Doctoral thesis in Biotechnology

Centrifugal microfluidics-based point of care diagnostics at resource limited settings

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Centrifugal microfluidics-based point of care diagnostics at resource limited settings

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Public defense of dissertation

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This is the true joy in life, the being used for a purpose recognized by yourself as a mighty one; the being a force of nature instead of a feverish, selfish little clod of ailments and grievances complaining that the world will not devote itself to making you happy.

I am of the opinion that my life belongs to the whole community, and as long as I live it is my privilege to do for it whatever I can.

I want to be thoroughly used up when I die, for the harder I work the more I live. I rejoice in life for its own sake. Life is no "brief candle" for me. It is a sort of splendid torch which I have got hold of for the moment, and I want to make it burn as brightly as possible before handing it on to future generations.

- George Bernard Shaw
Advancements in medical diagnostics have allowed us to understand the underlying mechanism and treat the root cause for many diseases which had been causing morbidity and mortality up until this point in human history. Furthermore, many of the standard diagnostic procedures have now been transformed to provide answers at or near the point-of-care. However, the effects of these positive developments have not trickled down to the parts of our society which are considered underdeveloped and lack the necessary infrastructure and facilities. Diagnostics in such resource limited settings still lag behind and fail to provide the requisite healthcare.

In order to translate the diagnostic solutions designed for central laboratories to resource limited settings, there are certain challenges that need to be addressed, such as portability, reduction in cost and ease-of-use, while keeping the sensitivity and specificity at the required level. The work presented in this thesis focuses on addressing some of these issues by using microfluidics to develop diagnostic platforms that are suitable to be used in resource limited settings.

In paper I, a very low-cost and simple centrifugal microfluidic platform was developed to be used in settings which do not have a reliable supply of electricity. The platform uses a smartphone as a source of power and the sensors of the phone for speed control.

In paper II, a portable and low-cost diagnostic platform was developed for multiplexed detection of biomarkers based on centrifugal microfluidics. The platform uses colorimetric detection and a simple readout method which does not require a spectrophotometer for quantification.
In paper III, a platform was developed for COVID-19 diagnostics which combines centrifugal microfluidics with a novel bead-based strategy for signal enhancement. The platform uses fluorescent detection with a smartphone readout and has the capability to process up to 20 samples at the same time.

In paper IV, as a follow up of paper III, a more advanced platform was developed for COVID-19 diagnostics which allows the operator to carry out nucleic acid amplification in a completely automated manner, from adding the sample to getting the final result.

In paper V, an alternative method for detection of SARS-CoV-2 was developed using electrochemical biosensing. This work combines the electrochemical technique with a flexible printed circuit board for a rapid, amplification-free and label-free detection of target SARS-CoV-2 sequences.

**Keywords:** microfluidics, centrifugal microfluidics, point-of-care, low-cost, diagnostics, agarose beads, immunoassays, colorimetry, fluorescence, cytokines, nucleic acid amplification, isothermal amplification, COVID-19, portable, smartphone, resource limited settings.
Framsteg inom medicinsk diagnostik har gjort det möjligt att förstå och behandla många sjukdomar som tidigare varit en betydande orsak till dödlighet. Trots framstegen har inte alla delar av samhället haft tillgång dessa diagnostiska verktyg på samma sätt, särskilt i resursbegränsade miljöer i låg- och medelinkomstländer. Detta har lett till en ojämlik tillgång till sjukvård. Den senaste utvecklingen inom mikrofluidik möjliggör utveckling av så kallade patientnära analysverktyg till en fraktion av kostnaderna i traditionella labb-baserade tester. För applikationer i resursbegränsade miljöer krävs diagnostiska lösningar som är portabla, kostnadseffektiva och användarvänliga samtidigt som de har hög känslighet och specificitet. I denna avhandling har vi jobbat med framtagande av mikrofluidik-baserade diagnostiska plattformar som är lämpliga för resursbegränsade miljöer. Syftet är att kunna göra avancerade tester på platser där sjukvårdstjänster tidigare varit otillgängliga eller kostsamma att etablera. För att lösa de tekniska utmaningarna har flera nya tekniker utvecklats, bland annat en centrifugalmikrofluidik-baserad plattform.

Centrifugalmikrofluidik är en teknik för att hantera små mängder vätskor med hjälp av en roterande skiva, liknande CD/DVD skivor men i detta fall finns det mikrofluidiska kanaler mönstrade i skivan för att möjliggöra analys. När skivan roterar skapas centrifugalkraft som används för att flytta och manipulera vätskor i dessa kanaler för att utföra ett antal steg som är nödvändiga för att göra bioanalys av olika prover. Det finns olika applikationer för denna teknik inom biologi och vi har utvecklat olika typer av metoder i denna avhandling. I ett av projekten kombinerade vi centrifugalmikrofluidik med en mobiltelefon för att utveckla en diagnostisk plattform som använde mobiltelefonen som strömkälla, analysering av provresultat samt som sensor för att kontrollera rotationshastigheten av rotorn som driver disken med analysen.

Sammanfattningsvis har vi inom denna avhandling utvecklat ett antal patientnära analysmetoder som riktar in sig på utmaningarna i resursbegränsade miljöer. Det är viktigt att utvecklar tekniker som kan användas i dessa miljöer, där infrastruktur och faciliteter är begränsade eller saknas helt. Med hjälp av den senaste teknikutvecklingen inom mikrofluidik tror vi att det är fullt möjligt att utveckla diagnostiska plattformar som är kostnadseffektiva och användas där de behövs som bäst, på ett innovativt sätt.
Preface

The primary focus of this thesis is to employ microfluidic technologies to develop low-cost diagnostic solutions for use at point-of-care in resource limited settings. The thesis provides an overview of the current diagnostic practices, a brief introduction to microfluidics, the working principle of diagnostic tests at a molecular level and a summary of the work. The thesis is divided into five chapters.

Chapter 1 gives an introduction to point-of-care diagnostics and discusses the shortcomings of the healthcare system in the context of resource limited settings and motivates the need for developing low-cost point-of-care diagnostic solutions.

Chapter 2 gives an introduction to the field of microfluidics, and outlines the different types of flow control methods. It also discusses the different types of signal detection and readout strategies.

Chapter 3 gives an overview of different diagnostic tests which are of high significance for resource limited settings and how they work at a molecular level.

Chapter 4 provides a summary of all the publications and manuscripts appended to this thesis.

Chapter 5 contains concluding remarks and provides a future outlook.

Stockholm, May 2023
Ahmad Saleem Akhtar
List of appended papers

Paper I
A smartphone powered centrifugal microfluidic platform for point-of-care diagnostics in resource limited settings

Noa Lapins *, Ahmad S. Akhtar*, Indradumna Banerjee, Inês F. Pinto, Aman Russom
Submitted to IEEE Sensors Journal (Under review)

Paper II
A portable and low-cost centrifugal microfluidic platform for multiplexed colorimetric detection of protein biomarkers

Ahmad S. Akhtar, Ruben R.G. Soares, Inês F. Pinto, Aman Russom
Analytica Chimica Acta 1245 (2023) 340823
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Paper III
Sample-to-answer COVID-19 nucleic acid testing using a low-cost centrifugal microfluidic platform with bead-based signal enhancement and smartphone read-out

Ruben R. G. Soares, Ahmad S. Akhtar, Inês F. Pinto, Noa Lapins, Donal Barrett, Gustaf Sandh, Xiushan Yin, Vicent Pelechano and Aman Russom
Lab Chip, 2021,21, 2932-2944
DOI: 10.1039/d1lc00266j
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Paper IV
Fully automated centrifugal microfluidic platform for COVID-19 diagnostics with computer vision-based readout

Ahmad S. Akhtar, Noa Lapins, João M. Moura, Luis Paula, Adriano Pedro, Fabio Martins, Duarte Mota, Inês F. Pinto, Marco Martins and Aman Russom
Manuscript

Paper V
Flex Printed Circuit Board Implemented Graphene-Based DNA Sensor for Detection of SARS-CoV-2

Samar Damiati, Sindre Søpstad, Martin Peacock, Ahmad S. Akhtar, Inês Pinto, Ruben R. G. Soares, and Aman Russom
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Respondent's contribution to appended papers

Paper I
Major part in the experimental execution, data analysis, and manuscript writing

Paper II
Major part in the experimental execution, data analysis, and manuscript writing

Paper III
Major part in the experimental execution, and a minor part in data analysis and manuscript writing

Paper IV
Major part in the experimental execution, data analysis, and manuscript writing

Paper V
Minor part in the experimental execution, data analysis, and manuscript writing
Contents

List of Abbreviations ................................................................. XV

Chapter 1: Point-of-care diagnostics ....................................... 18
  Evolution of disease diagnostics ........................................... 18
  Access to healthcare ............................................................ 19
  Diagnostics in resource limited settings ................................. 20
  Need for point-of-care diagnostics ....................................... 21
  Recent advances in point-of-care technology ......................... 23
    Benchtop point-of-care devices ......................................... 23
    Handheld point-of-care devices ........................................... 25
  Lessons from the COVID-19 pandemic .................................... 26

Chapter 2: Microfluidics ......................................................... 30
  Introduction ........................................................................... 30
  Flow control methods ........................................................... 32
    Passive flow control .......................................................... 32
    Active flow control ............................................................ 36
  Signal transduction methods .................................................. 40
    Optical detection ................................................................... 40
    Electrochemical detection ..................................................... 42
  Signal readout methods ........................................................ 45
    Smartphone based readout .................................................... 45
    Artificial intelligence-based readout ...................................... 47
Chapter 3: Diagnostic tests used in resource limited settings ......................................................... 50

  Nucleic acid amplification .......................................................... 50
    Polymerase chain reaction .......................................................... 50
    Isothermal amplification methods ........................................... 52

  Immunoassays .............................................................................. 58
    Enzyme linked immunosorbent assays (ELISA) .............................. 59
    Lateral flow immunoassays (LFIA) .............................................. 63

Chapter 4: Present investigations ............................................... 66

  Paper I: A smartphone powered centrifugal microfluidic platform for point-of-care diagnostics in resource limited settings ........................................................................... 68

  Paper II: A portable and low-cost centrifugal microfluidic platform for multiplexed colorimetric detection of protein biomarkers ................................................................. 72

  Paper III: Sample-to-answer COVID-19 nucleic acid testing using a low-cost centrifugal microfluidic platform with bead-based signal enhancement and smartphone read-out ......................................................................................................................... 77

  Paper IV: Fully automated centrifugal microfluidic platform for COVID-19 diagnostics with computer vision-based readout ..................................................................................... 83

  Paper V: Flex Printed Circuit Board Implemented Graphene-Based DNA Sensor for Detection of SARS-CoV-2 ................................................................. 87

Chapter 5: Concluding remarks ............................................... 90

Acknowledgements ................................................................. 92

References .................................................................
List of Abbreviations

