DNA BIOSENSORS BASED ON INTEGRATED ISOTHERMAL AMPLIFICATION-DETECTION STRATEGIES

Jonathan Sabaté Del Río

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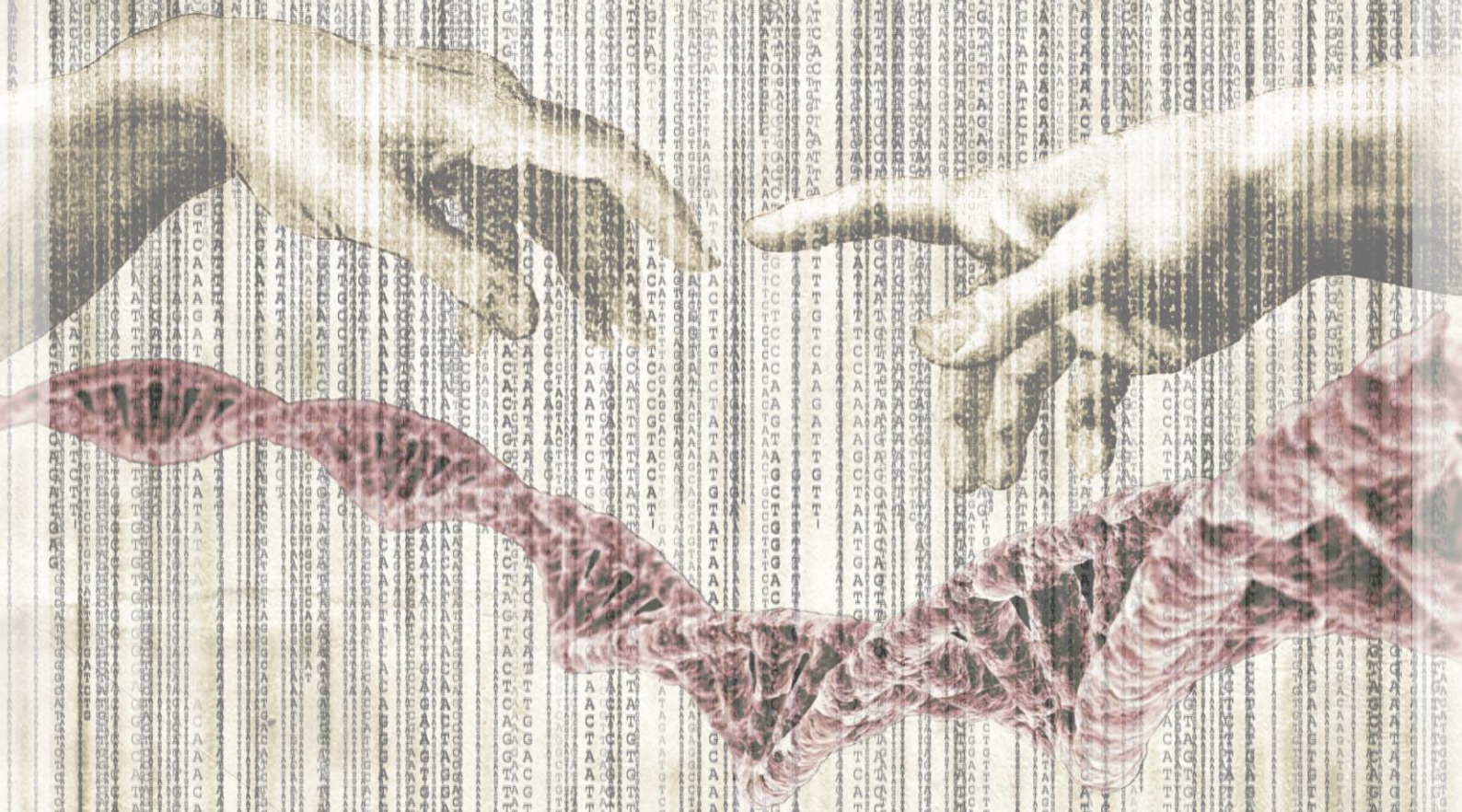

Jonathan Sabaté del Río

Doctoral Thesis

UNIVERSITAT ROVIRA i VIRGILI
Department of Chemical Engineering

Tarragona, 2015

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Doctoral Thesis supervised by

Dr. Ciara K. O'Sullivan

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CERTIFIES:

That the Doctoral Thesis entitled: "DNA biosensors based on integrated isothermal amplification-detection strategies" submitted by Jonathan Sabaté del Río in order to achieve the Degree of Doctor, has been carried out under my supervision, at Department of Chemical Engineering at Universitat Rovira i Virgili and that it fulfils all the requirements to be eligible for the International Doctorate Award.

Tarragona, September 1st, 2015

Dr. Ciara K. O'Sullivan

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- *Bueno, al fin nos vamos* – dijo Frodo.
Cargaron los bultos sobre los hombros, tomaron los bastones, y doblaron hacia el oeste de Bolsón Cerrado.

- *¡Adiós!* – dijo Frodo mirando el hueco oscuro y vacío de las ventanas. Agitó la mano, y luego se volvió; y (como siguiendo a Bilbo) corrió detrás de Peregrin, sendero abajo. Saltaron por la parte menos elevada del cerco y fueron hacia los campos, entrando en la oscuridad como un susurro en la hierba–.

El señor de los anillos
J. R. R. Tolkien

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Summary

The main goal of this Doctoral Thesis is to present alternative approaches in the field of DNA biosensors, designing and building new detection platforms that combine the amplification and quantification of targeted DNA while overcoming some of the current limitations. In order to achieve this objective, a variety of different strategies of effective solid-phase immobilisation strategies and isothermal enzymatic amplification have been explored to achieve lower detection limits with rapid and easy to execute assays.

This work presents a convenient, rapid, simple, easy to integrate yet robust biosensing detection platform that can bring new ideas for the integration of nucleic acid tests in point of care devices through the use of lab on a chip solutions. Ultimately, the vision underlining this work is to humbly contribute to the concept of decentralisation, allowing molecular diagnostics to move away from laboratories, providing bioanalytical information *in situ*.

The Thesis has the following structure: first, there is an introduction to the topic that describes the vision and the current trends in the field of molecular diagnostics, the role of isothermal DNA amplification and biosensors in this framework, the current state of the art and the objectives of the thesis. Chapters 2-6 report the work performed to achieve the specific objectives of this Doctoral Thesis: the use of solid-phase recombinase polymerase amplification strategy, as a hybrid concept that combines DNA amplification and detection, the optimisation of this idea, the detection of real samples, the exploiting of the surface chemistry and DNA to overcome the limitations found, and the use of ring resonators for the label-free and real-time monitoring of the RPA mechanism. The conclusions and the future prospects are finally detailed at the end of the Thesis.

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Resumen

El objetivo principal de esta Tesis Doctoral es presentar un distinto enfoque y alternativa de los sensores de ADN para diseñar y fabricar un nuevo tipo de plataforma que combine la amplificación con la detección de ADN, a la vez que se superan algunas de las limitaciones existentes en el campo. Para conseguir dicho objetivo, se presentan una serie de estrategias distintas basadas en explotar nuevas estrategias de inmovilización en fase sólida y amplificación enzimática para conseguir mejores límites de detección mediante ensayos rápidos y fáciles de ejecutar.

El trabajo expuesto representa un método rápido, simple y fácil de integrar a la vez que es una plataforma de biodetección que aporta nuevas ideas para la integración de los ensayos de ADN en dispositivos intervención en el sitio de interés a través de soluciones miniaturizadas o "lab on a chip". La visión que se persigue en última instancia a lo largo de este trabajo es la humilde contribución en este concepto ligado a la descentralización, permitiendo que el campo de diagnóstico molecular se mueva fuera del laboratorio para proveer información bioanalítica *in situ*.

La Tesis ha sido estructurada de la siguiente manera: primero, una introducción al tema de discusión que describe la actual visión y tendencias en el campo de los diagnósticos moleculares, el rol que poseen las estrategias de amplificación isotermas de ADN y los biosensores en general dentro del marco de trabajo descrito, el trabajo ya realizado y publicado en dicho campo, y los objetivos de la Tesis. Los capítulos 2-6 describen el trabajo realizado para conseguir los objetivos que se han perseguido en la Tesis Doctoral: el uso de estrategias de amplificación con polimerasas y recombinasas en fases sólidas como concepto híbrido que combina la amplificación y la detección de ADN, la optimización de esta idea, la detección de muestras reales, la explotación de los límites de la química de superficie y del ADN para desarrollar estrategias que suplan las limitaciones encontradas y el uso de "ring resonators" para monitorizar la evolución de dicho mecanismo sin marcadores y en tiempo real. Finalmente, las conclusiones y las perspectivas de futuro son descritas en detalle al final de la Tesis.

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Chapter

Introduction

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1.1 Introduction

This chapter presents a brief vision of future to understand the ongoing trends and challenges in the field of molecular diagnostics, and how isothermal DNA amplification biosensors can contribute to this goal. A short introduction to molecular diagnostics and scientific background dealing with nucleic acid tests is given. Biosensors are then presented as an interesting solution in this specific framework, discussing the principles involved in the fabrication of integrated amplification and detection platforms. Then an extensive research of the state of the art has been carried out to highlight the specific needs that have to be addressed. And finally, the objectives of the thesis are outlined.

1.1.1 A glance to the future

"Imagination is the first step in creation whether in words or trifles. The mental pattern must always precede the material form." – William W. Atkinson.

The quote from Atkinson exemplifies why the gift of imagination, along with the curiosity for understanding nature, is the flame that ignites the creative spirit in wonderers. Applied scientists need to be among these kind of enlightened spirits, not only because they need to know basic science, but because it is their burden to envision the future and shape the reality accordingly to accomplish their dreams. The process of designing the future, understanding design as the ultimate activity that translates an idea into reality, must be an unlimited act of imagination. It has been proved that hindering this process by limitations and constraints always leads to poorer end results. So it is a very healthy exercise to wonder how the future will be, and how we as scientists can humbly contribute a little bit to change the world we live in, adapting it to our vision. We should thus consider the future as a playground for our creativity, full of potential and opportunities. When we imagine how the future will unfold, it is easier to think in terms of trends, ideas or concepts instead of technologies, to see what is happening right now in our society, what motivates the ongoing transformations, identify the current needs and analyse how we are approaching these new problems to grasp the future.

1.1.2 Decentralisation of information

A major ongoing trend is the decentralisation of information at all of its levels, from data acquisition, to processing and decision making. In the future we will transition from bureaucratic hierarchies to technology driven networks, a change that has to deal on how information is organised. Bureaucratic hierarchies are the way the world is organised nowadays, *i.e.* in a pyramidal system in which the lower levels provide information to the cusp, where decisions are

made, and then these decisions flow back down to the lower levels where changes are made. Although it is an effective way of organisation, it is not particularly efficient, due to the fact that information needs to flow through many levels until it is processed, a decision based on this information is taken and an action is carried out. Currently, we live in an era of intercommunication of information and more efficient ways of dealing with this information flow process are appearing, thus changing the traditional organisational approach. Thanks to the internet and smartphones we can now live constantly sharing information in virtual environments that are able to organise services accordingly to our needs and criteria, without the direct intervention of centralised organisations. For instance, if we look at how traditional journals gather, process, and disseminate news and information to an audience, we see the base of the organisational pyramid composed by many journalists who gather that information and report it to a team of editors who decide what is worth to be published, then edit that information and submit it to the publisher. It is a rather inefficient, slow and constrained process that is centralised in one organism and therefore, the decision making is in most of the cases subject to private policies and interests. Internet, however, has no centralised governance in either technological implementation or policies for access and usage; thus each constituent network sets its own policies, resulting in most traditional communications media, including telephony and television, being reshaped or redefined by the internet, giving birth to new services such as Internet telephony and Internet television. Newspaper, book, and other print publishing are adapting to website technology, or are reshaped into blogging and web feeds. The entertainment industry, including music, film, and gaming, was initially the fastest growing online segment. The Internet has enabled and accelerated new forms of human interactions through instant messaging, Internet forums, and social networking. Online shopping has grown exponentially both for major retailers and small artisans and traders. Business-to-business and financial services on the Internet affect supply chains across entire industries. The old organisation model of our society is expiring, and the change is affecting our world and society in the way we interact, entertain, learn or make business, thus decentralisation allows information to be more free, obeying the own users' needs and not private interests.

A similar process as that described above is envisioned for objects instead of people, in order to build a global infrastructure network called the internet of things and that is expected to be a major trend in the future. Thanks to miniaturisation, higher computing capabilities and integration of sensors, regular objects can become smart objects, *i.e.* autonomous physical/digital objects augmented with sensing, processing, and network capabilities. A nice example of a smart

object is the self-driving car,[38] currently popularised by the Google's car, a vehicle that is capable of driving passengers to a specific location completely autonomously and only requiring a small intervention from the passengers. The concept behind the internet of things is to allow these smart objects to sense the environment, interpret what is occurring, but most importantly, to intercommunicate with each other and exchange information with people.[39] Following the example of the self-driven vehicle, the internet of things would allow one of these cars to find a parking place immediately via communicating with smart parking places, or communicate with other cars in case there is a medical emergency inside the vehicle, so the others could move aside to allow it to pass. The impact of such vision in reality is unknown, but apart from the benefits that smart objects themselves can offer, like better efficiency in terms of performance and energetic consumption, usefulness and adaptability, safety, etc. many different business opportunities will arise, as happened before with the advent of the internet. For instance, if we had a vehicle fleet composed by smart self-driven cars, it would not make sense to own a personal car anymore. Considering that, 90 % of a regular car's life-time is spent in the parking, there would be much more profitable ways to use a self-driven car as a common service like a taxi fleet, thus using it 90 % of the time. As this phenomena will grow in amplitude, we could start talking not only about smart object, but smart homes,[40, 41] smart medicine,[42] smart infrastructures,[43] smart cities[44] and ultimately, a smart world. However, before this vision can become a reality, there are also important social, economic and ethical implications that need to be addressed. There is an open question regarding the privacy and ownership of the data, moreover confidence in our environment will fade as nearly everything would be able to gather information from us.[45]

1.1.3 Personalised medicine through molecular diagnosis

In this context of decentralisation of information at the level of smart devices, the combination of nanotechnology, biotechnology and molecular biology together will revolutionise the field of health, wellness and healthcare through decentralised and personalised medicine.[46, 47] The concept, which encompasses the use of risk algorithms, molecular diagnostics, targeted therapies and pharmacogenomics, aims to provide people with information of their stable and dynamic genomic profile for an individualised therapy in order to facilitate rapid and correct health-care decisions. Molecular diagnosis describes a class of diagnostic tests that assess a person's health literally at a molecular level, detecting and measuring specific genetic sequences, including genes and single nucleotide polymorphisms (SNP) or the messenger RNA (mRNA) and proteins they express that are related to specific diseases. Analysis at molecular level could not only detect a specific disease in a person, but also predict if it is

predisposed to develop it and which treatment is likely to be more effective. Development strategies for personalised medicine aims to impact the course of the disease at six major points: disease predisposition, screening, diagnosis, prognosis, pharmagenomics and monitoring (Figure 1-1). Therefore, understanding of the underlying molecular mechanisms in the genome through molecular diagnosis is a critical step in the early stages of disease evolution.

