Microfluidic Point-of-care Diagnostics for Global Health

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Mechanical and Industrial Engineering
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Abstract

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In this thesis, microfluidic diagnostic technologies are developed for sample collection, sample processing, and detection and analysis for disease diagnosis, from initial conception to field validation in Vietnam. The most impactful contributions of this thesis consist of a total analysis system called the lab-in-a-pen for hepatitis B screening (Chapter 3), and strategies for advancing the diagnostic capabilities of paper-based assays (Chapters 4 and 5). The lab-in-a-pen offers comparable diagnostic performance as conventional testing in an easy-to-use format, with potential to transform the current rapid test market. An electrokinetic phenomenon, called ion concentration polarization (ICP), is leveraged to improve the detection sensitivity of paper-based assays, up to 40-fold improvement in signal intensity of a fluorescent tracer and 5-fold improvement in the detection limit for a model protein. The ICP approach is further developed for direct DNA analysis in paper, to detect hepatitis B virus DNA without prior viral load amplification and to clinically assess human sperm DNA integrity for informing male fertility potential. Clinical outcome using the ICP approach was 100% in agreement with that of the conventional method. Chapters 6 and 7 summarize my earlier work on developing modular units for sample collection and processing, as envisioned by Grand Challenges Canada. These units provide capabilities to transport and process milliliter sample volumes, enabling downstream multiplexed analysis. Microfluidic diagnostic technologies show promise for enabling low cost and scalable point-of-care diagnostics.
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Figure 7.5: Comparison between the white blood cell counts of the plasma filtered with the devices and separated using a centrifuge. The inset shows the white blood cell counts of the whole blood prior to filtration. The device filtered out an average of 96.9% of the white blood cells.

Figure 7.6: Comparison of the biochemistry testing results between plasma derived from the devices and the centrifuge for the four biomarkers, (a) ALT, (b) AST, (c) urea, and (d) creatinine. Reference ranges for the centrifuge are shown by the dashed lines, and reference ranges for the devices – scaled to accommodate adsorption levels averaged over all samples - are shown by dot-dashed lines (scaled 63% for ALT, 87% for AST, 94% for urea, and 28% for creatinine). Urea and creatinine have two dashed lines and two dot-dashed lines to represent the lower and upper values of their reference range and scaled range, respectively.

Figure 8.1: Paper-based assay for human sperm analysis. a Different layers of the device: reservoirs in layer 1, reagents and porous membrane in layer 2, and a lamination cover in layer 3. b Imaging process with representative images of pristine and tested devices. c Reaction used to measure sperm concentration. d Setup used to measure sperm motility. MTT is a tetrazole dye which is metabolized by live sperm, producing insoluble formazan.

Figure 8.2: ICP-based assessment of human sperm DNA integrity. ICP simultaneously concentrates and separates single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). The dye employed causes ssDNA to fluoresce red and dsDNA to fluoresce green.
Chapter 1

Forward

1.1 Motivation

Disease is a global issue, affecting millions of people worldwide. Disease burden can be measured by the Disability-Adjusted Life Year (DALY) [1], which is the number of years lost due to ill-health, disability, or early death. Specifically, it is the sum of years lost as a result of living with the health condition and the years lost due to premature death, as shown in Figure 1.1a. Infectious diseases, such as the human immunodeficiency virus (HIV), are among the top ten leading causes of global DALYs and account for ~17% of the total global DALY (Figure 1.1b). Since the beginning of the HIV epidemic in the 1980s, 78 million people have been infected and 39 million people have died from the disease [3]. Similarly, chronic hepatitis B affects more than 240 million people worldwide, with an estimated 780,000 deaths per year [4]. Disease prevalence and burden tend to be highest in developing countries [5]. Access to conventional healthcare (i.e. centralized hospitals and laboratory testing facilities) is limited in these areas, critically impeding the dissemination of relevant diagnoses and treatments to target populations. Therefore, increasing access to relevant healthcare can significantly reduce disease burden in developing countries, and in remote areas of developed countries such as Canada [6].

Barriers to healthcare access are generally associated with the high cost of infrastructure and equipment, and skilled personnel required to perform testing. Drawing on lessons from the recent Ebola virus epidemic in West Africa, Bill Gates identified a number of factors that resulted in the slow response to the epidemic, including the lack of relevant laboratory equipment and shortage of trained personnel [7]. The detection method of choice was quantitative polymerase chain reaction, which required expensive laboratory equipment and skilled technicians not widely available in the affected regions [7]. Gates strongly emphasized the need for low cost, field-deployable, and scalable diagnostic tools that can be used for both normal and epidemic circumstances. This call for better diagnostics is not exclusive to Ebola, but applies to all disease burdens.
Figure 1.1: Global disease burden and the Disability-Adjusted Life Year (DALY). a Expression for DALY and its quantitative measurement. Image by Radio89, distributed under the Creative Commons Attribution-Share Alike 3.0 Unported license. b DALY percentages of the top 10 diseases contributing to global DALYs. Infectious diseases account for ~17% of the total global DALYs (highlighted in pink). Reproduced with permission from the National Institute of Mental Health, US.
Microfluidic technologies show promise for accessible and scalable testing of infectious diseases [8], [9]. These small-scale fluidic systems offer exceptional capability in sample processing and analysis at lower cost and material requirements than their laboratory counterparts [10], [11]. Many of these systems have been employed for immunosensing [12], [13] and nucleic acid testing [14], [15] of highly prevalent diseases such as HIV, hepatitis, tuberculosis, and malaria. One major caveat of these technologies, however, is that they tend not to translate out of academic laboratories. Recently, there has been a shift in the culture of microfluidic technology development, positioning the end user at the centre of development. Specifically, the development of diagnostic technologies for the developing world has been guided by the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users) principle introduced by the World Health Organization [5].

In keeping with the ASSURED principle, current themes for developing microfluidic diagnostic technologies include using low cost, alternative substrate materials (e.g. paper, thread, thermoplastics) [16]–[19], leveraging equipment-free fluid transport (e.g. capillary flow) [20]–[23], integrating multiple processes into enabling formats [11], and exploiting well-established consumer technology (e.g. mobile phones and smartphones [24]). Recent work in these areas will be provided in detail in Chapter 2. The ultimate goal of these efforts is to provide simple yet effective healthcare at the point of care.

1.2 Thesis overview

This thesis describes the development of diagnostic technologies for infectious disease detection in resource-limited settings, leveraging ‘paper-based’ microfluidic formats. Here, ‘paper-based’ is an encompassing term to include formats that use paper or porous membranes as a functional component (e.g. chromatography paper, nitrocellulose membrane, plastic membranes). The work is largely motivated by a collaboration with the National Hospital for Tropical Diseases (NHTD) in Hanoi, Vietnam and funding from Grand Challenges Canada.

Chapter 2 provides a summary of the relevant concepts used in this work. It begins with a commentary on the challenges associated with microfluidic technology development. A critical review of pertinent ‘paper-based’ microfluidic technologies is then presented, highlighting recent advances in the field.
The following Chapters 3-7 are not organized chronologically. Rather, they are organized as below to best communicate the core of the thesis contributions. Specifically, Chapters 6 and 7 were earlier work. Chapter 3 describes the development of a lab-in-a-pen testing system for hepatitis B virus screening. Finger-prick based sample collection, blood filtration, and analyte detection via paper-based test strips are completely integrated into an easy-to-use pen format. Patient testing with devices was completed at NHTD. This work was published in *Lab on a Chip* [25].

Chapter 4 presents a method to enable post-wetting analyte concentration and transport in paper-based assays, by leveraging an electrokinetic phenomenon called ion concentration polarization. This work was published in *Analytical Chemistry* [26] and highlighted in ‘Our choice from the recent literature’, *Nature Nanotechnology* 9, 660 (2014).

Chapter 5 extends on the work in Chapter 4, demonstrating direct and simultaneous DNA preconcentration, separation, and detection in nitrocellulose membrane. The approach is applied to hepatitis B virus DNA detection and sperm DNA integrity assessment. This work has been submitted for publication to *Nature Nanotechnology*, NNANO-15061197.

Chapter 6 describes the development of a hand-powered sample collection and transport module with an expandable membrane pump and patient-to-chip interface. This work was published in *Biomicrofluidics* [27].

Chapter 7 describes the development of a capillary flow-driven membrane-based blood filtration device for large sample volumes, producing purified plasma suitable for multiplexed analysis. Devices were tested with patient samples at NHTD. This work was published in *Biomicrofluidics* [28].

Finally, Chapter 8 summarizes the diagnostic technologies developed in this thesis and provides a discussion of future work.
Chapter 2

Introduction

2.1 Microfluidic technology development

Microfluidic technologies have shown exceptional potential for a number of biomedical applications, as biosensors for diagnostics [10], [29]–[32] to organotypic models for tissue engineering [33]–[38]. In the context of diagnostics, the successful development and commercialization of microfluidic technologies depends on several groups, namely the device developers, the end users, and potential companies that will manufacture and distribute the product. Each group has distinct and often conflicting expectations of the target technology, as shown in Figure 2.1. Developers, for the most part, are university academics and are driven by technical innovation [39]. End users, on the other hand, are less concerned about the intricacies of a device. They just want it to work, while being inexpensive, simple, and safe to use. Moreover, the overarching agenda of companies is to make profit. The primary challenge, then, is to create a microfluidic device that satisfies all three parties (i.e. one that hits the ‘sweet spot’ indicated by the star in Figure 2.1). A core strategy for success is to put the user at the center of development; that is, engage the user at every stage of the process from conception to prototyping to field testing.

From a technical perspective, simple and familiar device designs can expedite user uptake. Simplicity is advantageous in relation to ease of use and enabling untrained end users to perform self-testing. Glucose testing for diabetes and home pregnancy tests are examples of successful commercial technologies that enable untrained end users to perform diagnostic testing. Both technologies use procedures that do not require extensive training to perform (i.e. finger prick and urine collection), and provide simple, easy-to-interpret readout either via a digital reader or colorimetric signals in a paper-based assay. Additionally, familiarity (in both design and operating procedure) reduces the time required to achieve proficiency by the end user. In the context of manufacturing, familiarity enables companies to leverage existing processes to produce devices at scale and reduce the device’s time-to-market. For example, the coupling of microfluidic technology to mobile phones [24], [40] leverages a well-established consumer market, potentially expediting uptake of such coupled technologies.
Figure 2.1: Expectations of microfluidic diagnostic technologies by the developer, end user, and company. The challenge is creating a product that meets the expectations of all three groups; that is, hitting the ‘sweet spot’ (i.e. the star).

Safety is another key factor to be considered, especially in the context of field use. Contamination from biological samples needs to be minimized. Once a sample is collected, it needs to be well-contained within the device to reduce the risk of exposure to the end user. If sharps are used to collect the sample, a suitable containment method needs to be employed [25], [27]. Integration of device components can simultaneously address issues with safety, portability, and complexity (i.e. required number of processing steps) [25], [41], [42].

Cost is perhaps the defining factor in technology development. From the perspective of the developer, material and operating costs need to be minimized so that the final device is affordable to the end user. This is typically achieved by using low cost materials (e.g. paper, thread, and plastic), process integration, minimizing reliance on external equipment, and leveraging consumer technology. Low cost, however, does not necessarily appeal to companies because a device that costs less than 1 USD may not be profitable. Therefore, strategies that fit the agendas of both the developers and companies need to be explored.

Clinical validation with patient samples is also critical to translating prototypes to a final product. Access to patients is often limited, which is why most microfluidic diagnostic technologies do not pass the proof-of-concept stage. Drawing on lessons from my research [25], [28], there can be drastic differences in spiked samples used for proof-of-concept and real patient samples (e.g. varying viscosities of blood samples due to disease state or lifestyle). Therefore, collaborating with clinicians is essential to the successful development of diagnostic technologies.
2.2 Paper-based microfluidics

Paper as an analytical testing platform dates back to the 1940s – 1950s with paper chromatography [43], paper electrophoresis [44], and diagnostic dipsticks for diabetes [45]. The ubiquitous lateral flow assay (e.g. home pregnancy test) first emerged in the 1980s [46] and has remained the ‘gold standard’ for rapid testing. Patterning of paper with hydrophobic materials can be traced back to the early 1900s [47]. However, the modern paper-based microfluidic format was first popularized by Whitesides’ group in 2007, which was termed the microfluidic paper-based analytical device or µPAD [48]. Since then, there has been tremendous effort in developing paper-based microfluidic diagnostic technologies [49]–[51]. Several factors motivate the surge in paper-based applications: (1) paper is inexpensive and ubiquitous; (2) paper is compatible with biological samples; (3) paper can be easily modified and manipulated; (4) paper can be safely disposed of by incineration, and (5) paper is scalable [49]–[51]. The following sections summarize fluid transport in paper-based assays, advances in flow control and analyte manipulation, strategies for sample preparation and processing in paper, paper-based total analysis systems, and the role of consumer technology in paper-based microfluidics.

2.3 Fluid transport in paper-based assays

Fluid transport in paper is a passive process, enabled by capillary action. Capillary action or wicking within the paper matrix is a result of the interplay between cohesive and adhesive forces. Cohesive forces, such as surface tension, arise due to intermolecular attraction between fluid molecules at the liquid-air interface, while adhesive forces result from intermolecular attraction at the liquid-fiber interface. The surface properties of the paper matrix dictate adhesion and consequently, the degree of wetting. Modification of the surface can either increase or decrease wetting as indicated by the contact angle at the liquid-fiber interface.

Fluid transport in paper can be modeled using theoretical concepts for transport phenomena in porous media: the Lucas-Washburn equation and Darcy’s law. The Lucas-Washburn equation was originally derived to describe one-dimensional capillary flow in a parallel bundle of cylindrical tubes [52], and can be extended to fluid transport in porous media such as paper [53] (Figure 2.2). The equation relates wetted length to wicking time and is given as:

$$L = \frac{\sqrt{D \cos \theta}}{4 \mu} \sqrt{t}$$  \hspace{1cm} (1)
where $L$ is the distance travelled by the fluid front, $\gamma$ is the liquid-air surface tension, $D$ is the average pore diameter in the paper, $\theta$ is the liquid-fiber contact angle, $\mu$ is fluid viscosity, and $t$ is time. Equation (1) assumes constant cross-sectional area, uniform pores, no impurities within the paper matrix, unlimited reservoir volume, and no effect on wicking from patterned hydrophobic channel walls. Any violation of these conditions can result in deviations between experimental observations and the analytical expression. Recently, Elizalde et al. [54] developed and experimentally validated a modified form of equation (1) that addresses transport in paper with non-uniform cross-sectional area. Their work provides a method to predict flow behavior in arbitrarily shaped paper-based assays, extending the predictive power of the traditional Lucas-Washburn equation. Similarly, Hong and Kim [55] analytically and experimentally characterized a modified form of equation (1) that includes the effects of wax boundaries on fluid transport. They demonstrated that hydrophobic wax boundaries in patterned paper decrease the rate of wicking as compared to unpatterned paper.

Darcy’s law is a phenomenologically derived equation that describes flow through porous media [56]. It is used to characterize wicking rate (flow rate) in paper [53] and expressed as:

$$Q = -\frac{KW}{\mu L} \Delta P \quad (2)$$

where $Q$ is the volumetric flow rate, $\kappa$ is the permeability of the paper, $WH$ is the cross-sectional area perpendicular to flow, $\mu$ is fluid viscosity, $L$ is the length of the paper, and $\Delta P$ is the pressure difference along the length of the paper. For a paper-based system with n-connected sections of varying geometry, the volumetric flow rate through the fluidic circuit can be modeled using an electrical circuit analogy (Figure 2.3):
\[ Q = -\frac{\Delta P}{\sum_i \frac{\mu L_i}{\kappa W_i H_i}} \quad \leftrightarrow \quad I = \frac{V}{\sum_i R_i} \] (3)

where \( Q \) is the volumetric flow rate which is equivalent to electric current \( I \), \( \frac{\mu L_i}{\kappa W_i H_i} \) is the fluidic resistance of the \( i \)th section (a function of fluid/paper properties and geometry) which is equivalent to Ohmic resistance \( R \), and \( \Delta P \) is the pressure difference across the fluidic circuit which is equivalent to electric potential difference \( V \). In keeping with the analogy, the properties of series and parallel electrical circuits can be applied to their equivalent fluidic circuits. For example, flow rate in a series fluidic circuit is the same along the each section (i.e. \( Q = Q_1 = Q_2 = \ldots = Q_n \)), where the equivalent fluidic resistance is equal to \( R_1 + R_2 + \ldots + R_n \).

Figure 2.3: Electrical circuit analogy (Ohm’s law) for fluid transport in a paper-based fluidic circuit. The volumetric flow rate in each section is equal to the total pressure difference over the fluidic resistance of the section. The equivalent resistance for both circuits is the sum of all the resistances in series.
2.4 Flow control in paper-based assays

Flow control is essential to fluidic processes. Enabling advanced fluid transport in paper-based assays has the potential to broaden their application. This section summarizes current strategies for coordinating and timing the delivery of samples and reagents, and strategies for controlled analyte manipulation (i.e. concentration, mixing, and separation).

2.4.1 Timing and programmable fluid delivery

A number of timing strategies have been demonstrated in the literature. The most direct method is to control capillary flow in the paper matrix (based on the theoretical concepts described in section 2.3), which has also been employed in channel-based microfluidics [20], [41], [57], [58]. Yager and colleagues demonstrated timed and sequential delivery of sample and reagents in two-dimensional (2D) paper-based networks by using legs of different lengths (i.e. $t \propto L^2$ from the Lucas-Washburn equation in section 2.3) [53], [59], [60]. By networking multiple legs, the authors achieved multistep processing analogous to the sequential pipetting/washing steps required for conventional immunoassays (e.g. enzyme-linked immunosorbent assay or ELISA), without the use of external benchtop equipment. This timing method has since been incorporated in their work on developing ‘automated’ paper-based assays for disease diagnosis [61]–[63].

Chemical and physical modifications of the paper matrix have also been leveraged for timing the delivery of fluids. Timing via chemical modification generally involves using additives to change the permeability of the paper matrix. Lutz et al. introduced soluble sugar barriers in paper as time delays [64]. Sugars are typically non-reactive and thus, unlikely to affect assay chemistries. The length of the time delay can be programmed by changing the concentration of the applied sugar solution. The authors successfully applied this concept in a paper-based assay for detecting malaria. Similarly, Noh and Phillips used paraffin wax to meter samples in a three-dimensional (3D) µPAD [65]. By patterning varying amounts of wax in a metering layer (i.e. changing the available wetting area of the layer), the authors were able to modulate flow to a downstream detection region. They applied their approach for the time-based detection of biomarkers, where the metering layer was used as a timer to signal assay completion [66], [67]. Other groups have also demonstrated controlled alteration of paper permeability by patterning wax of varying colours [68] and brightness [69], both of which correlate to the amount of wax embedded in the paper matrix. Chemical barriers have also served as bridges, connecting paper-based channels [70], [71]. These bridges act as shut-off valves, stopping flow once they are
eroded. The time required to erode the bridges can be programmed by changing its composition or by changing its mass. In terms of physical barriers, Toley et al. employed cellulose absorbent pads to increase the total fluidic resistance in a paper-based channel [72]. Pads were placed in direct contact with the channel, creating a parallel flow path and delaying fluid delivery to its target region. The length and thickness of the pads were changed to produce tunable flow rates. The authors recently extended this initial work to include expandable and movable pads for fluid switching and rerouting capabilities [73].

Strategies that utilize external actuation for modulating fluid transport in paper have also been demonstrated. The simplest actuation scheme involves the manual movement of paper structures within an assay; for example, a paper cantilever that is pushed by the operator to connect adjacent channels [74]. Shin et al. programmed the delivery of fluids by constricting flow in manually pressed channels [75]. A unique method for ELISA was demonstrated by Liu et al., where the multistep ELISA process was completed by sliding a paper-based assay through a series of reagent and buffer zones [76]. The strategies described here are simple and require no external instrumentation, however, constant operator engagement may increase human error. A more sophisticated timing valve based on a ferromagnetic paper cantilever has been realized [77]. The opening/closing of the cantilever is controlled by an external electromagnet, which is triggered by an ionic resistor embedded in a timing channel within the assay. Although more precise than manually controlling fluid delivery, the additional electromagnetic components may undermine the simplicity of the paper-based assay.
Figure 2.4: Flow control strategies in paper-based assays. 

a) A malaria diagnostic assay employing legs of different lengths for the sequential and automated delivery of reagents. Adapted with permission from [62]. Copyright 2012 American Chemical Society.

b) A valving toolkit with expandable paper-based components for on/off switching and fluid rerouting. Adapted from [73] with permission from The Royal Society of Chemistry.

c) Paper-based channels connected with an erodible bridge as a shut-off valve. Adapted from [71] with permission from The Royal Society of Chemistry.

d) A diagnostic device for semi-quantitation of enzymes using a fluidic timer. Adapted with permission from [67]. Copyright 2013 American Chemical Society.

e) A cantilever switch actuated by timed electromagnetic actuation. Adapted from [77] with permission from The Royal Society of Chemistry.
2.4.2 Analyte concentration and transport

Despite the many advantages of paper-based assays, poor detection sensitivity remains an issue. A commonly used approach for enhancing detection sensitivity is to amplify the colorimetric signal intensity of labeled targets in paper test strips. Fu et al. employed a commercially available gold enhancement solution in which gold nanoparticles accumulate on gold nanoparticle-conjugated targets, increasing overall nanoparticle density and subsequently, the color intensity [78]. They achieved 6-fold improvement in signal intensity [78] and 4-fold improvement in the limit of detection (LOD) of bovine serum albumin [61]. In a similar manner, Hu et al. improved the colorimetric signal intensity of their assay by leveraging dense gold nanoparticle aggregates conjugated with probes to capture target DNA sequences [79]. Other methods for analyte concentration include the use of volatile solvents and aqueous two phase flow. In the work by Yu and White, target analytes were concentrated in a lateral flow assay by the evaporative wicking of a solvent, achieving 24-fold improvement in the signal readout [80]. Chiu et al. demonstrated the accumulation of target biomarkers in the leading salt-rich portion of their two phase solution, which was further concentrated by the flow of a lagging polyethylene glycol-rich portion [81]. Using this approach, they achieved a 10-fold improvement in the LOD of the protein transferrin.

