Advanced Technologies in Rapid and Multiplex Detection of Nucleic acid

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by
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This thesis is dedicated to my loving parents,

who guided me to where I am today. I am truly thankful for having you in my life.

This work is also dedicated to my husband, Mostafa,

who has been a constant source of love, support and encouragement.
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Lena Gorgannezhad

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Principal Supervisors: Prof. Nam-Trung Nguyen, A/Prof. Helen Stratton
Abstract

Nucleic acids are key macromolecules of living organisms transferring genetic inheritance from one generation to the next. From how a living individual is created to how it interacts with external factors, all and all, can be found in the nucleic acid sequences inside every single cell of every organism. Therefore, the analysis of nucleic acids sequences is a critical capability for cancer and pathogen diagnoses, genotyping, and disease monitoring. To date, numerous methods have been used to detect both characterised and uncharacterised mutations and sequence variations. However, the detection of low amounts of mutant genes in the presence of high levels of wildtype sequences is still a challenge, and existing technologies has room for improvement. The classical approaches for nucleic acid detection and analyses mainly include DNA sequencing and the polymerase chain reaction (PCR). Although these methods with high analytical performance and reliability have facilitated the interrogation of the nucleic acids, some key obstacles such as the need of labelling, high costs for routine clinical use, slow turnaround time for giving results, the complexity of operation, and the inability to dually detect the genetic mutation in one step have limited their applications. To avoid drawbacks of the traditional approaches, a number of chip-based methods leverage electrochemical readouts or microfluidics to identify nucleic acids. However, there is still an unmet need for a less complex, rapid, low-cost, sensitive and accurate method to enable nucleic acid analysis even in resource-poor settings. The overall objective of this PhD thesis is to develop simple, inexpensive and accurate platforms for nucleic acid evaluation.

To achieve the aforementioned goal, the first attempt was to develop a lab-on-a-chip platform for cancer diagnosis by detection of circulating tumor nucleic acids (ctNAs) in plasma samples of cancer patients. ctNAs are fragmented DNA released from cancerous cells and tumours into the bloodstream of patients with cancer. Tumour-specific (epi-)genetic alterations in ctNAs are assumed to reflect tumour burden and could be of high value for
cancer diagnosis, prognosis, and management. In the first part of the thesis, I developed a new electrochemical assay for the detection of FGFR2:FAM76A fusion gene in ctNAs extracted from ovarian cancer patients. The assay was based on the high electrocatalytic activity of a new class of superparamagnetic graphene-loaded iron oxide nanoparticles. Electrochemical detection demonstrated a limit of detection (LOD) as low as 1.0 fM, high specificity and excellent reproducibility.

In the second part of the thesis, I designed and developed a real-time and quantitative PCR system for microbial source tracking (MST) in water samples. MST is a DNA-based technology that enables water-quality managers to identify sources of faecal pollution in environmental waters. Most of the MST methodologies typically require specialized and costly equipment, elaborated and time-consuming operations as well as trained personnel. Here, a simple, low-cost, and sensitive platform was implemented on a microfluidic array chip. The array was successfully used for the real-time PCR-based multiplex detection of three human-associated MST markers (H8, Gen bac III, UidA). The PCR mixture was loaded into an array of channels in a single step utilising capillary filling without the need for liquid handling instruments. The array was then integrated with our custom-made thermal cycling and optical detection system. By employing the fabricated platform, the LOD of 71.8 DNA copies/μL was achieved for Gen bac III sequence. In summary, we introduced a sensitive, simple and economical real-time and quantitative PCR system for MST in water samples.

In a further study, I investigated how a nucleic acid amplification setup can be miniaturised. To reach this goal, I utilised liquid marbles as an ideal biochemical microreactors for targeted amplification of the NAs. Liquid marbles are formed by encapsulating microscale volume of liquid with a thin layer of hydrophobic particles. Miniaturization of the nucleic acids (NAs) amplification process inside a liquid droplet
provides several advantages upon routine methods, such as reducing reagents consumption and contamination possibility, easy handling of liquids, eliminating the usage of disposable plastic consumables for carrying out biochemical reactions. However, one of the major concerns in liquid marble applications is the high rate of evaporation through the porous walls during the thermal cycling step. To eliminate the evaporation, I used core-shell beads synthesized from a composite liquid marble as a NAs amplification micro reactor comprising two non-miscible liquid droplets forming a spherical shape and a coating of hydrophobic powder. The shell liquid was then polymerised into a solid after exposure to blue light, converting the liquid marble into a core-shell bead. Fabricated core-shell beads were extended to explore their potential as a versatile bioreactor for phylogrouping of the *E.coli* strains. In general, this platform provided easy manipulation and storage of sample, elimination of the evaporation, and sample protection from possible external contamination. Moreover, this simple and effective method presented a sensitive and inexpensive way to track NAs.

In conclusion, this research endeavour presents a step forward towards the adaption of the selected group of tools and technologies, for the development of assays that can be applied as powerful alternatives to conventional tools used in molecular diagnostic. These technologies have the potential to revolutionise the NAs-based diagnostic approaches, by providing sensitive, rapid, accurate, and inexpensive platforms for point of care devices and in-field tests.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Lena Gorgannezhad

(Sign)

Date 10.03.2020
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Publications during candidature

Peer-reviewed publication


Under review


Conference Poster presentation


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Chapter 1: Introduction

1.1 Nucleic acid-based cancer screening

Cancer-specific fatality has scarcely decreased in decades, despite an exponential increase in studies about cancer pathogenesis and huge investment in the development of treatments. According to world health organization (WHO), cancer is the second primary cause of mortality worldwide, accounting to an expected 9.6 million deaths, or one in six deaths, in 2018 [1]. In 2016, 45,782 people died from cancer in Australia and it is estimated that it will increase to 49,896 deaths in 2019 [2,3]. Stomach, lung, colorectal, prostate, and liver cancer are the most widely recognized types of cancer in male, whereas breast, colorectal, cervical, ovarian, lung, and thyroid cancer are the most prevalent among females. Cancer care represents a considerable and quickly rising medicinal services cost, as the number of individuals recognised to have cancer is ascending because of ageing, changing lifestyle and environmental risk factors. The total healthcare cost of all cancers in direct health system costs of Australia is over $6 billion per annum [4,5]. Early cancer detection in people who have access health services more often can remarkably reduce the burden of cancer and save millions of lives and dollars [6]. Bearing in mind the commonality of cancer and the high survival rate if diagnosed at an early stage, there is a need to understand the fundamental principles of cancer biology and subsequent discovery of related markers for early cancer detection.

Cancer encompasses an enormous spectrum of diseases, where abnormal cells rapidly divide and spread to other parts of the body [7]. Depending on the type of the cancer cells, tumours can be divided into benign (unable to metastasize) or malignant (able to invade normal tissues). Cancers are further defined and classified by their tissue, or organ of origin [8]. A wide range of biological studies have been conducted to assess whether existing biomarkers can be recommended for
further exploration in clinical context. The World Health Organization [9] has defined biomarker as “any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease”. More especially in oncology research, biomarker is a biological indicator present in tumour tissues or body fluids and encompass a wide variety of molecules [10]. These marker molecules can be divided into two basic groups; either genomic (DNA/RNA) or phenotypic (morphology and protein expression) [11]. The aim of the cancer biomarker research is to develop sensitive, reliable, low-cost, efficient detection and screening strategies for measuring the risk of cancer indication in a specific tissue or, alternatively, evaluating the risk of cancer progression or potential response to therapy [12]. In order to obtain these biomarkers “solid biopsy” is not always useful since it is invasive and cannot depict current tumor dynamics or sensitivity to the treatment. However, “liquid biopsy” from body liquids has great potential in cancer diagnosis.

Over the past decades, much more attention has been focused on cell-free Nucleic acids (NAs) as the ideal analyte in liquid biopsy studies. Most cell-free NAs in body fluids is shed from dying cells. Basically, the quantity of cell-free NAs in plasma samples of normal individuals or patients with early-stage cancer is 3-9 ng/ml. This amount can even increase up to 10 times in metastatic patients, providing a high level of mutant NAs for oncology investigations. Circulating tumor NAs (ctNAs) are fragmented NAs in the bloodstream that originate directly from cancerous cells and tumours [13]. ctNAs reflect the same pattern of mutations as that of primary tumour. However, the amount of ctNAs detected in plasma varies in patients with the same tumour type, size, and stage. These distinct features of ctNAs have shown promise for development of personalised cancer analysis [14].

1.2 Nucleic acid-based water monitoring
Water scarcity is one of the main worldwide crises facing us today. 785 million people (1 in 9) lack access to safe water, comprising 144 million individuals who are using just surface water. According to WHO statistics 2017, at least 2 billion people use drinking water sources contaminated with faeces. Lack of safe drinking-water and sanitation can cause faecal-oral infections. 3.4 million people, mostly children under 5 years old, die from water-associated diseases per annum [15,16]. Monitoring the bacteriological quality of water is required to control the presence and concentration of microorganisms that can be the source of waterborne disease outbreak. The evaluation of microbiological quality of water is usually conducted using indicator organisms, particularly Escherichia coli and Enterococci. These microorganisms are primary indicators of faecal contamination in water resources [17]. They usually can be found in faeces and unable to reproduce outside the intestine. Though, these enteric microorganisms lead to just mild infections, their presence can demonstrate the potential presence of other more pathogenic and dangerous organisms [18]. The standard levels of indicator organisms are defined by existing legislations. In spite of reliable estimates of the health risks using above-mentioned indicators still there is a limitation remained. Since these organisms are not inevitably host-specific and they can reproduce in humans, farm animals or wildlife, they often result in a vague estimate of health risks in aquaculture, irrigation, and recreational waters [19,20]. To remove this obstacle, the evaluation of faecal contaminants should be conducted in parallel to identify the sources of pollution.

Microbial source tracking (MST) is a group of techniques designed to identify the sources of faecal contamination in water [21]. The analytical approaches employed in MST more frequently include molecular analysis of DNA or RNA to determine the animal source of the target organism. The theory underlying these types of MST methods is taking the advantage of genetic alterations of microorganisms after adaption to the specific host. Bacterial colonization in particular host (e.g., cow, bird, humans, …) can result in the production of bacteria strains with a
genetic “fingerprint” unique to that host. These “fingerprints” can be detected in the bacteria present in water samples and used as source identifier [22,23].

1.3 Current methodology in nucleic acid analysis

Accessing to the information of the genome and transcriptome provide precious molecular identity useful in cancer studies and microbial source tracking [24,25]. In molecular identification assays, it is almost necessary to seek sequences of interest using probes. Probe is referred to a short single stranded DNA or RNA molecule that can be used to scan and identify target sequences. The reason underlying this identification is hybridization between probe and its complimentary match sequence [26]. Probe can be artificial or from a biological source. Chemical modification of the probe (i.e. adding phosphate, carboxyl, thiol, biotin, etc) can enhance its efficiency and specificity [27]. To date, a wide range of probes have been designed with the ability to attach or capture NAs of interest for isolation procedures or detection assays. NAs detection systems taking the advantage of probes can be categorized into the two main groups of amplification-free, and amplification-based systems.

This thesis proposes three novel approaches for sensitive and effortless detection of NAs in amplification/amplification-free based platforms.

Amplification-free probe-based methods that leverage fluorescence, colourimetric, electrochemical, and electronic detection represent potent tools for the identification of NAs [28]. However, there is still a gap for improved techniques which are simple, sensitive, cost-effective, and amenable to use in resource-restricted area. The first part of the thesis relies on development of an amplification-free, highly sensitive, uncomplicated, rapid and inexpensive electrochemical method for the detection of ctNAs. Compered to amplification-free methods, amplification-based strategies are most extensively used in practice. These methods also use probes commonly known
as ‘primers’ for starting of an extension reaction by enzyme from targeted sequence in NAs. Targeted amplification results in replication of a DNA sequence of interest up to $10^9$-fold to obtain target concentration high enough to be detected by conventional methods such as gel electrophoresis, and staining [29]. Much-quoted example of targeted amplification is polymerase chain reaction (PCR). Nevertheless, there are some difficulties that hinders their broad application. For example, all targeted amplification strategies in conventional platforms are sensitive to cross-contaminations and subsequently false-positives results. Thus, trained personal and clean laboratory space are needed to increase the sensitivity of the detection assay. Also, these methods need an expensive thermal cycler [29-31]. With this problem statement, it is clear that there is an urgent need to develop suitable NAs detection platforms that have comparable performance to conventional PCR but eradicating its demerits. The second and third parts of this thesis interduce new methodologies based on microfluidics and liquid marbles for targeted amplification of NAs and their further analysis.

1.4 Research questions and objectives

The principal aim of this thesis is to develop simple, inexpensive, and sensitive platforms for NAs identification in cancer and water studies. To achieve this aim, the work has been divided into following specific goals.

(i) **Development of a diagnostic method to detect ctNAs in plasma of cancer patients based on electrochemistry.**

Among the emerging biomarkers for liquid biopsy of cancer patients, ctDNA has been proving itself as a non-invasive real-time biomarker. Diagnostic and prognostic potential of this potent biomarker has been realized upon the wide range of cancer screening studies. The main advantage of using this biomarker is that it is readily available in serum, urine, saliva and there is
no need for invasive biopsies. Additionally, it has been proven that ctDNA can also be found in body fluids of cancer patients who are in early stage, leading to early detection of the cancer and saving lives. Thus, the first research question of this thesis is: “How to develop a simple and cost-effective method based on ctNAs for cancer detection and monitoring?”

(ii) Development of a NAs detection platform for microbial source tracking in water samples based on microfluidics.

Maintenance of the microbiological quality and safety of water systems is imperative, as exposure of these systems with faeces of human and animal can lead to the public health risks. Understanding the origin of faecal contamination is of paramount importance in evaluating the associated health risks as well as planning for the required actions. Faecal indicator microorganisms such as known bacteria are commonly employed as potent markers in water quality assessment. Conventional approaches for detecting bacteria include cell culture and various NAs amplification techniques. However, these approaches mostly require sophisticated equipment, multistep procedures, and trained personnel. These limitations hamper the broad applications of these approaches particularly in resource-limited areas. Advanced methodologies such as microfluidics allow MST studies to shift focus from conventional amplification platforms to direct analysis of NAs. Microfluidic platforms are able to provide distinct merits for NAs studies such as rapid target detection, low reagent and sample consumption, low costs, process integration, and the capacity for automation. This leads to the second research question: “How to develop a microbial source tracking approach in water studies employing microfluidics?”

(iii) Development of a NAs detection platform for *E.coli* phylogrouping based on liquid marbles.
The identification of *E.coli* species members/phylogroups using classical phenotypic data is an overwhelming task. Such species/phylogroup identification is nevertheless highly required for epidemiological studies. Because it has been proven that there is a relation between the species/phylogroup and lifestyle. The scientific community mostly employ multiplex PCR-based method for identification and phylogrouping of *E.coli* strains. However, this method is prone to false-positives results. Thus, designing, fabricating and installing *de novo* devices and methodologies in a plug-and-play fashion are required. Among the proposed platforms, liquid marble has been introduced as a promising reactor for NAs amplification due to providing several advantages such as the ease of production and manipulation. The third research question is therefore: “Can we take the advantages of liquid marble in NAs amplification for phylogrouping of *E.coli* strains?”

1.5 Proposed methodologies

This thesis presents novel NAs detection platforms that could serve as candidates to eliminate common challenges in conventional methods. This section briefly describes of the methods that were employed in this thesis.

As mentioned previously, the first aim of the thesis is the development of an electrochemical platform for early detection of cancer. Our proposed method used a specific ovarian cancer biomarker and highly porous superparamagnetic nanoparticles. Among ovarian cancer biomarkers ctRNA has attracted great attention due to harbouring cancer specific molecular alterations and providing a minimally invasive tool for cancer diagnosis, prognosis, and survival prediction. However, ctRNA is a challenging analyte owing mainly to its extremely low abundance.
Therefore, specific detection of ctRNA against a background of bulk cell free nucleic acids (DNA, RNA) population present in clinical samples, adding a further layer of predicament mainly due to scarcity of specific molecular markers as well as the lack of the sensitive and specific bioassays. To eliminate these unmet challenges, we selected FGFR2:FAM76A gene fusion as an ideal marker gene, whose expression can cause up to 50% increase in proliferation of the cultured ovarian cancer cells. Using this gene fusion, we could efficiently exploit the translational potential of ctRNAs in ovarian cancer detection [32,33]. we also used a new class of superparamagnetic iron-oxide nanoparticles that were coated with graphene (Fe$_2$O$_3$@GO nanoparticles). Using the intrinsic (i) affinity intention graphene and nucleic acids and (ii) electrocatalytic activity of Fe2O3@GO nanoparticles towards the [Ru(NH$_3$)$_6$]$_{3+}$/[Fe(CN)$_6$]$_{3-}$ electrocatalytic cycle, our assay enabled the sensitive detection of ovarian cancer in an early stage.

We used a microfluidic-based platform to achieve the second goal and to address the research question. As previously stated, the second objective of this thesis is microbial source tracking in water samples based on nucleic acid detection. The hypothesis is that simultaneous detection of multiple targeted genes in one array can prevent tedious, manual PCR mixture loading into individual PCR reaction tubes. Most of the reported PCR-based microchip devices require an expensive liquid dispensing robot and pneumatic pressure source, as well as trained technicians [34]. Recent advances in microfluidics have enabled the development of new array devices without the need to any external pump. Also, due to several other associated features, such as low cost of preparation and operation, fast analysis, high sensitivity, and minimum infrastructure requirements, these devices have been used for the multiplex detection of contaminants. We aimed to combine the strengths of microfluidics and the amplification of specific markers using PCR to develop an inexpensive and portable device for sensitive and efficient real time monitoring of target genes. With this method, we could simultaneously detect three human-associated MST markers: Escherichia coli (E.coli) (H8), human-specific bacteroidals (Gen bac III), E.coli (UidA).
To circumvent some of the challenges raised for the second objective, elucidation for the third aim of the thesis was presented. We used a new class of NAs detection platform to eliminate problems such as sample evaporation and leakage during thermal cycling step. To this end, liquid marble technology and photopolymerization were employed to fabricate a new generation of NAs amplification reactors termed “core-shell beads”. The approach was used to perform multiplex PCR by integrating a series of single amplifications for phylogrouping of E.coli species. Four core-shell beads were synthesized and used for simultaneous detection of four marker genes. A custom-made thermal cycler was also developed to provide thermal cycling of the beads. The emitted fluorescent intensity by the fabricated beads was evaluated, resulting in phylogrouping of the E.coli strains.

1.6 Thesis framework

The present thesis is divided into seven chapters; Chapter 1: Introduction, Chapter 2: Published paper 1 (Critical review 1): Circulating tumor DNA and liquid biopsy: opportunities, challenges, and recent advances in detection technologies; Chapter 3: Published paper 2 (Critical review 2): Microfluidic-based nucleic acid amplification systems in microbiology ; Chapter 4: Published paper 3 (Research article1): Detection of FGFR2:FAM76A fusion gene in circulating tumor RNA based on catalytic signal amplification of graphene oxide-loaded magnetic nanoparticles; Chapter 5: Published paper 4 (Research article2): Microfluidic array chip for parallel detection of waterborne bacteria; Chapter 6: Submitted for publication (Research article3): Potential application of core-shell beads in phylogrouping of E.coli strains; Chapter 7: Conclusion and future perspective.

Chapter 1 introduces the aims, background and significance of this research.
**Chapter 2** presents a comprehensive literature review focusing on biology, clinical potential and application of ctDNA biomarker. In addition to the conventional ctDNA analysis platforms, the review drew a comprehensive appraisal of recent developments of ctDNA detection methodologies. The major challenges and limitations associated with both conventional and advanced strategies were highlighted. This chapter provides the answer to the question, why ctNAs have been chosen as a cancer biomarker for the research described in the subsequent chapter.

**Chapter 3** is a critical literature review describing existing microfluidic-based NAs amplification systems in microbiology. From the basic studies to the latest advances in each field were discussed comprehensively. Moreover, an overview of the most commonly used techniques for NAs analysis, and the major technical challenges of existing methods, were presented. The conclusion introduces more potent and credible approaches in microbial NAs analysis.

**Chapter 4** reports the development of a new amplification-free electrochemical detection assay for the detection of FGFR2:FAM76A fusion gene in ctRNA extracted from ovarian cancer patients. The assay is based on the electrocatalytic activity of a new class of superparamagnetic graphene-loaded iron oxide nanoparticles. The level of adsorbed ctRNA on unmodified/modified carbon electrodes is detected electrochemically in the presence of ferricyanide redox system.

**Chapter 5** describes the design and development of a highly sensitive microfluidic-based platform for NAs scanning. A sandwiched glass–polydimethylsiloxane (PDMS)–glass microchip containing an array of reactors was fabricated for real-time PCR-based analysis of three
waterborne bacteria. A home-made real-time PCR instrument was also constructed to provide thermal cycling to the microfluidic device. The applicability of the method was tested and validated in microbial faecal source tracking (MST) studies of water samples.

**Chapter 6** presents a simple and efficient liquid marble-based system for NAs detection. In this method we synthesized core-shell beads from a composite liquid marble as a reactor for carrying out amplification procedure. Resulting core-shell beads were used to perform several single PCR of targeted genes simultaneously. The feasibility and sensitivity of approach were successfully examined by phylogrouping of *E.coli* strains.

**Chapter 7** summarises the thesis and provides a perspective on the future works.

Figure1.1. depicts schematically the framework of this thesis.
Figure 1. 1. The structure of the thesis and the relationship between the chapters published as research papers.
1.7 References


Statement of contribution to co-authored published paper

This chapter is in a form of a co-authored published paper. The bibliographic details of the co-authored paper, including all authors, are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

My contribution to the published paper involved:

- Literature review
- Manuscript preparation
- Responding reviewers

(Sign)

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(Date) 18/3/2020

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(Date) 18/3/2020

(Sign)

Supervisor: Prof. Nam-Trung Nguyen

(Date) 18/3/2020
Chapter 2: Circulating tumor DNA and liquid biopsy: opportunities, challenges, and recent advances in detection technologies

Abstract

Cell-free DNA (cfDNA) refers to short fragments of acellular nucleic acids detectable in almost all body fluids, including blood, and is involved in various physiological and pathological phenomena such as immunity, coagulation, aging, and cancer. In cancer patients, a fraction of hematogenous cfDNA originates from tumors, termed circulating tumor DNA (ctDNA), and may carry the same mutations and genetic alterations as those of a primary tumor. Thus, ctDNA potentially provides an opportunity for noninvasive assessment of cancer. Recent advances in ctDNA analysis methods will potentially lead to the development of a liquid biopsy tool for the diagnosis, prognosis, therapy response monitoring, and tracking the rise of new mutant sub-clones in cancer patients. Over the past few decades, cancer-specific mutations in ctDNA have been detected using a variety of untargeted methods such as digital karyotyping, personalized analysis of rearranged ends (PARE), whole-genome sequencing of ctDNA, and targeted approaches such as conventional and digital PCR-based methods and deep sequencing-based technologies. More recently, several chip-based electrochemical sensors have been developed for the analysis of ctDNA in patient samples. This paper aims to comprehensively review the diagnostic, prognostic, and predictive potential of ctDNA as a minimally invasive liquid biopsy for cancer patients. We also present an overview of current advances in the analytical sensitivity and accuracy of ctDNA analysis methods as well as biological and technical challenges, which need to be resolved for the integration of ctDNA analysis into routine clinical practice.

This chapter has been published as:
2.1. Introduction

Circulation bound tumor DNA could potentially serve as a “liquid biopsy”, thus avoiding conventional tumor tissue biopsies. Tissue biopsies are not only hazardous for patients, but often clinically unfeasible and unable to encompass the temporal and spatial heterogeneity of the cancer cell genetic/epigenetic landscape. Liquid biopsy, on the other hand, allows for repeat blood sampling, thereby providing an insight into the evolutionary dynamics of the cancer. Moreover, as ctDNA originates from multiple tumor sites, its analysis may be able to provide a more comprehensive snapshot of intra-tumor clonal heterogeneity compared to single-site tissue biopsies. However, since ctDNA comprises only a very small fraction of the blood-borne cfDNA, specific identification of ctDNA among bulk cfDNA is a daunting task. Therefore, the need for finding characteristic features/markers, which could be used to distinguish ctDNA from cfDNA, has been highlighted. In this context, the hypothesis of tumor-specific mutations in ctDNA was proposed.[1] Sorenson et al. presumed that ctDNA in plasma should reflect the same pattern of mutations as that of primary tumor.

This assumption was proved by detecting tumor-specific k-RAS mutations in ctDNA samples obtained from pancreatic adenocarcinoma patients.[1] Subsequently, various other cancer-associated genetic and epigenetic aberrations such as loss of heterozygosity (LOH),[2,3] gene amplifications,[4,5] cancer derived viral sequences,[6–9] promoter hypermethylation,[10–12] and single-nucleotide mutations[13–17] have been successfully detected in ctDNA. A direct and consistent correlation between tumor load and ctDNA has been established in a wide range of malignancies.[18,19] Consequently, ctDNA could serve as an important biomarker for early diagnosis of cancer,[20–23] minimal residual disease monitoring,[24,25] as well as monitoring the response to chemotherapy, clonal evolution, and possible development of resistance.[26] Despite recent advances in ctDNA detection and
analysis methods, our current understanding of ctDNA biology is far from complete and its implementation as a clinical cancer biomarker needs more intensive research. Therefore, there is an urgent need for robust, sensitive and specific analytical approaches. [27] In recent years, chip-based technologies capable of analyzing plasma ctDNA without prior information about genomic characteristics of the tumor have been developed to exploit the potential of this non-invasive biomarker. DNA clutch probes (DCPs), [28] surface plasmon resonance (SPR) [29] and surface enhanced Raman scattering (SERS) [30] are some of the prominent examples of chip-based methods. These methods have the advantage of being enzyme free, highly sensitive, and low cost and requiring less testing times, and thus hold the potential to overcome various hurdles in ctDNA research. The biology of ctDNA has been reviewed previously. [31,32] Moreover, many recent reviews on applications of ctDNA [33–37] as well as ctDNA detection approaches [31] have been published. Many aspects of ctDNA biology have been unraveled since the publication of previous reviews. Coupled with the ever-expanding repertoire of excellent methods and devices being invented for this purpose, an updated, critical, and comprehensive overview of all aspects of ctDNA research is needed. This review provides a state-of-the-art view on ctDNA biology and its clinical significance and utility in cancer diagnostics, prognostics, and theranostics. More importantly, the review highlights the technical developments and challenges that have been addressed for ctDNA detection and analysis.

2.2. ctDNA as liquid biopsy: potential and applications

2.2.1. ctDNA in cancer diagnosis

Early, sensitive, and accurate diagnosis is considered sine qua non in cancer management as it can guide effective therapeutic interventions as well as substantially improve patient outcome and survival. [38] Despite years of research, diagnosing cancer at
early stages with high sensitivity and minimum risk of overdiagnosis is indeed an onerous task. The quest for more potent and credible biomarkers is still ongoing. Over the years, ctDNA analysis as a minimally invasive approach has made a remarkable contribution to this quest. Initial observations indicated that cfDNA concentration in plasma of cancer patients is substantially higher as compared to that in healthy controls as well as those with benign disease. [38,40]

Common analytical approaches for quantification of plasma DNA levels are spectrophotometry, [40] colorimetric DNA quantification, [41,42] use of dsDNA binding fluorescent dyes like SYBR Green I [43] and Pico Green® [44] as well as quantitative polymerase chain reaction (PCR) for β-globin gene. [45] These recent studies highlighted that cfDNA concentrations in cancer patients may be influenced by various tumor-related variables such as stage, grade, aggressiveness, etc. and can thus be potentially used to assess tumor burden. However, limited diagnostic information can be obtained by measuring changes in absolute cfDNA levels alone, [46] as increased plasma DNA levels have also been observed in various chronic diseases such as viral hepatitis and lupus erythematosus. [40] Moreover, human lymphocytes release nucleoprotein complex, whose excretion is particularly enhanced under chronic immune stimulation conditions, including cancer. [40] Various subsequent meta-analyses failed to establish a consistent correlation between cfDNA concentration and any of the tumor variables mentioned above. [27] Therefore, later approaches of the combination of cancer-specific alterations and the quantification of plasma cfDNA levels are more reliable for cancer diagnostics. [47]

Amidst an overwhelming excess of non-tumor cell-free DNA molecules, a more sophisticated approach for specific detection and quantification of ctDNA involves surrogate markers such as tumor-specific molecular aberrations, micro- satellite alterations, and
promoter methylation changes as well as amplification of specific genes like hTERT. Real-time PCR-based hTERT quantification is one of the earliest and prominent approaches and has been used widely.\[23\] Mutations in the KRAS gene are among the molecular alterations most frequently used for specific detection and quantification of ctDNA. Cancer diagnosis based on detection of KRAS mutations in cfDNA has shown its significance in early diagnosis, as KRAS mutations have been detected in healthy controls up to 2 years before the diagnosis of cancer.\[48\] Similarly, TP53 mutations have also been detected in the cfDNA of healthy subjects on the average 20.8 months before the cancer diagnosis.\[48\] Microsatellite alterations are also among tumor-related markers detected frequently in ctDNA. Since the publication of two first-of-a-kind reports in 1996, \[3,49\] a series of studies over the years have confirmed the high correlation between microsatellite alterations in tumor tissues and those detected in circulating DNA.\[50–52\]

Cancer-specific epigenetic aberrations like promoter hypermethylation can be readily detected in ctDNA and may prove useful to decipher tumor biology as well as its clinical analysis. SEPT9 gene methylation is a well-known colorectal cancer (CRC) marker and its detection in ctDNA is the first FDA-approved blood-based screening test for CRC.\[53,54\] A recent meta-analysis showed that the performance of SEPT9 gene methylation-based CRC screening in symptomatic patients is better than that of protein biomarker-based diagnosis or fecal immunochemical testing.\[55\]

2.2.2. Prognostic significance of ctDNA analysis: monitoring MRD and predicting patient outcome

Relapse or recurrence is one of the major challenges in cancer treatment owing mainly to minimal residual disease (MRD), residual tumor components that remain after curative surgery or chemotherapy. Although MRD is routinely monitored for hematological
malignancies with well-characterized pathognomonic chromosomal lesions, like BCR-ABL gene fusion in chronic myelogenous leukemia patients.[56] Very few effective and reliable markers for MRD monitoring in solid tumors are currently available.[57] Surgical removal of solid tumors provides an opportunity for its comprehensive molecular analysis and thus identification of reliable and personalized markers, which can be then utilized in minimally invasive assays for further monitoring of the relapse.[58] In the past few years, application of ctDNA as a prognostic marker for MRD evaluation has gained particular attention.[59–62] CtDNA has often been detected in patients post-surgery or after therapy with curative intent, even though the patients were tested negative for other clinical signs or biomarkers. Such ctDNA-positive patients are at higher risk of relapse compared to the ctDNA-negative group. [57,59,60,63,64] The correlation between ctDNA detection and outcomes such as overall survival (OS) and disease-free survival (DFS) has been explored. Post-surgery/treatment elevated ctDNA levels have been consistently observed across various types of cancers to be associated with poor outcomes such as reduced OS and DFS rates, even though the majority of subjects with increased ctDNA levels seldom exhibit any other clinical evidence of disease.[59,62–66] Further studies have demonstrated the superiority of the ctDNA biomarker in terms of reliability and sensitivity compared to other biomarkers such as carcinoembryonic antigen (CEA) for monitoring metastatic colorectal cancer (mCRC).[60] These studies highlight the MRD monitoring capability of ctDNA analysis. However, the majority of the studies exploring the potential of ctDNA analysis are based on the detection of point mutation/s in one or a set of cancer-specific genes. In contrast to the somatic point mutations, which may be shared by various tumor types, chromosomal rearrangements are highly tumor specific and thus may represent a unique “fingerprint” that can be exploited for cancer detection and monitoring. Recent studies based on massively
parallel sequencing (MPS) allowed for the characterization of personalized landscapes of chromosomal rearrangements among cancer patients, with application in prediction of the development of metastasis as well as post-surgery recurrence (Table 2.1). [67,68]