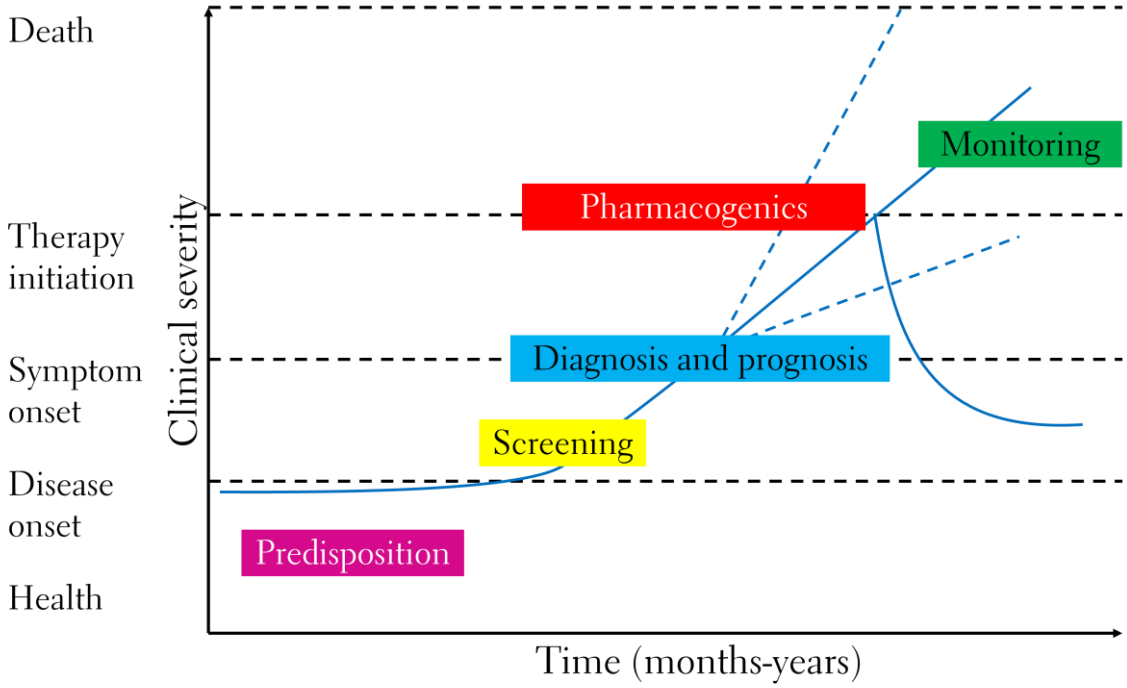

Figure 1-1. Research, intervention and personalised medicine opportunities at stages of a hypothetical disease.

1.2 Nucleic acid tests

The field of molecular diagnostics is represented primarily by the nucleic acid tests (NAT). A NAT is a molecular technique used for genotyping of human diseases, detection and identification of infections through a variety of diagnostic tests that analyse the presence in a sample of a particular nucleic acid sequence, gene expression, copy number, rearrangements of chromosomes, mutations, etc.[48]

Nucleic acids are biopolymers of nucleotides formed of three components: a pentose sugar (ribose for the ribonucleic acid (RNA) or deoxyribose for the deoxyribonucleic acid (DNA)), a phosphate group and one of the following nitrogenous bases: adenine (A), guanine (G), cytosine (C), thymine (T), uracil (U) o hypoxanthine (H) (Figure 1-2).

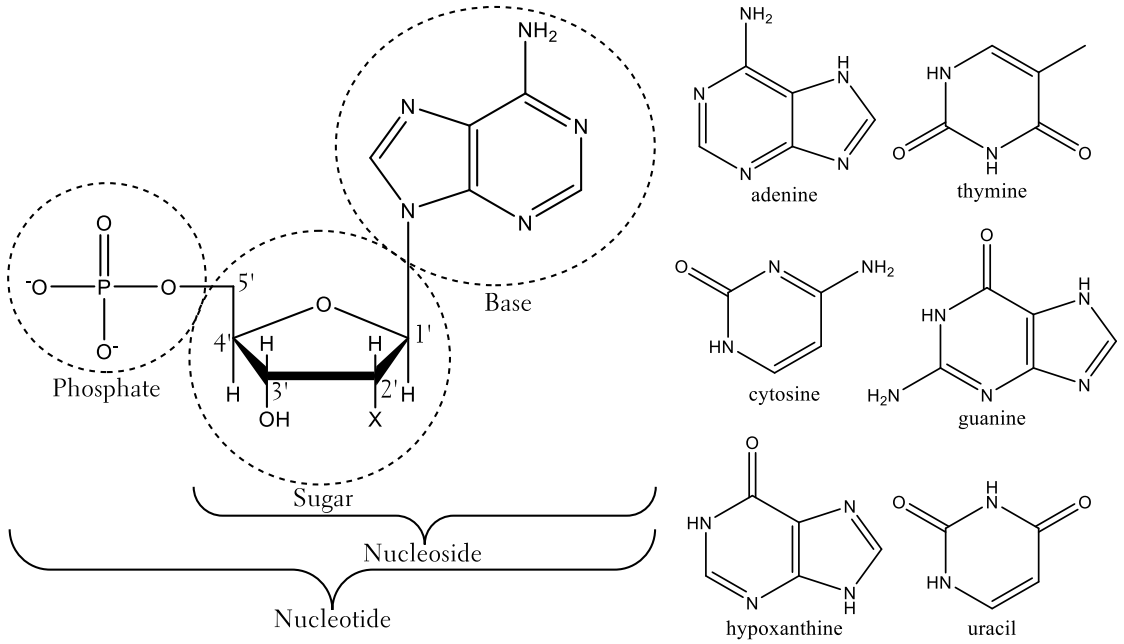

Figure 1-2. Structure of a nucleotide. -X is -H in DNA (2'-deoxyribose sugar) and -OH in RNA (ribose sugar), and natural nucleobases.

Different pairs of these bases, guanine-cytosine and adenine-thymine, exhibit affinity through hydrogen bonding patterns in a process called Watson-Crick base-pairing complementarity (Figure 1-3).

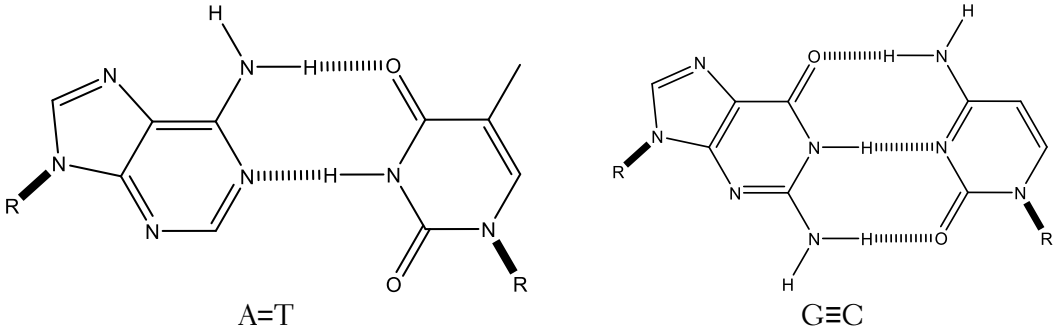

Figure 1-3. Watson-Crick base-pairing complementarity.

This phenomena leads to the formation of supramolecular self-assembly of nucleotides from nucleic acid strands to form a discrete helical supermolecule duplex structure in a process known as hybridisation (Figure 1-4).

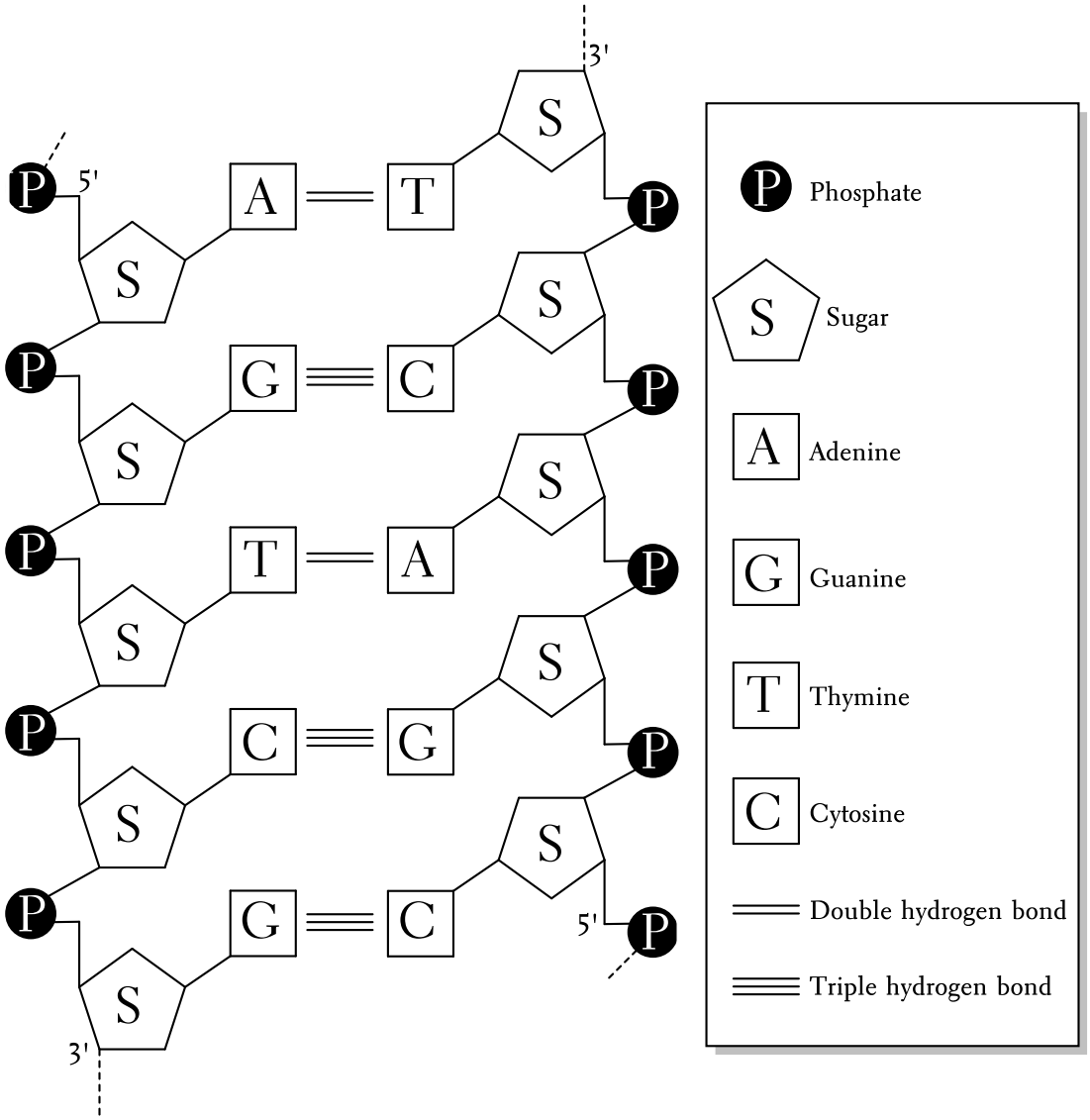

Figure 1-4. Hybridisation between two complementary DNA strands to form a duplex structure.

Nucleic acids are essential in all known forms of life and hold the function of encoding, transmitting and expressing genetic information, thus becoming an ideal and versatile target.[49] Along with proteins, nucleic acids are amongst the most exciting biopolymers in science, not only because of their major function in the process of life and their direct implication in modern biology and medicine, but also due to the fact that as a building block it holds a potential huge impact in the fields of nanotechnology, material science, molecular computing and bioanalysis. Nucleic acids can also be used for biorecognition, in the field of bioanalysis of pathological agents including virus, bacteria, fungi, genetically modified organisms, etc. It is of great importance in forensics for parental testing, cadaver identification and criminal investigation. Furthermore, in healthcare NAT are used for genetic disease profiling and patient stratification.

The number of achievements in the field of DNA and their implications in our knowledge of life is vast (Table 1-1), and research effort into the development of DNA diagnostic tools has increased dramatically.

Table 1-1. Brief overview of important nucleic acid test milestones.

