Doctoral Thesis in Fibre and Polymer Science

Sample-to-answer paper-based nucleic acid amplification tests

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Abstract

Nucleic Acid Amplification Tests (NAATs) with PCR technology to amplify DNA, are the golden standard for infectious disease diagnostics, but they require benchtop instruments and trained users to be performed. For this reason, we all had to send PCR test to centralized laboratories during the Covid-19 pandemic. A year into the pandemic, home-based antigen paper-based tests became available for Covid, but these were not as sensitive, so PCR tests had to be used still. This development emphasized the need for technologies that enable NAATs with superior sensitivity to be performed at home. There are three technological advanced that could make such tests possible: 1) Paper based devices, called paper microfluidics, have been developed to enable more advanced steps of testing without laboratory equipment. These paper-based system incorporate advanced functionality and multiple reaction steps. 2) New DNA amplification techniques, called isothermal amplification, have been developed which, contrary to PCR, can be run without a thermocycler, enabling DNA amplification to be carried out even inside a paper. 3) Several methods to detect DNA have been shown using paper.

One step that is still largely unsolved in NAATs is the sample preparation step, hindering the development of fully paper-based NAATs. In sample preparation, nucleic acids are extracted from bacteria or virus, usually using reagents harmful to DNA amplification. These steps are therefore complicated and require several washing steps and heating, and are therefore difficult to integrate into paper.

In this thesis, we used a simple, cost-effective, and scalable method to incorporate sample preparation in paper, thus taking NAATs towards point of care. We solve this problem by immobilizing enzymes that are used for sample preparation on nitrocellulose paper. The immobilized enzymes remain functional and can be used for biochemical reactions, while they are strongly bound to the paper. This method enables the separation of these enzymes from the sample, protecting downstream sensitive reactions of DNA amplification and eliminates the need for high temperature deactivation or washing steps. Specifically, we show that the
enzyme achromopeptidase can do cell lysis from the *Staphylococcus epidermidis* bacteria, a common pathogenic gram-positive bacterium, and use its DNA in further reaction to perform a sample-to-answer paper-based NAAT. These NAATs employed a low temperature amplification step called Recombinase Polymerase Amplification (RPA) and DNA detection with a lateral flow strip.

We further show the enzyme proteinase K, also immobilized on paper, can digest RNase in saliva samples, an enzyme that breaks down RNA leading to false-negative results. This results enabled an easy sample preparation step towards saliva viral DNA self-testing.

Finally, in this work we developed a paper microfluidic system that can carry out an enzyme-linked oligonucleotide assay, which demonstrated much higher sensitivity in detecting amplified DNA than conventional lateral flow assays. In summary, these results provide solutions towards high-performing, affordable and instrument-free paper-based NAATs home-testing.
Sammanfattning


Ett steg som fortfarande inte är helt löst är provberedningssteget för NAAT. I provberedningen extraheras nukleinsyror från bakterier eller virus, vanligtvis genom användning av reagenser som är förstörr DNA-amplifiering. Provberedning är därför komplicerade. Dom kräver flera tvättsteg och uppvärmning och är därför svåra att integrera i papper.

I det här arbetet använde vi en enkel, kostnadseffektiv och skalbar metod för att integrera provberedning i papper. Våra resultat tar oss ett steg närmare DNA självtestning. Vi löser problemet med provberedning genom att immobilisera relevanta enzymer på nitrocellulosapapper. Dessa immobiliserade enzymer förblir funktionella och kan sedan utföra biokemiska reaktioner, samtidigt som de är starkt bundna till papperet. Denna metod möjliggör separation av provberedningssenzymer från, andra känsliga reaktioner såsom DNA-amplifiering. Samtidigt elimineras

Vi visar vidare att enzymet proteinas K också kan immobiliseras på papper för att sedan förstora RNas:er, ett enzym som bryter ner RNA och som finns i humana salivprover. Möjligheten att använda papper för att ta bort RNAs från saliv öppnar vägen mot självtestning av salivirus-DNA, som influenza virus.

Slutligen, utvecklade vi ett pappersmikrofluidik system som kunde utföra en enzymkopplad oligonukleotid test. Denna test visar mycket högre känslighet för detektering av amplifierat DNA än vad en kommersiell paperbaserad flödesremsa visar.

Sammanfattningsvis har denna avhandling visat några olika lösningar som för oss närmare prisvärda och instrumentfria pappersbaserade DNA tester för hemanvändning.
List of Publications

This thesis is based on the following peer-reviewed journal publications and manuscripts:

Nitrocellulose-bound achromopeptidase for point-of-care nucleic acid tests.
Scientific Reports, 11(1), 1-7.

**Paper II:** Chondrogiannis, G., Réu, P., Hamedi, M. M. (2022)
Paper-Based Bacterial Lysis Enables Sample-to-Answer Home-based DNA Testing
Advanced Materials Technologies

**Paper III:** Chondrogiannis, G., Toldrà, A., Martin Hanze, Hamedi, M. M.
Paper-based RNase digestion towards viral nucleic acid self-tests
Manuscript

**Paper IV:** Toldrà, A., Chondrogiannis, G., Hamedi, M. M.
3D paper microfluidic devices for enzyme-linked assays, and its application to DNA analysis
Manuscript
The contribution of Georgios Chondrogiannis to the appended papers is described below:

I. First Author: Conception, methodology, experimental work, data analysis, writing.
II. First Author: Conception, methodology, experimental work, data analysis, writing.
III. First Author: Conception, methodology, experimental work, data analysis, writing.
IV. Second Author: Part of methodology, experimental work, data analysis, writing.
Contributions not included in this thesis

Portable electroanalytical nucleic acid amplification tests using printed circuit boards and open-source electronics  
Anna Toldrà, Alar Ainla, Shirin Khaliliazar, Roman Landin, Georgios Chondrogiannis, Martin Hanze, Pedro Réu Carvalho, Mahiar Max Hamedi, Analyst, 10.1039, 2022.

Electroanalytical Paper-Based Nucleic Acid Amplification Biosensors with Integrated Thread Electrodes  

Electrochemical Detection of Genomic DNA Utilizing Recombinase Polymerase Amplification and Stem-Loop Probe  
Shirin Khaliliazar, Liangqi Ouyang, Andrew Piper, Georgios Chondrogiannis, Martin Hanze, Anna Herland, Mahiar Max Hamedi, ACS Omega, 2020.

