells to form the in-well filters.

Characterization of pore size. As the microbeads are spherical, we can refer to the extensive literature studying the packing of spherical objects. The densest possible packing of spheres is hexagonal close packing in which the packing fraction is 0.74 and the pore diameter is given by:

\[ D_P = 0.154 D_s \]

Where \( D_p \) is the diameter of the pores and \( D_s \) is the volume of the spheres. For 5 µm diameter beads, assuming hexagonal close packing, the pore diameter is 0.77 µm which is sufficiently small to trap a bacterium (~1 µm). In reality, the spheres would pack in an assembly close to random close packing which has a slightly looser packing with a packing fraction of 0.637, which causes a distribution in pore sizes, but does not change the diameter of the smallest pores. This calculation is consistent with the high resolution images below. Using ImageJ, we measured the pore size and found a similar minimum pore size of approximately 0.8 µm.
Figure 7.2.7: Characterization of the in-well filter. (A) Image of the filter bead bed acquired using optical microscopy. (B) Illustration showing the effect of bead size and packing on pore size. The diameter of the largest particle, represented in red, capable of fitting through spherical beads with hexagonal close packing is 0.77 µm. (C) Optical microscope image showing a close up of the beads. The pore size in this image is approximately 0.8 µm which is consistent with the calculations. (D) When microbeads are not used, bacteria are captured with low efficiency. (E) Capture efficiency of *E. coli* as a function of flow rate. Error bars represent standard error.

**Measuring filter stability.** To measure the stability of the microbead filters, we injected 100 µL of microbeads at 20 µL/min into a version of the device without the in-well electrodes. We blocked the outer-channel inlet and the inner-channel outlet which forces the fluid through the wells. After stopping the flow, we acquired microscope images over the course of 1 hour. We found that the microbeads were stable over the course of 1 hour. Although a few beads did
become dislodged from the filters, this does not affect the electrochemical measurements as the electrodes are offset by 200 µm from the filter.

**Figure 7.2.8: Characterization of filter stability.** A series of optical images showing the stability of the filter over time.

**Eluting bacteria from filters for capture efficiency measurements.** To calculate the capture efficiency of the in-well filters, we eluted the captured bacteria and incubated off-chip on agar plates. To elute the bacteria, we inject buffer while directing the fluid flow backwards through the filters. This is accomplished by blocking the outer-channel inlet and the inner-channel outlet. The backflow of buffer forces bacteria out of the filters back towards the inlet. The eluent was cultured overnight at 37°C and the colonies were counted.
Figure 7.2.9: Eluting bacteria for off-chip culture. (A) Bacteria can be eluted by flowing buffer from the outlet towards the inlet and directing the flow backwards through the filters. (B) Image of *E. coli* expressing green fluorescent protein (GFP) captured in the in-well filter before and after applying a backflow.

Figure 7.2.10. Effect of electrodeposition and surface fouling on the on-chip electrodes. (A) Electrochemical scans on-chip of 1 mM resazurin before and after electrodepositing Au for 30 s. Electrodeposition increases the electrode surface area and thus the magnitude of the current. (B) Effect of surface fouling on the electrodes. After incubating for one hour, we observe a slight signal decrease when scanning on-chip with 1 mM resazurin. Currents are normalized to the maximum current.
**Time required for antibiotics to inhibit bacterial metabolic activity.** In order to choose a suitable incubation period for the susceptibility test, we studied the time required for the antibiotics to begin inhibiting bacterial metabolic activity. To study this, we used a high concentration of bacteria in order to determine the minimum time required for the bacteria to exhibit differential metabolic activity in response to the tested antibiotics. *K. pneumoniae* at $1 \times 10^5$ cfu/$\mu$L were incubated at 37°C in the presence of ampicillin and ciprofloxacin at 100 $\mu$g/mL in LB media and 1 mM resazurin. The increase in fluorescence induced by the conversion of resazurin by metabolically active bacteria was recorded. We find that the signal from the sample incubated with ciprofloxacin is suppressed within 30 minutes indicating that the antibiotic rapidly inhibits the metabolism of *K. pneumoniae*. As this strain of *K. pneumoniae* is resistant to ampicillin, the fluorescence increases as *K. pneumoniae* convert resazurin. These results indicate that the chosen incubation period of 60 minutes for the rapid on-chip susceptibility test is sufficiently long for the bacteria to exhibit differential metabolic activity in response to the tested antibiotics.

![Graph](image.png)

**Figure 7.2.11:** Time required for antibiotics to hinder the metabolic activity of bacteria. *K pneumoniae* at at $1 \times 10^5$ cfu/$\mu$L were incubated with 1 mM resazurin and ampicillin and ciprofloxacin at 100 $\mu$g/mL. When incubated with ciprofloxacin, the signal is supressed compared to that from ampicillin, indicating that ciprofloxacin reduces the metabolic activity of *K pneumoniae*. This difference in metabolic activity is detectable within 30 minutes indicating the antibiotics rapidly inhibit the metabolism of the bacteria.
We found good correlation between our measurements and standard assays with $r^2$ values of 0.81 and 0.82 for *E. coli* and *K. pneumoniae* respectively. Outliers were removed using a Modified Thompson Tau test.