AFB — Aflatoxin B1
AIDS — Acquired Immuno Deficiency Syndrome
ART — Antiretroviral Therapy
ASSURED — Affordable, Sensitive, Specific, User-friendly, Rapid and Robust
BIP — Backward Inner Primer
BLP — Backward Loop Primer
BSA — Bovine Serum Albumin
CAD — Computer Aided Design
CBW — Chemical and Biological Warfare
CD — Compact Disc
CE — Counter Electrode
hCG — human Chronic Gonadotropin
CHO — Chinese Hamster Ovary
CIMA — Competitive Immunometric Assay
CMOS — Complementary Metal-Oxide-Semiconductor
COVID — Coronavirus Disease
CRP — C-reactive protein
DALY — Disability-Adjusted Life Year
DC — Direct Current
DI — Deionized
DNA — Deoxyribonucleic Acid
DPV — Differential Pulse Voltammetry
EDC — 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ELISA — Enzyme Linked Immunosorbent Assay
EOF — Electroosmotic Flow
ESC — Electronic Speed Control
EUA — Emergency Use Authorization
EW — Electrowetting
FDA — Food and Drug Administration
FIP — Forward Inner Primer
FLP — Forward Loop Primer
FPCB — Flexible Printed Circuit Board
HCP — Host Cell Proteins
HIV — Human Immunodeficiency Virus
HRP — Horse Radish Peroxidase
HSL — Hue Saturation Lightness
IFN — Interferon
IL — Interleukin
INR — International Normalized Ratio
LAMP — Loop mediated isothermal amplification
LDH — Lactate Dehydrogenase
LED — Light Emitting Diode
LFA — Lateral Flow Assay
LFIA — Lateral Flow Immunoassay
LOD — Limit of Detection
MMOF — Magnetic Metal Organic Framework
MNP — Magnetic nanoparticles
NAAT — Nucleic Acid Amplification Test
NBNM — \( N \)-benzyl-\( N \)-methylethanolamine
NC — Nitrocellulose
NEAR — Nicking Enzyme Amplification Reaction
NHS — N-Hydroxysuccinimide
dNTP — Deoxynucleotide Triphosphate
OTA — Ochratoxin A
PBS — Phosphate-Buffered Saline
PC — Personal Computer
PCR — Polymerase Chain Reaction
PDMS — Polydimethylsiloxane
PEG — Polyethylene Glycol
PLP — Padlock Probe
PMMA — Poly(methyl methacrylate)
POC — Point-of-care
PS — Polystyrene
PSA — Pressure Sensitive Adhesive
PT — Prothrombin Time
RCA — Rolling Circle Amplification
RCP — Rolling Circle Product
RE — Reference Electrode
REASSURED — Real-time connectivity, Ease of specimen collection, Affordable, Sensitive, Specific, User-friendly, Rapid and Robust
RGB — Red Green Blue
RIA — Radioimmunoassay
RNA — Ribonucleic Acid
RPA — Recombinase Polymerase Amplification
RPM — Revolutions Per Minute
RSV — Respiratory Syncytial Virus
RT — Reverse Transcription
SARS — Severe Acute Respiratory Syndrome
SSB — Single-stranded binding protein
TMB — Tetramethylbenzidine
TNF — Tumor Necrosis Factor
TP — Treponema pallidum
WHO — World Health Organization
Chapter 1: Point-of-care diagnostics

Evolution of disease diagnostics

During the course of human history, disease diagnostics has evolved continually. The word “diagnosis” comes from the Greek word “diagnōsis” meaning to discern or distinguish. The earliest known physicians in human history are thought to have lived in ancient Egypt around 2600 BCE relying on physical examination and observation of the symptoms [1]. Hippocrates, born in the 5th century BC, has been called the father of medicine and is credited with the introduction of a holistic approach into medicine [2]. He is also credited with the introduction of humoral pathology, a belief that the human body is composed of four “humors” or fluids (blood, phlegm, yellow bile and black bile), and any disease or illness is caused by an imbalance of these fluids [3]. This theory dominated medicine until the emergence of modern scientific method, when such theories were replaced by more evidence-based methods for diagnosis owing to a better understanding of human anatomy and role of microorganisms in infections. In the 16th century, Antony van Leeuwenhoek observed the microorganisms under a microscope for the very first time but it took another two hundred years, until Robert Koch and Louis Pasteur were able to demonstrate that microorganisms cause infections [4, 5]. We have come a long way and advancements in molecular biology and genetics have helped us understand the underlying mechanisms and treat many different diseases or conditions which were deemed incurable a few decades ago. Despite advances in diagnostics, equal access to quality healthcare for everyone, which is a fundamental human right according to the World Health Organization’s (WHO) constitution, still eludes majority of the human population [6]. Figure 1.1 gives an overview of the disparity in terms of burden of disease in different regions of the world.
In today’s day and age, healthcare systems around the world vary a lot in terms of structure and available resources. However, generally speaking, in any healthcare system the first point of contact for the patients is termed as primary care [8]. Primary care refers to a general practitioner or a family doctor, and the focus of primary care providers is to treat common illnesses. Secondary care is the level above primary care and offers more specialized diagnosis and treatments [9]. Secondary care refers to facilities with specialized doctors such as urologists, cardiologists, neurologists and surgeons. The patients are usually referred to secondary care by the primary care providers. Tertiary care is regarded as the highest level in this classification and refers to doctors in highly specialized facilities where one would receive advanced medical procedures and treatments [9].
Depending on the infrastructure in a country, the ease of access to different levels of healthcare system can be very different for a person living in a remote rural area when compared with a person living in the capital or a major city of the same country. In case of a critical health condition, rapid and accurate diagnosis at the primary care setting, followed by administration of appropriate treatment at secondary or tertiary care setting, can often mean the difference between life and death. Increased access to diagnostics has been accredited to controlling the HIV epidemic in Uganda leading to a 67% reduction in prevalence between the years 1991 and 2001 [10]. Between 2000 and 2016, a 61% reduction in deaths due to HIV was observed in Africa as a result of more accessible diagnostics [11]. According to WHO, scaling up of primary health care interventions in low and middle-income countries could save 60 million lives and result in an increase of 3.7 years in average life expectancy by 2030 [12].

**Diagnostics in resource limited settings**

With the technological advancements of the last few decades, diagnostic methods have improved in terms of reliability and turnaround time as a result of increased automation in laboratories. Diagnostic testing has become an essential part of modern medicine, and its significance is increasing even more due to the threat posed by antimicrobial resistance [13, 14]. However, these advanced technologies are mostly centralized and require substantial infrastructure in terms of skilled personnel and expensive lab equipment [15].

Resource limited settings are characterized by a lack of medical personnel, ill-equipped laboratories and issues related to supply chain management [16]. In such settings, allocation of human and economic resources to diagnostic testing is generally not a priority [17]. These limitations make the transfer of diagnostic guidelines from the developed world to resource limited settings difficult, thus
making standard diagnostics inaccessible to a majority of the population living in remote areas [15]. In the absence of accurate diagnosis, patient treatment is initiated based on clinical signs alone, which can result in misdiagnosis [18].

According to statistics by WHO for the year 2019 [19], 7 out of the top 10 causes of deaths globally were noncommunicable diseases. However, the scenario changes drastically for low-income countries. In low-income countries, 6 out of the top 10 causes of death are communicable diseases with HIV/AIDS, malaria and tuberculosis being in the top 10. Figure 1.2 shows the difference in terms of total burden of disease by cause in the African region and the world as a whole. It is evident that low- and middle-income countries are affected more by communicable diseases as compared to the rest of the world. Most of these diseases are treatable provided they are diagnosed accurately [20]. It is estimated that rapid and more accessible diagnostics for four infectious diseases (bacterial pneumonia, syphilis, malaria, and tuberculosis) alone can prevent more than 1.2 million deaths in the developing world [13]. In order to overcome this disparity, WHO and other health organizations have been motivating the need for innovative diagnostic methods which are applicable to resource limited settings.

**Need for point-of-care diagnostics**

Point-of-care (POC) refers to diagnostic devices or platforms which can be operated, by the end-user themselves or by the healthcare worker deployed in the field or at a health post or peripheral laboratory, with minimal to no training [21, 22]. In the context of resource limited settings, WHO defined some guidelines to evaluate POC tests and termed it as the “ASSURED” criteria which means an ideal POC test should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to the user [20, 23, 24]. In 2019, it was suggested that due to recent developments in digital healthcare, the criteria should be updated
POC tests that fulfill all or some of the above-mentioned criteria can enhance diagnostic testing capability in any healthcare setting. POC tests can be cost-effective, both for the healthcare system and the patients, by reducing the cost per test and reducing the number of referrals to laboratories. POC tests can provide rapid results which means that appropriate treatment can be started at an earlier stage. Getting accurate and quick diagnosis also reduces the risk of spreading the infection as the infected patient can be isolated at an earlier stage. Furthermore, POC tests can provide access to healthcare services in remote rural areas which would, otherwise, be only available in a laboratory as it requires specialized equipment and personnel. Personalized medicine is another emerging benefit of POC tests which can enable healthcare workers to provide personalized treatment to the patients addressing their specific health issues.

Studies have shown that point-of-care tests for HIV have facilitated widespread screening and resulted in an earlier initiation of antiretroviral therapy [26, 27]. In Mozambique, the use of POC tests for CD4+ T cells count increased the rate of initiation of
antiretroviral therapy [28]. On the other hand, rapid tests for malaria have increased the testing rate by 40% and as a result reduced inappropriate antimalarial treatment [15]. Similarly, point-of-care tests for tuberculosis have been reported to have had a positive impact in terms of increase in case detection and reduction in time to initiate treatment [29, 30].

**Recent advances in point-of-care technology**

There is a myriad of point-of-care devices available in the market which are being currently sold commercially to be used at clinics or hospitals or directly by the end-user at home. These devices can vary in terms of their mode of operation or the underlying detection mechanism but generally, any point-of-care device will have some or all of the following features:

- Reagent storage
- Sample delivery mechanism
- Signal transduction
- Signal readout
- Control and communication interface

The commercially available point-of-care devices can be divided into two broad categories as below:

**Benchtop point-of-care devices**

Benchtop POC devices are similar in design and form to the conventional lab instruments. However, as they are targeted to be used in the field or at small clinics, they are smaller in size to make them more portable and have reduced complexity so they can be used by non-technical staff. The benchtop POC devices consist of single-use cartridges and a main instrument. The cartridge has pre-stored reagents and integrated fluidics for automated sample preparation and testing. The main unit has the control mechanism
for sample processing, heating (when required) and signal detection combined with a signal readout.

BioFire® FilmArray® (BioMerieux) is a multiplex PCR test that uses pouches combined with a benchtop analyzer. The pouch has freeze-dried reagents for integrated sample preparation, amplification and detection. BioFire® FilmArray® is available with panels for detection of respiratory and gastrointestinal infections, among other diseases [31]. Cepheid’s GeneXpert® is a real-time PCR technology that uses cartridges with pre-stored reagents which are processed on GeneXpert® systems [32]. Abbott’s ID NOW™ is a molecular point-of-care testing device that uses an isothermal technique, i.e. nicking enzyme amplification reaction (NEAR) for nucleic acid amplification. The sample is mixed directly in the ID NOW™ sample receiver where the lysis of virus and subsequent nucleic acid amplification takes place using the point-of-care ID NOW platform [33]. Cobas b 101 system (Roche Diagnostics) offers HbA1c, lipid panel and CRP testing at point-of-care. The blood sample is directly applied from the finger to a small disc shaped cartridge which is then processed in the benchtop device [34]. Afinion 2™ (Abbott) is another compact and rapid analyzer for quantification of multiple analytes such as HbA1c, Lipid panel and CRP. It uses test cartridges combined with a Afinion 2™ analyzer [35]. Piccolo Xpress (Abaxis) uses self-contained single use discs which can contain up to 14 tests for comprehensive metabolic profiling from 100 μL of blood or plasma sample [36]. Some of the examples of commercially available benchtop point-of-care devices can be seen in Figure 1.3.
Handheld point-of-care devices

Handheld POC devices come in the form of lateral flow assays (LFA) or dipsticks. These devices are portable in the true sense and can be used by the patients at home or by clinicians at patient’s bedside. Depending on the type of test, the sample delivery method to the device can vary. It can be in the form of a drop of blood applied directly to the chip, it can involve dipping the test strip into the sample or mixing the sample in an extraction buffer before applying it to the test strip.
Abbott’s BINAXNOW™ is an immunochromatographic assay which is available to be used for detection of malaria, influenza A&B and respiratory syncytial virus (RSV) [37]. OraQuick Advance® (OraSure technologies) is a rapid HIV test that can be used to provide information about the status of a patient in a single visit [38]. CoaguCheck® XS Plus (Roche Diagnostics) is a handheld device which serves as an anticoagulation monitor and tests prothrombin time/international normalized ratio (PT/INR) [39]. BD Veritor™ Plus System (BD Diagnostics) is a small handheld device which is used for rapid antigen detection of respiratory viruses [40]. Dual Path Platform (DPP®) technology (Chembio Diagnostics) is a lateral flow test that allows multiplexing capability [41]. It has tests for various infectious diseases, tropical diseases and respiratory diseases. It is used in combination with a small handheld analyzer which can read and provide results at the point-of-care. Some of the examples of commercially available handheld point-of-care tests can be seen in Figure 1.4.