The strategies described above demonstrate advanced manipulation of analytes, beyond the current capabilities of conventional lateral flow assays. However, they rely on capillary action generated in the remaining dry portions of the paper matrix. Once a paper-based assay is saturated, further analyte concentration or transport is generally not possible. To achieve post-wetting manipulation, the most relevant available techniques include the application of external (acoustic, thermal, and electric) fields. Surface acoustic waves (SAW) have been applied to the mixing of fluids in paper [82]. The SAW mechanism involves atomizing fluid at the end of a Y-channel via acoustic signals generated by a transducer. Atomization creates a negative pressure gradient in the channel, drawing and uniformly mixing fluid at the same time. In comparing their approach with capillary flow-based mixing, the authors observed consistently higher mixing indices for their SAW method. Wong et al. recently demonstrated that analyte concentration can be achieved by continuously evaporating fluid from the end of a wetted paper-based assay via localized heating [83]. Using this approach, the authors were able to concentrate a tuberculosis biomarker, lipoarabinomannan, up to 20-fold without compromising its detectability.

As aforementioned, the application of electric fields in paper dates back to the 1950s with paper electrophoresis [44]. Electrokinetic transport in paper-based microfluidic assays is more
recent, and presents an opportunity to translate advanced capabilities to these assays for broad application. Moghadam et al. leveraged isotachophoresis (*i.e.* the concentration and separation of ionic compounds based on an ion mobility gradient) to concentrate ionic analytes in lateral flow assays up to 900-fold [84]. They also demonstrated two orders of magnitude improvement in the detection limit of a model analyte (immunoglobulin G) with analytical sensitivity comparable to laboratory-based ELISA [85]. Around the same time, Rosenfeld and Bercovici developed a similar isotachophoretic approach to concentrate analytes in paper-based assays, achieving up to 1000-fold signal amplification [86]. Electrophoretic separation has also been translated to the paper-based microfluidic format [87]–[89]. Another electrokinetic phenomenon that has been used in paper is ion concentration polarization (ICP) [26], [90]. ICP occurs at the interface of ion-selective nanochannels and microchannels, where an applied electric field causes the formation of ion depletion and enrichment zones [91], [92]. These ICP effects have been leveraged for water desalination [93], [94], and biomolecular concentration [95], [96] and separation [97], [98] in channel-based microfluidic devices. Gong et al. demonstrated the first application of ICP in paper-based assays for concentration and directional transport of target analytes [26] (detailed in Chapter 4). They recently extended their approach to DNA analysis for detecting hepatitis B virus DNA and assessing sperm DNA integrity (detailed in Chapter 5). The ICP approach is particularly attractive for several reasons: (1) ion-selective nanomaterial can be readily patterned into paper, enabling nanofluidic transport; and (2) ICP offers simultaneous concentration and separation capabilities without the requirement of additional electrolytes.

A potential caveat of employing electric fields in paper is the requirement of an external high voltage source, which can undermine the simplicity of paper-based assays. Portable battery-based high voltage supplies are available [99] and have been employed for paper-based electrokinetic systems [84]. To mitigate the requirement of high voltage, Luo et al. developed an origami-based electrophoretic assay [89]. High electric field strength was achieved with low voltages (10 V) by folding the paper-based assay into a thin stack, effectively reducing the distance between electrodes. Moreover, Joule heating from applied electric current can be detrimental to paper-based electrokinetic systems, where uncontrolled fluid evaporation dries the paper matrix and hinders analyte transport. This effect was observed by Gong et al. during successive cycles of transporting analytes back and forth in their paper-based ICP system [26]. Evaporation from Joule heating was mitigated by placing a glass cover slip on the sample region, preventing analyte loss. In a similar manner, Rosenfeld and Bercovici directly insulated the
bottom side of their isotachophoretic assay with patterned wax and the top side with tape, minimizing the evaporative surface area of the assay [86]. Moghadam et al. employed a different strategy, whereby the main sample channel in their isotachophoretic assay was continuously replenished with fluid from adjacent reservoirs [84]. In summary, the application of electrokinetic phenomena in paper-based assays has advanced their diagnostic capability and analytical sensitivity for broad application, without compromising the inherent simplicity of the assays.

Figure 2.5: Analyte concentration and transport in paper-based assays. a A two-phase solution strategy for concentrating analytes in the salt rich portion. Adapted from [81] with permission from The Royal Society of Chemistry. b Evaporative concentration of analytes by continuously generating a dry region of the paper matrix with a heater. Adapted with permission from [83]. Copyright 2014 American Chemical Society. c Isotachophoretic concentration of analytes. Adapted from [86] with permission from The Royal Society of Chemistry. d Analyte concentration and directional transport enabled by nanoporous membranes and ion concentration polarization. Adapted with permission from [26]. Copyright 2014 American Chemical Society.

2.5 Sample preparation in paper-based assays

A typical diagnostic testing protocol consists of sample collection, sample preparation, analyte detection, and data analysis. Conventional sample preparation can involve multiple processing
steps, potentially taking several hours to complete. Laboratory equipment that are routinely used for processing samples, such as centrifuges, are costly and rely on infrastructure that may not be readily available in developing regions. In the context of blood sample preparation, plasma separation is often the first step prior to downstream analysis. Subsequent preparation can include lysing of the cellular components for nucleic acid purification or direct purification from separated plasma. The sections below provide examples of paper-based assays for plasma separation and nucleic acid purification.

There are two main categories of paper-based plasma separation: (1) chemical-based separation using reagents to induce red blood cell aggregation \[100\]–\[104\] and (2) porous membrane-based separation leveraging size exclusion of blood cells \[28\], \[105\]–\[108\]. For the former approach, Yang et al. devised an all-paper device embedded with agglutination antibodies (anti-A, B) \[102\]. Upon addition of a whole blood sample, red blood cells aggregate and are immobilized within the paper matrix, while plasma wicks into surrounding detection zones. Similarly, Ge et al. used anti-D antibodies to agglutinate red blood cells for plasma separation in their origami-based immunoassay \[101\]. These agglutination methods demonstrate effective plasma separation comparable to conventional centrifugation, however, the specific antibodies employed may not be applicable to all human blood types. Moreover, each agglutination-based assay would potentially require multiple antibodies to be dried within the paper matrix to accommodate a range of samples, increasing the overall cost of the assay. A more universal approach to red blood cell aggregation has been demonstrated by leveraging salt solutions. Nilghaz and Shen developed a salt functionalized separation device that generates an osmotic pressure gradient upon addition of whole blood, which subsequently induces red blood cell crenation and aggregation \[104\].

As an example of membrane-based plasma separation, Songjaroen et al. developed a membrane-paper hybrid assay consisting of a porous filtration membrane connected in a lateral flow configuration with chromatography paper \[105\]. The assay was strategically patterned with wax such that the sample zone resided in the filtration membrane and the detection zones in the paper. Based on size exclusion, blood cells are trapped in the membrane with plasma transported to the detection zones. Vertical flow membrane-based filtration has also been demonstrated in 3D paper-based assays. In the 3D paper-based assay developed by Vella et al., a filtration membrane was stacked on top of a detection layer, covering three separate detection zones \[106\]. Successful detection of target biomarkers in all three zones was achieved via application of a single 15 µL
finger prick-based blood sample, demonstrating the multiplexing capability of the vertical flow approach. This approach has been translated to the clinic [109]. In comparison to the lateral flow format, this approach also offers the advantages of faster reaction times due to shorter transport distances and smaller material footprint as a result of stackable fluidic layers. Several groups have also used filtration membranes within plastic microfluidic devices [28], [107], [108]. Gong et al. demonstrated milliliter-scale whole blood filtration using a large area membrane placed on top of a network of capillary flow-driven channels [28] (detailed in Chapter 7). The device was able to produce up to 350 µL of purified plasma per 1 mL of whole blood, suitable for multiple biochemical and immunological tests. Devices were field tested in Vietnam showing comparable performance with centrifugation. The membrane-based approach is robust and versatile. It does not necessitate the use of additional reagents as in the chemical-based methods, and membranes can be readily integrated into both paper-based and traditional channel-based microfluidic systems.

Conventional nucleic acid (DNA/RNA) analysis techniques, such as polymerase chain reaction (PCR), are considered the gold standard in infectious disease diagnosis, providing much lower LODs than serological-based tests at the onset of infection. Nucleic acid sample preparation, however, is both resource- and time-intensive. The development of convenient and practical methods for nucleic acid extraction and purification in paper has significant implications for point-of-care molecular diagnostics. Govindarajan et al. demonstrated an origami-based approach to extract and purify pathogenic bacterial DNA [110]. Although low cost and suitable for processing raw samples without complex instrumentation, the overall process requires the operator to complete ten manual steps, potentially increasing the risk of human error. Membranes, such as the Fusion 5 membrane developed by GE for lateral flow formats, have been integrated in a plastic device for DNA extraction and amplification [111]. Fluid handling for the device still relied on laboratory syringe pumps, and the amplification process required a conventional thermal cycler. Recently, Byrnes et al. devised a strategy to purify and concentrate DNA in one processing step using chitosan embedded in a lateral flow assay [112]. Below pH 6.3, the primary amine groups on chitosan become protonated, suitable for capturing negatively charged target DNA. When target DNA flows through the chitosan embedded region, they are immobilized and concentrated (up to 13-fold here). DNA is subsequently eluted for downstream processing by increasing the pH to above 6.3, at which the charge on chitosan returns to neutral. This work is
particularly exciting because the approach greatly simplifies the sample preparation needed for DNA analysis and demonstrates potential to be directly integrated in existing lateral flow assays.

![Figure 2.6: Sample preparation in paper-based assays.](image)

2.6 Paper-based total analysis systems

The ‘holiest grail’ for microfluidic diagnostic technology is a fully-integrated system that combines sample collection, sample preparation, analyte detection, and analysis; that is, a microfluidic total
analysis system (µTAS) [11]. This section summarizes progress towards the development of paper-based total analysis systems (paperTAS).

Process integration offers the advantages of reduced material and operating costs, reduced complexity in device operation, and improved scalability. Gong et al. developed a paperTAS for hepatitis B virus testing in the format of a pen, inspired by the ubiquitous ball-point pen [25]. The pen-based device was divided into two compartments: one compartment housed the sample collection mechanism (a standard lancet embedded in tubing for finger prick) and the other contained a paper-based platform comprised of a collection pad, filtration membrane for plasma separation, and lateral flow test strip for biomarker detection. Here, the familiarity of the pen format was leveraged to improve ease of use and scalability. Li et al. demonstrated a similar concept in a paperTAS for measuring glucose and cholesterol [113]. The device consisted of a finger press-activated microneedle for sample collection, and paper-based components for plasma separation and analyte detection. Downstream analysis in the device was automated upon initial activation of the microneedle, simplifying its overall operation. Recently, a ‘paper machine’ for the molecular diagnosis of E. coli DNA was developed by Connelly et al. [114]. The ‘paper machine’ is a magnetic device with a sliding magnetic strip containing paper discs for sample processing and analyte detection. Operation of the device consists of sliding the magnetic strip through the magnetic device, stopping at designated regions for sample preparation, isothermal DNA amplification, and endpoint detection via a camera phone. More recently, Rodriguez et al. developed a sample-to-answer molecular diagnostic paperTAS for the detection of Influenza A virus RNA [115]. The system achieved a clinically relevant LOD of $10^6$ copies/mL (10-fold improvement over existing rapid diagnostic tests) without the use of laboratory instrumentation. Viral RNA extraction and purification from patient samples, in situ isothermal amplification, and endpoint detection were all completed in paper-based components.
Figure 2.7: Paper-based total analysis systems. a A scalable pen-based device for hepatitis B testing with integrated finger prick-based sample collection, whole blood filtration, and endpoint detection via a paper-based test strip. [25] - Adapted by permission of The Royal Society of Chemistry. b A finger press-activated microneedle for sample collection integrated with on-chip sample collection, filtration, and colorimetric detection of blood glucose and cholesterol. Adapted from [113] with permission from The Royal Society of Chemistry. c A ‘paper machine’ for molecular diagnostics of pathogenic bacterial (E. coli) DNA. DNA sample preparation, isothermal amplification, and endpoint fluorescence detection are all contained within the device. Readout is quantified by a handheld UV source and camera phone. Adapted with permission from [114]. Copyright 2015 American Chemical Society.

An exciting advancement in the development of µTAS has been the coupling of these technologies to handheld consumer products, namely mobile phones (e.g. camera phones and smartphones) [24], [40], [116]–[119]. Mobile phone technology is particularly attractive as analytical systems owing to their advanced computing and imaging power, vast market base, and familiarity (to both the end user and manufacturer). They are quickly becoming a quintessential component for point-of-care diagnostics. In the context of coupling paper-based assays with mobile phones, the simplest approach is to leverage the imaging capabilities of the phone for signal acquisition [120], [121]. Captured images can be processed with the phone, on a separate computer, or delivered to a central database (telemedicine). A more integrated strategy includes leveraging accessories that attach to the phone for quantitation of signals in paper-based assays [122]–[126]. Commercial lateral flow test readers for iPhones (Detekt Biomedical LLC) and Android phones (Holomic LLC) are also available. Lee et al. developed an iPhone accessory with
a test strip and an accompanying application for measuring serum vitamin D levels [126]. Colorimetric analysis of vitamin D concentrations on the test strip produced results equivalent to conventional ELISA, demonstrating the applicability of the system for sensitive self-diagnosis of micronutrient levels. In addition to colorimetric analysis, quantitative fluorescence imaging on mobile phones has also been developed [127] and is commercially available (HRDR-300 Fluorescent Reader, Holomic LLC), enabling analytical measurements in paper-based assays that rival benchtop fluorescence microscopes. Thom et al. developed a paper-based assay with an integrated fluidic battery to power an on-chip fluorescent assay for detecting and measuring β-D-galactosidase [127]. The assay was coupled to a smartphone with a fluorescence imaging adapter, allowing measurement of β-D-galactosidase concentrations down to 700 pM.

Other handheld devices (e.g. glucose meters [128]) and wearable devices (e.g. Google Glass [129]) have also been adapted for paper-based assays. The Google Glass approach conceptualized by Feng et al. was used for both qualitative and quantitative measurement of HIV and prostate cancer biomarkers, respectively [129]. The overall system provided a hands-free means to detect target analytes, store information on a centralized server, and track the spatiotemporal pattern of tests in real-time. In the advancement of consumer technologies for paper-based (and traditional channel-based) microfluidics, developers need to keep in mind possible limitations of these technologies. For instance, the availability of higher end smartphones can vary from country to country, potentially limiting the computing and imaging capabilities required for diagnostics. The application of these technologies also needs to be appropriate; that is, mobile phones may be more suited to low-to-mid volume testing that does not require consistent use of battery power. Nonetheless, the coupling of (paper-based) microfluidics with consumer technology has exciting potential to transform the diagnostics landscape in the developing (and developed) world.
Figure 2.8: Paper-based microfluidics coupled with consumer technology. **a** A diagnostic accessory containing a test strip that can be attached to an iPhone to measure serum vitamin D levels. A dedicated application on the phone quantifies image captured results. Adapted from [126] with permission from The Royal Society of Chemistry. **b** Quantitative fluorescence measurement and analysis of β-D-galactosidase in a self-powered paper-based assay using a smartphone adapter. Adapted from [127] with permission from The Royal Society of Chemistry. **c** Google Glass employed as a lateral flow test reader, enabling quantitation and real-time spatiotemporal mapping of possible infections. This is an unofficial adaptation of an article that appeared in an ACS publication [129] (http://pubs.acs.org/doi/abs/10.1021/nn500614k). ACS has not endorsed the content of this adaptation or the context of its use.

2.7 Summary

This chapter provided an overview of topics relevant to the work presented in this thesis. Specifically, paper-based microfluidic technologies are well-positioned to provide inexpensive, sensitive, and scalable diagnostic testing in both developing and developed countries. Technical advances in all areas of paper-based diagnostic testing (sample collection, sample preparation, and analysis) have been demonstrated and extend the capabilities of these assays for broad application. There are now a number of advanced fluid handling strategies available for precise and controlled operation of assays. The sensitivity of paper-based assays is also being
significantly improved. Sample preparation, which is typically the bottleneck of diagnostic testing, may no longer be a barrier as innovative solutions continue to be developed. Perhaps, the most exciting aspect going forward is the realization of paper-based total analysis systems, and the ongoing integration with consumer technology.
Chapter 3

Lab-in-a-pen: a diagnostics format familiar to patients for low-resource settings

This Chapter was published in the journal *Lab on a Chip* [25] and reproduced by permission of The Royal Society of Chemistry. The Candidate was the first author for this work and played a primary role in the design of experiments, execution of experiments, data collection and analysis, and writing of the manuscript. Additional authors include: Dr. Brendan D. MacDonald, Dr. Trung Vu Nguyen, Dr. Kinh Van Nguyen, and Prof. David Sinton. Their efforts were integral to the publication of this work and are gratefully recognized and appreciated.

3.1 Introduction

The World Health Organization’s ASSURED principles (i.e. Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users) [5] are guidelines for developing microfluidic point-of-care diagnostic devices for low-resource settings. Many devices have shown promise for point-of-care testing [9], [10], [12], [49], [50], with application to specific bioanalyses including whole blood processing [27], [28], [42], [102], [105], [130], [131], biochemistry [16], [106], [109], [128], [132], [133], immunochemistry [31], [41], [76], [134], [135], and nucleic acid analysis [110], [136], [137]. However, most devices are still costly and complex, making them less practical for low-resource settings.

Simplicity is advantageous in diagnostic devices particularly in relation to ease of use and enabling untrained end users to perform self-diagnosis. Glucose self-tests for diabetes and home pregnancy tests are examples of successful commercial technologies that enable untrained end users to perform diagnostic testing. Both technologies incorporate procedures that do not require extensive training to perform (i.e. finger prick and urine collection), and provide simple, easy-to-interpret readout either via a digital reader or via colorimetric signals in a paper-based strip. Currently, there is a disconnect between microfluidic device output and user uptake because most devices are not ‘simple and cheap and invisible’ [39], as preferred by end users.

Safety (i.e. biological sample and sharps containment), process integration, and equipment-free operation are also key factors that influence the adoption of a technology. Biological sample and sharps containment reduces the risk of harmful exposure to the user and
environment. Integration of processes (i.e. sample collection and processing, and analyte detection) reduces the number of procedures and time required to run a test. Equipment-free operation ensures that devices are portable and can operate on a standalone basis.

Cost is a major consideration in the context of microfluidic devices for low-resource settings. Devices need to be low cost (~1 USD or less per test) and amenable to mass manufacturing to be deliverable to end users on a large scale. The irregular and bulky format of most microfluidic devices is not conducive for mass manufacturing. The challenge remains to develop devices in formats that can (1) leverage established low cost manufacturing processes [39], [138] and (2) incorporate existing low cost commercial assays; both aspects leading to greatly reduced cost and potential for scale-up. In the context of patient-administered drug delivery, the pen has been a very successful format (e.g. auto-injectors for treatment of anaphylaxis (EpiPen) and fertility (piOna)). Pen-based lancing devices (e.g. ACCU-CHEK® Multiclix lancing device) are also commercially available and widely used for self-diagnosis.

In this paper, we present a low cost, simple and integrated device for paper-based medical diagnostics in low-resource settings called the lab-in-a-pen. Finger pricking, sample collection and processing, and analyte detection are integrated into a pen format – one of the cheapest mass manufactured mechanical products. This approach ensures safety and is simple enough to be used by untrained end users across multiple settings. The pen format is amenable to existing manufacturing infrastructure. We characterize sample wicking in the lab-in-a-pen using porcine whole blood. The clinical diagnostic utility and usability of the lab-in-a-pen is demonstrated by direct testing of patients for Hepatitis B surface antigen (HBsAg) and Hepatitis B ‘e’ antigen (HBeAg) by medical staff at the National Hospital for Tropical Diseases in Hanoi, Vietnam.

3.2 Experimental

3.2.1 Device design and fabrication

The lab-in-a-pen is a plastic- and paper-based device with integrated sample collection, whole blood filtration, and multiplexed diagnostics. The components of the lab-in-a-pen are illustrated in Figure 3.1a and an assembled device is shown in Figure 3.1b. The current form of the lab-in-a-pen is a proof-of-concept design that mimics the ubiquitous retractable pen already manufactured at scale. A standard lancet (Bayer Microlet) was stripped of its original plastic casing and secured into tubing with epoxy, forming the lancing mechanism. Prior to use, the lancet was sterilized with 70% ethanol and sealed with its original cap. Housing for the device
components was cut from transparent polycarbonate tubing (1800 mm x 13 mm x 13 mm, McMaster Carr, Aurora, OH, USA), with each housing measuring 100 mm in length. Brackets, a divider, and the diagnostic cartridge were cut to fit inside the housing from sheets of 1.5 mm thick poly(methyl methacrylate) (PMMA) (Plastic World, Toronto, ON, Canada) using a CO₂ laser (Universal Laser Systems Inc., Scottsdale, AZ, USA). The collection pad (Cellulose Absorbent Pad, Pall Corporation, East Hills, NY, USA), plasma membrane (GR VIVID™ Plasma Separation Membrane, Pall Corporation, East Hills, NY, USA), and paper assay (Grade 1 Chr, Whatman – Part of GE Healthcare, Piscataway, NJ, USA) were cut to size and attached onto the diagnostic cartridge using Scotch double-sided tape and lamination (Figure 3.1c). The diagnostic cartridge is designed to be interchangeable, where a single or multiple paper assays or commercial rapid tests can be incorporated.

The lab-in-a-pen was assembled by first securing the brackets and divider into the housing with permanent double-sided tape. A spring was then placed between the brackets followed by the lancing mechanism, as shown in Figure 3.1c. The divider minimizes the risk of physical damage to the diagnostic cartridge during finger prick with the lancing mechanism. It also allows for parts to be interchanged, if so desired. The diagnostic cartridge was attached to the housing with permanent double-sided tape. Finally, caps were used to seal the ends of the housing.