DNA and RNA extraction using paper and enzymes  
Georgios Chondrogiannis, Réu, P., Hamedi, M. M.  
Swedish patent PRV, SE 2230302-8, 2022.
Abbreviations

ACP: Achromopeptidase
CDNA: Complementary DNA
COVID-19: Coronavirus Disease 2019
Cq: Quantification cycle
ACP: Achromopeptidase
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunoSorbent assay
ELONA: Enzyme-linked oligonucleotide assay
FITC: Fluorescein isothiocyanate
HRP: Horse radish peroxidase
LFA: Lateral flow assays
NAAT: Nucleic acid amplification test
PCR: Polymerase chain reaction
PDMS: poly(dimethylsiloxane)
PMMA: poly(methyl-methacrylate)
POC: Point-of-care
qPCR: Quantitative PCR
RPA: Recombinase Polymerase Amplification
RT-PCR: Reverse transcription PCR
RT-qPCR: Quantitative reverse transcription PCR
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SDS: Sodium dodecyl sulfate
μPAD: Microfluidic paper-based analytical devices
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1 Introduction

Infectious diseases caused by pathogenic organisms such as viruses, bacteria, fungi, parasites, and prions, have always had significant impact on human lives and societies.\(^1\) In fact, this problem first occurred since humans started to live in settlements and to grow food and domesticate animals.\(^2\) Some ancient examples are the Justinian plague in 541 and the Black Death in 1348.

Their severity has escalated in modern times due to the enormous human population and especially due to the accumulation of the population in large cities and the increased global mobility. As a result, there is a rising rate of local and global outbreaks of infectious diseases. In addition, the overuse of antibiotics has also led to new strains of antibiotic resistant bacteria.\(^3,4\) All these issues require better global diagnostics.

The gold standard for molecular disease diagnostics is nucleic acid amplification tests (NAATs). These tests are currently mostly only at modern laboratories. The subject of this thesis is to advance the technologies for NAATs to bring them closer to the patient for point-of-care testing and even home-testing.

1.1 Infectious diseases, history and early diagnostic methods

In this work, the focus is on diagnostics of infectious diseases caused by bacteria and viruses. Not all bacteria are pathogenic to humans, but they cause a variety of diseases on humans such as Tuberculosis, Anthrax, Tetanus, Pneumonia, Cholera, methicillin-resistant \textit{Staphylococcus aureus} infection, the Bubonic Plague and more. Diseases such as the common cold, AIDS, Ebola, Influenza and the Coronavirus Disease 2019 (COVID-19) are caused by viruses.
Bacterial disease, history and overview

Bacteria were discovered nearly 400 years ago with the invention of the microscope, when Robert Hooke described the structure of molds using microscopes and later Leeuwenhoek discovered bacteria in 1676. In 1884 Robert Koch postulated that bacteria were the causal relation between microbe and disease. Koch postulated that “The microorganism must be isolated from a diseased organism and grown in pure culture”

There are two main categories of bacteria regarding their cell wall; gram-positive and gram-negative. This characterization is based on a reaction called Gram stain. Gram stain is a differential staining process that causes gram-positive bacteria to appear purple and gram-negative pink. Gram-positive bacteria have a thicker cell wall that mainly consists of several layers of one type of molecule; peptidoglycan. They also have teichoic acids in the cell wall that give them a negative charge. The cell wall of gram-negative bacteria is not as thick and consists of layers of different materials. Peptidoglycan is a polysaccharide made of two sugar molecules and some amino acids, and it is responsible for the rigidity of bacterial cells. Sheets of peptidoglycan are formed by long chains next to each other, which are also cross-linked to provide two-dimensional strength.

Viral diseases, history, and overview of early diagnostics

Viruses were first described in 1989 by Beijerinck with the discovery of the Mosaic virus. It was originally difficult to differentiate viruses from toxins and work by Louis Pasteur, Emile Roux and Alexander Yersin in late 1800s led to identifying that toxins caused diphtheria. Here they used filtration to differentiate bacteria from toxins, which later were identified as viruses.

Unlike bacteria, viruses are not considered cells nor life forms because they lack some essential properties including the ability to replicate without infecting a host organism. The first methods to detect virus indirectly was developed by George Hirst on 1941 with the
‘hemagglutination assay’. Here, receptors on the surface of red blood cells bind the hemagglutinin glycoprotein on the surface of a virus (mostly influenza but also other viruses) creating an interconnected network red blood cells and virus particles. This creates a suspended distribution, typically viewed as a diffuse reddish solution. When virus concentration is too low, the red blood cells cannot form the network and instead settle to the bottom of the well.

Culture-based methods for disease diagnostics

Traditional culture-based methods have been used for infectious disease diagnostics. These rely on culturing the microorganisms from a patient’s sample in vitro and identifying the microorganisms by multiple-step analysis of the organism’s biochemical properties.

Culture-based methods have a high cost associated with manual labor of highly trained personnel and large laboratory footprint. They are also not effective for patients under antibiotic treatment. Cultures require sensitive and expensive materials from animal and plant sources, and even living tissue in the case of viral cultures. Culturing of bacteria often includes at least overnight incubation steps, thus the results cannot be available until a few days after sample collection. Apart from the cost, time and laboriousness of culture-based methods, there are some organisms that do not survive in laboratory environment. Furthermore, the biochemical properties based on the phenotype of different pathogens are sometimes sufficient to distinguish them, or highly specialized equipment and expertise might be sometimes necessary. Finally, culture-based methods require more space, longer incubation periods, and multi-step detection procedures, all of which are difficult to automate and miniaturize for large scale operations.

Poor diagnostics lead to complications in healthcare. The old methods of culturing cells for bacterial infections lead to problems like unnecessary use of broad spectrum antibiotics for the safety of the patient is costly, can cause adverse health effects and contribute to the rising problem of antibiotic resistance. Culture methods require days or weeks for pathogen growth and are not suited for automation, and can thus not be
used for POC diagnostics. Many viral and infectious disease could also not be detected because of the low sensitivity of the tests.

It was not until the development of Enzyme-Linked ImmunoSorbent Assay (ELISA)\textsuperscript{12} and later DNA amplification, which we describe in more detail in chapter 2.2, that viruses could be directly detected in diagnostics.
1.2 Nucleic Acid Amplification Tests (NAATs)

**History of the Polymerase Chain Reaction (PCR)**

PCR was invented in 1983 by Kary Mullis. The method uses a genetically modified DNA polymerase enzyme to generate a large number of copies of the target DNA, while starting from small amounts, down to even a single copy. Specifically, the method uses short oligonucleotides with sequence complementary to the target DNA, called primers. High temperature is used to denature the target DNA so that primers can bind and anneal with their targets in a subsequent step with lower temperature. DNA polymerase then extends the primers by adding complementary nucleotides, thus copying the DNA. This reaction is repeated and exponentially produces millions of copies of the short target DNA sequence, called amplicons. The heating and cooling requires a thermocycler, a benchtop instrument capable of rapid heating and cooling at precise temperatures.