**Lessons from the COVID-19 pandemic**

The COVID-19 pandemic has changed the world in many different ways, and one such example was the evolution in the role of diagnostics. The role of diagnostics had been limited to identifying the cause of illness but during the COVID-19 pandemic, it was used as preemptive tool to support public health measures and control the spread of the disease [42]. According to WHO, there have been more than 750 million cases and more than 6.5 million deaths as a result of it [43]. During the early stages of the pandemic, developed countries were able to scale up the laboratory-based testing and contain the spread of virus to some extent. However, as the facilities got overwhelmed by the unprecedented burden of testing, focus was shifted on development and deployment of point-of-care diagnostics. As a consequence of interest from both the public and private sector, targeted solutions towards SARS-CoV-2 diagnosis were developed in a short amount of time [44].
Nayak et al. described the “POC ecosystem” as having technological and non-technological components, and both of these play a major role in developing and providing access to good quality POC diagnostic tests [45]. On the technological side, there is a need for investment in diagnostic research and development to come up with innovative strategies for assay chemistries, assay integration and data analytics. On the non-technological side, problems associated with clinical workflow, regulatory guidance and legislation need to
be addressed in order to remove the hurdles in transferring the technological advancements to the market and the end-user.

Within the technological side of the POC ecosystem, traditionally the focus has been on the development of core technological components such as assay chemistry and integration of assay steps using microfluidics. However, the COVID-19 pandemic brought to attention the importance of having smart and connected instruments which can integrate into the existing healthcare system and share relevant data in a secure and automated way with key stakeholders. In order to stop the spread of an ongoing pandemic, the data generated by decentralized diagnostic testing can be used for surveillance and adopting appropriate public health measures [46, 47]. However, the use of point-of-care tests outside the traditional healthcare settings, such as at-home POC tests, can also pose a challenge to such centralized collection of data. Therefore, developers of new point-of-care tests need to consider solutions for collection and reporting of data based on their specific use case. In this context, smartphones can play an important role as an enhancement to POC tests [44].

On the non-technological side, the shortage of reagents, trained personnel and infrastructure led to an increase in reliance on POC diagnostic tests in certain settings by the clinicians. From the regulatory point of view, “emergency use authorization” or EUA enabled diagnostic tests to be developed and deployed within a matter of weeks. The pandemic also saw huge investment by the governments in development of diagnostic technologies to help mitigate the spread of the virus. A key lesson to be learnt here is that the government’s support for novel diagnostic technologies should not be limited to time of crisis. The continued support of the government for research and development, creating diagnostic workflows and having a testing infrastructure in place is required to be prepared for the next pandemic.
Another key lesson learnt from the COVID-19 pandemic was regarding the inequitable access to healthcare facilities and the fact that these inequalities are further exacerbated in situations like a pandemic [42]. According to SARS-CoV-2 test tracker from FIND, only 38.8% of the tests administered worldwide were used in low- and lower-middle- income countries, even though these countries comprise 76% of the world population [48]. The Lancet Commission on diagnostics found out that 47% of the world population has little to no access to diagnostics and identified digitalization, point-of-care diagnostics and democratization of diagnostics as three main approaches that have the potential to transform diagnostics in the coming years [49].
Chapter 2: Microfluidics

Introduction

Microfluidics is the handling and analysis of fluids constrained in structures that are between sub-millimeter to micrometer scale. This translates to fluids in the microliter to nanoliter range, generally [50]. The benefit of working with fluids at such small scale is on one hand, miniaturization of the device itself, and on the other hand, the physical behavior of the fluid [51]. The physics at such small scale is dominated by effects which are not so dominant at the macroscale, and thus allows for new possibilities and functionalities [52].

Some of the effects that become dominant at the microscale are laminar flow, diffusion, surface area to volume ratio, surface tension and fluidic resistance which are discussed briefly below:

Laminar flow

When the fluid is flowing at microscale, typically the viscous forces will dominate the inertial forces and the fluid exhibits laminar flow which is a condition in which velocity of a particle in a fluid stream is not a random function of time [52, 53]. This type of flow is characterized by a dimensionless number called Reynolds number (Re) which is a ratio between inertial forces and viscous forces. A Re < 2300, generally, indicates a laminar flow and as the number becomes greater than 2300, the flow is considered to be turbulent [52]. Reynolds number is calculated by

$$Re = \frac{\rho \nu D_h}{\mu}$$  \hspace{1cm} (1)

where $\rho$ is the fluid density, $\nu$ is flow velocity of the fluid, $D_h$ is hydraulic diameter of the channel and $\mu$ is the dynamic viscosity.
Diffusion

Diffusion is a process in which a particle moves from an area of higher concentration to an area of lower concentration as a result of Brownian motion. In one dimension, diffusion of a particle is modeled as

$$d = \sqrt{2Dt}$$  \hspace{1cm} (2)

where $d$ is the distance traveled by diffusion, $D$ is the diffusion coefficient of the particle and $t$ is time.

Surface Area to Volume Ratio

As we move from macroscale to microscale, the surface area to volume ratio increases by orders of magnitude [52]. Surface forces (viscosity, interfacial tension, capillary forces) change with the square of the length and volume forces (inertia, gravitational force) change with the cube of the length. Therefore, a 10-fold decrease in one dimension, translates into a ~100-fold decrease in the surface forces and a ~1000-fold decrease in the volume.

Surface Tension

Surface tension is another effect that becomes dominant at microscale. A liquid exhibits surface tension because of the cohesion between liquid molecules at the liquid-gas interface [52]. Because of the dominance of surface tension at microscale, capillary forces become significant and are expressed as the ratio of viscous to interfacial forces.

Fluidic Resistance

Fluidic resistance in a channel is given by

$$R = \frac{\Delta P}{Q}$$  \hspace{1cm} (3)

where $R$ is the resistance of the channel, $\Delta P$ is the pressure differential across the channel and $Q$ is the flow rate [52].
For a circular channel, the fluidic resistance can be calculated as

\[ R = \frac{8\mu L}{\pi r^4} \]  

(4)

where \(\mu\) is the fluid viscosity, \(L\) is the channel length and \(r\) is the channel radius [52].

Owing to the properties of a fluid at microscale, as discussed above, the use of microfluidics leads to a reduction in cost of reagents, reduction in the time for assays, reduction in the volume of sample needed and the possibility to get maximum information from the sample [54]. As a result, microfluidic devices which are commonly referred to as lab-on-a-chip (LoC) have found applications in areas such as biotechnology, pharmaceutics, environmental science and healthcare, among others [50, 55].

**Flow control methods**

For any microfluidic device, flow control plays a key role in its operation [56]. In the context of microfluidics, flow control refers to moving the fluid from one chamber of the chip or device to another chamber in a controlled manner. There are different mechanisms to achieve fluid flow at a microscale and they are generally divided into two main categories: passive flow control and active flow control.

**Passive flow control**

Passive flow control refers to flow control methods that do not require any external power source or driving force and instead rely on the geometry and design of the device combined with fluid properties [56]. Devices with passive flow control have the advantage of not having any moving part or peripheral equipment, but they lack the control to carry out complex fluid manipulation as the flow cannot be controlled in real time [57]. Some common methods for passive flow control are presented below:
Capillary force

Passive flow control using capillary force is one of the most common methods used in microfluidics [58]. Capillary force is used as a driving mechanism with porous substrates such as paper, generally. The intermolecular forces between the liquid and the porous substrate cause the liquid to flow in the narrow spaces [59]. In such devices, liquid flow is a function of the wetting properties and feature size of the porous material [60]. Capillary flow can also be used as a driving mechanism in hydrophilic materials, such as glass and silicon or other solid materials after chemical or gas treatment to render them hydrophilic [58].

Ahi et al. reported a capillary driven microfluidic chip for detection of human chorionic gonadotropin (hCG) protein from urine samples using an immunoassay. The chip, shown in Figure 2.1 (A), consists of four chambers connected via microchannels. The interface between the microchannels and chambers forms a capillary burst valve allowing the loading of chambers with different reagents without any overflow. Magnetic metal organic framework (MMOF) nanoparticles modified with anti-hCG antibodies are used for preconcentration of hCG from urine samples. The MMOF nanoparticles are moved through different chambers using a magnet to complete the immunoassay [61].

Lateral flow assays, which employ capillary force driven flow, are one of the most common tests that are being used in healthcare, food safety and environmental monitoring because of their ease of operation and low cost [62]. Conventionally, LFAs have been used with a colorimetric readout using naked eye which provides only a qualitative result, e.g. pregnancy test, and have only been used for applications where high sensitivity is not required. However, recently a lot of efforts have been made to improve the sensitivity of LFAs by improving the detection mechanisms [63].
Gravity-driven

Gravity-driven flow is one of the simplest methods of passive flow control in which two reservoirs are connected through microchannels and the height difference between them allows the liquid to flow under the action of gravity [64]. Xiang et al. used a gravity driven microfluidic chip for quantification of aflatoxin B1 (AFB1) in food samples. The chip uses competitive immunometric assay (CIMA) on a gravity-driven chip which has four regions containing labeled target capture antibody, reaction channel, beads conjugated with antigen for competitive immunity and output signal. The layout of the chip is shown in Figure 2.1 (B). The fluid flow in the chip is controlled by manipulation of the tilt angle [65].

Surface tension

Surface tension of the liquids can also be used as a driving force where two droplets of different sizes connected by a closed channel can cause the liquid inside to move because of the difference in their surface tension [58]. Groot et al. used surface tension driven microfluidic platform for hanging droplet culture. Hanging droplet technique is a common method for 3D culture of tissues. This work combines open microfluidic with hanging droplet culture to provide an easy-to-use platform for 3D tissue culturing. The device consists of two hanging droplets which are connected and the pressure difference between the two droplets allows fluid flow from one droplet to the other as shown in Figure 2.1 (C). It enables long term culture of shear sensitive cells [66].

Finger actuated

Another approach for passive flow control is to apply pressure using fingers. Such devices need the user to apply pressure at a spot in order to initiate the liquid flow [64]. Park et al. presented a finger actuated microfluidic device for blood cross-matching test. Blood cross-matching test is used to identify if the blood of the donor is
Figure 2.1: Examples of passive flow control methods in microfluidics. (A) Capillary force driven microfluidic chip for detection of hCG protein in urine samples. (B) Gravity driven chip for detection of aflatoxin B1 in food samples. (C) Surface tension driven system for hanging droplet 3D tissue cultures. (D) Finger actuated device for cross-matching test before blood transfusion. Adapted from [61, 65-67] with permission.
compatible with the blood of the recipient. The schematics of the device is shown in Figure 2.1 (D). With a few finger actuation steps, the blood plasma is separated from the whole blood and cross-reacted to check for transfusion stability. The device is designed to not be affected by the pushed depth of the pressure chamber to minimize user-dependent errors [67].

**Active flow control**

Active flow control refers to flow control methods that employ an external power source or driving mechanism to achieve fluid flow. The fluid can be driven through electrical, mechanical or centrifugal force [56, 59]. Active flow control methods, although being more complex to implement, provide a solution to the problems faced by passive control [57].

**Mechanically-driven**

A very commonly used method in laboratories for active flow control is the use of syringe pumps [56, 68]. A syringe pump uses a stepper motor to push the plunger of a syringe which is connected to the microfluidic chip through a conduit. As the plunger of the syringe moves, the fluid inside the syringe gets pushed through the conduit into the microfluidic channel. The syringe pump can also be used as a negative pressure pump where the conduit is connected at the outlet and the inlet is connected to one or more reservoirs with fluids of interest. The syringe can be filled with any buffer or liquid and instead of pushing the plunger of the syringe, it is pulled to create a negative pressure in the channel which allows the fluid from the reservoir to flow into the channel. The syringe pump can be used to control the flow based on flow rate or the volume dispensed.

As syringe pumps are usually bulky and expensive, their application in portable microfluidic devices has been limited [62]. Zhang *et al.* developed a plug-and-play syringe pump for use in low-cost point-of-care testing applications. The pump uses a compression spring
mechanism for driving fluid and a flow regulatory chip with three passive valves for controlling the flow rate. Irrespective of the pressure due to the spring compression force, flow regulation is realized through the flow regulatory chip. The schematics and design of the portable syringe pump are shown in Figure 2.2 (A). The portable plug-and-play syringe pump was used for particle separation in an inertial microfluidic chip [69].