Year	Milestone
1952	Electophoresis
1967	DNA ligase
1969	Fluorescence *in situ* hybridisation
1970	Restriction enzymes Reverse transcriptase
1972	Cloning
1975	Southern blot
1977	DNA sequencing
1980	Restriction fragment length polymorphisms concept
1982	P-element-mediated manipulation of the fly genome Whole genome shotgun
1983	Restriction fragment length polymorphisms realisation Polymerase chain reaction
1985	DNA fingerprinting
1987	Yeast Artificial Chromosomes Site-directed mutagenesis of the mouse genome
1988	Chromatin Immunoprecipitation
1990	Basic Local Alignment Search Tool
1992	Bacterial Artificial Chromosomes
1995	Microarray technology
1998	Sequencing by synthesis Full-length complementary DNA (cDNA) technologies
2002	Launch of UCSC Genome Browser
2003	DNA assembly programs
2004	Ensembl project. An example of a gene annotation tool
2005	HapMap Sequencing by ligation/polony sequencing
2006	Genome-wide maps of DNA methylation

From the discovery of DNA by Friedrich Miescher in 1869,[50] the rediscovery of Mendel's work led in 1900 to a better understanding and definition of the heredity concept.[51] The role of chromosomes in this process was established in 1902 by Walter Sutton while observing the segregation of chromosomes during meiosis,[52] and the word "*gene*", as well as "*genotype*" and "*phenotype*" to differentiate between the genetic traits of an individual and its outward appearance was coined by Wilhelm Johannsen in 1909 to describe the Mendelian unit of heredity.[53] In 1941 George Beadle and Edward Tatum's experiments on the red bread mould showed that genes act by regulating distinct chemical events, and proposed a direct link between each gene and enzyme. In 1943 William Astbury obtained the first X-ray diffraction pattern of DNA revealing they must have a periodic structure; and finally, in 1953, the structure of DNA was elucidated by Francis H. Crick, James D. Watson, Maurice Wilkins, Rosalind Franklin and many other contributors. Three years later, Crick proposed an explanation of the genetic

information flow within a biological system (replication, transcription and translation) in what is considered the central dogma of molecular biology, although this postulation was not published until 1970.[54] In 1966 Marshall Nirenberg figured out how the genetic code allow nucleic acids with 4 nucleotides to determine the order of 20 kinds of amino acids in proteins. DNA sequencing was discovered by Frederick Sanger's group and Alan Maxam and Walter Gilbert independently in 1975. In 1982, the GenBank was formed, an open public database where scientists begin submitting DNA sequence data. The polymerase chain reaction was invented in 1983 by Kary Mullis, allowing researchers to rapidly produce billions of copies of specific DNA sequences, revolutionising the field of DNA research. These achievements, among many others, resulted in the launch of the human genome project in 1990, a plan to sequence the whole human genome, all 3.2 billion letters, in 15 years. The first full human chromosome was sequenced in 1999, one year later 90 % of the human genome was already sequenced with a 99.9 % accuracy and finally in 2003 the whole human genome was sequenced opening a new era for unveiling and understanding all the information contained. Finally, the recent findings of how complex and important is the non-coding DNA as regulatory neighbours will allow us to understand human disease at the level of the molecules that are involved, thus dramatically changing the practice of medicine by leading to the development of new drugs, as well as to genetic testing to allow stratification for individualised treatments. All these achievements have been possible thanks to molecular techniques, and the desire for reaching a further understanding have also motivated the development of new ones. These techniques have also contributed to a wide number of milestones in nucleic acid research history, from the first techniques of electrophoresis to the recent genome-wide maps of DNA methylation.[55]

Working with nucleic acids also allows more possibilities than working with traditional enzyme or antibody assays that must be taken into account:

- Nucleic acids are stable in non-physiological conditions and solvents including ethyleneglycol, methanol, formamide, dimethyl sulfoxide (DMSO) or acetamide by forming hydrogen bonds, opening more opportunities for analysis in different environmental conditions.
- Chemical synthesis of nucleic acids yields high purity, reproducible batches and avoids ethical problems related to animal-based production.
- Specific base sequences can be easily produced in the laboratory by the means of enzymatic or solid-phase chemical synthesis, and are widely commercially available. Moreover, artificial nucleic acids with analogues in the backbone analogues,[56-59] the bases,[60, 61] as well as engineered brand new bases with specific pairing[62] and labelling by addition of a wide variety of functional groups allow nucleic acids to be adapted in

order to meet particular needs and become a flexible tool while maintaining its functionality.

- There is a large pool of enzymatic tools available for manipulation of nucleic acids including polymerases, ligases, telomerases, endonucleases, nicking enzymes, etc.
- Their small size allows efficient immobilisation while offering reusability and long-term stability.

1.2.1 Polymerase chain reaction

The field of DNA technology was revolutionised by the development of a method for the amplification of low number of DNA copies from samples. The polymerase chain reaction, developed by Kary Mullis in 1983,[63] provided a system that was able to exponentially amplify desired DNA sequence regions in a rapid and cost-effective manner. PCR permits amplification of short sequences of nucleic acids even in samples containing only minute quantities of nucleic acids with a polymerase enzyme, deoxyribose nucleoside triphosphates (dNTPs) and two primers that define the genetic region that will be amplified. Polymerases are enzymes that catalyse the polymerisation of a polynucleotide as a complementary form to a template strand. The final product of this reaction is a double helix composed of the template and the newly synthesised complementary polynucleotide. The thermostable Taq is an enzyme able to withstand the protein-denaturing conditions, *i.e.* high temperatures. A primer is a short strand that is complementary to a specific sequence in the template chain. There are three basic steps in this method (Figure 1-5). First, the original double stranded DNA (dsDNA) must be denatured, that is, the strands of its helix must be unwound and separated by heating to 90 to 96 °C. The second step (at about 50 to 55 °C) consists of primer annealing in which the primers bind to their complementary sequences in the single stranded DNA (ssDNA) molecules. The third step is the synthesis of a complementary nucleic acid strand mediated by the polymerase, at 72 °C, in a process referred to as elongation. Starting from the 3'-end of the primer, the polymerase reads the template strand, inserting complementary nucleotides that form phosphodiester bonds between each base. The product is two new double helices, each composed of one of the original strands plus its newly assembled complement. This cycle can be repeated many times, the amount of final product molecules being 2^n, where n is the number of cycles. As each cycle takes 1 min to 3 min, millions of copies of the original DNA can be generated in about 1 h.

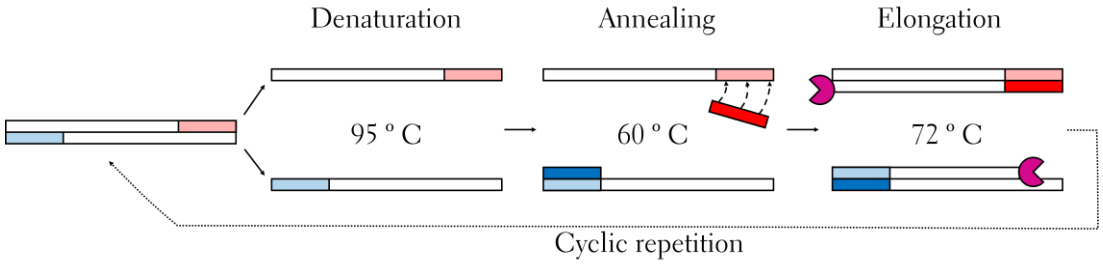

Figure 1-5. Schematic of the three steps in the polymerase chain reaction: melting, annealing and elongation.

A real-time polymerase chain reaction, *i.e.* PCR amplification with simultaneous real-time detection of targeted DNA molecule, is also possible by using either a method based on fluorescence detection of a non-specific fluorescent dye that intercalate with dsDNA during amplification[64] or a sequence-specific fluorescent-labelled DNA probe[65] that permits detection only after hybridisation with its complementary sequence.

1.2.2 DNA Microarrays

In the human genome project, DNA microarrays were extensively used for molecular screening of genetic sequences with great success due to the fact that simultaneous, multiplex and quantitative information could be achieved. The first use of microarray technology was early in 1982, for screening sequences expressed in a mouse colon tumour,[66] but the field rapidly experience huge growth during the following decades. Microarray technology consists of a collection of microscopic DNA probe spots arranged on a solid surface, the probes being small fragments of DNA that are complementary to a section of a specific genomic DNA sequence. Probe-target hybridisation is usually detected and quantified by using labelled targets to determine relative abundance of nucleic acid sequences in the sample (Figure 1-6.A) or by an indirect sandwich assay using a complementary labelled probe (Figure 1-6.A).[67, 68]

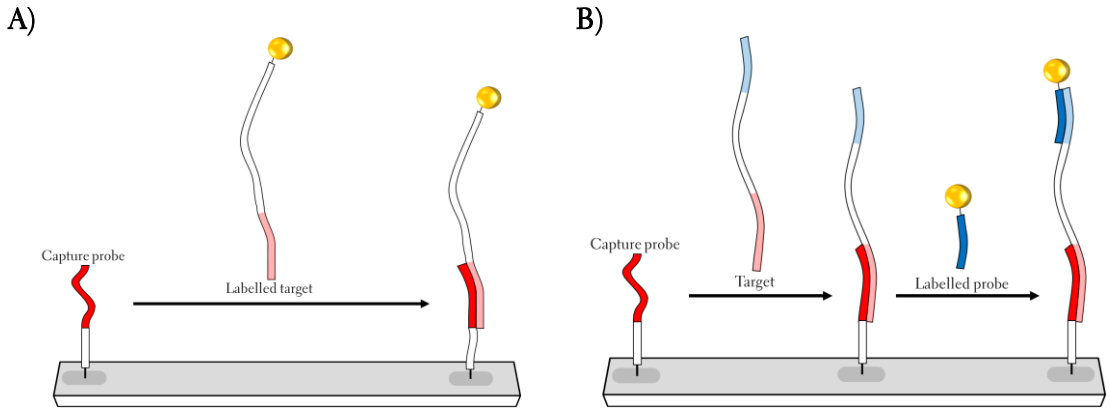

Figure 1-6. Microarray detection principle scheme using direct or sandwich-type labelling. A) Direct label of the DNA target. B) Sandwich-type label, indirect labelling of an additional complementary probe to the target.

1.2.3 Point-of-care testing

The reason some of the NAT still represent a gold standard in molecular diagnosis after many years in use is because they are robust, provide an effective solution in terms of performance, are well standardised and are commercially available. However, as the world's needs evolve to face new situations, it is evident that new scenarios require novel solutions and, to a large extent, traditional NAT techniques cannot meet these requirements.[69] For instance, there is currently a huge demand to provide rapid, *in situ* and cost-effective analytical solutions in low-resource-scenarios; however, traditional NAT are expensive, time-consuming, require trained laboratory personnel, laboratory facilities and therefore are not ideal for these scenarios.

The idea to move the analytical laboratory *in situ* to provide faster analytical results, thus facilitating rapid decision making, is based on the general trend of decentralisation (Figure 1-7), and is increasingly feasible idea through the advances in miniaturisation and microfluidics presented in lab-on-chip solutions. These point of care (POC) devices need to be smart objects, with the idea to make them self-sufficient and capable of handling all the analytical process: automated sample preparation, analysis, data evaluation and generation of analytical results.

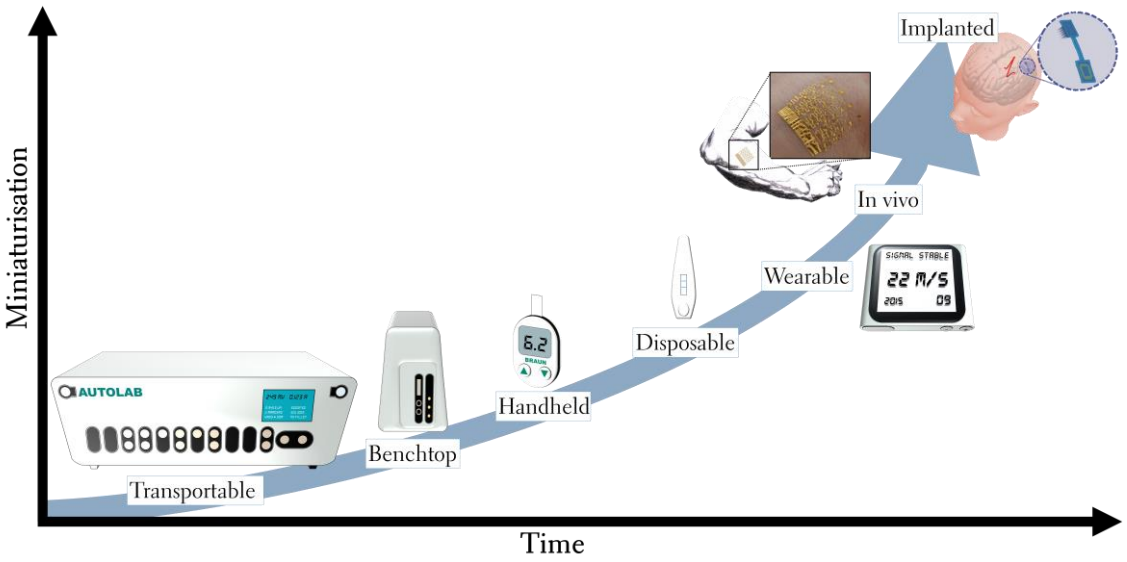

Figure 1-7. Decentralisation from transportable instruments to in-vivo monitoring.

We already have smartphone applications that measure heart rate,[70] body temperature, track personal exercise, the quality of sleep, detect flu outbreaks, and allow people to be aware and monitor their health, decentralising and minimising the need for use of the health service.[71] However, the sensors used in these devices are not yet able to provide biochemical information, and this is the reason why telemedicine is not yet a reality. Current lab-on-chip solutions allow molecular diagnosis to be implemented in point-of-care devices, thanks to nanotechnology and

biotechnology, providing biochemical information from real samples, *in situ* and in real-time. Although this decentralisation process of the analytical process is moving molecular diagnosis instrumentation out of the laboratories into portable[72] or hand-held devices,[73] best represented by the successful glucose meter,[74] the commercial implementations are modest, at best. Apart from moral and ethical reasons we are still far from achieving *in vivo* biosensing. Few biosensors have been reported for *in vivo* monitoring,[75-77] including wearable[78] and tattoo-based sensors,[79, 80] though most are only prototypes being implemented at a research stage, and there is a gap between what we have successfully achieved and the real integration of these systems in the market for wearable or *in vivo* devices.[81, 82]

1.2.4 Bio-microelectromechanical systems (Bio-MEMs)

Bio-MEMs can be defined as "devices or systems, constructed using techniques inspired from micro / nanoscale fabrication, that are used for processing, delivery, manipulation, analysis, or construction of biological and chemical entities". Other terms commonly used to refer to bio-MEMS include lab-on-a-chip (LOC) and micro-total analysis systems (μ-TAS).[83] The concept is intimately framed into life sciences, including the areas of diagnostics, therapeutics, hybrid biodevices, bio-inspired materials, etc. The success of this concept in the area of diagnostics relies largely on the effective integration of microfluidics, miniaturisation, electronics,[84] and biosensors[85] to generate self-sufficient devices capable of extracting bioanalytical information from real samples. A large number of applications have been presented,[86-88] and the current state of the art is slowly being introduced in the market as commercial products.

The enormous amount of genetic information brought by extensive genome sequencing has raised the need for simple, rapid, cost-effective and high-throughput miniaturised and mass-producible analytical devices to address the growing market of molecular diagnostics, thus accomplishing the basic criteria for decentralised DNA testing. Biosensors are expected to play an important role in the trend of decentralisation of molecular diagnostics by bridging the gap existing between traditional NAT and the definitive integration of POC devices through LOC solutions.[89] All in all, biosensors represent a new approach to molecular diagnostics as they provide an intrinsic miniaturisation over the traditional NAT, surpassing their drawbacks, and allowing easier, faster and cheaper results whilst keeping high sensitivity and specificity of detection (Table 1-2).

Table 1-2. Comparison of the characteristics between traditional NAT and biosensors.

NAT	Biosensors
Time consuming	Rapid, real-time detection
Expensive	Cost-effective
Laboratory monitoring	Portable (*in situ* monitoring)
Trained laboratory personnel	Simple use
High-tech equipment	Simple apparatus
Extensive sample preparation	Limited sample preparation
More organic solvent consumption	Less organic solvent consumption
Commercial availability	Limited commercial availability
Standarised	Non-standarised
Sensitive	Sensitive
Selective	Selective
Reusable	Reusable

1.3 Biosensors

The word sensor has a Latin root "*sentire*", which means "to perceive" originated around the 14th century. Therefore, a sensor is a device that is sensitive to input stimuli and can transform that information into a useful output signal that is proportional to the magnitude of the event.[90, 91] There are many different kinds of sensors based on the event they are able to detect, including temperature, pressure, sound, light, humidity, gases, motion, acceleration, displacement, etc.