**The use of PCR towards in NAATs**

After the invention of PCR, which as noted in the introduction was not originally meant for disease monitoring, it has become the gold-standard method in molecular disease diagnostics, in addition to its use in many other areas. PCR-based detection of pathogens is naturally better suited for timely and accurate detection of infectious diseases than culture-based detection with biochemical tests.\(^{13}\)

The first step of a NAAT is sample preparation in which the DNA from bacteria or RNA from virus has to be extracted and purified. Sample preparation is a critical part which is currently hard to implement at the point of care and will be discussed in more detail in the following chapter.

For diagnostics based on RNA, such as in the case of viruses with RNA as genetic material and cancer biology, the target RNA needs to first undergo reverse transcription by the enzyme reverse transcriptase. This enzyme was discovered independently by David Baltimore and Howard Termin in
retroviruses. The combination of these methods led to Reverse transcription PCR or RT-PCR. The enzyme reverse transcriptase produces cDNA from target RNA in an initial step followed by PCR or typically quantitative PCR is performed with the cDNA as template.

Following PCR, or RT-PCR, the large amount of amplified DNA, copied from the target nucleic acids in the sample, can be detected via another method, such as gel electrophoresis. Another much faster method, which is currently the main technique used in laboratories for NAATs, is quantitative PCR (qPCR) enabling the detection and quantification of the target DNA sequence simultaneously. One method of qPCR utilizes oligonucleotide probes that emit fluorescence when DNA amplicons form and it is measured after each cycle. qPCR thermocyclers make use of a camera to capture the fluorescent readout, and thus do not require downstream analysis such as gel electrophoresis. The results can be monitored at real-time and are quantitative and highly sensitive, allowing for safer conclusions. This significant advancement reduced time, laboriousness, and contamination risks, while increasing the throughput of NAATs.

**Point-of-care NAATs based on PCR their use today**

Today, qPCR is the gold standard of NAATs. The workflow includes pathogen inactivation for safety reasons, nucleic acid extraction and purification from the sample, and finally nucleic acid amplification and detection by qPCR or RT-qPCR. In larger scale operations, production-scale instruments and robotic equipment is used to enable higher throughput of these steps, especially in the inactivation and nucleic acid extraction parts of the workflow. During the COVID-19 pandemic, hospital and external laboratories around the world were equipped with supplies and instruments needed for detection of SARS-CoV-2 by RT-qPCR in an unprecedented scale.

Unfortunately, during the COVID-19 pandemic, the development towards point-of-care NAATs had not matured, so PCR testing was performed in centralized laboratories, and was not available to the extent needed
especially in the first months of the pandemic. This led to enormous cost, billions of dollars globally, and it might have played a role in the unveiling global financial crisis. Nevertheless, this event had three very important implications for NAATs: i) It made PCR testing a known and accepted notion for almost everyone globally, lowering the bar for its acceptance. ii) It showed the lowest limit for the cost of a test to be around 100 USD, which is still at least 10-100 times higher than it should be for home testing. iii) It fast-tracked some alternative technologies to PCR, for point-of-care testing which we will describe a bit more below.

Towards point-of-care NAATs

The need for bulky and costly thermocyclers is a known disadvantage of PCR that hinders the use of NAATs for low resource areas or for low-scale purposes such as home testing. Recent advances towards miniaturization of PCR thermocyclers have led to more portable instruments and even though they generally have lower throughput, it is not so important for POC applications.

MiniPCR, for example, is a thermocycler that weighs 0.45 kg and is being used for research outside the laboratory,\(^\text{17}\) at schools for demonstration purposes and even in the international space station.\(^\text{18}\) Digital PCR and droplet digital PCR have achieved miniaturization of sample volume and reaction time, by splitting the sample into subsamples or droplets.\(^\text{19}\) Chip-based PCR systems perform PCR on chips produced affordably and in scale by micromachining with integrated heating and other functions. The chip-based PCR instruments are highly portable and affordable when compared with conventional ones.\(^\text{20}\)

The most promising step taken to simplify PCR has been the development of so isothermal DNA amplification techniques. These are new amplification techniques which do not require the advanced thermal cycling of PCR. Instead isothermal techniques generally amplify the DNA at a constant temperature.
Currently, a number of different isothermal DNA amplification methods exist\textsuperscript{21} which could be used for POC testing. Because of these technological advancements, thermal cycling and accurate temperature control is not needed in isothermal DNA amplification. This is an important step towards affordable and disposable NAATs, since it enables simple resistant heaters, or chemical heaters, or even body heat to be sufficient for DNA amplification. Three of the most common isothermal amplification methods are:

1. **Loop mediated isothermal amplification\textsuperscript{22}** uses four or up to six primers which offers very high specificity, since they recognize six to eight sequences of the target DNA. Two of these primers are designed to form DNA loops that enable exponential amplification rounds in which primers anneal to the loops and are extended DNA polymerase. This method is performed at 60-65 °C, which is higher than other methods and the primer design process is complicated.

2. **Rolling Circle Amplification** uses a circular target DNA sequence as template to extend a primer continuously producing a long single stranded DNA complementary to the DNA target.\textsuperscript{23,24} It is one of the few DNA amplification methods that can be performed at room temperature or up to 37 °C.

3. **Recombinase Polymerase Amplification\textsuperscript{25}** (RPA) is performed at 37 °C up to 42 °C in only 20 minutes and requires only two primers, making it one of the most suitable options for rapid home-testing. In this thesis we have focused mainly on RPA because of all the advantages that we motioned.
The enzyme recombinase and single-stranded DNA binding proteins assist in annealing the primers to the target DNA when it is double-stranded. Strand-displacing polymerase extend the primers to form amplicons and the process is repeated exponentially without the need for thermal cycling (Figure 1).

Other isothermal amplification techniques include; nucleic acid sequence-based amplification, helicase-dependent amplification, strand displacement amplification and more.
Sample preparation

The central part of this thesis has been the implementation of sample preparation, which is the first step towards home-based NAATs. We therefore discuss here briefly the structure of viruses and bacteria and methods to prepare the sample for nucleic acid testing.

The genome of bacteria is typically a large, circular and closed double-stranded DNA molecule arranged in a structure called chromosome and located inside the bacterial cell. Generally, bacterial cells have a cell wall surrounding their cell membrane which is responsible for the structural strength of the cell.\(^{30}\)

Virus genomes can be either DNA or RNA, single stranded or double stranded. Viruses cannot synthesize proteins unless they infect a cell host. Their genome is inside a shell made of repetitive protein molecules, the capsid. The capsid and genome together are called nucleocapsid which has a symmetrical shape. The shape, size and chemistry of viruses is highly variable, and some of them have additional structural layers surrounding the nucleocapsid.

NAATs target the nucleic acids present in a sample, which can be extracellular, but are often located in cells or in viral nucleocapsids. For this reason, nucleic acid extraction is the first step, in which the nucleic acids are released from the cells or viral particles.