**Optimization of in-line urine sample processing.** To test undiluted urine on chip, we devised a method to remove large particulates from urine while allowing bacteria to pass through the filter. We tested various pre-filter sizes to ensure that bacteria spiked in whole urine could be recovered. We spiked *E. coli* at $1 \times 10^2$ cfu/µL into whole urine and passed 100 µL of the urine through the pre-filters with various pore diameters. We plated the filtrate on agar plates and incubated the plates overnight at 37°C. We counted the number of bacterial colonies and found that using a 10 µm pre-filter, nearly 75% of bacteria could be recovered directly from whole urine.
Figure 7.2.13. Effect of pre-filter size on recovery of bacteria spiked in undiluted urine. *E. coli* can be efficiently recovered directly from urine using a pre-filter with a 10 μm pore diameter.
Electrochemical scans acquired on chip after introducing *E. coli* spiked in urine at 100 CFU/µL and subsequently introducing 1 mM resazurin and an antibiotic. Samples were incubated for 60 min at 37°C. *E. coli* were incubated with (A) 100 µg/mL ampicillin, (B) 1 µg/mL ampicillin, (C) 100 µg/mL ciprofloxacin, and (D) 1 µg/mL ciprofloxacin. In the presence of 1 µg/mL ampicillin, the magnitude of the signal is reduced as that concentration of ampicillin is insufficient to inhibit the metabolism of *E. coli*. Electrochemical scans were acquired on-chip with the on-chip Au reference electrode. Peaks are shifted to more negative potentials when compared to using the Ag/AgCl reference electrode.

7.2.1 References

7.3 Supporting Information for Chapter 4

**Calculation of mRNA diffusion.** To model the diffusion of analytes toward the sensor under varying lysis conditions, we created a model in COMSOL model consisting of single *E. coli* inside a well that contains a 20 µm sensor (Figure 7.3.1). Each *E. coli* contained multiple copies of rpoβ, a 4000 bp mRNA molecule with a diffusion constant of approximately $1.25 \times 10^{-12}$ m$^2$/s.$^1$ We assumed this mRNA had a copy number of 1400 as calculated previously.$^2$ At the moment of lysis ($t = 0$), we assumed all intracellular analyte molecules are released and that the flux of the analyte towards the sensor is due solely to diffusion. The diffusion profile in time was calculated using Fick’s Second Law:

$$\frac{dc}{dt} = D \nabla^2 C$$  \hspace{1cm} (1)

Where $C$ is the concentration profile and $D$ is the diffusion constant. The diffusional flux of molecules at the sensor surface was simulated using COMSOL and the results are plotted in Figure 4.1B.

To calculate the upper bound of the number of molecules accumulated at the sensor, $N$, we assumed any target analyte molecule that reaches the sensor will bind irreversibly and used the following equation:$^3$

$$N(t) = \int_0^t J(\tau) d\tau$$  \hspace{1cm} (2)

Where $J$ is the total flux (molecules s$^{-1}$) and $t$ is time (s).
Using the simulations of the diffusional flux in time, we used equation 2 to calculate the molecules accumulated at the sensor surface over time. The results are plotted in Figure 4.1C.

Figure 7.3.1: COMSOL model used for diffusion calculations consisting of a 20 micron hemispherical sensor at the bottom of a 120 µm wide and 25 µm deep well. An *E. coli* cell, 50 microns from the sensor, containing target mRNA is lysed. The normalized concentration at of target analytes 300 s after lysis plotted.

Figure 7.3.2: Fluorescent images of lysis in phosphate buffer. *E. coli* expressing GFP (green) uptake propidium iodide (red) after lysis in phosphate buffer with 20 V pulses.
Figure 7.3.3: Representative differential pulse voltammagrams (DPVs) from *E. coli* lysed on-chip. Solid lines indicate after hybridization and dashed lines indicate before hybridization.

Figure 7.3.4: Representative differential pulse voltammagrams (DPVs) from *E. coli* lysed off-chip. Solid lines indicate after hybridization and dashed lines indicate before hybridization.
Figure 7.3.5: Representative differential pulse voltammograms (DPVs) of oxidation of Fe(CN)$_6^{4-}$ at a probe-modified electrode before and after applying on-chip potential pulses in various buffers. Increases in magnitude of the oxidation current after applying potential pulses indicates probe is removed. Chips were washed in PBS before scanning and all scans are performed in 2.5 mM Fe(CN)$_6^{4-}$ and 0.1x PBS. Solid line indicates after applying a potential. A dashed line indicates before applying a potential.

Figure 7.3.6: Cyclic voltammetry of a bare NME electrode in 50 mM H$_2$SO$_4$. 
7.3.1 References


7.4 Supporting Information for Chapter 5

**Figure 7.4.1: Effect of electroless deposition.** Electrodes were immersed in 30% H$_2$O$_2$ before and after dipping in a platinum solution for 25 minutes (no potential was applied). Bubbles do not form after 10 minutes in either case, indicating there is no appreciable electroless deposition.

**Figure 7.4.2: Setup used for electrochemical sensing.** The NME acts as the working electrode and the Au mesh readout electrode acts as the counter electrode. A platinum wire serves as an electronic bridge between the two solutions.
Figure 7.4.3: Effect of hydrogen peroxide concentration on bubble growth. We measured the effect of hydrogen peroxide concentration on bubble growth after 2 minutes in peroxide solution for various applied currents. By tuning the peroxide concentration it is possible to control the rate of bubble growth. When 3% peroxide is used, no bubbles form after applying a 1 nA deposition current.
<table>
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**Table 7.4.1: Parameters used to calculate time to visual appearance.** Parameters used in calculating the time required to induce a visible change using various methods.
Appendix B – Publications