Table 2.1. Summary of key studies about prognostic potential of ctDNA in cancer

<table>
<thead>
<tr>
<th>Patient population</th>
<th>Marker in ctDNA</th>
<th>Outcome</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic colorectal cancer (mCRC) patients (n = 31)</td>
<td>KRAS mutations and RASSF2A hypermethylation</td>
<td>- Association of KRAS mutations and RASSF2A hypermethylation in ctDNA with decreased disease-free survival at one year in patients with MCRC receiving chemotherapy</td>
<td>[69]</td>
</tr>
<tr>
<td>MCRC patients (n=58)</td>
<td>KRAS mutations, p16 gene promoter methylation</td>
<td>- Two-year survival was only 48% in CRC patients with ctDNA, in comparison with 100% in those without.</td>
<td>[57]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Verification of high diagnostic accuracy of KRAS mutations in ctDNA for MCRC detection with sensitivity of 87.2% and specificity of 99.2</td>
<td></td>
</tr>
<tr>
<td>Patients with single sporadic colorectal carcinomas (n=46)</td>
<td>TP53 mutations</td>
<td>- Association of TP53 mutations in ctDNA with advanced clinical stage and liver metastases, but not lymph node metastases, tumour size or vascular invasion.</td>
<td>[70]</td>
</tr>
<tr>
<td>Patients who underwent curative resection for CRC (n=150)</td>
<td>TAC1 and SEPT9 methylation</td>
<td>- Introduction of methylated TAC1 and SEPT9 in serum ctDNA as an independent predictor of tumour recurrence post-surgery and cancer specific mortality</td>
<td>[71]</td>
</tr>
<tr>
<td>Patients with esophageal squamous cell carcinoma (n=209)</td>
<td>Hypermethylation of the MutS Homolog 2 (MSH2) promoter</td>
<td>- Identification of hypermethylation in promoter reign of MSH2 in primary tumor specimens of 101 of 209 patients (48%) with esophageal squamous cell carcinoma, of which 77 patients (76%) had matching findings in plasma ctDNA. - Prediction of reduced disease-free survival post esophagectomy</td>
<td>[72]</td>
</tr>
<tr>
<td>Patients with APC</td>
<td></td>
<td>- Association between levels of</td>
<td>[73]</td>
</tr>
<tr>
<td>Disease</td>
<td>Biomarker(s)</td>
<td>Results/Findings</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
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<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>esophageal adenocarcinoma (n= 52)</td>
<td>hypermethylation</td>
<td>Hypermethylated APC gene in ctDNA and decreased survival in esophageal adenocarcinoma.</td>
<td></td>
</tr>
<tr>
<td>Patients with Hepatocellular carcinoma (HCC) before operation (n=79), patients with liver cirrhosis (n=20), and healthy volunteers (n=20).</td>
<td>Microsatellite instability of D8S258 and D8S264</td>
<td>-Prediction of overall and 3-year disease-free survival in HCC. -Association of D8S258 with tumour stage, tumour differentiation and vascular invasion.</td>
<td>[74]</td>
</tr>
<tr>
<td>Patients with invasive epithelial ovarian carcinoma (EOC)(n=164), patients with benign ovarian neoplasms (n=49), and unaffected age-matched controls (n=75)</td>
<td>b-globin, b-actin and GAPDH</td>
<td>-High level of preoperative ctDNA in patients with EOC which is an independent predictor for survival probability in ovarian cancer patients.</td>
<td>[75]</td>
</tr>
<tr>
<td>Patients with colorectal cancer (n = 18)</td>
<td>APC, KRAS, TP53, PIK3CA</td>
<td>ctDNA level reflects the total systemic tumor burden and could be applied to monitor tumor dynamics over surgery or chemotherapy. ctDNA is a much more reliable and sensitive indicator compared to other current standard biomarkers.</td>
<td>[60]</td>
</tr>
<tr>
<td>Patients with metastatic colorectal cancer (n = 108)</td>
<td>KRAS, BRAF</td>
<td>Relation between the quantitative measures of ctDNA and the tumor specific KRAS mutation in ctDNA. High concordance between KRAS status in primary tumor and ctDNA.</td>
<td>[63]</td>
</tr>
<tr>
<td>Patients with breast cancer (n = 147), healthy controls (n = 35)</td>
<td>TP53</td>
<td>Similar molecular signature in tumor and ctDNA in 61 of the 142 patients during 58 months of assessment. ctDNA can be utilized as a prognostic marker of the overall survival of breast cancer patients.</td>
<td>[64]</td>
</tr>
<tr>
<td>Patients with early-stage breast cancer (n = 29)</td>
<td>PIK3CA</td>
<td>Detection of PIK3CA mutations in primary tumor samples and ctDNA from patients with early-stage breast.</td>
<td>[59]</td>
</tr>
</tbody>
</table>
Early breast cancer patients (n = 5) | Panel of 273 genes | Detection of minimal residual disease (MRD) in breast cancer noninvasively Prediction of metastatic relapse with high accuracy Demonstration of intra-tumor genetic heterogeneity Appearance of resistance mutations in ctDNA many months prior to the development of clinical resistance in the metastatic setting | [62]

Patients with primary gastric adenocarcinoma (n = 202) | XAF1 | High frequency of XAF1 methylation in ctDNA of cancer patients could be a good biomarker for diagnosis, prognosis and recurrence of gastric adenocarcinoma | [66]

Patients diagnosed with primary breast cancer (n = 20) | 237 tumor-specific chromosomal rearrangements | ctDNA monitoring is highly accurate for prediction of postsurgical recurrence | [67]

Patients with high-grade serous OC stage III–IV (n = 10) | Genomic rearrangements | Panel of individualized junctions derived from ctDNA provides a promising tool for assessment of disease surveillance and therapeutic response monitoring Selected chromosomal junctions were detected in eight of the ten patients | [68]

### 2.2.3. Dynamic cancer genotyping and prediction of response to therapy

The role of tumor genotype in determining therapeutic response is well established. Over the past years, patient management methods have shifted the focus from empirical decision making based on patient's clinicopathological features to the genotype-informed biomarker-driven treatment strategies and personalized targeted therapies. Intratumor heterogeneity
leads to temporal and therapy induced clonal evolution, which may subsequently result in the development of acquired resistance to previously effective therapies. Therefore, dynamic monitoring of the clonal genetic composition of tumors has been increasingly recognized as a cornerstone strategy for improving patient outcomes. In this context, liquid biopsies can be used not only for real-time monitoring of the response to therapies but may also help to identify the emergence of resistant subclones.[58] Numerous studies have shown that the decline in plasma ctDNA levels is observed after therapy.

Thus, ctDNA can be used as a biomarker for monitoring the response to radio and chemotherapies as well as surgical resection.[17,76,105,106] On the other hand, a contrasting observation, i.e. increase in plasma ctDNA concentration post-therapy, has also been reported to be an indicator of successful therapy in some cases. Increased ctDNA levels reflect increased cell death which in turn is indicative of the effectiveness of the therapy.[106] In addition to monitoring the dynamic changes in ctDNA levels, analysis of changing patterns of cancer-specific biomarkers in ctDNA may serve as an early quantitative metric for prediction and monitoring of cancer treatment response.[77] Biomarker-driven decision making in non-small cell lung cancer (NSCLC) patients is one of the areas where ctDNA based analysis has shown particular translational significance. Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI) therapy in NSCLC patients is known to be influenced by mutations in the EGFR gene.[78] Certain activating mutations in the EGFR kinase domain increase the responsivity of patients towards the EGFR TKIs gefitinib or erlotinib.[78,79] On the other hand, EGFR-T790M mutation causes secondary resistance to gefitinib and erlotinib.[80] Therefore, genotype-informed therapeutic decisions in NSCLC patients are critically important. The utility of ctDNA analysis for therapy response prediction and determination of patient suitability has been demonstrated in a large number of studies [see ref. 81 and the references therein]. Although various studies showed that the
sensitivity of detecting EGFR mutations in plasma may be as low as 60–65%, these lower sensitivities were probably due to the limited analytical sensitivity of the method employed.[58] Similarly, different types of EGFR mutations have shown variable rates of concordance between plasma and tissue levels. For example, in a phase III trial of osimertinib, concordance between plasma and tissue levels of EGFR-T790M mutation was found to be very low.[82] Nonetheless, a large number of studies showed that the correlation between plasma mutation status and response rates to therapy is almost similar to that of tumor tissue mutation status and therapy response rates.[82–85] Therefore, both European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have approved ctDNA-based mutation testing to select appropriate therapies for NSCLC patients.[86,87]

Mutations in KRAS and BRAF genes are known to confer resistance to EGFR-based therapies in mCRC patients. Therefore, similar to EGFR testing in NSCLC patients, analysis of the mutational status of these genes in mCRC patients has been explored as a minimally invasive tool for prediction of response to therapy.[88] ctDNA-based KRAS mutation analysis has been applied to monitor the evolution of resistance to therapy in real time. Relative changes in the fractional concentration of various tumor mutations in ctDNA may indicate clonal evolution, which in turn may be correlated with the emergence of resistance to therapy.[89] Remarkably, ctDNA based analysis has been shown to be able to detect the emergence of resistance-conferring mutations months before progression is clinically obvious.[16,90] These studies highlight the significance of a minimally invasive ctDNA-based analysis approach for dynamic genetic monitoring of cancers to identify and predict the emergence of therapy resistance. Monitoring of tumor dynamics is an important application of ctDNA. Plasma ctDNA levels depict total systemic tumor burden level and fluctuations in ctDNA levels postsurgery are related to the extent of surgical resection. These ctDNA levels are reduced upon resection of the entire tumor and may increase again as new
lesions become obvious.[60] Analysis of ctDNA genomic profiles has also helped reveal the tumor dormancy (Table 2.2).[91]

Table 2.2 Summary of key studies using ctDNA to monitor response to therapy

<table>
<thead>
<tr>
<th>Patient population</th>
<th>Method</th>
<th>Outcome</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women with metastatic breast cancer (n=30)</td>
<td>-Targeted sequencing</td>
<td>-Greater dynamic range for ctDNA and its better correlation with tumor burden than CA 15–3 or CTCs. - Increases in ctDNA reflection of progressive disease - Early diagnosis of cancer 5 months before imaging confirmation.</td>
<td>[92]</td>
</tr>
<tr>
<td></td>
<td>-Microfluidic digital PCR or TAm-Seq.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian and breast cancer (n=38)</td>
<td>-Deep Sequencing Tumor DNA (TAm-Seq) and screen of 5995 genomic bases for low-frequency mutations</td>
<td>-Identification of mutations in ctDNA at allele frequencies as low as 2%, with sensitivity and specificity of &gt;97%. - Identification of the origin of metastatic relapse in a patient with multiple primary tumors. - Monitoring tumor dynamics and tracking 10 concomitant mutations in plasma of a metastatic breast cancer patient.</td>
<td>[17]</td>
</tr>
<tr>
<td>Early breast cancer patients undergoing neoadjuvant chemotherapy (n=55)</td>
<td>-Massive parallel sequencing on tumor to identification somatic mutations, then designed personalized digital PCR assay.</td>
<td>-Baseline ctDNA (presurgery) was not predictive of disease-free survival. - Introducing ctDNA as a significant predictor of early relapse by detection of ctDNA in post-surgical sample or during serial samples.</td>
<td>[62]</td>
</tr>
<tr>
<td>Breast cancer (n=20)</td>
<td>-MethDet-56 technique for evaluating methylation in ctDNA</td>
<td>-Change of methylation patterns in ctDNA after surgery and tamoxifen treatment, especially after combined treatment suggesting ctDNA for monitor treatment.</td>
<td>[77]</td>
</tr>
<tr>
<td>Patients with primary breast cancer treated with surgery (n=20). patients with long-term disease-free</td>
<td>-Whole-genome sequencing then development of personalized ddPCR assays across breakpoint junction.</td>
<td>-Accurate discriminating between patients with (93%) and without (100%) eventual recurrence. - Clinical detection of metastasis in 86% of patients with average lead time 11 months.</td>
<td>[67]</td>
</tr>
</tbody>
</table>

27
<table>
<thead>
<tr>
<th>Study Description</th>
<th>Results/Methods</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients undergoing radiation therapy (n= 15)</td>
<td>- Quantification of human and mouse nucleic acids in plasma samples using real-time PCR.</td>
<td>[93]</td>
</tr>
<tr>
<td>Patients with histologically proven metastatic NPC treated with first-line chemotherapy (n= 127)</td>
<td>- Real-time quantitative PCR system</td>
<td>[94]</td>
</tr>
<tr>
<td>Volunteers for screening of early Nasopharyngeal carcinoma (n= 1318)</td>
<td>- Real-time PCR system</td>
<td>[95]</td>
</tr>
<tr>
<td>Lung cancer patients with EGFR T790M who had resistance to EGFR tyrosine kinase inhibitors (EGFR-TKI). (n= 89)</td>
<td>- The mutation-biased PCR and quenching probe (MBP-QP) method</td>
<td>[96]</td>
</tr>
<tr>
<td>Subjects undergoing multimodality therapy for colorectal cancer (n= 18)</td>
<td>- BEAMing (beads, emulsion, amplification and magnetics)</td>
<td>[60]</td>
</tr>
</tbody>
</table>
| Women with metastatic breast cancer who were receiving systemic therapy (n= 30) | - Microfluidic digital PCR assay  
- Tagged-amplicon deep sequencing | -Identification of Somatic Genomic changes  
-Quantification of ctDNA in Plasma  
-Monitoring of Multiple Somatic Genomic changes in ctDNA  
-High sensitivity of ctDNA in comparison with CA 15-3 and CTC  
-Tumor Monitoring | [97] |
|---|---|---|---|
| Breast cancer patients and eight healthy female controls. (n= 65) | -Real-time quantitative PCR | - Detecting and monitoring breast cancer using genomic analysis of ctDNA isolated from plasma  
-Different SNP profiles in cfDNA between patients with breast cancer and healthy female controls  
-Heterogeneous copy number variations (CNVs) in Plasma and tumor DNA  
-Plasma SNP/CNV changes during routine follow-up | [91] |
| Patients with metastatic melanoma (n = 93) | -Sensitive allele-specific PCR | Mutant BRAF V600E ctDNA can be identified in >90% of stage IV melanoma patients  
Kinetic changes in ctDNA levels can provide early information about responding and nonresponding patients as early as 2 weeks after the initiation of therapy, which enables clinicians to modify or change the treatment procedure accordingly | [98] |
| Patients with metastatic colorectal cancer (n = 108) | -ARMs PCR | Correlation between the status of ctDNA and pKRAS  
Quantitative changes in ctDNA and pKRAS over the course of treatment  
Changes of mutational levels during treatment | [88] |
| Patients with colorectal cancer (n = 28) | -BEAMing, qPCR | KRAS mutations were detectable in 38% of patients  
The emergence of KRAS mutations was very consistent, usually occurring between 5 and 6 months during treatment | [89] |
Patients with advanced breast, ovarian and lung cancers (n = 6) - Sequencing of cancer exomes

| Tracking the genomic evolution of metastatic cancers during treatment | Correlation of increased level of mutant alleles and resistance to therapy | Exome-wide analysis of ctDNA | [16] |

2.3. Challenges in detection and translational application ctDNA

Although ctDNA holds promise for the development of safe and effective cancer detection and monitoring methods, several major challenges still remain. Here we present an overview of the biological and technical challenges that have so far hampered the introduction of ctDNA analysis into routine clinical practice, along with a brief discussion on possible ways to circumvent these hurdles.

2.3.1. Biological challenges

2.3.1.1. High fragmentation

One of the most crucial challenges in cfDNA assessment is its highly fragmented nature, which not only makes its isolation challenging but also affects the accuracy of its quantitation.[99] Moreover, DNA fragmentation reduces the number of analyzable or intact DNA copies thus making analysis such as amplification or sequencing difficult.[100,101] Another confounding feature of the ctDNA compartment of circulating cfDNA is the wide variability in the fragment sizes. This wide variability can be attributed to the variety of mechanisms underlying the dead cancer cells. cfDNA in healthy individuals mostly arises as a result of apoptosis, producing smaller fragments with uniform size distributions of around ~185–200 bp.[27,61,102–104] On the other hand, necrosis and autophagy, which are common causes of cell death in solid tumors, produce fragments of less uniform sizes.
because of random and incomplete DNA digestion.[105] ctDNA fragments as small as 134–144 bp have been measured in a mouse xenograft model,[106] while DNA fragments longer than 10 000 base pairs have also been observed.[61] Additionally, the degree of fragmentation may even increase with increasing tumor size.[107] As a result of the high fragmentation, loss or reduction in the number of DNA molecules harboring the genetic marker of interest may occur, thereby jeopardizing the accuracy of ctDNA analysis. Moreover, from a methodological point of view, fragmented DNA may be difficult to analyze due to potential loss of DNA characteristics being exploited to design a particular assay, e.g. loss of the primer binding site may hamper PCR amplification.

2.3.1.2. Low abundance

The other important biological challenge that needs to be considered is the low abundance of cfDNA, which is usually 5–10 ng ml⁻¹ of plasma in healthy individuals.[108] Although this quantity in cancer patients may increase up to 50 times the normal levels, the percentage of ctDNA within the bulk cfDNA is usually low.[108] In most of the early-stage cancer patients, the level of ctDNA may be as low as one genome equivalent in 5 ml of plasma. Therefore, the detection of ctDNA in plasma volumes sampled routinely is extremely difficult and increased volumes of blood samples are required.[109] Moreover, tumor heterogeneity may influence mutant allele frequency (AF) thus making identification of target genotype masked within a large background of normal cfDNA and heterogenous ctDNA a formidable challenge. Recent advances in library preparation and massively parallel sequencing (MPS) technologies,[110,111] as well as digital genomic methods[112,113] have improved the sensitivity of ctDNA detection, quantification, and molecular characterization.
2.3.1.3. Difficulty in direct analysis of ctDNA.

Among the two most common methods of nucleic acid analysis, amplification and hybridization, the latter offers high sequence specificity and is therefore particularly suited for cancer specific molecular alteration-based analysis of ctDNA. However, the double stranded structure of DNA is a key hurdle in this respect as efficient denaturation of dsDNA into ssDNA molecules suited for hybridization analysis requires multiple processing steps and is time-consuming. Moreover, renaturation of ssDNA molecules also affects the efficiency of hybridization-based analyses.[114] To obviate this hurdle, various methods have been proposed. Noh et al. proposed a sensitive electrochemical method for direct detection of dsDNA using alkaline phosphatase labelled Zn-finger proteins. [114] More recently, DNA clutch probes (DCPs) have been used for sensitive and specific electrochemical detection of ctDNA. DCPs prevent renaturation of denatured DNA molecules, thus facilitating efficient hybridization analysis.[28]

2.3.1.4. Low stability of cfDNA

The half-life of cfDNA in circulation has been measured to be 4–30 minutes.[115] Yu et al. evaluated the kinetics of fetal cfDNA clearance in postcaesarian women by applying massive parallel sequencing. The results showed biphasic clearance mechanisms with half-lives of about 1 h for the rapid phase and a second phase of 13 h.[116] While the kinetics and fate of cfDNA, particularly cell-free fetal DNA, are better understood, very few studies have addressed the clearance rate and mechanism of ctDNA. Nonetheless, the half-life of ctDNA post-resection has been shown to be 1.5 to 2 hours.[60] cfDNA clearance is influenced by a multitude of factors which further affect the reliability of cfDNA measurements. Multiple mechanisms are involved in the release, degradation, and clearance of cfDNA. Various forms of circulating DNA (exosome associated, nucleosomal DNA, etc.) exhibit different levels of
stability.[117,118] Moreover, the variable levels of DNase activity as well as the adsorption of cfDNA on the surface of blood cells also affects its stability and clearance time.[119,120] And lastly, a multitude of mechanisms and organs, such as the spleen, liver, and kidneys, are involved in cfDNA clearance as well as uptake and degradation by phagocytes.[116,121] Consequently, diagnostic studies based on measuring the amount of cfDNA present insufficiently robust and consistent results.[122]

2.3.2. Technical challenges

2.3.2.1. Sample processing and choice of sample source

The concentration of cfDNA can be affected by various preanalytical factors that need to be standardized such as blood collection, serum or plasma processing, the time interval between collection and centrifugation, the storage conditions, centrifugation force, and cryopreservation conditions.[123–126] It has been reported that the time interval between venipuncture and sample processing and the storage temperature affect cfDNA concentration. However, the length of time delays post-collection which significantly affect cfDNA concentration is still debatable. Some reports have shown a significant increase in cfDNA levels within 4 hours of collection while others suggest that any significant increase does not appear before 24 hours of storage.[127] Nonetheless, swift sample processing is a prerequisite for reliable cfDNA analyses and may pose a significant challenge under field conditions. On the other hand, storage temperature may also affect cfDNA concentration. However, it has been observed that storage at +4 to RT does not significantly alter cfDNA concentration. Similarly, gentle and careful blood drawing and handling is recommended during processing samples for cfDNA analysis as sample agitation may cause hemolysis and subsequent increase in cfDNA levels.[128] Choice of sample source is also a significant factor that contributes to the variations in measured cfDNA quantities. The cfDNA
concentration in serum can be 2–24-fold higher than in plasma due to in vitro lysis of leukocytes during coagulation/fibrinolysis.[123,129] A 2004 study by Taback et al. found that cfDNA quantity in serum samples was considerably higher than that in the paired plasma samples, which even further increased if the serum was obtained by letting the blood samples clot overnight at 37 °C. The authors thus reasoned that cell lysis and destruction during the clotting may be the reason behind increased cfDNA concentrations in serum compared to plasma. Such “interference” from DNA supposedly originating from blood cells may hamper cfDNA quantification based tumor screening, particularly in early-stage patients, as well as obscure minute ctDNA amounts and thus hinder detection of tumor-specific mutations, etc.[130] Many laboratories thus propose the use of plasma instead of serum for ctDNA analysis.[131] In addition to the choice of sample source and storage time, several other preanalytical factors should be taken into account in order to develop reliable ctDNA-based clinical tests. For example, the choice of anticoagulant also affects the stability of cfDNA as well as contamination from genomic DNA of blood cell origin. EDTA has been shown to be a better choice in this regard compared to citrate and heparin. [132] The choice of anticoagulant may also depend on the downstream analytical method intended to be employed.[127] While a few studies have compared the performance of sample collection tubes for cfDNA analysis,[133,134] such data are currently lacking in the field of ctDNA analysis and more studies will be needed to characterize the impact of this preanalytical factor on the reliability of ctDNA analysis.[127] While the lack of consensus and well-established standard operating procedures (SOPs) in regarding preanalytical sample processing warrant more in-depth evaluation of these factors, recent developments in the near-patient and point-of-care devices may also help control the variability in ctDNA test results that emanates primarily from the time delays between sample collection and processing.[135] Recent advances in microfabricated chip-based devices are expected to
bring the aim of integrating ctDNA analysis in routine oncological testing closer to reality.[136–138]

2.3.2.2. Low efficiency of the cfDNA extraction procedures

The available concentration of cfDNA in body fluids is very low. Therefore, a sensitive and specific method needs to be designed for extraction of cfDNA from samples. A variety of protocols for cfDNA extraction have been reported such as isolation with organic solvents, use of the various commercial kits, and the use of magnetic beads.[125,126,139,140] The earliest report of Mandel describing the existence of cfDNA in human blood could isolate ∼5.4 μg mL⁻¹ DNA from blood of healthy individuals.[141] Later studies by Stroun et al. reported the isolation of 150 ng mL⁻¹–12 μg mL⁻¹ cfDNA from plasma of cancer patients.[40] However, modern column-based methods can isolate much less DNA as compared to the aforementioned amounts. Schmidt et al. reported that the amount of isolated cfDNA can be increased by as much as 8 times the routine column-based methods by their salting-out DNA isolation protocol.[142] However, the loss of small DNA fragments (<100 bp) during these isolation processes is still a crucial challenge.[142] Recovery of smaller DNA fragments is particularly important in ctDNA analyses. Due to the high level of fragmentation and wide range of fragment sizes observed in cancer patient samples, these smaller fragments may potentially harbor characteristic tumor-specific targetable molecular alterations, such as mutations.[60] A probable solution for the mentioned problem is designing a method to capture all cfDNA fractions with any length.[143] A new generation of plasma/serum cfDNA purification kits have been developed with a high affinity for all sizes of DNA, including very low MW fragments.[144] In spite of the various advances and improvements that have been made to cfDNA/ctDNA purification protocols, substantial amounts of cfDNA/ctDNA are still lost at the purification stage.
Furthermore, during the separation of plasma from blood, the samples need to be essentially subjected to centrifugation and it has been shown that high centrifugation speeds lead to the ex vivo release of DNA from blood cells.[145] Therefore, there has been a growing interest in the development of methods which obviate the need to subject blood samples to tedious DNA purification procedures.[146] Advancements in microfluidics and nanotechnology open up new cfDNA isolation methods with better yields. For example, Sonnenberg et al. reported dielectrophoresis-based cfDNA isolation. However, their method uses a very small volume of sample, whereas high throughput is desirable due to the low abundance of ctDNA.[147,148] On the other hand, nanochip and nanowire-based methods have also been reported which carry out the recovery of cfDNA from the plasma, followed by their release by switching the oxidation state of the conducting polymer.[149,150] These innovations hold great promise for improving the overall sensitivity of ctDNA detection approaches, thus paving the way for their clinical integration.

2.3.2.3. Limited comparability of different quantification methods

Different cfDNA quantification methods such as spectrophotometric approaches and sensitive fluorometric methods using different dyes as well as various PCR-based assays with different targets have been reported.[129,140,151,152] However, the results are not the same because these assays target total or only amplifiable DNA.[153] For example, the target sequences in PCR-based methods should be as short as possible to allow the detection of ctDNA fragments.[154,155] On the other hand, in fluorometric assays, one of the favourable methods for analysis of total cfDNA levels, there is no limit for DNA length.[43,156] Currently there is a lack of studies conducting direct comparisons between various cfDNA analysis approaches; thus optimal methods for reliable cfDNA quantification and efficient recovery for molecular analysis are poorly defined.[157] There is an urgent need to develop
consensus SOPs and standardized methods for cfDNA/ctDNA analysis taking into consideration all of the possible analytical and preanalytical factors that may influence the test outcomes.[127]

### 2.3.3. Clinical challenges

The development of a ctDNA biomarker-based cancer detection and monitoring method requires large-scale clinical studies and ample evidence not only for the efficiency of the method and reliability of the marker but also for the clinical utility of the developed test. For each targeted tumor type, several hundreds of cancer patients may need to be analyzed. To verify that variants are cancer-defining, the test should also evaluate cfDNA profiles from large numbers of healthy individuals as controls. Continuous clinical follow-up of these two groups is also necessary to distinguish false positive signals from true positives [158,159]

### 2.4. Methods for ctDNA detection

The analysis of ctDNA is challenging and requires sensitive techniques due to the very low ctDNA percentage in total cfDNA (<1.0% in many cases) and low AF of targetable molecular alterations.[13,14] Various approaches have been used for ctDNA analysis including Sanger sequencing or pyrosequencing,[160] DNA-binding dye-based fluorometric assay, [161] or fluorescent spectrophotometry.[162] However, these methods are capable of detecting ctDNA fragments only in patients with advanced stage disease or high tumor burden as large quantities of ctDNA are present in the blood of these patients.[160] Recent advancements have made it possible to detect extremely low quantities of ctDNA, thus facilitating their clinical usage.[16,39,109] Methodologies being used for ctDNA analysis can be broadly categorized as targeted and untargeted approaches. Targeted approaches detect specific known molecular alterations Targeted ctDNA detection methods have attracted more
attention due to their ability to overcome common challenges associated with the ctDNA analysis such as short fragment length, low quantity of blood, and low diagnostically relevant allele frequency.[163] On the other hand, untargeted approaches do not require a prior knowledge of any specific genetic/epigenetic changes present in primary tumors and usually employ whole genome or whole exome sequencing. Such approaches are particularly important for the discovery of novel disease markers. In addition to sequencing, other prominent untargeted approaches include digital karyotyping, personalized analysis of rearranged ends (PARE), etc.[163,164] Despite the important roles that untargeted methods may have played in deciphering the tumor heterogeneity as well as discovery of novel drug targets, their clinical utility may be hampered by various shortcomings such as difficulty in detection of rare mutations and requirement for large quantity of samples as well as high associated costs.[33]

Targeted approaches, however, are generally more clinically applicable and can be further divided into three subcategories: PCR-based, [165–167] digital PCR-based [17,39,59,62,92] and sequencing-based technologies. [39,168]

2.4.1. Amplification and sequencing-based approaches

PCR-based technologies are easy to use and of low cost. However, most of these methodologies have low sensitivity and are only capable of analyzing a limited number of genomic loci. Various PCR-based protocols have been reported over the years including amplification refractory mutation system (ARMS-PCR),[63] bidirectional pyrophosphorolysis activated polymerization (bi-PAP),[169] MIDI-activated pyrophosphorolysis (MAP),[166] etc. While ARMS-PCR is based on allele specific primers which can only generate an amplified product if the target allele is present, both MAP and bi-PAP utilize oligonucleotides that are blocked at the 3’ end by a dideoxynucleotide. As these
primers bind their cognate templates, the pyrophosphorolysis activity of DNA polymerase removes the 3’ dideoxynucleotide, thus allowing strand extension. Bi-PAP uses a pair of opposing primers with one overlapping nucleotide at the 3’ end. [169] On the other hand, MAP is MIDI (microinsertions/deletions/indels) activated and primers used in MAP have multiple mismatches with wild-type sequences. [166] Despite being rapid and cost-effective, the performance of conventional PCR-based methods regarding detection of low AF alterations is limited. Moreover, in many cases high error rates are associated with these methods. Digital PCR-based (dPCR) methods are known to be comparatively more sensitive at mutation detection. Prominent dPCR based methods employed for ctDNA detection include BEAMing (beads, emulsion, amplification and magnetics), droplet digital PCR (ddPCR), and microfluidic digital PCR. [170] dPCR-based methods have shown much improved limits of detection (LODs) compared to conventional PCR-based methods (ARMS LOD: 0.05–0.1% vs. ddPCR LOD 0.001%). [46] and are thus particularly useful for detection of low AF alterations. [35]

BEAMing is a dPCR-based method (Fig. 2.1) and is considered to be the most sensitive among targeted approaches with detection accuracy as high as 4.3 in 100 000. [171] In comparison, the accuracy of ddPCR is 1 in 10 000. [108] Using emulsion PCR and flow cytometry, BEAMing can effectively detect rare mutations with AF less than 0.01%. [172] This method is sensitive enough to directly quantify the error rate of DNA polymerases used for PCR. Moreover, BEAMing does not require any specialized instrument setup and can be carried out using broadly accessible apparatus like a magnetic stirrer, thermocycler, and flow cytometer. Besides this simplicity, the sensitivity can be easily amplified just by evaluation of more beads. The use of recyclable beads which contain variant alleles is another remarkable advantage of this method in comparison with other common techniques. [170]
Figure 2. 1. Schematic representation of BEAMing. 1: Magnetic beads covalently conjugated with streptavidin are attached to biotinylated oligonucleotides (oligos). 2: A mixture of chemical components needed for PCR, primer-bound beads and template DNA are mixed together with an oil/detergent mix in order to produce microemulsions. Red and green templates show two template molecules with different sequences. 3: PCR cycleing of the microemulsions. During this procedure if a DNA template and a bead are present in a single microemulsion, the bead-bound oligos play as primers for amplification. 4: Broking microemulsions and purification of beads using magnet. 5: Incubation of the beads with oligos which can distinguish between the sequences of the different types of templates. In order to mark the bound hybridization probes, fluorescently marked antibodies are used, which presents the beads containing PCR product as red or green after laser excitation. 6: Using flow cytometry the red and green beads can be counted [170]. (Reproduced from mentioned reference with kind permission of related journal).
Droplet dPCR (ddPCR) is also based on the principle of dPCR, where individual DNA molecules are dispersed into thousands of droplets (Fig. 2.2). Using fluorescently labelled TaqMan probes, droplets containing wild-type or mutated DNA strands can be identified and enumerated on a flow cytometry apparatus.[173] The sensitivity of a ddPCR system relies on the number of droplets.[112,160] Commercially, the QX200 Droplet dPCR System as one of the commonly used droplet dPCR devices has the ability to produce 20 000 nanolitersized droplets. This number of droplets has further been increased up to 10 million picoliter-sized droplets using the RainDrop dPCR System (RainDance Technologies). However, there are some limitations associated with the ddPCR method such as difficulty in producing droplets. Moreover, droplet processing is time-consuming.[112,174] Various targeted deep sequencing approaches have been utilized to analyze specified genomic regions in ctDNA and have proved to be highly sensitive.
Figure 2.2. Schematic illustration of droplet digital PCR: (a) Samples and droplet generation oil are transferred to droplet generator cartridge. (b) Using a vacuum in the droplet well, 1 nL droplets are produced. (c) Droplets which are stabilized by surfactant are transferred to a 96-well PCR plate. (d) The thermal cycler is used to perform droplet PCR amplification to endpoint. (e) In order to read droplet fluorescence, the plate is loaded onto a reader which sips droplets from each well and flows them single-file past a two color sensor. (f) Droplets are labeled as positive or negative which is attributed to their fluorescence amplitude. The number of positive and negative droplets is applied to calculate the concentration of the target and reference DNA sequences [125]. (Reproduced from mentioned reference with kind permission of related journal).
Tagged amplicon sequencing (TAm-Seq),[17] Ion-AmpliSeq,[175] cancer personalized profiling by deep sequencing (CAPP-Seq),[176] safe-sequencing system (Safe-SeqS)[39] and targeted resequencing[177] are some of the prominent deep sequencing-based ctDNA analysis methods. Amplicon sequencing is one of the main approaches for assessing mutated genes in specific genomic areas. Ion-AmpliSeq technology as a time and cost-effective method is one of the broadly applied deep targeted sequencing platforms and requires very small amounts of input DNA (as little as 10 ng). However, a high error rate in detection of small insertions and deletions limits its usage.[175] Usage of target capture-based platforms may reduce error rate but also require increased input (around 200 ng) of DNA.[178,179] Target capture-based platforms, such as cancer personalized profiling by deep sequencing (CAPP-Seq) are broadly applied for studying gene changes in cancers.[176] Various other remarkably sensitive sequencing approaches have been designed for ctDNA detection. For instance, in Safe-SeqS, each DNA molecule from the sample is tagged with a special identifier (UID) and is amplified. Thus, any molecular alteration present in the starting DNA template is amplified giving rise to a family of DNA molecules with the same alteration/mutation (UID family). A supermutant UID family in which \( \geq 95\% \) of family members have the same mutation is subsequently identified through MPS.[180]