Chemical sensors are specific kinds of sensors that can provide information about the (bio)chemical composition of their environment and transform that information into an output signal that can be monitored and quantified. The information extracted from the output signal may be qualitative, *e.g.* if a specific kind of analyte is present or not; or quantitative, by giving the concentration of the analyte.[92]

Chemical sensors have a minimum of two components: a **receptor** and a **transducer**. The receptor is a recognition element with specific sites that can interact selectively with the analyte, (*i.e.* the target of interest that needs to be detected). A transducer is a device that converts one form of energy to another form of energy. In a biosensor, the transducer is an element that transforms a characteristic (bio)chemical event into an output signal that can be processed (Figure 1-8).

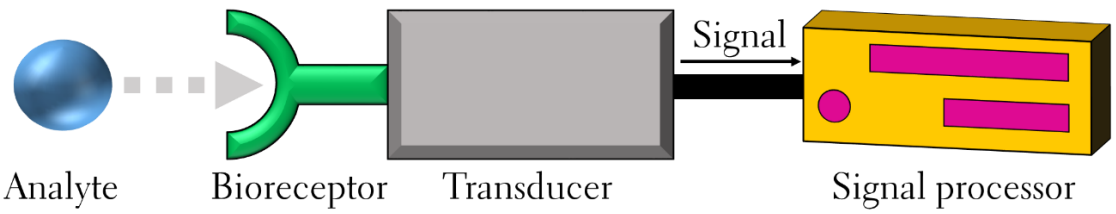

Figure 1-8. Schematic representation of the parts from a biosensor.

A biosensor is described by the International Union of Applied Chemistry (IUPAC) as is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction element.[93] Although a more general and simpler definition might be: "Biosensors are chemical sensors in which the recognition system utilises a biochemical mechanism".[94] The biochemical mechanism includes the use of biological material like peptides or proteins, lipids, liposomes, nucleic acids, whole cells, or even more complex systems like a plant or animal tissue.[90]

Biosensors can be classified regarding the recognition process:[95]

- Catalytic biosensors recognise, bind and chemically convert the analyte in a reversible reaction, thus yielding a product.
- Affinity biosensors rely on the reversible formation of an affinity complex between the analyte and the bioreceptor.[96]
- DNA biosensors (genosensors) is based on the hybridisation mechanism between two complementary DNA sequences as recognition mechanism.

Although this is a broad classification for biosensors, catalytic and affinity biosensors using DNA as receptors, *i.e.* DNAzymes and aptasensors respectively,[97, 98] should not be confused with DNA biosensors (or genosensors). In this thesis we will focus only on the description of genosensors. The solid-phase recombinase polymerase amplification-detection process can be regarded as a catalytic reaction due to the fact that the receptor (primers) and the analyte produce an amplified product by on the surface of the transducer by the use of enzymes.

Research publications related to DNA biosensors started growing in the late 1980s thanks to the invention of the Polymerase Chain Reaction (PCR) and has grown significantly during the last three decades due to the importance of and need for rapid, cheap and cost-effective gene detection systems in molecular diagnosis (**Figure 1-9**).

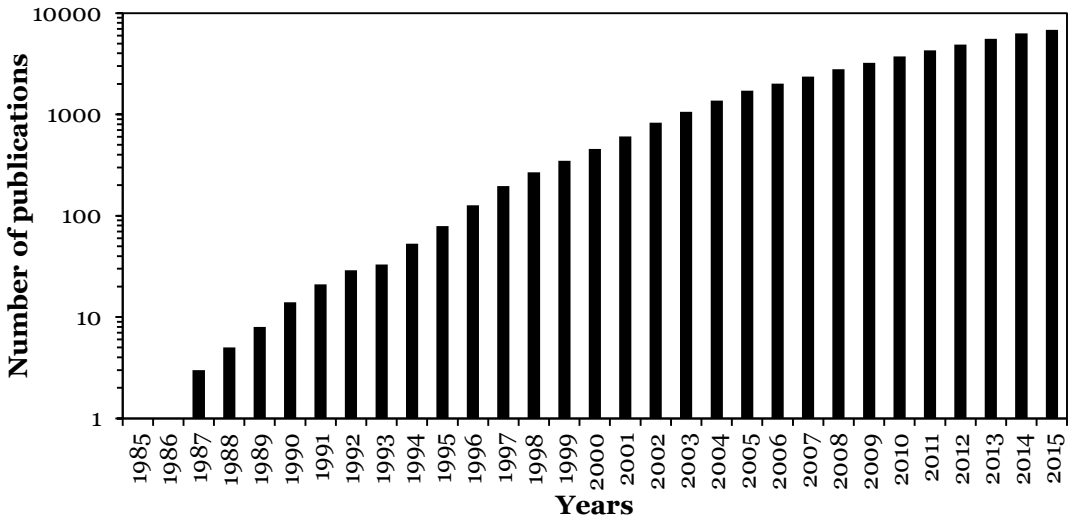

Figure 1-9. Number of papers published per year with the topic DNA biosensors.

Genosensors focus on the detection of relevant genetic sequences via hybridisation of the target with a complementary single stranded DNA chain receptor, that is linked and in close contact to the transducer, to form a double-stranded DNA duplex by Watson and Crick base-pairs (Figure 1-10).[69]

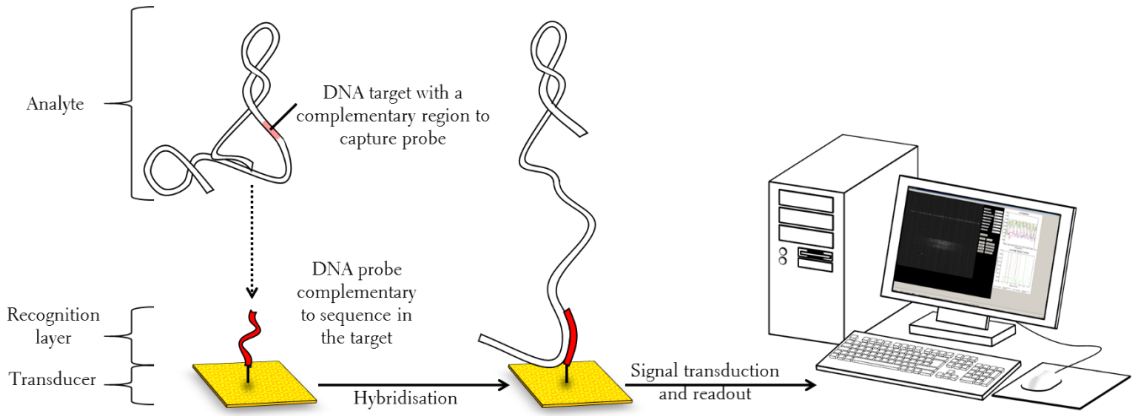

Figure 1-10. Hybridisation in a DNA biosensor. The hybridisation occurs between the ssDNA target and the complementary ssDNA probe immobilised on a transducer. The hybridisation event is transduced and the readout signal processed.

It is important to clarify that, as mentioned by Stakenborg *et al.*,[49] though the traditional definition of biosensors might include platforms like lateral flow strips or microarrays, these cannot be considered a true biosensors in the sense that the receptor element is not in intimate contact with the transducer.

The theoretical foundations regarding the quality parameters of biosensors used through the thesis, like the limit of detection or the sensitivity, are described in detail in the Appendix 4.

1.3.1 DNA immobilisation strategies

A large number of immobilisation strategies have been developed to anchor the single stranded DNA probes on the surface of the transducer, ranging from simple physiosorption to Langmuir–Blodgett films to direct and covalent immobilisation of biomolecules on surfaces using self-assembled monolayers or coupling to polymers. Each of these methods has their advantages and disadvantages. For example, adsorptive coupling (physisorption) is still widely used today in bioanalysis for microtitre plate-based assays (Enzyme-linked Immunosorbent/Oligonucleotide Assays, ELISA/ELONA) and for lateral flow strips. In both test formats, a regeneration of the immobilised component (antibody molecules) is not necessary. On the other hand, if a higher functional availability of the biomolecules is required, together with the possibility of regeneration and a low susceptibility to nonspecific binding, as in the case of biosensors and biochips, it is very likely that covalent attachment will yield superior results over physisorption. The main disadvantages of simple adsorption, including limited accessibility of the recognition sites of the biomolecules (for example due to interactions between the DNA bases) and desorption processes, can largely be avoided with covalent coupling methods.

The immobilisation step for the DNA probe is essential to achieve high sensitivity and selectivity while minimising nonspecific adsorption, and achieving stability and uniformity of immobilised probe layer. These monolayers form spontaneously by adsorption of suitable components from a diluted solution directly onto a surface. The formation of ordered and orientated monomolecular layers by spontaneous adsorption from a diluted solution is called self-assembling and the respective layers are called self-assembled monolayers (SAM) or self-organised monolayers. The selection of the substrate for immobilisation depends on the intended application. For example, SAMs are often used for the development of biosensors with electrochemical, piezoelectric, or optical detection. Glass and silica are typical materials for optical sensors, whereas gold is preferred for electrochemical sensors because of its outstanding inert properties. On the other hand, only certain chemicals can be used to form self-organising layers on these substrates.

Formation of SAMs from thiol compounds on gold surfaces

A well-researched and popular class of monolayers is based on the strong adsorption of thiols (R – SH), disulphides (R – S – S – R) and sulphides (R – S – R) onto metal surfaces. Although thiols, disulphides, and sulphides strongly align with a number of different metals including silver, platinum or copper, gold is usually the substrate of choice because of its inert properties and the formation of a well-defined crystal structure. The absence of impurities and

contaminants from the gold substrate is preferable but not absolutely essential, as thiols are able to displace contaminating compounds from the gold due to the high affinity of the sulphur groups to gold.[99]

Thiols, sulphides, and disulphides are dissolved in a sufficiently pure solvent and then applied onto the cleaned gold surface. As a first step, the sulphur donor atoms align strongly with the gold (typical bond energy 126 kJ mol^{-1})[100] and initiate the self-assembling process of the alkyl thiols on the gold surface. The respective bond between the sulphur and the gold is non-covalent. A novel structure is formed in which each sulphur atom is bonded co-ordinatively with three gold atoms, resulting in a lattice spacing of 0.4995 nm (as compared to an unmodified gold lattice with a spacing of 0.2885 nm). Thiol groups are deprotonated ($RSH + Au \rightarrow RS - Au + e^- + H^+$),[100] and for disulphides the S–S bond is cleaved during the process of adsorption. The sulphur is in a sp3-hybridised configuration, which is also the explanation for the tilting of the thiol chains from the surface level by 20–40° (Figure 1-11).[100] Furthermore, the axis of the alkyl chains are twisted by approximately 55° against the surface level.[100, 101] In a second step, the tail-tail interactions (non-binding interactions such as van der Waals, repellent, steric, and electrostatic forces) between the molecules are responsible for the parallel alignment of the molecules on the gold surface. In doing so, the tail groups form a crystalline film. Above all, van der Waals forces between the methyl groups of the hydrocarbon chains are responsible for the orientation and stabilisation of the monolayer.

The two-step mechanism for the formation of SAMs described above is reflected by the two-phase formation kinetics of monolayers: the diffusion-controlled adsorption is followed by the slower crystallisation process. Densely packed monolayers form in less than one hour from a diluted solution of alkane thiols, but it can take several days until this monolayer reaches a well-ordered state.

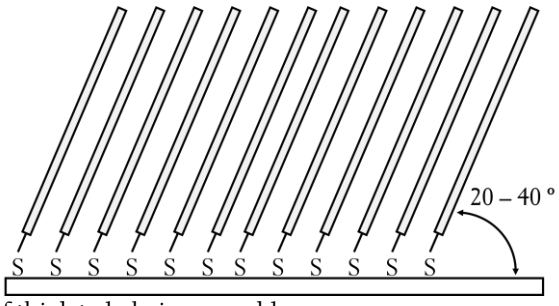

Figure 1-11. SAM tilting of thiolated chains on gold.

The terminal groups of the heterobifunctional thiol compounds are important for the interaction of the monolayers with biomolecules. For this purpose, several functionalised thiols

are used to couple biomolecules with the monolayer, for example amino-, carboxyl- and hydroxyl-terminated thiols. As long as the terminal groups (*e.g.* -NH_2, -OH) are relatively small (< 5 ˚A),[102] they have little or no influence on the orientation of the monolayers.

In addition to uniform monolayers formed using a single thiol compound, mixed SAMs are used for the immobilisation of DNA probes in order to avoid known disadvantages of uniform monolayers. With this strategy, the surface is more efficiently covered and non-specific binding of biomolecules is reduced, and the approach can also be useful for creating lateral spacing and controlling DNA probe density, to direct the orientation of the probes during the immobilisation or to suppress non-specific binding of analytes on the surface (Figure 1-12).

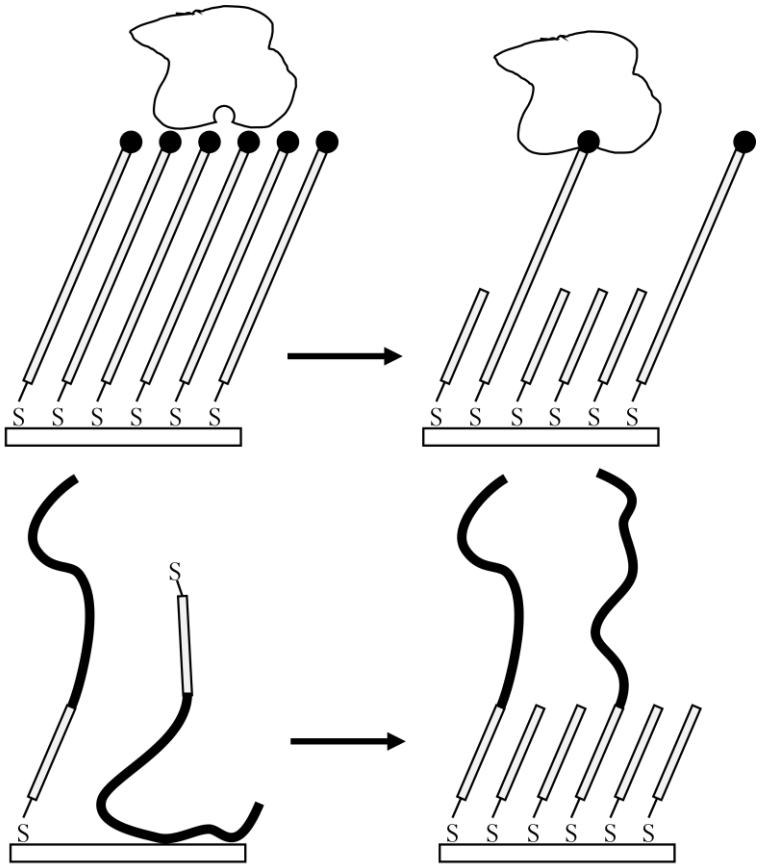

Figure 1-12. Schematic illustration of benefits from using mixed SAMs.