The general operation of the lab-in-a-pen consists of removing the end cap from the lancing end of the device, removing the cap from the lancet, sterilizing the patient’s finger for pricking, deployment of the lancet by a quick press of the button on top of the lancing mechanism, formation of a blood drop (~30 µL) on the patient’s finger, and collection of the blood drop into the device via contact with the collection pad. Importantly, finger prick collection is used as opposed to venous draw collection since it requires less skill and equipment, and enables full containment of the sample within a single device.
Figure 3.1: Lab-in-a-pen. (a) Schematic of the lab-in-a-pen in exploded view. A standard lancet (Bayer Microlet) is integrated into the pen tubing and used for finger prick. The brackets are used to guide the lancing mechanism in a straight path when it is deployed by pushing the cover at the top of the tubing. The divider separates the lancing mechanism from the diagnostic cartridge, which minimizes the risk of physical damage to the collection pad, plasma membrane, and paper assay during a finger prick. It also allows for interchangeability of lancing and diagnostic components, if so desired. The end caps ensure that the components of the device are properly contained before and after use. (b) Images of assembled lab-in-a-pen. The diagnostic cartridge is outlined by the dashed box on the “Diagnostic side.” (c) Enlarged view of the lancing mechanism and diagnostic cartridge.

3.2.2 Analysis of wicking in the paper-based test platform

The configuration and size of the collection pad and plasma membrane affects the wicking behaviour in the paper assay. It is crucial to determine the configuration and size that provides sufficient sample wicking for diagnostic tests. Three configurations were assessed: lateral, vertical-lateral, and vertical. To determine the best configuration, experiments were conducted to measure the distance travelled by filtered plasma in the paper assay and to observe potential red blood cell (RBC) leakage into the paper assay. For each experiment, the collection pad and plasma membrane volume capacities were fixed at 50 µL and 15 µL, respectively.

Sample wicking behaviour in the paper assay was also evaluated for different sizes (i.e. volume capacities) of the collection pad and plasma membrane. Specifically, the evaluation was conducted for collection pads with volume capacities of 30 µL and 50 µL for both low and high absorbencies, at plasma membrane volume capacities of 10, 15, 30, and 50 µL. For the above
experiments, 50 µL of undiluted, anticoagulated porcine whole blood (Lee Biosolutions, St. Louis, Missouri, USA) was used, the paper assay width was fixed at 5 mm, and images were taken with a desktop scanner (CanoScan 9000F, Canon Inc., Ōta, Tokyo, Japan) after 5 minutes of sample wicking.

The effect of blood hematocrit and initial sample volume on sample wicking in the paper assay was also characterized using porcine whole blood. Porcine whole blood was centrifuged, after which the blood cells and plasma were placed into different vials. Stock porcine whole blood with a hematocrit percentage of 80% was created using the separated cells and plasma. This stock was further diluted to hematocrit percentages of 10% to 70% for testing, since this percentage range represents physiological values found in human males and females [139]. For each hematocrit percentage, initial sample volumes of 20, 30, and 40 µL were investigated. These volumes represent finger prick volumes from the literature18 and measurements during finger prick-based testing at the National Hospital for Tropical Diseases. For each test, the width of the paper assay was fixed at 5 mm and the wetted assay length was measured after 5 minutes of sample wicking.

3.2.3 Patient testing at the National Hospital for Tropical Diseases
To demonstrate the diagnostic utility and usability of the lab-in-a-pen, devices were used for direct testing of patients at the National Hospital for Tropical Diseases in Hanoi, Vietnam. Commercial rapid tests for the detection of HBsAg and HBeAg were incorporated into the devices. The results from the lab-in-a-pen testing were compared to the results of conventional immunology testing for each patient. All tested patients provided written consent prior to testing and the work was performed by a nurse following regulatory testing standards (i.e. ISO 2001:2008 and ISO 15189:2007). Patients and medical staff also provided relevant feedback on the suitability of the lab-in-a-pen as a diagnostic device via patient-, nurse-, and doctor-specific surveys.

For testing, the nurse first sterilized the patient’s finger then pricked the finger using the lancing mechanism in the device. After ~30 µL of sample formed on the patient’s finger, the nurse collected the sample using the collection pad in the device. Test results were read and recorded ~10 minutes from the onset of wicking in the commercial rapid tests.
3.3 Results and Discussion

3.3.1 Comparison with existing methods

Figure 3.2 illustrates the features of existing rapid test methods as compared to the lab-in-a-pen. Most rapid tests are rated for use with whole blood; however, plasma or serum is the preferred sample type by users. Without an on-strip mechanism to filter whole blood, blood cells can reduce the sensitivity of the test. For instance, at the National Hospital for Tropical Diseases, the procedure involves: venous draw into a tube, centrifugation, removal of plasma with a pipette, and deposition on the test strip – all requiring trained staff and hardware available only in the hospital. In contrast, the integrated plasma membrane in the lab-in-a-pen filters out the blood cells and provides plasma for downstream detection. In addition, the integrated lancing mechanism and collection pad ensures that patient samples are safely collected and well-contained within the device, without the necessity of an external pipetting tool (and associated handling). Furthermore, the lab-in-a-pen efficiently utilizes a single sample volume for parallel testing (~50 µL for three test strips in Figure 3.2), whereas each commercial test strip can only detect one biomarker and requires ~50 µL of sample for each strip. The reduction in required sample volume for the lab-in-a-pen test format is enabled by the smaller dimensions of the test strips (~1.5 mm in width per test strip) used in the diagnostic cartridge and by integration of the sample collection and blood filtration processes. Control lines are shown for both methods with HBsAg, HBeAg, and Hepatitis B surface antibody (HBsAb) in Figure 3.2.
Figure 3.2: Comparison with existing methods. The current protocol requires venous draw, centrifugation, and transfer of plasma to the test strip. In contrast, the lab-in-a-pen testing does not require any additional sample collection or processing. Sample and sharps are well-contained in the device. The time-to-result for both commercial and lab-in-a-pen testing is ~10 min. Centrifuged porcine plasma was used to test the commercial rapid tests (top image), and porcine whole blood was used to test the diagnostic cartridge (bottom image). Test regions are outlined by the red dashed box and labelled “T”, and control regions by the grey dashed box and labelled “C”. Both the commercial and lab-in-a-pen tests indicate control lines for HBsAg, HBeAg, and HBsAb tests.

3.3.2 Analysis of wicking in the paper-based test platform

Wicking behaviour in the paper assay can be tailored by changing the configuration and size of the collection pad and plasma membrane. The configuration also affects the quality of filtered plasma wicked into the paper assay. Comparing the three setups in Figure 3.3a, there is no substantial difference between the distance travelled by the plasma in the paper assay for all three setups (as sectioned by the dotted lines). However, RBC leakage occurs for the vertical setup, which can affect the signal readout in the paper assay. The lateral and vertical-lateral setups both provide filtered plasma without RBC leakage. Although there was no observable RBC leakage for the vertical-lateral setup, the direct contact of sample to the lip of the plasma membrane can potentially lead to wicking under the membrane into the paper assay. This risk of blood cell contamination in the paper assay is minimized for the lateral setup, which was selected for subsequent characterization of sample wicking in the lab-in-a-pen and for patient testing.

In addition, the size of the collection pad and plasma membrane affects the wicking
behaviour in the paper assay. In Figure 3.3b, it is observed that the distance travelled by the plasma is greater for plasma membranes with lower volume capacities, with an optimal capacity at ~15 µL. Similarly, sample wicking in the paper assay is considerably better for the lower volume capacity collection pad, 30 µL as compared to 50 µL. In regards to absorbency, there is no substantial difference in the wetted assay length for low and high absorbency collection pads: ~11 mm for a 30 µL, low absorbency collection pad and ~13 mm for a 30 µL, high absorbency collection pad, at a plasma membrane capacity of 15 µL. The selection of the collection pad depends on the desired application. For low sample volumes obtained from finger pricks, a low absorbency collection pad is more conducive since low retention of collected sample maximizes plasma output via the plasma membrane for downstream analyte detection. In summary, the results in Figure 3.3b indicate that a low volume capacity collection pad in combination with a low volume capacity plasma membrane provides the optimal sample wicking behaviour in the paper assay for the integrated lab-in-a-pen device.

From Figure 3.3b, the wetted assay length of ~11 mm at an assay width of 5 mm provides wetted surface area sufficient for >10 colorimetric test spots (i.e. given that a colorimetric test spot is ~4 mm² in area [106]). This number of tests is adequate for a standard Hepatitis B panel consisting of three to four immunology tests and three or more biochemistry tests [140]. The capacity to pattern a number of different tests onto the paper assay enables the testing of multiple diseases per patient per device, improving information output for medical staff.

Sample wicking in the paper assay is also affected by the properties of whole blood, especially blood viscosity, and by the initial sample volume. Blood viscosity is related to blood hematocrit, which is the volume percentage of red blood cells in a total sample volume. The normal range for blood hematocrit is suggested as 40%–54% for males and 37%–47% for females [139]. However, the clinically relevant range for blood hematocrit is 10% to 70% [139], which can represent patients with anemia to patients with dehydration. In Figure 3.4, the wetted assay length decreases linearly as the hematocrit increases from 10% to 70%, but increases as more sample volume is available initially. For an initial sample volume of 20 µL at a hematocrit of 70%, the wetted assay length is ~5 mm for an assay width of 5 mm. For this extreme case of hematocrit, it is still possible to pattern multiple colorimetric test spots of 4 mm² given the wetted surface area.
Figure 3.3: Configuring and sizing the collection pad and plasma membrane based on sample wicking in the lab-in-a-pen. (a) Schematics of different setups for the collection pad and plasma membrane: lateral, vertical-lateral, and vertical. Corresponding experimental images for each setup are indicated by arrows, with the collection pad and plasma membrane volume capacities fixed at 50 µL and 15 µL, respectively. (b) Plot showing wetted assay length as a function of plasma membrane volume capacity for low (solid line) and high (dashed line) absorbency collection pads of volume capacities 30 µL (diamonds) and 50 µL (squares). The width of the paper assay was fixed at 5 mm for each test. Porcine whole blood was used for each test, 50 µL per test. Images were taken after 5 minutes of sample wicking.
3.3.3 Patient testing at the National Hospital for Tropical Diseases

Figure 3.5a shows patient testing with the lab-in-a-pen at the National Hospital for Tropical Diseases in Hanoi, Vietnam. The main objective of the testing was to evaluate the usability of the device and its social relevance in a low-resource setting. Direct survey feedback from patients and medical staff at the hospital provided the necessary input for both the usability and social relevance of the device. Specifically, patients were asked to comment on the level of pain experienced during finger prick (increasing from 1 to 10), their level of trust in the results from the device (increasing from 1 to 10), and a suitable time-to-result for the device (time in minutes). The nurse that performed the testing was asked to comment on the ease of use of the device (increasing from 1 to 10), the level of safety of the device (increasing from 1 to 10), the sufficiency of sample volume during collection (~30 µL?), and a suitable time-to-result for the device (time in minutes). On average, patients ranked their level of pain at 3 (i.e. low pain), level of trust at 8 (i.e. high trust), and desired time-to-result at 15 min. Most patients articulated their preference for the lab-in-a-pen testing as opposed to conventional venous draw-based testing in terms of pain. They also specified that they would readily use the device for home-based testing if it was currently available on the market. The nurse ranked ease of use at 8 (i.e. high ease of use), level
of safety at 8 (i.e. high safety), sufficiency of sample volume at ‘most of the time’, and desired
time-to-result at 15 min. The nurse (and doctors) additionally commented that the lab-in-a-pen
would be an asset for home-based practices, which is prevalent in Vietnam. In general, patients
and medical staff were receptive and enthusiastic about the lab-in-a-pen technology and eager to
provide input in furthering its development. The low cost, simple and integrated format of the
device provides a viable alternative for disease diagnosis both inside and outside of hospitals.

Another objective of the testing was to assess the diagnostic utility of the lab-in-a-pen. A
series of lab-in-a-pen devices were used for patient testing for HBsAg and HBeAg. Finger prick
with the lancing mechanism, sample collection, and whole blood filtration worked well for all
tested devices. Colorimetric signal readout in the commercial test strips was available for 7 tests;
an example of a valid test is shown in Figure 3.5b. Out of these tests, 5 patients were positive for
HBsAg but negative for HBeAg and 2 patients were negative for both HBsAg and HBeAg. These
results matched with the results of conventional immunology testing for each patient, as expected
of commercial tests (i.e. quality controlled tests). From the same batch of tests, 22 devices had no
signal readout due to poor pad contact between the plasma membrane and test strip, resulting in
insufficient plasma flow to the test strip. This mechanical issue was readily fixed. A separate batch
of (n=25) diagnostic cartridges were tested in the laboratory with porcine whole blood and showed
sufficient plasma flow for all 25 cartridges, demonstrating that the pad contact issue was resolved.
Further refinement of the current design and assembly of the device, especially in the context of
commercialization, can address manufacturing consistency for reliable plasma output.

Collectively, the positive feedback from the patients and medical staff, and the patient testing
results establish the suitability of the lab-in-a-pen as a diagnostic device for low-resource settings.

Lastly, in terms of cost, retractable pens are manufactured with a bulk (n > 1000) cost of
~0.10 USD per pen. The addition of the collection pad, plasma membrane, and paper-based assay
at bulk volumes is estimated to increase this base cost by a factor of ~3, indicating a total cost of
~0.40 USD per device. This estimated cost is well within the goal of 1 USD per test.
Figure 3.5: Patient testing at the National Hospital for Tropical Diseases in Hanoi, Vietnam. (a) A nurse using the lab-in-a-pen on a patient for Hepatitis B diagnosis. The patient’s finger was pricked followed by blood collection into the device (inset). (b) Results of a lab-in-a-pen test demonstrating complete sample wicking into the diagnostic cartridge. The tested patient is negative for HBeAg and positive for HBsAg, as shown by the outlined test and control lines.

3.4 Conclusions

In this paper, we developed a low cost, simple and integrated device for medical diagnostics in low-resource settings called the lab-in-a-pen. Sample wicking in the lab-in-a-pen was characterized using porcine whole blood, demonstrating that the sample collection and processing components can be tailored to produce different wicking rates. Also, the potential to pattern multiple test spots on the paper assay translates to multiplexed diagnostics on a single device. In-field patient testing with the lab-in-a-pen at the National Hospital for Tropical Diseases in Hanoi, Vietnam established the proof-of-concept of the device for point-of-care testing. Positive feedback from patients and medical staff at the hospital confirmed the social relevance of the device. Low cost and simple microfluidic diagnostic devices have the potential to transform the current rapid test market in low-resource settings.
Chapter 4

Nanoporous membranes enable concentration and transport in fully wet paper-based assays

This Chapter was published in the journal *Analytical Chemistry* and reprinted with permission from [26]. Copyright 2014 American Chemical Society. The work was highlighted in ‘Our choice from the recent literature’, *Nature Nanotechnology* 9, 660 (2014). The Candidate was the first author for this work and played a primary role in the design of experiments, execution of experiments, data collection and analysis, and writing of the manuscript. Additional authors include: Mr. Pei Zhang, Dr. Brendan D. MacDonald, and Prof. David Sinton. Their efforts were integral to the publication of this work and are gratefully recognized and appreciated.

4.1 Introduction

Paper has been used as a platform for many micro- and nanoscale applications including paper-based electronics [141]–[145], plasmonics [146]–[149], and microfluidics [25], [49], [50], [132], [150]. Several factors motivate the recent surge in paper applications: (1) abundance and low cost, (2) compatibility with biological samples, (3) ease of fabrication and modification, and (4) ease of disposability [49], [50], [132]. In the context of paper-based microfluidic assays, the lateral flow assay is a well-established and historic format for routine point-of-care diagnostics [50]. Commercial lateral flow assays are available for testing a wide spectrum of biomarkers, requiring minimal expertise and equipment. Perhaps, the most widely used lateral flow assay is the home pregnancy test, enabling end users to self-test for pregnancy at their own discretion. Fluid transport (and associated analyte transport) in paper-based microfluidic assays occurs via passive capillary action and depends on the physical properties of paper (*i.e.* surface area, thickness, porosity, and pore size) [50], [151]. Flow control in paper-based assays is critical for enhancing detection sensitivity via analyte concentration, and for coordinating and timing the delivery of samples or reagents.

Existing strategies for analyte concentration during paper wetting consist of chemical amplification using gold nanoparticles [61], [78], [79], molecular amplification using nucleic acid circuits [152], and lateral flow concentration leveraging the evaporation of a volatile solvent [80]. Strategies for controlled analyte transport during wetting include: varying the geometry and
dimensions of paper reservoirs and channels [53], [59]; actuating paper cantilevers to connect channels [74], [77]; sliding a paper test strip through a stationary reagent loading unit [76]; and adding soluble physical barriers to act as time delays [64], [65], [72] or as shut-off valves [70], [71]. These approaches have demonstrated enhanced flow control, however, they rely on capillary forces generated in remaining dry portions of the paper matrix.

Once a paper-based assay is saturated, further analyte concentration or transport is generally not possible. To achieve post-wetting manipulation, the most relevant available techniques are electrokinetic in nature. Electrophoretic separations have been demonstrated in fully wet paper [44]. Droplet movement has also been demonstrated with electrowetting on a dielectric paper surface [153]. Concentration and directional transport of analytes using electrokinetic phenomena have yet to be demonstrated. Ion concentration polarization (ICP) is another promising approach for analyte manipulation within the electrokinetic toolbox. ICP occurs at the interface of microfluidic and ion selective nanofluidic channels under an applied electric field [91]. An ion depletion region forms at the interface, which repels charged particles [154]. ICP has been used extensively for analyte concentration [95], [96], [155]–[158] and separation [97], and water desalination [93], [94] in conventional microfluidic channels. ICP induced pH changes at the micro-nano interface has also been studied in detail [159]. This phenomenon has yet to be leveraged in alternative microfluidic formats, such as paper, for analyte concentration and controlled analyte transport. The potential for patterning nanoporous membranes within microporous paper presents an opportunity to build ICP functionality into paper-based assays.

Here, we demonstrate active concentration and transport of analytes in fully wet paper-based assays by leveraging nanoporous material (mean pore diameter ≈ 4 nm) [160] and ion concentration polarization. Two classes of devices are developed – an external stamp-like device with the nanoporous material separate from the paper-based assay, and an in-paper device patterned with the nanoporous material. Experimental results demonstrate up to 40-fold concentration of a fluorescent tracer in fully wet paper, and directional transport of the tracer over centimeters with efficiencies up to 96%. In a diagnostic application, in-paper devices are used to concentrate fluorescein isothiocyanate conjugate bovine serum albumin (FITC-albumin) and bromocresol green dye, extending their limits of detection from ~10 pmol/mL to ~2 pmol/mL and from ~40 µM to ~10 µM, respectively.
4.2 Experimental Section

4.2.1 Device classification
Two classes of devices are developed – an external stamp-like device with the nanoporous membranes separate from the paper-based assay, and an in-paper device. Nanoporous membranes are embedded into a silicone rubber layer in the external devices to serve as the membrane-paper interface for inducing ICP. Nanoporous membranes are directly patterned into the in-paper devices, serving two important functions: (1) they act as hydrophobic barriers, much like wax, to define fluidic reservoirs and channels, and (2) they form the micro-nano interface necessary for ICP. The utility of the nanoporous membranes patterned into paper is similar to that of the external devices, where the presence of paper fibers does not compromise membrane function (i.e. cation selective transport through the nanoporous membranes is maintained in both cases).

4.2.2 Fabrication and operation of the external devices
Schematics for the external concentration and transport devices are shown in Figure 4.1a and 4.1b, with the central ICP phenomenon depicted in Figure 4.1c. The fabrication and assembly procedures for the external devices are detailed below and also in Figure 4.8 in Supporting Information. The geometry for the external devices was first drawn in AutoCAD and then cut into the relevant layers using a CO2 laser printer (Universal Laser Systems Inc., AZ, USA). The Nafion nanoporous membrane layer was fabricated by cutting through 0.5 mm thick silicone rubber (McMaster Carr, OH, USA) with laser writing power of 50% and speed of 2.0%, producing ~0.3 mm wide through-cuts after 15 passes. Through-cuts for buffer reservoirs were micromachined into 3.0 mm thick poly(methyl methacrylate) (PMMA) (Plastic World, Toronto, Canada) with laser writing power of 100% and speed of 1.0% after one pass, having an enlarged geometry of the through-cuts in the silicone layer (Figure 4.1a and 4.1b). Through-holes for bolts were also micromachined into the silicone and PMMA layers for device assembly. After these cutting steps, the prepared silicone was elevated onto rulers and a pipette was used to fill the through-cuts with 20 µL of Nafion 117 solution – 5% in a mixture of lower aliphatic alcohols (Sigma Aldrich, MO, USA), followed by heating in an oven for 30 min. at 65 °C. The silicone layer was then placed in a Petri dish and immersed in 0.25x Tris-acetate-EDTA buffer (TAE buffer, 10 mM Tris-acetate and 0.25 mM EDTA at pH 8.2) for 30 min. to hydrate the hardened Nafion. The silicone and PMMA layers were assembled together using bolts and nuts to form a complete device.
To operate, the assembled external device is simply brought into contact with the paper-based assay, buffer reservoirs are filled with 0.25x TAE buffer, and the voltage applied, as shown in Figure 4.1 and Figure 4.8 in Supporting Information. Importantly, the silicone layer containing the embedded nanoporous membranes prevents direct mixing of the sample in the assay with buffer in the buffer reservoirs. Under an applied voltage, ions migrate within the assay to their respective counter-electrodes. Figure 4.1c depicts this phenomenon in the cross-section of the whole device. Cations in the assay migrate toward the negative electrode (cathode) and travel through the cation selective Nafion nanoporous membrane into a buffer reservoir, while anions (e.g. fluorescent tracer and all other negatively charged species in solution) migrate toward the positive electrode (anode) through the assay, stopping at the membrane-paper interface. The efflux of cations from the anodic side of the nanoporous membrane causes anions to also vacate the vicinity to conform to electrical neutrality, forming an ion depletion region. This ion depletion region pushes and concentrates anions toward the anode. Conversely, enrichment of anions occurs at the cathodic side of the nanoporous membrane due to the influx of cations. In the external concentration device, enrichment is leveraged at the inner membrane and ion depletion at the outer membrane. The geometry of the membranes is symmetric for the transport device, where direction is controlled by adjusting the polarity of the membranes. We envision the external devices to be used as portable sample preparation tools in the field or clinic where conventional laboratory equipment may not be readily available. For example, the external concentration device can be used to pre-concentrate sample in an assay in lieu of centrifugation and sample extraction. These devices can be used multiple times when properly sterilized after each use (i.e. contact surface is rinsed with deionized water, wiped dry, and then wiped with ethanol).
Figure 4.1: External devices for analyte concentration and transport. Schematics of the external (a) concentration and (b) transport devices. Nafion nanoporous membranes are embedded into the silicone layer to trigger ICP at the membrane-paper interface. (c) Cross-section of the devices and paper-based assays depicting ICP under an applied voltage. Ion depletion occurs at the anodic side of the nanoporous membrane to push analytes toward the anode.