Recently, duplex as a new PCR method based on Safe-SeqS has been applied by Chen et al. which can detect ctDNA in quantities as low as 0.1 ng mL\(^{-1}\). This approach with a novel internal standard could reduce variation and allow for more sensitive, accurate, and constant quantitative measurements of ctDNA.[181] On the other hand, TAm-Seq demonstrated amplification and deep sequencing from as few as individual copies of fragmented DNA.[17] Although methods like TAm-Seq and Ion-AmpliSeq are cost- and time-effective, a higher error rate is a big drawback in their usage (0.1% and 1–2%, respectively). Other methods like CAPP-Seq and Safe-SeqS have ventured to overcome this limitation. The error rate for the
CAPP-Seq and Safe-SeqS approaches has been recorded as 0.1% and $9.1 \times 10^{-6}$, respectively.[176,180] Despite various advantages, targeted approaches examine merely a few loci, and mutations or other genetic alterations in regions located outside mutational hotspots are missed. Targeted resequencing of genes known to be associated with tumorigenesis and progression has been adopted as one of the solutions to this hurdle.[182]

On the other hand, in the past few years untargeted approaches have gained significance for comprehensive characterization of genetic and epigenetic alterations in ctDNA instead of limiting the analyses to predefined or existing mutations.[16] These thriving untargeted approaches detect broad ctDNA alterations across wide genomic regions.[110,111,183] Sensitive mutation detection using sequencing (SiMSen-Seq) is a simple multiplexed PCR-based method for barcoding of cfDNA, which applies molecular barcodes to facilitate the detection of ultra-rare variants and reduce sequencing errors introduced during next-generation sequencing (NGS) library construction. By adopting various strategies such as reduced primer concentrations, extended PCR extension times, and hairpin-protected barcode primers, sequence variants with allele frequencies as low as <0.1% using DNA input (<50 ng) can be detected.[184]

The same limit of detection for low-frequency mutations on ctDNA was achieved in the recently reported method of UltraSEEK. In this approach, single-base extension is performed after multiplex PCR mutation-specific using chain terminators labeled with a moiety for solid phase capture. Subsequently, the captured products are interrogated by applying mass spectrometry. This method showed unique advantages over existing approaches such as alleviating the need for allele-specific PCR, annealing temperature constraints, or several step reactions for interrogation of variants.[185] Recently, Song et al. developed a nuclease-assisted minor-allele enrichment method using overlapping probes (NaME-PrO) for ctDNA detection which enables routine mutation detection at 0.01–
0.00003% abundance. This method can improve detection limits for all endpoint detection technologies.[186] Personalized analysis of rearranged ends (PARE)163,167 and digital karyotyping [187] are among the prominent untargeted assays. PARE is a PCR-based approach for detection of genomic rearrangements in ctDNA.[163,164] Digital karyotyping (Fig. 2.3) is another untargeted approach used to analyze new genomic regions and uncharacterized chromosomal changes in human tumors.[187–189] Incorporation of massively parallel sequencing (MPS) to ctDNA genomic profiling has led to many ground breaking discoveries. However, under conventional operating conditions, MPS could not detect mutations lower than 2% abundance. More recently the accuracy of the method has been improved via molecular barcoding and multiplex sequence readout.[184] Whole-genome rearrangements and CNAs in ctDNA have been reported as two truthful tumor biomarkers with high sensitivity and particularity.[91,167] In this regard, whole-genome/exome sequencing of ctDNA has led to the discovery of various novel cancer-associated genomic alterations including single nucleotide variants (SNV), copy number alterations (CNAs) and structural changes of DNA (Table 2.3).[13,16,164,191]

2.4.2. Chip-based approaches

Although the aforementioned strategies (Fig. 2.4A) have led to a significant progress in ctDNA detection, some key obstacles limit their clinical application. While sequencing-based approaches involve complicated operations and slow turnaround times, they are also prohibitively expensive for optimal translational usage. On the other hand, PCR-based methods are prone to sequence-specific amplification biases and require sample preprocessing, thus significantly increasing cost and time of analysis.[216] Consequently, low-cost platforms which involve simple operation and are able to accurately detect specific
ctDNA mutations directly from serum or blood are best suited for routine clinical use. Chip-based platforms are ideal candidates for this purpose due to their capacity for automation and also because they are affordable, user-friendly and require only a minuscule amount of sample.

Figure 2. 3. A graphical presentation of the digital karyotyping method. Colored boxes: genomic tags; small ovals: linkers; large blue ovals: streptavidin-coated magnetic beads. See
text for further information [182]. (Reproduced from mentioned reference with kind permission of related journal).
Table 2.3. Summary of ctDNA detection methods using sequencing technology.

<table>
<thead>
<tr>
<th>Principle of detection</th>
<th>Method</th>
<th>Features</th>
<th>Type of alteration</th>
<th>Advantages and limitations</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted approaches</td>
<td>PCR-based</td>
<td>ARMS</td>
<td>3’ nucleotide of PCR primer needs target mutant gene for extension</td>
<td>Known point mutations</td>
<td>Detection of PIK3CA mutations in ctDNA of 28% of patients with metastatic cancer Evaluation in real time (within 48 h)</td>
</tr>
<tr>
<td></td>
<td>SNPase-ARMS</td>
<td>qPCR</td>
<td></td>
<td>Application of two distinct polymerases to amplify target DNA and prevent amplification of non-target DNA Detection of 1 mutated DNA in 200 000 wild type Identification of target DNA in all melanoma patients</td>
<td>[190]</td>
</tr>
<tr>
<td></td>
<td>Bi-PAP</td>
<td></td>
<td></td>
<td>Detection of ctDNA in most of the patients with metastatic uveal melanoma (20 of 21 tested patients) Detection of a single mutated molecule per reaction, whereas 104 copies of normal DNA were not distinguished Failed to detect less than 1% mutant DNA diluted in normal DNA</td>
<td>[169]</td>
</tr>
<tr>
<td></td>
<td>qPCR</td>
<td></td>
<td></td>
<td>85% and 73% specificity</td>
<td>[191]</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>Detecting MIDIs which comprise about 15% of mutations</td>
<td>Known point mutations</td>
<td>Ultra-high analytical selectivity of one mutant molecule per more than 1 billion wild-type molecules and an analytical sensitivity of one mutant molecule per reaction Analytical sensitivity of $10^{-7}$ Applicable for large-scale, high throughput “gene pool” analysis</td>
<td>[166]</td>
</tr>
</tbody>
</table>
| Method          | Coamplification at lower denaturation temperature and HRM | Known point mutations | Detection of KRAS mutations in 21.3% of tumors  
Diagnostic sensitivity and specificity of 0.96  
No significant increase of KRAS mutation load in patients with advanced lung cancer compared with patients at early stages | [192] |
|-----------------|---------------------------------------------------------|-----------------------|-------------------------------------------------------------------------------------------------|------|
| PCR/SERS        | Application of SERS nanotags to address the limitations of fluorescence-based detection | Known point mutations | Detection of as few as 10 mutant alleles from a background of 10,000 wild-type sequences (0.1%)  
Providing single-tube multiplex assay  
Sensitive as ddPCR | [193] |
| SiMSen-Seq      | Usage of barcodes to uniquely tag individual target cfDNA molecules | Target sequence       | Reduced sequencing errors during NGS library construction  
Robust detection of ultra-rare variants  
Reduced primer concentrations  
Elongated PCR extension times  
Hairpin-protected barcode primers  
Detection of sequence variants at or below 0.1% allele frequency  
Works with low DNA input (<50 ng) | [184] |
| mmPCR-NGS       | mmPCR followed by NGS                                     | CNVs                  | Identification of clonal and subclonal CNVs in ctDNA  
Detection of CNVs with average allelic imbalances as low as 0.5%  
Five times more sensitive than whole-genome sequencing methods  
Limitations: needing to haplotype information and deficiency in distinguishing between copy number gains and losses at low average allelic imbalance | [194] |
| Ultra-SEEK      | Multiplex PCR followed by a mutation-specific single-base extension | Panel of 26 clinically relevant | High throughput, multiplexed, ultrasensitive mutation detection of mutant sequence mixtures as low as 0.1% minor allele frequency  
The performance is equivalent to ddPCR and superior to | [185] |
<table>
<thead>
<tr>
<th>Method</th>
<th>Targets</th>
<th>ARMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digital PCR-based BEAMing</td>
<td>DNA templates amplified separately on beads and then quantified</td>
<td>Feasible 0.01% sensitivity 100% concordance between BEAMing and standard sequencing Detection of ctDNA with very low percentage of mutant genes [195, 196]</td>
</tr>
<tr>
<td>Multiplexed dPCR</td>
<td>Varying the concentration of the fluorescent probes to differentiate mutations on the basis of fluorescence intensity</td>
<td>75% sensitivity and 100% specificity Detect as few as 3 copies of the mutant allele Test for the 4 hotspot mutations in one single reaction [197]</td>
</tr>
<tr>
<td>ddPCR</td>
<td>DNA templates amplified separately in water-in-oil droplets then quantified</td>
<td>Identification of several exon mutations 93.3% sensitivity, 100% specificity Accurate mutation detection in ctDNA at single molecule sensitivity Discrimination of somatic mutant rearranged sequences down to 0.01% tumor DNA content (one rearranged sequence per10 000 wild-type sequences) Feasibility of ctDNA analysis for patients with early stage Difficulties such as studying just a few hotspot mutations, time-consuming [59, 62, 113, 198–201]</td>
</tr>
<tr>
<td>Fluidic PCR-based</td>
<td>Evaluation of determined gene copies by calculating the ratio in panel</td>
<td>Detection and quantification of rare mutations (0.02–9.26% abundance) Demonstration of the presence of intratumor molecular heterogeneity in early-stage lung tumors [202, 203]</td>
</tr>
<tr>
<td><strong>Targeted deep sequencing-based technologies</strong></td>
<td><strong>TAm-Seq</strong></td>
<td><strong>Primers generate amplicons that tile regions of interest in short segments, followed by a two-step amplification process then sequencing</strong></td>
</tr>
<tr>
<td><strong>Safe-SeqS</strong></td>
<td><strong>Uses single molecule barcoding on one or both strands before PCR amplification, followed by sequencing to reduce sequencing error</strong></td>
<td><strong>Point mutations</strong></td>
</tr>
<tr>
<td><strong>Ion-AmpliSeq</strong></td>
<td><strong>Assessing mutant genes in particular genomic regions</strong></td>
<td><strong>Known point mutations</strong></td>
</tr>
<tr>
<td><strong>Targeted resequencing</strong></td>
<td><strong>Targeted resequencing</strong></td>
<td><strong>A panel of genes</strong></td>
</tr>
<tr>
<td><strong>(CAPP Seq)</strong></td>
<td><strong>Usage of biotinylated oligonucleotide selector probes</strong></td>
<td><strong>Rearrangements, copy number alterations</strong></td>
</tr>
<tr>
<td>Method</td>
<td>Preparation</td>
<td>Known point mutations</td>
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<tr>
<td>------------------------</td>
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<td>------------------------</td>
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<tr>
<td>Duplex</td>
<td>Preparation of internal standard sequence using plasmid vector, then amplification of internal standard and targeted gene</td>
<td>Known point mutations</td>
</tr>
<tr>
<td>Targeted NGS</td>
<td>Description by Couraud and using the IonTorrent or other platforms</td>
<td>Point mutations, insertions/deletions, and rearrangements and CNVs</td>
</tr>
<tr>
<td>Untargeted approaches</td>
<td>PARE</td>
<td>Genome wide rearrangements, structural alterations in gene regions</td>
</tr>
<tr>
<td>Digital karyotyping</td>
<td>Short sequence tags from specific genomic loci isolated and enumerated</td>
<td>Copy number, chromosomal changes, amplification</td>
</tr>
<tr>
<td>Method</td>
<td>Process Description</td>
<td>Findings</td>
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<tr>
<td>-------------------------------</td>
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<tr>
<td>Whole-genome sequencing</td>
<td>Purification of ctDNA and generating paired-end libraries for whole-genome sequencing, then analyzing each library</td>
<td>Appropriate to genomic changes with high allele frequencies Expensive Detection of ctDNA in most of the patients The sensitivity and specificity of this approach are related to the amount of sequence</td>
</tr>
<tr>
<td>Shallow whole-genome sequencing</td>
<td>A combination of shallow whole-genome sequencing with personalized droplet digital PCR, analytical methods, and a bioinformatics pipeline</td>
<td>Rearrange ment, copy number alterations Discrimination of patients with eventual metastasis from those with long-term disease-free survival with 93% sensitivity and 100% specificity Detection of mutant gene in allele frequency of 3.7 ± 1.6%</td>
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**Abbreviations:** ARMS, Amplification Refractory Mutation System; bi-PAP, bidirectional pyrophosphorolysis-activated polymerization; MAP, MIDI-Activated Pyrophosphorolysis; MIDIs, Detecting microinsertions/ deletions/indels; BEAMing, Beads, Emulsion, Amplification, Magnetics; ddPCR, Droplet digital polymerase chain reaction; TAm-Seq, Tagged-Amplicon deep Sequencing; Safe-SeqS, Safe-Sequencing System; CAPP-Seq, Cancer personalized profiling by deep sequencing; PARE, Personalised Analysis of Rearranged Ends.

Figure 2. 4. Typical strategies for ctDNA detection. A) Analyzing plasma ctDNA applying massively parallel sequencing and digital genomic technologies. WGS, whole genome sequencing, WES, whole exome sequencing, TS, targeted sequencing [208]. B) The clamp chip for the electrochemical evaluation of mutant cell free nucleic acids (cfNAs). The sample is used to the PNA probe-modified microsensor, and merely the target mutated cfNAs hybridize to an immobilized PNA probe [199]. C) Unified nanoplasmic biosensor system for methylated ctDNA detection which comprises dark-field microscope, spectrograph, and CCD camera for brightness, assemblage, and signal conversion of Rayleigh scattering spectra [34]. D) The SWNT-based SERS assay coupling with RNase HII-assisted amplification [35]. (Reproduced from mentioned references with kind permission of related journals).
In particular, microfabricated devices or chips that incorporate ctDNA detection based on electrochemical readout have attracted considerable attention as they are amenable to high levels of multiplexing and sensitivity.[136–138] As discussed earlier (section 3.1.1), whereas cfDNA in healthy subjects is usually of uniform size (~185–200 bp), fragments as large as >10 000 bp have been observed in cancer samples. Therefore, DNA integrity is considered a differential cancer biomarker and has been exploited for ctDNA analysis. Confocal spectroscopy can detect femto- to nanomolar quantities of DNA, which corresponds to the range of cfDNA levels usually encountered in clinical samples. A further modification to confocal spectroscopy platforms which creates sheet-like observation volumes and detects digital fluorescence bursts spanning over the cross section of a microchannel using a cylindrical lens, cylindrical illumination confocal spectroscopy (CICS), was used for quantification and size analysis of cfDNA in a microfluidic platform (μCICS).[217]

The assay demonstrated its capability to size fragments ranging from 564 bp to 27.5 kbp and was subsequently used to detect ctDNA in early- and late-stage lung cancer patients. A noteworthy feature of this assay was that it enabled single-molecule ctDNA detection and sizing directly from serum samples without the need for any preprocessing or expensive reagents. The only reagent used in this experiment was a DNA intercalating dye, thus providing a simple, rapid, and low-cost platform for ctDNA analysis.[217] Dielectrophoretic (DEP) manipulation and analysis of biological samples offers great advantages due to its non-destructive and fast operation. Consequently, a wide range of DEP devices have been developed for isolation and detection of micro- and nanoparticulate biomarkers such as CTCs, cfDNA, viruses, etc. However, DEP manipulation of biomolecules in high conductance biofluids such as blood is challenging and sample dilution is required. Sonnenberg et al. introduced a DEP microarray chip, which consisted of individually addressable platinum microelectrodes with uniform interelectrode spacing. This arrangement of microelectrodes
gives rise to DEP high- and low field regions. The electrokinetic device used in these experiments can be operated at 20 volts peak to peak (Vpk–pk) at 10 kHz. Under these conditions more polarizable DNA molecules experience positive DEP and are concentrated into high-field regions on microelectrodes while less polarizable biomolecules such as cells experience negative DEP and move to the low-field regions. The device was used to isolate high molecular weight cfDNA directly from the blood of chronic lymphocytic leukemia (CLL) patients. Although the device may be useful for rapid cfDNA isolation directly from unprocessed blood (10 min) or plasma (25 min) subsequent analysis and characterization of isolated DNA is reliant on conventional PCR- and sequencing-based methods. Nonetheless, low volume requirements (5 μl), fast processing time, and minimally destructive DNA isolation capability make the method useful for integration into cfDNA analysis workflows.[148] Yang et al. reported a microfluidic device for cfDNA quantification which concentrated Pico Green labelled cfDNA fragments electrophoretically into a gel by applying direct current.[218]

Recent advances in materials science are opening up new avenues in biosensing platforms. Graphene aerogel (GA) is considered as the world’s least dense solid and has found useful applications in microsensing platforms due to its high conductivity and electronic interaction. On the other hand, the shape of nanoparticles has been shown to significantly alter the enhancement factor (EF) experienced by adsorbed analytes. Gold nanostars (GNSs) are a prominent example in this context which show unique thermoplasmonic and electromagnetic properties owing to their multiple sharp and pointed branches, and their application in biosensing has been widely explored. A method was recently reported for cfDNA detection which combined GNS with nitrogen-doped multiple GA (N-doped MGA/GNS) to make an electrochemical sensing platform with enhanced capacity to form an electroactive conjugate with dsDNA.[219] The strength of the differential
pulse voltametric (DPV) signal is directly proportional to the concentration of dsDNA, thus making the device capable of electrochemical cfDNA detection. Although the device has so far only been used for proof-of-concept demonstration of its cfDNA detection capability, the hybrid biosensor reportedly exhibited much better sensitivity compared to the previously reported DNA sensors.[219] Although sensitive, inexpensive, and facile, the chip-based methods and platforms discussed in the preceding paragraphs are only capable of measuring cfDNA levels or differentiating ctDNA from an overwhelming background pool of cfDNA using an arbitrary fragment size based marker. Efforts have also been made to devise platforms capable of more specific ctDNA detection. The study by Dias et al. used relative quantification of shorter and longer Arthrobacter luteus (ALU) repeat fragments (shorter 115 bp fragment ALU115 and longer 247 bp fragment ALU247) to assess the DNA integrity index, a measure commonly used for ctDNA detection.[220] An array of magnetoresistive (MR) sensors integrated into a chip was used for probe-based capture and quantification of ALU repeats.[220] Although this proof-of-concept report only demonstrated the capacity of the device to assess the DNA integrity index, its ability to measure target fragments up to picomolar concentrations suggests that the device may further be applicable for detection of low allele frequency biomolecules.

In this context, several novel electrochemical sensors aiming to interrogate single-nucleotide polymorphisms (SNPs) and methylated nucleotides on ctDNA have been developed.[221,222] One of the earliest electrochemical ctDNA detection methods was demonstrated by Xiao et al. This method offered SNP detection in complex samples such as serum. This method is based on the usage of a single self-complementary methylene blue (MB)-labeled DNA probe possessing a triple-stem structure. In the absence of an exactly matched target, the triple-stem probe creates an uneven, rigid, 21-base duplex, preventing electron transfer between the MB redox label and the Au electrode, while in the presence of a
target, the triple-stem structure is deformed, creating a flexible single-stranded segment and enabling efficient collisions between the MB and the electrode which increases the observed faradaic current. This sensor is sensitive, stable, reusable, and does not require target modification, postwashing, or the addition of exogenous reagents. The sensor can effectively distinguish the 64 nM matched target in a serum sample comprising a significant excess (4 μM) of single-base mismatched targets in 30 min.[221] One of the foremost challenges of ctDNA detection is screening a small amount of SNPs or methylated nucleotides in biological samples, which usually contain long DNA fragments (e.g., kilobases). To avoid this obstacle, different amplification methods such as hybridization chain reaction (HCR) and polymerase chain reaction (PCR) have been used.

More recently, Li et al. employed the aforementioned triplex DNA structure and hybridization chain reaction (HCR) in an amplified colorimetric method for selective and sensitive detection of ctDNA. This approach utilized HCR amplification followed by the use of triplex-forming oligonucleotides (TFOs) to interrogate the dsDNA products of HCR and synergistically create the asymmetrically split G-quadruplex structure. Due to its high peroxidase-like activity, hemin was used to label the resulting structure. Subsequently, G-quadruplex/hemin complex catalyzed the oxidation–reduction reaction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS2−) and H2O2, and yielded the green radical anion (ABTS') and H2O. A consequent color change was detected by the naked eye or with a spectrophotometer. The satisfactory accuracy, reproducibility and detection limit of 0.1 pM were achieved by this method.[222] In addition to the combination of electrochemistry with HCR for ctDNA detection, some other studies based on PCR have been developed. Since conventional assays based on PCR are usually restricted in detection sensitivity and specificity owing to non-specific amplification interference including primer dimers and off-target amplification, designing methods for targeted amplification is necessary. In this
context, Chen et al. reported accurate electrochemical interrogation of circulating methylated DNA from plasma samples employing a paired-end tagging and amplification approach. In this method, the first two DNA primers each labeled with digoxigenin (Dig) and biotin at the 5′ end were designed to discriminate methylated DNA from the unmethylated one and its subsequent amplification. The resulting paired-end tagged amplicons were incorporated on the electrode covered with anti-Dig. This modification on the electrode enhanced the efficiency of target capturing by avoiding biotin from the interface. Next, avidin-HRP molecules were captured by the biotinylated amplicons, reducing hydrogen peroxide in the presence of TMB to generate a quantitative electrochemical signal. This signal is directly correlated with the amount of methylated DNA in the sample. The method was able to detect as little as 40 pg of methylated genomic DNA and as low as 1% methylation level. It attained a very high clinical sensitivity of 91% (10/11 patient samples).[223]

In a similar approach, this sequential discrimination–amplification strategy was used for circulating methylated DNA detection with single-copy sensitivity. In this method a novel nanostructured capture probe, termed self-assembled tetrahedral DNA, was immobilized on the surface of a gold electrode to capture amplicons. Tetrahedral DNA significantly enhanced the assay performance compared to the existing neutral capture-probe-based assay. This is because these probes notably increase target hybridization and reduce the non-specific adsorption of amplification by-products owing to the inflexible scaffold, ordered orientation and well controlled spacing. The hybridization event resulted in a measurable electrochemical signal applying an enzymatic amplification. Using this sensitive detection technique, interrogation of as few as one methylated DNA molecule in the presence of a 1000-fold excess of unmethylated alleles was obtained.[224] Apart from amplification-based strategies, several other novel approaches have been devised for direct and ultrasensitive detection of ctDNA. For example, Das et al. described an electrochemical clamp assay that could directly
detect mutant circulating nucleic acids in patients’ serum. By employing an assortment of oligonucleotides, selective binding of mutated sequences to a chip-based electrochemical sensor is facilitated. Electrochemical sensors used in this method could successfully detect mutations within 15 minutes with high sensitivity and specificity [225] (Fig. 2.4B). The electrochemical clamp assay provided a useful way for detecting tumor-specific circulating RNAs; however, the appeared to be unsuitable for ctDNA detection, probably due to its inability to target double-stranded molecules. The method was further modified for ctDNA analysis by using DNA clutch probes (DCPs). DCPs are pairs of ssDNA molecules that inhibit re-association of denatured ssDNAs and make one of the two strands in dsDNA molecules accessible for hybridization to probes (Fig. 2.5). Furthermore, DCPs also deactivate closely related sequences present in solution, thus facilitating preferential association of mutated sequence-based hybridization events with chip-based sensors. [28] The results demonstrated that this method can distinguish 1 fg μL−1 of a selected mutation against a background of 100 pg μL−1 of wild-type DNA. In other words, the method can detect mutation down to 0.01%.[28]
Figure 2.5. Schematic of clutch probe method for detecting ctDNA. (A) Detection approach. First, ssDNA formation by denaturation of double helices DNA by 90 °C. Prevention of ssDNA strands reassociation using DNA clutch oligonucleotide probes. The PNA clamps obstruct wild-type target ssDNA and the mutated target ssDNA stays without hybridization. (B) Chip-based detection. NMEs are prepared with PNA probes which can match to mutated target DNA. Just the complementary mutated targets attach to the probe. Lastly, after hybridization of predetermined sequences and washing, the generation of signal from individual sensors was recorded in the existence of an electrocatalytic reporter system using differential pulse voltammetry. (C) Sensor chip layout. Sensors were arranged on a microchip where patterned holes supply a template for the electrode position of gold. The inset depicts cross-section of the chip at hole. (D) A picture of a nanostructured microelectrode sensor represented by scanning electron microscopy [33]. (Reproduced from mentioned reference with kind permission of related journal).

Various nanoplasmonic biosensors have been used to detect DNA targets. [226–228] The said approach is based on localized surface plasmon resonance (LSRP), which happens when conduction electrons close to the AuNP surface sustain united oscillations coupled to a
The LSPR shift is related to the size, structure, and the surrounding dielectric environment of the AuNPs. Therefore, nanoplasmonic biosensors have high sensitivity for detecting changes in the refractive index within the surface plasmon band. Nguyen and Sim reported a nanoplasmonic platform for specific ctDNA detection that used peptide nucleic acid (PNA) probes coupled to gold nanoparticles (AuNPs). The PNA probes used a targeted 69 bp PIK3CA fragment and tumor-specific mutations (E542K and E545K) (Fig. 2.4C). Capture of ctDNA fragments by probes generated an LSPR peak shift, showing the primary response. Subsequently, immunogold colloids were used to detect methylation at the captured PIK3CA fragment. The findings of this report showed that this biosensor can distinguish the hot-spot mutations and epigenetic changes on the ctDNA even at a very low concentration.

In another sensitive electrochemical platform developed by Chu et al., a thin layer of molybdenum disulfide (MoS2) was combined with graphene through hydrothermal and ultrasonic methods. DNA probes were immobilized onto the surface of nanosheets. Consequently, this immobilization can change the pulse voltammetric (DPV) response of the guanine bases; thus, DNA can be detected without fluorophore labelling or enzyme amplification steps. This electrochemical sensor is capable of detecting ctDNA quantities as low as 10–16–10–13 M.[231] More recently, Zhou et al. have reported the detection of mutant ctDNA fragments occurring at frequencies as low as 0.3 fM from 5.0 μL of sample volume (Fig. 2.4D). In this strategy, DNA-mediated surface-enhanced Raman scattering (SERS) of single-walled carbon nanotubes (SWNTs) was applied for highly sensitive detection of a broad range of ctDNAs in human blood. By applying the predetermined triple-helix molecular switch (THMS) as both a molecular identification unit and a signal amplification unit, along with T-rich ssDNA-mediated SERS enhancement of SWNTs, the method could detect various ss ctDNAs in human blood.[30]
2.5. Conclusion and future perspectives

We have thoroughly reviewed ctDNA as a non-invasive cancer biomarker with applications from diagnosis to prognosis to monitoring tumor evolution and response to therapy\cite{20,232} and recent advances in ctDNA detection strategies. We have also addressed the main methodological deficiencies of these strategies. All recently applied NGS-based approaches that rely on sensitivity of amplification are restricted by the error rate of DNA polymerases, which is regarded to be 0.01%. However, the advent of the third-generation sequencing methods will reduce the limitations related to PCR amplification. Additionally, since many tumor types lack recurrent genetic alterations, revealing specific cancer markers and improved analytical and diagnostic sensitivity of genome-wide analyses are required. Furthermore, intratumor heterogeneity needs more attention.\cite{233} As it is not obvious that ctDNA is representative of all metastatic cell clones placed at various sites or whether ctDNA represents DNA from distinct subclones, more clinical, molecular, and imaging studies as well as detailed functional experiments are required to analyse clinical progression and/or therapeutic resistance in better detail. Altogether, many attempts to develop appropriate tools for a thorough analysis of tumor genomes from ctDNA have been made. Most laboratory processes are too timeconsuming and expensive for the actual implementation in a diagnostic setting. Ideally, ctDNA analysis methods should be capable of genome-wide coverage, that too with high sensitivity and low cost. Future developments in sequencing technology and chip design are expected to achieve this idea.
2.6. References


150. H. Lee, et al., Theranostics, 2018, 8, 505–517.

Statement of contribution to co-authored published paper

This chapter is in a form of a co-authored published paper. The bibliographic details of the co-authored paper, including all authors, are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

My contribution to the published paper involved:

- Literature review
- Manuscript preparation
- Responding reviewers

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Chapter 3: Microfluidic-Based Nucleic Acid Amplification Systems in Microbiology

Abstract

Rapid, sensitive, and selective bacterial detection is a hot topic, because the progress in this research area has had a broad range of applications. Novel and innovative strategies for detection and identification of bacterial nucleic acids are important for practical applications. Microfluidics is an emerging technology that only requires small amounts of liquid samples. Microfluidic devices allow for rapid advances in microbiology, enabling access to methods of amplifying nucleic acid molecules and overcoming difficulties faced by conventional. In this review, we summarize the recent progress in microfluidics-based polymerase chain reaction devices for the detection of nucleic acid biomarkers. The paper also discusses the recent development of isothermal nucleic acid amplification and droplet-based microfluidics devices. We discuss recent microfluidic techniques for sample preparation prior to the amplification process.

This chapter has been published as:
3.1. Introduction

The steady increase of human life expectancy over the last century largely reflects the many advances in controlling bacterial infections. A range of areas in modern life, such as drinking and recreational water supply, industrial food production, pharmaceutical production, and point-of-care clinical diagnostics, require the identification of bacteria. Rapid and accurate bacterial detection is crucial for timely interventions including preventive vaccination and antimicrobial therapy [1]. However, conventional detection methods cannot entirely meet the need for rapid and accurate bacterial detection. For example, semi-quantitative plate culture has been the gold standard for bacterial identification, which is based on their morphological and metabolically characteristics. Although this method is well established and reliable, its major disadvantages are a long assay time, weak detection of non-culturable bacteria, low positive rate, and inefficient differentiation of bacteria at the level of strains or species [1,2].

More recently, state-of-the-art immunological and molecular diagnostics have been used for microbial detection. Immunological diagnostics are based on specific biomarker antibodies and optical sensing to detect the corresponding bacteria. Some drawbacks still exist with these methods, such as low or lack of signal, high background noise, and inconsistent results between replicate samples or controls [3,4]. Molecular diagnostics has been further expanded to proteomic and genomic methods such as mass spectrometry (MS), polymerase chain reaction (PCR), isothermal amplification, and high throughput next generation sequencing (NGS) [5–8].