Silanisation

In general, the silanisation of hydroxyl-terminated substrates such as silica or glass is an effective method, which is used quite often for chemical modification of the substrate surface for the immobilisation of biomolecules. The main focus for silanisation procedures is once again the use of self-organising silane-monolayers. The properties of the monolayer depend on the

chemical structure of the silanisation reagent, the density of silanol groups available on the surface, and the physical surface structure on a nanoscale level.

The exact mechanism of silanisation depends on the reaction conditions. It is generally accepted that silanisation in a liquid solution is a three-step process.[103] In a first step, the previously mentioned silanes form silanetriols by hydrolysis in the presence of water, on the surface or in the solvent. These silanetriols attach themselves by physiosorption via hydrogen bonds onto the substrate surface. Subsequently, the silanol groups react with the free hydroxyl groups on the surface according to a SN_2 reaction mechanism (Figure 1-13). Similar to thiol SAMs, non-specific interactions can be reduced by mixing polyethylene glycol (PEG) terminated silanes into the monolayer.

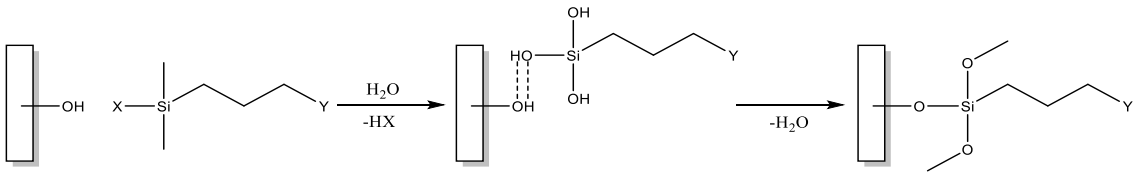

Figure 1-13. Scheme of the silanisation mechanism.

1.3.2 DNA functionalisation strategies

In general, nucleic acid modifications offer many functional groups that are suitable for coupling purposes. Using these moieties, a DNA probe can be coupled covalently via its functional groups to SAMs by a large number of suitable coupling compounds and reactions. A selection of such procedures is given in Table 1-3.

Table 1-3. Methods for covalent coupling.

Anchor group	Binding group	Reaction
-COOH	$-NH_2$	Via carbodiimide
epoxide	$-NH_2$, -SH, -OH	Direct
$-NH_2$	$-NH_2$	Glutaraldehyde
acid hydrazide	-CHO	Direct
-COR	$-NH_2$	Reductive amination
-OH	$-NH_2$	With bromcyane
chloromethyl group	$-NH_2$	Direct
-Azide	-Alkyne	Huisgen cycloaddition

A coupling method quite often used is the binding between avidin (respectively streptavidin) and biotin. Streptavidin and avidin have a very high affinity for biotin ($K_D = 10^{-15}$ M). Therefore, this combination is a suitable method for the stable attachment of biomolecules onto surfaces. Usually, the biological component is coupled to the small biotin molecule, often via a flexible spacer.[104]

1.3.3 Transduction mechanisms

Once the target DNA has been captured onto the genosensor surface, a range of different approaches can be used for transducing the biorecognition event. Electrochemical and optical transduction are by far the most commonly used mechanisms in genosensors and offer a wide variety of detection techniques (Figure 1-14).[49, 105]

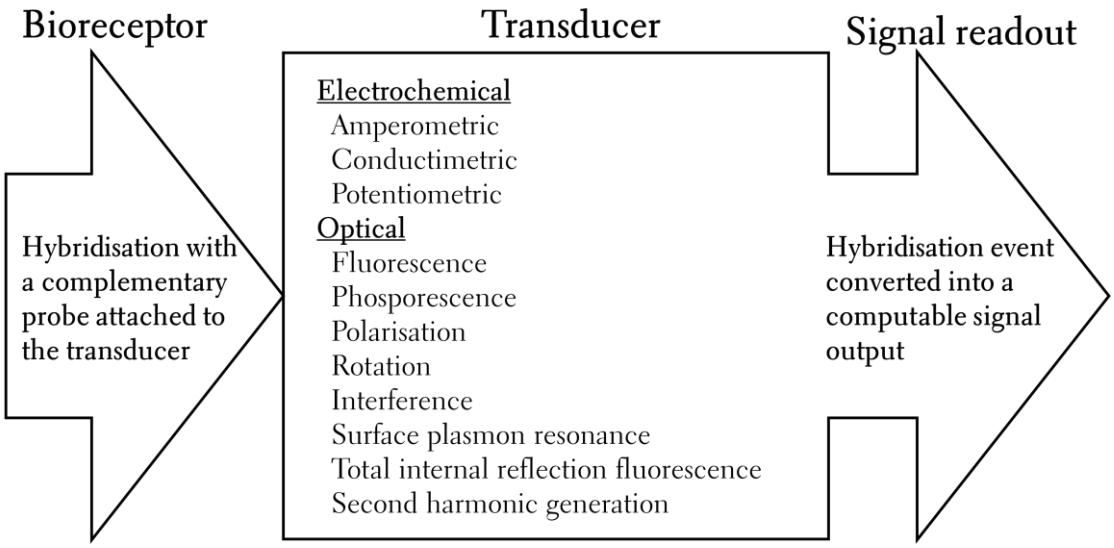

Figure 1-14. Transduction mechanisms typically employed in DNA biosensors.

Electrochemical methods utilise various electrochemical responses to measure changes in the electrical properties of the biological recognition element. Amperometric biosensors are based on monitoring the current associated with oxidation or reduction of an electroactive species involved in the recognition process. The current produced is linearly proportional to the concentration of the electroactive product, which in turn is proportional to the non-electroactive enzyme substrate. Transducers based on optical detection techniques have also been used in the field of biosensors. These may employ linear optical phenomenon, including fluorescence, phosphorescence, polarization, rotation, interference, surface plasmon resonance (SPR), total internal reflection fluorescence or second harmonic generation. Advantages of optical techniques involve the speed and reproducibility of the measurement.

1.3.4 Label *vs.* label-free methods

The majority of the monitoring methods for hybridisation events require the application of a label to detect the molecular recognition between a ligand and its receptor, though label-free strategies exist for both optical and electrochemical platforms. Although techniques that employ labels are highly sensitive due to the analytical characteristics of the label applied, the concept of direct detection interactions offers potential simplicity (Table 1-4) due to the fact that

the labelling steps involved in the indirect techniques impose additional time and cost constraints, and can in some cases interfere with the molecular interaction by blocking a binding site or leading to false negative responses.

Table 1-4. Comparative table of label *vs.* label-free detection methods.

Labelled	Label-free
Utilise electroactive signal generating labels	Detects a physical change in the system as a result of the biomolecular recognition
Labelled biosensors are more sensitive due to the amplification afforded by the enzymatic reaction or the electroactive label	Less sensitive especially to molecular recognition involving small molecules
Requires additional steps and increases the probability of error	Require fewer steps
Electroactive label may require high redox potential, which may destroy the selectivity of the biorecognition elements	Suitable for both in situ and ex situ measurements
	May facilitate the regeneration of the electrode surface using selected potential modulation

Direct label-free transduction of DNA hybridisation involves the measurement of physicochemical changes occurring on the surface of the transducer. Although this method can be achieved in electrochemical systems through DNA reduction[106] or electrochemical impedance spectroscopy methods,[107] it is usually reported for optical platforms involving surface plasmon resonance sensors in multiple formats, including Mach-Zehnder interferometers,[108] resonant interferometers,[109, 110] or surface-enhanced Raman scattering[111] to cite a few. The evanescence wave is an electromagnetic wave generated when light is completely reflected within the sensor surface in a phenomenon called total internal reflection. Evanescence wave penetrates beyond the optical interface into the lower reflection medium and interacts with the analyte allowing real-time and label-free monitoring capabilities due to the fact that the output signal is directly associated with adsorbed molecular mass.[112]

Electrochemical DNA sensors can detect the hybridisation through the charge transfer current between certain electron mediators that enable the reversible exchange of electrons with the electrode's surface. Typical mediators are $Co(phen)_3^{3+}$,[113, 114] osmium complex,[115] $Fe(CN)_6^{3-/4-}$,[116] $Ru(bpy)_3^{3+/2+}$,[117] ferrocene[118] and methylene blue.[119] The charge transfer process is usually monitored by electrochemical impedance spectroscopy, chronocoloumetry or voltammetric methods.

The electrochemical detection of DNA via redox-active indicators, such as DNA-intercalating and groove-binding compounds, possess a much higher affinity for the resulting hybrid compared to the single stranded probe. Accordingly, the concentration of the indicator at the electrode surface varies when hybridisation occurs, resulting in changed electrochemical signals.[120, 121] Commonly used indicators in fibre-optic and planar waveguide based sensors

employ the use of fluorescent labelled probes,[122] fluorescent intercalating dyes like ethidium bromide,[123] thiazole orange derivatives,[124] 3,3',5,5'-tetramethylbenzidine dihydrochloride[125] Alexa Fluor[126] and Cy5-labelled nucleotides.[127] In electrochemical platforms, frequently used compounds include organic dyes like Hoechst 33258[128, 129] ,methylene blue,[119, 130, 131] and organic drug small molecules including daunomycin,[132] doxorubicin[133] and anthraquinone[134] and metal-cation compounds such as like $Co(phen)_3^{3+}$[135] and $[Ru(NH_3)_6]^{3+}$.[136, 137]

Enzyme amplified strategies for DNA biosensors are widely used in electrochemical systems because they provide signal amplification that provides higher sensitivity, although additional reagents are usually required during the detection, thus confining the strategy solely for end-point detection. Among the most commonly used enzymes is horseradish peroxidase (HRP), which, for example, can catalyse the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H_2O_2, and the substrate can be later interrogated either by colorimetric[138] or electrochemical methods.[139] Glucose oxidase has been also used to electrochemically detect the hybridisation event[140, 141] and other enzymes including alkaline phosphatase or catalase are common.

1.4 Isothermal amplification techniques

Nucleic acid amplification is one of the most valuable tools in nucleic acid detection because it can amplify fewer than 10 target copies, significantly improving assay sensitivity. As mentioned previously, the importance of this technique and the impact on the specific field of DNA biosensors can be observed in the number of papers published per year since the introduction of PCR. Needless to say that, without PCR, not only the field of biosensors, but the whole field of DNA technology would be almost inexistent. However, the traditional nucleic amplification approach PCR, requires thermal cycling instrumentation, expertise and the technique is inherently confined to diagnostic laboratories. New techniques known as isothermal nucleic acid amplification overcome the current limitations of PCR by lowering the temperature cycling requirements.[142, 143]

1.4.1 Loop-mediated isothermal amplification (LAMP)

LAMP was first described in 2000 and developed by the Japanese Eiken Chemical Co, Ltd. (Tokyo).[144] The reaction employs a DNA polymerase with strand displacement activity and a set of two or three primer pairs that recognise 6 or 8 distinct sequences on the target DNA under isothermal conditions (60 °C to 65 °C). An additional pair of "loop primers" can further

accelerate the reaction by enabling the generation of a stem-loop DNA for subsequent complex LAMP cycling including self-priming reactions.

1.4.2 Nucleic acid sequence-based amplification (NASBA)

NASBA was first described in 1991,[145] The reaction requires an initial heating step of 95 °C (for DNA as template) or 65 °C (for RNA) to prepare accessible single strands, before the amplification takes place at a constant temperature of 41 °C. During the initial phase, reverse DNA primers containing a T7 promoter region, bind to any available target sequence in the sample. The primers are extended by the reverse transcriptase. The resulting RNA-cDNA hybrids are degraded by the activity of RNase H, leading to cDNA single strands. A forward DNA primer hybridises to these targets forming a new template, which can be elongated by the reverse transcriptase. This step integrates the T7 promoter region into the produced DNA, allowing a T7 RNA polymerase to bind, generating complementary copies of RNA. During the cyclic process, each synthesised RNA will initiate a new round of duplication, leading to exponential amplification in 90 minutes.

1.4.3 Helicase-dependent amplification (HDA)

HDA, firstly described in 2004,[146] makes use of the naturally occurring process of DNA replication. A helicase unwinds the target DNA strand at a temperature of 37 °C to circumvent the heat-induced denaturation step of PCR. The MutL protein stimulates the helicase unwinding, whilst single stranded binding (SSB) proteins prevents re-hybridisation of the separated ssDNA targets. The primers can hybridise to the free ssDNA and a DNA polymerase subsequently extends them. This exponential reaction can produce million-fold copies of target DNA in 60 to 120 min.

1.4.4 Strand Displacement Amplification (SDA)

The SDA reaction, which can be performed over a broad temperature range (37 °C to 70 °C), was already described in the early nineties applying multifunctional primers, both having target sequences for the directed hybridisation to the DNA strand of interest and a restriction site for endonucleases.[147] After the heat induced strand separation of the dsDNA, the primers bind sequence-specifically introducing a restriction site into the product. Bumper primers, which bind adjacent to the first primer, are elongated by a polymerase with strand displacement activity, releasing the first single stranded amplicon. In a second step, the reverse primer, including a nickable restriction site, is elongated. An endonuclease cleaves the restriction sites only at one strand, because thiol-modified nucleotides are incorporated to prevent cutting of the whole

dsDNA strand. The free 3'-end is subsequently extended, displacing the new single stranded copy molecule. This process of nicking and displacing leads to exponential amplification of DNA.

1.4.5 Rolling circle amplification (RCA)

RCA, first described 1998,[148] exploits the excellent strand displacement activity of a Phi29 bacteriophage polymerase on target molecules. The isothermal reaction allows for the amplification of a single stranded DNA to generate a continuous catenated product of up to 0.5 Mbases. Padlock probes are linear oligonucleotides containing two target specific sequences designed to circularise after hybridisation and subsequent ligation. The dual recognition in combination with a ligation reaction ensures specificity of detection. After that, the circular padlock probe serves as a template for the polymerase, which continuously elongates the product and displaces the generated strand.

1.4.6 Recombinase Polymerase Amplification (RPA)

The RPA[149] is particularly impressive as compared to all other isothermal amplification techniques as saturation is typically achieved within 5 to 10 minutes at an optimal constant temperature of 37 °C and no initial thermal or chemical melting of DNA is required, making it ideal for POC tests (Table 1-5).[143]

Table 1-5. Summary of isothermal nucleic acid amplification techniques.