4.2.3 Fabrication and operation of the in-paper devices

Schematics for the in-paper concentration and transport devices are shown in Figure 4.2a and 4.2b, with the ICP phenomenon depicted in Figure 4.2c. The in-paper devices are the two-dimensional analogues of the external devices, where nanoporous membranes are directly patterned into the paper. The patterned nanoporous membranes serve (1) to act as hydrophobic barriers, much like wax, to define fluidic reservoirs and channels, and (2) to create the micro-nano interfaces necessary for ICP. The in-paper devices were fabricated from 0.18 mm thick Whatman No.1 chromatography paper (Sigma Aldrich, MO, USA). A Xerox ColorQube™ 8570N color
printer (Xerox Canada, ON, Canada) was used to pattern the wax boundary, and the patterned paper was heated at 150°C for 2 min to allow penetration of the wax through the thickness of the paper. Nanoporous membranes were patterned into the paper by manually depositing Nafion 117 solution via a pipette, ~3 µL of Nafion per device. Devices were then soaked in 0.25x TAE buffer for 30 min to hydrate the membrane. Following membrane hydration, devices were air dried at room temperature, and re-hydrated on use. The morphology of hydrated Nafion has been well-characterized in the literature [160], where the mean pore diameter is ~4 nm, well-suited to inducing ICP phenomena. Importantly, the sulfonic acid groups in Nafion provide a net negative surface charge in the pores [160], enabling the selective transport of cations through the membrane during ICP. The analytes used here, as specified below, are negatively charged at their respective pH values and do not pass through the cation selective membranes patterned into the in-paper devices, but rather migrate within the paper matrix and concentrate at the membrane-paper interface when ICP is induced, as shown in Figure 4.2c.

Operation of these devices is the same as the external ones: a voltage is applied across the device at the buffer reservoirs (as depicted in Figure 4.2a and 4.2b) to trigger ion depletion in the sample region bounded by the nanoporous membranes (as depicted in Figure 4.2c). The in-paper approach offers advanced functionality in a disposable unit at low cost and potential for scale-up. The added cost of Nafion in paper is very low at ~0.015 USD per device, given a material cost of ~0.005 USD/µL (Sigma Aldrich, MO, USA). The total material cost per device, using No. 1 chromatography paper, is ~0.03 USD (a detailed breakdown of material costs and fabrication time requirements are provided in Table 4.1 and Table 4.2 in Supporting Information). We envision an extension in functionality of currently available lateral flow assays and paper-based colorimetric assays by integrating nanoporous membranes into these assays using the approach described above. Specifically, substantial improvement in the sensitivity of existing assays can be potentially achieved using our concentration method.
Figure 4.2: In-paper devices for analyte concentration and transport. Schematics of the in-paper (a) concentration and (b) transport configurations. Fluidic regions are defined by Nafion nanoporous membranes and wax patterned into the paper. (c) Cross-section of the in-paper devices illustrating ICP under an applied voltage. Ion depletion occurs at the anodic side of the nanoporous membrane to push analytes toward the anode.

4.2.4 Preparation of fluorescein, protein and dye solutions

Fluorescein solution was prepared by mixing fluorescein in 0.25x TAE buffer (10 mM Tris-acetate and 0.25 mM EDTA at pH 8.2). The solution was adjusted to a working pH of 9.0 using 1x NaOH solution. This adjustment was done to minimize loss in the emission intensity of fluorescein during experimentation, where intensity can drop for pH < 8 [161]. Tests were conducted at both pH 8.2 and 9.0 with no observable differences in the buffer capacity, as indicated by consistent fluorescein migration in both cases. Fluorescein isothiocyanate conjugate bovine serum albumin (FITC-albumin) was diluted with 0.25x TAE buffer into molar concentrations ranging from 2 pmol/mL to 30 pmol/mL (i.e. 2, 4, 8, 11, 15, and 30 pmol/mL). Bromocresol green dye was diluted with 0.25x TAE buffer into molar concentrations ranging from 10 µM to 100 µM (i.e. 10, 25, 50, and 100 µM). All reagents were obtained from Sigma Aldrich.
4.2.5 Data acquisition and analysis
An inverted fluorescence microscope (DMI 6000B, Leica Microsystems Inc., ON, Canada) was used to capture images of fluorescein concentration and transport, and FITC-albumin concentration. Image sequences were taken with a CCD camera (Orca AG, Hamamatsu, NJ, USA). Captured fluorescence images were background subtracted using a dark field image. A desktop scanner (CanoScan 9000F, Canon Canada Inc., ON, Canada) was used to image bromocresol green dye concentration. All captured images were processed in ImageJ and quantified using Excel and Mathematica.

4.3 Results and Discussion
4.3.1 Active concentration and transport using the external device
Experimental results for fluorescent tracer concentration using the external concentration device are shown in Figure 4.3. For the circular device geometry (overlaid in Figure 4.3a), an ion depletion region pushes the tracer radially inwards from the outer membrane, while enrichment occurs at the center, underneath the inner membrane. The diameters of the inner and outer membranes are fabricated as 0.12 mm and 8 mm, respectively. For an applied voltage of 50 V, a 15 µL sample of fluorescein solution is concentrated in a 10 mm by 10 mm paper sheet after 3 minutes (Movie 1 in Supporting Information). In Figure 4.3a, the diameter of the focused area is 1.2 mm from an initial diameter of 8 mm, which equates to a surface area ratio of 44-fold. Specifically, 44-fold provides an upper limit on the concentration factor possible with this geometry. The maximum peak concentration factor, in Figure 4.3b, is 40-fold (91% of the upper limit), as quantified by the ratio of final to initial peak fluorescence intensity.

Figure 4.3a shows several notable time points: (1) fluorescein enrichment at the center is first observed at \( t = 15 \) s, (2) concentration due to ion depletion becomes visible at \( t = 50 \) s (i.e. propagating wave from the outer membrane), (3) ion depletion and enrichment regions merge at \( t = 105 \) s (also specified in Figure 4.3b by the left dashed line), and (4) concentration reaches saturation at \( t = 155 \) s (also specified in Figure 4.3b by the right dashed line). For the intensity profiles in Figure 4.3c, outer peaks at \( t = 105 \) s denote the merging of the ion depletion and enrichment regions. These peaks become pronounced at the end of the concentration process at \( t = 155 \) s, resulting in a concentrated ring. Collectively, the results in Figure 4.3 demonstrate a substantial improvement in signal intensity after analyte concentration in fully wet paper.
Figure 4.3: Active concentration using the external device. (a) Contrast enhanced images of fluorescent tracer concentration under an applied voltage of 50 V. The location of the outer membrane is indicated by the dashed circle and the inner membrane by the dot. (b) Time evolution of peak concentration factor (i.e. ratio of final to initial peak fluorescence intensity). The maximum value reaches 40-fold at $t = 155$ s. Merging of the ion depletion and enrichment regions is indicated by the left dashed line at $t = 105$ s. The peak concentration factor starts to plateau at $t = 155$ s, as indicated by the right dashed line. (c) Intensity profiles at selected time points, as indicated. Scale bars are 2 mm.
Experimental results for fluorescent tracer transport using the external transport device are shown in Figure 4.4. For an applied voltage of 50 V, a 5 µL sample of fluorescein solution is transported multiple cycles in a 6 mm by 3 mm paper strip (Movie 2 in Supporting Information). The sample is initially concentrated at an average rate of 1.6 mm/min from the cathode to the anode, as shown in Figure 4.4a. Subsequent transport cycles in predetermined directions are achieved by simply switching the polarity of the membranes (i.e. switching the onset location of ion depletion), as shown in Figure 4.4b and 4.4c. Notably, the repeated transport is achieved over the ~6 mm distance (total of ~18 mm) with minimal loss. The full width at half-maximum (FWHM) values for the initial and two subsequent transport cycles are 0.38, 0.34, and 0.26 mm. Thus, from an initially focused sample, the peak is broadened as it translates through the paper and then refocused with negligible broadening and little net loss.

Transport efficiency is quantified by comparing the area under the curve (AUC) of the final state in each cycle to the AUC of the final state in the initial concentration step (i.e. comparison of AUC in state IV in Figure 4.4b and 4.4c to the AUC in state IV in Figure 4.4a). A transport efficiency of ~96% is observed for the first cycle, decreasing to ~88% after the second cycle. The average transport rate also decreases from 0.8 mm/min to 0.6 mm/min for the first and second cycles, respectively. These small reductions in transport efficiency and rate can be attributed to lowered ionic current (i.e. ion migration) as a result of fluid evaporation from the paper, as well as some inherent retention of tracer in the paper. Overall, the results in Figure 4.4 demonstrate the effective transport of analytes over centimeter distances with minimal loss, enabled by external Nafion nanoporous membranes.
4.3.2 Active concentration and transport using the in-paper device

Experimental results for fluorescent tracer concentration using the in-paper concentration device are shown in Figure 4.5. Analogous to the circular geometry of the external concentration device (Figure 4.1), the in-paper device has an outer semi-circular membrane enclosing the sample region.
(with a diameter of 8 mm). For an applied voltage of 50 V, a 15 µL sample of fluorescein solution is concentrated after 8.5 minutes (Movie 3 in Supporting Information). In Figure 4.5a, the area of the concentrated spot is 1.3 mm² compared to an initial area of 32 mm², giving a surface area ratio of 25-fold. The measured maximum peak concentration factor based on fluorescence intensity is 22-fold (i.e. ~88% of the upper limit), as shown in Figure 4.5b. Thus, concentration efficiency of both the in-paper and external approaches are high (~88% here as compared to ~91% for the external device). Together, the results in Figure 4.5 demonstrate the concentration of analytes using the in-paper approach, with substantial improvement in signal intensity. Notably, nanoporous membrane material patterned into paper can both define flow regions and enable ICP concentration.

A noteworthy alternative to the No. 1 paper employed here is nitrocellulose membrane, which is commonly used in commercial assays. For completeness, we patterned nanoporous membranes into nitrocellulose membrane to concentrate fluorescent tracer, resulting in a 16-fold concentration comparable to the 22-fold concentration achieved in No. 1 paper (full details are provided in Figure 4.9 in Supporting Information).
Figure 4.5: Active concentration using the in-paper device. (a) Contrast enhanced images of fluorescent tracer concentration under an applied voltage of 50 V. Patterned inner and outer nanoporous membranes are indicated by the rectangle and arc, respectively. Patterned wax boundaries are indicated by the dashed lines. (b) Time evolution of peak concentration factor. The maximum value reaches 22-fold at $t = 510s$. Merging of the depletion and enrichment regions is indicated by the dashed line at $t = 420s$. (c) Intensity profiles at selected time points, as indicated. Scale bars are 2 mm.
Experimental results for fluorescent tracer transport using the in-paper transport device are shown in Figure 4.6. For an applied voltage of 50 V, a 5 µL sample of fluorescein solution is transported multiple cycles in an 8 mm by 3 mm paper channel (Movie 4 in Supporting Information). The sample is initially concentrated at an average rate of 0.9 mm/min, as shown in Figure 4.6a. Following this step, the concentrated sample is transported multiple cycles by switching the polarity of the membranes, as shown in Figure 4.6b and 4.6c. This back and forth transport is achieved over the ~8mm distance (total of ~24 mm) with relatively little loss, albeit more than in the external device case. Specifically, the FWHM values for the initial and two subsequent transport cases are 0.79, 0.84, and 0.82 mm.

Transport efficiencies of ~88% and ~65% are observed for the first and second cycles, respectively (with transport efficiency as defined earlier). A key cause of the reduction in transport efficiency is fluorescent tracer left behind during each transport cycle, as indicated by the remaining peaks at the cathodic side in Figure 4.6b and 4.6c. We attribute these remaining peaks to evaporation, and specifically, sample stranded as a result of evaporation. To verify the role of evaporation here, a separate test was conducted with a glass cover slip to minimize fluid loss in the sample region. The results of the test yielded consistent transport efficiencies of ~90% and average transport rates of 1.1 mm/min for both the first and second cycles (Figure 4.10 in Supporting Information). Thus, the in-paper device can be run without a cover slip as demonstrated in Figure 4.6; however, if longer run times and/or higher transport efficiencies are required, a cover slip evaporation barrier is recommended. Collectively, the results in Figure 4.6 demonstrate the directional transport of analytes in paper, over centimeter distances, using patterned nanoporous membranes.
Figure 4.6: Active transport using the in-paper device. (a) Initial concentration of fluorescent tracer from the cathode (blue band, ‘-’) to the anode (red band, ‘+’) at a rate of 0.9 mm/min, under an applied voltage of 50 V. (b) The concentrated band is transported to the opposite side upon switching membrane polarities, at a rate of 0.8 mm/min and ~88% efficiency. (c) Membrane polarities are reversed again and the concentrated band is transported to the opposite side, at a rate of 0.6 mm/min and ~65% efficiency. Contrast enhanced images are shown for the transport process at indicated time points. Scale bars are 2 mm.
4.3.3 Protein/colored dye concentration using the in-paper concentration device

To demonstrate the diagnostic applicability of the devices developed here, the in-paper concentration device is used to concentrate protein \textit{(i.e.} FITC-albumin\textit{)} and dye \textit{(i.e.} bromocresol green dye\textit{)}. The geometry of the device is slightly modified such that wax boundaries are angled to provide better focusing in the sample region, as shown in Figure 4.7b. FITC-albumin is used as a model protein, enabling quantitative imaging at low concentrations, and the dye is a typical model of analytes in paper-based colorimetric assays as visualized with a desktop scanner.

The limit of detection (LOD) curves for FITC-albumin and bromocresol green dye before and after concentration are shown in Figure 4.7. The intensity value at each molar concentration is averaged over \(n = 3\) independent measurements with error bars representing one standard deviation. The LOD for each analyte is given as the molar concentration corresponding to the intensity value that is three standard deviations from the control intensity value. In Figure 4.7a, the LOD for FITC-albumin before concentration is \(\sim 10\) pmol/mL and \(\sim 2\) pmol/mL after concentration. Similarly in Figure 4.7b, the LOD for bromocresol green dye before concentration is \(\sim 40\) µM and \(\sim 10\) µM after concentration. These results demonstrate an extension of the limit of detection with implications for the growing field of paper-based (colorimetric) assays.

4.4 Conclusions

In conclusion, we demonstrated active concentration and transport of analytes in fully wet paper-based assays by leveraging nanoporous material and ion concentration polarization. This work is distinct from the rapidly growing literature on paper-based microfluidics in that it offers unprecedented analyte control, independent of wetting history and capillary forces. In the in-paper devices, this functionality was enabled by patterned nanoporous material that (1) acts as a hydrophobic barrier, much like wax, to define fluidic reservoirs and channels, and (2) creates the micro-nano interface necessary for ICP phenomena. Experimental results yielded up to 40-fold concentration and up to 96% transport efficiency of a fluorescent tracer. Directional transport of the tracer was achieved over centimeters with consistent peak shape and minimal loss. Most notably, the limits of detection for protein and colored dye were improved using the in-paper approach. Collectively, these results demonstrate a major advance in analyte concentration and manipulation for the growing field of low cost paper-based assays.
Figure 4.7: Concentration of a model protein and colored dye using the in-paper concentration device. (a) Limit of detection (LOD) curves for FITC-albumin before (dashed) and after (solid) concentration. The LOD before concentration is ~10 pmol/mL and ~2 pmol/mL after concentration. Device images are shown inset, with patterned membranes indicated by the arc and rectangle. (b) LOD curves for bromocresol dye before (dashed) and after (solid) concentration. The LOD before concentration is ~40 µM and ~10 µM after concentration. Device images are shown inset. Each intensity value is averaged over $n = 3$ independent measurements with error bars representing one standard deviation. Scale bars are 2 mm.
4.5 Supporting Information

4.5.1 Experimental

Figure 4.8 shows the fabrication and assembly procedures for the external devices and are detailed as follows: (1) cut defined geometry from AutoCAD into silicone and PMMA using a CO₂ laser printer, (2) elevate the silicone onto rulers and pipette Nafion solution into the through-cuts, followed by baking for 30 min. at 65 °C, (3) place the silicone in a Petri dish and hydrate in buffer for 30 min., (3) assemble silicone and PMMA layers using bolts and nuts, and (4) place assembled device on a paper-based assay, fill buffer reservoirs and apply voltage.

Figure 4.8: Fabrication and assembly procedures for the external devices.

4.5.2 Results and Discussion

Figure 4.9 shows the results for concentration of fluorescent tracer in nitrocellulose membrane patterned with nanoporous membranes. Under similar experimental conditions used for Whatman No. 1 paper (i.e. applied voltage of 50 V), fluorescent tracer is concentrated within the same timeframe of 8.5 minutes. The peak concentration factor reaches a maximum value of 16-fold as compared to 22-fold for the No. 1 paper. The concentration process, however, is comparatively faster as the concentration factor plateaus earlier in the nitrocellulose membrane. This difference can be attributed to the more uniform pore structure in nitrocellulose membranes and consequently, more uniform transport rates.
Figure 4.9: Active concentration in nitrocellulose patterned with nanoporous membranes. (a) Contrast enhanced images of fluorescent tracer concentration under an applied voltage of 50 V. Patterned inner and outer nanoporous membranes are indicated by the rectangle and arc, respectively. Patterned wax boundaries are indicated by the dashed lines. (b) Time evolution of peak concentration factor. The maximum value reaches 16-fold at $t = 210$ s. (c) Intensity profiles at selected time points, as indicated. Scale bars are 2 mm.
Figure 4.10 shows the results for the transport of fluorescent tracer using the in-paper device with a cover slip over the sample region. Fluid evaporation is minimized by the cover slip. By comparing the area under the curve (AUC) of the first and second cycles to the AUC of the initial concentration step, transport efficiencies of ~90% are quantified for both cycles. The total transport time for each cycle is 7.5 minutes which equates to a transport rate of 1.1 mm/min per cycle, over the ~8 mm distance.

Figure 4.10: Active transport using the in-paper device with a glass cover slip over the sample region. The final intensity profiles are plotted for the initial concentration step, and first and second cycles. Contrast enhanced images are shown for the transport process. Scale bars represent 1 mm.
Table 4.1 details the material costs for the external and in-paper devices. The total cost per device for all device classes is <0.50 USD, with the nitrocellulose membrane device at the highest cost of ~0.41 USD.

Table 4.1: Material cost of external and in-paper devices.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Product No.</th>
<th>Unit</th>
<th>Total cost (USD)</th>
<th>Unit cost (USD/cm²)</th>
<th>Device cost (USD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 chromatography paper (CHR)</td>
<td>Sigma Aldrich</td>
<td>WHA3001861</td>
<td>400 cm²/sheet 100 sheets/pk</td>
<td>55.40</td>
<td>0.001385</td>
<td>N/A</td>
</tr>
<tr>
<td>Nitrocellulose (NC) membrane</td>
<td>Sigma Aldrich</td>
<td>Z6709091</td>
<td>400 cm²/sheet 5 sheets/pk</td>
<td>160.60</td>
<td>0.0803</td>
<td>N/A</td>
</tr>
<tr>
<td>Nafion 117 solution</td>
<td>Sigma Aldrich</td>
<td>70160</td>
<td>25 mL</td>
<td>131.50</td>
<td>0.00526 (USD/µL)</td>
<td>0.0152</td>
</tr>
<tr>
<td>Solid ink, black</td>
<td>Xerox</td>
<td>108R00726</td>
<td>30 cm²/sheet ~1100 sheets/stick</td>
<td>84.99</td>
<td>0.002575</td>
<td>0.005562</td>
</tr>
<tr>
<td>Silicone rubber sheet</td>
<td>McMaster Carr</td>
<td>582TT21</td>
<td>900 cm²/sheet</td>
<td>14.77</td>
<td>0.016411</td>
<td>N/A</td>
</tr>
<tr>
<td>Acrylic sheet (PMMA)</td>
<td>Plastic World</td>
<td>AC3MM4x8</td>
<td>900 cm²/sheet</td>
<td>1.91</td>
<td>0.002122</td>
<td>0.028647</td>
</tr>
</tbody>
</table>

Total cost per device: 0.355396 0.02799 0.406782
Table 4.2 details the fabrication time required for the external and in-paper devices. The in-paper devices can be fabricated in <60 min. and the external device in 74 min.

Table 4.2: Fabrication time required for external and in-paper devices.

<table>
<thead>
<tr>
<th>Task</th>
<th>Time required (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>External</td>
</tr>
<tr>
<td>Wax printing</td>
<td>N/A</td>
</tr>
<tr>
<td>Wax melting</td>
<td>N/A</td>
</tr>
<tr>
<td>Nafion patterning</td>
<td>3</td>
</tr>
<tr>
<td>Nafion baking</td>
<td>30</td>
</tr>
<tr>
<td>Nafion hydration</td>
<td>30</td>
</tr>
<tr>
<td>Silicone micromachining</td>
<td>5</td>
</tr>
<tr>
<td>PMMA micromachining</td>
<td>5</td>
</tr>
<tr>
<td>Silicone to PMMA assembly</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total time required per</strong></td>
<td><strong>74</strong></td>
</tr>
</tbody>
</table>

N.B.: Wax printing and melting, and Nafion baking and hydration can be done in parallel for up to 40 devices for the 20 cm x 20 cm No. 1 paper and NC membrane sheets used.
Chapter 5

Direct DNA analysis with nanofluidic paper-based analytical devices

This Chapter has been submitted to *Nature Nanotechnology*. The Candidate is the first author for this work and played a primary role in the design of experiments, execution of experiments, data collection and analysis, and writing of the manuscript. Additional authors include: Mr. Reza Nosrati, Dr. Maria C. San Gabriel, Prof. Armand Zini, and Prof. David Sinton. Their efforts were integral to the preparation of this work for publication and are gratefully recognized and appreciated.