MS has emerged as a powerful approach for analysing the molecular structure and molecular weight of analytes. Using the characteristic mass spectral fingerprints directly from intact bacteria, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
(MALDI-TOF MS) has been used as a rapid method to identify the microbial species [9,10]. This technique can only identify highly abundant and purified bacterial samples, such as single colonies on a plate culture, and thus is still limited by the time-consuming procedure and low positive rate of bacterial culture [11]. PCR is one of the most well-known techniques in molecular biology for amplifying segments of DNA. Targeting the desired gene sequences, synthetic primers corresponding to these sequences can be designed to amplify the targets using the polymerase enzyme [12]. To implement conventional PCR, a few repeated temperature cycles are needed. In contrast, isothermal amplification eliminates the thermo-cycling steps, reducing the cost and improving the overall assay quality. Therefore, isothermal amplification is more convenient than PCR for laboratories with limited technical capacity [13,14]. However, isothermal amplification has several challenges such as the dependence of sensitivity on annealing temperature, nonspecific amplification, RNase contamination, RNA with secondary structures or high GC (guanine-cytosine) content, PCR-borne mutations, pipetting errors, false negative results, cost, and time [15].

High-throughput NGS can be used to determine the whole genome of bacteria for highly veracious microbial typing [16] or determine the overall microbiota composition of a given sample without needing to culture the bacteria [17]. However, the huge amount of data produced by NGS leads to difficulties in managing and storing the results in clinical laboratories. Additionally, NGS approaches require enrichment, amplification, and labelling steps, causing the experiment to be both time- and cost-consuming, as well as increasing the possibility of false positive results [18]. Despite the broad use of traditional molecular techniques, there is still a research gap in the design, construction, and installation of de novo devices and pathways for improve and accelerate the detection of microbial nucleic acids.

In the past decade, microfluidic handling systems with microchambers and microchannels have been developed for a range of practical applications, particularly for
nucleic acid (NA) analysis in microbiological assays [19,20]. Microfluidic devices have several benefits over conventional macroscale counterparts, such as laminar flow, the minute amount of reagents required, relatively fast mixing, low thermal inertia, and rapid heat transfer [21,22]. Due to the small size of microchannels, flow is mainly laminar and stable. Two or more streams flowing in a microchannel do not mix together. Without further engineering intervention, mixing relies entirely on molecular diffusion [23]. The small size and volume of microfluidic devices reduce the amount of sample and reagent in use, resulting in low operation cost [20]. The short diffusion length is another critical advantage of microfluidic tools as it improves reactions between enzymes and nucleic acids, thereby reducing assay time [24]. Heat and mass transfer can be well controlled in microfluidic devices. The small device size leads to a high surface to volume ratio and consequently a rapid mass and heat transfer rate. Thus, for a PCR process with many heating and cooling cycles, the total time can be significantly reduced [25].

In addition to the above features, distinct advantages, such as portability, automation, high throughput, and the ability to integrate multiple elements on a single chip, provide promising opportunities for on-chip analysis of microbial nucleic acids [26]. Various microfluidic chip-based portable nucleic acid analysers have been developed for the detection and identification of nucleic acids. Kopp et al. developed the first flow-through microfluidic PCR device based on silicon as the device material [27]. Glass and silicon were common materials for microfluidic devices in the early 1990s. Due to the relatively large design footprint and the corresponding cost, the device materials have been gradually shifting to polymers, such as polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), and cyclic olefin copolymer (COC). The most recent PCR-based or isothermal amplification-based devices are composed of polymers. Whereas conventional PCR allows for the qualitative detection of NA sequences, microfluidic-based PCR allows for real-time and
highly sensitive quantification of DNA down to the single molecule level, particularly with
digital PCR using dispersed droplets as sample compartments. However, some bottlenecks
still remain to be overcome [28].

Firstly, PCR-based microdevices require accurate thermal cycling of the sample. The fast
temperature transition and the thermal homogeneity inside the PCR mixture critically affect
the total run time, the number of thermal cycles, the efficiency, and the specificity of the
amplification reactions. The requirement for accurate thermal control restricts the choice of
materials for microfluidic devices, increasing their fabrication costs [29]. Secondly, during
the amplification process, enzymes can be inhibited by a variety of compounds such as
hemoglobin, humic acid, and cellular debris. Thus, microfluidic PCR systems require sample
preparation steps, such as cell lysis and nucleic acid purification, to remove debris and
unwanted compounds. Loop-mediated isothermal amplification (LAMP) avoids these issues
and has been widely used for nucleic-acid-based amplification of a variety of pathogens.
LAMP is highly sensitive, specific, and rapid, and is less susceptible to typical PCR
inhibitors. The results generated by LAMP can be detected by the naked eye, and therefore
do not need sophisticated instruments [30,31]. However, some bottlenecks still limit the
application of LAMP-based microfluidics. Careful optimization of loop primers is necessary
for reproducible and sensitive target detection. A relatively cold environment is needed for
the experiments due to the instability of Bst Polymerase (a LAMP-specific enzyme) and
deoxynucleotide triphosphate (dNTP) in warm and even room temperatures [32].

Recently, droplet-based microfluidics and digital microfluidics led to new
developments of on-chip PCR devices. PCR within a single droplet is inexpensive, well-
controlled, reproducible, and has high throughput as well as low contamination. Thus,
droplet-based microfluidics has attracted considerable attention from the commercial world,
particularly for digital PCR, which can be used to directly quantify and clonally amplify
nucleic acids strands, making it more precise than PCR. In this approach, the sample is separated into a many partitions and the amplification is performed in each partition individually.

Digital microfluidics is a subclass of droplet-based microfluidics, dealing with discrete droplets. Digital microfluidics has huge potential due to its excellent capacity in running established chemistries and protocols in a single droplet. Splitting and merging of droplets in digital microfluidics allow for the implementation of sample preparation on the same platform [33,34]. There is interest in integrating various functions, such as sample preparation, amplification, and detection, in the same automated microfluidic device to reduce handling time and to prevent sample contamination [35,36]. Consequently, droplet-based microfluidics and particularly digital microfluidics play critical roles in designing advanced and robust nucleic acid amplification assays. Table 3.1 summarizes the existing relevant review papers, which mainly discuss microfluidic-based NA amplification in microbiology.
Table 3. Summary of previous review articles addressing the microfluidic-based bacterial nucleic acid (NA) amplification approaches in chronological order.

<table>
<thead>
<tr>
<th>Main Focus of the Review</th>
<th>Year</th>
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<tr>
<td>Review of few studies reporting microfabrication of PCR in microbiology [37]</td>
<td>2007</td>
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<tr>
<td>A comprehensive review paper of miniaturized isothermal nucleic acid amplification [38]</td>
<td>2011</td>
</tr>
<tr>
<td>A review on the general use of microfluidics in bacterial pathogens monitoring with less focus on amplification methods [39]</td>
<td>2014</td>
</tr>
<tr>
<td>A review of works based on centrifugal microfluidic platforms for NA detection and amplification in microbial samples [28]</td>
<td>2015</td>
</tr>
<tr>
<td>A review of a few studies on microfluidic PCR for bacterial pathogens identification [40]</td>
<td>2015</td>
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<tr>
<td>A review describing applications of droplet microfluidics in microbiology [41]</td>
<td>2016</td>
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<tr>
<td>A comprehensive review focusing on detection of microorganisms using microfluidic-based analytical approaches [42]</td>
<td>2018</td>
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<tr>
<td>A review of a few studies on micro-scale bacterial NA amplification [43]</td>
<td>2018</td>
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Although the majority of past review papers focused on just one microfluidics-based approach for NA amplification, no comprehensive review has covered all approaches, particularly for microbiology applications. The present paper provides a comprehensive overview of the most popular microscale NA amplification platforms for bacterial detection based on conventional PCR and LAMP. The paper introduces the seminal studies, followed by the latest advances in each field. Limitations and drawbacks of the techniques are discussed to identify research gaps with recommendations for future developments.
3.2. Spatial Polymerase Chain Reaction

If the sample moves continuously in a microchannel through regions with fixed temperatures, thermal cycling occurs spatially. Thus, this category is referred to as spatial or continuous-flow PCR devices. Advantages in heat transfer make this concept attractive for designing microfluidic PCR devices. Because only the sample mass receives periodic heating and cooling, the thermal inertia of the system decreases. The time interval between temperature transitions is therefore correlated with the sample flow rate and the thermal equilibrium time, enabling a quick thermal response and short reaction time [20,44]. Over the past decade, microfluidic devices for spatial PCR with serpentine and oscillating designs have been developed for rapid and simple detection of pathogenic bacteria.

3.2.1. Serpentine Design

Most spatial PCR devices use several heaters to provide multiple temperature regions for the corresponding PCR temperatures, i.e., initialization, denaturation, annealing, and extension regions [45]. In 1998, Kopp et al. reported the first attempt to amplify a 176-bp fragment from the DNA gyrase gene of Neisseria gonorrhoeae using a serpentine continuous-flow on-chip PCR [27]. Three copper blocks define three temperature zones that are maintained at 95 °C, 77 °C, and 60 °C (Figure 3.1A). The sample is pumped through a single serpentine channel etched in a glass chip, resulting in heating or cooling of the sample through the predefined temperature zones. The channel geometry and the arrangement of the channel over the temperature zones are two critical parameters for optimising the total reaction time on the chip. At the fixed flow rate, the residence time of a given temperature zones is determined by the channel cross-section and the length of the channel section. For instance, with a fixed channel cross-section, the length of the extension region is designed to
be longer than that of the denaturation or annealing regions [46] to increase the duration of the extension step in PCR. The transition time between two temperature levels is determined by the transition regions of the channel. To minimize the transition time, a smaller channel cross-section with a smaller width could be designed. Li et al. fabricated a PCR microdevice comprising a serpentine microchannel with various widths and a constant depth to amplify 90-bp *Bacillus anthracis* DNA fragments (Figure 3.1B). By changing the widths of the channel, the transitional time was decreased remarkably [47].

The other significant challenge of using serpentine channels for spatial PCR is heat management without thermal cross-talk. The devices need enough space between the heaters to provide sufficient thermal insulation, making the overall footprint relatively large. The longer channel over the narrow temperature zone requires extra loops, which also enlarges the footprint of the PCR device [46]. Since controlling multiple temperature zones on a single microfluidic device is challenging, reducing the required temperature zones and the number of heaters was a possible solution. Toward this idea, molecular-level interactions in various temperature zones have been investigated. Once the sample reaches the required temperature, the denaturation and annealing reactions occur almost immediately within one second, and the extension rate is on the order of 60–100 bases per second [48]. The investigation revealed that extension reactions even occur during the transition between annealing and denaturation temperatures. Thus, a holding time is not necessary if there are only a few amplification targets. Several studies on continuous-flow PCR with only two temperature zones have demonstrated rapid amplification cycle, high efficiency, high specificity, and low assay cost [49–51].

Fernández-Carballo et al. [6] reported a serpentine continuous-flow PCR with only two heaters below the chip (Figure 3.1C). Each heater consists of an aluminum heating block, a cartridge heater, a thermocouple, and a programmable temperature controller. The
temperature control system was accompanied by an optical system for the real-time fluorescence detection of *Chlamydia trachomatis* and *Escherichia coli* O157:H7. The chip was designed with two inlets for the sample and the qPCR master mix, which are mixed in a long microchannel. The gradual movement of mixed reagents through serpentine channel leads to the reaction. First, the sample passed through a 95 °C zone for activation of polymerase and melting the DNA double strands. Next, the reagents were thermally cycled for 40 times between 95 °C and 62 °C for annealing, DNA synthesis, and denaturation. Recently, Trinh et al. [52] reported a serpentine continuous-flow PCR device composed of a glass-polytetrafluoroethylene (PTFE)-glass (GPG) sandwich and two heaters for denaturation and annealing/extension (Figure 3.1D). The GPG consists of two parallel glass slides holding a serpentine PTFE tube in-between. The chip is mounted on top of two heating copper locks. The glass slides are able to conduct the heat from the heaters to the PTFE microchannel without much loss. The GPG chip successfully amplified the specific sequences of *Salmonella* spp/ and *E. coli* O157:H7. Although the above-mentioned designs provided fast amplification reaction, simple fabrication, and portability for molecular detection of pathogens, further reduction to a single heater and creating the appropriate reaction regions for PCR are future remaining challenges [53].

For instance, Chen et al. [45] proposed the fabrication of a PDMS-based PCR device employing a single heater to detect *Coxiella burnetii*. By integrating one heater onto the chip centre and two heat pipes on the two sides of the chip, five temperature zones were created within the small footprint of the chip. The major achievement of this design is the significant reduction of the chip size and the thermal control cost. However, external and large pumps, such as syringe pumps, were necessary to continuously deliver the sample in all of the above-mentioned designs, leading to complex operations and preventing the further miniaturisation of the whole system. To solve this bottleneck, miniature pumping systems were integrated
with the microfluidic devices [54,55]. Even though these micropumps efficiently controlled
the sample flow, they still need external energy for running, which still causes a large overall
footprint of the system. Passive capillary pumping could solve the problem of bulky power
supply [56,57]. The capillary force of the liquid/air interface could be applied as a driving
force for delivering the sample through microfluidic device without the need for an active
micropump.

Tachibana et al. [58] designed a self-driven serpentine continuous-flow PCR device in
a Si/glass microfluidic device for the amplification of 16S ribosomal DNA (rDNA) of *E. coli*
(Figure 3.1E). The PCR solution only needs to be dropped onto the inlet, and then is
autonomously transported by capillary force. The limitation of capillary-driven microfluidics
is that both the capillary pressure at the fluid front and the viscosity of the solution change
periodically due to the different temperature zones. This problem was solved by using an
extremely long PCR microchannel. The length and design of the microchannel were
optimised for the capillary pressure and the viscosity. In their further study, the team used a
non-ionic surfactant for coating the wall of the microchannel to increase the capillary force,
resulting in fast quantitative detection of *E. coli* and pathogenic *E. coli* O157 [59]. Most
reported devices are similar to the original serpentine design of Kopp et al [27].
Figure 3. 1. Dynamic PCR devices. (A) (a) Schematic illustration of a chip for flow-through PCR. Three temperature zones are stabilized at 95 °C, 77 °C, and 60 °C using thermostated copper blocks. The sample is pumped into a single channel etched in the glass chip. (b) Layout of the microfluidic device. The device has three inlets for carrying the sample/buffer and one outlet [27]. (B) a) A schematic presentation of the thermally-optimized 20-cycle continuous-flow PCR microfluidic device. b) A top view of the microchip. c) One cycle of the microchannel with different widths [47](C) (a) Schematic presentation of the chip. (1) Mixing zone. (2) Polymerase activation zone. (3) Thermal cycling zone. (b) Image of the chip [6]. (D) A schematic of on-chip amplification and on-site detection of amplicons using a GPG microdevice [52]. (E) Schematic illustration of a device for self-propelled continuous-flow PCR: (a) concept diagram, (b) cross-sectional view of device, and (c) picture of device [58]. Reproduced from the mentioned references with permission from the related journals.
3.2.2. Oscillating-Flow Design

Oscillatory microfluidics was introduced as the simplification of digital microfluidics. The oscillating-flow PCR method combines the cycling flexibility of stationary-chamber-based PCR and the fast dynamics of continuous-flow PCR [60]. Oscillating or bidirectional flow PCR was first described by Chen et al. in 2007 [61]. Since then, oscillatory-flow PCR has been one of the most favourable concepts due to advantages including simple system configuration, number/dwell time flexibility, simple application in real-time detection, large footprint reduction, and ability to amplify multiple samples in parallel [60].

In an oscillating-flow device, the PCR reagents are transported back and forth through a single channel spanning the various temperature zones. Due to the decreased surface inhibitory effect in oscillating-flow PCR, the channel is significantly reduced to a single straight channel, which only requires a smaller temperature zone and therefore keeps the device footprint to a minimum [62]. In the last decade, the oscillatory-flow PCR concept has been implemented in various substrate materials (silicon, glass, and polymers), with different heating (metal/ceramic heating blocks, Peltier heater, and film heaters) and pumping concepts (e.g., external pumps, and integrated micropumps) [60].

Oscillating-flow PCR devices still have shortcomings that limit their usage. For example, the concept only suits one DNA target in a single reaction solution; therefore, the detection throughput is low. Consequently, the detection speed is slow for multiple DNA targets. The concept is particularly not suitable for time-sensitive or throughput-sensitive diagnostics such as foodborne pathogens and related biowarfare agents. To overcome the throughput drawback, Zhang et al. [60] implemented oscillatory-flow multiplex PCR amplification in parallel in multiple channels, enhancing the detection throughput (Figure 3.2A). An array of independent parallel channels placed across the three temperature zones
was able to perform multiplexed PCR of foodborne bacterial pathogens like *Listeria monocytogenes, E. coli O157:H7*, and *Salmonella enterica*. In another study, the oscillation-flow concept was implemented with a droplet reactor to achieve high-speed amplification (Figure 3.2B). Employing interfacial chemistry, a water-in-oil droplet was created by allowing an oil-water plug to flow through a polytetrafluoroethylene (PTFE) capillary. The resulting droplet serves as the reactor for oscillating-flow PCR and provides a stable reaction environment including fast reagent mixing and minimum surface adsorption. The efficiency of the device was evaluated using the amplification of the *New Delhi metallo-beta-lactamase (NDM-1)* gene [62].
Figure 3. 2. (A) Images of the oscillating-flow PCR microfluidic device. (a) The system consists of a precision syringe pump (1), glass syringe (2), lift table (3), silicon tube-based connector (4), support plate (5), copper block with a glass cover (6), thermocouple sensor (7), cartridge heater (8), and PTFE capillary tube (9). (b) The temperature control and measurement system [60]. (B) Schematic illustration of the oscillation-flow instrument. (a) Heating module (1), syringe pump (2), PT 100 sensor (3), relay (4), distributed multichannel controller (5), electric power source (6), and computer (7), and a magnified illustration of a heating module. (b) PC cover (1), grooved aluminum plate (2), PT 100 sensor (3), 6 aluminum plate holders (4), and Peltier heating element (5) [62]. Reproduced from the mentioned reference with permission from the related journal.
3.3. Transient PCR

The transient approach introduces the PCR solution into a single or multiple reaction chambers. The chambers are then subjected to repeated heating and cooling processes corresponding to the thermocycling profile. Since the sample is stationary and its temperature varies over time, the amplification approach is referred to as transient PCR. In this class of PCR devices, the temperature profile does not depend on the channel design; thus, the heating and cooling protocols and consequently the PCR thermal cycling condition can be modified. Sample transport is not needed and therefore there is no necessity for pumps. However, this device cannot be operated continuously, so the sample throughput is restricted. The common transient PCR platforms for microbial NA analysis are centrifugal microfluidic devices, laboratory discs, and arrays.

3.3.1. Centrifugal Microfluidic Devices

Centrifugal microfluidics exploits centrifugal force and capillary force to control the liquid flow. Both forces are accessible as centrifugation is typically present in a rotating system and capillary force is dominant in microscale devices [63,64]. This group of devices are designed in the format of compact discs (CD) that house the reaction chambers and other components for the PCR [65]. The reaction mixture moves outward toward the edge of the disc, pushing air our, facilitating on-disc liquid storage [66]. Assembling CD-based microfluidics with on-board power provides the required thermal cycling for PCR [67]. Centrifugal microfluidics has several advantages over the conventional stationary microfluidics. First, the centrifugal driving mechanism provides a closed fluidic platform without the need for external pumps. Second, the elimination of any bubbles that may cause serious problems is particularly easy due to the scalable buoyancy in centrifugal microfluidics. Third, residual liquids that may be trapped due to surface forces can be easily removed from channels and chambers by adjusting the rotation speed. Forth, varying the
rotation speed of the disc offers a combination of microfluidic sample preparation steps such as liquid mixing, metering, aliquoting, switching, valving, and storage. These advantages of centrifugal microfluidic devices make them a potential candidate for bacteria detection [68].

Early centrifugal microfluidic CD-like devices were composed of silicon/glass with 24 serpentine channels and 313 microchambers (volumes of 1.5 nL) and were used for selective detection of *S. enterica* from a mixture of *S. enterica* and *E. coli*. The sample was placed in an inlet port and distributed over the microchambers by centrifugal force (Figure 3.3A). PCR was performed by thermocycling the entire disc. After PCR, the fluorescence intensity was evaluated by placing the device into an image analyser [69]. The inlet and outlet were sealed tightly with a butyl gum polymer, which prevents evaporation of the sample and effectively keeps the microchannel moist during the PCR cycles.

Amasia et al. reduced the evaporation in centrifugal microfluidic devices by freezing the liquid next to the PCR chamber and integrated pumping, valving, and thermoelectric heating/cooling in the system for the amplification and detection of *Bacillus anthracis* genes (Figure 3.3B). Three thermoelectric modules were used for thermocycling (one module) and for freezing small volumes of the PCR buffer in the channel network (two modules) to serve as ice valves. These ice valves were able to block the linkage channel between the PCR chamber and the outlet hole [70]. Although this method was successful at sealing the thermocycling chamber and preventing sample evaporation, controlling the temperature distribution between the thermoelectric heater and the ice-valve is challenging and requires complex design. In another attempt, Strohmeier et al. designed a cartridge for the detection of food borne pathogens such as *L. monocytogenes*, *Salmonella typhimurium*, enterohemorrhagic *E. coli*, *Staphylococcus aureus*, *Citrobacter freundii*, and *Campylobacter jejuni* [71]. This cartridge included qPCR reaction chambers for integrated positive and negative controls and was sealed with a pressure sensitive adhesive tape (Figure 3.3C).
Czlilwik et al. developed a passive microfluidic vapor-diffusion barrier to reduce pressure enhancement over PCR thermocycling [72].

Figure 3. 3. (A) Microfluidic design of a compact disc (CD) device and schematic illustration of single cell isolation: (1) a large number of microchambers align along a channel, (2) the cells flow through the microchambers and (3) are spread into individual microchambers [69]. (B) (a) Schematic graph of the multi-layered centrifugal disc. The disc is comprised of five layers of hard plastic: A. polycarbonate sheet, B. pressure sensitive adhesive tape (PSA), C. polycarbonate sheet, D. polycarbonate film, and E. polycarbonate film. (b) (Right) Image of the integrated centrifugal microfluidic platform for pumping, valving, and thermocycling of fluid. (Left) Close-up of the actuated thermal platform showing the location of the central thermocycling TE and two ice-valve TEs. (c) Schematic presentation of the hardware details and fluidic process for the integrated CD system [70]. (C) Schematic illustration of one microfluidic structure: (a) elution buffer and PCR mastermix are loaded to the inlets, (b) the elution buffer is transported into two elution buffer chambers while the PCR mastermix is distributed into metering fingers, (c) the PCR mastermix aliquots are gated into amplification chambers which filled with primers, (d) DNA is added to one of the sub-volumes, (e) each
subvolume is aliquoted into several aliquots, (f) PCR is started, and (g) the Lab Disk is mounted to a custom-made holder [71]. Reproduced from the mentioned references with permission from the related journals.

The other critical component of this group of devices is the heater that regulates the temperatures for amplification. Heaters can be categorised as direct contact and non-contact types. Although many studies used direct contact heating to maintain the temperature for the amplification protocol [69,70,73], the elaborate designs and the rotating platform complicate their implementation. In contrast, non-contact heating allows the device to move while maintaining the temperature. Non-contact methods are based on microwave [74], magnetic induction [75], and infrared (IR) heating [76]. Microwave and induction heating need complex electronic components such as high frequency oscillators with high power inputs. IR heating has been used for rapid amplification of NA in microfluidic devices due to the quick heating and cooling of the sample [77]. Recently, a centrifugal microfluidic system with IR heating was developed for NA-sequence-based amplification reaction, targeting the tmRNA transcript of Haemophilus influenzae. In addition to the temperature control, this platform was also equipped with a control system for the rotation speed and positioning system of the reaction chamber for heating and fluorescence detection [66]. Table 3.2 provides an overview of centrifugal microfluidic platforms for the detection of various bacteria over the last few years.

Table 3.2. Summary of key studies using centrifugal microfluidics to identify bacteria.

<table>
<thead>
<tr>
<th>Target Bacteria</th>
<th>Sensitivity</th>
<th>Time (cycles)</th>
<th>Detection Technology</th>
<th>Heating Technology</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Below 10 copies of DNA per well</td>
<td>110 min (50 cycles)</td>
<td>FAM-labeled hydrolysis probes; real-time fluorescence</td>
<td>Air mediated in commercially available PCR thermocycler</td>
<td>[78]</td>
</tr>
<tr>
<td>Pathogen</td>
<td>Target (Procedure)</td>
<td>Detection</td>
<td>Temperature Control</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>----------</td>
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<td>-----------</td>
<td>---------------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Less than 7 copies per sample</td>
<td>17 min (10 cycles) primary PCR; 52 min (50 cycles) main PCR</td>
<td>FAM-labeled hydrolysis probes; real-time fluorescence detection</td>
<td>Air mediated in commercially available PCR thermocycler [79]</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>Amplification of one gene from single cell</td>
<td>8.33–20.83 min (20–50 cycles)</td>
<td>FAM-labeled hydrolysis probes; post-PCR fluorescence detection</td>
<td>Contact [69]</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus anthracis, Bacillus cereus</em></td>
<td>Not mentioned</td>
<td>53 min (35 cycles)</td>
<td>Off-chip (analysis of PCR products by gel electrophoresis)</td>
<td>Contact; with thermoelectric modules [70]</td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes, Salmonella typhimurium, EHEC, S. aureus, Citrobacter freundii, and Campylobacter jejuni</em></td>
<td>0.1 pg DNA per well for <em>Salmonella</em> and <em>Listeria</em></td>
<td>Around 2 h (50 cycles)</td>
<td>FAM-labeled hydrolysis probes; real-time fluorescence detection</td>
<td>Air mediated in commercially available PCR thermocycler [71]</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Not mentioned</td>
<td>Around 54 min (30 cycles)</td>
<td>Agarose gel pre-stained with ethidium bromide</td>
<td>Convection of hot air and ambient air in commercially available PCR thermocycler [72]</td>
<td></td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Fluorescence sensitivity down to 100 CE with tmRNA</td>
<td>70 min (Isothermal)</td>
<td>FAM-labeled beacon probes; fluorescence detection</td>
<td>Non-contact infrared (IR) heating [66]</td>
<td></td>
</tr>
<tr>
<td>24 Pneumonia-related pathogens</td>
<td>As few as 10 copies</td>
<td>45 min (Isothermal)</td>
<td>Real-time fluorescence detection</td>
<td>Contact [73]</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Detection Value</td>
<td>Analytical Time</td>
<td>Method</td>
<td>Contact</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>--------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>$10^2$ colony-forming unit per millilitre</td>
<td>15 min (Isothermal)</td>
<td>Real-time fluorescence detection</td>
<td>[67]</td>
<td></td>
</tr>
<tr>
<td>E. coli O157:H7, S. typhimurium, and <em>Vibrio parahaemolyticus</em></td>
<td>380 copies</td>
<td>60 min (Isothermal)</td>
<td>Colorimetric detection using eriochrome black T; naked eye</td>
<td>[80]</td>
<td></td>
</tr>
<tr>
<td>E. coli O157:H7, S. typhimurium, and V. parahaemolyticus</td>
<td>500 copies</td>
<td>60 min (Isothermal)</td>
<td>Colorimetric detection using eriochrome black T; naked eye</td>
<td>[81]</td>
<td></td>
</tr>
<tr>
<td>E. coli, Salmonella spp, and <em>Vibrio cholerae</em></td>
<td>$3 \times 10^{-5}$ ng·μL$^{-1}$</td>
<td>60 min (Isothermal)</td>
<td>Calcein colorimetric method; smart phone</td>
<td>[65]</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3.2. Lab Disk

The further development of centrifugal microfluidics, particularly for bacteria detection, is integrating and automating the different analysis steps into a single device. This group of devices is known as “Lab Disk” [82]. A Lab Disk includes cell lysis, DNA extraction, amplification, and detection, in an integrated and automated format, to provide “sample in, answer out” for the identification of bacteria. Some pioneering platforms have already been developed for attaining this goal. For instance, Czilwik et al. [83] reported the integration of lysis, DNA extraction, and nested PCR in a single centrifugal microfluidic disk. Different types of bacteria (*Staphylococcus warneri, Streptococcus agalactiae, E. coli,* and *Haemophilus influenza*) were first lysed by adding chemical reagents. Next, binding, washing, and elution of DNA were conducted using magnetic beads. Lastly, the purified DNA was amplified and identified via nested real-time PCR. Despite simplifying the detection assays, the turnaround time of the complete analysis time was approximately 3 h
and 45 min. Roy et al. [84] reported another device for the identification of *Bacillus atropheus* with integrated mechanical cell lysis, PCR, amplicon digestion, and microarray hybridization steps. The assay needed 2 h and 10 min to complete. With a single PCR chamber, this device was not suitable for multiplexing. Keller et al. reported multiplexed real-time PCR of *E. coli* employing a centrifuge-thermopneumatic fluid control system that uses the temperature-induced partial vapor pressure change of an enclosed gas volume for valving and aliquoting [82].

Despite the considerable progress in this field, improvement is still required. Yan et al. [85] recently developed a robust and user-friendly Lab Disk with a multiplexed detection ability of six types of bacteria. Compared to existing devices, this device has provided remarkable progress. For example, bacteria lysis is performed by only rotating a pair of magnets to generate bead-beating, while the chip remains stationary. The on-chip assay is rapid and the results can be interpreted through fluorescence detection or by the naked eye. Zhang et al. [86] developed a fully hand-powered centrifugal microfluidic platform for diagnostics of six pathogenic bacteria. This Lab Disk relies on zeolite-based purification of nucleic acids, loop-mediated isothermal amplification, and visual detection of the fluorescent signals. In addition, the flow actuation inside the device is enabled by a simple manual pull-out operation of the rack of the centrifuge, resulting in high-speed rotation of the disc and efficient mixing of preloaded sample/reagent fluids.

### 3.4. Array

Over the last decade, microarrays have been reported as a high-throughput platform for simultaneous detection of multiple gene targets [87]. The sensitivity of microarray technology is low, and compared to real-time PCR, the results are more variable [88]. Besides the microarray technology for parallel analyses of multiple bacteria, several designs
have been created to integrate PCR with microarray techniques [89,90]. Most of these designs integrate solid-phase PCR with the microarray platform. However, solid-phase PCR is less efficient than solution-phase PCR [91]. Yauk et al. assessed genomic DNA microarray, amplified DNA microarray (PCR followed by microarray), and real-time PCR assays for their suitability for the identification of waterborne pathogens [88]. The attained results revealed that the real-time PCR assay is approximately 108 times more sensitive than the genomic DNA microarray and about 70 times more sensitive than the amplified DNA microarray [87,88]. Even though real-time PCR is still the gold standard for validation of the data generated by microarrays, it has limited capability to perform multiplex analyses of multiple samples and assays (<384 wells plates). To solve this problem, Roche Applied Science (Indianapolis, IN, USA) has commercialised the LightCycler® 1536 Real-Time PCR System, uses utilises a 1536 multi-well plate. This system still needs a robotic liquid handling instrument to load the samples. Many efforts have been made to design PCR-based devices as a high-throughput platform for a larger number of samples.

Most of the first-generation devices need manual PCR mixture loading into individual reaction wells in a PCR array chip. For example, Nagai et al. developed a microchamber array in silicon for picoliter PCR using manual sample loading [92]. Solutions for avoiding manual sample loading include the expensive robotic liquid dispensing robot system or the immobilization of primers in a matrix [92–94]. Matsubara et al. used a nanoliter dispensing robotic system for loading PCR mixtures into a microchamber array [93]. In another work, primers were immobilized in a rectilinear array of 3072 holes in a stainless-steel plate. The array was then sealed inside a cassette using UV-curable epoxy [94]. This chip is commercially available from BioTrove (Open Array® DLP Real-Time qPCR System, Woburn, MA, USA). This system could detect multiple bacteria from environmental water [95]. Although this platform offers a solution to high-throughput real-time PCR, loading
samples to the device requires the differential surface treatment of the steel plate, making the chip fabrication complex and expensive.

Besides sample loading, the other significant challenge in designing these devices is sealing the chip. Uncured PDMS, mineral oils, pressure-sensitive adhesive tapes, and microvalves have been widely used for sealing microreactors [96–98]. Some PCR array chips have open or unsealed reactors to reduce the complexity of fabrication and operation [99,100]. Ramalingam et al. used open reactors for the detection of waterborne pathogens such as *Pseudomonas. aeruginosa*, *Aeromonas. hydrophila*, *Klebsiella. pneumoniae*, and *Staphylococcus. aureus*. Recently, Fluidigm introduced a microarray for multiplex detection of bacteria, supporting a large number of simultaneous reactions with simple use [101,102].