**Electrically-driven**

Another method used for fluid manipulation in microfluidics is called electroosmotic flow (EOF). This type of flow is based on the property that an electrical double layer (Debye layer) is formed at the interface of liquid and solid. This double layer is the result of the inherent charge of the solid surface and the ions in the solution achieving an equilibrium close to the surface of the solid. When an electric field is parallel to the channel, the ions experience a force towards or away from the electrode and drag the liquid along which results in net motion of bulk fluid inside the channel [70]. Another method for liquid manipulation using electric field is called electrowetting (EW). In EW the wettability of a liquid on a surface is changed by changing the applied potential and droplet manipulation such as formation, splitting and merging can be achieved [62]. Li et al. presented a microfluidic device that uses a conductive substrate and droplets with ionic surfactants. As a potential is applied, the ionic surfactants inside the droplet move away or towards the surface, resulting in a change in the contact angle [71].

Coudron et al. presented a fully integrated digital microfluidic platform for carrying out an immunoassay in an automated manner using electrowetting. They demonstrated automated detection of four classes of chemical and biological warfare (CBW) agents. The digital microfluidic platform integrates all the steps for a chemiluminescent immunoassay. The assay is performed in a faraday cage to avoid any electromagnetic interference. A vertically
movable magnet controlled by a microcontroller and a DC motor is used for magnetic separation. The overview of the setup for the chip is shown in Figure 2.2 (B). For droplet actuation, a command is sent from the PC to a microcontroller to switch on specific channels at the drive voltage. The microcontroller connects the high drive voltage with the selected channels and disables it after a specified time [72].

**Centrifugal force**

One way to achieve active flow control while using minimal external equipment is the use of centrifugal force. This approach is commonly called lab-on-a-disc or centrifugal microfluidics. Centrifugal microfluidics only requires a rotor to achieve the driving force for fluid flow [68]. The microfluid chip, in this case, is shaped like a compact disc (CD) with microfluidic chambers and channels on it. As the disc is rotated, the fluid gets pushed in the radially outward direction. The flow rate of the fluid is a function of rotational speed, channel geometries and radial position [56]. Different types of valves can be integrated in the design to achieve sequential flow of fluids in order to perform multi step assays.

As centrifugal microfluidic devices require only a motor for their operation, they are particularly suitable for development of portable devices to be used in the field which have all the components (heating, detection, etc.) for immunoassays or nucleic acid testing integrated. Liu et al. developed one such platform for bacterial detection using loop mediated isothermal amplification. The microfluidic disc has a lysis chamber and a reagent chamber which lead into a mixing chamber before being aliquoted into reaction chambers with pre-stored primers as shown in Figure 2.2 (C). The output signal is colorimetric and it is detected using a color sensor [73].
Michael et al. presented an alternative way to generate centrifugal force by using a fidget spinner for point-of-care diagnosis of urinary tract infection as shown in Figure 2.2 (D). The device allowed on site detection of infection in 50 min with a readout using naked eye. The device was also used for antimicrobial testing [74]. Similarly, Zhang et al. used a hand powered centrifugal microfluidic platform for sample to answer nucleic acid testing [75]. Lin et al. used a similar concept for chitosan bead-based ELISA [76].

Figure 2.2: Examples of active flow control in microfluidics. (A) Design and schematics of a portable plug-and-play syringe pump. (B) Overview of the electrowetting chip setup for automated immunoassay to detect chemical and biological warfare (CBW) agents. (C) A portable centrifugal microfluidic platform for bacterial detection using loop mediated isothermal application. (D) A centrifugal microfluidic chip inspired by fidget spinner for detection of urinary tract infection. Adapted from [69, 72-74] with permission.
Signal transduction methods

In any chemical or biological assay, a signal transduction strategy is needed to convert the chemical or biological event happening at the microlevel into a signal which can be measured using a sensor in order to get a qualitative or quantitative output. In microfluidics, as the volumes of reagents and samples are reduced considerably, this task becomes even more challenging requiring signal transduction strategies and sensors that can register and measure signals at a microscale.

Optical detection

One of the most widely used detection strategy in microfluidics is optical detection. Optical detection, as the name suggests, measures some form of light signal. The optical detection methods can be further subdivided into the following categories:

Absorbance

Absorbance based detection methods are based on the property of the light that it loses its energy as it passes through a sample as shown in Figure 2.3 (A). The absorption of light energy passing through a medium follows the Beer-Lambert law which can be expressed as:

\[ A = \varepsilon lc \] (5)

where \( A \) is the absorbance, \( \varepsilon \) is the absorption coefficient of the attenuating species, \( l \) is the optical path length and \( c \) is the concentration of the attenuating species.

According to (5), a smaller optical path length, which generally is the case in microfluidics, would result in less attenuation of light and the decrease in the light energy would be directly proportional to the concentration of the analyte being measured. A commonly used strategy is to use Horse Radish Peroxidase (HRP) enzyme
conjugated to detection molecule, which generates a colorimetric signal, when it reacts with a suitable substrate, which is visible to the naked eye and can be quantified by measuring absorbance at 450 or 630 nm.

**Fluorescence**

Fluorescence is the property of a substance to emit a light wave of a certain wavelength when excited by another light wave, which is different from the emission wavelength. At the atomic level, this is a result of absorption of light energy by the electrons which get excited to a higher energy level. As the electrons go back to the stable energy state, the difference in the energy levels is emitted as a photon. A schematic representation of this phenomenon is shown in Figure 2.3 (B). The relation between energy of the emitted photon and its wavelength is expressed as:

$$ E = \frac{hc}{\lambda} \quad (6) $$

where $E$ is the energy of the light wave, $h$ is the Planck’s constant, $c$ is the speed of light and $\lambda$ is the wavelength of light. According to (6) the wavelength of emission would be higher than the excitation wavelength as it has lower energy. For fluorescence-based detection, detection molecules are labelled with fluorescent dyes. Since different fluorescent dyes can have different excitation and emission wavelengths, it provides a method for multiplexing in the same chamber which can be resolved based on the use of appropriate optical filters.

**Chemiluminescence**

Chemiluminescence is the phenomenon in which a photochemical signal is generated as a result of a chemical reaction. In this case, the signal is generated when an exothermic reaction excites a molecule to a high energy state and as it relaxes back to its ground state, it emits a photon within the visible or near infrared region \[77\]. A schematic representation of this process is shown in Figure
2.3 (C). The intensity of the emitted light can be correlated to the concentration of the analyte. There is no need for an excitation source like fluorescence detection, which eliminates any noise or background emission. A common method to generate chemiluminescent signals is using Horse Radish Peroxidase as an enzyme to catalyze the oxidation of Luminol by Hydrogen Peroxide.

![Optical detection methods](image)

Figure 2.3: Schematic representation of the optical detection methods. (A) During absorption, the wavelength of the light remains the same while the intensity is reduced after passing through the sample. (B) Representation of excitation of an electron from ground state to an excited state when excited by a light of specific wavelength followed by emission at a wavelength larger than excitation owing to loss of energy due to vibrations. (C) Representation of excitation of an electron from ground state to excited state as a result of a chemical reaction and subsequent emission as it relaxed to its ground state.

**Electrochemical detection**

Electrochemical detection makes use of the chemical/biological changes taking place at or close to the surface of an electrode which is then quantified by translation into an electrical signal. The change is measured in terms of current (I), voltage (V), resistance (R), conductance (G) or phase shift (θ) [78].

Three-electrode system is one of the most common setups used for electrochemical measurements [79, 80]. It consists of three electrodes: (i) Reference Electrode (RE), (ii) Counter Electrode (CE) and (iii) Working Electrode (WE) as shown in Figure 2.4 (A). The reference electrode is electrically isolated and has a constant
potential. The counter electrode is used to complete the current loop and the working electrode is where the redox reaction takes place and a signal is generated. WE and CE are immersed in the solution under investigation and RE is electrically isolated but connected through a salt bridge [80].

When an electrode with an excess charge is placed in a solution, an electrical double layer is formed at its surface as shown in Figure 2.4 (B). The inner part of the double layer, called inner layer, is formed as a result of oppositely charged ions balancing the excess charge on the electrode. The second layer, known as diffuse layer, is made up of oppositely charged ions with a concentration that decreases exponentially with distance. In an electrochemical process, the analyte moves from the bulk solution to the electrical double layer by diffusion, migration or convection and participate in a redox reaction [80]. The electrochemical techniques can be further subdivided into two broad categories:

Amperometry

Amperometry is a technique in which a constant potential is applied between the WE and CE and the current is measured. The current I is recorded as a function of time t. As the redox reaction takes place at the electrode surface, the current at WE is proportional to the flux of analyte to the electrode surface which, in turn, is linearly dependent on the concentration gradient of the analyte. Initially, the analyte near the double layer is depleted which results in a high current. As the current continues to flow, the region with reduced analyte concentration is extended further which results in a decline in current. The relationship between time and the magnitude of the current can be related to the concentration of analyte in the solution.

Voltammetry

Voltammetry is a more common technique used in electrochemistry. In voltammetry, an electric potential E is applied
between the WE and CE and the resulting current I is measured. Unlike amperometry, where a fixed potential is applied, E is varied as a function of time in case of voltammetry. As E becomes more positive, the analyte is oxidized and as E becomes more negative, the analyte is reduced. Both the steps, oxidation and reduction, have a peak current associated with them which can be used to determine the analyte concentration. Some types of voltammetry are linear sweep voltammetry, differential sweep voltammetry and stripping voltammetry.

Figure 2.4: (A) Illustration of electrical double layer formation when an electrode is placed in an electrolyte solution. (B) Simplified schematics of three electrode setup used in electrochemistry. Representative plots for (C) amperometry and (D) cyclic voltammetry. Adapted from [80] with permission.
Signal readout methods

At the completion of an assay, a signal is generated indicating presence or absence of the analyte of interest. In order to measure and quantify this signal, a signal readout method is needed. The signal readout method plays an important role in microfluidics-based assays as the sample and reagent volumes are reduced considerably compared to the macroscopic counterparts [81]. In the context of point-of-care diagnostics in resource limited settings, the signal readout method becomes even more important as its cost and practicality can greatly affect the overall applicability of any point-of-care solution [82].

Conventional signal readout methods inside the lab, such as microscopes and plate readers, are expensive and bulky which makes them unsuitable to be carried into the field. Furthermore, they require trained and skilled personal for operation which further limits their use in point-of-care diagnostics [83]. A simplified signal readout method can make it possible to carry out sample-to-answer diagnostic tests in the field because of their portability, low-cost and ease-of-use [82].

Smartphone based readout

Recent advancements in smartphone technology and the widespread usage, especially in low- and middle- income countries, has motivated the researchers to use smartphones for point-of-care diagnostics [84, 85]. Smartphones are being used in combination with microfluidic platforms for processing data, displaying results, transmitting information or instructions, controlling devices and as a power supply [86]. One area in which the smartphones have been used recurrently, is the detection module by making use of the complementary metal oxide semiconductor (CMOS) cameras [83, 87]. Smartphones are equipped with high resolution cameras which can be used for visual imaging [86].
Zheng et al. reported a microfluidic device that uses smartphone imaging for detection of *Escherichia coli* O157:H7 as shown in Figure 2.5 (A). The device uses magnetic nanoparticles (MNP) modified with capture antibodies and polystyrene microspheres (PS) which are conjugated with detector antibodies and catalases. The nanoparticles and microspheres are mixed with the sample in the mixing channel of the chip and the MNP-*E.Coli*-PS complex is captured in the separation chamber. Afterwards, hydrogen peroxide is added and it gets catalyzed by the catalases on the complexes. A mixture of gold nanoparticles (AuNPs) and crosslinking agent is mixed with the catalysate in the second mixing channel where a color change in AuNPs is observed. The color change from blue to red is detected and quantified using a smartphone app for Hue-Saturation-Lightness (HSL) measurement [88].

Sun *et al.* reported a portable microfluidic platform that uses smartphone-based fluorescence imaging for detection of viral and bacterial pathogens causing equine respiratory infections among horse population. The microfluidic chip is shown in Figure 2.5 (B). The system uses loop mediated isothermal amplification (LAMP) for nucleic acid detection in a multiplexed manner. Silicon microfluidic chips were used to carry out LAMP assays by having the primers dried on the chip. A custom-designed cradle was used for fluorescence imaging. Eight blue LEDs combined with a short pass optical filter were used as the excitation source. A long pass optical filter placed in front of the macro lens served as the emission filter allowing only the fluorescent emission of EvaGreen DNA-intercalating dye to reach the camera. The green channel of the RGB images was used for analysis [89].