5.1 Introduction

DNA analysis is fundamental to forensic science [162], genetics [163], and disease diagnosis [164]. Conventional DNA testing relies on methods generally limited to the laboratory. For example, the polymerase chain reaction (PCR) is the most commonly used method for viral load detection [164]. Similarly, the flow cytometry-based sperm chromatin structure assay (SCSA) is the gold standard for sperm DNA integrity assessment [165]. Nanotechnology and specifically, nanostructured sensors show potential for scalable analysis of DNA [11], [166]. Common classes of these sensors include nanopores [167], plasmonic nanohole arrays [168], single and arrayed nanoparticles [169], nanostructured microelectrodes [170], and nanowires [171]. These nanostructures have also been integrated into microfluidic and nanofluidic systems [11], [166], with demonstrated performance comparable to conventional DNA testing technologies. In all of these cases however, the nanofabrication in silicon and glass is a barrier to widespread application.

Paper is emerging as an inexpensive, versatile, and scalable platform for diagnostics, with microfluidic paper-based analytical devices (µPADs) as a central format [50], [51], [132], [172]. Recent advances in µPADs position these technologies for broad diagnostic application [26], [84], [86], [90], [112]. Specifically, the integration of nanomaterials into paper potentially offers enhanced detection capabilities [147], [173]. Nanoparticles are the most commonly employed nanomaterial in lateral flow assays and µPADs, and are mainly used as labels for colorimetric detection of DNA and other analytes [15], [173]. The plasmonic properties of these particles have also been exploited for label-free sensing in paper [80], [149], [174]. Graphene nanosheets have also been used for electrochemical sensing in paper-based devices [175]. The nanomaterials in
these applications are mainly used as static sensing elements. Leveraging nanomaterials for nanofluidic transport in paper would enable advanced control of target analytes, extending the current molecular diagnostic capabilities of paper-based devices.

Here, we demonstrate direct DNA analysis in nanofluidic paper-based analytical devices (nanoPADs), uniquely enabled by ion concentration polarization (ICP) effects at the interface of patterned nanoporous membranes in paper. ICP is an electrokinetic phenomenon caused by transport of ions through ion-selective nanostructures [91]. It has been applied to water desalination [93], [94] and biomolecular concentration in microchannels [95], [176]. We apply our nanoPAD approach to detect hepatitis B and assess male infertility. Hepatitis B virus (HBV) DNA targets in human serum are simultaneously preconcentrated, separated and detected in a single operation with a limit of detection (LOD) of 150 copies/mL. This LOD is achieved without prior PCR amplification of viral load, eliminating the need for thermal cycling. We clinically assess the DNA integrity of sperm cells in raw human semen samples by preconcentrating and separating denatured DNA from intact DNA. The percent DNA fragmentation results from the nanoPADs strongly correlate ($R^2 = 0.98$) with the conventional sperm chromatin structure assay (SCSA), and inform the same clinical outcomes.

5.2 Methods

5.2.1 Fabrication of nitrocellulose paper devices

Device designs were created in Microsoft PowerPoint and printed on nitrocellulose paper (mean pore diameters of 450 nm and 200 nm, Bio-Rad Laboratories Ltd., Canada) using a solid wax printer (ColorQube 8570N, Xerox Canada, Canada). Printed devices were heated in an oven at 150 °C for 5 minutes to allow the wax to penetrate through the thickness of the paper. Nafion perfluorinated resin solution (20% wt. in lower aliphatic alcohols and water, Sigma-Aldrich, US) was manually pipetted into each device, 0.5 $\mu$L of Nafion per device. Following this step, devices were immersed in deionized (DI) water for 30 minutes to hydrate the Nafion. Fabricated devices were covered in petri dishes at room temperature until use.

5.2.2 Preparation of fluorescent tracers and biological samples

Fluorescein and calcein were used as fluorescent tracers and were both acquired from Sigma-Aldrich, US. Stock solutions of fluorescein and calcein were prepared using DI water, with final concentrations of 2 $\mu$M and 1 $\mu$M, respectively. The pH values of the solutions were adjusted to
Synthetic hepatitis B virus (HBV) DNA (100 µL stock solution with concentration of 6.8 x 10^8 copies/mL, ATCC VR3232SD) was obtained from Cedarlane, Canada, containing fragments from the precore, core, polymerase, surface, and X regions of the viral genome. A 10 µL aliquot of stock solution was serially diluted seven times (at 10-fold per dilution) to concentrations of 10 to 10^8 copies/mL with DNase/RNase-free distilled water (Thermo Fisher Scientific Inc., US). Each of the eight dilutions were stained with Quant-iT PicoGreen dsDNA Reagent (Thermo Fisher Scientific Inc., US) at a volume ratio of 1:20. The same dilution and staining procedure was completed for stock HBV DNA using human serum from Cedarlane, Canada (final serum protein concentration of 3 mg/mL).

Human semen samples from patients and healthy donors (n = 7) were obtained by masturbation after 2-4 days of sexual abstinence at the Urology Research Laboratory, Royal Victoria Hospital, Canada. All donors signed and informed consent, and the information for this study remains confidential within the institution. Samples were incubated at 37 °C for 30 min to allow liquefaction. Computer assisted sperm analysis (CASA) was used to obtain standard semen parameters in accordance to World Health Organization guidelines. Semen samples were diluted to a concentration of 20 million sperm per milliliter (20M/ml) with HBS buffer (135 mM NaCl, 25 mM HEPES, 5 mM KCl, 0.75 mM Na2HPO4·2H2O, 12 mM Glucose, pH 7.4). To lyse the sperm cells, a 10 µL aliquot of the diluted sample was treated with 40 µL of 50 mM dithiothreitol (DTT), 50 µL of distilled water, and 300 µL of 8 M GuHCl in separate steps. The solution was vortexed for 30 s between each step and for 15 s after the final step. Single- and double-stranded DNA (ssDNA and dsDNA) in the sample were stained by adding 50 µL of SYBR Green to 50 µL of the sample and incubated at room temperature for 10 min. The resulting solution was diluted 500-fold in DI water and used immediately for testing.

**5.2.3 Sperm Chromatin Structure Assay**

Raw semen samples were diluted to a concentration of 10M/mL in TNE buffer (0.1 M TRIS buffer, 0.15 M NaCl, 1 mM EDTA, pH 7.4). After dilution, 200 µL of the sample was treated with 400 µL acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) for 30s. After 30 seconds, 1.2 mL of staining buffer (6 µg/mL AO, 37 mM citric acid, 126 mM Na2HPO4, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0) was admixed to the test tube. The sample
is placed into the MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with a 488 nm air-cooled laser with the sample flowing to establish excellent sheath/sample flow, and then at 3 min after AO staining, measurements are taken. A minimum of 5000 cells from two aliquots of each sample were analyzed at a flow rate of 100-200 events/sec by FACS scan interfaced with a data handler (CELLQUEST 3.1, Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA). WinList (Verity Softwarehouse Inc., Topsham, ME) was used to generate the cytogram (red vs. green fluorescence) and histogram (total cells vs. DFI) plots, as well as, % DFI readings. A mean of the two sperm % DFI values was reported. The variability of the replicate SCSA measures (% DFI) is less than 5%.

5.2.4 Data acquisition and analysis

For the experiments related to Figs. 1-3, an inverted fluorescence microscope (DMI 6000B, Leica Microsystems Inc., Canada) was used to visualize fluorescent tracers and stained HBV DNA with a L5 filter cube. Image sequences were captured using a CCD camera (Orca-AG, Hamamatsu Photonics K. K., Japan). For the clinical experiments related to Fig. 4, an upright fluorescence microscope (Axiophot, Carl Zeiss AG, Germany) was used to visualize stained sperm DNA with a green fluorescence filter. Image sequences were captured using a CCD camera equipped with the microscope. All captured images were processed in ImageJ and Adobe Photoshop. Data quantification was completed in Microsoft Excel and Mathematica.

5.2.5 Analytical modeling of the separation process

We use SR as a performance metric to evaluate the separation process and to determine practical operating conditions. SR is defined as the ratio of peak-to-peak distance between adjacent analyte bands to their average width [177]:

\[
SR = \frac{x_2 - x_1}{4\sigma_{avg}}
\]

(1)

where \(x_1\) and \(x_2\) are the peak positions of adjacent analyte bands 1 and 2, and \(\sigma_{avg}\) is the average width of the bands as approximated by normal fits. Jorgenson and Lukacs derived an expression for SR as a function of applied voltage for electrophoresis in glass capillaries, assuming molecular diffusion to be the only contributing factor to band broadening [178]:

\[
SR = \frac{1}{\sqrt{32}} \left( \mu_2 - \mu_1 \right) \left( \frac{V}{D_{avg}(\mu_2 + \mu_{eof})} \right)^{1/2}
\]

(2)
where $V$ is the applied voltage, $D_{avg}$ is the average diffusion coefficient of the analytes, $\mu_1$ and $\mu_2$ are the electrophoretic mobilities of analytes 1 and 2, $\bar{\mu}$ is the average electrophoretic mobility of the analytes, and $\mu_{eof}$ is the electroosmotic mobility of the bulk fluid. Using equation (2), SR for our nitrocellulose paper device can be calculated as a function of channel aspect ratio and residence time, given the following:

$$E = \frac{V}{L} \quad (3)$$

$$L = \bar{\mu} t \quad (4)$$

$$\bar{\mu} = (\bar{\mu} + \mu_{eof})E \quad (5)$$

where $E$ is the applied electric field, $L$ is the channel length, $\bar{\mu}$ is the average velocity of the analytes, and $t$ is time. By substituting equation (3) into (2) for $V$, we get SR as a function of channel length:

$$SR = \frac{1}{\sqrt{32}} (\mu_2 - \mu_1) \left( \frac{EL}{D_{avg}(\bar{\mu} + \mu_{eof})} \right)^{1/2} \quad (6)$$

We then substitute the channel aspect ratio, $L^* = length/width = L/W$, into equation (6) to yield:

$$SR = \frac{1}{\sqrt{32}} (\mu_2 - \mu_1) \left( \frac{EL^* h}{D_{avg}(\bar{\mu} + \mu_{eof})} \right)^{1/2} \quad (7)$$

where $h$ is constant width when $L^*$ changes with length or constant length when $L^*$ changes with width. In regards to residence time, we substitute equations (4) and (5) into equation (6) to yield:

$$SR = \frac{1}{\sqrt{32}} (\mu_2 - \mu_1) \left( \frac{E^2 t}{D_{avg}} \right)^{1/2} \quad (8)$$

Collectively, equations (2), (7), and (8) provide expressions for SR that can be experimentally characterized to determine practical operating conditions for separating different analytes.

### 5.3 ICP-based preconcentration and separation in the nanoPAD

The nanoPAD is nitrocellulose paper with a sample channel and reservoirs defined by patterned wax, and a region coated with cation-selective nanoporous Nafion (Figure 5.1a). Nanoporous Nafion is the main functional component in the device, enabling nanoscale electrokinetic transport of ions for inducing ICP. Nafion-coated regions of nitrocellulose paper with original mean pore diameters of 200 nm and 450 nm are shown in environmental scanning electron microscopy images in Figure 5.1b.
To operate the nanoPAD, buffer is deposited into each reservoir and sample is deposited into the sample channel. Once the device is fully wet, a voltage is applied to induce ICP, as illustrated in Figure 5.1c. Cations selectively migrate through the Nafion-coated region toward the cathode (i.e. negatively charged electrode) causing an ion enrichment zone at the cathodic Nafion interface. This efflux of cations causes anions to vacate the region due to electrical neutrality, forming an ion depletion zone. The depletion zone propagates and repels anions toward the anode (i.e. positively charged electrode). The net movement of anions in the channel is ultimately dictated by electrophoretic migration (EPH) toward the anode and electroosmotic flow (EOF) toward the cathode. Anions experience different electrokinetic forces along the channel as a result of local changes to the applied electric field and ionic concentration from ICP [92]. Specifically, lower ionic concentrations lead to higher EOF transport rates and vice versa [179]. EOF is dominant downstream of the depletion zone, causing net movement of analytes toward the depletion boundary in the cathodic direction. EPH is higher than EOF in the depletion zone, causing anions to migrate toward the depletion boundary in the anodic direction. The balance of these two opposing effects focuses ions at the depletion boundary. For a system with multiple anionic analytes, each analyte migrates at a different net velocity due to variations in their electrophoretic mobility, enabling separation following preconcentration.

We demonstrated three different operating modes using anionic fluorescent tracers (fluorescein isothiocyanate (FITC) – 332.3 g/mol and calcein – 622.6 g/mol) as model analytes: 1) transport without ICP; 2) preconcentration and separation with switching-polarity ICP; and 3) direct preconcentration and separation with ICP. For transport in the absence of ICP (mode 1), EOF was dominant over EPH everywhere, resulting in net migration of analytes toward the cathode with no preconcentration or separation effects (Figure 5.1d and Supplementary Movie 1). For preconcentration and separation with switching-polarity ICP (mode 2), a Nafion-coated region is employed to achieve both enrichment and depletion (Figure 5.1e). Specifically, ICP applied with the anode on the left preconcentrated anions at the Nafion interface via enrichment (t ≤ 40 s). Switching the polarity of the electrodes caused depletion to grow at the same interface, transporting and separating the preconcentrated analytes left-to-right (Supplementary Movie 2). In this mode, a droplet of bulk (non-fluorescent) fluid grew on the surface of the paper near the Nafion interface.
Figure 5.1: ICP-based preconcentration and separation in the nanoPAD. a) Device schematic with reservoirs (blue), a sample channel (purple), and a region coated with cation-selective nanoporous Nafion (white). b) Environmental scanning electron microscopy images of nitrocellulose paper with and without Nafion coating. c) Schematic of ICP phenomenon. An ion enrichment zone forms at the cathodic Nafion interface, while an ion depletion zone forms at the anodic interface. Net movement of analytes is governed by electrophoresis (EPH) and electroosmotic flow (EOF), in response to the local electric field strength, E. d) Operating mode 1 – transport without ICP. e) Operating mode 2 – preconcentration and separation with switching-polarity ICP. f) Operating mode 3 – direct preconcentration and separation with ICP. g) Separation resolution (SR) in modes 2 and 3. h) Effect of Nafion concentration (% w/v) on the separation process. Plotted points are the average of n = 3 measurements with error bars as one standard deviation. Images have been contrast adjusted for presentation (similarly in each case).

This accumulation of bulk fluid is a result of a mismatch between EOF transport rates through the paper and the Nafion-coated region. For direct preconcentration and separation with ICP (mode 3), no reverse-polarity preconcentration step was employed, and ICP was applied only in the forward direction (Figure 5.1f and Supplementary Movie 3). Analytes rapidly collected at the depletion boundary (t ≤ 30s) as a result of strong initial EOF (to the left), achieving early preconcentration without a reverse polarity. EOF was reduced as the electrically-insulating depletion zone grew, and the analytes were electrophoretically separated left-to-right. It is noteworthy that as the depletion boundary grows, the reduction in the electric field strength reduces both EOF and EPH (electric field diagram in Figure 5.1c); however, EOF is additionally reduced by the increasing ionic concentration ahead of the depletion zone. Although the
underlying electrokinetic phenomena are complex, the nanoPAD approach is robust and easy to use.

The separation quality achieved in modes 2 and 3 was quantified by a separation resolution (SR) metric, which is defined as the ratio of peak-to-peak distance between adjacent bands to their average width [177] (see Methods). SR values were comparable at ~1.1 for mode 2 and ~1.3 for mode 3, indicating that the reverse-polarity preconcentration step in mode 2 is not advantageous nor necessary (Figure 5.1g). We also investigated the effect of Nafion concentration (% w/v) on the separation process (Figure 5.1h). Cation transport increased with Nafion concentration, resulting in stronger ICP effects [180]. No separation of the analytes was detectable for 5% w/v Nafion due to weak ICP effects. Separation occurred at 10% w/v, and markedly improved at 20% w/v with a SR of ~1.3. For concentrations over 20% w/v, Nafion precipitated out of the solution and it was not suitable for patterning in nitrocellulose paper.

Figure 5.2 shows the characterization of operating parameters for the nanoPAD using FITC and calcein tracers as model analytes. There is good agreement between the experimental measurements and an analytical model [178] (see Methods). As plotted in Figure 5.2a, SR improved with increasing voltage up to 140 V. Similarly, SR improved for higher channel aspect ratios, when 1) channel length increased with constant channel width and 2) channel width decreased with constant channel length (Figure 5.2b). As with capillary electrophoresis [181], longer and narrower channels provide better separation performance. In regards to residence time, SR continuously improved until 180 s and plateaued thereafter (Figure 5.2c). Band broadening becomes more significant after 180 s due to increased Joule heating, reducing any gain in peak-to-peak distance (as shown by the inset images in Figure 5.2c). While these operating parameters may change based on the application, the trend would be similar and equally predictable using the analytical expressions derived here (see Methods).
Figure 5.2: Characterization of operating parameters for the nanoPAD. a) Separation resolution (SR) as a function of applied voltage, using devices of fixed geometry (channel length of 7 mm and width of 0.6 mm). SR improves with increasing voltage, up to 140 V (200 V/cm). b) SR as a function of channel aspect ratio (i.e. length/width), with a fixed field strength of 200 V/cm. Longer and narrower channels result in better SR. c) SR as a function of residence time, using an applied field of 200 V/cm and devices with channel aspect ratio of 12. SR improves with increasing residence time, up to 180 s where band broadening from Joule heating becomes limiting (inset images). Plotted points are the average of \( n = 3 \) measurements with error bars as one standard deviation. Images have been contrast adjusted for presentation.

5.4 PCR-free hepatitis B analysis

Workflows for both conventional and nanoPAD DNA analysis are shown in Figure 5.3a. The infrastructure-intensive nature of PCR is a major barrier to widespread DNA testing in both developed and developing regions [8]. As an alternative to increasing viral load through PCR, the nanoPAD leverages ICP preconcentration to intensify the signal of target sequences prior to separation. We demonstrate this process using HBV DNA, a hepadnavirus with a genome consisting of circular, partially double-stranded DNA (dsDNA) of \(~3200\) base pairs (bp) [182].

Figure 5.3b shows the separation of a DNA standard containing dsDNA fragments of 100 bp to 2000 bp (6 fragments in total). The nanoPAD had channel lengths of 7 mm and 10 mm (aspect ratios of 12 and 17, respectively), and mean pore diameters of 450 nm and 200 nm. Our separations compare well to an ideal case in 2% agarose gel, where 3 of 6 fragments (the 2000, 800, and 100 bp fragments) were resolved using the 7 mm channel devices and 4 of 6 fragments (the 2000, 800, 400, and 100 bp fragments) using the 10 mm channel devices, for both mean pore diameters (Figure 5.3b). For the intensity profiles in Figure 5.3c, the quality of each separation was quantified by the SR for the 2000 bp and 800 bp fragments. The SR values were comparable between all four separations, ranging from 0.5 to 0.7, with the highest resolution, and best alignment of fragments to the ideal case, achieved with the highest aspect ratio (10 mm) nanoPADs. Notably, the 200 nm nitrocellulose pore diameter correlates to the pore size of the recommended 2% agarose gel [183], enabling separation of the DNA standard with resolution comparable to conventional gel electrophoresis. Similarly, the 450 nm pore diameter device is representative of a 1% agarose gel [183], which is generally used to separate larger DNA fragments of 500 bp to 10,000 bp. These results demonstrate that the nanoPAD approach is a viable means to separate DNA.
Figure 5.3: PCR-free hepatitis B analysis. a) Comparison of conventional and nanoPAD approaches for HBV DNA analysis. No prior PCR amplification is required for the nanoPAD, eliminating the need for thermal cycling. b) Separation of a DNA standard containing dsDNA fragments ranging from 100 bp to 2000 bp (6 fragments in total), using 7 mm and 10 mm channel devices of two different mean pore diameters – 200 nm and 450 nm. The separations are compared to an ideal case in 2% agarose gel (image from Invitrogen). c) Intensity profiles are measured for the gel and the four nanoPAD cases using the region of interest (ROI) outlined by the yellow dashed box in b). d) Multiplexed analysis of the DNA standard, HBV DNA in water, HBV DNA in serum, and stock serum in a multichannel device. The intensity profile for each channel is measured using the ROI box. e) A limit of detection (LOD) of 150 copies/mL is achieved by measuring the peak intensity of the precore (preC) fragment at serially diluted HBV DNA concentrations in serum. The LOD is the HBV DNA concentration at three standard deviations from a base intensity of 0.20. Each data point is the mean of the normal fit to the intensity profile of the preC fragment, with error bars as one standard deviation. Images have been contrast adjusted for presentation (similarly in each case).

HBV DNA fragments (i.e. precore, core, surface, X, and polymerase) were simultaneously preconcentrated, separated and detected using a multichannel nanoPAD (Figure 5.3d). Each channel contained a different sample: M) the DNA standard molecular size marker; 1) HBV DNA in water (HBV water) as a positive control; 2) HBV in serum (HBV serum) as the target; and 3) stock serum as a negative control. DNA fragments in HBV water and serum were resolved after 10 minutes (at 150 V/cm) (Supplementary Movie 4). In comparing the intensity profile of HBV
serum to that of the DNA standard in channel M, the polymerase, surface, and precore fragments were resolved, having sizes of 2535 bp, 678 bp, and 87 bp [184], respectively. The fragment near the 400 bp mark likely contains both the X and core fragments, which have sizes of 463 bp and 555 bp [184]. Importantly, the separation produced a distinct profile for the HBV serum, which corresponds to the profile for HBV water (i.e. positive control) in both position and intensity. A 100-fold increase in the signal of the precore fragment was achieved following preconcentration and separation. The stock serum produced a relatively high fluorescence signal, as a result of PicoGreen (the DNA stain employed here) binding to serum proteins in the absence of DNA [185]. When DNA was present, however, PicoGreen preferentially bound to the DNA and the background fluorescence from serum proteins was less significant [185]. Collectively, these results demonstrate that the nanoPAD is amenable to multiplexed analysis and to amplified detection of target DNA in relevant biological fluids.