3. 5. Isothermal Amplification-Based Microfluidic Devices

Although PCR is the most popular approach for DNA amplification, the technique requires several thermocycles. Isothermal amplification of NA omits the thermocycling steps, resulting in low cost and high assay quality [38]. Over the last decade, a variety of isothermal methods have been developed for NA amplification such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), helicase-dependent amplification (HDA) [103], rolling circle amplification (RCA) [104], strand displacement amplification (SDA) [105], signal-mediated amplification of RNA technology (SMART) [106], nucleic acid sequence-based amplification (NASBA) [107], single primer-triggered isothermal amplification [108], and cross priming amplification (CPA) [109]. LAMP, RPA, and HDA have attracted significant attention from the microfluidics community.

3.5.1. RPA-Based Microfluidic Devices

Recombinase polymerase amplification (RPA) is a low temperature isothermal platform used to amplify target DNA consuming recombinase, a DNA polymerase, and
DNA-binding proteins. The approach was first reported in 2006 by Piepenburg et al. [110]. Recombinase–primer complexes mediate primer binding at the specific sequence of double-stranded DNA and displacement of the non-template strand at low temperature (37 °C). The displaced new strand is maintained by single-strand DNA binding proteins and the primer is extended by DNA polymerase. The resulting double-strand DNA products can each be copied, leading to exponential amplification [38,111].

RPA provides significant advantages over existing amplification methods such as low incubation temperature, ease of primer design, sensitivity, and high tolerance to impurities in sample [112]. Due to the robustness of its biochemistry, RPA has been integrated into microfluidic devices. Lutz et al. developed a fully automated centrifugal microfluidic cartridge including pre-stored liquid and dry reagents for RPA [113]. The fluidic cartridge can run up to 30 reactions simultaneously in separate 10-mL microchambers. This system was employed for the detection of the antibiotic resistance gene mecA of S. areus. The limit of detection (LOD) was less than 10 copies and the assay time was approximately less than 20 min. Hakenberg et al. designed a phase-guided passive microfluidic batch mixing chip for RPA. The device can be fabricated through an inexpensive approach that integrates dry film resist technology and direct wafer bonding [114]. This detection assay relies on phase-guided fluid handling, resulting in direct fluorescence detection from the chip after a one-minute mixing sequence. RPA has been also used in a droplet microfluidic chip. The microfluidic digital RPA slip-chip has been employed for the simultaneous running of over 1000 nL-scale RPA reactions in parallel [115]. The amplification process starts by adding a chemical initiator to each reaction compartment. During the experiment, precise temperature control is not required owing to RPA tolerance of fluctuations in the incubation temperature ranging from 37 to 42 °C. The generation of an amplified target material is monitored by fluorescence. The performance of this platform was validated by the successful amplification
of methicillin-resistant *S. aureus* genomic DNA. RPA has been growing in popularity in digital microfluidics. Recently, Kalsi et al. [116] performed RPA on a digital microfluidic device using magnetic beads and a pre-concentration unit. The sample with DNA is immobilised on the magnetic beads and then is introduced to a pre-concentration unit that interfaces with the digital microfluidic device. Next, the DNA-loaded beads are pulled through an immiscible oil/aqueous interface directly onto the digital microfluidic platform. The required temperature for amplification is just 39 °C. The final sample volume is 2 μL in a single step and the assay time is less than 30 min with a LOD of $10^4$ bacteria colony forming units (CFU)·mL$^{-1}$.

### 3.5.2. HDA-Based Microfluidic Devices

HDA is an isothermal amplification reaction working optimally at 65 °C and relying on DNA helicase activity. DNA helicase separates complementary strands of double-strand DNA to allow hybridization of target specific primers. The primers are then extended using DNA polymerase to produce target DNA copies. Each of the resulting products can then be copied, leading to exponential amplification of the target. The simplicity and high sensitivity of the reaction means HAD has potential for use in a microfluidic platform [38]. Ramalingam et al. [100] developed a real-time HDA microfluidic chip using PDMS and glass. During the fabrication of this sandwich structure, the HDA primers are dried onto the glass surface of each microchamber. This method allows for simple multiplex analysis of one single sample. The microfluidic chip was verified by successful HDA of target DNA at 62 °C. HDA has been also applied on disposable plastic cartridge [117]. Despite the distinct advantages, there are still some drawbacks that limit its wide application. For example, HAD has been reported to be prone to sample contamination. The non-specific amplification products is a concern when using this method [118].
3.5.3. LAMP-Based Microfluidic Devices

Among the aforementioned methods, LAMP [14,119] was demonstrated to be quicker and more stable, more sensitive, and more specific for NA identification. LAMP-based approaches produce approx. 50-fold more amplicon than PCR-based methods [120]. Using LAMP, the amplification of medium- to long-range template strands of NA (130 < bp < 300) is possible [121].

Most importantly, LAMP can amplify NA in complex substrates even in the presence of contaminants that typically hinder PCR reactions, such as blood components [122] or food ingredients [123]. LAMP-based approaches have high specificity due to implementation of four to six different primers that bind to specific sites on the template strand [124]. LAMP amplification is conducted at temperatures between 60 °C and 66 °C employing the Bst polymerase enzyme and high strand displacement activity [121]. However, RNA targets need extra reverse transcriptase enzyme to transcript RNA into cDNA before the amplification step. A number of works have been conducted on monitoring LAMP amplicons using microfluidics. These detection methods for LAMP amplicons can be divided into end-point colorimetric detection and real-time detection.

3.5.3.1. End-Point Colorimetric Detection

The colorimetric detection relies on production of magnesium pyrophosphate (Mg$_2$P$_2$O$_7$) as a by-product of the reaction between deoxynucleotide triphosphate (dNTP) and magnesium sulfate (MgSO$_4$). Magnesium pyrophosphate appears as a white precipitate in the reaction mixture, which increases its turbidity [13]. The presence of this component allows easy distinction of whether NA is amplified by LAMP. Although no extra instruments are needed, this approach relies on human interpretation of the colour. Different colorimetric dyes have been applied to detect the existence of amplicons, but these dyes should not hinder
the amplification, and the colour change should be easily detected by the naked eye. The dyes that have been applied for bacterial LAMP amplicon detection are listed in Table 3.3.

Table 3.3. Different dyes employed for bacterial Loop-mediated isothermal amplification (LAMP) identification.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Colour Before Amplification</th>
<th>Colour After Amplification</th>
<th>Prevents LAMP</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxynaphtol blue (HNB)</td>
<td>Violet</td>
<td>blue</td>
<td>no</td>
<td>[125]</td>
</tr>
<tr>
<td>HNB+SYBRGreen I</td>
<td>Orange-red</td>
<td>green</td>
<td>no</td>
<td>[126]</td>
</tr>
<tr>
<td>NeuRed</td>
<td>Light brown</td>
<td>pink</td>
<td>no</td>
<td>[127]</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Purple</td>
<td>red</td>
<td>no</td>
<td>[29]</td>
</tr>
<tr>
<td>Calcine</td>
<td>Yellow</td>
<td>green</td>
<td>no</td>
<td>[31,128,129]</td>
</tr>
<tr>
<td>SYBR GREEN</td>
<td>Dark orange</td>
<td>green</td>
<td>no</td>
<td>[130]</td>
</tr>
<tr>
<td>HNB Calcein</td>
<td>Purple</td>
<td>blue</td>
<td>no</td>
<td>[131]</td>
</tr>
</tbody>
</table>

Calcein and hydroxynaphthol blue (HNB) are the two frequently used dyes that do not interfere with the LAMP reaction [125]. Thus, these dyes can be added to the samples before starting the amplification reaction. Conversely, other dyes, such as propodium iodine, SYBR GREEN I, and Picogreen, have to be applied after amplification due to their inhibition effect on LAMP.

Over the last few years, many papers reported the integration of different functions in a simple platform such as paper [132–134]. Connelly et al. designed a paper-based microfluidic device for identification of the \textit{E. coli malB} gene [130]. Three layers of magnetic slips and a cellulose fibre network were used to construct this sliding-strip device, which enables the serial operation of cell lysis, DNA extraction, purification, LAMP amplification, and detection. SYBR GREEN I was used to detect the amplicons with a LOD of 5 cells in 80 μL of sample (Figure 3.4A). In another study, a hybridised paper/plastic microfluidic chip was manufactured for the detection of \textit{Neisseria meningitidis} applying calcein that showed a LOD
of 3 DNA copies in a 26-μL sample (Figure 3.4B) [129]. A microfluidic cassette comprising of two aluminum reels and a plastic ribbon with an array of chambers was developed to identify *E. coli* (30 CFU/mL) and *S. aureus* (200 CFU/mL) using HNB and calcein detectors, respectively (Figure 3.4C) [131].

Although SYBR GREEN I, calcein, and HNB still attracted considerable interest in recent years, leading to rapid development of LAMP on-chip [126,127], there are drawbacks that should be addressed. One main limitation is the difficulty of detecting weak LAMP fluorescence in a few microliters of sample with the naked eye. Channels and reaction chambers with heights of more than 800 μm are possible solutions for higher sensitivity [135–137]. Jiang et al. developed a microfluidic chip that has a staggered herringbone mixer (SHM) structure to perform rapid and efficient airborne bacteria capture and high-throughput LAMP analysis [31]. Using deeper channels and calcein, the amplicons could be observed by the naked eye (Figure 3.4D).

Xia et al. reported another solution that optimized the concentration of calcein for LAMP reactions on a rotate-and-react SlipChip to increase the sensitivity [128]. Under the optimized LAMP conditions, *Bacillus cereus*, *E. coli*, *S. enterica*, *V. fluvialis*, and *Vibrio parahaemolyticus* were identified with the naked eye. The team achieved a fluorescent signal-to-noise ratio of about five-fold and a LOD of 7.2 copies/μL genomic DNA. All the aforementioned methods and the most recent publications [126,127] confined their approaches to the application of Mg_{2}P_{2}O_{7} for naked-eye observation of the amplification; using gold nanoparticles (AuNPs) could be an alternative method. AuNPs depict a characteristic localized surface plasmon resonance absorption band (LSPR) in the visible spectrum of light, which depends on the interparticle space. The aggregation leads to a red shift originating a red-to-purple colour change [138]. Garrido-Maestu et al. reported microfluidic LAMP amplification using functionalised AuNPs for naked-eye detection of
Salmonella spp. in food samples. This method achieved a very low LOD of 10 CFU/25 g [29].

Figure 3. 4. Schematic illustration of sliding-strip device: (a) the exploded view and (b) the assembled device. (c) An image of a prototype concept device [130]. (B) Schematic depiction of the chip layout [129]. (C) Photograph of the cassette microfluidic device [131]. (D) Image of the airborne bacterial capture and LAMP system: (i) bacteria capture chip and (ii) LAMP chip [31]. Reproduced from mentioned reference with permission from the related journals.

3.5.3.2. Real-Time Optical Detection

The main concept behind optical LAMP detection is the production of Mg$_2$P$_2$O$_7$ as a side product of the LAMP polymerase reaction. Enhancement of magnesium pyrophosphate leads to the turbidity of the sample, which can be visualised with a turbidimeter [139], spectrophotometer [140], surface plasmon resonance (SPR) sensor [141], and real time [142] or fluorescent imaging by a charged coupled device (CCD) camera [143]. Amplicons can be
enumerated by plotting turbidity against amplification time. Stedtfeld et al. designed a valveless microfluidic device to detect multiple genes, including stx2 and eaeA of *E. coli* and mecA and vicK genes of *S. aureus* [144]. In this work, SYTO-81 dye was added to the reaction mixture before being loaded to the microchambers. The amplification was performed for an hour at a temperature of 63 °C. A LOD of 13 copies per sample (1 μL) was obtained using LED light at the bottom of each chamber.

Wang et al. reported the construction of a LAMP-based microfluidic device for the identification of methicillin-resistant *S. aureus* in applying a spectrophotometer [140]. Lysing, washing, and reaction chambers were integrated on a single chip. After thermal lysis of bacteria at 95 °C, the target DNA was recognized using specific probe-conjugated magnetic beads. Next, the target DNA was purified and LAMP reagents were added to the chamber. The amplicons were subsequently measured using a spectrophotometer. The entire sample treatment and amplification procedure was performed automatically, and a LOD of 10 fg/μL was achieved. CCD-based fluorescent imaging has also been applied for real-time monitoring of LAMP amplification to identify food- and water-borne pathogens (*Salmonella. enterica, Cryptosporidium. parvum, Campylobacter jejuni, Legionella. pneumophila, Escherichia coli. coli O157:H7, Vibrio. cholera*). SYTO-81 was used as the fluorescent dye, and real time imaging was conducted for *C. jejuni* 0414 gene detection. A single copy of a gene was distinguished within only 19 min [143]. Chang at al. used an optical photomultiplier (PMT) for multiplex detection of *Streptococcus galactiae* and *Aeromonas hydrophila*. The target DNA was amplified and optically identified within 65 min with a LOD of 20 copies in a 25-μL sample [145]. Chiu et al. reported an SPR-LAMP-based chip. Single-layer graphene was deposited on the surface of the Au SPR chip to capture *Tuberculosis bacillus* DNA [146]. Although label-free optical detection modalities are attractive for developing real-time detection of LAMP amplicons, these approaches need bulky and expensive readers.
Zhou et al. [147] used CapitalBio RTisochip-™ isothermal chip detection system for bacterial NA analysis in real time. This commercial platform was developed by CapitalBio Co. (Shanghai, China) and contains both LAMP amplification and an imaging system. The device can simultaneously detect 10 pathogenic bacteria in aquatic animals (Nocardia seriolae, Pseudomonas putida, Streptococcus iniae, Vibrio alginolyticus, Vibrio anguillarum, Vibrio fluvialis, Vibrio harveyi, Vibrio parahaemolyticus, Vibrio rotiferianus, and Vibrio vulnificus), with the LOD ranging from 0.40 to 6.42 pg per 1.414 μL and reaction time of less than 30 min. The CCD sensor has been the most common technique for real-time imaging [30,128,148]. Chen et al. reported a microfluidic in-gel loop-mediated isothermal amplification (gLAMP) for simultaneous detection of E. coli, Proteus hauseri, V. parahaemolyticus, and Salmonella subsp [148]. The emitted fluorescence was evaluated with an inverted fluorescence microscope equipped with a CCD camera. This simple and easy-to-operate system achieved a LOD of 3 copies/μL, which is comparable to existing platforms and has potential for point-of-care applications.

3.6. Design Considerations for PCR Devices

3.6.1. Sealing

Microfluidic devices are typically sealed to contain the sample in a predetermined volume, avoiding uncontrolled spreading of liquids, preventing contamination and biohazards, and decreasing evaporation. Despite recent progress, sealing of these microdevices remains a complex and laborious process requiring specific equipment and protocols. In recent years, attention focused on providing robust, versatile, and reversible sealing solutions that are compatible with cell and molecular biology protocols. A wide range of techniques for sealing microfluidic chips have been outlined in past reviews [149–151]. Based on the materials that are used in chip fabrication and the limitations imposed by their
application, sealing methods vary. PDMS and adhesive materials are the most commonly used materials in sealing devices for amplification.

PDMS, as the most popular material in the academic microfluidics community, is able to seal itself or other substrates both reversibly and irreversibly without an adhesive. Structured or flat layers of this elastomer can seal other flat materials such as silicon, glass, or plastics [152]. Uncured PDMS has been applied to seal inlets and outlets in PDMS-based microchips [136,153–156]. Although PDMS is an excellent material for rapid and easy sealing of many microfluidic devices, some drawbacks have restricted its wider usage. These limitations include the adsorption of hydrophobic samples, instability after surface treatment, swelling in organic solvents, water permeability, and inconsistency under high pressures [157,158].

An alternative approach is using adhesives for sealing microfluidic devices. Adhesive materials can overcome some of aforementioned problems. Pressure sensitive adhesive foil [83], polyolefin sealing foil [72], adhesive tape [80,86], adhesive sealing film [81], and UV adhesive [65] have been used. Sealing the inlets and ventilation holes with the above materials effectively prevented evaporation and contamination during the amplification reaction of bacterial NA. Sayad et al. investigated sealing solutions in an automatic centrifugal microfluidic platform for foodborne pathogen NA detection. In this system, the connection channel between the metering chamber and the amplification chamber was sealed to prevent liquid evaporation (Figures 3.5A,B) [65].
3.6.2. Valving

In addition to sealing, valving is the other critical issue that should be considered in designing a PCR device [159,160]. Under high temperatures, liquid sample may be lost due
to thermocapillary pumping and evaporation. Thus, robust valving is required [70].

Generally, microfluidic valves can be categorized into two groups: passive and active. Passive valves are designed within the microdevice due to fluid flow or modified surface chemistry. Active valves can be classified based on working principles such as capillary valves [161], pneumatic siphon valves [162], and film valves [163]. However, most of these methods can incidentally leak vapor or fluid into an unwanted area due to the absence of a physical barrier. Active valves eliminate this shortcoming by employing a mechanical or external energy source to open and close the microvalve. A variety of techniques have been used for the active valve platforms, including magnetic [164], thermo-pneumatic [165], frozen liquid [70], hydrogel [166], and paraffin wax [167]. Some of these techniques have shown high potential for use mostly due to their low cost, simple operation, and biocompatibility. For example, Koh et al. employed an in-situ gel photopolymerization to form local gel plugs in an integrated plastic microfluidic device to detect *E. coli* O157 and *S. typhimurium* [97]. Using this technique, convective flow of the PCR mixture into other regions was remarkably minimized. In another study, Liu et al. used paraffin as single-use valving material in a disposable microfluidic device to identify *E. coli* [168]. This valve can hold a pressure of 40 psi in a “closed” position.

In another approach, Liu et al. designed thermally actuated valves using a PDMS-expandable microsphere composite [169]. Before the amplification, the valves were heated, expanded, and the amplification reactor was sealed. The valves were able to tolerate a pressure up to 200 kPa without any significant leakage. This device was successfully used for the detection of *E. coli*.

More recently, Brennan employed an elastomeric pinch valve in a microfluidic cartridge system for *E. coli* identification with a leak pressure of 340 kPa [170]. In another study, Huang et al. reported a simple valveless and air-insulated microfluidic chip for detecting a
group of pneumonia-related pathogens such as *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Haemophilus influenzae*, *Legionella pneumophila*, *Chamydiae pneumoniae*, and *Mycobacterium tuberculosis* [73].

### 3.6.3. Detection

The common detection methods for amplicons are electrophoresis, real-time fluorescence analysis, turbidity, or colorimetry [13,125,131,138,171]. Among these methods, real-time fluorescence and colorimetric detection are the most widely used approaches for microfluidics due to their simplicity. Whereas fluorescent detection needs external equipment, colorimetric detection using metal indicators does not require extra detection instruments. Adding a metal indicator to the reaction solution can change the colour of the solution when the target gene is amplified. The colour change can be easily monitored with the naked eye. Oh et al. designed a centrifugal microfluidic device for multiplex identification of *E. coli* O157:H7, *S. typhimurium* and *V. parahaemolyticus* by loop-mediated isothermal amplification and colorimetric detection using Eriochrome Black T (EBT) [80].

EBT is a metal indicator that causes colour change by changing the Mg$^{2+}$ concentration. Due to the reduction of the Mg$^{2+}$ concentration over the amplification procedure, colorimetric detection can be a favourable choice for tracking the reaction. Similarly, this reagent was used for high-throughput screening of a group of foodborne pathogenic bacteria in another centrifugal device [81]. More recently, Sayad et al. reported a centrifugal microfluidic system with endpoint detection and identification of three pathogenic bacteria (*E. coli*, *Salmonella* spp, and *V. cholerae*), with eight strains each. Calcein, a synthetic fluorescein that emits a bright fluorescence, was used and further analysed via electronics interfaced with Bluetooth wireless transmission of the data to a smartphone [65].
3.7. Droplet-Based Microfluidics

Droplet-based microfluidic PCR is a technology that potentially provides fully programmable and automated PCR assays. Droplet-based microfluidics for the detection and the identification of pathogens presents several advantages over classical methods such as ultra-small sample volume, large number of droplet reactors, and ability to incorporate complex liquid handling protocols for these droplets. The large number of droplets allows the encapsulation of NA to follow a stochastic process. Single NAs are isolated from the bulk sample and are confined each in their own liquid compartment. A massively large number of individual droplets, even in the millions, could be evaluated, resulting in an extremely high throughput. Operations on droplets can be performed repetitively, allowing for more complex experimental protocols. Multiple manipulation tasks can be conducted on the same droplets [172–174] pre-and post-PCR: controlled droplet formation, merging, mixing with PCR reagents, splitting, sorting, and incubation [175,176]. Droplets are formed from two immiscible phases: the continuous phase (typically organic liquid-like oil in which droplets flow) and dispersed phase (the aqueous sample droplets). Aqueous droplets are commonly generated in a microchannel with the T-junction [177] or the flow-focusing configuration [178]. To date, the two droplet-based microfluidic technologies are continuous-flow and digital microfluidics. Compared to continuous-flow microfluidics, digital microfluidics implements the reaction protocols in a single droplet. Electrowetting and dielectrophoresis are common actuation techniques for digital microfluidics [33].

3.7.1. Continuous-Flow Microfluidic

Droplets are often in motion in continuous-flow droplet microfluidics. The physics of droplet formation and handling has been well studied [179–181]. Droplets can be incubated [182], split [183], and merged [184]. From the microbiological point of view, continuous-
flow droplet-based microfluidics allows for the distribution of the large volume of aqueous suspensions of microorganisms in the order of millilitres or more into droplets with volume ranging from pico- to microlitres. The droplets can subsequently be manipulated automatically. Droplets can be formed at a frequency up to more than ∼10,000 Hz with a dispersity of less than 2% [185]. Droplet fluorescence can be analysed at a speed up to 250,000 droplets per second [186]. Instead of primer solution, some continuous flow droplet-based PCR systems used primer-modified beads [187] or agarose droplets [188].

To generate droplets, capillary tubes have often been employed. For instance, Dorfman et al. [189] used a PFA capillary, coiled around a cylinder heater to encapsulate PCR mixture in 1 mL droplets. Hartung et al. reported the droplet generation within Teflon FEP tube and T-connectors [190]. Markey also used PTFE tubing coiled around the aluminum cylinder heaters and T-junction to produce droplets [191]. In a different layout, Ohashi et al. applied the external magnet to move droplets containing hydrophilic magnetic beads through different temperature zones in a reaction chamber [192].

3.7.2. Digital Microfluidics

Digital microfluidics relies on stationary or semi-stationary droplets. This technology enables generation, manipulation, and monitoring of droplets carrying single or a bulk of NA in a highly parallel and high throughput process. A large number of droplets can be produced via the surface-assisted approach [193,194]. Hydrophilic wells or through-holes are patterned on the substrate and trap the sample solution into a stationary droplet array. Fluorinated oil is generally applied to prevent evaporation during the thermal cycling process. Beneyton et al. [195] used a high-throughput droplet-based microfluidic platform for detecting and sorting of *E. coli* based on the enzymatic activity of CotA laccase. The analysis/screening format of the system enabled the analysis of the enzymatic activity in droplets at a frequency of 1,000 Hz.
and active sorting of droplets at 400 Hz. After cell growth and protein expression inside the droplets, a fluorogenic reagent was inserted. Fluorescence-marked droplet was then sorted, recultivated, and identified based on colorimetric assays.

Although selective cultivation is useful to enrich the target species of bacteria prior to sequence analysis, this method is not successful for bacteria that are not cultivable. Lim et al. [196] used a culture-independent strategy for sorting microbial cells based on genomic content. The fluorescence signal generated in droplets during PCR was applied for sorting and analysis of the specific gene sequences. In another study, encapsulation in droplets allows for massive amplification of *E. coli* while maintaining sequence accuracy and uniformity [197].

In addition to high-throughput digital microfluidics, devices with the ability to facilitate and accelerate the amplification procedure have also attracted considerable attention. Easley et al. [198] used a microfluidic genetic analysis system with sample-in–answer-out capability for *B. anthracis* identification in blood samples. A single syringe pump delivered the sample and reagents into the glass-chip for NA purification. Elastomeric membrane valves were employed for the isolation of each functional region of the device. Purified DNA and PCR reagents then entered to the 550-nL chamber for PCR amplification.

Hua et al. [199] presented a digital microfluidic platform for quick multiplexed real-time PCR of methicillin-resistant *S. aureus* and *Mycoplasma pneumoniae*. Fast PCR thermocycling was achieved by periodically shuttling the sample droplet between two fixed temperature zones in an oil-filled cartridge. The cartridge was composed of a printed circuit board.

One of the possible methods to accelerate the amplification experiments dealing with samples with large concentration differences is providing a wide dynamic range using digital microfluidics [200,201]. A wide assay dynamic range would increase the speed of
measurements by preventing sample serial dilution before PCR amplification. Most of the microfluidic digital PCR platforms use the strategy of increasing the number of compartmentalized microreactors to provide a wide dynamic range [194,202]. However, increasing the reaction number results in a significant challenge in the fabrication of high-density chambers. To overcome this hurdle, a multivolume digital PCR method has been reported, where multiple microreactors with different volumes are used. Compared with single-volume digital PCR platforms, the multivolume method considerably decreases the total number of reactors while preserving the same dynamic range [203]. To fabricate numerous chambers with a large volume range on one glass chip, complex multistep lithography and wet-etching techniques have been used to produce wells with different depths [204].

A critical concern in designing droplet digital platform is evaporation during thermal cycling. Bian et al. [96] used a mineral oil-saturated polydimethylsiloxane (OSP) chip for droplet digital PCR. The system provided droplet generation, amplification, and end-point fluorescence readout (Figure 3.6A) to identify *E. coli* O157:H7 and *L. monocytogenes*. Although the initial efforts led to sensitive detection of target sequences in bacteriology at single-molecule resolution, which is even higher than qPCR, there were still some drawbacks. For example, conventional digital microfluidics usually employs a relatively small number of fixed hard-wired electrodes. Consequently, the droplet manipulation operations were restricted to these electrodes patterned on the device. Over the last years, thin film transistor (TFT) electronics have been introduced as an ideal alternative [205]. These active matrix platforms have thousands of individually trackable electrodes allowing for simultaneous and independent manipulation of several droplets and running complex analytical processes. Kalsi et al. [206] presented a digital microfluidic system using TFT for detection of three genes that confer resistance in bacteria to antibiotics (*CTX-M-15, KPC*, and
In this assay, automated dispensing protocols were applied to generate droplets with nL-volume-comprising sample DNA, reagents, and controls. The reagents were then mixed, and isothermal DNA amplification of droplets was performed. Positive amplification was measured by fluorescence.

Though digital platforms can generate large quantities of droplets automatically, there are still some practical drawbacks. For example, droplet-to-droplet coalescence may occur if they contact each other [207] during the heating process [208]. One of the possible solutions for this problem is the use of a droplet array. Ma et al. [209] presented a novel microfluidic device capable of arraying emulsion droplets and conducting digital LAMP of vancomycin-resistant Enterococcus bacteria. The system was a combination of an emulsion droplet formation device with a hydrodynamic trapping array. After preparation of target NA and LAMP reagents, they were digitized on-chip into water-in-oil droplets using a flow-focusing configuration. Subsequently, droplets were hydrodynamically sorted into a droplet array. The method was able to produce uniformly sized droplets with a variation of less than 3%. Successful LAMP amplification and fluorescence detection of positive droplets were recorded with a fluorescence microscope, Figure 3.6B.

Although digitization of amplification has led to improved time to detection and direct quantification of NA without a standard curve, these methods mostly are limited to laboratories with trained personnel and expensive equipment. To overcome these hurdles, Byrnes et al. [210] reported a polydisperse droplet emulsions approach with a statistical correction for E. coli detection. The assay provides accurate quantification of droplet digital PCR and reverse transcriptase droplet digital PCR which makes it more powerful compared to commercially available devices such as BioRad’s ddPCR. This method overcomes a few practical restrictions of the BioRad system. For example, since the measurement of droplets
occurs regardless of size, there is no data loss due to improper droplet size. Also, it requires less equipment and time for running (Figure 3.6C).

Figure 3.6. (A) Droplet digital PCR workflow: (a) fabrication of mineral oil saturated PDMS (OSP) microfluidic chip, (b) generation of droplets, (c) on-chip amplification followed by fluorescence readout, and (d) data analysis [96]. (B) (a) Schematic depiction of the assay for emulsion droplet array-based digital LAMP analysis. (b) eExperimental set up for result analysis [209]. (C) Multiplexing in ddPCR [210]. (Reproduced from mentioned reference with kind permission of related journal).

Table 3.4 provides a brief list of the advantages and disadvantages of microfluidic-based amplification techniques which discussed above.
Table 3.4. Merits and demerits of microfluidic-based amplification platforms.

<table>
<thead>
<tr>
<th>Platform</th>
<th>Complexity</th>
<th>Sample Volume</th>
<th>Assay Time</th>
<th>Throughput</th>
<th>Sensitivity</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpentine [59]</td>
<td>- Several heaters and pumping are required - Complex channel design</td>
<td>0.35 μL</td>
<td>18 min</td>
<td>Low</td>
<td>0.031 pg/μL</td>
<td>On-site gene testing</td>
</tr>
<tr>
<td>Oscillating-flow [62]</td>
<td>- Pumping is required - Complex design - Low detection speed - Elaborate designs and rotating platforms - Complex electronic components</td>
<td>2 μL</td>
<td>12 min</td>
<td>Low</td>
<td>10 DNA copies</td>
<td>On-the-spot analysis</td>
</tr>
<tr>
<td>Centrifugal [66]</td>
<td>- Very complex design - Long analysis time - Mostly unsuitable for multiplexing</td>
<td>40 μL</td>
<td>70 min</td>
<td>Low</td>
<td>100 CE with tmRNA</td>
<td>Clinical application</td>
</tr>
<tr>
<td>Lab disk [86]</td>
<td>- Robotic liquid handling is required - Complex and expensive</td>
<td>4.8 μL of the sample plus preloaded primers and LAMP reagents</td>
<td>60 min</td>
<td>Low</td>
<td>$2 \times 10^2$ cells per μL</td>
<td>Nucleic acid diagnostics in resource-limited settings particularly in clinical stage</td>
</tr>
<tr>
<td>Array [102]</td>
<td>- Difficult naked eye detection</td>
<td>20 uL</td>
<td>~60 min</td>
<td>High</td>
<td>High</td>
<td>Water distribution systems, clinical field</td>
</tr>
<tr>
<td>LAMP-based [139]</td>
<td></td>
<td>600 nL</td>
<td>~70 min</td>
<td>Low</td>
<td>3 copies/μL</td>
<td>Application in point-of-care</td>
</tr>
</tbody>
</table>
3.8. Microfluidic Sample Preparation for Amplification

To fully integrate an amplification protocol onto a microfluidic device with sample-to-answer capability, the following processing steps have to be integrated: cell lysis, NA extraction and purification, amplification, and amplicon detection [211,212]. Several devices were reported to complete cell lysis and DNA extraction with PCR using silica-based separations or magnetic beads for extraction [213,214]. However, due to the inhibition effect of silica and some magnetic beads on amplification, the DNA should be eluted, usually with ethanol, which is a strong PCR inhibitor as well.

Oblath et al. [215] used a monolithic aluminium oxide membrane (AOM) in an integrated microfluidic device. The device was successfully used for the identification of...
methicillin-susceptible *S. aureus* and methicillin-resistant *S. aureus* in saliva. AOM is a porous material that can be applied for the extraction of DNA. The amount of extracted DNA relies on the size of the AOM’s pores, pH, and salt concentration of the solution. In this approach, bacteria were first lysed by heating. The resulting solution was then injected to the chip and filtered via the AOM to extract the DNA. By adding PCR reagents to each well, the chip was ready to be thermocycled. Ethidium monoazide (EMA) is another commonly used material for differentiating live and dead bacteria. EMA is a DNA staining fluorescent dye that can enter into the broken cell walls of dead bacteria and intercalate into double-stranded DNA. Since the EMA cannot enter live cellular membranes, it can be used for labelling the DNA of dead cells within a population of viable and dead cells [216]. This marker has been used in various integrated amplification microfluidics to detect bacteria such as methicillin-resistant *S. aureus* (MRSA) [216], *E. coli*, *S. aureus*, *P. syringae*, *Enterococcus* sp., methicillin-resistant *S. aureus*, and coagulase-negative staphylococci [217], and live bacteria for periprosthetic joint infection [218].

Magnetic beads are the other group of material that can be used to eliminate the presence of inhibitors in the sample and to increase the collection efficiency of target DNA. Particularly, nucleotide probe-conjugated magnetic beads have been employed to capture specific DNA fragments [219]. Chao et al. [220] used magnetic beads in an integrated microfluidic device for *Helicobacter pylori* detection. In this experiment, the surfaces of the magnetic beads were modified with 16S rRNA probes to capture the conserved DNA region of *H. pylori*. After capturing the target DNA, SYBRs Green I, as a fluorescent dye, was added in the PCR step to intercalate into the DNA fragments, resulting in a measurable fluorescence intensity in the developed microfluidic system.