Methods of extraction of nucleic acids

In both bacteria and virus, extraction of nucleic acids can be performed by physical methods or by solution-based methods. The main methods of extraction are:

1. Chemical lysis methods: include the alkaline method and the detergent lysis. The alkaline method uses high pH of about 11.5 to 12.5 to damage the cell membrane while SDS is also included in the mixture. This method is effective but time-consuming.\(^{31}\) Detergents are used to disintegrate the lipid bilayer of the cell membrane. This method is often combined with enzymes to achieve bacterial cell lysis,
because of the cell wall. SDS, Tween and Triton-X are some of the surfactants commonly used in lysis protocols. Chaotropic salts like guanidine, EDTA and urea are also typically included in lysis protocols.

2. Enzymatic lysis: Here the enzyme lysozyme can catalyze the breakdown of the bond between peptidoglycan molecules compromising the bacterial cell wall, and protecting the animal from infection. Once the cell wall has been damaged by lysozyme, water enters the cell which leads to cell lysis. Proteinase K is another enzyme often used in enzymatic cell lysis, or in combination with chemical lysis methods. Proteinase K can digest a wide range of proteins present both in the cell membrane and inside the cell. It is also often included in RNA extraction protocols to digest RNase, an enzyme responsible for the rapid degradation of RNA. Other enzymes used for cell lysis are lysostaphin, protease, zymolase and more. These can specifically lyse cells that are resistant to lysozyme and Proteinase K.

1. Osmotic shock: Here, the salt concentration around the cell is rapidly reduced and there is much higher salt concentration inside the cell than in its environment. Water then enters the cell rapidly and the cell bursts. This method is used for less stable mammalian cells but is also effective for some bacteria.\(^{32}\)

2. Thermal cell lysis: This is achieved by applying high temperature, close to boiling, to the sample. This method is effective for gram-negative bacteria, but not sufficient to release significant amounts of genomic DNA from gram-positive bacteria.\(^{33}\) Repeated freeze-thaw cycles is another method to damage cells and cause lysis,\(^{31}\) but it is time consuming and not preferred in diagnostic applications.

3. Mechanical lysis: Here, the cell is lysed by physically damaging the cell membrane or cell wall by powerful mechanical forces. Bead beating is a very common method used in laboratory scale, where beads are
mixed with the sample and agitated forcefully by a vortex or specialized equipment.\textsuperscript{34}

Nucleic acid purification

Following nucleic acid extraction, their isolation is the next step in sample prep. Here nucleic acids from the cell lysate are purified so they can be used for further analysis. The major techniques used today for purification include:

1. Solid-phase extraction: This uses the properties of glass fibers (silica) to bind DNA in the presence of high concentration of salts and releases it when the salt concentration is lowered. Proteins and other substances present in the lysate are then separated by centrifugation (or gravity, vacuum) and the nucleic acids can be washed with surfactants. The final step is elution of purified nucleic acids in water or buffer.\textsuperscript{35}

2. The organic extraction and precipitation method: This employs a phenol-chloroform solution and quinidine isothiocyanate to perform phase separation of nucleic acids which are in the aqueous phase from proteins in the organic phase.\textsuperscript{35} This method is effective and simple, but involves significant safety limitations as phenol is toxic and hazardous.\textsuperscript{36}

1. Paramagnetic beads method: Here beads can be used to first bind nucleic acids to their surface, and then be held in place using magnets while the content of the lysis is washed away, leaving the nucleic acids bound on the beads. Nucleic acid elution is the final step of this purification method.
1.3 Paper-based analytical tests and paper microfluidics

Microfluidic systems have been used to provide POC diagnostics outside of centralized laboratories eliminating the need for bulky equipment.\textsuperscript{37,38} Traditional microfluidics were based on silicon, and glass, and require advanced manufacturing methods. Later rubbers like poly(dimethylsiloxane) (PDMS), and plastics like poly(methyl-methacrylate) (PMMA), were used as alternatives to glass because they could be copied from melt to produce cheaper devices. The total systems that are needed for microfluidic systems are however still hardly portable or affordable, for example most of them require pumps and other auxiliary equipment.\textsuperscript{39} For these reasons, the field of paper microfluidics has appeared an alternative to typical microfluidics. Paper microfluidics promises to create simple and more affordable solutions for POC applications.

History of paper-based analytical devices

The first paper-based tests were dipsticks used to measure glucose in urine samples\textsuperscript{40}. A dipstick is a paper with stored reagents that is immersed in the liquid sample. The paper wicks the sample and a reaction of the sample with the reagents stored in the paper lead to a color change which can be observed by the human eye, just like in the pH paper. The most common dipstick test today is used to assess urine pH (Figure 2A), protein, glucose, red blood cell content\textsuperscript{41} and numerous other dipstick-based tests are now commercially available.\textsuperscript{42}
A more advanced version of the dipsticks tests appeared as the so called “lateral flow assays” (LFAs) (Figure 2B). In these paper-based tests the sample flows through different parts of a strip, that are made of different paper materials: a sample pad first prepares the sample for subsequent steps, the sample is then mixed with the conjugated particles in the conjugate pad, and migrates along a nitrocellulose paper membrane. The nitrocellulose contains conjugated particles bound to its surface in lines marking the detection and control zones. The sample passes through these detection zones, and accumulates at the absorbent pad, driven laterally by capillary flow.\textsuperscript{44} The entire system is often enclosed in a plastic cartridge for mechanical support and contamination prevention. One of the most common application of LFAs is the pregnancy test while they have also been used for detection in saliva, whole blood, serum, plasma, sweat and more.\textsuperscript{45}

In a direct lateral flow immunoassay, the target molecule binds to two complementary antibodies. If the target line appears, the result is positive. In competitive assays, the target binds to antibodies present in the test line, and hinders conjugate binding. The absence of target line indicates a positive test. Nucleic acid lateral flow assays use the same principles, but the target of the assay is a nucleic acid.

The most critical part of the LFA test is the ability of nitrocellulose that binds proteins in the detection lines,\textsuperscript{46} so they are immobilized while retaining both their activity as well as the wicking properties of the paper.
Nitrocellulose, or cellulose nitrate, is cellulose with nitrate groups having replaced most of the hydroxyl groups. Nitrocellulose is produced by nitric acid treatment of purified cellulose, and the nitrocellulose membranes with specified pores size used in healthcare applications are produced by phase inversion.\textsuperscript{46}

Nitrocellulose membranes were first used in biotechnology to immobilize RNA-DNA complexes.\textsuperscript{47} Later, a method to transfer DNA fragments from polyacrylamide gels for further analysis (Southern blot),\textsuperscript{48} and then RNA (Northern blot).\textsuperscript{49} Western blot is a method that includes the transfer of proteins from electrophoretic gels to nitrocellulose.\textsuperscript{50}

The first lateral flow test was commercially available in 1988 and made use of nitrocellulose to immobilize capture components of the test in specific bands of the membrane called test and control lines.\textsuperscript{51} Lateral flow tests have since revolutionized point-of-care testing thanks to their low cost and ease of use and distribution.