Zangheri *et al.* used a smartphone for chemiluminescence based detection of ochratoxin A in wine and coffee. Ochratoxin A (OTA) is a common mycotoxin that can contaminate food. Lateral flow immunoassays (LFIA) are used as a screening tool to detect OTA contamination. A low-cost and disposable cartridge, shown in
Figure 2.5 (C), integrating all the reagents and a nitrocellulose strip is used which can be operated by very simple manual steps and thus, can be used at the point-of-need. The analysis is based on competitive immunoassay format in which OTA in the sample and OTA conjugated with HRP compete for binding with anti-OTA antibodies on the nitrocellulose strip. Addition of Luminol/H$_2$O$_2$ substrate generates a chemiluminescent signal which is inversely related to the concentration of OTA in the sample. The signal is measured by taking images using 4-sec exposure time which are analyzed by ImageJ software [90].

Low et al. developed a smartphone-based electrochemical biosensor for detection of circulating microRNA-21 in saliva samples. The portable system uses a disposable screen-printed biosensor which is modified with reduced graphene oxide/gold composite. A circuit board was designed for electrochemical detection using the biosensor and a customized Android app communicates with the circuit through a Bluetooth module. For detection of miR-21, a synthetic ssDNA probe is immobilized on the electrode and as the miR-21 in the sample hybridizes with the probe, a decrease in the peak current is observed with increasing concentration of miR-21. The workflow of the assays and a schematic of the device is shown in Figure 2.5 (D). The mobile phone is used to send commands and perimeters to the circuit board for electrochemical sensing and the resulting data is sent back and displayed on the phone [91].

**Artificial intelligence-based readout**

An active area of research in microfluidics is the use of artificial intelligence for different applications. One such area in which artificial intelligence is being used is image processing and data analysis. Quite often, the analysis of data generated by imaging takes more time than the image acquisition itself [92]. Image-based artificial intelligence applications are being used for different tasks such as image enhancement, image reconstruction, colorimetric
quantification, classification, segmentation and denoising. Depending on the application, task to be performed and the amount of data to be analyzed, a traditional machine learning algorithm or a deep learning algorithm could be used [83].

Kühnemund et al. used machine learning algorithms for counting rolling circle products (RCPs) using a multi-modal mobile phone-based microscope. The setup was used for targeted DNA sequencing and *in situ* point mutation analysis. A 3D printed attachment was fitted with battery powered laser diodes for excitation. The fluorescence signal passed through a multi-band emission filter and was collected by a lens placed in front of the mobile phone camera. Images were acquired in lossless digital negative format with a 4-sec exposure. A machine learning algorithm was developed to count the RCPs from the acquired images [93].

Turbé et al. used deep learning algorithms to classify images of rapid human immunodeficiency virus (HIV) tests acquired in rural parts of South Africa as positive or negative. This combination of diagnostics with artificial intelligence and mobile connectivity has the potential to help in devising disease control strategies, preventing outbreaks and increasing efficiency of healthcare systems in low- and middle-income countries [94].

Holmström et al. used an artificial intelligence model based on deep learning for analysing microscope slides that had been digitised at the point-of-care. The diagnostic system was used for screening of cervical cancer in resource limited settings and as an alternative to conventional cytology screening which is quite labor intensive. The novel POC digital diagnostic system was tested at a rural clinic in Kenya, where the prevalence of cervical cancer is quite high. The developed system uses a portable whole-slide microscope scanner for digitisation of samples (Papanicolaou smears). The images are compressed and uploaded to a machine learning platform via 3G and 4G mobile networks. The results were then compared with
analysis of physical slides by trained pathologists. The results showed that the deep learning system has high accuracy compared to the visual interpretation of slides [95].

Figure 2.5: (A) Schematics of the microfluidic chip and the method of detection for *Escherichia coli* O157:H7. (B) Schematics of the portable platform used for smartphone-based fluorescence imaging to detect bacterial and viral pathogens. (C) Disposable cartridge used for smartphone-based chemiluminescence detection of ochratoxin A in wine and coffee. (D) Smartphone based electrochemical biosensor used for detection microRNA-21 in saliva samples. Adapted from [88-91] with permission.
Chapter 3: Diagnostic tests used in resource limited settings

As discussed in Chapter 1, conventional diagnostic methods have several limitations when it comes to their application at the point-of-care, especially in resource limited settings. Thus, miniaturization of these methods using microfluidics can circumvent these problems [96]. In this context, some common diagnostic tests which are of high significance for resource limited settings are presented below with a focus on microfluidics:

Nucleic acid amplification

Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are the molecules that exist in all living organism and contain the genetic information for synthesis of life [97, 98]. As a consequence, nucleic acids have become an important tool for pathogen detection and identification as each species has its own unique nucleic acid sequence [99]. A key step to achieve this is nucleic acid amplification to generate million fold copies of the target of interest [100]. Nucleic acid amplification techniques (NAATs) have revolutionized diagnostics and have also found widespread use in fields such as genetics, forensics and agriculture [101, 102]. NAATs have advantages such as higher sensitivity and specificity compared to traditional diagnostic methods such as immunoassays [97, 100].

Polymerase chain reaction

Polymerase chain reaction (PCR) is the earliest detection method used for nucleic acid amplification and to this day it remains the gold standard for nucleic acid-based diagnostics owing to its high sensitivity and specificity [97, 99, 100]. PCR was developed and reported for the first time by Kary Mullis in 1985 [103]. In 1992, Higuchi et al. reported the development of real-time PCR that
allowed the amount of product formed during PCR to be monitored in real-time [104]. Real-time PCR, also known as quantitative PCR (qPCR), made it possible to calculate the initial number of target molecules present in the sample [105].

Polymerase chain reaction can be used for amplification of single or double stranded DNA template. It requires two oligonucleotide primers specific to the target, dNTPs, thermostable polymerase and magnesium ions in the buffer. The reaction takes place in a cyclical manner in which three steps are repeated. These three steps are: (i) denaturation, (ii) annealing and (iii) elongation, as shown in Figure 3.1. Denaturation typically takes place at 95°C which causes the two strands of DNA to separate. The temperature is then lowered to around 50°C to allow the primers to anneal to the DNA template. For the final step of the cycle, temperature is raised to 72°C to allow polymerase enzyme to extend the primers using dNTPs [106, 107].

![Diagram](image-url)

**Figure 3.1:** Schematic illustration of a single PCR cycle. Adapted from [106] with permission.
Kopparthy et al. developed a microfluidic chip for PCR that uses oscillating flow through temperature gradients for continuous-flow PCR. The microfluidic chip has a simple channel geometry and uses a syringe pump to introduce sample into the channel and to achieve oscillatory flow through temperature gradients. Fluorescence signal is acquired in real time for detection and analysis. A heating system combined with a controller maintains the thermal gradient across different sections of the microfluidic chip. A sample to result time of 30 min was reported based on melting curve analysis [108].

**Isothermal amplification methods**

As explained in the last section, PCR requires precise thermocycling which needs complex and bulky instrumentation. This makes it unsuitable for use in the field or in resource limited settings and has limited the application of PCR based nucleic acid detection to centralized laboratories [97, 99, 101]. In order to overcome this limitation, isothermal amplification techniques have emerged as an alternative to PCR. Isothermal amplification, as the name suggests, requires a constant temperature for nucleic acid amplification. This simplifies the instrumentation requirements to a great extent and reduces the cost, thus making these techniques amenable to miniaturization for use in resource limited settings [109, 110]. Some commonly used isothermal amplification techniques are described below:

**Loop-mediated Isothermal Amplification (LAMP)**

Loop-mediated isothermal amplification was developed by Notomi et al. in 2000 [111]. LAMP uses a set of four (or six) primers which bind to six (or eight) specific target regions and it has been reported to achieve excellent specificity [112]. A set of LAMP primers includes two outer primers, described as forward outer primer (F3) and backward outer primer (B3), and two inner primers, described as forward inner primer (FIP) and backward inner primer (BIP). Two additional loop primers, called forward loop primer (FLP) and
backward loop primer (BLP) are also used to accelerate the amplification process [113].

The LAMP amplification process can be divided into two phases as shown in Figure 3.2. At the end of the initial phase, a dumbbell like structure is formed which serves as a template for the subsequent phase i.e., cyclic amplification. In the initial phase, the F2 of FIP hybridizes to F2c region of the target and F3 anneals to the F3c region of the template strand. Bst DNA polymerase starts synthesizing a complementary strand from the 3´ end of the F2 region in FIP. F3 primer anneals to the F3c region on the DNA strand and starts displacing FIP-linked complementary strand while synthesizing a complementary strand to the target DNA. A double strand is formed as a result of this. The FIP-linked complementary strand is displaced as a single strand and this strand forms a stem loop structure as the FIP also contains a F1c and F1 region at the 5´ end. This single strand serves as the starting point for BIP and the same process is repeated at the other end which results in a double stranded DNA and a single stranded BIP-linked complementary strand. The BIP-linked complementary forms a dumbbell like structure and serves as the starting structure for cyclic amplification. In the cyclic amplification phase, the dumbbell structure is converted into a stem-loop DNA as a result of self-primed DNA synthesis. F2 sequence in FIP hybridizes with F2c of the dumbbell structure and starts strand displacement DNA synthesis. A stem-loop structure is formed at the 3´ end because of B1 and B1c regions. Then DNA synthesis starts from 3´ of B1 and the FIP-linked complementary strand is released. This released single strand forms a dumbbell structure as the ends have F1-F1c and B1-B1c complementary regions. This dumbbell structure is a turn-over of the initial dumbbell structure and it undergoes the same process through BIP in which B2 anneals to the B2c on the loop and repeats the DNA synthesis process [113, 114].
Nguyen et al. developed an RT-LAMP based diagnostic platform that provides real time detection of SARS-CoV-2. The platform is powered by a battery, making it portable and uses Internet-of-
Things (IoT) for real time connectivity to transmit data to a smartphone through Wi-Fi. The platform uses an integrated microfluidic chip which has a lysis chamber and four detection chambers to detect three different targets and a negative control. The real-time fluorescence signal is measured through a CMOS camera and transmitted to the smartphone. The platform uses a Raspberry Pi 4 as control unit [116].

Rolling Circle Amplification

Rolling circle amplification (RCA), when combined with the use of Padlock Probes (PLPs), is a powerful and highly specific isothermal amplification method. It generally requires four components i.e., padlock probe (PLP), DNA or RNA ligase, Phi29 polymerase and deoxynucleotide triphosphate (dNTPs), to generate long single stranded products [117]. PLPs are linear oligonucleotide sequences which have sequences on either end that are complementary to the target sequence. The backbone of PLPs is a linker sequence which can be designed based on the functionality required [118]. As the PLPs hybridize with the target sequence, the nick in between the two ends is sealed by a ligase enzyme such as T4 DNA ligase. In the presence of a mismatch with the target sequence, the ligation is prevented making this method highly specific. After ligation, the target sequence is amplified using a polymerase enzyme such as Phi29 polymerase which is the most widely used polymerase in RCA applications [119]. The steps involved in rolling circle amplification process are shown in Figure 3.3. Upon amplification, the RCA products (RCPs) collapse and fold into a coil like structure. For visualization, fluorophore conjugated dNTPs or fluorophore-tethered complementary strands are used among other methods [117].

Na et al. presented a microfluidic device for rapid detection of multiples viruses within 15 min using rolling circle amplification (RCA). The device uses packed microbeads inside the channel and
the surface of the microbeads serves as the solid phase for RCA. Primers are immobilized on the microbeads and the template hybridizes with the primers. When the template hybridizes with the target, ligation takes place and RCA products are generated by Phi29 polymerase. As the RCA products get elongated, they form a DNA hydrogel inside the voids between packed microbeads. This hydrogel formation blocks the flow path through the bead-packed channel which can be visualized by using an ink. The sample and the ink are driven inside the channel by a vacuum pressure which is generated using a syringe [120].