The early detection of acute HBV infection requires detection of low levels of HBV DNA (10² to 10⁴ copies/mL [186]), generally necessitating PCR amplification. Such levels are present in serum after 4 weeks of infection, which is ~3 weeks prior to levels of serological markers (e.g. hepatitis B surface antigen) becoming detectable with immunoassays [186]. To evaluate the potential for early detection of HBV using the nanoPAD, we determined its LOD for HBV DNA in serum by measuring the intensity of the precore fragment at different HBV DNA concentrations. The results in Figure 5.3e show a LOD of 150 copies/mL, applicable to early detection of acute HBV infection. This low LOD was achieved without prior PCR amplification, and is comparable to that of commercial PCR systems (e.g. COBAS® AMPLICOR Analyzer) with a LOD of 50–100 copies/mL. In addition to early detection, the nanoPAD has potential to monitor chronic HBV infection. During HBV chronicity, viral load typically ranges from 10³ to 10⁵ copies/mL and represents active viral replication [182]. With a LOD of 150 copies/mL and a dynamic range up to 10⁸ copies/mL, the nanoPAD has potential to monitor HBV chronicity and evaluate antiviral therapies in real-time. Collectively, nanoPADs enable low cost (materials cost < 1 USD per test) and rapid (~10 minutes) HBV DNA analysis, with comparable performance to laboratory-based testing.

5.5 Clinical assessment of human sperm DNA integrity
Workflows for both conventional flow cytometry-based SCSA and the nanoPAD approach are shown in Figure 5.4a. SCSA measures the proportion of sperm cells (typically 5,000 to 10,000
sperm are evaluated) with DNA denaturation, and the results are expressed as percent DNA Fragmentation Index or % DFI [165]. A low % DFI predicts higher success rates in natural/assisted reproduction and lower risks of transmission of genetic defects to the offspring [187], [188]. The high capital and operating costs of flow cytometry are, however, barriers to widespread sperm DNA integrity assessment in both developed and developing regions.

Figure 5.4: Clinical assessment of human sperm DNA integrity. a) Comparison of conventional and nanoPAD approaches for assessing sperm DNA integrity. Contrast enhanced image showing preconcentration and separation of single- and double-stranded DNA (ssDNA and dsDNA, respectively) for patient P1 over 15 min at 150 V/cm. b) Intensity profile of the separated DNA. The percent DNA Fragmentation Index (% DFI) is calculated using areas under the normal fits. c) Comparison of % DFI results for patients P1 to P4 and donors D1 to D3. The nanoPAD results strongly correlate ($R^2 = 0.98$) with the clinical flow cytometry results, as shown in the inset. A % DFI threshold of 30% is used to determine clinical outcome and treatment recommendations. Error bars for the nanoPAD results represent one standard deviation of the normal fits. Clinical results are the average of two measurements with error bars as one standard deviation.

As an alternative to SCSA, nanoPADs were used to directly quantify % DFI of sperm cells in raw semen samples from patients and donors ($n = 7$; P1 to P4 and D1 to D3, respectively) at the Royal Victoria Hospital in Montreal, Canada. Specifically, single-stranded DNA (ssDNA) and dsDNA from lysed sperm cells were preconcentrated and separated over 15 minutes at 150 V/cm (a representative image is shown for P1 in Figure 5.4a). Intact and heavier dsDNA are concentrated at the depletion boundary, while fragmented and lighter ssDNA are concentrated further downstream. The intensity profile for this separation is plotted in Figure 5.4b, showing the overlapping ssDNA and dsDNA distributions, with SR calculated as ~0.5. The areas under the ssDNA and dsDNA normal fits were used to calculate % DFI (i.e. ssDNA area/total area).

Figure 5.4c compares the nanoPAD and SCSA results for both patients and donors. The nanoPAD % DFI results strongly correlate ($R^2 = 0.98$) with those of SCSA for values ranging from 4% to 97%. In the clinic, a value greater than 30% indicates poor male fertility potential,
and a recommendation for assisted reproduction technologies (ART) [188]. Here, P1 and P4 would be recommended for ART based on both the nanoPAD and SCSA results, while P2, P3, and all donor samples show suitable fertility potential. Collectively, these results demonstrate the nanoPAD approach as a promising method for scalable male infertility testing.

5.6 Conclusions
We demonstrated direct DNA analysis in paper by leveraging nanoscale electrokinetic transport at the interface of patterned nanoporous membranes. We applied our nanoPAD approach to detect HBV DNA in human serum and assess sperm DNA integrity in raw human semen, with comparable performance to the conventional gold standards. For hepatitis B testing, a LOD of 150 copies/mL was achieved with no prior viral load amplification, sufficient for early diagnosis of HBV. Multiplexed analysis of HBV was also demonstrated in a four-channel nanoPAD. For male fertility assessment, the % DFI results from the nanoPADs strongly correlated ($R^2 = 0.98$) with those of flow cytometry-based SCSA, providing identical clinical outcomes for tested patients and donors. NanoPADs enable inexpensive (materials cost < 1 USD per test) and rapid (~10 minutes) DNA analysis in a simple format suitable for widespread application.
Chapter 6

Hand-powered microfluidics: A membrane pump with a patient-to-chip syringe interface

This Chapter was published in the journal Biomicrofluidics and reprinted with permission from [27]. Copyright 2012, AIP Publishing LLC. The Candidate was the first author for this work and played a primary role in the design of experiments, execution of experiments, data collection and analysis, and writing of the manuscript. Additional authors include: Dr. Brendan D. MacDonald, Dr. Trung Vu Nguyen, and Prof. David Sinton. Their efforts were integral to the publication of this work and are gratefully recognized and appreciated.

6.1 Introduction

Fluid transport and flow control components, such as pumps, are required front-end components of lab-on-a-chip devices for the delivery and control of biological samples [189]. A range of micropumps have been developed for biomedical applications based on electrostatic [190], piezoelectric [191]–[193], electromagnetic [194], [195], electroosmotic [196], and pneumatic [197] actuation. Although these pumping mechanisms provide precise flow rates [198], they require electrical control and external electrical or air supply.

In the context of point-of-care diagnostics, electrically and pneumatically actuated pumps are not well-suited due to the requirement of external support equipment (e.g. a power or air supply). Point-of-care diagnostic devices must be low cost, portable, robust, and minimally instrumented, especially if they are to be deployed for public health in resource-poor, remote, or developing settings [8], [9]. In these settings, energy and material resources are often limited or too costly to acquire. The ideal strategy, then, is to integrate point-of-care diagnostic devices with on-chip pumping mechanisms that can leverage battery power [99] or human-power [31], [199], [200] and eliminate the necessity of external support equipment.

A number of on-chip pumping mechanisms have shown promise for point-of-care applications. Walker and Beebe [20] presented a passive pumping method based on the surface tension of a small droplet of liquid. Droplets of water were pipetted into open inlet and outlet ports with the inlet droplet having a larger curvature. The difference in capillary pressure induced flow in the channel [201]. Gervais and Delamarche [41] demonstrated an integrated, one-step
immunoassay that used a capillary pump for transport of blood serum. The capillary pump, placed at the end of the device, induced a negative capillary pressure to allow continuous flow of sample volume from an open reservoir. In another integrated immunodiagnostic device, Dimov et al. [42] used vacuum pressure to deliver a few microliters of whole blood from open ports to downstream analysis. They exploited the high gas-permeability of poly(dimethylsiloxane) (PDMS) to remove the trapped air within dead-ended channels. Similarly, Li et al. [202] developed a “place n play” modular pump based on the degassed-PDMS approach. Weibel et al. [203] also leveraged the properties of PDMS for storing and pumping fluids. PDMS compartments were deformed under fluid pressure and closed off by manual screw valves on the upstream and downstream sides. The isolated sample could be used later by opening the downstream screw valve. Chin et al. [31] used a syringe attached to an on-chip port via a metal spacer to induce negative vacuum pressure for transporting whole blood through an immunoassay. In a similar manner, Wu et al. [200] employed an air-filled hand-held syringe as a portable plastic pump for on-chip continuous-flow polymerase chain reaction (PCR). The air-filled syringe was connected to a continuous-flow PCR component via highly gas-permeable tubing. The flow of a sample plug was enabled by a pressure gradient created by the compressed air in the syringe and air escaping from the tubing. Another more recently developed hand-operated pump is the squeeze-chip by Li et al. [199]. In their device, fluid transport is dictated by finger-operated PDMS reservoirs with a network of check valves. When an operator presses on a reservoir, valves open and close to allow fluid movement downstream and refilling of the sample reservoir from an open port. These existing on-chip pumping mechanisms have addressed some important challenges in the design of pumps for point-of-care applications, such as low cost, portability, and minimal instrumentation. However, several key attributes are still lacking: (1) a robust patient-to-chip interface for safe sample collection that leverages established sample collection methods (e.g. syringe and needle), (2) biological sample containment to avoid exposure of the operator and/or environment to infectious agents, and to ensure sample integrity, and (3) capacity for high sample volume throughput.

In this paper, we present a device that incorporates an on-chip hand-powered membrane pump leveraging established syringe-based sample collection methods. This approach enables safe sample collection, sample containment, integrated sharps disposal, high sample volume capacity, and controlled downstream flow with no electrical power requirements. The working principle and design of the device is described, and the fabrication of the membrane pump is also
presented. Furthermore, the performance of the pump is established through experiments and characterized using an electrical circuit analogy.

6.2 Working principle and design

The device is comprised of several interconnected components for collecting and transporting fluids: a patient-to-chip syringe interface, membrane pump, fluidic resistor, and downstream component, as shown in Figure 6.1. The patient-to-chip syringe interface exploits existing sample collection methods (e.g. syringe and needle) to safely collect patient blood (or other bodily fluids) into the device. An operator injects the patient sample into the device by inserting the needle into the syringe interface. To reduce the risk of operator injury, the device should be braced against a solid surface such that only one hand is used for needle insertion. The injected patient sample is contained within the membrane pump, which deforms under the fluid pressure. The membrane pump stores the sample volume and the mechanical energy of the operator as it inflates. Subsequent deflation of the pump pushes the sample downstream, as regulated through a fluidic resistor.

A typical human thumb can apply a force of 70 N [204] resulting in an injection pressure of 4 MPa for a 1 mL BD plastic syringe. The potential for such high levels of operator-generated pressure indicate both (1) the need to isolate the operator from the downstream processes, and (2) the relatively large potential for mechanical work in the syringe injection process. The membrane pump is designed to separate the operator from downstream components such that subsequent flow is not dictated by the operator’s injection rate or hand strength. Instead, the fluid flow is controlled through prior engineering of the membrane pump’s pumping capacitance and the downstream fluidic resistance of the fluidic resistor.
Figure 6.1: (a) Image of the device with syringe and needle inserted. (b) Solid model of the device with all interconnected components: patient-to-chip syringe interface, membrane pump, fluidic resistor, and downstream component. An operator injects the patient sample into the device using the collection syringe directly. The sample is fully contained within the membrane pump, which inflates under the fluid pressure. Subsequently, the pump deflates and pushes the sample downstream, as regulated by the membrane characteristics and the fluidic resistor. (c) Exploded view of the device showing the PMMA and silicone layers. The top PMMA layer is 1.5 mm thick, the silicone layer is 1.6 mm thick, and the bottom PMMA layer is 3.0 mm thick.

6.2.1 Patient-to-chip syringe interface

The syringe and needle is the standard accepted method of collecting mL-scale volumes of patient blood. The device components connecting the syringe and needle to the chip consist of a silicone seal, guiding channel for the needle, and check valve, as shown in Figure 6.2a. During sample
injection into the device, the needle punctures the silicone seal and follows the guiding channel until it reaches the valve chamber. The guiding channel can be designed to accommodate any common needle length or diameter. After safely collecting the sample, the syringe can be unscrewed with the needle remaining in the chip for integrated sharps disposal. The check valve simply prevents sample backflow through the syringe interface following sample injection, as demonstrated previously with a membrane hole valve design [205]. As shown in Figure 6.2b, the through hole of the check valve lies within the silicone layer and only opens when there is sufficient forward pressure in the valve chamber pushing against the silicone. Backpressure from the membrane pump closes the check valve (Figure 6.2b).

Figure 6.2: Patient-to-chip syringe interface. (a) Magnified view showing the silicone seal, guiding channel for the needle, and check valve. (b) Schematic of the simple check valve enabled by the layered membrane pump design (open under forward pressure and closed under backpressure).
6.2.2 Membrane pump

The membrane elastically deforms and stores sample volume as it is injected into the device, as shown in Figure 6.3a. The volume stored under a deflected membrane is defined by the spherical cap equation:

\[ V = \frac{\pi \delta}{6} (3r^2 + \delta^2), \quad (1) \]

where \( V \) is the stored volume, \( r \) is the radius of the membrane pump, and \( \delta \) is the deflection of the membrane. The deflection of the membrane can be modeled analytically using solid mechanics theory for the deformation of circular, reservoir-bound elastic membranes [206]–[208]. It is a function of the geometry of the reservoir and membrane, the material properties of the membrane, and the applied fluid pressure:

\[ \delta = \left( \frac{3r^4}{16Eh^3} \right) (1 - \nu^2)p, \quad \text{for } \delta \leq h, \text{ (linear regime)} \quad (2) \]

\[ \delta = h \left( \frac{3r^4(1-\nu)}{8Eh^4} \right)^{\frac{1}{3}} p^{\frac{1}{3}}, \quad \text{for } \delta > h, \text{ (nonlinear regime)} \quad (3) \]

where \( h \) is the membrane thickness, \( E \) is the elastic modulus of the membrane material, \( \nu \) is the Poisson’s ratio of the membrane material, and \( p \) is the applied fluid pressure, as shown schematically in Figure 2.3b. The Poisson’s ratio for silicone elastomer is 0.5 [206], such that Eqs. (2) and (3) become:

\[ \delta = \left( \frac{9r^4}{64Eh^3} \right) p, \quad \text{for } \delta \leq h, \text{ (linear regime)} \quad (4) \]

\[ \delta = h \left( \frac{3r^4}{16Eh^4} \right)^{\frac{1}{3}} p^{\frac{1}{3}}, \quad \text{for } \delta > h, \text{ (nonlinear regime)} \quad (5) \]

Deflection behavior is linear for \( \delta \leq h \) and nonlinear for \( \delta > h \) with respect to applied fluid pressure. It is noted that these equations hold only when the diameter of the membrane pump is much greater than the membrane thickness, \( 2r \gg h \), a condition that is met by the pump developed here.
6.2.3 Pump characterization through an electrical circuit analogy

Connecting a fluidic resistor to the membrane pump allows for controlled flow of stored sample volume to downstream components. The flow of the sample volume is dictated by the total resistance of the fluidic resistor and downstream components as well as the fluid pressure in the membrane pump which changes with time. In terms of an electrical circuit analogy, the membrane pump with the fluidic resistor and downstream components form a resistor-capacitor (RC) circuit, where the membrane pump serves as a capacitor. The pressure and flow rate outputs from this fluidic RC circuit after loading can be defined by equivalent voltage and current output expressions for the electric RC circuit. These expressions are given as:

\[ p = p_0 e^{-t/\tau}, \quad (6) \]
\[ Q = \frac{p_0}{R} e^{-t/\tau}, \quad (7) \]
\[ \tau = RC, \quad (8) \]

where \( p_0 \) is the initial pressure in the membrane pump after loading, \( Q \) is the flow rate output at time \( t \), \( \tau \) is the characteristic time constant which governs the rate of decay in pressure and flow rate outputs, \( R \) is the total resistance of the fluidic resistor and downstream components, and \( C \) is the capacitance of the membrane pump. The capacitance is the derivative of the volume stored in

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**Figure 6.3**: Membrane pump. (a) Magnified view showing stored sample volume. (b) Schematic of the membrane pump inflated under fluid pressure.
the pump with respect to the applied pressure [209]. From this relationship, linear and nonlinear expressions for the pumping capacitance can be derived using Eqs. (1), (4), and (5):

$$ R = \frac{\partial V}{\partial p} = \left(\frac{3\pi}{8k}\right)\left[\left(\frac{3p}{4k}\right)^2 + r^2\right], \quad \text{for } \delta \leq h, \quad \text{(linear regime)} \quad (9) $$

$$ R = \frac{\pi}{6}\left(\frac{h^2}{k}\right)^\frac{1}{2}\left[r^2 p^{-\frac{2}{3}} + \left(\frac{h^2}{k}\right)^\frac{3}{2}\right], \quad \text{for } \delta > h, \quad \text{(nonlinear regime)} \quad (10) $$

$$ k = \frac{16Eh^3}{3r^4}, \quad (11) $$

where $k$ is the stiffness of the membrane (i.e. pressure required per unit deflection of the membrane center-point) [209]. The pump outputs become increasingly more steady as $\tau$ is increased. Increasing $\tau$ by increasing capacitance, $C$, can be achieved by enlarging the pump (i.e. increasing pump radius) and/or connecting multiple pumps in parallel. The total resistance of the fluidic circuit, $R$, can also be increased; however, this approach reduces flow rate, and may or may not be suitable for a given application. In summary, combinations of pumps and pump geometry can be tuned in a straightforward manner to engineer pump performance. Pumping capacitance, $C$, is a particularly useful metric indicating the volume output available at a given pressure.

6.3 Experimental

6.3.1 Device fabrication

The device was fabricated from several layers of poly(methyl methacrylate) (PMMA) and silicone, as shown in Figure 6.1c. Both PMMA and silicone are biocompatible, inexpensive in bulk, and easy to fabricate, which greatly reduces the total material and fabrication costs of the device. Based on the current prices of PMMA and silicone (McMaster Carr, Aurora, OH, US; Plastic World, Toronto, ON, Canada), and a typical chip size (with an area of 60 x 60 mm$^2$ per layer), the total material cost is ~ 0.20 USD/device.

The features of the device (e.g. channels, reservoirs, through holes) were micromachined into the PMMA and silicone layers via a CO$_2$ laser ablation system (Universal Laser Systems Inc., Scottsdale, AZ, USA). After micromachining, the PMMA layers were lightly polished with sandpaper to remove ablated residue, followed by cleaning in an ultrasonic bath (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 15 minutes. The silicone layer was rinsed with deionized water and isopropanol to remove residue from the laser ablation. The bonding of the PMMA and silicone layers was based on the protocol provided by Kim et al. [210], and we briefly summarize
the steps here: (1) prepare a 5% v/v aqueous solution of (3-Aminopropyl)trimethoxysilane (APTMS) (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) and heat to 85 °C, (2) expose surfaces of the PMMA layers to oxygen plasma for 1 minute and immediately immerse in the heated APTMS solution for 30 minutes, (3) remove the treated PMMA layers and dry with compressed air, and (4) expose each side of the silicone layer to oxygen plasma for 1 minute and sandwich between the treated PMMA layers in a heated press (Carver Inc., Wabash, IN, USA) at 85 °C under 1300 N for 60 minutes. The resulting device had a tensile bonding strength of about 1000 kPa [210].

6.3.2 Characterization of membrane pump

The pressure and flow rate outputs of a single membrane pump and multiple pumps connected in parallel were characterized using an experimental setup consisting of a syringe pump, pressure gauge, valves, and an analytical balance, as shown in Figure 6.4. The membrane pump(s) was evaluated for both deionized (DI) water and undiluted, anticoagulated, mouse whole blood (Cedarlane Laboratories Ltd., Burlington, ON, Canada). The mouse whole blood was used within three days upon reception as advised by the supplier. Fluid was injected by the syringe pump into the device via an on-chip port directly after the first valve as shown in Figure 6.4. Flow was then directed to the membrane pump(s) through on-chip fluidic channels, where in the case of multiple pumps connected in parallel the network was filled simultaneously. It is important to note that the multiple pumps within the chip do not require additional input from the operator; these pumps are connected by the fluidic channels. This inflated the pump(s) to an initial gauge pressure of ~ 1 atm (103.4 kPa), and then upstream and downstream connections were closed to maintain that pressure (Figure 6.4). After initially opening the downstream valve to allow flow, the valve was closed at fixed time intervals of 0.5, 1, and 2 minutes to collect and measure the mass output using the analytical balance. A volume output was calculated from the mass output using the density of the fluid (i.e. 1.0 g/mL for water and 1.1 g/mL for mouse whole blood). The volume output is the time-integral of the flow rate in Eq. (7). This relationship was used to calculate the characteristic time constant, \( \tau \), of the fluidic circuit. In Eq. (7), the total resistance, \( R \), and time constant, \( \tau \), are the only unknown variables given that the initial pressure, \( p_o \), was set to ~ 1 atm, which necessitates a system of two equations. The measured volume output at the three time intervals (0.5, 1, and 2 minutes) provided a system of three equations, where two equations were used to calculate \( \tau \) and the third was used as verification. The capacitance of the pump was determined
using Eq. (9) given the material properties and geometry of the membrane pump. Using this capacitance value and the calculated time constant, the total resistance, $R$, was ascertained using Eq. (8).

The pump pressure decay was measured directly; however, the results were unreliable due to instrumental error. Specifically, the pressure gauge contained a dead volume and a deformable diaphragm that acted as a pump with capacitance similar to the membrane pump. The result was experiments that overestimated the pumping capacitance significantly. For this reason, the gauge was closed off and the volume measurement approach was applied without real-time pressure measurement. Instead, pressure and flow rate outputs were calculated and plotted from the measured volume output of the pumps through Eqs. (6) and (7).

Red blood cell (RBC) lysis in the mouse whole blood was also investigated. Whole blood samples were observed with bright-field microscopy prior to and after pumping, which provided a rough estimate of RBC density. Shear stress in the device during flow was also calculated and compared to existing values required for lysing red blood cells.