In lateral flow tests, nitrocellulose is used to immobilize proteins, which in turn bind labelled target biomolecules. As a result, the latter are concentrated in a line, which we can observe directly with our eyes. Immobilized enzymes are also used to great extent in the field of industrial biotechnology. The enzymes that catalyze a reaction are immobilized so that they can be used continuously, to increase their stability, and to enable easier control of the reaction.\textsuperscript{52} In this thesis we extend the use of nitrocellulose by applying it to sample preparation, as will be discussed in the coming chapters.

Lateral flow assays are very successful commercially and have been used widely for pregnancy tests, drug tests, and at massive scale for COVID-19 testing as any reader most probably has done at least once. They offer, however, limited functionality and cannot accommodate more complicated assays that require multiple reactions with different reagents. For this reason, more advanced paper-based diagnostics devices have been developed in the last decades as will be described in the next chapter.
Next generation paper-based analytical devices

To extend the lateral flow tests, the field ‘paper microfluidics’ emerged. The idea of paper microfluidics to extend the lateral flow test in two particular ways:

i) By patterning hydrophobic patterns on a paper, we enable the formation of many separate zones or channels for liquid on the same surface. Advanced patterns of hydrophilic channels are needed to enable more complex assays on paper-microfluidics. These designs are more complicated and need to have high resolution and reproducibility. The simplest method to define patterns on paper is by cutting. Simple scissors or a razor can be used for cutting but these methods are time-consuming and have low resolution and reproducibility. Craft cutter printers can be used for fast and reproducible fabrication, while laser-cutters offer higher resolution. An alternative to cutting is to design a pattern on paper to create defined hydrophilic channels. This was initially done using photolithography, similar to traditional microfluidics fabricated in the cleanroom. Less demanding alternative photolithography methods and chemical modification of cellulose were used for paper microfluidic fabrication in the coming years. A simpler and popular fabrication method is wax printing, where wax is applied on paper in some pattern, and then melted into the paper by heat. The use of commercial wax printers increased the versatility and popularity of wax printing for paper microfluidics, but these printers are now no longer available commercially. More recently, methods based on inkjet printing were developed. Methods that avoid the usage of strong organic solvents are safer and more environmentally friendly.

ii) By stacking many layers of papers (the most common substrate materials are filter paper, chromatography paper, nitrocellulose membrane) one can create vertical flow in 3D microfluidic systems. These paper-based microfluidic systems have been termed microfluidic paper-based analytical devices (μPADs). μPADs enable complex multi-step assays owing to the advanced channel designs, control of low and reagent storage and the stacking of several layers of materials. These 3D μPADs
offer an additional way to control flow by including removable hydrophobic barriers between hydrophilic layers. Different materials can be used to perform different tasks, such as filtration of crude blood sample. The fibrous material of μPADs enables the storage of reagents necessary for the assay. The stored reagent can for example be dissolved by the fluid during testing, and thus no active reagent addition is needed. Reagents can be added on μPADs by simple pipetting, reagent pencils, or by inkjet printers, which yield rapid and reproducible results.

The use of multiple layers is very important as it allows for more advanced multistep reactions: for example, one layer could be used for a protein binding, like in the LFA assay, another layer used for another chemical reaction and a third layer used as a filtering step. μPADs can thus be used to detect a variety of chemical and biochemical analytes. Many paper-based tests are immunoassays targeting antigens or antibodies. Lateral flow immunoassays are used commercial in massive scale for the detection of human chorionic gonadotropin hormone (pregnancy test) and SARS-CoV-2 infection. Enzyme immunoassays have also been performed on paper (ELISA).

Detection of target nucleic acids relies on the use of oligonucleotides with sequence complementary to the target. Typically, one or more of the oligonucleotides are labelled and can be detected either in a colorimetric, fluorescent, or electrochemical assay. Colorimetric detection most commonly employs gold nanoparticles, and can be performed rapidly without any equipment, since the results can be interpreted by naked eye. We have for example used this capability in Papers 2 and 4 to demonstrate multistep paper-based colorimetric detection related to NAATs. Fluorescent detection of nucleic acids can be performed on μPADs using labelled DNA oligonucleotide probes, or DNA intercalating dyes, and other methods. Electrochemical detection in paper-based diagnostics is commercially used in scale for the quantitative measurement of glucose in blood. Electrochemical detection can be integrated with electronics to provide automated quantitative readings. Electrochemical μPADs able to detect various viral and bacterial
pathogens have been presented. Both immunoassays and nucleic acid assays can be coupled with electrochemical detection.
1.4 Paper-based NAATs

NAATs consist of sample preparation, DNA amplification and DNA detection. Each of these steps requires today bench-top instruments and many manual steps performed by a trained technician. To enable the use of molecular diagnostics in the society, at resource-limited areas, and even at home, the fusion of NAATs with μPADs has been suggested.

Since paper microfluidics is versatile, and can integrate a variety of different reactions and functions while minimizing the use of external equipment, it has been used as a platform for to integrate all the steps of NAATs.

Integration of sample preparation

As discussed previously, in sample preparation for conventional NAATs, sample preparation for paper-based NAATs also consists of two main parts: nucleic acid release from the target pathogen, and nucleic acid purification. The first step is used to access the nucleic acids that are located inside bacterial cells or viruses. Some NAATs can yield sufficient sensitivity by detecting only free nucleic acids. In the case of infectious disease diagnostics, however, sensitivity is crucial and cell lysis is typically considered necessary.

Purification is needed because the sample can sometimes contain substances that can have inhibitory effect on DNA amplification or interfere with the nucleic acid detection method used in the test. In central laboratories, nucleic acids are purified from all components present in the sample including proteins, lipids and polysaccharides that originate from the pathogen itself, but also from other components present in the sample from the patient or from environmental samples. This is a multi-step process that requires fluid handling and separation techniques, which can be difficult to integrate into paper-based systems. Some of the isothermal amplification techniques, described in chapter 2.2, are quite tolerant to some of these impurities. For example, RPA can
be performed using crude lysate as sample\textsuperscript{75} and rolling circle amplification can even be performed inside living cells.\textsuperscript{76}

Impurities from body fluids or environmental samples, such as blood and soil, can have a larger impact on DNA amplification, so there is a need to perform partial purification. An example of paper-based partial purification that can be readily integrated in paper-based NAATs, is plasma filtration from whole blood.\textsuperscript{77}

Sample preparation methods that are integrated in paper-based NAATs currently in the literature are done either by: a) conventional means in articles that focus on amplification and detection, b) tube-based methods adapted for POC usage, or c) performing sample preparation using paper. Here, a brief description of categories b) and c) is included, as these represent sample preparation methods that can be directly used in POC scenarios or have the potential with further development.