Method	Preferred template	Amplicon type	Performance	LOD (copies)	Amplification temperature (Initial temp.) (°C)	Primers required	Interventions (temperature/ reagent additions)	Multiplex demonstrated	Detection
LAMP	ssDNA	Concatenated DNA	109 – fold in 60 min	~5	60-65 (95)	4-6	2	Yes	Intercalating DNA dye, fluorescence probe, SPR, turbimetric, lateral flow dipstick
NASBA	RNA	RNA	Detection within 60 min	1	41 (65/95)	2	2	Yes	Fluorescence, electrochemicasl, molecular beacons
HDA	dsDNA	dsDNA	1010 – fold in ~100 min	1	64	2	1	2 plex plus internal control	Fluorescence, visual detection, electrochemical
SDA	ssDNA	dsDNA	107 – fold in 2 h	1	37 (95)	2 DNA/RNA chimeric primers + 2 bumper primers	2	-	Fluorescence, lateral flow dipstick, electric-field-driven immunoassay
RCA	Circular ssDNA	Concatenated ssDNA	109 – fold in 90 min	10	30-65 (95)	1	2	-	Fluorescence, electrophoretic, flow cytometry, AGE, AFM
RPA	dsDNA	DNA	Detection in 20 min	1	37	1	-	Yes	Propietary fluorescence probe, lateral flow dipstick, visual detection, electrochemistry, ring resonators

The method couples isothermal recombinase-driven primer targeting of the template material with the strand-displacement DNA synthesis. A RPA cycle is initiated by the binding of a recombinase to the primers in the presence of Adenosine triphosphate (ATP). The resulting protein-DNA complex is highly efficient at scanning dsDNA to identify homologous sequences in targeted DNA sequences. The recombinase proteins then facilitate the strand-inversion and the formation of a D-loop structure where the primers are introduced at the cognate site of the template, leaving the 3'-end of the oligonucleotide accessible to a strand displacing DNA polymerase whose displacement activity creates a single strand, which is stabilised by single stranded binding proteins. The polymerase elongates the primer according to the template sequence and exponential amplification from just a few target copies is rapidly accomplished by the cyclic repetition of this process (Figure 1-15).[150, 151]

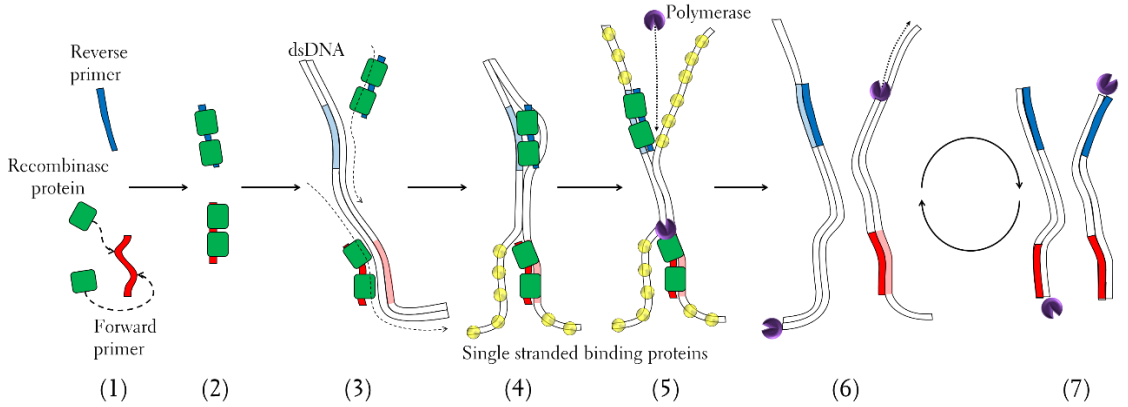

Figure 1-15. Recombinase polymerase amplification in solution. (1) Primers conjugate with recombinase proteins (2) to form oligo-protein complexes. (3) The complex scans dsDNA for homologous sequences, (4) conjugating the oligo-protein complexes with double stranded targets at the cognate sites, introducing the primers by a strand-displacement mechanism and stabilising the displaced strand with single stranded binding proteins. (5) Polymerase starts DNA amplification at the free 3'-end sites from the primers. (6) Forward and reverse primers are elongated and (7) the process is repeated cyclically through exponential amplification

The application of RPA in the field of molecular diagnostics has garnered rapidly increasing interest since the first report of the technique in 2006 by Piepenburg *et al.*,[149] with a rising number of publications detailing a wide variety of different applications of the technique (Figure 1-16).

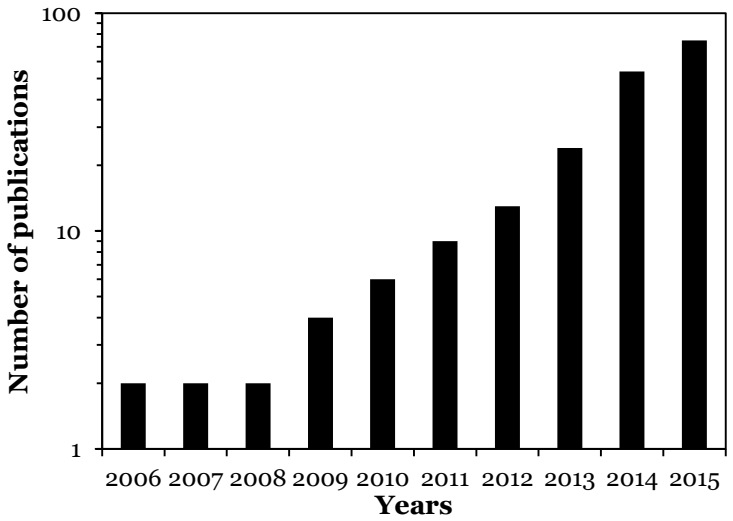

Figure 1-16. Number of papers published per year with the topic recombinase polymerase amplification.

Fluorescence detection methods using EvaGreen binding dye,[8] or sequence-specific fluorophore-quencher probes (exonuclease fluorescent probes (EFP)),[1-5, 7, 9-16, 20, 21, 23, 24, 30, 32, 34, 35] along with lateral flow strips,[3, 7, 17, 26, 29, 31-33, 36] configure the majority of the reported systems for the detection of nucleic acid sequences from real samples using RPA that can be considered POC/*in situ* assays. Lateral flow strips is the go-to option in low resource scenarios as it is a cost-effective solution with a non-instrumented and simple visual detection system where a qualitative end-point assay is sufficient, and fluorescence detection systems can provide real-time monitoring where quantitative measurement is required. However, both approaches are not compatible with true multiplexing, and to this end some reports have detailed efforts to overcome this limitation, relying on purpose-made systems based on arrays. Examples include DNA arrays on epoxy-silanated glass slides, and amplifying with fluorescently-labelled reverse primers using a microarray scanner for the end-point reading.[27] Another alternative reported was a ring resonator setups which was able to achieve multiplex, label-free and real-time monitoring but due to its complexity and cost, this approach is unlikely to be implemented in POC devices.[6, 152, 153] Microarrays nested in Digital Versatile Disc (DVD) platforms, using both labelled[22, 25] or label-free[37] detection strategies have also been reported to be capable of multiplex amplification. However, the amplification and the detection steps are still performed in separate places and a fully integrated platform has not yet been developed. Kim *et al.* reported on a lab-on-disc device that integrates the nucleic acid extraction step with the recombinase polymerase amplification, but the detection step is performed separately in flow strips.[18] A full review of the published analytical techniques using RPA is displayed in Table 1-6.

Table 1-6. Comparative table of RPA analytical methods published.

Target	Type	Label	Amplification	Detection	Platform	Ref.
Mycoplasma capricolum	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[2]
Foot-an-mouth virus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[4]
Mycobacterium tuberculosis	DNA	-	On-chip fluidics with thermoelectric cooler	Wavelength shift	Ring resonator chip	[6]
Aptamer-based bio-barcode	Aptamer	EvaGreen	Not described	Fluorescence	Microtiter plate	[8]
L. interrogans.	DNA	EFP	Real-time PCR detection system	Fluorescence	Real-time PCR detection system	[10]
Phytophthora	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[12]
Influenza virus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[13]
Dengue virus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[16]
Salmonella	DNA	Specific kit	Lab-on-disc device with laser-based temperature controller	Visual indication	Lateral flow strip	[18]
Fungi	DNA	Biotinylated reverse primer	Incubation chamber	Visual colour change after enzymatic reaction	Plastic slides	[20]
Salmonella and *Cronobacter*	DNA	Digoxigenin primer, and biotinTEG probe	Oven	Colour change	DVD	[22]
Group B *streptococci*	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[23]
GMO	DNA	Digoxigenin labelled primer	Oven	Optical transmission after enzymatic reaction	DVD	[25]
3 pathogenic bacteria	DNA	Fluorescent labelled reverse primers	Hybridisation chamber	Fluorescence	Microarray scanner	[27]
HIV proviral target DNA	DNA	Fluorescent or hapten-labelled primer	Heat by sodium acetate mixtures	Lateral flow strip	Lateral flow strip	[29]
Cryptosporidium scpecies	DNA	Fluorescein isothiocyanate and biotin-labelled primers	Incubation chamber	Visual indication	Lateral flow strip	[31]
HIV proviral target DNA	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[32]
Mycobacterium tuberculosis	DNA	Hapten-labelled primer	incubation chamber	Visual indication	Lateral flow strip	
	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[34]
Plasmodium falciparum	RNA	Fluorescent and digoxigenin labelled primers	Incubation chamber	Visual indication	Lateral flow strip	[36]

Target	Type	Label	Amplification	Detection	Platform	Ref.
White spot syndrome virus	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[1]
Plum pox virus	RNA	Biotin and carboxyfluorescein-labelled primers	Portable heat block	Visual indication	Lateral flow strip	[3]
	RNA	TAMRA-labelled fluorogenic probe	Fluorometer	Fluorescence	Fluorometer	
Schmallenberg Virus and Bovine Viral Diarrhea Virus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[5]
Dengue virus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[7]
	RNA	Biotin and fluorescent-labelled antibodies	Heat block	Visual indication	Lateral flow strip	
Shiga toxin–producing *E. coli*	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[9]
GMO	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[11]
Francisella tularensis	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[14]
Dengue virus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[15]
Giardia	DNA	Biotin and fluorescent-labelled antibodies	-	Visual colour change	Lateral flow strip	[17]
Allergens, GMO, pathogenic bacteria and fungus	DNA	Digoxigenin labelled primer, biotinylated probes	Oven	Visual colour change in microtiter plate reader	Microtiter plate, ELISA format	[19]
Staphylococcus aureus	DNA	EFP	Fluorometer	Fluorescence	Fluorometer	[21]
Middle East Respiratory Syndrome Coronavirus	RNA	EFP	Fluorometer	Fluorescence	Fluorometer	[24]
Chlamydia trachomatis and *H. sapiens* GAPDH	DNA	Biotin and carboxyfluorescein-labelled primers	Incubation chamber	Visual indication	Lateral flow strip	[26]
Penaeus stylirostris	RNA	-	Incubation chamber	Visual indication	Gel electrophoresis	[28]
Bacteria containing blaCTX-M-15	DNA	EFP	Integrated heater	Microscope imaging of fluorescence	Active matrix electrowetting-on-dielectric Thin Film Transistor	[30]
Little cherry virus	RNA	EFP	Fluorometer	Visual indication	Lateral flow strip	[33]
Yam mosaic virus	RNA	EFP	Real-time PCR instrument	Fluorescence	Real-time PCR instrument	[35]
Salmonella	DNA	Digoxigenin labelled primer	Oven	Change in optical density	DVD	[37]

1.5 Recombinase polymerase amplification in combination with DNA biosensing detection

One of the crucial limitations of regular DNA biosensors is their inability to directly hybridise and detect dsDNA, which is the common form in genomic DNA from real samples. Therefore, most of the analytical methods include an additional sample pre-treatment step for generating ssDNA that adds complexity to the assay, irreproducibility, increases the analysis time required and usually need the use of additional reagents and/or instruments like. Some of these solutions include thermal denaturation, labelling of the target, triplex formation, asymmetric PCR or the use of exonuclease digestion combined with magnetic beads for separation. Additionally, the high temperatures required for denaturing dsDNA, or the temperature cycles produced in PCR amplification techniques could risk the stability of the weak bonding of DNA.[154]

An alternative method to circumvent these limitations is the use of an isothermal amplification system like recombinase polymerase amplification that not only amplifies the amount of genetic material thus increasing the sensitivity, but also facilitates hybridisation between the target and the probe and the target denaturation step with enzymes is constant and at low temperature.

1.6 Objectives

The general objective of this doctoral thesis is the development of a detection platform exploiting a combined strategy of isothermal solid-phase amplification technique with genosensor detection for a simple, sensitive and rapid analysis of genetic material.

This general objective is divided in the following specific objectives:

- To demonstrate solid-phase isothermal recombinase polymerase amplification is able to successfully amplify DNA from bulk solution through the immobilisation of primers on the transducers and be detected by an electrochemical or an optical platform.
- To apply solid-phase recombinase polymerase amplification concept for the electrochemical detection of genomic DNA from pathogens in the analysis of real samples.
- To explore the selectivity of solid-phase recombinase polymerase amplification to successfully perform multiplex analysis of different nucleic acid targets.

The main added value of this thesis is that it presents a flexible solution for detecting DNA with biosensors, exploiting a general concept of solid-phase amplification and detection, thus integrating two nucleic acid tests, PCR and microarrays, in a single device.

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UNIVERSITAT ROVIRA I VIRGILI
DNA BIOSENSORS BASED ON INTEGRATED ISOTHERMAL AMPLIFICATION-DETECTION STRATEGIES
Jonathan Sabaté Del Río

Chapter

Electrochemical detection of *Francisella tularensis* genomic DNA using solid-phase recombinase polymerase amplification

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Electrochemical detection of *Francisella tularensis* genomic DNA using solid-phase recombinase polymerase amplification

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2.1 Abstract

Solid-phase isothermal DNA amplification was performed exploiting the homology protein recombinase A (recA). The system was primarily tested on maleimide activated microtitre plates as a proof-of-concept and later translated to an electrochemical platform. In both cases, forward primer for *Francisella tularensis holarctica* genomic DNA was surface immobilised via a thiol moiety and then elongated during the Recombinase A mediated amplification, carried out in the presence of specific target sequence and reverse primers. The formation of the subsequent surface tethered amplicons was electrochemically monitored using a HRP-labelled DNA secondary probe complementary to the elongated strand. The amplification time was optimised to amplify even low amounts of DNA copies in less than an hour at a constant temperature of 37 °C, achieving a limit of detection of $3.3 \cdot 10^{-14}$ M ($2 \cdot 10^5$ copies in 10 µL). The system was demonstrated to be highly specific with negligible cross-reactivity with non-complementary targets or primers.