Figure 6.4: Experimental setup used to determine the characteristic time constant of the fluidic RC circuit (shown magnified in the current view).

6.4 Results and Discussion

6.4.1 Linear versus nonlinear pump deflection

Linear pump deflection behavior is generally preferred over nonlinear behavior (as described in Eqs. (4)-(5) and Eqs. (9)-(11)), particularly where transport can influence downstream diagnostics. The pumping capacitance for linear and nonlinear deflection was plotted against applied pressure for given pump geometry and material properties, as shown in Figure 6.5, using Eqs. (9)-(11). The plot illustrates that the capacitance steadily increases in the linear deflection regime; however, it drops sharply at a transition pressure ($p_t$) when the deflection shifts to the
nonlinear regime. This transition pressure decreases with increasing pump radius for fixed membrane thickness. The lowered pumping capacitance for nonlinear deflection effectively shortens the characteristic time of the fluidic circuit and thus decreases the steadiness of sample flow. For this reason, the membrane pump was designed such that linearity was maintained up to the applied working pressure of ~ 1 atm.

Figure 6.5: Pumping capacitance for linear and nonlinear deflection. The capacitance steadily increases when deflection is linear, however, it drops sharply at a transition pressure \( p_t \) as the deflection becomes nonlinear. For different pump radii (3.5 mm (solid), 4.0 mm (dashed), and 4.5 mm (dot-dashed)) at a fixed membrane thickness of 1.6 mm and elastic modulus of 1600 kPa, the transition pressure decreases with increasing radius. The transition pressures for each pump are indicated in the figure.

Different thicknesses of the silicone layer were employed to experimentally test the linearity of the membrane pump. Figure 6.6 shows the deflection-pressure relationship of the membrane pump for the two different membrane thicknesses, 0.5 mm and 1.6 mm, at a fixed pump radius of 2.5 mm. The solid and dashed lines in Figure 6.6 are curve fits of the experimental data. For applied pressures up to 103.4 kPa and greater, the deflection of the thicker 1.6 mm membrane remained linear, while the thinner 0.5 mm membrane showed nonlinear behavior beyond \( \delta = h = 0.5 \text{ mm} \), as predicted by theory. The 1.6 mm thickness for the silicone layer, which showed linear behavior throughout the range, was used for all subsequent experimental characterization.
Figure 6.6: Deflection-pressure relationship of the membrane pump for two different membrane thicknesses, 0.5 mm (dashed) and 1.6 mm (solid), at a fixed pump radius of 2.5 mm. For the 0.5 mm thick membrane, linear deflection transitions to nonlinear deflection beyond $\delta = 0.5$ mm, while there is no transition for the 1.6 mm thick membrane. The inset images show the central portion of the pumps as viewed from the side at 20.7 kPa, 55.2 kPa, and 103.4 kPa for the 0.5 mm thick membrane (left), and at 83.6 kPa and 138.1 kPa for the 1.6 mm thick membrane (right). The dashed lines indicate the base of the spherical cap.

An upper bound on the pump radius was calculated using Eq. (4) to ensure the deflection of the membrane pump remained linear up to the applied pressure. The applied pressure was set to $\sim 1$ atm (103.4 kPa), and the maximum deflection was set equal to the membrane thickness (1.6 mm) to maintain linear deflection. The hardness of the silicone, as quantified by the shore durometer, was 40A (McMaster Carr), which correlates to an elastic modulus of $\sim 1600$ kPa [211]. Solving Eq. (4) using these defined parameters yielded an upper bound of 4.8 mm for the pump radius.

6.4.2 Material delamination

Material delamination can lead to leakage and discrepancies in the sample flow. To prevent delamination while maintaining linear deflection, we calculated a lower bound on the pump radius using Eq. (4). The delamination pressure was 1000 kPa (i.e., tensile bonding strength of PMMA and silicone layers [210]), the maximum linear deflection was the thickness of the membrane (1.6 mm), and the elastic modulus was 1600 kPa, which yielded a lower bound of 2.7 mm on the pump radius. Taking into consideration both the lower and upper bounds on the pump radius, 2.7 mm
and 4.8 mm, a radius value of 4.5 mm was selected for subsequent testing to maximize the volume throughput while maintaining linearity.

6.4.3 Measurements of pumping pressure and flow rate

Pump performance as governed by the characteristic time constant, $\tau = RC$ (Eq. (8)), was measured and characterized by: (1) varying the total resistance, $R$, of the downstream fluidic circuit at a fixed capacitance, and (2) varying the total capacitance at a fixed resistance for both DI water and undiluted, anticoagulated mouse whole blood. Figure 6.7 shows the pressure and flow rate outputs of the membrane pump becoming more steady as the fluidic resistance increases, and this is shown quantitatively by the increase in the characteristic time constant from 21 to 63 s for water and from 53 to 97 s for whole blood from small to large fluidic resistor, respectively. However, increasing the total resistance while fixing the total pumping capacitance and volume capacity greatly reduces the sample volume throughput (e.g. reduction in initial flow rate from about 125 $\mu$L/min to about 40 $\mu$L/min for water and from about 50 $\mu$L/min to about 25 $\mu$L/min for whole blood from small to large fluidic resistor, respectively). The flow rate also becomes more steady as the viscosity of the pumped fluid increases. The high viscosity and hematocrit of the whole blood increased the total resistance of the devices although the dimensions of the channels and downstream connections remained the same. The induced high resistance is related to the shear rate in the channels, where at low shear rates the viscosity of the whole blood appears to be higher than it would be at high shear rates [212]. This property of blood flow can be leveraged to even out the flow rate output produced by the membrane pump. Although the form of the pressure/flow rate decay will in general be as shown in Figure 6.7, required flow rates and total downstream fluidic resistance (including diagnostic channels) will be application specific.
Figure 6.7: Comparison of pressure and flow rate outputs for DI water and mouse whole blood for different total fluidic resistances. (a) Pressure and (b) flow rate outputs of the membrane pump for DI water for three different fluidic resistors: small (solid), medium (dashed), and large (dot-dashed) with total resistances of 0.8 kPa min/µL, 1.7 kPa min/µL, and 2.5 kPa min/µL, respectively. The characteristic time constants of the small, medium, and large fluidic circuits were 21 s, 42 s, and 63 s, respectively, with a fixed total capacitance of 0.4 µL/kPa. (c) Pressure and (d) flow rate outputs for mouse whole blood using the same three devices as for DI water. The high viscosity and hematocrit of the whole blood increased the total resistances to 2.2 kPa min/µL, 3.2 kPa min/µL, and 4.0 kPa min/µL, resulting in time constants of 53 s, 74 s, and 97 s for small, medium, and large resistors, respectively. The characteristic time constants were calculated using volume outputs at time intervals of 0.5 minutes, 1 minute, and 2 minutes (as indicated by arrows) given the volume is the time-integral of the flow rate.

For a fixed downstream fluidic resistance, pumping capacitance and volume capacity can be increased by connecting multiple membrane pumps in parallel. This approach was used rather than enlarging a single pump (i.e. increasing pump radius) to maintain linearity for each pump in the network (for membrane thickness, $h = 1.6$ mm and maximum pump radius, $r_{max} = 4.8$ mm). Enlarging a single pump decreases the transition pressure at which the pumping capacitance drops (Figure 6.5). Figure 6.8 shows that the pressure and flow rate outputs become more steady without any decrease in initial throughput as more membrane pumps are added to the parallel network. For water, the measured characteristic time constant increases from 22 to 121 s from the single pump to six pumps in parallel, respectively, and the volume output increases from about 40 to 240
µL. Similarly for whole blood, the characteristic time constant increases from 52 to 321 s from the single pump to six pumps in parallel, respectively, while having the same volume output as water. The observed linear scaling of the characteristic time constant is consistent with theory. The multiple membrane pump strategy thus provides a simple yet powerful way to scale the throughput of the device and enhance the flow control at the same time.

Whole blood samples before and after pumping were viewed under bright-field microscopy for the highest initial flow rates (i.e. small resistor and six pumps in parallel having initial flow rates of about 50 µL/min). For both cases, there were minimal differences in the cell density of blood samples before and after pumping. The maximum shear stress in the devices is ~ 8 Pa given the maximum initial flow rate of 50 µL/min, the cross-sectional area of the channels (~ 0.02 mm²), the hydraulic diameter of the channels (~ 0.22 mm), and the viscosity of the mouse whole blood (~ 5 mPa s) [213]. This value is much lower than the shear stress required to lyse RBC’s, 100 Pa [214]. Therefore, the membrane pump is capable of delivering viable cells (and plasma or sera) downstream for cell-based testing and analysis.

In contrast to existing on-chip pumping mechanisms [41], [42], the membrane pump can provide larger sample volume and higher sample volume throughput for downstream analysis. Specifically, up to 240 µL and about 25 µL/min after 3 minutes of operation for both DI water and mouse whole blood (Figure 6.8b and 6.8d) as compared to previous on-chip pumping methods with sample volume of ~ 5 µL and a maximum flow rate on the order of 10 µL/min. The flow rate output of the membrane pump is also comparable to those of the electrically and pneumatically actuated pumps having maximum flow rates ranging from 10 µL/min to several mL/min [198], [215]. The output here, however, is generated without any electric power or air supply requirements, which makes the device more suited to point-of-care applications in developing settings. The price for this all-mechanical operation is variability in output flow rate over time. As demonstrated here, this variability can be tuned through engineering resistance and particularly pump capacitance, while maintaining operation within the linear deflection regime.
Figure 6.8: Comparison of pressure and flow rate outputs for DI water and mouse whole blood for different total pumping capacitances. (a) Pressure and (b) flow rate outputs of the membrane pump for a single pump (solid), three pumps in parallel (dashed), and six pumps in parallel (dot-dashed) with total capacitances of 0.4 µL/kPa, 1.2 µL/kPa, and 2.4 µL/kPa, respectively. The characteristic time constants for the single, three, and six pumps in parallel are 22 s, 62 s, and 121 s, respectively, at a fixed total resistance of 0.8 kPa min/µL. (c) Pressure and (d) flow rate outputs for mouse whole blood using the same three devices as for DI water. The high viscosity and hematocrit of the whole blood increased the total resistance to 2.2 kPa min/µL for the same total capacitances, resulting in time constants of 52 s, 147 s, and 321 s for the single pump, three pumps in parallel, and six pumps in parallel, respectively. The characteristic time constants were calculated using volume outputs at time intervals of 0.5 minutes, 1 minute, and 2 minutes (as indicated by arrows) given the volume is the time-integral of the flow rate. The pump networks are shown schematically in the figure.

6.5 Conclusions

A device consisting of an on-chip, hand-powered, membrane pump using a robust patient-to-chip syringe interface was presented. This strategy offers safe sample collection, sample confinement, integrated sharps disposal, and controlled sample volume throughput. The membrane pump captures the operator’s mechanical work and delivers fluid to downstream components in a controlled manner. The maximum flow rate output of the membrane pump was comparable to those of electrically and pneumatically actuated pumps, with the advantage of no electrical power
or air supply requirements. The pumping capacitance and volume capacity of the membrane pump was scaled by connecting multiple pumps in parallel. Our approach demonstrated higher sample volume throughput over existing on-chip pumping mechanisms. We anticipate the application of this device to point-of-care applications requiring biologically safe pumping mechanisms in developing settings, exploiting a readily available power source: the human hand.
Chapter 7

Field tested milliliter-scale blood filtration device for point-of-care applications

This Chapter was published in the journal *Biomicrofluidics* and reprinted with permission from [28]. Copyright 2013, AIP Publishing LLC. The Candidate was the first author for this work and played a primary role in the design of experiments, execution of experiments, data collection and analysis, and writing of the manuscript. Additional authors include: Dr. Brendan D. MacDonald, Dr. Trung Vu Nguyen, Dr. Kinh Van Nguyen, and Prof. David Sinton. Their efforts were integral to the publication of this work and are gratefully recognized and appreciated.

7.1 Introduction

A hallmark of microfluidic point-of-care devices has been the ability to use low sample volumes [189]. There are a number of devices that can produce test results using only a few microliters of fluid [41], [42], [216], [217]. However, larger sample volumes are often required for the detection of analytes with low concentration or the incorporation of multiple diagnostic tests on a single device.

In the case of diseases with dilute markers, larger sample volumes are required to ensure that sufficient analyte is present for reliable detection and quantification. In addition to having a volume with sufficient analyte, transport of analyte to the sensor can also be an issue [193], [218]. Fortunately, these transport issues can be addressed by large volume pumping mechanisms [27], [193], [200], [202], [219] and by nanostructured sensors such as flow-through nanohole arrays that enable rapid transport [220], [221], and analyte concentration [222] within the sensing element. While sensor design can aid in the collection of analyte from the sample, the need for large initial sample volumes is fundamental for dilute markers.

In cases where a large number of parallel tests are required to properly assess a patient, larger initial sample volumes are also required. For example, to fully assess a patient suffering from chronic hepatitis B, three to four separate immunodiagnostic tests are generally required in addition to three or more biochemistry tests[140]. A comprehensive hepatitis B analysis chip would require all of these tests multiplexed on one device, each requiring on the order of 10 – 100
µL of sample. Enabling multiplexed tests on point-of-care devices poses new challenges and will require mL-scale upstream sample collection and preparation.

A number of on-chip sample collection and preparation devices have been developed to incorporate the separation process. Traditionally plasma is separated from whole blood through centrifugation; however, this approach does not translate well to point-of-care diagnostics. Most previous on-chip separation devices are designed for finger prick collection and have input blood capacities ranging from 1 to 300 µL [41], [42], [107], [223]–[229]. Obtaining blood from a finger prick in an average adult finger provides between 10 and 20 µL [106]; therefore, there is a practical limit on the amount of blood that can be collected from a patient through finger pricks. Successful blood filtration was demonstrated in a laboratory setting using animal (mouse, rat, and rabbit) blood [223], [225], [226], [228], [230], and human blood [42], [107], [224], [227], [231]. For testing with human blood samples the blood was typically spiked with analyte and/or diluted to facilitate the separation process [42], [224]. Most notably, Homsy et al. [107] developed an on-chip whole blood filtration element that was validated for clinical studies by measuring the adsorption of interleukins through the filtration element. Their device was capable of separating 12 µL of plasma from 100 µL of undiluted whole blood in approximately 10 minutes. Importantly, the work of Homsy et al. [107] demonstrated the suitability of microfluidic on-chip whole blood filters as sample preparation units for clinical studies, for low plasma volume output (~10 µL). On-chip sample preparation with mL-scale whole blood and in-field efficacy with clinical blood samples have not been demonstrated.

Commercial blood filtration systems are also available, such as the in-line Blood Filter (Bemedical Filtration Corp., Plano, TX, USA) and Rapid Plasma Separation Device (patent pending – Advanced Microdevices Pvt. Ltd., Ambala Cantt, India). The in-line Blood Filter is designed for use in cardiopulmonary bypass procedures to remove microemboli greater than 40 µm in diameter (e.g. aggregates of platelets and red blood cells). It is not suitable for the removal of individual red and white blood cells (average sizes of 7 µm and 15 µm in diameter, respectively), which is required for blood plasma production. In contrast, the Rapid Separation Device is designed specifically for plasma production; however, its sample volume capacity and plasma output are low (< 1 mL and < 25µL, respectively).

In this paper, we present a low cost and equipment-free blood filtration device capable of producing plasma from mL-scale blood samples and demonstrate its clinical application for hepatitis B diagnosis. We report the results of in-field testing of the device with undiluted,
anticoagulated whole blood samples from patients at the National Hospital for Tropical Diseases in Hanoi, Vietnam. The plasma generated by the device is compared with plasma generated by centrifugation for red and white blood cell counts, liver enzyme and metabolite levels (e.g. alanine transaminase, aspartate transaminase, urea, and creatinine), and hepatitis B antigen and antibody levels (e.g. hepatitis B “e” antigen, hepatitis B “e” antibody, and hepatitis B surface antibody) related to hepatitis B examinations. The diagnostic tests selected for this study represent the standard hepatitis B panel performed at the hospital. This device provides a practical front-end sample processing method for point-of-care microfluidic diagnostics, enabling integration with multiplexed downstream tests and dilute analyte detection tests.

7.2 Experimental

7.2.1 Device Design and Fabrication

The blood filtration device is designed as a front-end modular sample preparation unit with the capacity to be integrated with downstream on-chip components or as a cartridge into a universal diagnostics system. This approach, as envisioned by our granting agency Grand Challenges Canada in partnership with the Bill and Melinda Gates Foundation, leverages the “plug and play” nature of a universal diagnostics system where the user can tailor the system to a specific need or application by exchanging different cartridges.

The device consisted of a bottom layer of poly(methyl methacrylate) (PMMA) with hydrophilic channels to transport the plasma, a membrane layer for blood filtration, a layer of silicone rubber to prevent leakage, and a top layer of PMMA which provided structural support and formed the top of the syringe port and collection area, as shown in Figure 7.1. The syringe port is shown offset from the chip to facilitate location of the input port during insertion, and it is analogous to the patient-to-chip syringe interface presented in our previous work [27]. In practice, the syringe port can be oriented vertically such that the syringe is inserted into the device without requiring the operator to manually brace the device, as shown in Figure 7.1a. This approach ensures safe, hands-off, bench-top operation of the device. To facilitate testing in the hospital where blood samples were provided in vials, a simple opening in the top PMMA layer was provided for pipetting, in lieu of the syringe port. This method was used at the National Hospital for Tropical Diseases for all subsequent testing of the devices with patient samples. Plasma was collected from the extraction port. In a universal diagnostics system, an integrated device would
not require an extraction port since it could be connected directly to another component for downstream on-chip diagnostic testing.

The bottom layer was constructed of 3 mm thick PMMA micromachined by a CO₂ laser (Universal Laser Systems Inc., Scottsdale, AZ, USA). The channels were cut in the pattern shown in Figure 7.1b using the laser, which resulted in V-shaped channels approximately 350 µm wide and 900 µm deep. After laser cutting, the PMMA pieces were placed in an oven to anneal at 85°C for at least 30 minutes.

Figure 7.1: Schematics of the blood filtration device. (a) Assembled chip shown with syringe for injecting patient blood sample. The syringe port can be oriented vertically such that the syringe is inserted into the device without requiring the operator to manually brace the device. (b) Expanded device illustrates details for each layer.

To facilitate the transport of the filtered plasma along the channels and away from the bottom of the filtration membrane, the channels were coated with Pluronic® F-108 (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), which is hydrophilic and reduces protein adsorption [232]. Contact angle tests with deionized water demonstrated a decrease in the contact angle from 75° for native
PMMA to 25° for the PMMA coated with Pluronic® F-108. The coatings were tested for longevity and no measurable decrease in capillary flow performance was found over a 4 week testing period; therefore, the coatings were deemed suitable for devices being shipped to Vietnam. Immediately following one minute of oxygen plasma treatment (PDC-32G Harrick Plasma, Ithaca, NY, USA), an aqueous solution of 0.01 g/mL Pluronic® F-108 was applied directly into the channels by a pipette (approximately 70 µL). The coated PMMA pieces were then baked in an oven at 85°C for at least 16 hours (usually overnight). After baking, the PMMA pieces were partially covered with tape, so that only the coated channels were covered, to prepare for the bonding stage, which is described below.

The membrane selected for this device was the GR VIVIDTM Plasma Separation Membrane (Pall Corporation, East Hills, NY, USA). It is a hydrophilic asymmetric polysulfone membrane designed for plasma filtration from whole blood. The membrane is approximately 330 µm thick and the capacity of blood filtration is listed by the manufacturer as 40-50 µL/cm² membrane area. The membrane was cut into the shape shown in Figure 7.1b using a scalpel and a stencil. The resulting membrane area for this device was approximately 15 cm². The specified loading volume of blood is 750 µL; however we found from experimenting with volumes up to 1 mL of blood that a membrane of this size is capable of filtering more blood than the specified amount. The efficiency of the membrane for plasma yield is listed as greater than 80%.

A silicone layer was placed on top of the membrane to prevent leakage and to seal the syringe port. The silicone rubber sheet was 1.6 mm thick (McMaster-Carr, Elmhurst, IL, USA). The pattern shown in Figure 7.1b was micromachined using the CO₂ laser after which the silicone was washed thoroughly with water and isopropanol. To seal the membrane, the method of Maltezos et al. [230] using PDMS was attempted; however, it was not possible to prevent PDMS in liquid form from transporting along the membrane surface and into the pores, which clogged the membrane and blocked blood transport. This difference in performance could be due to a difference in the membrane material; the specific membrane used by Maltezos et al. [230] is no longer obtainable from Pall Corporation. Here, a silicone layer was employed to provide an effective seal when bonded using the procedure described below.

The top PMMA sheet was constructed of 1.5 mm thick PMMA, which was micromachined by the CO₂ laser. After laser cutting the PMMA pieces were placed in an oven to anneal at 85°C for at least 30 minutes. The roles of this layer are to (1) form the top portion of the syringe port, (2) seal the blood chamber, (3) provide confinement of the patient sample, and (4) provide
structural support for the device. For testing in the hospital where blood was provided in vials, this top PMMA sheet was cut in the same pattern as the silicone rubber layer so the blood could be applied directly onto the membrane by a pipette.

The layers of the device were bonded using (3-aminopropyl)trimethoxysilane (APTMS, 97%, Sigma-Aldrich Co. LLC., St. Louis, MO, USA). An aqueous mixture of 5% v/v APTMS was pre-heated to 85°C in an oven. The bottom PMMA layer was treated with oxygen plasma for one minute then the pre-heated APTMS was applied to the surface with a pipette (approximately 80 µL), taking care not to apply fluid on the tape-covered channels. The coated PMMA pieces were baked in an oven at 85°C for at least 5 minutes, and the tape was removed. The same procedure was applied to the top PMMA layer. The silicone layer was treated with oxygen plasma for one minute on each side and the complete unit was assembled as shown in Figure 7.1b. The layers were compressed in a heated press (Carver, Wabash, IN, USA) for at least 40 minutes at 85°C under a force of approximately 1300 N. The assembled chip was manually checked for bonding quality then wrapped in aluminum foil and sealed in a plastic bag until use. The time between manufacture in the lab at the University of Toronto and testing at the National Hospital for Tropical Diseases was between seven and twelve days. After shipping, no changes in the sealing or wettability were detected.