**Tube-based sample preparation**

This category includes sample preparation method using chemicals and enzymes in tube. These methods are similar to chemical cell lysis in laboratory-based NAATs, but the protocols have been adapted to require less bulky instruments. The latter is often achieved by the use of paper to capture nucleic acids and enable purification by washing steps and transport of nucleic acids to the next stage of the test. Cell lysis is done by commonly using chemicals such as sodium hydroxide, guanidine thiocyanate,\textsuperscript{78} Triton-X100,\textsuperscript{79} dithiothreitol,\textsuperscript{80} sodium dodecyl sulfate,\textsuperscript{81} Proteinase K,\textsuperscript{82} Buffer AVL.\textsuperscript{83} These compounds, are often used in combinations and their major problem is that they inhibit enzymatic DNA amplification which is the next step of the NAAT. For this reason, they often need to be washed away prior to the amplification step which introduces additional steps and difficulty in integration.

**Sample preparation using FTA™ paper**

Sample preparation in paper is often achieved using the commercial substrate ‘Whatman® FTA™ card technology’,\textsuperscript{84} widely used in sample preparation within centralized lab testing for diagnostics, forensics and
research applications. This technology involves proprietary chemicals impregnated on a paper card. The chemicals then, in a single process that involves drying, lyse cells and denature proteins. The cards bind nucleic acids and preserve them over a long period of time. When the dried samples have been transported to a laboratory equipped for PCR testing, the cards are washed by a buffer included in the kit and water to remove chemicals that are detrimental to enzymatic DNA amplification. The DNA is then eluted from the cards for further processing, or parts of the card itself containing DNA is added in the PCR assay. This product has been used extensively for sample preparation in paper-based NAATs (Figure 3).70,72,85

Figure 3: Sample-to-answer NAAT with Whatman® FTA™ sample preparation, loop mediated amplification, and fluorescent DNA detection. Adapted and reprinted with permission from Reference70. © 2015 American Chemical Society.

Paper microfluidics are a means to integrate all steps of NAAT, including sample preparation, in an automated way, to make the test simple enough for home-testing. Early efforts to integrate sample preparation relied on the already established and effective FTA technology. To achieve this, they had to integrate drying steps and washing steps with different
buffers either manually,\textsuperscript{86,87} or in an automated fashion by including advanced valving systems (Figure 4).\textsuperscript{88}

Figure 4. A highly integrated sample-to-answer NAAT with advanced valves for fluid control. Adapted and reprinted with permission from Reference\textsuperscript{88}. © 2017 Royal Society of Chemistry.

Use of enzymes and heat deactivation

Enzymatic cell lysis has also been applied to paper-based NAATs. As discussed previously, enzymes such as lysozyme can lyse cells and release their nucleic acids for NAATs. These enzymes also inhibit DNA amplification like the chemicals mentioned in the previous section, but they can be deactivated by high temperature for a few minutes. Enzymatic lysis combined with heat deactivation of the enzyme was first shown to be an effective method of sample preparation for gram-negative bacteria, gram-positive bacteria and viruses, while enabling DNA amplification without further purification.\textsuperscript{89} The same group presented a chemical heater capable of providing heat for the deactivation of the enzyme, as a method to avoid the use of power and electronic temperature control.\textsuperscript{89} Finally, they developed a battery-powered, sample-to-answer, paper-based NAAT with enzymatic lysis for the
detection of methicillin-resistant *Staphylococcus aureus* (Figure 5). The use of enzymes that can be deactivated by heat for sample preparation allows for simpler integration and automation than washing steps, but introduces the need for high temperature.

Figure 5. Integrated sample-to-answer NAAT with enzymatic sample preparation. Adapted and reprinted with permission from Reference 91. © 2016 Royal Society of Chemistry.

**Integration of nucleic acid amplification**

As discussed previously, PCR needs for precise temperature control and thus for instrumentation and power supply, making its integration into POC tests difficult. This is why the development of isothermal DNA amplification techniques was instrumental in enabling affordable paper-based NAATs. These methods can amplify DNA at a constant temperature that is lower than the temperatures needed for PCR.

In this thesis, we have focused on RPA as the amplification method of choice as it is rapid, easy to design and adapt for detection of new pathogens, and operates at body temperature. The integration of the RPA
method in paper-based devices has been demonstrated by several groups.\textsuperscript{92} Basically this step requires storage of the RPA reagents in the correct paper matrix. The amplification then occurs inside the paper when the sample wicks into the paper in paper microfluidic systems. This part of the implementation into paper-based devices is considered solved in the context of this work. Instead, the focus of this thesis has been to eliminate the bottleneck for sample-preparation prior to isothermal RPA amplification. This bottleneck will be discussed below.

\textbf{Integration of nucleic acid detection}

The final step of NAATs is the detection of target nucleic acids relies on the use of oligonucleotides ideally using a sequence complementary to the target. Typically, one or more of the oligonucleotides are labelled and can be detected either in a colorimetric, fluorescent, or electrochemical assay.

Colorimetric detection can be done using the same principles as that used for the commercial lateral flow assay tests. Here the most commonly employed method uses gold nanoparticles attached to the amplified DNA, and subsequently captured by binding on the lateral flow nitrocellulose. The band can then be read by naked eye,\textsuperscript{68} eliminating the need for detection equipment (similar to the antigen tests). In paper 2, we employ this method as the final step for a sample-to-answer paper based NAAT.

Other methods include fluorescent detection of nucleic acids can be performed on \( \mu \)PADs using labelled DNA oligonucleotide probes,\textsuperscript{87} or DNA intercalating dyes,\textsuperscript{75} and other methods.

For digital readout, electrochemical detection can be used. Electroanalytical paper-based diagnostics is already commercially in use at large scale for the quantitative measurement of glucose in blood. Electrochemical detection can be integrated with electronics to provide automated quantitative readings. Electrochemical \( \mu \)PADs able to detect various viral\textsuperscript{76,77} and bacterial\textsuperscript{78,79} pathogens have been presented. Both
immunoassays and nucleic acid assays can be coupled with electrochemical detection.

The bottleneck of sample preparation

The integration of drying and washing steps for nucleic acid purification without manual steps has been shown successfully but the valving systems used are fragile and might complicate the production, transport and packaging of these home-based tests. Many of the proposed solutions have eliminated manual washing solutions with valving systems, but are not fully automated since they still require activation of the valves by the user. The use of enzymes for cell lysis that can be deactivated by exposure to high temperature is a procedure easier to automate than washing steps.