Recombinase Polymerase Amplification

Recombinase polymerase amplification (RPA) was developed by Piepenburg et al. in 2006 [121]. RPA is an isothermal amplification method which typically takes place at 37°C. The process, shown in Figure 3.4, is similar to PCR but two proteins i.e., recombinase and single-strand DNA binding proteins (SSB) are used as an alternative to heat denaturation [122]. To initiate the process, forward and reverse primers combine with recombinase proteins to form recombinase-primer complexes. These complexes then scan the dsDNA for homologous sequence and strand invasion by the primer takes place at the cognate site. The displaced strand is stabilized through ssDNA binding proteins. The polymerase enzyme then extends the primers resulting in two copies. These two dsDNA copies go through the same process to produce more copies and as the cycle is repeated, the target sequence is amplified exponentially. The amplification process usually takes around 20 min [123].
Kong et al. presented a wearable microfluidic device for detection of HIV-1 DNA using recombinase polymerase amplification. The wearable device uses body heat for RPA and a smartphone-based fluorescence readout. A microfluidic device using PDMS was designed to go around the wrist and allow maximum contact and heat transfer and a thermal insulation band was used to reduce any temperature variations. The device showed amplification down to 100 copies/mL within 24 min. For fluorescence-based readout, a
detection setup consisting of an excitation/emission filter and a dichroic mirror was developed [124].

**Shortcomings of isothermal amplification methods**

As already discussed, isothermal amplification methods provide advantage over PCR when it comes to use in resource limited settings, but each of them also has some disadvantages associated with them. For example, the complicated primer design for LAMP makes the primer development quite time consuming and as there are strict requirement in terms of distance between the binding sites and melting temperatures, which might results in having to settle for a suboptimal primer design [125]. Additionally, the temperature required for LAMP (65°C) is relatively higher compared to other isothermal amplification methods. RPA kits are currently being sold by only one company which could have an impact on its pricing and also limits the flexibility of kit formulation [123]. RPA has also been reported to be prone to non-specific amplification in water controls [121, 125, 126]. RCA is a linear amplification process which means it needs a longer time for amplification, requires multiple steps of operation and its performance has not been evaluated extensively with different sample matrices [118, 127].

**Immunooassays**

Immunooassay is an analytical technique that uses the antibody-antigen interaction to detect an analyte of interest. An antibody, also known as immunoglobulin, is the most important part of an immunooassay. There are five types of immunoglobulins present in humans *i.e.*, immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin E (IgE) and immunoglobulin D (IgD). Out of these, IgG is the most commonly used antibody in diagnostics. An IgG molecule is Y-shaped having four polypeptide chains. Two of the identical polypeptide chains located on the inside are called heavy chains (H) and two identical polypeptide chains on the outside are called light chains (L). Each L
chain is made up of a variable region (V<sub>L</sub>) and a constant region (C<sub>L</sub>), whereas each H chain is made up of one variable region (V<sub>H</sub>) and three constant regions (C<sub>H1</sub>, C<sub>H2</sub> and C<sub>H3</sub>). The V<sub>H</sub> and V<sub>L</sub> regions form the antigen binding sites on each arm of Y-shaped IgG which implies that each IgG molecule has two antigen binding sites [128].

Immunoassays are being used for detection and quantification of proteins in fields such as diagnostics, food and environment safety, proteomics and pharmaceutical research [96]. Two of the most common types of immunoassays in the context of POC diagnostics are presented below:

**Enzyme linked immunosorbent assays (ELISA)**

Initially, immunoassays were performed using a radioactive label for detection e.g., iodine-131 and termed radioimmunoassay (RIA). In 1970s, Peter Perlmann and Eva Engvall conceptualized and developed enzyme linked immunosorbent assays (ELISA) in which they used an enzyme as the reporter label. Since its development, ELISA has become the gold standard in diagnostic immunoassays [129]. The different types of ELISA based on the protocol and the type of solid support used are described below:

**Types of ELISA**

There are four types of ELISA i.e., direct, indirect, sandwich and competitive which are explained below:

**Direct ELISA**

In direct ELISA the antigen to be detected is coated on the surface of the microtiter plate or some other solid surface and incubated with an enzyme coated primary antibody which binds to the antigen as shown in Figure 3.5 (A). After incubation, the unbound antibodies are washed away and a signal is generated by using a suitable substrate.
Indirect ELISA

Indirect ELISA, as shown in Figure 3.5 (B), is an extension of direct ELISA in which a secondary antibody conjugated with an enzyme is used to bind to an unconjugated primary antibody and produces a signal with an appropriate substrate.

Sandwich ELISA

In sandwich ELISA, the solid surface is first coated with a capture antibody which has affinity for the antigen to be detected. This is followed by incubation with the sample containing the antigen to allow the antigen to bind to the immobilized antibodies. Afterwards, detector antibodies tagged with enzymes are incubated to bind to the antigen which is immobilized on the surface owing to its affinity to the capture antibody and a signal is generated by adding a substrate. A schematic representation of sandwich ELISA is shown in Figure 3.5 (C).

Competitive ELISA

In competitive ELISA, the antigen to be measured in the sample and another version of the same antigen which has been conjugated with an enzyme label are added simultaneously to compete for the same binding spots on the antibodies as depicted in Figure 3.5 (D). Having a high concentration of the antigen in the sample would not allow the enzyme conjugated antigen to bind to those spots and they will get washed away. Thus, producing a low signal. Conversely, a low concentration of antigen in the sample would allow the enzyme conjugated antigen to occupy more of the available binding spots on antibodies, which in turn would produce a high signal [130].
The solid substrate is used for immobilizing antigens or capture antibodies for carrying out ELISA. Some common substrates used for ELISA in microfluidics are presented below:

**Planar surface**

Planar surface of a microfluidic channel is a commonly used substrate for immobilization of antibodies because of its simplicity. The antibodies can be immobilized directly on the surface through physical adsorption but it might lead to reduced activity because of denaturation and such type of binding is highly dependent on external conditions (type of material or pH). To circumvent this, the planar surfaces are often functionalized to immobilize antibodies using bioaffinity or covalent linking [131]. Kim et al. present a microfluidic immunoassay for POC testing using a computer-based fluid vent control to allow enhanced reaction time. The microfluidic device, shown in Figure 3.6 (A), has a working principle similar to a lateral flow immunoassay (LFIA) as it uses detector antibodies conjugated with fluorescent beads. The biotinylated capture antibodies are immobilized on the PMMA surface by plasma treatment of PMMA surface followed by EDC/NHS coupling and then cross-linking of streptavidin to amine reactive NHS ester. The flow in the device is started by opening the vent and stopped once to allow sufficient reaction time with the immobilized capture
antibody and then opened again to wash away the unbound species. The limit of detection (LOD) of the device was similar to a sandwich fluorescence immunoassay in a microtiter plate. [132]

**Magnetic bead based**

Using three dimensional surfaces, such as microbeads, provide an advantage in terms of higher surface area when compared to planar surfaces. This implies that more antibodies can be immobilized to capture more of the target analyte. Diffusion length is also reduced compared to a planar surface due to reduced distance between the beads, leading to a higher analytical sensitivity. Using microbeads in microchannels adds a level of complexity compared to planar surfaces as the beads need to be maneuvered. In this regard, magnetic beads are an attractive solution as their movement can be controlled from the outside using a magnet [131]. Coarsey *et al.* developed a flow-free magnetic actuation platform to carry out ELISA in an automated manner for detection of HIV-1 p24 antigen. The device does not require any external pump but instead uses a magnetic actuation platform controlled through Arduino microcontroller. The device consists of diamond shaped chambers for aqueous solutions separated by ellipse shaped oil wells as shown in Figure 3.6 (B). The alternating aqueous and oil phase prevent the mixing of different solutions while allowing the magnetic beads to move from one well to the next under the effect of external magnet. The final signal was colorimetric and an image-based quantitative method was used that can be implemented on a smartphone [133].

**Porous bead based**

The use of porous microbeads (such as cross-linked agarose beads) provides all the advantages of using 3D surfaces mentioned in the previous section with the added advantage that the use of nanoporous microbeads in microchannels can lead to an increase in the surface area to volume ratio by a factor of 50 [134]. A multiplexed microfluidic cartridge using agarose beads as solid
support for immunoassays was developed for at-line monitoring of mammalian cell culture processes in biopharmaceutical production as shown in Figure 3.6 (C). The PDMS microfluidic device consists of 4 columns which were packed with agarose beads and used for quantification of IgG, Chinese hamster ovary host cell proteins (CHO HCPs) and lactate dehydrogenase (LDH). The signal readout was colorimetric and a flatbed scanner was used for quantification purposes. The device provided comparable performance to commercial ELISA kits while using lower amount of reagents and having minimal handling times [135].

Lateral flow immunoassays (LFIA)

Lateral flow immunoassays are low-cost, rapid and easy to operate, providing qualitative results that can be seen through the naked eye. These factors make LFIA particularly suitable for POC diagnostics [136]. Home pregnancy test was the first example of a successful lateral flow test as it gained widespread acceptance for use at home. This was followed by development of lateral flow tests for diagnosing infectious diseases at POC. Over the years, lateral flow tests have been used for diseases such as malaria, zika, dengue, HIV, hepatitis and, more recently, COVID-19 [137].

A standard lateral flow test is in the form of a strip and constructed using nitrocellulose (NC) membrane. This is the membrane on which the antibodies are immobilized and through which the sample flows and a reaction takes place. A sample pad, made from a material that has low affinity for proteins, is used for loading the sample. The sample then flows into a conjugate pad that has the labelled biorecognition elements which will bind to the target analyte. As the sample continues to flow through the NC membrane, it passes through the test line and the control line. The test line has antibodies specific to the target of interest and as the sample flows through the test line, the target conjugated with the biorecognition element will bind to the immobilized antibody and produce a red
zone in the test strip. The control line has another immobilized antibody or biomolecule, independent of the target, which indicates successful completion of the test. The last part of the lateral flow test is an absorbent pad which absorbs excess fluid and prevents backflow of the sample. All these components are supported on a backing card and placed inside a plastic housing [137-139].

Rong et al. developed an integrated platform for POC diagnosis of infectious diseases using fluorescent lateral flow assay in a multichannel test cartridge shown in Figure 3.6 (D). The lateral flow assays were developed in sandwich immunoassay format for four infectious diseases. A test cartridge was designed which can hold four test strips and is fed by a common inlet channel that distributes and delivers the sample to all four test strips under the action of centrifugal force. A low-cost integrated platform was developed for sample delivery, optical signal acquisition and data analysis. The platform was tested for detection of HIV, Treponema pallidum (TP), hepatitis C virus antibody and hepatitis B virus surface antigen and verified using clinical serum samples [140].
Figure 3.6: (A) Schematics of the microfluidic device with PC based fluid vent control. Different regions of the device are shown with a schematic representation of immunoassay steps that take place in those regions. (B) Schematics of the microfluidic device for flow-free magnetic bead based automated ELISA. (C) Schematics of the microfluidic chip used for porous bead-based multiplexed detection of key proteins in mammalian cell culture process. (D) Schematic illustration of the test strips, multi-channel cartridge and integrated platform for point-of-care diagnosis of infectious diseases. Adapted from [132, 133, 135, 140] with permission.
Chapter 4: Present investigations

The primary aim of the research work presented in this thesis is to develop low-cost diagnostic platforms for point-of-care diagnostics in resource limited settings. There is an unmet demand for cheap, portable and integrated platforms for molecular diagnostics and immunoassays. In order to accomplish this goal, microfluidics was used as the main building block and devices for carrying out diagnostic tests were developed around it.

In **Paper I**, a low-cost microfluidic platform was developed to be used in extreme point-of-care settings that do not have a stable and reliable supply of electricity. The platform combines centrifugal microfluidics and a simple cardboard structure to serve as the housing. The motor used for rotating the centrifugal disc is powered by the battery of the mobile phone and the mobile phone also serves as a sensor for measuring the speed of rotation.

In **Paper II**, a portable and low-cost centrifugal microfluidic platform was developed for multiplexed detection of cytokines. The platform uses agarose beads as solid phase for sandwich immunoassay and allows detection of up to three targets from the sample. Each disc can process six samples in parallel. The colorimetric signal is measured using a simple setup consisting of LEDs and photodiodes which are controlled by a microcontroller and programmed to record the signal while the disc rotates. This allows for the measurement of signal without needing any external equipment. The total cost of this platform is less than 50 USD.