2.2 Introduction

The polymerase chain reaction (PCR) is a widely used DNA amplification technique to increase low amounts of genetic material, where during the amplification of target DNA, thermal cycling is required, and precise temperature control is crucial to the efficiency of the PCR, and becomes even more important when attempting to amplify several DNA targets in a single reaction. Recently, isothermal enzymatic DNA amplification systems have emerged including nucleic acid sequence-based amplification (NASBA),[1] loop-mediated isothermal amplification (LAMP),[2] rolling circle amplification (RCA),[3] helicase-dependent amplification (HDA),[4] and recombinase polymerase amplification (RPA).[5] RPA is particularly impressive as saturation is typically achieved within 5 to 10 minutes at an optimal constant temperature of 37 °C and no thermal or chemical melting of DNA is required.

RPA is based on the use of an enzymatic mixture of polymerases and DNA recombination proteins. Recombinase proteins assemble along ssDNA present in solution, *e.g.* primers, to form a stable helical filament. The resulting protein-DNA complex is highly efficient at scanning dsDNA to identify homologous sequences in targeted DNA sequences. The recombinase proteins then facilitate the strand-inversion and the formation of a D-loop structure where the primers are introduced at the cognate site of the template, leaving the 3'-end of the oligonucleotide accessible to a strand displacing DNA polymerase. The polymerase elongates the primer according to the template sequence and exponential amplification from just a few target copies is rapidly accomplished by the cyclic repetition of this process.[6, 7] To date, several examples of RPA have been reported, including the amplification of DNA from bacteria and/or viruses.[8-10]

However, RPA does suffer from some drawbacks, such as the production of DNA by-products of random sequences due to the formation of primer-dimers when the target DNA is present in low concentration. To overcome this problem tetrahydrofuran (THF)-modified primers and more complex enzymatic solutions are required, which has considerably hindered the application of RPA in multiplexed amplification. A facile means of addressing this drawback could be achieved by immobilising the forward primer on a specific surface, providing a support for solid-phase amplification and multiplexing capability by functionalising with different surface primers in an array format (Figure 2-1.A).[11, 12] Detection of the amplified primers is carried out optically or electrochemically after a series of steps of denaturation, hybridisation with a biotin labelled primer and conjugation with a streptavidin-HRP protein that catalyses the oxidation of a TMB substrate (Figure 2-1.B).

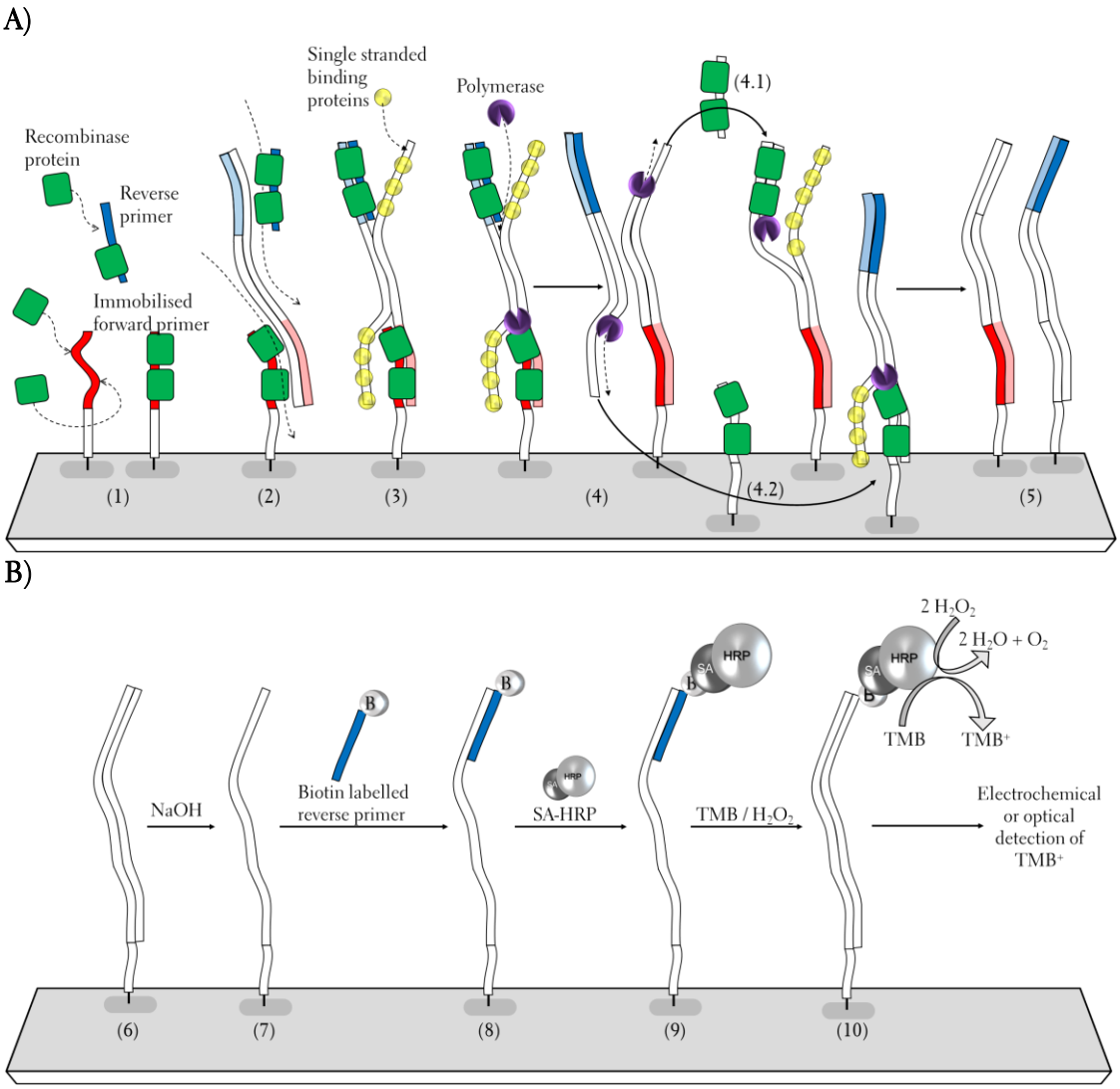

Figure 2-1. **A)** Schematic representation of Solid-phase RPA. (1) Recombinase proteins form a complex with forward and reverse primers, (2) scan dsDNA for cognate sites and (3) introduce the primers in the template by a strand-displacement mechanism. (4) The polymerase initiates primer elongation at their 3'-ends and exponential amplification is achieved by cycling of this process either in (4.1) liquid or (4.2) solid-phase. (5) Reverse primers end up ligated with the elongated forward primers in solid-phase. **B)** Detection protocol. (6) The solid-phase amplified product is denatured with NaOH, (7) hybridised with biotin labelled reverse primer, (8) and conjugated with streptavidin-HRP. (9) TMB / H_2O_2 substrate is added and (10) TMB^+ is detected by either optically or electrochemically.

We report a demonstration of isothermal solid-phase RPA of *Francisella tularensis.* A proof of concept of the approach is first demonstrated on microtitre plates using an enzyme linked oligonucleotide assay (ELONA) with optical detection, and subsequently transferred to an electrochemical platform (Figure 2-2). The time required for maximum amplification was optimised and the specificity of the approach evaluated using non-specific DNA templates.

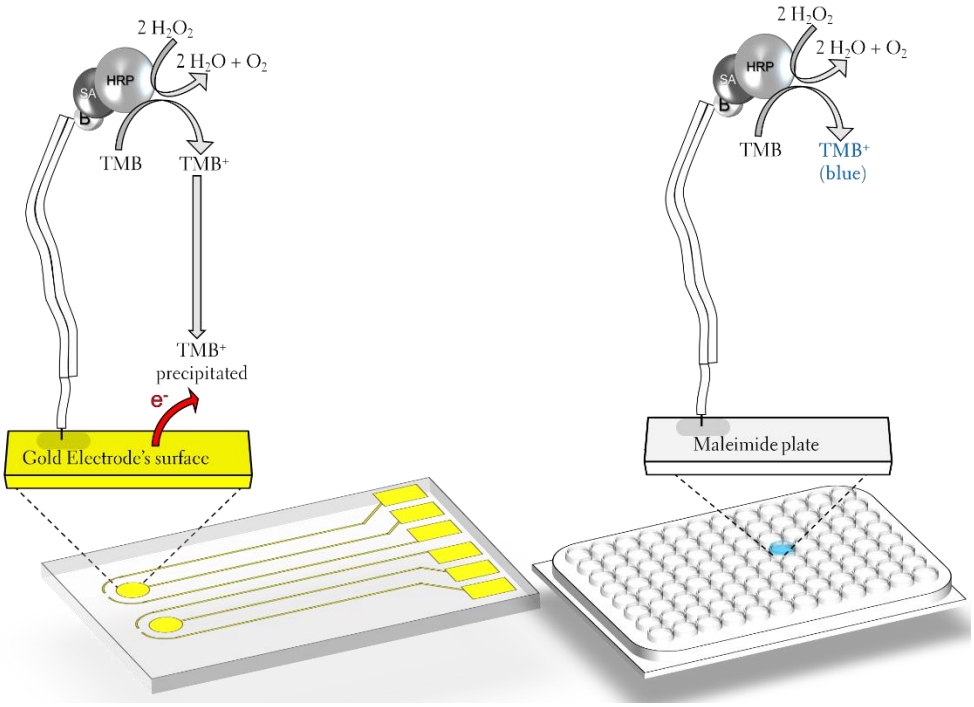

Figure 2-2. Electrochemical and optical detection strategies of solid-phase RPA products.

2.3 Materials and methods

RPA kit TwistAmp® Basic obtained from TwistDx Ltd. (Babraham, United Kingdom). Maleimide activated plates were obtained from ThermoScientific (Madrid, Spain). The HRP substrate formulation TMB enhanced one component HRP membrane was purchased from Diarect AG (Germany), GelRed™ Nucleic Acid Gel Stain (Biotium, Barcelona, Spain) and low range ultra agarose gel powder was from Bio Rad Laboratories S.A. (Barcelona, Spain). 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DSP) was obtained from Fluka (Barcelona, Spain). 6 mm thick polymethylmethacrylate (PMMA) was purchased from La Indústria de la Goma (Tarragona, Spain), Double-sided medical grade adhesive foil from Adhesive Research (Ireland) and all other chemicals were obtained from Sigma Aldrich S.A. (Barcelona, Spain) and used as received.

Synthetic oligonucleotides designed for the identification of the pathogenic bacteria *Francisella tularensis holarctica*[13] were purchased as lyophilised powder from Biomers.net (Ulm, Germany), reconstituted in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain) and used without further purification (Table 2-1).

Table 2-1. List of oligonucleotide sequences and their respective modifications.

Name	Nucleotide sequence (from 5'-end to 3'-end)
F. tularensis forward primers	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCC (regular, 5'-SH-$(CH_2)_6$-T_{30}- and NH_2-$(CH_2)_6$-T_{30}-)
F. tularensis reverse primers	CGCTACAGAAGTTATTACCTTGCTTAACTGTTA (regular and 5'- biotin)
F. tularensis DNA sequence	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCCAGAAGGTTCTA AGTGCCATGATACAAGCTTCCCAATTACTAAGTATGCTGAGAAGAA CGATAAAACTTGGGCAACTGTAACAGTTAAGCAAGGTAATAACTTC TGTAGCG
Non-complementary primer (HPV45E6)	GACAAACGAAGATTTCACAGCATAGC (5'-SH-$(CH_2)_6$-T_{30}-)
Non-specific DNA target (CD24)	GGGTTCCCTAAGGGTTGGACAAGTAACTCCTCCCAGAGTACTTC CAACTAATCCAACTAATGCCACCACCAAGGCGGCTGGTGGTGCCC CTGCATCTACAGGCTCGTATATGTATCTAGATTGGATCTTGCTGG CGCGTCC

All the DNA solutions were aliquoted and diluted to convenient concentrations using Milli-Q water unless otherwise stated. Double stranded DNA templates were prepared by mixing equal volumes of complementary strands, heating at 95 °C for 10 minutes and letting the solutions cool down to room temperature gently.

2.4 Experimental

2.4.1 Colorimetric detection of *Francisella tularensis* genomic DNA by solid-phase RPA on 96 wells microtitre plates

Immobilisation of forward primer

Microtitre plates were prepared by pipetting 50 µL of 200 nM thiolated forward primer prepared in 10 mM phosphate buffered saline (PBS, pH 7.4) into the wells of a maleimide coated microtitre plate and left to incubate overnight at room temperature. The plates were subsequently thoroughly washed with PBS containing 0.05 % (v/v) Tween 20 (PBS-T20) and any remaining maleimide groups were blocked with 100 µM 6-mercapto-1-hexanol in water (200 µL per well) for 1 hour before washing the plate in PBS-T20.

Solid-phase RPA on 96 wells microtitre plates

Solid-phase RPA on microtitre plates was performed in 50 µL DNAse free water using a TwistAmp Basic kit, 240 nM reverse primer, template DNA, 14 mM magnesium acetate and 1X rehydration buffer. All reagents except for the template and magnesium acetate were prepared in the master mix, which was distributed into each of the primer functionalised maleimide wells. Subsequently, 13.2 µL of *Francisella tularensis* template and 2.5 µL of magnesium acetate were pipetted into the wells to initiate the amplification reaction. The reaction took place at 37 °C for 1 hour after which the amplification product present at the well surface was denatured for 3

minutes by adding 200 µL of 100 mM NaOH and thoroughly washing with PBS. The resulting extended DNA was labelled by pipetting 50 µL of a 20 nM biotin-labelled reverse primer per well, followed by a 30 minute incubation step at room temperature. Following another washing in PBS, 50 µL of 0.5 nM streptavidin-HRP (SA-HRP) was added and left to incubate for 30 minutes at room temperature. After a final washing step, the presence of the HRP-labelled probe hybridised to the elongated primer *i.e.* the surface immobilised amplicon, was measured by adding 50 µL of TMB substrate, followed 5 minutes later by the addition of 50 µL of 1 M H_2SO_4 (Figure 2-1.B). The absorbance was read at 450 nm (SpectraMax 340PC384, bioNova científica s.l., Spain). Negative controls consisted of (i) the absence of template DNA in the RPA mix, (ii) using a different DNA target sequence (CD24) and (iii) the immobilisation of a non-related primer (HPV45E6).

To assess the sensitivity of the assay, amplification was carried out with different starting concentrations of *Francisella tularensis* genomic DNA (10 nM, 1 nM, 0.1 nM, 0.01 nM, 0.001 nM). Finally, the assay time was optimised by varying the amplification time (0, 10, 20, 30, 40 and 60 minutes).