Ultimately, sequential washing steps or high temperature are only required for the step of sample preparation in paper-based NAATs. Isothermal DNA amplification techniques can be performed at mild temperatures that do not need power and they are single pot reactions. Similarly, the simplest DNA detection methods do not need a power source or additional steps of fluid handling. For these reasons, sample preparation is arguably the limiting step in the implementation and broad usage of paper-based NAATs. In this work, we proposed paper-based sample preparation methods that eliminate the need for washing steps or high temperature deactivation and show their use towards paper-based NAATs for both bacterial and viral detection.
2 Experimental rationale, results, and discussion

The present thesis focuses on addressing the bottleneck of sample preparation that was described in the previous chapter and on increasing the sensitivity of paper-based NAATs. This chapter is divided into four sections and provides an overview of the experimental work performed for this thesis, the rationale of the methods and experimental setups used, and a summary of the results obtained and the conclusions we can draw from them.

2.1 Enzyme immobilization on nitrocellulose (Paper 1)

Sample preparation in paper-based NAATs has been logically adapted from conventional NAAT sample preparation. Steps of the conventional process have been omitted or simplified. Chemical lysis has been the preferred method, since they require less external equipment and power source than mechanical and other methods. The chemicals used for cell lysis, however, are detrimental to downstream enzymatic reactions and need to be removed. Paper has been used in many cases to bind nucleic acids and facilitate washing steps or even concentration. An alternative method was to use enzymes, such as achromopeptidase (ACP), that if deactivated by high temperature, do not need to be removed. ACP has been used to lyse gram-positive bacteria that are resistant to lysozyme, as well as gram-negative bacteria and viruses.\(^{93,94}\)

In paper 1, we used nitrocellulose membrane, as a substrate to immobilize ACP so that it can be physically and simply separated from the sample after lysis. To achieve this, we first needed a method to effectively immobilize ACP on paper. We chose simple drying of enzyme from solution to nitrocellulose as the simplest way that requires the least amount of process steps and equipment, ensuring that the method is scalable. It is known that ACP is an RPA inhibitor as it digests the enzymes necessary for the reaction.\(^{95}\) An important question in this work was whether ACP could be immobilized on nitrocellulose to allow RPA, and
whether immobilized ACP is active and can be used for sample preparation.

To assess if the enzyme can be immobilized by this method, we performed RPA in a tube in the presence of ACP-paper in the tube. We analyzed the RPA products analyzed by gel electrophoresis and found that ACP immobilized on paper indeed had no significant impact on RPA. ACP in solution however as expected completely inhibited RPA, even at very low concentrations, indicating that ACP itself was able to digest RPA enzymes under these conditions.

Next, we indirectly investigated the stability of the bond between ACP and nitrocellulose. Densitometric analysis of target band intensity showed quantitatively that agitation did not release enough ACP to induce negative effects on RPA. To confirm that ACP immobilized on nitrocellulose remains active, and thus useful for sample preparation in paper-based NAATs, we examined its effect on RPA master mix over a longer period. We incubated the RPA master mix in a tube containing ACP paper for 60 minutes.

Apart from the effect of ACP paper on RPA, we investigated the stability of the immobilization method in a more direct manner by chip-based protein electrophoresis. ACP mixture contains proteins 30-50 kDa, and the respective bands could be observed. When compared with water in which ACP paper had been agitated thoroughly, the target band intensity was lower and no bands appeared at the target size when water in which ACP paper had been incubated for 60 minutes. These results show that ACP is strongly bound to nitrocellulose and does not leak into the solution when agitated or left to diffuse for at least hour which is enough for the NAAT tests.
2.2 Bacterial lysis by immobilized ACP (Paper 2)

After establishing a simple method of immobilizing ACP on nitrocellulose and showing that it remains active and is able to digest proteins in the RPA mixture, we then set out to use it for sample preparation towards paperbased NAATs in Paper 2. The potential of enzymatic bacterial lysis by immobilized enzymes is that it can allow for sample preparation without washing steps or high temperature. Specifically, we tested whether ACP paper can be used for enzymatic cell lysis of *S. epidermidis* and release genomic DNA for amplification. *Staphylococcus epidermidis* is a bacterial pathogen that often infects hospital patients, and can also serve as a model for *Staphylococcus aureus* and other gram-positive pathogens.

To investigate the effect of ACP paper on bacterial cells, we evaluated the amount of amplifiable DNA present in a suspension with lysed cells compared with untreated cells. For this purpose, we chose qPCR as it is highly sensitive and quantitative. The target DNA was detected at significantly lower Cq values for ACP paper treated samples, compared to untreated ones, suggesting that there was significantly more amplifiable DNA in the treated samples. We also performed enzymatic lysis in solution following a protocol already adapted for POC NAATs. The results showed no significant difference in the DNA released from ACP in solution compared to ACP immobilized on nitrocellulose.

Encouraged by these results, we went further and implemented ACP lysis in a sample-to-answer NAAT using RPA and lateral flow detection. We added bacterial culture diluted to 200 CFU/μl (a relevant concentration for many samples) with buffer in a lysis tube containing ACP paper and performed lysis at 37 °C. Next, we added 5 μl of lysate as template into a tube with RPA mixture. After 30 minutes RPA at the same temperature, we diluted the RPA product and detected the amplified DNA with lateral flow dipsticks.

Since both sample preparation with ACP paper and RPA can be performed at 37 °C, this is the only heating requirement for a sample-to-answer
To enable instrument-free NAAT, we used inexpensive, off-the-shelf hand warmers instead of a benchtop heater. To further simulate point-of-care testing conditions, we also used a swab to collect 5 μl diluted bacterial culture and transfer it to the lysis tube. We otherwise prepared the lysis tube as described previously and placed it in a chemical heater. qPCR results showed significantly higher amplifiable DNA in samples treated with ACP paper compared to samples with plain paper.

We then performed lysis as described above using a swab for sample collection and a disposable chemical heater for 40 minutes’ incubation. With the use of a disposable pipette to add 5 μl lysate to the RPA tube and performed RPA using a benchtop heater and lateral flow dipstick detection. The results showed significantly higher target band intensity for the positive samples compared with the negative ones.
2.3 Low-temperature nuclease digestion (Paper 3)

RNases are enzymes that digest RNA and can be found in any sample including saliva, urine, blood etc. These enzymes are a major problem for amplification methods that detect RNA, for example for RNA research and diagnostic tests for retroviruses. In conventional NAATs, some of the approaches used to prevent RNA degradation are the use of additives that inhibit RNase activity and storage of samples at -80 °C. In point-of-care testing, these additives need to be washed away or diluted heavily.