Another on-chip DNA purification technique is solid-phase based DNA collection. Ha et al. [221] employed an integrated thermoplastic microdevice for solid-phase based NA
purification. The prepared chip consisted of three microchannels for washing solution, DNA purification, and amplification. Polycarbonate (PC) was the chip material. The surface of PC was treated with amine-bearing polyethyleneimine (PEI) to make it hydrophilic. After sealing the device, the microchannel walls were coated with epoxy-terminated poly(dimethylsiloxane) (PDMS) (epoxy-PDMS). Chambers functionalised with amine were used for capturing NA. The microdevice was successfully assessed by detection of genetically modified E. coli O157:H7. The same team also used glass beads to perform solid-phase based on-chip DNA purification to detect E. coli [222].

Kim et al. [223] employed a solid phase reversible immobilization (SPRI) method for NA extraction in a high-throughput automated microfluidic system. This platform was used for whole-genome shotgun (WGS) sequencing of M. tuberculosis and soil micro-colonies. Another possible alternative to the aforementioned methods is the separation and enrichment of bacteria before amplification. Ohlsson et al. [224] developed an integrated microchip with acoustic separation, enrichment, and PCR detection of bacteria from blood. The blood sample was first processed in an acoustophoresis chip to remove red blood cells. Next, the remaining bacteria-containing plasma proceeded into a glass capillary where the bacteria were trapped and enriched onto suspended polystyrene particles. The trapped bacteria were subsequently washed and released into a polymeric device containing dried PCR reagents for amplification (Figure 3.7).

Recently, Ip et al. [225] used modified magnetic beads in an integrated microfluidic platform to identify live M. tuberculosis (TB). The device comprises four identical diagnostic sets including reaction chambers, positive chambers, negative chambers, and components for automatic liquid handling. Bacteria were trapped using magnetic beads modified with TB-specific markers. Since the magnetic beads capture both live and dead TB, propidium monoazide (PMA) was used in the second step for differentiating these cells. PMA is able to
preferentially bind to dsDNA. This photoreactive dye is unable to penetrate the membrane of viable bacteria, so it can be applied for selectively capturing dead bacteria. After washing unbound bacteria and unnecessary reagents, bacteria were lysed and the resulting DNA was used for amplification and fluorescence detection. Yu et al. [226] used mannose-binding lectin (MBL)-coated magnetic beads in an integrated microfluidic system to isolate methicillin-resistant *S. aureus* and *E. coli*. MBL is a liver-derived serum protein with the ability to identify carbohydrate patterns on a broad range of pathogens, and particularly bacteria. Using this device, the entire process including bacteria isolation, on-chip amplification, and fluorescent signal detection is completed within 1 h.

![Figure 3. 7. Bacteria were separated from red blood cells using acoustic separation, enriched, and then released to dry-reagent PCR chips for detection [224]. Reproduced from the mentioned reference with permission from the related journal.](image)

### 3.9. Conclusions and Perspectives

Remarkable progress has been achieved in the field of microfluidics. Since its beginning, microfluidics applications are becoming increasingly more relevant to life sciences and medicine. One of the reasons for this achievement is due to the unique chemical and physical processes that occur on the microscale.
Microfabrication has much to offer to microbiologists, particularly in NA detection and identification without a major investment of time and resources. Since the first successful PCR in microfluidic devices was reported, a variety of techniques have been developed. The techniques provide rapid analysis, automation, high throughput, high specificity, high accuracy, portable type, low cost, and convenience. However, a number of time and space domain PCR devices are still unable to provide simple user interfaces. Fabrication facilities and skilled hands are required to handle these types of devices. The complexity is a significant barrier for PCR users, preventing them from adapting microfluidic devices. Only a few instances, such as the Lab Disk, leverage the advantages of microfluidics and provide the simple use. Future studies, particularly in spatial and transient PCR, should focus on making the microfluidic approaches easily accessible for users by simplifying of the fabrication process and consequently reducing the final price.

LAMP is a promising alternative to PCR and can reduce technical complexity for microfluidic devices. LAMP approaches have a higher robustness against temperature variations and inhibition compared to PCR. LAMP-based approaches still require vigilant optimization of loop primers for reproducible and sensitive target detection.

Microfluidics-based digital PCR has become a promising alternative to conventional amplification platforms, which allows absolute quantification with high precision without the requirement for standard curves. Digital PCR is based on a compartmentalization of NA molecules into individual volumes from bulk solution. Although digital PCR is being employed broadly, the impact of the technology could be extended. To attain this success, studies should be performed to allow for simple, automatic, high-throughput, and multiplexed digital PCR. Commercial integrated amplification platforms also have advantages, such as high throughput, simple operation, and low cross contamination. Even though experiment expenses could be decreased by a reduction in sample volumes, the cost advantages are not
still significant enough for most end users. Consequently, simplification of digital PCR processes and reduction of required equipment is necessary, for example, by simplifying the sample pre-treatment method to shorten the assay time and further improve the detection limit.

Alternative approaches to NA detection, such as DNA arrays and next generation sequencing, have been used widely in the last years, providing another perspective for designing microfluidic-based detection systems. However, both arrays and sequencing methods often require a minimum amount of NA and thus need a PCR sample preparation step. Hence, integrating PCR and NA arrays or sequencing methods on the same microfluidic device could be an interesting approach for future research.
3.10 References


93. Matsubara, Y.; Kerman, K.; Kobayashi, M.; Yamamura, S.; Morita, Y.; Tamiya, E. Microchamber array based DNA quantification and specific sequence detection from a


115. Shen, F.; Davydova, E.K.; Du, W.; Kreutz, J.E.; Piepenburg, O.; Ismagilov, R.F. Digital isothermal quantification of nucleic acids via simultaneous chemical initiation of


Statement of contribution to co-authored published paper

This chapter is in a form of a co-authored published paper. The bibliographic details of the co-authored paper, including all authors, are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

My contribution to the published paper involved:
- Literature review
- Experimental set-up
- Manuscript preparation
- Responding reviewers

First author: Lena Gorgannezhad

Corresponding author: Dr Muhammad J.A. Shiddiky

Supervisor: Prof. Nam-Trung Nguyen
Chapter 4: Detection of FGFR2:FAM76A Fusion Gene in Circulating Tumor RNA Based on Catalytic Signal Amplification of Graphene Oxide-loaded Magnetic Nanoparticles

Abstract

Circulating tumor nucleic acids (ctNAs) are directly adsorbed onto the GO-NPFe$_2$O$_3$ surface through promising biomarkers for minimally invasive cancer graphene-RNA affinity interaction. The electrocatalytic assessment. The FGFR2: FAM76A fusion gene is one of signal was achieved by the reduction of surface-attached the highly promising ovarian cancer biomarkers detect- ruthenium hexaammine(III) chloride which was further able in ctNAs. Herein, we introduce a new amplification- amplified by using the ferricyanide redox system. Our free electrochemical assay for the detection of assay depicted an excellent detection sensitivity down to FGFR2:FAM76A fusion gene in ctNAs extracted from 1.0 fM, high specificity and excellent reproducibility ovarian cancer patients. The assay relies on the electro- (% RSD= < 5 %, for n= 3). The analytical performance catalytic activity of a new class of superparamagnetic of our method was validated with standard qRT-PCR graphene-loaded iron oxide nanoparticles (GO-NPFe$_2$O$_3$). analysis. We believe that this newly developed assay After isolation and purification, the target RNA was would be practically applicable in clinical research.

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4.1. Introduction

Circulating tumor nucleic acids (ctNAs) have emerged as promising biomarkers for diagnosis, prognosis, treatment response prediction, as well as assessment of tumor stage [1]. As the access to circulating nucleic acids is minimally invasive, ctNA analysis may replace painful, expensive, and time-consuming tumor tissue biopsy [2]. However, ctNA detection is an exigent task primarily due to their extremely low abundance in serum/plasma and low allele frequencies of targeted molecular alterations. Moreover, highly fragmented nature makes their isolation and analysis difficult [2a]. Somatic genomic rearrangements such as translocation, deletion, or inversion can lead to joining together of previously separate genes. The resulting hybrid or “fusion genes” can induce cancer initiation and progression by altering expression levels or functionality [3].

Fusion genes are exceptionally powerful cancer biomarkers, as in most cases they are pathognomonic in contrast to other more promiscuous genetic lesions, like point mutations, which can be found in several types of cancers. Fusion genes have been detected in various cancers, such as breast [4], lung [5], prostate [6], and ovarian [7] and have attracted a great deal of attention from the research community in recent years. Ovarian cancer is the seventh most common cancer among females worldwide and causes more than 150,000 deaths annually. Several fusion genes have been identified in ovarian cancer and occur frequently in high-grade serous carcinoma (HG-SC). A previous study identified 45 fusion genes in ovarian cancer, however primarily due to high level of heterogeneity, none of the fusion genes occurred in more than one patient [8]. Nonetheless, transcriptome sequencing has identified fusion genes ESRRAC11 or f20 [9] and CDKN2D-WDFY2 [10] in 15% and 20% of the patients, respectively, thus suggesting some gene fusion events may play a critical role in HG-SC development. Fusion between the exon 17 of FGFR2 gene and the exon 2 of FAM76A gene (FGFR2: FAM76A) has also been reported in ovarian cancer, whose expression can cause up to 50% increase in proliferation of the cultured
ovarian cancer cells [7]. Moreover, as it was shown that FGFR2: FAM76A could be detected noninvasively in ctNAs, this gene fusion event can be considered as a promising minimally invasive biomarker for the detection of ovarian cancer. Current ctNA detection methodologies have largely been limited to polymerase chain reaction (PCR) [11] or sequencing [12]. Though sequencing is robust and sensitive, high cost and long analysis time makes it particularly unsuited for routine clinical use [13]. On the other hand, PCR based methods are amenable to biological interference and require sample preprocessing [2b]. Electro-chemical approaches represent attractive alternatives for the analysis of ctNAs owing to their low cost, relatively high sensitivity, specificity and capacity for multiplex detection [14]. Over the past few years, various new electrochemical strategies have been introduced to improve the detection sensitivity of ctNAs, such as DNA concatamer-based biosensor [15], DNA nanostructure-based biosensor [16] and nanocomposite-based biosensors [17]. Nevertheless, these technologies are partially hampered by the requirements of signal amplification, complex surface modification and necessity of target labeling. Moreover, analysis of patient samples (heterogenous) for target biomarker is challenging using these methodologies due to their limited selectivity. These issues have been addressed using probe-based approaches. DNA probes are single-stranded DNA used for detecting the presence of target sequences on particular DNAs/RNAs based on the complementary base-pairing phenomenon. The hybridization between DNA probes and sample DNAs/RNAs can result in double-stranded hybrid structure, which is necessary to initiate sequence targeting process. The strength of this hybridization is attributed to different factors such as: nucleotides composition and particularly the genomic G+C content of DNA probes, base pair mismatches distribution within the hybrid duplexes structure, concentrations of ingredients (i.e. DNA probe, target nucleic acids, salt,…), and reaction temperature [17c-d]. The length of DNA probe is also one of the major elements affecting hybridization efficiency. Although the long probes generate better signal
intensity compared to the short ones, they are prone to cross-hybridization between one probe and several targets [17e].

Recently, Das et al. reported a novel electrochemical clamp assay using DNA clutch probes (DCPs), which could directly detect mutated circulating nucleic acids in patient serum [18a]. Although this elegant approach is enzyme-free and sensitive, the complexity of clamp fabrication limits its broad application. On the other hand, sensitive and selective label-free electrochemical ctRNA sensing platforms based on nanomaterials such as MoS2/graphene have emerged as a new class of biosensors. For example, Chu et al. used synthetic oligonucleotide probes immobilized on MoS2/graphene nanosheets for the detection of E542K mutation in PIK3CA gene [17a]. Similarly, Koo et al. have reported the detection of prostate cancer specific fusion gene by combining isothermal amplification and label-free readout [19a]. This paper reports a new method for sensitive and specific detection of FGFR2: FAM76A fusion gene via electrocatalytic signal amplification. To the best of our knowledge, this assay is the first amplification-free approach for electrochemical detection of FGFR2: FA- M76A fusion gene in ctRNA. To this end, target FGFR2: FAM76A RNA were magnetically isolated and purified using probes complementary to the gene fusion junction. According to our previous studies [6a-b], a suitable short DNA probe with good analytical performance in optimum reaction condition was used for ctRNA detection. Purified target RNAs were subsequently directly adsorbed onto the graphene surfaces of a GO-NFe2O3 through graphene-RNA affinity interaction followed by electrocatalytic reduction of surface-attached ruthenium hexaammine (III) chloride ([Ru(NH3)6]3+). The electro- catalytic signal was further amplified by using the ferricyanide ([Fe(CN)6]3−) system coupled with the [Ru(NH3)6]3+ system. Our assay was successfully tested in plasma samples of patients with ovarian cancer.
4.2. Experimental Sections

4.2.1. Reagents and Materials

Analytical-grade reagents and chemicals were used in this assay, unless mentioned otherwise. Hexaammineruthenium (III) chloride and phosphate buffer saline (PBS) tablets were obtained from Sigma-Aldrich (Australia). Tris was purchased from VWR Life science (Australia). UltraPure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used throughout. Synthetic RNA, primers and capture probes were obtained from Integrated DNA Technologies (Coralville, IA, USA) and sequences are depicted in Table 4.S1. Screen-printed carbon electrodes (SPCE) (DRP-150) were purchased from DropSens (Spain). In the three-electrode system, working (4 mm diameter), counter and reference electrodes were carbon, platinum and silver-modified respectively.

4.2.2. Synthesis and Characterization of GO-NPFe₂O₃

The synthesis and characterization of GO-NPFe₂O₃ has been reported previously [20]. In brief, the synthesis procedure involves three major steps; 1) preparation of graphene oxide (GO) sheets using Hummer’s method, 2) preparation of Prussian blue (PB) nanoparticles, and 3) mixing the GO solution with the PB solution in the specific weight ratios of 25 : 75. Then, the mixture was sonicated, aged for 24 h and dried at room temperature. Finally, the mesoporous GO-NPFe₂O₃ were achieved by calcining the GO/PB powders at 400 °C with a heating rate of 1 °C/min⁻¹. For characterizing of GO-NPFe₂O₃, SEM, wide-angle XRD and N₂ adsorption-desorption isotherms studies have been performed (see Ref. 20 for details). As discussed in Ref 20, the original 2D morphology of the GO sheet is well preserved while the PB nanoparticles are located within the stacked GO sheets interlayer spacing. The surface area and pore volumes were calculated to be 120.5 m²/g and 0.384 cc/g by the BET and BJH methods, respectively.
4.2.3. Study Group and Samples

Staged samples (cross sectional) were collected at the Ochsner Baptist Medical Center and obtained via the UQ Centre for Clinical research (UQ IRB 2016000300). Plasma samples were obtained in accordance with the declaration of Helsinki and approved by the Ethics Committee of The University of Queensland and the Ochsner Medical Center (New Orleans, USA). Plasma was separated from whole blood by centrifugation (2000 g × 10 min at Room temperature) and stored at -80 °C until further analysis. Ovarian cancer samples were collected prospectively and assigned according to the histotype classification (e.g., stage I, and stage III) and stored to -80 °C in the Biobank units. Only patients with epithelial ovarian cancer high-grade serous subtype (n = 5) and benign controls (n = 5) were included in this study.

4.2.4 RNA Extraction from Clinical Plasma Samples

Total RNA was extracted from 200 μL volume of each plasma sample using plasma/serum RNA purification mini kit 55000 (Norgen Biotech Corp, Canada) according to the manufacturer’s recommendations. The extracted RNA was was diluted by adding 20 μL elution buffer. The kit provided a reliable purification and subsequently RNA with high quality and purity which is necessary for successful RNA detection platforms. Nanodrop spectrophotometric analysis (BioLab, Ipswich, MA, USA) was performed to quantify and verify the purity of extracted RNA. The concentration of RNA was recorded in ng/mL and stored at -80 °C until assayed.

4.2.5 Probe Hybridization and Magnetic Isolation

In order to perform probe hybridization, extracted total RNA from serum samples were adjusted to the required concentration in 5 μL of 5X saline sodium citrate (SSC) buffer (pH 7) and 10 μL of 10 μM biotinylated capture probes [(5’-TGA-AAG-GAA-AGG-AAC-]}
ATA-TGT-TTG-TTT-TAC-A-3'-Biotin). Underlined and bold are complementary to FGFR2 and FAM76A sequences, respectively]. The mixture was then heated to 65°C for 2 min for linearization of RNA. The mixture was placed on mixer for 1 h at room temperature to allow capture probe hybridization to FGFR2:FAM76A RNA. For magnetic isolation of FGFR2:FAM76A RNA, 10 μL of streptavidin-labeled Dynabeads (MyOne Streptavidin C1 (Invitrogen) magnetic beads) were first washed two times with washing and binding (B&W) buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl) and resuspended in 10 μL of B&W buffer. After this step, the mixture was added to the prepared capture probes-FGFR2:FAM76A RNA (as mentioned above) and incubated on a mixer for 30 minutes at room temperature to allow the magnetic beads to be labelled with capture probes. Finally, the magnetic beads with attached capture probes- FGFR2:FAM76A RNA were isolated using a magnet, washed two times with B&W buffer, and resuspended in 10 μL of RNase-free water. This mixture was heated for 2 min at 95 °C, and instantly placed on a magnet to collect the supernatant, which contained the captured FGFR2:FAM76A RNA. Subsequently, 5.0 μL of the released FGFR2:FAM76A RNA was diluted with 10 μL of 5X SSC buffer (pH 7.0) for electrochemical measurements. The assay was also repeated for wrong target with uncomplimentary sequence (5'-AGAGAAAACUUCCUCACCGGGUCGGUC-3') to test the specificity of the designed probe.

4.2.6 Adsorption of Isolated RNA on modified SPCE

The SPCE was washed with an excess amount of Milli-Q water and positioned onto a permanent magnet in such a way that the working electrode surface was centered to the magnet (Figure 4.S1). 4 μg of GO-NPFe₂O₃ nanoparticles were employed on the electrode surface and allowed to attach magnetically. The modified SPCE was then washed with 10 mM PBS to remove unattached or loosely attached particles from the electrode surface before conducting subsequent steps for electrochemical readout.
4.2.7 Electrochemical Detection

CHI650 electrochemical workstation (CH instrument, USA) was used in all the electrochemical experiments. The effective SPCE surface area was determined using the Randles-Sevcik equation (eq. 1) as described previously [21, 22].

\[ i_p = (2.69 \times 10^5) n^{3/2} AD^{1/2} CV^{1/2} \]  

(1)

where \( i_p \) is the peak current (A), \( n \) is the number of electrons transferred (\( Fe^{3+} \rightarrow Fe^{2+}, n = 1 \)), \( A \) is the effective area of the electrode (cm\(^2\)), \( D \) is the diffusion coefficient of [Fe(CN)\(_6\)]\(^{3-}\) (taken to be \( 7.60 \times 10^{-5} \text{cm}^2\text{s}^{-1} \)), \( C \) is the concentration (mol cm\(^{-3}\)), \( v \) is the scan rate (Vs\(^{-1}\)). For detecting ctRNA, 5 \( \mu \text{L} \) of magnetically isolated target ctRNA were adsorbed directly onto the GO-NPFe\(_2\)O\(_3\) modified SPCE followed by the 10 min incubation to attach ctRNA onto the graphene oxide moiety of GO-NPFe\(_2\)O\(_3\). The electrode was then incubated with 50 \( \mu \text{L} \) of 50 mM [Ru(NH\(_3\))\(_6\)] for 20 min and washed twice with the PBS. The chronocoulometry (CC) measurement was carried out in 10 mM Tris buffer (pH 7.4) under conditions described previously [22]. In the case of redox cycle; the CC data was obtained by using 10 mM Tris buffer with 4 mM potassium ferricyanide solution [Fe(CN)\(_6\)]\(^{3-}\). The number of cationic redox molecules electrostatically associated with the surface-attached anionic phosphate backbone provides a measure of the amount of ctRNA adsorbed onto the GO-NPFe\(_2\)O\(_3\)/SPCE surface. The total charge \( Q \) at a time ‘t’ can be expressed by the integrated Cottrell equation (eq. 2) as described previously [22].

\[ Q = \frac{2nFAD_0^{1/2}C_0^{1/2}t^{1/2} + Q_{ad} + nFAR_0}{\pi^{1/2}} \]  

(2)

The amount of RuHex attached onto the electrode surface is denoted by \( \Gamma_0 \) while charge obtained by the adsorption of target RNA, also known as surface excess, is represented by...
nFA $\Gamma_0$. CC curves were constructed by plotting the charge flowing through the ctRNA-attached electrode versus square-root of time ($t^{1/2}/\text{s}^{1/2}$). $Q$ and $Q_{dl}$ were estimated from the intercepts of the two curves at $t=0$ for the RuHex-treated (i.e., ctRNA-attached electrode was incubated with 50 mM RuHex for 20 min) and untreated (i.e., in the absence of RuHex) electrodes respectively. Therefore, $Q$ represents the total charge comprising both the Faradic and non-Faradic (capacitive) charges. The corresponding charge of RuHex (electrostatically bound to surface confined RNA) and the surface density of ctRNA can be calculated by the eqn (3) and (4), respectively [14a].

\[
Q_{ctRNA} = Q - Q_{dl}
\]

\[
\Gamma = \frac{(Q_{RNA} \times N_A/nFA)(z/m)}{A}
\]

where, $n$ is the number of electrons involved in the reaction ($n=1$), $A$ is the working electrode area, $N_A$ is the Avogadro’s number, $m$ is the number of nucleotides in the ctRNA, and $z$ is the charge of redox molecules (for RuHex, $z=3$).

For Figure 4.3, using the equation (3) and (4), the surface density of ctRNA on the electrode surface were calculated to be $3.57 \times 10^{13}$ and $5.74 \times 10^{12}$ molecules cm$^{-2}$ for 1.0 nM and 1.0 fM of ctRNA respectively.

**4.2.8 Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR)**

First strand cDNA synthesis was performed using miScript Reverse Transcription kit (Qiagen, Germany). Sensi FAST SYBR No-ROX Kit (Biloine, UK) and gene specific primers (Table 4.S1) were used for RT-qPCR analysis. Each qPCR reaction was performed in a total volume of 20 µl and contained approx. 100–150 ng of cDNA template and 10 mM each of forward and reverse primers. qPCR for fusion gene was performed on CFX96 (Bio-Rad) thermocycler with the following conditions; initial denaturation 95 ºC for 2 min followed by 40
cycles of 95 °C for 10 s, 47 °C for 30 s, and 72°C for 20 s (extension). The expression level of fusion gene was normalized against the GAPDH housekeeping gene (qPCR annealing temperature 55°C). All samples were run in triplicate and no-template control was included in each assay.

4.3. Results and Discussion

4.3.1. Assay Principle

Using FGFR2:FAM76A as a model we report here an amplification-free assay for detecting fusion gene ctRNAs from clinical plasma samples with high detection sensitivity and specificity. The assay principle is schematically outlined in Figure 4.1 and described in detail in experimental section. Target RNAs were hybridized with complementary biotinylated probes. Target-probe hybrids were captured through streptavidin coated magnetic beads followed by magnetic isolation and heat release of captured ctRNAs. The released FGFR2:FAM76A ctRNA were allowed to adsorb onto the GO-NPFe2O3 modified screen-printed carbon electrode (SPCE/GO-NPFe2O3) via RNA-graphene affinity interaction. This adsorption process follows conventional physisorption mechanism and involves the direct interaction of nucleotides with graphene oxide surface through van der Waal (vdW) forces [14d, 23], where the interaction between the individual nucleobases and the graphene (i.e., adsorption on graphene) is controlled by the polarizabilities of the nucleobases [23-24]. In order to quantify the amount of adsorbed FGFR2:FAM76A ctRNA, GO-NPFe2O3 modified electrode surface was interrogated in chronocoulometric analyses with [Ru(NH3)6]3+. The [Ru(NH3)6]3+ acts as a promising indicator for measuring the amount of nucleic acid (e.g., DNA, RNA) on the electrode surface due to its electrostatic affinity for the phosphate groups of the RNA backbone. The further enhancement of surface bound [Ru(NH3)6]3+ was achieved by coupling [Ru(NH3)6]3+ with [Fe(CN)6]3- redox system [25]. The electrochemically reduced ([Ru(NH3)6]3+ to [Ru(NH3)6]2+) Ru2+ was re-oxidized
by solution phase \([\text{Fe(CN)}_6^{3-}]\). This cycling triggers multiple turnovers of \([\text{Ru(NH}_3)_6^{3+}\] resulting in significant signal amplification. Thus, the amount of CC charge generated by the redox couple comprising \([\text{Ru(NH}_3)_6^{3+}\] and \([\text{Fe(CN)}_6^{3-}\] system should have a clear correlation with the concentration of ctRNA. As schematically shown in Figure 4.1, target ctRNA absorbed on modified electrode in the presence of electrocatalytic cycle generates higher CC charge (i.e. charge density/mCm\(^2\)).

Figure 4.1. Assay principle for quantification of ctRNA. Total cell-free RNA was extracted from plasma samples using a commercially available kit. Target fusion gene ctRNA was separated and purified magnetically from the bulk of RNA and adsorbed directly on the GO-NPFe\(_2\)O\(_3\) modified screen-printed carbon electrode (SPCE). A significant electrocatalytic signal amplification was attained via the chronocoulometric (CC) charge interrogation of target ctRNA-bound \([\text{Ru(NH}_3)_6^{3+}\]/[\text{Fe(CN)}_6^{3-}\] electrocatalytic assay system. Inset: typical electrocatalytic cycle showing the electrostatically attached target ctRNA that generates higher CC charge in comparison to without electrocatalytic cycle.
4.3.2 Effect of Electrode Surface Modification

To evaluate the effect of nanoparticles, we investigated our assay with the same amount of FGFR2:FAM76A ctRNA (10 pM) using GO-NPFe$_2$O$_3$-modified and unmodified SPCE (bare electrode). As can be seen in Figure 4.2 and Figure 4.S2, negligible amount of total charge density (Q/mCcm$^{-2}$) was obtained from bare SPCE (SPCE/Bare). This data indicates that there was very insignificant adsorption of target ctRNAs as well as Ru$^{3+}$ onto the bare SPCE electrode. The negligible amount of charge observed in our experiment was probably due to the non-faradic component of the response at the electrode. In a control experiment (Control; SPCE/NPs/ PBS), almost 3-times higher total charge was obtained at the GO-NPFe$_2$O$_3$-modified SPCE electrode (6.0 versus 2.2 mCcm$^{-2}$) (Figure 4.S2). The relatively larger Q at the GO-NPFe$_2$O$_3$-modified SPCE can be explained by the fact that the presence of GO-NPFe$_2$O$_3$ on the surface of the SPCE could facilitate the adsorption of some redox molecules ([Ru(NH$_3$)$_6$]$^{3+}$) that could be electrochemically reduced at the GO-NPFe$_2$O$_3$/SPCE electrode [14a]. After the adsorption of target FGFR2:FAM76A ctRNA onto the GO-NPFe$_2$O$_3$-modified SPCE, we performed our assay in absence (ctRNA/ GO-NPFe$_2$O$_3$/SPCE was not incubated with [Ru(NH$_3$)$_6$]$^{3+}$) and presence (ctRNA/GONPFe$_2$O$_3$/SPCE was incubated with [Ru(NH$_3$)$_6$]$^{3+}$) of [Ru(NH$_3$)$_6$]$^{3+}$. In the absence of [Ru(NH$_3$)$_6$]$^{3+}$ (referred as Qdl, e. g., SPCE/NP/mRNA), 10 pM of target ctRNA resulted an approximately 5.2 mCcm$^{-2}$ of charge. In the presence of [Ru(NH$_3$)$_6$]$^{3+}$ this value was calculated to be 16.2 mCcm$^{-2}$. This could be explained by the fact that, in presence of [Ru(NH$_3$)$_6$]$^{3+}$, a stoichiometric amount of Ru$^{3+}$ was bound with the phosphate backbone of surface attached ctRNA and thus an equivalent amount of CC charge was generated.
Figure 4. 2. Specificity of the Assay. Charge density data for SPCE/Bare (without NP modification), control (i.e., NP modified electrode without RNA), Q_{dl} - the non-Faradic charge in the absence of RuHex, non-complementary FAM-134B, target mRNA (ctRNA) Inset; corresponding CC curves (Q vs. t_{1/2}). Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (%RSD= <5%, for n=3).

4.3.3 Specificity of the Assay

To assess the assay specificity and efficiency of capture probe for isolating FGFR2:FAM76A ctRNA, we also performed our assay using a noncomplementary FAM-134B mRNA (i.e., SPCE/NP/FAM-134B mRNA). As can be seen in Figures 4.2 and 4. S2, a very small increase in the charge density was obtained for FAM-134B mRNA when compared to the control data (e.g. 6.6 versus 6.0 mCcm\(^{-2}\) Figure 4.S2), indicating that our assay is not significantly affected by non-specific mRNAs binding present in the samples. Notably, 10 pM of target ctRNA generated
approximately three-times higher charge than that of noncomplementary FAM-134B (16.2 versus 6.6 mCcm$^{-2}$).

These data clearly demonstrated the high specificity of our assay in isolating and subsequent electrochemical detection of fusion genes. Most of the conventional biosensors involve hybridization of target to the complementary probes bound to the surface of electrodes.

However, interference from non-specific molecules is one the major shortcomings of such a two-dimensional capture approach. In comparison, our assay relies on selective capture of target RNA through complementary probes followed by magnetic bead-based isolation. Intimate mixing of target-probe hybrids with magnetic beads enhances the efficiency of capture. On the other hand, washing, and purification steps avoid matrix effect of complex biological samples and thus reduce non-specific interferences. While the ability to discriminate between fully-matched and uncomplimentary sequence was investigated using our assay, other studies have presented even very sophisticated methods for specificity evaluation. More recently, some high-performance biosensors with ability to distinct fully complementary hybridization and mismatch hybridization (as few as a single base-pair) between probe and target DNA have been developed. For example, Kumari et al. [18c] demonstrated an electrochemical affinity DNA biosensor that is able to detect one, two, and three base pair mismatches. The method is based on current changes which are caused by the hybridisation between ideal capture and target DNA, leading to selective detection of target DNA. Moreover, several other methods such as quantum dots-based fluorescence resonance energy transfer technique [18d], and nanoparticle based colorimetric approach [18e], have been used for single-base mismatch DNA detection.
4.3.4 Effect of Electrocatalytic Signal Amplification

To enhance the sensitivity of our assay, we coupled the [Ru(NH3)6]3+ redox system with another redox system, [Fe(CN)6]3−, and measured the amount of CC charges corresponding to the surface bound ctRNA. [Ru(NH3)6]3+ / [Fe(CN)6]3− is a widely used redox system to achieve sensitive electrochemical detection of biomolecules [18b,26]. In this system, [Ru(NH3)6]3+ acts as an electron acceptor and is thereby reduced at the electrode surface under the applied reduction potential. The [Fe(CN)6]3− present in the bulk solution re-oxidizes (as [Fe(CN)6]3− acts as a stronger oxidant in the system) the [Ru(NH3)6]2+ back to the [Ru(NH3)6]3+, resulting in the generation of enhanced electrocatalytic signal [22]. As can be seen in Figure 4.2, in the presence of [Ru(NH3)6]3+ / [Fe(CN)6]3− system the control (SPCE/NP/Buffer) electrode generated higher amount of charge compared to that of without electrocatalytic system (20.0 versus 6.0 mCcm−2). The noncomplementary FAM-134B mRNA and Qdl generated almost similar charge responses to that of the control. For target ctRNA, approximately four-time higher charge density was obtained under electro-catalytic system (63.0 versus 16.2 mCcm−2) than what was observed in the absence of [Fe(CN)6]3−. It is also worth mentioning that a significant enhancement in CC response was found with the electrocatalytic system (control vs target mRNA, 20.0 versus 63 mCcm−2 in Figure 4.2) compared to that without electrocatalytic cycle (control vs target mRNA, 6.0 versus 16.2 mCcm−2 in Figure 4.S2). This high signal response of the target mRNA compared to controls and non-specific mRNA clearly demonstrates the enhanced functionality and specificity of our assay.