In **Paper III**, a sample-to-answer diagnostic platform was developed for COVID-19 diagnosis which combines centrifugal microfluidics, a novel bead-based signal enhancement strategy and mobile phone readout method for detection of SARS-CoV-2 virus directly from heat inactivated nasopharyngeal swab samples without any prior RNA extraction step. The detection is based on an
isothermal amplification method (LAMP) that requires a temperature of 65°C for 30 mins. The platform can process up to 20 samples in parallel and costs less than 250 USD.

In Paper IV, following up on the work in paper III, a fully automated centrifugal microfluidic platform was developed that allows the automation of multiple steps for nucleic acid based COVID-19 diagnostics. The user can predefine a complete protocol starting from heating, rotation of the disc to end the reaction followed by end-point detection. The detection mechanism is fluorescence based in this case but instead of a smartphone, an integrated camera module is used combined with computer vision to automatically provide the answer.

In Paper V, an alternative method is presented for the detection of SARS-COV-2 directly from a sample in an amplification-free and label-free detection method using electrochemical sensing. This work combines the electrochemical sensing with flex printed circuit boards by integrating electrodes directly onto the printed circuit boards. This allows for an easy-to-use and sensitive detection method which can be used at the point-of-care. As a proof of principle, SARS-CoV-2 target sequences were detected by hybridization with complementary capture sequences immobilized on the surface of the electrode.
The goal of this paper was to develop a very simple, portable and low-cost diagnostic platform that can be used in settings which suffer from a lack of basic infrastructure such as continuous power supply. To tackle this issue, the electric power stored in the battery of a mobile phone was used to power a small DC motor which can then be combined with centrifugal microfluidics for point-of-care diagnostics. The developed platform uses a cardboard housing which is easy to cut and assemble in the field as shown in Figure 4.1. The components used here are a small DC motor, a lens for imaging, a potentiometer for speed control and a cable to connect the mobile phone with the motor.

Figure 4.1: (a) The mobile lab disc platform with step wise instructions to assemble it. (b) Features on the disc added to serve as a tool for measuring speed of rotation (c) Using the mobile phone and the platform to image chamber on the disc.
The developed platform was tested with two common diagnostic tests *i.e.*, a sandwich immunoassay and a hematocrit test. Figure 4.2 shows the schematics and the exploded view of the disc designs used for each of the tests. For sandwich immunoassay, interleukin-2 (IL-2) was chosen as a model target. In order to demonstrate partial automation of the assay steps, we used capillary valves for sequential release of liquids already stored on the disc. The detection chamber in the disc was coated with capture antibodies for IL-2 followed by blocking. The sample and the detector antibody solution were mixed and loaded onto the disc and incubated for 30 mins. The washing buffers and the TMB substrate solution were also loaded onto the disc at the same time for sequential release later on. After 30 mins, the sample solution was emptied from the chamber at 300 RPM followed by the flow of two washing solutions which happen at 700 RPM and 900 RPM, respectively. At 1400 RPM, the TMB substrate solution flowed the detection chamber and the signal was allowed to develop. For hematocrit test, a simple structure was used which meters the added blood sample to a specific volume allowing the extra volume to go into the waste chamber.

![Figure 4.2: Schematic diagram and exploded view of the centrifugal discs designed for (a) IL-2 sandwich immunoassay and (b) hematocrit test.](image-url)
In order to achieve sequential flow in centrifugal microfluidics, it is important to have feedback regarding rotational speed of the disc as the centrifugal forces are modulated by changing the rotational speed. In order to achieve a simple method for speed control, without the use of any external circuitry, the embedded sensors in the phone were used to measure the speed of the rotation. One of the methods involved using the magnetometer of the phone combined with a small disc magnet embedded on the disc. As the disc rotates and passes the magnetometer, it registers a signal and calculates the frequency of rotation. Another method used the microphone of the phone to measure the acoustic signal generated by the rotating disc with a small hole on one end, which produces sound of a particular frequency according to the frequency of rotation. This sounds signal is measured through the microphone and the frequency of rotation is calculated. The comparison of smartphone-based tachometry with standard laser-based tachometry is shown in Figure 4.3.

![Graph a)
Rotational speed vs Voltage (V)](image)

![Graph b)
Current draw vs Voltage (V)](image)

**Figure 4.3:** Characterization of speed control methods. (a) Graph demonstrating how the rotational speed and current draw changes as the applied voltage is increased. (b) Comparison of smartphone-based tachometry techniques with standard laser-based technique.

Figure 4.4 shows the comparison of calibration curves obtained for IL-2 sandwich immunoassay performed on a conventional 96-well plate and our developed platform. Both the curves show a linear response but the LOD achieved on the platform was higher than the
one achieved on the conventional 96-well plate. Figure 4.5 shows the results for hematocrit tests where blood samples from six healthy donors were tested using the platform.

![Comparison of calibration curves](image1)

**Figure 4.4:** Comparison of calibration curves obtained for IL-2 sandwich on a conventional 96-well plate and the developed platform. The error bars represent standard deviation of three independent measurements.

![Images and graphs](image2)

**Figure 4.5:** (a, b) Before and after images of processing a blood sample on the disc for hematocrit measurement. (c) Characterization of centrifugation time for hematocrit measurement. (d) Hematocrit measurement of 6 blood samples.
Our immune system is always working using different pathways and employing different cell types to put up an effective defense against the invading pathogens. One such type of cells is called cytokines, which are signaling molecules that carry out effective communication between different parts of the immune system. An increase or decrease in the concentration of these cytokines in our system can be indicative of some underlying health issues. Therefore, detection and quantification of panels of cytokines can provide an overview of the state of the immune system. The gold standard method used for this purpose is Enzyme Linked Immunosorbent Assay, which usually takes 4-6 hours to complete and requires trained professionals and expensive instruments. For these reasons, there is currently a lack of portable and low-cost diagnostic solutions for detection of cytokines at point-of-care.

The goal of this paper was to develop an integrated platform for multiplexed detection and quantification of cytokines at point-of-care. To achieve this goal, centrifugal microfluidics and streptavidin coated agarose beads, serving as a solid phase for sandwich immunoassay, were used in combination. As shown in Figure 4.6 (A), the centrifugal disc consists of three layers and is made from PMMA sheets. Each disc consists of 6 structures and each structure has 3 serially arranged bead columns connecting the inlet chamber with the waste chamber. The bead columns can be packed with beads conjugated with biotinylated capture antibodies of up to 3 different targets. Following the packing of the beads, the sample solution is mixed with HRP-conjugated detector antibodies of the relevant target molecules and flowed through the bead columns where the target molecules can bind to the corresponding capture and detector antibodies. This is followed by a washing step and
colorimeric signal generation upon flowing of TMB (3,3',5,5'-Tetramethylbenzidine) substrate.

In order to simplify the detection process, a colorimetric detection method and a simple detection strategy involving light emitting diodes (LEDs) and photodetectors was used. Figure 4.6 (B) shows that the transparent PMMA sheets were painted in black prior to fabrication and this was done to aid the colorimetric detection process by blocking any light going around the region of interest. In the absence of such blocking method, an optical setup consisting of a very precise mask and placement of the disc close to the light source and photodetector would be needed, which would complicate the construction of the platform and make it less user-friendly while also increasing the cost substantially. Figure 4.6 (C) shows the schematics of the integrated platform having a cost of around 50 USD.

In order to characterize our developed platform, three cytokines i.e., TNF-α, IFN-γ and IL-2 were chosen to serve as a model panel. As a first step, the assay conditions were optimized for the three targets in single-plex format to maximize the signal-to-noise ratio. This included optimizing the concentrations for capture antibodies and detector antibodies. After optimizing the assay conditions, concentration curves for the three targets were obtained and limits of detection were determined. The results are shown in Figure 4.7 (A, B and C).

After obtaining the concentration curves in single-plex format, the platform was tested for multiplexed detection. In this case, the three bead columns on the disc were packed with beads conjugated with capture antibodies for all three targets, respectively. Similarly, the sample solution was mixed with detector antibodies of all three targets. In order to check for potential cross-reactivity, the sample solution was spiked with the target molecules in different concentrations and the results are shown in Figure 4.8 (A-E). In
order to check the performance of the integrated platform with a patient sample, the target molecules were spiked into undiluted and serially diluted plasma samples. While the undiluted plasma sample resulted in high background signal, the signals after 5-fold dilution were similar to those observed in buffer.

![Diagram](image)

Figure 4.6: (A) Schematic representation of the design and structure of the centrifugal disc and the immunoassay strategy used for detection of cytokines. The disc consists of a 1 mm top layer, 50 μm pressure sensitive adhesive as middle layer and 2 mm bottom layer. The different colors of the beads represent beads conjugated with three different capture antibodies for multiplexed detection. (B) A transparent square window on the top disc and a transparent bead column align to create a small region on top of the middle of the bead column allowing light to only pass through the beads. (C) Schematic diagram and exploded view of the integrated centrifugal microfluidic platform.
Figure 4.7: Concentration curves for the three targets (A) TNF-α, (B) IFN-γ and (C) IL-2. The error bars are representative of standard deviation of three independent measurements.
Figure 4.8: Multiplexed detection of cytokine targets and comparison with concentration curves. (A) [TNF-α] = 0 ng/mL, [IFN-γ] = 0 ng/mL, [IL-2] = 0 ng/mL; (B) [TNF-α] = 30 ng/mL, [IFN-γ] = 30 ng/mL, [IL-2] = 30 ng/mL; (C) [TNF-α] = 30 ng/mL, [IFN-γ] = 0 ng/mL, [IL-2] = 0 ng/mL; (D) [TNF-α] = 0 ng/mL, [IFN-γ] = 30 ng/mL, [IL-2] = 0 ng/mL; (E) [TNF-α] = 0 ng/mL, [IFN-γ] = 0 ng/mL, [IL-2] = 30 ng/mL. The error bars represent standard deviation of three independent measurements in buffer.
Paper III: Sample-to-answer COVID-19 nucleic acid testing using a low-cost centrifugal microfluidic platform with bead-based signal enhancement and smartphone read-out

There is an unmet demand for rapid, sensitive and specific detection of virus at point-of-care, generally, and in resource limited settings, specifically. The lack of such diagnostic solution results in thousands of deaths every year due to viral pathogens such as Zika, Dengue, Ebola and Human Immunodeficiency Virus (HIV). During the COVID-19 pandemic, it became evident that an effective screening and testing strategy is of immense importance in order to devise a contingency plan against a global pandemic.

The goal of this paper was to develop a low-cost integrated microfluidic platform that can be used for direct detection of SARS-CoV-2 virus in heat-inactivated nasopharyngeal swab samples. In order to achieve this, a combination of centrifugal microfluidics and a one-pot isothermal amplification method with a novel bead-based signal enhancement strategy were used and a platform was developed that costs around 250 USD and allows the user to process up to 20 samples within one hour and check the results using a smartphone readout. The isothermal amplification method used in this work was loop mediated isothermal amplification (LAMP) and the beads used for signal enhancement were agarose beads functionalized with N-benzyl-N-methylethanolamine (NBNM) multimodal ligands.

The platform, as shown Figure 4.9 (A, B), consisted of three modules namely (i) centrifugal module (ii) heater module and (iii) detection module. The centrifugal module consisted of a small DC motor that served as the rotor to rotate the centrifugal microfluidic disc. The speed and protocol of rotation were controlled by the electronic speed controller (ESC) of the DC motor combined with an Arduino Uno microcontroller. The heater module consisted of two 30W mat
heaters which were used to heat two copper plates respectively and arranged like a sandwich to heat the disc from top and bottom. A PMMA housing structure was used to enclose the mats and the copper plates. The detection module consisted of an 80W blue laser diode (λ=450 nm) which served as the excitation source combined with a Kapton film serving as an emission filter.