Proteinase K is widely used to digest nucleases and facilitate other functions in nucleic acid purification protocols. Proteinase K is however an inhibitor to DNA amplification and needs to be either removed or deactivated. In paper 3, we used the enzyme immobilization method described previously to bind Proteinase K on nitrocellulose (proteinase paper) and simplify its separation prior to downstream processes. First, we showed that the enzyme is strongly bound to the paper, and allows RPA to be performed directly after nuclease digestion by proteinase paper.

Next, we investigated whether proteinase K paper could digest pure RNase A. To this end, we used the commercial kit “RNase Alert v2” to measure RNase activity detection using fluorescence emission. We digested the RNAse using proteinase K paper in a tube containing 20 μl RNase solution at 50 °C. RNase A treated with proteinase paper displayed significantly lower RNase activity than the untreated sample, but similar activity as nuclease-free water. Plain nitrocellulose paper however had no significant effect on RNase activity. These findings show that immobilized Proteinase K can digest RNase A under these conditions.

It is known that saliva contains RNases which is a problem for the detection of retroviruses by NAATs in saliva samples. Here, we designed a NAAT where the sample was saliva collected by a swab, diluted, and then spiked by Influenza Virus A genomic RNA. We incubated this sample at 50 °C for 30 minutes and used it as template for RT-RPA, which was performed at 42 °C for one hour. Gel electrophoresis analysis showed no
amplification for the artificial sample, and appeared indistinguishable from the negative control. These results showed that RNases present in human saliva digest RNA and could lead to false negative results.

For this reason, we investigated whether proteinase paper could digest the variety of RNases present in human saliva, which is a complex sample matrix often used in real-world diagnostics. Indeed, the RNase activity was significantly suppressed in samples treated with proteinase K paper compared to untreated samples. For example, saliva sample diluted 1:16 had no significantly higher RNase activity than nuclease-free water, showing that these conditions fully deactivate RNases present in human saliva.

Paper 3 shows in summary that proteinase K immobilized on nitrocellulose can be used for sample preparation in NAATs targeting retroviruses in human saliva, to protect RNA from RNases present in the sample.
2.4 Enzyme-linked DNA detection (Paper 4)

Papers 1, 2, 3 introduce simplified, and effective sample preparation methods that enables point-of-care NAATs with very affordable and fully disposable equipment, and without manual washing steps. In paper 2, a sample-to-answer NAAT was also presented utilizing sample preparation with immobilized enzymes, isothermal amplification (RPA), and a lateral flow nucleic acid detection. This detection method is simple, off-the-shelf as it relies on commercially available nitrocellulose lateral flow dipsticks with immobilized protein strips. It does however have limited sensitivity as it relies solely on DNA amplification to increase the signal in positive samples.

Enzyme-linked oligonucleotide assays (ELONA) are more sensitive nucleic acid detection methods, since alongside DNA amplification they include an additional step of signal amplification using enzymes. The most commonly used enzyme is horseradish peroxidase (HRP), which reacts with TMB in the presence of hydrogen peroxide, generating a blue color.

To facilitate the implementation of this method we developed a 3D-μPAD that implemented ELONA. The method started with an RPA reaction performed in tubes. We designed the assay to produce amplicons that had FITC on one end and biotin in the other. We could then further bind these amplicons to commercially available magnetic beads with a streptavidin coated surface and to antiFITC-HRP conjugates, generating magnetic bead complexes.

A part of the innovation here was to then use nitrocellulose membrane to retain the magnetic bead complexes as these were added to the paper-device. This allows for the washing of the excess enzyme conjugates with an absorption pad collecting the waste. We could then execute the DNA detection step utilizing a paper layer which had immobilized TMB on its surface. The addition of hydrogen peroxide allowed the TMB in one paper layer to react with the beads in the other paper enabling the first paper-based ELONA. This ELONA assay displayed 70 times higher sensitivity.
when we compared its LOD side by side to the commercial lateral flow dipstick.

This paper shows an important step towards implementing more sensitive DNA detection in paper-based NAATs. It also demonstrates that established materials like beads, that are commonly used in labs, can be integrated into 3D µPADs.
3 Conclusions and future work

The main conclusion of this work is that immobilized enzymes can be used to facilitate necessary functions of sample preparation, i.e., bacterial cell lysis, and nuclease digestion, in point-of-care NAATs. Importantly, sample preparation by immobilized enzymes can be performed at mild temperature and does not require multiple manual washing steps or advanced valves and equipment. Unlike previous methods, sample preparation by immobilized enzymes does not need any instruments or systems beyond what is already necessary for paper-based NAATs. Hence, the work presented here can eliminate the bottleneck of sample preparation. Additionally, we present a method to integrate an ELONA for detection of amplified DNA in NAATs with increased sensitivity using 3D paper microfluidics.

In this work, several methods are performed in a tube format. For example, enzyme-treated papers are included in a tube where the reactions occur, and the test user needs to open the tube and use a disposable pipette to proceed with the test. Although this is not a complicated process and the general public is now accustomed to performing self-tests with some liquid handling (SARS-CoV-2 antigen tests), it is still not ideal. An ideal a paper-based test kit is envisioned to have eliminated all user steps after sample collection and addition to the paper-based device.

The sample-to-answer test presented in this work utilized lateral flow detection, which as discussed has limited sensitivity. This sensitivity can perhaps be improved by methods such as sample concentration by binding of nucleic acids on glass fiber, which can be readily integrated. More advanced detection techniques, such as paper-based ELONA and the recently developed CRISPR-based detections have demonstrated superior sensitivity and specificity, and can be integrated to improve the test.

Apart from integration and optimization of the paper-based NAAT, such tests need to be thoroughly validated and proven with extensive clinical
studies. The prototypes that we show here are not sufficient to establish these tests as equivalent to the conventional NAATs. Clinical tests performed in the field can locate areas of improvement and provide proof for the decision makers of healthcare. Due to the nature of these tests, it is important that they are tested outside of the laboratory and by untrained users. Clinical studies often use deactivated samples kept in the freezer, but to include sample preparation in the test, the use of fresh, untreated samples is necessary. The results ideally need to be compared with the widely accepted golden standard, qPCR and RT-qPCR.

For field deployment of paper-based NAATs and use in scale by the general public, further issues need to be addressed. The complete integrated version of the device needs to provide mild heating, prevent evaporation, prevent contamination of the test components from environmental components. Another important aspect of NAATs for self-testing which is often undiscussed is the risk of contamination from positive samples: If the amplified DNA is released to the environment after use, subsequent tests of the same disease may give false positives. This problem could be addressed by enclosing the device in a shell designed accordingly.

Finally, the reliance on paper-based self-tests widely in healthcare and pandemic necessitates, the instructions and rules to the users of the test need to be clear and easy to follow even for those less accustomed in using technology. This could be achieved by a software application with recorded instructions and live online assistance if necessary.
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