7.2.2 Blood cell count, biochemistry, and immunology testing

Device testing was undertaken at the National Hospital for Tropical Diseases in Hanoi, Vietnam. Patient blood samples were delivered in vials and the volume was approximately 2 mL of blood. In most cases 3 mL of blood were drawn from the patient and the tests ordered by their doctor required 1 mL, leaving 2 mL ±0.5 mL of blood available for testing. These volumes varied due to variability between patients and the practices of individual health care providers. Since there was a high probability that the samples were infected with hepatitis B and a possibility with HIV or other tropical diseases, extreme care was exercised during the testing. Only hospital-based medical personnel handled the samples and performed the tests. The testing focused on hepatitis B, an infectious inflammatory illness of the liver. Hepatitis B is prevalent in Vietnam and the National Hospital for Tropical Diseases specializes in diagnosis, treatment, and care for patients infected with the hepatitis B virus.

Biochemistry testing was performed for alanine transaminase (ALT), aspartate transaminase (AST), urea, and creatinine levels. ALT and AST are enzymes associated with liver
parenchymal cells; therefore their levels in plasma provide an indication of liver health. The blood urea nitrogen test is a measure of the amount of nitrogen in the blood in the form of urea. Ammonia is converted into urea by the liver; therefore, urea levels in plasma can indicate the ability of the liver to remove ammonia from the blood stream. Chronic hepatitis B infection can cause liver failure and inhibit the liver from removing ammonia from the blood, which can lead to severe health problems, such as the development of hepatic encephalopathy. Hepatitis B can also result in kidney damage resulting from the deposition of immune complexes in kidney tissue, which leads to increased toxin levels in the blood. Creatinine is one such toxin, which is primarily filtered out of blood by the kidneys. Patients with hepatitis B and high levels of creatinine may be recommended for dialysis to reduce the toxin levels in their blood stream. Testing of these four biomarkers is essential for diagnosing the health status of hepatitis B patients.

Immunology testing yielded positive or negative readings for hepatitis B “e” antigen (HBeAg), hepatitis B “e” antibody (HBe Ab), and hepatitis B surface antibody (HBs Ab). A positive test result for HBeAg 3 to 6 weeks after onset of symptoms indicates an acute active infection at its most infectious period, and means that the patient is infectious. Persistence of HBeAg beyond 10 weeks shows progression to chronic infection and infectiousness. During the acute stage of infection the conversion from HBeAg to HBe Ab indicates that the patient is combating the infection. A positive test for HBs Ab 1 to 4 months after onset of symptoms indicates recovery and subsequent immunity to hepatitis B. HBs Ab can neutralize hepatitis B and provide protection against infection. For this reason, a positive HBs Ab test could also indicate that the patient received a vaccination for hepatitis B. Collectively, these three immunodiagnostic tests reveal much about the hepatitis B infection, particularly with respect to the time of infection and the patient’s immune system response.

The procedure for blood filtration using our devices was performed by a designated doctor at the hospital. Blood was first delivered to the membrane by a pipette. The amount of blood delivered was 0.8 – 1 mL, in one continuous batch. A clean pipette was used to collect the plasma from the extraction port, withdrawn in small (~20 µL) batches at a steady, unhurried pace, until there was no plasma. For manual extraction, the collection time was dependent on the efficacy and speed of the pipette withdrawal and varied considerably. For an integrated device, where manual extraction is unnecessary and continual wicking to downstream components is assumed, we have used the experimental observations to estimate a delivery time of ~5 minutes for the plasma volumes used in our study. Plasma collection is shown in Figure 7.2.
The blood remaining in the vial (~1 mL) was separated using a centrifuge (Hettich Universal 320, Tuttlingen, Germany) and plasma was extracted from the vial with a pipette. The plasma from the device and the centrifuge were labeled and taken for the hematology, biochemistry, and immunology testing. The hematology testing (CBC) was performed for red blood cell (RBC) and white blood cell (WBC) levels. The same technician tested all of the samples. The volume requirements for each of the tests are summarized in Table 7.1. The specifications of the methods used for the testing are summarized in Table 7.2.

Table 7.1: Volume of plasma required for tests commonly performed on hepatitis B patients at the National Hospital for Tropical Diseases.

<table>
<thead>
<tr>
<th>Test type</th>
<th>Analyte</th>
<th>Plasma volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematology</td>
<td>RBC/WBC</td>
<td>50</td>
</tr>
<tr>
<td>Biochemistry</td>
<td>ALT</td>
<td>10 – 15</td>
</tr>
<tr>
<td>Biochemistry</td>
<td>AST</td>
<td>10 – 15</td>
</tr>
<tr>
<td>Biochemistry</td>
<td>Urea</td>
<td>10 – 15</td>
</tr>
<tr>
<td>Biochemistry</td>
<td>Creatinine</td>
<td>10 – 15</td>
</tr>
</tbody>
</table>

Figure 7.2: Collection of plasma from a device during testing at the National Hospital for Tropical Diseases, Hanoi, Vietnam.
Table 7.2: Methods and test kits used for testing.

<table>
<thead>
<tr>
<th>Company</th>
<th>Model</th>
<th>Cell count</th>
<th>Biochemistry</th>
<th>Immunology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sysmex - Japan</td>
<td>XS 1000i</td>
<td>Olympus - Japan</td>
<td>Elecsys 2010</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hitachi – Japan</td>
</tr>
</tbody>
</table>

Test kits

1. Cell Pack (Japan)  
2. Stromatolyser-4DL (Singapore)  
3. Stromatolyser-4DS (Singapore)  
4. Sulfolyser (Japan)  
1. AST (OSR6109)  
2. ALT (OSR6107)  
3. Urea-Urea nitrogen (OSR6134)  
4. Creatinine (OSR6178)  
1. HBeAg  
2. Anti-HBe  
3. Anti-HBs (ROCHE)

7.3 Results and Discussion

A different patient blood sample was used to test each of the 34 devices. The collected plasma output was subsequently tested for remaining red and white blood cells, biochemistry and immunology. Results for each patient and each test were compared with traditional centrifugation sample preparation, as described below.

7.3.1 Volume of filtered plasma

The maximum theoretical yield for 800 µL of whole blood on the membrane is 350 µL of plasma, with a membrane efficiency of 80%. The device has a dead volume of ~ 70 µL; therefore, the maximum theoretical yield for the device is 280 µL of plasma. The volume of plasma produced from each of the 34 devices is summarized in Figure 7.3. There is a wide range of results, and we attribute the discrepancy to both inter-patient and inter-device variation, specifically the deviation in fluid properties between patient blood samples (i.e. viscosity), the manual plasma extraction process, and the inconsistencies of the hydrophilic coating inherent to individual devices. The engineering factors can be overcome with refined manufacturing and downstream integration, but some variation is unavoidable due to inherent differences in fluid properties of patient blood samples.
Figure 7.3: Volume of plasma collected for the 34 devices tested. Initial raw blood volumes were 0.8 – 1 mL, with a maximum expected yield of 280 µL. Variability is largely due to the deviation in fluid properties between patient blood samples (i.e. viscosity), the manual plasma extraction process, and the inconsistencies of the hydrophilic coating inherent to individual devices.

7.3.2 Blood cell count results

Blood cell counts were performed to obtain a quantitative measure of the effectiveness of the devices at filtering out both red and white blood cells from the whole blood. The samples were analyzed successfully except for sample number 11, in which there was an error for the cell counter. The results for the red blood cell counts are summarized in Figure 7.4. The device outperformed the centrifuge in 31 out of 34 cases, with lower red blood cell counts. In comparison to the whole blood levels, shown in the inset of Figure 7.4, the device filtered out an average of 99.9% of the red blood cells. Most biochemistry tests require cell-free plasma (i.e. 99% or better removal of red blood cells) to minimize unwanted matrix effects [233]. The results of the red blood cell counts indicate that the device can produce plasma with purity suitable for biochemistry testing. The results for the white blood cell counts are summarized in Figure 7.5. The device output was comparable to that of the centrifuge process in most cases with the exception of three anomalous results (these higher white blood cell levels did not impact the biochemistry and immunology results, as detailed later). In comparison to the whole blood levels, shown in the inset of Figure 7.5, the device filtered out an average of 96.9% of the white blood cells. Collectively, these results demonstrate successful filtration of both red and white blood cells using the device,
with output levels comparable to (and in the case of red blood cells, improved over) the current centrifugation process.

Figure 7.4: Comparison between the red blood cell counts of the plasma filtered with the devices and separated using a centrifuge. The inset shows the red blood cell counts of the whole blood prior to filtration. The device outperformed the centrifuge machine in 31 out of 34 cases, with lower red blood cell counts. The device filtered out an average of 99.9% of the red blood cells.

Figure 7.5: Comparison between the white blood cell counts of the plasma filtered with the devices and separated using a centrifuge. The inset shows the white blood cell counts of the whole blood prior to filtration. The device filtered out an average of 96.9% of the white blood cells.
7.3.3 Biochemistry results

Biochemistry testing was performed to demonstrate the capability of the devices as upstream blood filtration units for downstream biomolecular diagnostics. For each patient/device, the biochemistry results using plasma obtained from devices were compared to those obtained from the centrifuge. The reference values used by the National Hospital for Tropical Diseases and test precision information for the four biomarkers investigated in this study are listed in Table 7.3. The objective of this testing was to assess the degree of molecular adsorption in the device, and particularly any impact on diagnostic outcome. Due to the high internal surface area of the device, some molecular adsorption by the device is expected; however, it is critical that levels of adsorption do not preclude downstream detection, and the degree of adsorption is consistent and predictable.

Table 7.3: Reference ranges and test precision for biochemistry tests at the National Hospital for Tropical Diseases, Hanoi, Vietnam.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Reference range</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>≤ 40 U/L</td>
<td>0.79 U/L</td>
</tr>
<tr>
<td>AST</td>
<td>≤ 37 U/L</td>
<td>1.06 U/L</td>
</tr>
<tr>
<td>Urea</td>
<td>2.5 – 7.5 mmol/L</td>
<td>0.15 mmol/L</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Male: 62 – 120 µmol/L</td>
<td>2.29 µmol/L</td>
</tr>
<tr>
<td></td>
<td>Female: 53 – 100 µmol/L</td>
<td></td>
</tr>
</tbody>
</table>

The results for the biochemistry tests are presented in Figure 7.6, which illustrates that the levels from the devices were consistently lower than those from the centrifuge. In Figure 7.6 some of the samples have no biochemistry results displayed since there was an insufficient amount of plasma to perform the test. In order to examine the relevance of the reduced biomolecule levels the reference ranges listed in Table 7.3 were scaled according to the difference between the mean values of the two data sets. For ALT, the levels using plasma from the device were, on average,
63% of those from the centrifuge, AST were 87%, urea were 94%, and creatinine were 28%. The reference ranges for the centrifuge from Table 7.3 are shown in Figure 7.6 by dashed lines, and the scaled ranges for the devices are shown as dot-dashed lines. Urea and creatinine have two dashed lines and two dot-dashed lines to represent the lower and upper values of their reference range and scaled range, as shown in Figure 7.6c and 7.6d, respectively. These relative reference ranges enable comparison of the diagnoses resulting from the plasma derived from the device versus the centrifuge.

An analysis of the diagnostic results from the biochemistry tests allows for a qualitative understanding as to the effectiveness of the devices for upstream plasma separation for each of the biomarkers. In Figure 7.6a we see that for ALT the centrifuge results indicate 8 patients above the reference range, and the device results correlate well with those of the centrifuge with the exception of one disagreement above the scaled range (sample #6 in the upper left quadrant) and two disagreements below the scaled range (samples #16 and #19 in the lower right quadrant).
Figure 7.6: Comparison of the biochemistry testing results between plasma derived from the devices and the centrifuge for the four biomarkers, (a) ALT, (b) AST, (c) urea, and (d) creatinine. Reference ranges for the centrifuge are shown by the dashed lines, and reference ranges for the devices – scaled to accommodate adsorption levels averaged over all samples - are shown by dot-dashed lines (scaled 63% for ALT, 87% for AST, 94% for urea, and 28% for creatinine). Urea and creatinine have two dashed lines and two dot-dashed lines to represent the lower and upper values of their reference range and scaled range, respectively.

In Figure 7.6b we see that for AST the centrifuge results indicate 9 patients above the reference range, and the device results correlate well with only one disagreement above the scaled range (sample #12). In Figure 7.6c we see that for urea the centrifuge results are similar to the device results with agreement for the test below the reference range (sample #3). Considering the error precision of the urea test, there were only a few results below the scaled range where the device disagreed with the centrifuge results (samples #1, #7, #8, #12, and #17). In Figure 7.6d we see that there is little correlation between the centrifuge and device results for creatinine (reference ranges and scaled ranges shown on plot are for males), where ten results for the device disagreed with those of the centrifuge (i.e. two results above the bounds of the scaled range but within the bounds of the reference range and eight results below the bounds of the scaled range but within the bounds of the reference range). These results indicate that the device adsorbs a significant and inconsistent amount of creatinine. Collectively, these results demonstrate that for ALT, AST, and urea the device adsorbs a consistent and predictable amount of the biomolecules, and is thus suitable as an upstream plasma filtration unit for downstream testing of these biomarkers.

7.3.4 Immunology results

Immunology testing was performed to demonstrate the suitability of the device for use with hepatitis B immunoassays. Specifically, antigens and antibodies associated with hepatitis B were compared for each patient’s plasma prepared by both the device and the centrifuge. For the 29 immunology tests performed, only the HBe Ab test for sample #23 resulted in a differing test result between the plasma obtained from the device and the centrifuge. Both of these results were close enough to the cut-off value that under normal clinical conditions a follow-up test would be recommended. In this case, it is likely that the test result from the device is more accurate since the patient was positive for HBeAg, and thus unlikely to also be positive for HBe antibodies. Collectively, these 29 test results demonstrate that the device is effective for use as an upstream plasma filtration component for downstream hepatitis B immunodiagnostic testing.
7.4 Conclusions

A device capable of filtering plasma from mL-scale blood samples was presented. A batch of devices was tested in the field with clinical blood samples from patients at the National Hospital for Tropical Diseases in Hanoi, Vietnam. Hematology testing confirmed that the devices were capable of filtering out red and white blood cells, and the plasma collected from the devices contained lower red blood cell counts than plasma obtained from a centrifuge. Biochemistry testing demonstrated that the devices can be used as upstream plasma filtration components for testing ALT, AST, and urea levels. Immunology testing demonstrated that the devices can be used as upstream plasma filtration components for testing HBe antigens and antibodies, and HBs antibodies. The device provides a simple and practical front-end sample processing method for point-of-care microfluidic diagnostics, enabling integration with multiplexed downstream tests.
Chapter 8

Conclusions and future work

8.1 Summary of thesis contributions

This thesis described the development of microfluidic diagnostic technologies for sample collection, sample processing and detection and analysis for disease diagnosis.

8.1.1 Lab-in-a-pen

In Chapter 3, a total analysis system, call the lab-in-a-pen, was developed for hepatitis B screening. Sample collection, whole blood filtration, and endpoint detection in paper-based test strips were provided in an easy-to-use format. Field testing with patient samples was conducted in Vietnam, demonstrating successful detection of hepatitis B virus antigens in patients. In comparison to the other technologies developed in this thesis, the lab-in-a-pen shows the most potential for commercialization. In particular, the familiarity of this system to both end users and manufacturers can expedite its uptake. However, several technical aspects of the device need to be further optimized as part of the commercialization process. These aspects include the inclusion of an indicator or automatic shut-off when sufficient sample volume has been collected, a locking mechanism for the lancet to prevent accidental injury, and more robust assembly of the diagnostic platform to reduce poor contact between components. A larger clinical study is also needed; specifically, more devices need to be tested per patient to obtain a more representative analysis of its performance.

The lab-in-a-pen was envisioned for self-diagnosis, where the end user could be empowered to conduct their own screening as similar to glucose monitoring for diabetes. The exact fit of the lab-in-a-pen for self-diagnosis depends on the target disease, whether or not there is a need to conduct home-based testing for the disease, and the willingness of the end user to self-diagnose. These questions all need to be addressed as a part of the commercialization process. Nonetheless, the lab-in-pen can be readily used in the clinic for the rapid screening of patients.

8.1.2 Ion concentration polarization and paper-based assays

In Chapters 4 and 5, ion concentration polarization was used to improve the detection sensitivity of paper-based assays and to analysis DNA in paper. This approach offers unprecedented analyte
control, independent of wetting history in the paper matrix. For analyte concentration, up to 40-fold improvement in signal intensity of a fluorescent tracer and 5-fold improvement in the detection limit of a model protein were achieved.

ICP capabilities were extended to DNA analysis for the detection of hepatitis B virus DNA and assessment of sperm DNA integrity. A detection limit of 150 copies/mL was achieved for BV DNA without prior viral load amplification, sufficient for early diagnosis of hepatitis B. For the assessment of sperm DNA integrity, the % DFI results from the paper-based devices strongly correlated ($R^2 = 0.98$) with those of the conventional test, providing identical clinical outcomes for tested patients and donors.

Ion concentration polarization offers exceptional analytical capability for paper-based assays. A key issue, however, is reduced target specificity for both protein concentration (Chapter 4) and DNA analysis (Chapter 5). To improve specificity, the most relevant approach is similar to that used in commercial lateral flow assays: capture and detection probes are pre-embedded in the paper-based assay. This approach selectively captures and labels targets that complement the antibody or nucleic acid probe embedded in the assay. By leveraging the directional transport capability of ICP, high efficiency of target-probe hybridization can be achieved by cycling the sample back and forth over the detection region. Portable power is also an issue that needs to be addressed. Currently, the power requirement of the ICP approach is on the order of microwatts and more specifically, the voltage requirement is up to 140 V. These requirements are achievable with a battery-powered high voltage supply with a DC-to-DC converter (< $50 USD, Mouser Electronics Inc.). Joule heating needs to be further investigated, especially its potential effect on blood cell lysis. Overall, ICP offers exciting potential to enable advanced diagnostics in paper-based assays, positioning these low cost platforms for broad application.

8.1.3 Milliliter-scale sample collection and processing
In Chapters 6 and 7, modular units were developed for milliliter-scale sample collection and processing. A hand-powered membrane pump was engineered to store and deliver fluid to downstream components in a controlled manner by leveraging the mechanical strength of the operator. Pumping capacitance and volume capacity was scaled by connecting multiple pumps in parallel. This approach offers safe sample collection, sample confinement, and sharps storage, exploiting a readily available power source: the human hand.
A membrane-based capillary flow-driven blood filtration device was developed for plasma separation. Devices were field tested at the National Hospital for Tropical Diseases. The purity of plasma extracted from the devices contained lower red blood cell counts than plasma obtained from a centrifuge (99.9% removal of red blood cells). The removal efficiency of white blood cells was 96.9%. This lower efficiency for white blood cells is likely due to leaking around the filtration membrane. Further optimization and prototyping is needed to investigate this potential source of error. Extracted plasma samples were used for subsequent biochemical and immunological tests related to the diagnosis and monitoring of hepatitis B, showing comparable biomarker levels as plasma from centrifugation. Overall, the blood filtration device provides suitable plasma separation for downstream multiplexed analysis, having potential for integration into a larger total analysis system.

8.2 Additional markets for microfluidic diagnostic technologies

In addition to infectious disease detection, the microfluidic diagnostic technologies developed in this thesis have potential applicability for other global health sectors, including fertility, food safety, water monitoring, and environmental analysis. In the context of fertility, my colleague, Reza Nosrati (lead author), and I have ongoing work in the area of paper-based technologies for human semen analysis. One such paper-based assay is a home male fertility test analogous to the home pregnancy test for women; however here, male fertility potential is determined through the measurement of sperm viability and motility (Figure 8.1). A metabolic reagent embedded in the paper produces a colorimetric signal for live sperm cells and is used to inform sperm viability. Sperm motility is measured in a similar manner, where viable sperm cells swim through a porous membrane to interact with the reagent in a separate reaction zone. Clinical testing of devices has been conducted at the Royal Victoria Hospital in Montreal, showing comparable performance with conventional methods and promise towards realizing a home male fertility test.
Another assay we are developing leverages the ICP capabilities in paper demonstrated in Chapters 4 and 5. Specifically, ICP is used to assess sperm DNA integrity by separating single-stranded (damaged) DNA from double-stranded (intact) DNA, as shown in Figure 8.2. Sperm DNA is labelled with a dye that fluoresces red for damaged DNA and green for intact DNA. A percent DNA fragmentation index (i.e. proportion of damaged DNA to total DNA) is determined based on these fluorescent signals and quantifies the degree of DNA damage in a patient sample. In contrast to the direct separation method employed in Chapter 5, differentiation of fluorescent signals here provides higher specificity and improves the overall analysis of sperm DNA integrity. The proposed method has been evaluated in a clinical setting against the conventional flow cytometry-based method, demonstrating potential for low cost and scalable assessment of sperm DNA integrity.
8.3 Whole cell analysis using ion concentration polarization

As demonstrated in Chapters 4 and 5, and in section 8.2, ICP is a versatile electrokinetic method for molecular analysis. To date, there has been minimal application of ICP for whole cell analysis in microfluidic devices, which I envision as a potential area of future work. Human cells possess surface charges that are dictated by proteins and glycoproteins embedded within their cell membranes; for example, red blood cells have a net negative surface charge due to sialic acid groups present in their cell membranes [234]. These surface charges, as measured by zeta potential, are directly linked to the maturity and health state of the cells [235]. Therefore, ICP can be potentially leveraged to distinguish different populations of cells for cell-based studies. As a specific application, ICP can be leveraged to enumerate and purify cancer cells from patient whole blood samples, as normal, cancerous, and red blood cells all have different zeta potentials [235], [236].

In summary, microfluidic diagnostic technologies show exciting potential for a number of global health applications. Paper-based assays are well-suited for low cost diagnostics. Incorporating advanced capabilities into these assays has broadened their applicability. Going forward, the challenge lies in translating these proof-of-concept prototypes into viable commercial products.
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