The centrifugal microfluidic disc consisted of 20 structures as shown in Figure 4.10 (A). Each structure has a main channel with a LAMP region where the isothermal amplification takes place and the bead region where NBNM beads are pre-packed and dried. The main channel is connected with a waste chamber through a 50 μm channel that allows the liquid to go through the beads and into the waste chamber but stops the beads. Figure 4.10 (B) shows the working principle of the disc. The inlet and the waste chamber holes are sealed after adding 10 μL of sample and heated for 30 min at 65°C. The disc is then rotated up to 6000 RPM which forces the solution through the beads into the pneumatic waste chamber. As the disc is slowed down to 0 RPM, the pressure from the pneumatic chambers pushes the liquid back into the main channel. This cycle is repeated four times.
Figure 4.9: Different modules of the centrifugal microfluidic platform used for LAMP-based diagnostics. (A) Photographs of the module from the front side with the lid removed. The lid contains a lens on the top side and a Kapton film on the bottom side. Zoomed in image shows the DC motor that rotates the disc and a laser diode that shines on the disc and serves as the light source for fluorescence detection using a smartphone. (B) Zoomed in image shows the assembled heater module and the schematics shows an exploded view of the heater module. The heater is separate from the centrifugal and detection module and the disc is manually transferred from the heaters to the centrifugal module for further processing. The graph shows the temperature profile of the disc and the metal plates.
The bead-based signal enhancement strategy uses agarose beads, with an average pore size of 100 nm, functionalized with NBNM multimodal ligands which combine anion exchange with other interactions such as hydrophobic, hydrogen bonding, etc. These beads are generally used in chromatography to capture host cell DNA or plasmid DNA. In this work, the beads were used to separate the specific signal caused by the LAMP amplification product from the non-specific signal caused by intra- and cross-primer hybridization. As shown in Figure 4.11 (A), the beads show a selectivity for short oligonucleotides in the range of 25-100 bp with
a cutoff around 400 bp. Figure 4.11 (B) gives a visual representation of the effect of beads on the signal.

![A.](image1.png)

**A.**

- Negative Input
- Negative Output
- Positive Input
- Positive Output

![B.](image2.png)

**B.**

- Ladder Input
- Ladder Output

**Figure 4.11:** (A) Capillary electropherogram of LAMP mixes before and after flowing through the beads in the presence and absence of $10^6$ SARS-CoV-2 RNA fragment copies (top) and DNA ladder having a range of 25-1000 bp (bottom) (B) The effect of NBNM beads on the signal-to-noise ratio. In the absence of beads, the negative sample has a fluorescence signal similar to the positive sample. In the presence of beads, the non-specific signal caused by intra- and cross-primer hybridization is captured by the beads. All the photos were taken with a smartphone camera.
The developed platform was characterized by testing a dilution series of SARS-CoV-2 RNA fragments in DI-water. This helped in optimizing the time needed for amplification without any false positive results, determine the limit of detection of our system and testing the smartphone-based readout method. The developed platform was then tested for detection of SARS-CoV-2 from 131 heat inactivated nasopharyngeal swab samples in viral transport media. The sensitivity and specificity of our developed platform was compared with a benchtop thermocycler and the results are shown in Figure 4.12.

Figure 4.12: Sensitivity and specificity of our integrated platform compared with a benchtop thermocycler. “RT-LAMP” refers to LAMP performed on a benchtop thermocycler and “LAMP-disc” refers to LAMP performed on centrifugal disc on our platform. For RT-LAMP, (1) and (2) refer to two replicates. Filled and dash boxes refer to RT-LAMP and LAMP-disc, respectively. MedCalc software (Ostend, Belgium) diagnostic test evaluation calculator was used to calculate the mean and Clopper-Pearson 95% confidence intervals.
The main goal of this paper was to build on the work done in paper III and develop a fully automated centrifugal microfluidic platform for COVID-19 diagnostics. In this work, using the bead-based signal enhancement strategy of paper III, a platform was developed that can carry out all the assay steps in automated manner without the need for any user intervention to move the disc from the heater or imaging at the end of the assay.

This work uses a new design of the microfluidic channel on the centrifugal disc that is based on one time flow of the sample solution through the beads instead of back-and-forth flow as shown in Figure 4.13 (A and B). The microfluidic structure consists of a loading chamber and a detection chamber which serve as the liquid and air reservoir respectively before the rotation of the disc. As the disc is rotated, the centrifugal force pushes the liquid from the loading chamber into the detection chamber and the air trapped inside the detection chamber gets pushed into the loading chamber. This results in the sample solution ending up in the detection chamber and staying there irrespective of how long the rotation is continued as shown in Figure 4.13 (C). This design greatly simplified the requirements for the type of motor that needs to be used as one time flow of the liquid through the beads can be achieved at a much lower rotational speed as well. Each disc consists of 26 structures.

The integrated centrifugal platform has dimensions of 200 × 160 × 125 mm (L × W × H) as shown in Figure 4.13 (D). The platform consists of a ring-shaped PCB heater which works on the principle of Joule heating and generates heat as current passes through the conductor inside the PCB. In the middle of the ring-shaped PCB heater is a mounting structure which serves as the slot for mounting
the centrifugal discs. The mounting structure also has AruCo codes printed on its top which help with identification of the channel using computer vision algorithm. The mounting structure can be moved vertically up or down using a lifting mechanism controlled by a servo motor. The centre of the mounting structure is attached to the shaft of a stepper motor which rotates the structure and the disc mounted on it.

Figure 4.13: (A) Schematics of disc assembly showing the three layers of the disc. (B) 3D model of the microfluidic structure after the disc has been assembled. (C) Top view of the integrated centrifugal microfluidic platform showing the Raspberry Pi 4 computer and the ring-shaped PCB heater. (D) Side view of the integrated platform showing the location of the camera and a zoomed in image of the laser angled to shine on the detection chamber.
For computer vision-based end point analysis of the assay, a Raspberry Pi Camera Module 2 is used in combination with a Raspberry Pi 4 computer. The sensor of the camera module is covered with a Kapton film to serve as an emission filter for the fluorescence signal. The video feed from the camera is processed by the RPi4 computer in which it first detects the AruCo markers and then measures the RGB signal in the detection chamber relative to the position of the AruCo marker. Based on the intensity of the signal in the green channel, a decision is made whether the signal is positive or negative. At the completion of signal acquisition, a compilation of results from all the channels is displayed on the screen.

The developed platform was characterized by comparing its performance to a benchtop Mic® qPCR cycler. A dilution series of ORF1ab RNA fragments spiked in nuclease free water and artificial saliva samples was first tested on the Mic® qPCR cycler and then the same dilution series was tested on the integrated platform in an automated manner. The automated protocol for loop mediated isothermal amplification (LAMP) started with heating the PCB heater to 90°C and then bringing the disc in contact with the heater. The temperature was kept at 90°C for the 25 sec and then maintained at 65°C for next 30 mins. At the end of the assay, the disc is lifted from the heater and rotated at 1500 RPM to push the sample mix through the beads and into the detection chamber. This is followed by end point analysis using computer vision algorithm. The compiled results are shown in Figure 4.14.
Figure 4.14: Results for LAMP assay for a dilution series of ORF1ab RNA fragments in water performed on (A) Mic® qPCR cycler and (B) integrated centrifugal microfluidic platform. (C) and (D) show results for LAMP assay for a dilution series of ORF1ab RNA fragments in artificial saliva samples in Mic® qPCR cycler and integrated platform respectively.
The goal of this paper was to explore the possibility of an alternative method for signal readout at point-of-care after nucleic acid amplification. In the previous papers, fluorescence and colorimetric readout methods were used combined with simple optical setups for microcontroller or smartphone-based readout. In this work, we combined electrochemistry with a flex printed circuit board for rapid detection of target sequences in a sample. Electrochemical readout methods have been used in POC devices because of their simplicity, low-cost, selectivity and sensitivity. Integrating electrodes directly on to printed circuit boards have the potential to provide mass-manufactured biochemical sensors at a low cost.

As shown in Figure 4.15, the flex printed circuit board having graphene electrodes was functionalized with streptavidin as the first step. Graphene is a 2D sheet of carbon atoms which is used in many biosensing platforms owing to its favourable properties such as high conductivity and biochemical stability. In the second step, biotinylated probes were immobilized on the streptavidin coated electrodes using the strong affinity interaction between streptavidin and biotin. In the third step, the surface of electrode was blocked using BSA to prevent any non-specific interaction. In the fourth step, the sample containing the target sequence was added to the surface of the electrode to allow for hybridization resulting in a detectable signal.
In order to prevent evaporation during the incubation and measurement step, a small microfluidic chip was fabricated having a small sealed chamber for the electrode and sample as shown in Figure 4.16. The chip consisted of two PMMA layers which could align and seal using 4 magnets on each corner of the two layers. A Nitrile rubber O-ring was added on each layer of PMMA to result in a sealed chamber in the middle of the chip. Two holes were added on the top layer to allow access to the electrode for adding or removing samples.

Figure 4.16: (A) CAD drawing for the microfluidic chip. (B) Schematic of the microfluidic chip assembly. (C) FPCB and microfluidic chip before assembly (1) and after assembly (2).
In order to test the performance of the DNA sensor, DPV response was calculated for different concentrations of the target sequence for SARS-CoV-2 in PBS. The results are shown in Figure 4.17 (A). The current increases with increasing target DNA concentrations and showed a linear response. Furthermore, the selectivity of the immobilized capture sequence was tested by introducing a sample with a non-complementary DNA sequence. The signal observed in case of complementary sequence was 20% higher compared to non-complementary sequence as shown in Figure 4.17 (B).

![Figure 4.17](image-url)
Chapter 5: Concluding remarks

The aim of the work presented in this thesis is to use microfluidics for development of low-cost solutions for point-of-care diagnostics in resource limited settings. In this work, conventional diagnostic tests, such as nucleic acid amplification and immunoassays, have been adapted and implemented to be performed using microfluidics. The microfluidic platforms developed in this work are a step towards facilitating diagnostics in resource limited settings, by making platforms that are portable, easy to use and low-cost compared to the traditional laboratory based diagnostic equipment.

In paper I, an extremely low-cost centrifugal microfluidic platform is developed which uses a cardboard housing and a small DC motor that can be powered using a mobile phone. The idea behind developing such a platform is to enable carrying out of simple diagnostic tests in areas which do not have a reliable supply of electricity. In paper II, a low-cost and portable centrifugal microfluidic platform is developed for multiplexed detection of biomarkers using agarose beads as a solid phase for sandwich immunoassay having a simple colorimetric readout without the need for any external equipment. In paper III, a platform was developed for nucleic acid amplification-based diagnosis of COVID-19. The platform combines centrifugal microfluidics and loop mediated isothermal amplification (LAMP) and uses a novel bead-based signal enhancement strategy to allow for a low-cost fluorescence-based detection using a smartphone. In paper IV, the platform developed in paper III was improved by making the platform fully automated that allows the user to carry out the whole test at the press of a single button and uses a computer vision-based readout method. In paper V, an electrochemical detection method is explored as an alternative to optical detection methods presented in the previous papers.
The different platforms presented in this work have different levels of complexity and provide different levels of functionality based on the application they are used for and the cost of development. This demonstrates that a trade-off exists between the cost of the platform and the level of automation and functionality that can be implemented on it. Therefore, in order to design a fit-for-purpose diagnostic platform, it is important to take into considerations various factors such as the appropriate test in terms of sensitivity and specificity, the level of expertise of the targeted end-user and the type of settings in which the diagnostic platform will be used.

In conclusion, the field of microfluidics, in general, and centrifugal microfluidics, in particular, has the potential to fulfill the gap between conventional diagnostic methods and their application at point-of-care in resource limited settings. The work presented in this thesis is a step towards realization of this goal as it tries to address some of the common problems faced in this regard. A limitation of this work is the application and testing of the developed platform in the field, outside the research settings. It can be envisaged that such studies will provide useful information from the end-user perspective in terms of the ease-of-use and robustness of the developed platforms. Another limitation of the work is the lack of testing with pre-stored lyophilized reagents. For any test to be used in resource limited settings effectively, the need for a cold chain needs to be eliminated. Therefore, it is important to test the platforms with pre-stored reagents to achieve true sample-to-answer diagnostic tests.
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References


[28] I. V. Jani et al., "Effect of point-of-care CD4 cell count tests on retention of patients and rates of antiretroviral therapy


References


[126] A. Aebischer, K. Wernike, B. Hoffmann, and M. Beer, "Rapid genome detection of Schmallenberg virus and bovine viral
References