4.3.5 Sensitivity of the Assay

To test the sensitivity and reproducibility of our assay, various concentrations of magnetically isolated and purified synthetic target RNA ranging from 1.0 fM to 1.0 nM were adsorbed onto the GO-NPFe2O3-modified electrode surface (Figure 4.3). It is important to mention that, these concentrations are not the total RNA concentrations of the original samples. They
belong to the extracted ctRNA of the original samples that have been diluted. In fact, ctRNA is a very small portion of the total RNA that can be identified using designed probes.

As shown in Figure 4.3A, the CC charge generated by $[\text{Ru(NH}_3)_6]^{3+}/[\text{Fe(CN)}_6]^{3-}$ electrocatalytic cycle increases with increasing RNA concentration. In comparison, negligible signal was observed in the No-Target (NoT) control experiment. The linear regression equation was calculated to be $y$ (charge density, mC/cm$^2$) = 8.047 (amount of RNA) + 0.1134, with a correlation coefficient (R2) of 0.97154 (Figure 4.3B). The limit of detection (LOD) was estimated to be 1 fM which is clearly distinguishable from that of control electrode. The LOD is determined by the analysis of samples with defined concentrations of ctRNA and by establishing the minimum amount, where the ctRNA can be identified. To report a reliable LOD each data point represents the average of three independent trials, and error bars show the standard deviation of measurements. We could not get result with a concentrations lower than the reported LOD.

Without the catalytic cycle step (i.e., using only $[\text{Ru(NH}_3)_6]^{3+}$, we obtained 100 times less sensitive LOD (1 fM versus 100 fM) (Figure 4.S3A). For the system without the catalytic cycle step, the linear regression equation was estimated to be $y$ (charge density, mC/cm$^2$) = 4.147 (amount of RNA) + 1.0106, with a correlation coefficient (R2) of 0.99773 (S3B). The high sensitivity of our assay may be attributed to the large exposed surface area of graphene oxide within the GO-NPFe$_2$O$_3$ nanoparticles, which leads to a relatively larger amount of RNA being adsorbed onto the GO-NPFe$_2$O$_3$ - modified SPCE via the RNA-graphene oxide affinity interaction. This also allows a relatively larger amount of $[\text{Ru(NH}_3)_6]^{3+}$ ions to be electrostatically bound to surface confined RNA. Moreover, GO-NPFe$_2$O$_3$ nanoparticles can increase electrocatalytic signal of Ru$^{3+}$/Ru$^{2+}$ process. The LOD of our method is higher than some of the earlier electrochemical fusion gene assays [17b, 27]. Although some other
studies have reported similar LODs (Table 4.S2) the usage of these methods is hindered mainly because they involve enzyme-based amplification [28] and complicated surface functionalization steps (the extra steps involved in the immobilization and hybridization of DNA probes on electrodes for capturing target DNA) [29], or both of them [30]. It is also important to note that only a few electrochemical methods can offer relatively higher sensitivity [31]. However, clinical application of these methods may also be restricted due to the complexities in sensor fabrication steps. In contrast to these, our assay is relatively simple and does not require any immobilization of probe on the electrode surface and thus simplifies the detection method by avoiding the complex chemistry underlying each step of the sensor fabrication.

Figure 4. 3. (A). Typical CC curves (Q vs. t^{1/2}) for the 1.0 fM–1.0 nM of synthetic ctRNA (with [Ru(NH₃)₆]^{3+}/[Fe(CN)₆]^{3-} electrocatalytic assay system) . (B) Corresponding calibration plot. Q_{ctRNA} (corresponding charge of target ctRNA bound to surface bound RuHex) = Q-Q_{dl}. The concentration of RuHex and ferricyanide is 50 µM and 4.0 mM respectively. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (%RSD= <5%, for n=3).
4.3.6 FGFR2:FAM76A ctRNA Detection in Clinical Plasma Samples

To assess the applicability of our assay in analysing complex clinical samples, we used this amplification-free electrochemical method for the analysis of FGFR2:FAM76A ctRNA levels in total RNA extracted from the clinical plasma samples of 5 HG-SC (P1- P5), and 5 benign patients for ovarian cancer as controls (B1–B5). Our assay detected all the cancer samples to be FGFR2:FAM76A positive, and CC signal generated by our assay could distinguish different FGFR2:FAM76A levels in clinical samples as depicted in Figure 4.4A. The FGFR2:FAM76A-positive samples showed higher CC charge density as compared to benign samples, which indicates upregulation of FGFR2:FAM76A in ovarian cancer patients. This clinical data represents a very good reproducibility of our assay (relative standard deviation, %RSD= <5%, for n=3) for analyzing differential expression pattern of FGFR2:FAM76A genes in ovarian cancer sample. We also validated the results of our electrochemical analysis by determining expression of FGFR2:FAM76A fusion gene in a subset of clinical samples through standard RT-qPCR method. Fusion gene expression was normalized against the expression of GAPDH and data is presented as Dcq. As can be seen in Figure 4.4B, qPCR results show a trend similar to those of electrochemical analysis, demonstrating the accuracy and potential of our assay.

Furthermore, results of our assay were also consistent with published reports describing the presence of FGFR2:FAM76A in ovarian cancer patients [7]. The clinical significance of our assay is broad, as it is useful for detecting different gene fusions in RNA/DNA by changing the capture probe sequences, which will pave the way for comprehensive diagnosis and personalized treatment. The assay is a portable, cost affordable, non-enzymatic and amplification-free alternative to current amplification-based approaches for fusion gene detection. This method avoids PCR, which is prone to sequence specific
amplification biases and requires sample pre-processing, thus significantly increasing cost and time of the analysis.

Our method removes complicated cleaning and sensor fabrication, tedious experimental protocols, and costly fluorescence readout instruments. In addition, our method uses commercially available and disposable SPCE electrodes, which are cheap and avoid the utilization of typical electrochemical cells, counter and reference electrodes, thereby providing a relatively simple platform with highly reproducible results. Most importantly, the method uses GO-NPFe$_2$O$_3$ nanoparticles to modify electrode. Due to the synergistic effects of Fe3O4 nanoparticles and graphene with a large surface area and excellent electron transfer ability, the obtained nanocomposite significantly improves the sensing behaviour for RNA detection. Owing to the sensitivity, simplicity, inexpensive and portable nature of our method, the proposed approach is ideal for developing a clinically friendly FGFR2:FAM76A assay.
Figure 4. (A). Analysis of patient samples. Corresponding QRNA obtained for five benign ovarian cancer patients (B1–B5) and five patients of high-grade serous subtype ovarian cancer (P1–P5) (B). RT-qPCR ctRNA expression profile in plasma samples. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (%RSD= <5%, for n=3).

4.4. Conclusions

We have presented the first amplification-free and enzyme-free electrochemical assay for the detection of FGFR2:FAM76A ctNAs using a new class of iron oxide nanoparticles. The assay demonstrates a high sensitivity due to (i) high surface area and catalytic activity of GONPFe$_2$O$_3$ nanoparticles and (ii) the use of the [Ru(NH$_3$)$_6$]$^{3+}$/[Fe(CN)$_6$]$^{3-}$ electrocatalytic cycle for signal amplification. Moreover, this method involves a good level of specificity due to functionalized magnetic dynabeads based target capture. We successfully examined the assay performance in clinical samples obtained from benign and high-grade ovarian cancer.
patients, with good reproducibility (%RSD= <5%, for n=3). We envisage that due to its flexibility, our method may find broad application in ctNA based fusion gene detection in a variety of cancers.

4.5. Supplementary materials

4.5.S.1. Supplementary tables

Table 4.S.1. List of the oligonucleotide sequences applied in this assay.

<table>
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<tr>
<th>Target genes and primers</th>
<th>Oligonucleotide sequences (5′–3′)</th>
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<tr>
<td>Synthetic FGFR2-FAM76A Fusion sequence</td>
<td>5′-U-GUA-AAA-CAA-ACA-UAU-GUU-CCU-UUC-CUUUCA-3′</td>
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<tr>
<td>Biotinylated capture probes of FGFR2-FAM76A Fusion</td>
<td>5′-TGA-AAG-GAA-AGG-AAC-ATA-TGT-TTG-TTT-TACA-3′-Biotin</td>
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<tr>
<td>FGFR2- FAM76A Fusion forward</td>
<td>5′-GGATAAAGGAAGAGATTGCAC-3′</td>
</tr>
<tr>
<td>FGFR2- FAM76A fusion reverse</td>
<td>5′-TGTGGGAGTTAAGTAAGAACT-3′</td>
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</table>

Table 4.S.2. Some available methodologies for different fusion genes detection.

<table>
<thead>
<tr>
<th>Method</th>
<th>Fusion gene</th>
<th>LOD</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan encapsulated quantum dots platform</td>
<td>BCR-ABL</td>
<td>2.56 pM</td>
<td>27a</td>
</tr>
<tr>
<td>Quantum dots self-assembly platform</td>
<td>BCR-ABL</td>
<td>1.0 pM</td>
<td>27b</td>
</tr>
<tr>
<td>Graphene sheets, polyaniline and AuNPs based DNA sensor</td>
<td>BCR-ABL</td>
<td>2.11 pM</td>
<td>32</td>
</tr>
<tr>
<td>Polymerase assisted multiplication</td>
<td>BCR-ABL</td>
<td>2 fM</td>
<td>28</td>
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</tbody>
</table>
coupling with quantum dot tagging

<table>
<thead>
<tr>
<th>Indicator-free DNA hybridization biosensor with a graphene-based nanocomposite</th>
<th>BCR-ABL</th>
<th>2.6 fM</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlled deposition of functionalized silica coated zinc oxide nano-assemblies at the air/water interface</td>
<td>CML</td>
<td>0.1 fM</td>
<td>31a</td>
</tr>
<tr>
<td>DNA biosensor based on aldehyde-agarose hydrogel modified glassy carbon electrode</td>
<td>BCR-ABL</td>
<td>4.0 pM</td>
<td>31b</td>
</tr>
<tr>
<td>A sandwich-type electrochemical biosensor using locked nucleic acids on gold electrode</td>
<td>CML</td>
<td>10 fM</td>
<td>30</td>
</tr>
</tbody>
</table>

4.5.S2. Supplementary figures

Figure 4.S.1. Schematic representation of the electrode preparation. a) The magnet and screen-printed carbon electrode (SPCE); b) SPCE centered and attached onto the magnet; and c) the GONPFe2O3 NPs were attached magnetically onto the SCE surface throughout the whole experiment
Figure 4.5.2. Specificity of the Assay. Corresponding charge density data without electrocatalytic cycle (in the presence of only [Ru(NH3)6]3+) for the SPCE/Bare (without NP modification), control (i.e., NP modified electrode without RNA), Qdl- the non-Faradic charge in the absence of RuHex; non-complementary FAM-134B, and target mRNA (ctRNA). Inset: corresponding CC curves (Q vs. t1/2). Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for n = 3).
Figure 4.S.3. (A). Typical CC curves (Q vs. $t_1/2$) for the 100 fM-1.0 nM of synthetic ctRNA (mRNA) without electrocatalytic cycle. (B) corresponding calibration plot. $Q_{ctRNA}$ (corresponding charge of target ctRNA bound to surface bound RuHex) = $Q - Q_{dl}$. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD == <5%, for n = 3).
4.5. References


Statement of contribution to co-authored published paper

This chapter is in a form of a co-authored published paper. The bibliographic details of the co-authored paper, including all authors, are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

My contribution to the published paper involved:

- Literature review
- Experimental set-up
- Manuscript preparation
- Responding reviewers

(Sign) [Signature]

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(Date) 18/3/2020

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(Sign) [Signature]

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(Date) 18/3/2020
Chapter 5: Microfluidic Array Chip for Parallel Detection of Waterborne Bacteria

Abstract

The polymerase chain reaction (PCR) is a robust technique used to make multiple copies of a segment of DNA. However, the available PCR platforms require elaborate and time-consuming operations or costly instruments, hindering their application. Herein, we introduce a sandwiched glass–polydimethylsiloxane (PDMS)–glass microchip containing an array of reactors for the real-time PCR-based detection of multiple waterborne bacteria. The PCR solution was loaded into the array of reactors in a single step utilising capillary filling, eliminating the need for pumps, valves, and liquid handling instruments. Issues of generating and trapping bubbles during the loading chip step were addressed by creating smooth internal reactor surfaces. Triton X-100 was used to enhance PCR compatibility in the chip by minimising the nonspecific adsorption of enzymes. A custom-made real-time PCR instrument was also fabricated to provide thermal cycling to the array chip. The microfluidic device was successfully demonstrated for microbial faecal source tracking (MST) in water.

This chapter has been published as:
5.1. Introduction

The faecal pollution of water is one of the major threats to human health. Understanding the origin of faecal pollution is necessary for identifying public health risks, devising effective management, and preventing further pollution [1]. To date, water resource managers responsible for monitoring microbiological water quality have a limited number of conventional tools available. Cultivation-based approaches for standard faecal indicator bacteria enumeration (SFIB) [2] are time-consuming and not informative about pollution sources.

To overcome the drawbacks of the traditional SFIB method, molecular microbial source tracking (MST) approaches have been developed over the last few years and can link faecal pollution to its origin [3,4]. The polymerase chain reaction (PCR) and quantitative PCR (qPCR) have been widely used as tools for specific nucleic acid-based tests [5,6], particularly for the detection and quantification of source-associated genetic markers in water samples [7]. Over the years, multiplex PCRs and microarrays have been introduced as the two powerful high-throughput genomic technologies [8]. Simultaneous detections of multiple waterborne bacterial pathogens have been reported employing a multiplex PCR system [9]. However, multiplex PCRs requires accurate primer designs and an optimal reaction mixture to avoid the probability of primer dimer formation that may result in the preferential amplification of certain targets [10]. Microarrays are another alternative method for analysing the pathogen content in wastewater. Microarrays provide quick detection of multiple target genes of multiple organisms simultaneously due to its ability of screening a large number of sequences [11]. Even though microarrays have been broadly used for simultaneous detection, there are still limitations hindering their applications such as a low sensitivity and variability of the result as compared to those of real-time PCRs [12].
Platforms capable of integrating PCRs with microarray techniques have been introduced as a probable solution to alleviate the existing difficulties in the application of microarrays. Some of these systems integrate solid-phase PCRs with microarrays. However, recent studies have indicated that the solid-phase PCR efficiency is less than that of the solution-phase PCR [13].

Recently, loop-mediated isothermal amplification (LAMP) method has also attracted a considerable attention by providing an analytical solution to some of the drawbacks associated with existing amplification approaches. Compared to traditional PCR/qPCR, LAMP is able to amplify DNA with high specificity and rapidity under isothermal conditions. Despite simplicity in operation and reduced thermal budget, this method faces to several challenges, limiting its wide application. For instance, nonspecific amplification, and variable sensitivity based on the provided temperature are two main concerns that need to be addressed [14]. Given all the advantages and disadvantages of isothermal PCR, quantitative PCR (qPCR) is still a gold standard for the quantification of DNA/RNA and is still considered as the method of choice in molecular biology. The method relies on real-time monitoring of amplification of the nucleic acid allowing its quantification. The change in fluorescence intensity over the amplification procedure is measured by a real-time PCR thermal cycler. Commercially available real-time PCR instruments integrate temperature cycling and an optical unit [15].

Recent advances in microfluidics have enabled the development of new cost-effective array devices for the multiplex detection of contaminants. The main advantages of microfluidics-based array platforms are the lower cost of purchase and operation, fast analysis, high sensitivity, and minimum infrastructure requirements [16]. However, there are still some bottlenecks in the design and operation of these systems. Most PCR-based microfluidic devices need to load the PCR mixture into reaction wells using costly liquid-
dispensing robots or pneumatic pressure sources, or the immobilization of primers in a solid matrix [17–19].

The development of manual liquid loading methods without handling robots or external pumps is challenging for microfluidic arrays. The manual liquid loading process comprises nucleic acid sample loading into the reaction wells, followed by the isolation of these wells to prevent cross-contamination. Recently, a few research teams have successfully used micropumps in conjunction with an array of valves for performing PCRs on microfluidic chips. Liu et al. [19] developed a microfluidic device that integrates thousands of hydraulic valves and pneumatic pumps, allowing the distribution of 2 µL of PCR mixture among 400 independent reactors. The implementation of valves and pumps lead to complex fabrication and operation processes and may also increase the size of the chip. The sealing of a microfluidic array is another significant challenge that needs to be addressed. Based on the materials that are employed in chip fabrication, sealing methods require specific equipment and protocols. Polydimethylsiloxane (PDMS), pressure-sensitive adhesive foil, adhesive sealing film, and ultraviolet (UV) adhesive are the most commonly used materials for sealing devices for the PCR [7]. In addition, a microPCR array chip with open reactors has also been reported [20,21].

In this context, we present a simple microfluidic PCR array. We used a glass–PDMS–glass configuration to reduce water loss during PCR thermal cycling. The first novelty is the well-designed closed microreactors that restrict bubble generation during PCR, which is one of the main factors affecting amplification efficiency. Channel dimension, rounded corners and a smooth internal surface were key elements in fabricating bubble-free microreactors. By the robust surface modification of the PDMS, the absorption of proteins/nucleic acids on the surface of this material was limited. The loading process only required two pipetting steps to introduce the sample and oil. The other novelty of our platform is the confinement of the
sample inside the chamber employing mineral oil and sealant. Sealant was used to enclose sample loading/air venting ports to prevent liquid movement and evaporation. The dimensions of the sealant were 5 cm length, 1 cm width, and 1 mm height. Using this technique, we could perform amplification and detection steps simultaneously in approximately the same position, thus decreasing the final sample volume and increasing the signal-to-noise ratio. There was no need for additional components or external equipment for the operation. Although some commercial vendors such as ChipShop in Germany are providing microfluidic devices for PCR, the high cost of the products often limits the usage of this technology. However, we were able to offer a relatively inexpensive (∼AUD $10 per chip) platform for PCR. Furthermore, in contrast to commercial chips, our fabricated array reactors were preloaded with primer pairs which make the microdevice ready to use. Finally, the capability of the array chip was verified by the simultaneous detection of three human-associated MST markers: *Escherichia coli* (*E.coli*) (H8), human-specific *bacteroidals* (*Gen bac III*), *E.coli* (UidA).

**5.2. Materials and Methods**

**5.2.1. Chip Design**

Figure 5.1 shows the proposed microfluidic PCR chip. The device was designed as an array for the parallel detection of three human wastewater-associated MST markers. The array chip, including microreactors, sample loading ports and waste channels, inlet and outlet bridge channels, and air venting ports, was made of polydimethylsiloxane (PDMS) and glass. A non-ionic surfactant, Triton X-100 (TX-100) (Sigma Aldrich, St. Louis, MO, USA), was used as a surface modifier. The surfactant provides a great reduction in contact angle and prevents the absorption of macromolecules onto the PDMS surface [22]. Contact angle measurements and UV-Vis studies were carried out to evaluate the effect of TX-100 on the
wettability changes of the pure PDMS (Figures 5.S1 and 5.S2). Over the chip fabrication step, different microreactors were loaded with dry primer pairs for the simultaneous detection of targeted MST markers.

The loading channel was employed for distributing the PCR mixture containing DNA templates into the array of microreactors. Loading the PCR mixture into microreactors purges out the air inside the device through the air venting ports, facilitating liquid flow. After filling the microreactors, the extra liquid samples inside the microreactors were isolated from each other. The inlet and air venting ports were sealed with a sterile mineral oil and a commercially available sealant to avoid sample movement and evaporation during the PCR thermal cycling process. The smooth internal surface of the microreactors restricts the generation and trapping of air bubbles during sample loading and PCR thermal cycling. Scanning electron microscopy (SEM) results are shown in Figure 5.S3.

The existence of air bubbles inside the microreactors pushes the liquid out due to bubble expansion. Furthermore, air bubbles also have a negative effect on PCR efficiency by creating various temperature zones in microreactors. Figure 5.1B shows the image of the microfluidic array chip, including an array of seven microreactors for the PCR. The chip contains three microreactors connected to a common loading channel for the target sample (positive control reaction; PC), three microreactors connected to another common loading channel for a no-template control reaction in the presence of a wrong target (NTC1), and a single microreactor for a no-template control reaction with water (NTC2). Typical dimensions of the array chip are as follows. The bridging channels are 10 mm long, 300 µm wide, and 500 µm high. The microreactors are 10 mm long, 1 mm wide, and 500 µm high, resulting in a volume of 5 µL. Reactors 1–3 were pre-loaded with dried primer pairs, which are specific for EC H8, Gen bac III, and UidA, respectively. If a PCR mixture containing a bulk of standard DNA templates (for E.coli H8, Gen bac III, and UidA) was loaded into the
array, the mixture would fill the microreactors. During the amplification process, the primer pairs specifically hybridised to their desired targets. Reactors 4–6 were also pre-loaded with dried primer pairs (similar to reactors 1–3), to perform the NTC1 for the corresponding reactions. For NTC1, the PCR mixture without the standard DNA template, which contains a DNA template from *Staphylococcus aureus*, was loaded into the NTC1 loading port. Reactor 7 was also pre-loaded with one dried set of primer pairs to perform the NTC2. For NTC2, the PCR mixture containing just water was loaded into the array to check the possible contamination in that.
5.2.2. Chip Fabrication

The microfluidic array chip described above is a glass–PDMS–glass sandwich configuration. Figure 5.1A schematically illustrates the fabrication process of the array chip. AutoCAD (Autodesk Inc., San Rafael, CA, USA) was used to design the mould for making the PDMS layer. The PMMA module was prepared by the micro-milling method (LPKF Laster& Electronics Proto Mat S 43 at Nanjing University of Science and Technology, Nanjing, China). After the completion of the module, a PDMS pre-polymer (Dow Corning Sylgard 184, Dow Corning, Midland, MI, USA) and a cross-linker were mixed in a 10:1 ratio by weight [23]. Subsequently, Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) at a 0.5% weight percentage was added to liquid PDMS [22]. Later, the mixture was degassed in vacuum for around 20 min. Subsequently, the mixture was poured over the mould with a thickness of 1 mm and degassed in vacuum for 10 min and cured at 67 °C for 3 h. After being completely cured, the PDMS was peeled off the mould, and the venting ports were punched. The resulting PDMS layer was washed with acetone, isopropanol, and milliQ water and dried at 67 °C for 30 min. Another PDMS mixture with a pre-polymer/cross-linker ratio of 10:1 by weight was spread on two acetone-washed glass slides and then bonded to the PDMS layer. Finally, the device was cured at 67 °C for 48 h to enhance the bond between the PDMS part and the glass layers. The mixtures of forward and reverse primers for each marker were loaded to the preferred microreactor by pipetting through the air venting ports. The primers were then dried by annealing the chip at 67 °C for 10 min.
5.2.3. Chip Operation

Figure 5.2 shows the basic operation procedure of the chip. The hydrophobic nature of PDMS avoids the liquid solutions flowing easily into the microreactors through capillary filling. The addition of 0.5% of Triton X-100 into PDMS resolved this problem. The resulting reduction in contact angle allows the PCR mixture to flow into the reactors and channels by capillary action [22]. The loading process does not need an external pump. After a single step of manual pipetting of the PCR solution into the loading port, the capillary force completed the fluidic operation process, and the extra PCR mixture was collected in the outlet pool. The sealing performance of the chip was checked using food colouring. Figure 5.2 shows that the reactors were well sealed and isolated from each other: POC (blue), NTC1 (red), and NTC2 (green).

The smooth inner surfaces of the microreactors prevented bubbles from forming and being trapped during sample loading. Trapped bubbles are one of the key challenges in designing a PCR chip [24]. This smoothness was the result of the microreactor design with curved corners and the use of liquid PDMS prepolymer as an adhesive material to bond the cured PDMS layer and glasses. The absence of bubbles during the loading step was clearly confirmed by a fluorescent dye test, Figure 5.2B.
Figure 5.2. Liquid sample loading and isolation of the reactors. (A) Test with food colouring; (B) test with fluorescent dye.

After filling the microreactors, sterile mineral oil was added into the input and gas venting ports to hold the sample in microchambers and to avoid their movements during thermal cycling. The oil also reduced the evaporative loss of the sample. Finally, a commercially available sealant was used to completely seal the ports. Figure 5.3 schematically outlines the operating procedure of the microdevice.
5.2.4. Real-Time Polymerase Chain Reaction (PCR) Instrument

A customised thermal cycler was developed to run the PCR thermal cycles on the proposed microfluidic chip. A $5 \times 5 \times 2 \text{ cm}^3$ aluminium block embedded with a 60 W cartridge heater (Core electronics, Kotara, Australia) was used as the thermal cycling platform. A LM35 temperature sensor (Core electronics, Kotara, Australia) was attached to the aluminium block using heat-conductive glue to monitor the temperature. The thermal cycling platform was mounted on a Peltier thermoelectric cooler (TEC-12706, Aus electronics, Chipping Norton, Australia), which was, in turn, attached to a heat sink-cooling fan assembly, as shown in Figure 5.4. The temperature of the platform was controlled by a proportional-integral-derivative (PID) algorithm implemented in an Arduino UNO microcontroller board. The thermal cycler was programmed to run the following temperature cycles: Initial denaturation at 95 °C for 45 s followed by 35 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The thermal ramping rate of the custom-made thermal cycler was 0.20 K/s during heating and 0.26 K/s during cooling. The temperature
difference between the aluminium block and the sample inside the chip was measured using a pre-calibrated negative-temperature-coefficient thermistor (Figure 5. S4A, B).

Figure 5. 4. Experimental setup of the PCR thermal cycling platform.

5.2.5. Bacterial Samples and PCR Experiment

Thirty millilitres of faecal slurry from the mixed human samples was spun at 4 °C for ten minutes at 5000 rpm to concentrate the sample. The total DNA was extracted from the pellet after mixing with 10 parts ASL buffer using the QIAamp DNA stool mini kit according to the instructions of the manufacturer (QIAGEN, Victoria, Australia). The resulting DNA extracts were stored at −20 °C until actual use. The resulting DNA was used as the template DNA to synthesise the standard DNA for desired gene sequences. First, the concentration of DNA was estimated by a nanodrop instrument (BioLab, Ipswich, MA, USA) and diluted to reach a concentration of around 100 ng/µL. Next, one PCR was run for each set of primers in the presence of the template DNA. The sequences of forward and reverse primers are listed in
Table 5.1. The PCR solution (20 µL) contained 2.5 µL forward primer, 2.5 µL reverse primer, 2.5 µL DI water, 2.5 µL template DNA, and 10 µL Gotaq® green master mix (Promega, Madison, WI, USA). The mixture was placed in a conventional PCR instrument (Biorad CFX Connect, BioRad, Hercules, CA, USA) with the thermal cycling condition of initial denaturation at 95 °C for 45 s followed by 35 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The resulting amplicons, which were synthetic DNA for the desired gene, were then ready to be used for on-chip amplification. Three synthetic DNA samples were mixed together to prepare a usable template DNA for on-chip amplification. The bulk of DNA plus water and SsoFast EvaGreen supermixes (Bio-Rad, USA) was then added to the chip, which had the primer pairs immobilised in all reactors beforehand. Next, the chip was placed on top of a custom-made thermal cycler to tolerate the thermal cycling condition. The results were verified via both fluorescence measurement and agarose gel (1.5%) electrophoresis followed by exposure under a UV transilluminator (Bio-Rad).

<table>
<thead>
<tr>
<th>Target Organism</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>PCR Product Size (bp)</th>
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<td>GenbacIII-F</td>
<td>GGGGTTCCTGAGAGGAAGGT</td>
<td>129</td>
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<tr>
<td></td>
<td>GenbacIII-R</td>
<td>CCGTCATCCTTCACGCTACT</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>H8-F</td>
<td>ACAGTCAGCGAGATTCTTC</td>
<td>177</td>
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<td>(E. coli)</td>
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<td>E. coli</td>
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<tr>
<td></td>
<td>UidA-R</td>
<td>GAGCGTCGCAGAACATTACATT</td>
<td></td>
</tr>
</tbody>
</table>

5.3. Results
5.3.1. Simultaneous Detection of Microbial Faecal Source Tracking (MST) Markers

Using Microfluidic Device

The two bacteria of *Escherichia coli (E. coli)* and *Bacteroidales* were selected to demonstrate the function of the chip. *E. coli* and *Bacteroidales* are gram-negative bacteria
flourished in the intestine with properties that both positively and negatively affect the host [25]. Infection with some species of this bacteria, either untreated or with delayed treatment, can lead to infections in the host [26,27]. The high level of *E. coli* and *bacteroidales* in the gut of humans and animals has made them two of the most important faecal indicator bacteria in microbial source tracking (MST) assays in waters. We selected two target genes for *E. coli* (UidA, H8) and one target gene for *Bacteroidales* (Gen bac III) to demonstrate the function of the chip. The performance of the chip was examined by detecting sequences of Gen bac III, UidA, and H8 in a bulk of standard DNA extracted from *Bacteroidales* and *E. coli*. The microdevice was loaded with PCR solution containing a predefined concentration of DNA templates. The temperature of the chip was controlled by the custom-built portable and programmable PCR apparatus described in the previous section. The numerical values of fluorescent intensities of the amplicons at various time intervals were captured by a camera and evaluated using ImageJ (National Institutes of Health, Bethesda, MD, USA) [28], an open-source software. Next, the fluorescent intensity values of the samples inside the microchambers of the device were normalised. Normalisation of the fluorescent intensities was achieved by the following formula [29]:

\[
I^*_{st} = (I_{st} - I_{s0})/I_{max},
\]

where *I*<sub>st</sub> is the fluorescent intensity of the sample in each microchamber measured at a given time, *I*<sub>s0</sub> is the fluorescent intensity of that sample at the start of thermal cycling, and *I*<sub>max</sub> is the maximum fluorescent intensity measured among all the samples.

Figure 5.5 presents the amplification curve from microreactors on the device. The normalised fluorescent intensity increases with increasing cycle number in all microchambers containing positive controls (PC). The increase in fluorescent intensity of the PCR solution indicates a positive polymerase chain reaction within the microreactors. The microreactors containing negative controls of the PCR mixture, for the wrong target (NTC1) and for water
(NTC2), show no significant fluorescence over thermal cycling. This demonstrates the specificity of the PCR inside the microreactors. Maximum fluorescence was observed in the microreactor containing primer pairs for detecting the Gen bacIII gene. The reason can be attributed to the high concentration of this sequence (71,800 copies/µL) in the bulk of the template DNA sample. The level of maximum fluorescence signal was less in microreactors with H8 and Uid A primer pairs due to the smaller amount of DNA copies of 52,300 and 13,600 copies/µL, respectively.

The threshold line is the maximum level of fluorescence that can be defined as background. The first cycles of the run show the initiation phase. In this phase, all PCR mixture components were in abundance, but the number of amplicons present in each reaction was negligible to produce a fluorescent signal that exceeds the threshold line. By progressing the experiment, the amount of fluorescence signal will exceed the threshold line. The cycle threshold (Ct) is the cycle number at which the fluorescent signal reaches the threshold line [30]. The values of Ct for the PCR with primer pairs of Gen bacIII, H8, and UidA were recorded as 23, 25.2, and 31.8, respectively. Figure 5.5 also clearly shows the PCR efficiencies of the different fragments. Multiple parameters can affect the PCR efficiency and specificity. For instance, reagents concentration, presence of PCR inhibitors in sample, primer design, secondary structures, amplicon length, G+C content, were found to play a significant role in setting up an efficient PCR platform [31].
Figure 5.5. Amplification plot for on-chip detection of Bacteroidales and Escherichia coli (E. coli). Positive controls (PC): Bulk of standard templates of DNA containing GenbacIII (71,800 copies/µL), H8 (52,300 copies/µL), and UidA (13600 copies/µL) sequences. Negative controls (NTC): NTC1 (template DNA from Staphylococcus aureus), NTC2 (no template DNA; water).

5.3.2. Performance Characteristics of the Developed Microfluidic Device

We measured the limit of detection (LOD) to examine the performance of the microdevice on our real-time PCR instrument. In this experiment, LOD is defined as the lowest concentration of the nucleic acid that can be reliably detected with our microfluidic device. The LOD measurement was performed by the DNA dilution series of the Gen bac III sequence ranging from 718,000 to 71.8 DNA copies/µL. In this experiment, the microreactors were preloaded with Gen bac III primer pairs. Each concentration was then added separately to the specific channel through the air venting ports. Figure 5.6 shows the on-chip real-time PCR of the serially diluted Gen bac III sequence carried out on our customised thermal cycler instrument.
We observed that the trends in fluorescent intensities in all concentrations increased with increasing PCR cycles. In comparison, no signal was detected in the negative target control (NTC1). The LOD was estimated to be 71.8 copies (Figure 5.6A). The regression equation was calculated to be $y$ (threshold cycle) $= -3.36 \log\text{(copy numbers)} + 39.276$, with a correlation coefficient ($R^2$) of 0.99 (Figure 5.6B). The PCR efficiency of the primer pair was 98.44%, which was calculated using the following equation:

$$E = 10^{-1/\text{slope}} - 1.$$ 

These high levels of $R^2$ and the amplification efficiency value confirm the successful PCR and amplification of the template DNA in our platform. The LOD of our method is comparable to some microfluidic array systems reported previously [32]. Although some other studies have reported better LODs, their method required more complicated and expensive chip fabrication processes [33,34]. However, our platform with the reported LOD, is still one of ideal candidates for nucleic acid analysis in water studies. Based on a study that has been conducted in three different climatic zones in Australia, E. coli mean concentrations in pooled human wastewater data sets from determined zones were $3.2 \times 10^6$ gene copies/ml [35], demonstrating the applicability of our method.
Figure 5.6. (A) Amplification plot for 10-fold serially diluted standard template DNA (Genbac III sequence); NTC1 (template DNA from Staphylococcus aureus). (B) Standard curve for 10-fold serially diluted standard template DNA.

5.3.3. Validation of Real-Time PCR Using Gel Electrophoresis

To validate our optical measurements in another platform, we used gel electrophoresis for on-chip amplicons analysis. In this experiment, the resulting amplicons after PCRs were pipetted out through the air venting ports and loaded on a gel which was preloaded with a ladder (100–20,000 bp). Figure 5.7A shows the result of the simultaneous amplification of standard templates of DNA containing GenbacIII (71,800 copies/µL), H8 (52,300 copies/µL), and UidA (13,600 copies/µL) sequences on the developed microdevice. The banding pattern analysis on the gel revealed three distinct bands of 129, 177, and 68 bp for GenbacIII, H8, and UidA, respectively. These bands were reported before using some off-chip methods.
[36–38]. The appearance of no significant bonds in the negative controls for untargeted samples such as *Staphylococcus aureus* and water (NTC1 and NTC2) demonstrates the specificity of our platform.

Figure 5.7B presents the resulting amplified amplicons of the serially diluted GenbacIII sequence. The band intensities gradually decrease for lanes from left to right, as the amounts of template DNA (copy numbers) were reduced in the PCR mixture. No significant change was observed for NTC1.

![Figure 5.7B](image)

Figure 5. 7. Gel electrophoresis image of on-chip PCR products after 35 cycles. (A) Illustration of GenbacIII, H8, UidA, and negative controls (NTC1, NTC2). (B) Image of serial dilution of GenbacIII sequence.

### 5.4. Conclusions

We designed and developed a real-time and quantitative PCR system for nucleic acid detection. The system is based on a disposable chip specifically designed to be compatible with our custom-made thermal cycling and optical detection system. The advantages of our
chip are simple preparation and application, low cost, parallel detection of various marker genes, capillary flow-based sample loading, and no need for external pumps or valves. Evaporative loss and PCR efficiency, which are two significant technical challenges in designing the chips, were successfully addressed. We used glass on the top and at the bottom of the PDMS layer, and oil on both sides of the microreactors, to capture the PCR sample and prevent its movement. Furthermore, we used sealant to close the input/air venting ports. Bubble generation was another challenge in chip design and can decrease the PCR efficiency. We solved this issue with bubbles by employing rounded corners and a smooth internal surface for the microreactors. We also demonstrated that the surface modification of PDMS using a surfactant (Triton X-100) can effectively prevent undesired protein adsorption and subsequently improve PCR amplification. The function of the chip was verified with successful microbial source tracking for three human faecal markers. Future works would be extended to develop chips with other materials to facilitate chip sealing or even omit the sealing step.

5.5. Supporting information

S1. The contact angle measurements were carried out to analyze the wettability changes of chips (Fig.5.S1). Fabricated chips using pure PDMS, and modified PDMS (0.5% TX-100) were employed. The effect of washing step on surface characterization was also investigated. A droplet of water was placed on desired chip and images were captured at 10-min interval. Images were then evaluated using imageJ (software). The results demonstrated that the initial contact angles (time = 0 min) for pure PDMS and washed PDMS+Surfactant were very close 117° and 115.8° respectively. However, unwashed PDMS+Surfactant showed low contact angle of 113° due to the excess amount of surfactant. To study the time-dependent wettability changes of surfaces the experiment were performed in 10 min duration.
Within several minutes, the contact angles of all surfaces started to decrease. However, the decrease of contact angle for washed PDMS+Surfactant was more than pure PDMS 83° and 90.5° respectively. Unwashed PDMS+Surfactant showed the least contact angle (72.8 °).

Figure 5.S1. Contact angle evaluation of the chips.

**S2.** The UV-Vis study was performed to optically test the transparency of the chips. The optical property of pure/modified PDMS was characterized by measuring transmittance with a UV/Vis spectrophotometer. The spectra were obtained for samples over a wavelength range of 200-700 nm. The result showed that the transmittance of the pure PDMS was decreased by adding 0.5% surfactant. However, based on our real time experiment results and a published study [22] this surface modification has negligible effect on fluorescence transit. Also, washing chip had no significant impact on the transparency of PDMS+surfactant.
S3. The surface morphology of the chip was investigated by scanning electron microscopy (SEM) using a JSM-6510LV with an acceleration voltage of 15 kV. It can be found that edges and surface are smooth enough to prevent bubble generation during loading.
The temperature difference between the aluminium block and the sample inside the chip was measured. A pre calibrated negative temperature coefficient (NTC) thermistor (Build Circuit, Australia) was inserted between the PDMS upper layer and the glass bottom layer of a microfluidic chip similar to the one explained in the manuscript as shown in the Fig. 5.S4A. A small incision was made on the top PDMS layer of the microfluidic chip to insert the NTC thermistor. This incision was later closed using a UV curing polymer and the thermistor was placed intact. It has to be noted that it is not possible to put any thermal conductive paste between the thermistor and sandwiching layers of PDMS (on top) and glass (at the bottom) in this arrangement. So, the heat transfer efficiency to the sensor might be a little less than the actual scenario and the temperature reading inside the chip might be a little less than the actual values. The micro controller was programmed to heat up the aluminium block to a constant temperature and the corresponding temperature inside the microfluidic chip was measured using the thermistor. The results of the experiments are depicted below (Fig. 5.S4B). The temperature readings from the inside part of microfluidic chip is satisfactory considering the minor heat transfer inefficiency of the measurement setup. The average temperature difference between the aluminium block and the inside of the microfluidic chip was observed to be 2.3±0.8 K over the temperature ranges under consideration. The reading provides evidences for the satisfactory heat transfer between the aluminium block and the PCR sample inside the microfluidic chip.
Figure 5.S4. Comparison of the aluminium block temperature and the sample inside the chip.

5.6. Ethical approval
Ethical approval for the study the effectiveness of molecular assays in detecting human faecal pollution, and the effect of freezer storage on human faecal samples was done by the ethics committee through Griffith University office of research. Ref No. BPS/01/13/HREC.
5.7. References


Statement of contribution to co-authored submitted paper

This chapter is in a form of a co-authored submitted paper. The bibliographic details of the co-authored paper, including all authors, are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

My contribution to the published paper involved:

- Literature review
- Experimental set-up
- Manuscript preparation
- Responding reviewers

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(Date) 18/3/2020

(Date) 18/3/2020

(Date) 18/3/2020

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Chapter 6: Potential application of core-shell beads in phylogrouping of *E. coli* strains

**Abstract**

Current multiplex polymerase chain reaction (PCR) methods are effective tools for simultaneous detection of target genes. Nevertheless, their application has been restricted due to the intrinsic interference and competition among primer pairs during amplification. Performing several single PCRs instead of a multiplex PCR is a simple way to overcome this obstacle. Despite this fact, there are still major technical challenges in designing new generation of single PCR microreactors such as providing small volume operation, rapid thermal cycling, and preventing evaporation during amplification. We report a simple and efficient liquid marble-based platform to perform multiplex PCR by integrating a series of single amplifications. Four core-shell beads containing special polymer coating and PCR solution were synthesized by liquid marble technology and photopolymerization. Each bead was able to detect one type of targeted gene. A custom-made thermal cycler was fabricated to perform thermal cycling of these core-shell beads. Based on fluorescent intensity emitted by the fabricated beads, phylogrouping of the *E.coli* strains was performed. This platform can be a promising alternative tool for multiplex nucleic acid analyses due to its simplicity, feasibility, high-throughput capacity. This method also reduces the cycling time and addresses evaporation/contamination of the sample during amplification.

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Lena Gorgannezhad, Kamalalayam Rajan Sreejith, Melody Christie, Jing Jin, Chin Hong Ooi, Mohammad Katouli, Helen Stratton, Nam-Trung Nguyen. Potential application of core-shell beads in phylogrouping of *E.coli* strains. Submitted to Analyst.
6.1. Introduction

The *Escherichia coli* (*E. coli*) is a bacterium that is frequently found in the intestine of humans and warm-blooded animals. While most strains of *E. coli* are harmless, some strains are pathogenic and can cause certain infectious diseases such as pneumonia, urinary tract infections, diarrhea, gastrointestinal, and extra-intestinal infections [1,2]. Based on phylogenetic studies, *E.coli* strains can be classified to four main groups (A, B1, B2, and D) [3,4], and distribution within these groups appears to correlate with the host origin of the strains. Phylogroups B2 and D have been demonstrated to include extraintestinal virulent strains, expressing several virulence factors (e.i. proteins) [5,6]. However, phylogroups A and B1 include mostly human and animal commensal strains [7-9].

Over the past few decades, a wide range of conventional genotyping methods based on the polymerase chain reaction (PCR) have been employed for the characterisation of *E.coli* phylogroups such as pulsed-field gel electrophoresis (PFGE) [10], enterobacterial repetitive intergenic consensus (ERIC)-PCR [11,12], random amplified polymorphic DNA (RAPD) alone [13] or combined with biochemical fingerprinting [14], ribotyping [15], and multiplex phylogrouping PCR [16,17]. The major bottlenecks in employing these conventional approaches are the time-consuming and elaborate laboratory procedures and equipment. PFGE is digestion of bacterial nucleic acids with rare-cutting restriction enzymes followed by separation of the large DNA fragments. This digestion may result in DNA patterns with a relatively low number of bands. The ease of interpretation in PFGE makes it a handy method for the genotyping of bacterial isolates. Nevertheless, the usage of this method has been limited due to the long processing time required for isolation and restriction of DNA prior to electrophoresis (5-6 days) [18].
ERIC-PCR is a rapid, sharp and profitable fingerprint method for discriminating different types of strains. However, its large number of protocol steps impacts its reproducibility and speed. Also, it needs gel electrophoresis as a mandatory step for identification [19,20]. RAPD is a popular genotyping approach. This method amplifies large template of genomic DNA using short and arbitrary primers, generating highly polymorphic fragments profiles as microbial identification fingerprints. These profiles can be quickly analyzed without PFGE [21]. Even though, several normalization and standardization algorithms have been developed to solve the reproducibility problem of this method [22]. PCR ribotyping, is a molecular technique for bacterial phylogrouping that uses information from rRNA-based phylogenetic analyses [23].

Multiplex (tri/tetra plex) is another common PCR-based molecular technique for E.coli phylogrouping. In this method, the specific primers are simultaneously used for the amplification of 4 genes whose presence/absence produces a genotype which identifies an isolate to be a member of one of 8 different phylogroups.[4,24]. Among the above-mentioned approaches, PCR ribotyping and multiplex PCR have been adopted in many laboratories as the method of choice for E.coli typing over the past few years. But there are still some obstacles that limit their application. For example, the agarose gel-based DNA separation technique is a necessary step for both assays. There are some hardships in the conventional agarose gel-based technique application as well, such as inter-laboratory variation and interpretation of banding patterns, time-consuming and complex process, and need for skilled personnel and expensive equipment [25,26].

Microfluidics has been also successfully adapted for nucleic acid amplification [27]. These lab-on-a-chip systems have gained popularity owing to their flexibility for
real-time analysis, automation, integration, miniaturization, portability, and multiplexing. Microfluidic chips or arrays also have many other distinct advantages which make them attractive to researchers. For example, the high throughput and low consumption of the sample make them cost effective [28-31]. Nonetheless, one approach cannot meet every need. There are major drawbacks in their development and application in nucleic acid amplification such as complex fabrication processes in clean room facilities, difficulty in sealing/valving, evaporation, and incompatibility with rapid processing of large sample volumes. Also, chip design and fabrication require specialized instrumentation and expertise [32]. In this context, there is a need for a novel real-time amplification method for phylogrouping complying with features, such as low cost, high speed, simple operation/interpretation, and high sensitivity.

Liquid marble, a liquid droplet encapsulated by hydrophobic powder, can be an alternative to address difficulties of conventional methods. The particles coating the surface of the liquid droplet prevent direct contact between the core liquid and surfaces outside the marble, thus reducing contamination [33]. Liquid marble can then maintain its stability on a solid surface, leading to easy handling of liquids [34]. Since the shells of liquid marbles are made from loose particles, they can be easily opened. These penetrable shells allow materials such as reagents to be inserted into or extracted from the liquid marble. Taking advantage of the small dimension of liquid marbles, the reagents consumption can be reduced [35,36]. Liquid marble has been employed for a wide range of chemical and biological [37]. More recently, Sreejith et al [38] have used liquid marble as a potent bioreactor for DNA amplification. In this method, polytetrafluoroethylene (PTFE) were used as the hydrophobic coating. However, due to the evaporation of the PCR mixture through the porous walls of liquid marbles the duration of thermal cycling was limited. The lower number of thermal cycles may
negatively impact on the amplification efficiency. To reduce the evaporation, this group have also employed a composite liquid marble as a bioreactor [39].

Herein, we report an amplification-based detection assay using four core shell beads to perform phylogrouping of E. coli strains. The PCR solution was inserted to a photopolymer droplet, forming a spherical bead. The resulting liquid droplet was embedded by a hydrophobic/oleophobic powder. The powder and shell liquid not only provide sterile condition for the core liquid (PCR mixture), but also remarkably reduce evaporation. Moreover, solidification of the polymer under blue light can transform the liquid marble into a core-shell bead, leading to easy manipulation and storage of the sample.

6.2. Method and materials

6.2.1. General procedure for the fabrication of core shell bead

The synthesis and characterization of the core shell bead has been reported previously [39]. In brief, the synthesis procedure involved four main steps. First, a super amphiphobic silicon monolith known as “marshmallow like gel” [40] was crushed and used as a super amphiphobic powder bed. Next, 0.05 g of camphorquinone and 0.06 g of 94 ethyl-4-(dimethylamino) benzoate were added to 10 g of Trimethylolpropane trimethacrylate (TRIM) by a magnetic stirrer at 600 rpm for 2 minutes. The prepared mixture was used as the photo polymer liquid. Subsequently, 20 µl of the photopolymer was nested on the powder bed using a micropipette. Next, 2 µl of the PCR solution containing templated DNA was injected into the photopolymer droplet. The obtained droplet was then slowly rolled in the powder bed to create a protective amphiphobic surface (Fig. 6.1A, steps 1-3). Finally, a photopolymerization step in presence of blue light resulted in solidification of the
polymer and encapsulation of the master mix solution (Fig. 6.1B). To push the inner liquid droplet (PCR solution) towards the center of the outer droplet (photo polymer), the photopolymerization process was performed in a motorized cylindrical drum and rotated at 140 rpm for 5 minutes. The powder on the surface of the bead was washed using mineral oil to obtain a transparent core-shell bead. The diameter of the bead was 1-2 mm.

Figure 6. 1. Operation of PCR based on a core-shell bead: (A) (1) Deposition of photopolymer droplet on top of super amphiphobic powder surface. (2) Embedding PCR solution into the deposited droplet. (3) Covering the droplet by amphiphobic powder. (B) Photo polymerization of the droplet rotating at 140 rpm in a motorized drum under blue light. (C) Transferring the generated core shell bead on top of a custom-built thermal cycler to process amplification. (D) Discriminating the core shell beads containing amplicons based on fluorescent intensity.
6.2.3. Thermal cycler construction

A custom-built thermal cycler was developed to provide required thermal cycling conditions for core shell beads during PCR experiment [39]. To reach this goal, an aluminium block (A 20 mm× 20 mm ×15 mm) embedded with a cartridge heater (5-mm diameter and 15-mm length, Core electronics) was employed as the thermal cycling platform. The aluminium block was clamped onto a Peltier thermo electric cooler (40 mm × 40 mm × 3.5 mm. TEC-12706. AUS Electronics). The entire platform was in turn attached to a heat sink-cooling fan assembly (12 V, 3300 rpm, 70 mm × 70 mm × 25 mm) as shown in Fig. 6.1C. For precise thermal cycling, a proportional-integral-derivative (PID) algorithm implemented in an Arduino UNO microcontroller board. The thermal cycler was programmed to run the following temperature cycles: initial denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 5 s and annealing at 59 °C for 20 s; 5 min incubation at 72°C; infinite hold at 12°C.

6.2.4. DNA amplification

*E. coli* strains were isolated on nutrient agar plates from faeces samples in a clinical pathology lab. Single colonies were picked and grown overnight at 37 °C in nutrient broth. Isolates were stored at -80°C until use in nutrient broth with 30% glycerol. The isolates were then used for phylo-grouping experiment using single PCR and quadruplex PCR assay according to the method reported by Clermont et al. [41]. The sequences of forward and reverse primers for the desired target genes are listed in Table 6.1. For single PCR, the PCR mixture (20 µl) contained 2.5 µl forward primer (10 µM), 2.5 µl reverse primer (10 µM), 2.5 µl DI water, 2.5 µl isolate (template DNA), 10 µl Syber green. A volume of 2 µl of the mixture was inserted into the
polymer and after photopolymerization it was placed on top of the customized thermal cycler to tolerated the cycling condition of initial denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 5 s and annealing at 59 °C for 20 s; 5 min incubation at 72°C; infinite hold at 12°C. For the quadruplex PCR, briefly, amplification was performed in a 20-µl PCR mixture including 0.3 µl chuA.1b, 0.3 µl chuA.2, 0.5 µl yjaA.1b, 0.5 µl yjaA.2b, 0.5 µl TspE4C2.1b, 0.5 µl TspE4C2.2b, 1 µl AceK.f, 1 µl ArpA1.r, 10 µl Syber Green master mix, 2.4 µl DI water, 3 µl Template DNA. The concentration of stock primers was 50 µM. The mixture was placed in a conventional PCR instrument (Biorad CFX Connect) for thermal cycling. The resulted amplicons were subsequently run on 2% agarose gel electrophoresis and followed by exposure of the UV transilluminator (Bio-Rad).

Table 6. 1. Primer sequences and sizes of amplicons used in the phylo-grouping experiment.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer ID</th>
<th>Sequence (5ʹ-3ʹ)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chuA</td>
<td>chuA.1b</td>
<td>ATGGTACCGGAACACCAAC</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>chuA.2</td>
<td>TGCCGCCAGTCCCCAGACCA</td>
<td>211</td>
</tr>
<tr>
<td>yjaA</td>
<td>yjaA.1b</td>
<td>CAAACGTGGAAGTGGCAAGGA</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>yjaA.2b</td>
<td>AATGCCTCTCCACCCCTG</td>
<td>400</td>
</tr>
<tr>
<td>TspE4C2</td>
<td>TspE4C2.1b</td>
<td>CACTATCGTAAAGGTGCACCC</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>TspE4C2.2b</td>
<td>AGTTTATCGTGCGGTTGTCG</td>
<td>211</td>
</tr>
<tr>
<td>arpA</td>
<td>AceK.f</td>
<td>AACGCTATCGGCTGCCAGCCTG</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>ArpA1.r</td>
<td>TCTCCCCATACCCGTACGCTA</td>
<td>400</td>
</tr>
</tbody>
</table>

6.2.5. Optical detection

For real-time analysis of the amplification, we developed a fluorescent detection platform as shown in Fig. 6.2. The fluorescent excitation wavelength of the microreactor was 450-490 nm (Blue light) and the recording emission range was 520-560 nm (Green). To provide the source of fluorescence excitation, a blue LED light source (450-490nm) was used (1500mCd, Jaycar, Australia). Fluorescence measurement was performed at the end of the desired thermal cycle and the
fluorescent signal was collected by a vertically mounted CMOS camera (Edmund Optic EO-5012C) attached with a 0.5× telecentric lens (Edmund Optics-63074). A green optical filter (520-560 nm) was also used for filtration of emitted light from amplification microreactor to enhance the signal to noise ratio. The intensity of the emitted light was the indicator of amplification efficiency.

![Figure 6. 2. Schematic of the experimental setup](image)

6.3. Results and discussion

6.3.1. Assay principle

Using a core-shell bead as a microreactor, we report here an amplification-based assay for detecting marker genes in samples and phylogrouping *E. coli* strains. The preparation of the core-shell bead is schematically outlined in Figure 6.1 and described in detail in section 2. For the phylogrouping experiment, 4 core-shell beads were fabricated. Each bead contained 2 µl PCR solution with a particular set of primer
to detect one marker gene. The resulting core-shell beads were subsequently placed on top of a custom-made thermal cycler to tolerate thermal cycling condition of 94 °C for 4 min; 30 cycles of 94 °C for 5 s and 59 °C for 20 s; 72°C for 5 min; infinite hold at 12°C. A separator made of PDMS was used to prevent sticking the beads during thermal cycling step. The experiment was performed in a dark laboratory environment. A Blue LED light was employed to emit light, matching well with the central wavelength of the beads. The light was manually turned on in intervals of 5 cycles. The emitted light by the beads was filtered using a green optical filter, which was finally collected by a CMOS camera attached with a 0.5X telecentric lens. Experiments were repeated four times comprising one for positive control and three for unknown samples.

6.3.2. Phylogrouping using core shell beads

To assess the applicability of our assay in analyzing clinical (control/unknown) samples, we used core-shell beads of A, B, C, and D for the analysis of chuA, arpA, yjaA, TspE4C2 genes respectively. The numerical values of fluorescent intensities of the amplicons inside core shell beads were evaluated using ImageJ, an open source software. The values were then normalised by following formula:

\[
I_{sc}^* = (I_{sc} - I_{s0}) / I_{max}
\]

where \(I_{sc}\) is the fluorescent intensity of a sample measured at a given cycle, \(I_{s0}\) is the fluorescent intensity of that sample at the start of thermal cycling and \(I_{max}\) is the maximum fluorescent intensity measured among all the samples. Fig 6.3. presents the amplification curves from core-shell beads. Normalised fluorescent intensity increases
with increasing cycle number in all beads containing target gene and related primer set. The increase of fluorescent intensity of the PCR solution demonstrates positive polymerase chain reaction within the bead. Lack of target gene inside bead, depict no significant fluorescence over thermal cycling. A genotype corresponding to the presence/absence of the four genes in four beads is determined for each strain. The phylogrouping of a positive control (EC RBH2) using beads is shown in Fig 6.3.I.

Beads of A, C, and D show positive amplification; however, bead B has no significant amplification. The obtained genotype (++++) indicates chuA +, arpA -, TspE4C2 +, yjaA +, and subsequently an isolate is assigned to a phylo-group of B2. In addition to one positive control, three unknown samples were also evaluated using this assay and the phylo-groups are depicted in Table 6.2.

A desired experiment for another unknown sample was also repeated three times to show the reproducibility of the assay and the results were presented in Fig 6.4.
Figure 6. Amplification plot and phylogrouping of E. coli strains using core shell beads. Positive control (I), Unknown samples (II, III, IV); A, B, C, and D Beads containing specific set of primers to detect chuA, arpA, TspE4C2, yjaA genes individually; Images of core shell beads are provided as inset.

Table 6.2 Genotypes for assigning E. coli isolates to phylo-groups.

<table>
<thead>
<tr>
<th>Experiment ID</th>
<th>Bead A (chuA)</th>
<th>Bead B (arpA)</th>
<th>Bead C (TspE4C2)</th>
<th>Bead D (yjaA)</th>
<th>Phylo-group</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>B2</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Unknown</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Clade I or II</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Validation of phylogrouping by gel electrophoresis

To verify the phylogrouping results of the samples in another platform, we used quadruplex PCR and gel electrophoresis. Fig 6.5. shows the result of Sybr-Green quadruplex PCR of positive control (EC RBH2), negative control (water), and unknown samples. The banding pattern analysis on gel revealed three distinct bands of 288 bp, 211 bp, and 152 bp for chuA, yjaA, and TspE4C2 genes respectively. Lane 2 assigns to B2 strain of *E.coli* that possess chuA, yjaA, and TspE4C2 genes. *E.clade I/II* strain just shows amplification of yjaA gene (lane 5). Remaining genotypes (lanes 4 and 6), cannot be classified in any distinct phylo-group. These results present a very good validation for the accuracy of the amplification using core-shell beads.
6.4. Conclusion

We report a simple and efficient core-shell bead-based platform to perform multiplex PCR by integrating a series of single amplifications. Four core-shell polymer beads containing PCR mixtures were prepared by liquid marble technology and photopolymerisation process. A custom-made thermal cycler was developed to perform thermal cycling of the core shell bead. Thermal cycling of the PCR microreactor beads was performed and the phylogrouping of the *E. coli* strains were determined. This method potentially resolves the obstacles of conventional multiplex PCR in a single tube. The novelty of this work is developing sustainable bio-labs, by introducing an alternative platform to conventional phylogrouping methods that generate several tonnes of plastic annually. Furthermore, a variety of challenges have hindered the application of multiplex PCR such as intrinsic interference and competition among primers, requiring optimized multiplex PCR protocols, and
difficulty in detection of a large number of targets simultaneously. Our novel assay displayed the following advantages: (i) Very easy fabrication and operation; (ii) Fully sealed PCR microreactor that eliminates volume loss due to evaporation of the samples during thermal cycling and also the risk of contamination; (iii) Reducing the PCR mixture volume to 2 µl, which can reduce the cycle time. In summary, the core shell bead-based platform reported here is capable of full PCR assay functionality due to its feasibility, high-throughput capacity, and simplicity. This PCR micro reactor can be widely used in multiplex nucleic acid analyses for clinical or environmental diagnostics and basic research.

6.5. Ethical approval

E. coli strains were isolated by a clinical pathology lab (Detay in patoloji laboratory, Ankara, Turkey) as part of their routine analyses of clinical samples they receive. No clinical samples were directly collected from any patients nor the isolated strains were part of any projects and therefore there is no need for ethics approval in further testing them.
6.6. References


Chapter 7: Conclusion

The overall focus of this PhD study was to design and develop simple and inexpensive platforms for the analysis of nucleic acids (NAs). The thesis presents electrochemical, microfluidic, and liquid marble-based platforms for detecting NAs in clinical and environmental samples with high sensitivity and specificity. Chapter 1 provides the background of the research topic and highlights the specific motivation for the work. Chapter 2 presents an extensive and critical literature review, focusing on the significance, and potential role of ctNAs as non-invasive cancer biomarkers from diagnostic to prognostic application as well as providing an overview of the latest developments in NAs analysis techniques. This chapter further describes the major technical and biological challenges of existing ctNAs detection techniques. The extensive literature review presented in Chapter 3 further provides an overview of the up-to-date developments in microfluidic-based NAs analysis platforms in microbiology. This chapter specially emphasises the recent progress in microfluidics-based devices for polymerase chain reaction and isothermal nucleic acid amplification. The major concerns in designing microfluidic devices for NAs amplification are also addressed. Chapter 4 reports an electrochemical detection method for direct quantification of the ctRNA present in the plasma of cancer patients using modified commercially available screen-printed electrodes. Chapter 5 introduces a microfluidics-based platform for the real-time PCR-based detection of multiple waterborne bacteria. Chapter 6 reports a liquid marble-based platform for phylogrouping of the *E.coli* strains through integrating a series of single PCR reactions.

In brief, the thesis describes the design and development of three novel platforms for the rapid, simple and inexpensive analysis of NAs. Considering the well-established significance of ctNAs as invasive cancer biomarkers, leveraging the advantages of screen-
printed electrodes and electrochemical readout, we developed a simple, low-cost, and amplification free electrochemical method for detecting targeted ctRNA in ovarian cancer patients. To increase the sensitivity of the assay, the novel nanoporous Fe$_2$O$_3$@GO nanomaterials were utilised. Taking advantage of superparamagnetism, great surface loading capacity, and high electrocatalytic activity of these nanoparticles, an electrochemical (chronocoulometric) readout strategy was developed for the detection of FGFR2:FAM76A fusion gene in ctRNAs, with an ultrasensitive detection limit of 1.0 fM. The main benefit of the current assay is that all the quantification steps are performed on a single modified electrode, resulting in simple fabrication of the device and less sample consumption, which is suitable for point-of-care settings.

Furthermore, we addressed two main improvements for the next platform development: providing sensitive and simultaneous detection of several target genes. In this regard, we designed and developed a quantitative real-time PCR system based on microfluidics for NAs monitoring in microbial source tracking studies on environmental samples. We used a sandwiched glass-PDMS-glass microfluidic chip containing an array of reactors for detection of multiple targeted genes. Since the required primers are preloaded within the reactors in the chip fabrication step, the chip is ready to use and there is no need for additional pipetting steps. The microchip loading process is based on capillary filling which eliminates the need for elaborate and costly liquid handling instruments. Due to the special design and smooth internal surfaces of microreactors/channels we could eliminate bubble generation during loading step, which is one of main concerns in the development of the PCR microchip. The usage of surfactant in surface modification of PDMS removes undesired enzyme adsorption and results in a higher PCR efficiency. As one of the major challenges in the application of PDMS-based microchips at elevated temperatures is the evaporative loss of sample, we used mineral oil on both sides of reactors, two layers of glass
on top and the bottom of the device, and a layer of sealant to close the venting ports. A real-
time amplification instrument was also manufactured and integrated with the system to
provide thermal cycling to the microchip. Employing this detection platform, we could detect
three human-associated MST markers (H8, Gen bac III, UidA) and reach a detection limit of
71.8 DNA copies/µl that clearly demonstrates a high sensitivity of this approach.

As one of the major challenges in NAs detection research is designing an easy and
efficient platform for multiplex amplification, the main goal of our final assay was to develop
a method not only provides a sensitive detection, but also avoids complex and time-
consuming operations. To achieve this goal, we moved on to report a novel liquid marble-
based platform for the NAs analysis where marble technology and photopolymerization were
integrated under one assay protocol. In this assay, four core shell beads containing polymer
and PCR solution were synthesized and employed for simultaneous detection of four marker
genes in phylogrouping of E.coli strains. These fabricated beads were then transferred on a
custom-made thermal cycler to undergo the required thermal condition for amplification. The
emitted fluorescent intensity from the beads was captured by a camera and then used as an
evidence for successful amplification procedure. The real-time evaluation of NAs presence in
this assay indicates a great potential for the development of a user-friendly and cost-effective
assay in resource-limited settings, where sophisticated equipment and skills may not be
available. Unlike conventional multiplex amplification methods, our approach does not
require to mix several primer pairs in one reaction which subsequently prevents intrinsic
interference and competition among primers. Taking advantage of sealing PCR solution with
polymer, we could eliminate the contamination risk as well as sample loss due to evaporation
during thermal cycling. Our approach requires a very small amount of PCR solution (2µl)
that leads to a reduction of thermal cycling time. Simplicity in fabrication and operation as
well as high throughput capacity make this platform a potent alternative for existing multiplex amplification techniques.

The investigations carried out in this thesis represent a step forward in the adaption of advanced technologies as NAs analysis tools that can be employed in molecular diagnostics. We believe that the methods presented here have a huge potential for commercialization due to the relatively inexpensive platforms by eliminating the expensive equipment utilization and manual labour. Programmable NA amplification platforms bring us even closer to fully automated system, which help to save cost and time effectively. Among the presented approaches, Core-shell bead is more promising for NA amplification due to its superior sensitivity, feasibility, and low sample consumption. However, further studies can be also conducted on automation of this platform to make it more user-friendly for high-throughput analysis. Mass fabrication of the beads for simultaneous analysis of a large number of samples is labour intensive. Thus, automation of bead preparation step and the partitioning of the PCR solution and inserting it into the fabricated beads can increase the speed and efficiency of the assay. Moreover, this platform has the potential to be fully-integrated with other systems to perform the entire analytical protocol including sample preparation, DNA extraction, amplification, and optical detection for the identification of bacteria in many basic research and translational applications. We hope that, the current effort toward the development of high-performance NAs detection techniques will result in the next-generation platforms that can be routinely used for NAs analysis for both clinical and environmental applications.