2.4.2 Electrochemical detection of *Francisella tularensis* genomic DNA by solid-phase RPA on electrodes

Electrode preparation

The electrodes were designed to have a set of two working electrodes, one for specific measurements and the other to be used as a control, and each of these working electrodes was surrounded by their respective counter and reference electrodes (Figure 2-3.A). These electrodes were fabricated in clean room facilities by UV mask photolithography and sputtering of Ti and Au layers of 10 nm and 150 nm, respectively, on a glass substrate. Prior to chemical modification, the electrodes were rinsed with Milli-Q water and ethanol, blown dry with N_2 and plasma treated for 5 minutes in O_2:Ar plasma (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w at 35 mm working distance) (ATC Orion 8-HV, AJA International Inc. USA).

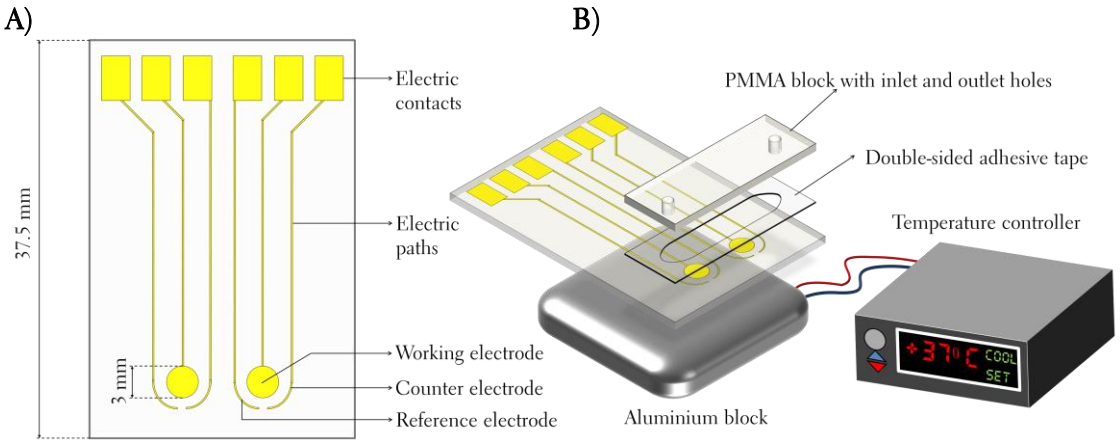

Figure 2-3. A) Design of the electrode with their respective counter and reference electrodes. B) Schematic image of the ensemble: the PMMA block, the adhesive foil, the electrodes and the aluminium block heater.

The electrodes were functionalised overnight at room temperature by immersion in 5 mM DSP in chloroform,[14] subsequently thoroughly rinsed in ethanol, dried in a stream of nitrogen and kept under vacuum until used. For the immobilisation of the Ft forward primer, 10 µL of the amino-terminated DNA prepared at a concentration of 10 µM in 150 mM NaH_2PO_4/Na_2HPO4 at pH 8.5 was deposited onto each specific electrode and incubated in a saturated humidity chamber for 1 hour and rinsed with Milli-Q water. Primer sequences were not immobilised on the control electrodes. The remaining active groups were blocked with 0.1 M ethanolamine hydrochloride (pH 8.5) for 30 min. Finally the electrodes were washed in Milli-Q water, dried with N_2 and stored at 4 °C until needed.

Solid-phase RPA on electrodes

Microfluidics were fabricated using medical grade double-sided adhesive and PMMA cover plates using a CO_2 laser marker (Fenix, Synrad, USA) to precisely define channels dimensions, as well as inlets and outlets in the PMMA cover plates (Figure 2-3.B). Functionalised electrodes and adhesive-PMMA assemblies were aligned and pressure bonded to produce a 10 µL microfluidic channel to carry out the RPA and electrochemical measurements.

An amplification enzymatic mix solution was prepared and used as described for the solid-phase RPA protocol described in Section 2.4.2 and distributed in different eppendorfs. The DNA template and the magnesium acetate solution was then added, mixed briefly and injected into the microfluidic channels of the set-up. The constant temperature was achieved by using a purpose-built heating stage controlled by LabView (Figure 2-3.B).

Electrochemical detection

All electrochemical measurements were performed with a potentiostat/galvanostat PGSTAT 12 Autolab controlled with the General Purpose Electrochemical System (GPES). To carry out the electrochemical measurements each of the microfluidic channels of the set-up were flushed following RPA with 100 µL of Tris-buffered saline (TBS) to remove all the amplification mixture, and subsequently washed with 100 µL of NaOH 0.1 M for 3 minutes to denature the DNA and then flushed again with 100 µL of TBS. Biotin-labelled reverse primer (10 µL) prepared in TBS was then injected in each of the channels and the electrodes were kept in a humidity box for 30 minutes at room temperature to produce the hybridisation. Subsequently, each channel was flushed with 100 µL of TBS and filled with 10 µL of 0.5 nM streptavidin-HRP prepared in TBS and kept in a humidity box for 30 minutes. Finally, the channels were flushed with 100 µL of TBS and filled with 10 µL of TMB enhanced one component HRP membrane. Fast chronoamperometry was used to detect the oxidation of precipitated TMB by the HRP label, by applying two consecutive potential steps at 0 V for 0.01 sec and -0.2 V for 0.5 sec to each electrode sequentially, and taking the current readout at the end of the second step (Figure 2-1.B).

2.5 Results and discussion

2.5.1 Solid-phase RPA on 96 wells microtitre plates

As a proof-of-concept for the solid-phase RPA mechanism maleimide activated microtitre plates were used to immobilise the forward primer. The mechanism was demonstrated to function and amplification of genetic material and elongation of surface tethered primers was achieved. A selectivity study was carried out to investigate the ability of the RPA to differentiate between a non-complementary primer on the surface (HPV45E6), the absence of target or the presence of a non-complementary target (CD24).

Figure 2-4.A shows the absorbance reading recorded at the end of the assay for the specific amplification of *Francisella tularensis* genomic dsDNA and the controls, using a starting DNA concentration of 2.64 nM ($8{\cdot}10^{10}$ copies) for both. The assay yielded a large absorbance for the specific amplification with negligible response for the controls. The solid-phase approach retains the specific sequences on the surface while the random amplified DNA in solution is washed away during the process, thus avoiding the use of complex engineered primers and other enzymes.[5] The kinetics of the RPA mechanism was evaluated in order to optimise the reaction

at 37 °C, showing an exponential amplification after 10 minutes and reaching saturation after 35 minutes for an initial target concentration of 2.64 nM ($8 \cdot 10^{10}$ copies) (Figure 2-4.B).

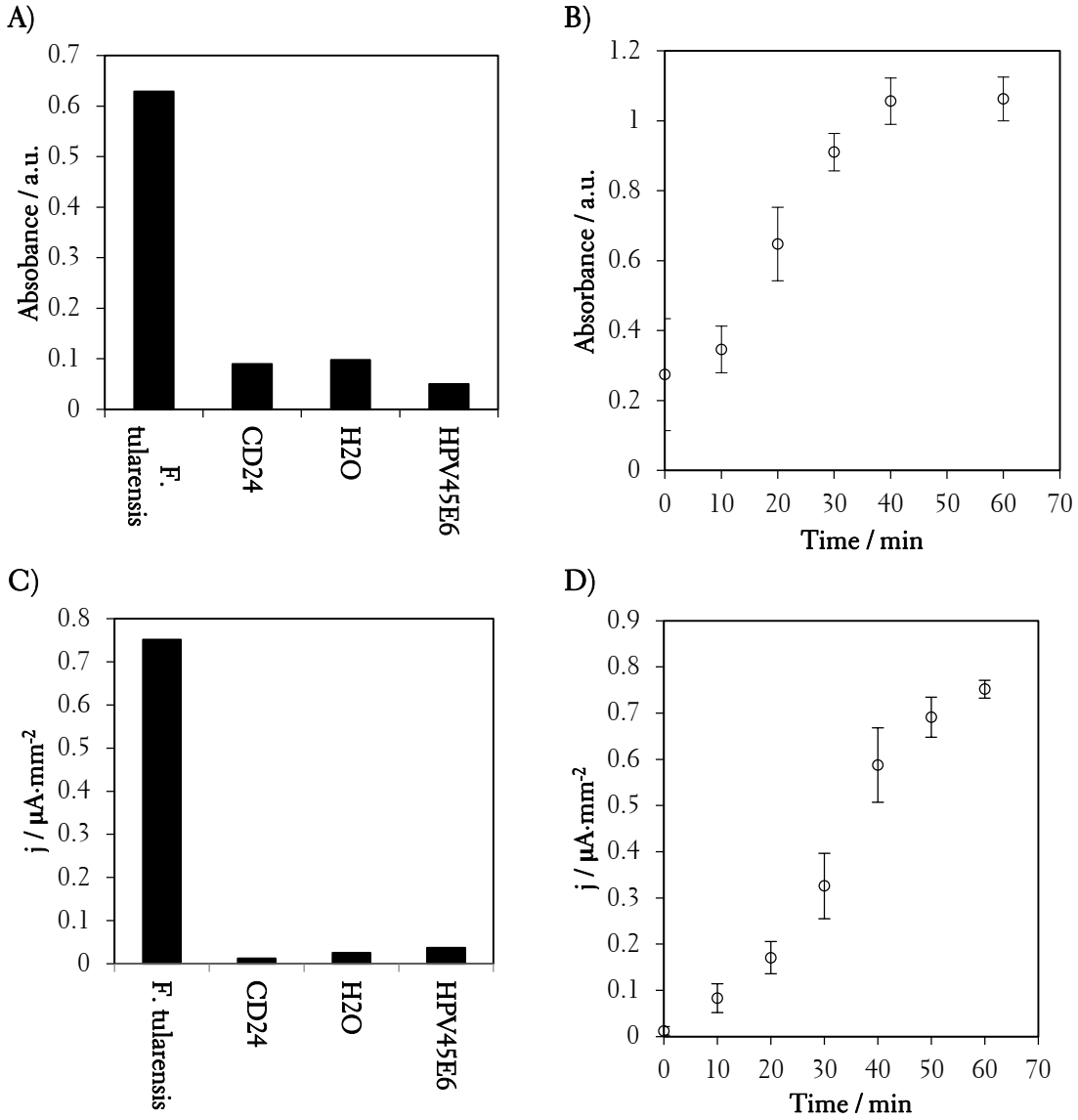

Figure 2-4. Solid-phase RPA A) Demonstration of solid-phase RPA with controls (CD24 = non-specific template DNA; H_2O = water blank; HPV45E6 = non-specific immobilised primer) on maleimide plates and B) on gold electrodes. C) Optimisation of time required for maximum amplification on maleimide plates and D) on gold electrodes.

Figure 2-5.A shows the calibration plot achieved using different concentrations of starting DNA template, achieving a linear range that spans at least five orders of magnitude. The limit of detection (LOD), defined as the concentration of the analyte at the mean blank plus three times the standard deviation of the blank, using a logarithmic regression was $1.3 \cdot 10^{-13}$ M ($4 \cdot 10^{6}$ copies in 50 µL).

2.5.2 Solid-phase RPA on electrodes

Once solid-phase RPA had been demonstrated using the microtitre plate format, the system was successfully transferred to an electrochemical platform. A higher current density (j), was obtained for the electrodes modified with specific forward primers whilst negligible response was observed for the controls, using an initial concentration of 2.64 nM ($8{\cdot}10^{10}$ copies), for both specific and controls (Figure 2-4.C). The electrochemical platform showed a better signal-to-noise ratio as compared to the assay performed on maleimide microtitre plates, although the time required for an optimum amplification (Figure 2-4.D) was slightly longer. As can be seen in Figure 2-5.B, the calibration curve of the electrochemical platform spans six orders of magnitude. The LOD using a logarithmic regression, was $3.3{\cdot}10^{-14}$ M ($2{\cdot}10^{5}$ copies in 10 µL).

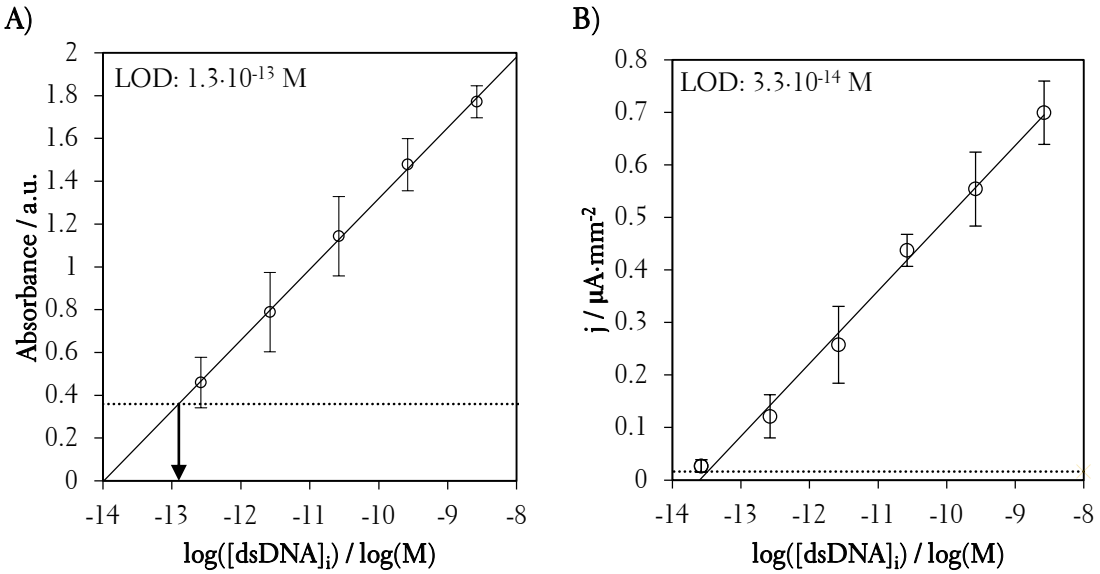

Figure 2-5. Calibration plot using different amounts of starting DNA (n = 5) on A) maleimide plates and B) on gold electrodes.

2.6 Conclusions

We have demonstrated isothermal solid-phase RPA using both optical and electrochemical measurement for the rapid detection of a low number of DNA copies (10^{5} copies), achieving detection limits of $1.3{\cdot}10^{-13}$ M and $3.3{\cdot}10^{-14}$ M, respectively. Furthermore, the solid-phase approach overcomes the limitations present in regular RPA-based analysis, where the production of by-product DNA sequences can hinder the final analysis and thus requires the need for specific THF-based engineered primers. As the developed approach obviates the need to use these engineered primers, traditional PCR primers can be used, further highlighting the simplicity and genericity of the system reported here. Current work is focused on

electrochemical isothermal solid-phase real-time PCR with multiplex detection of different genetic markers using a microelectrode array.

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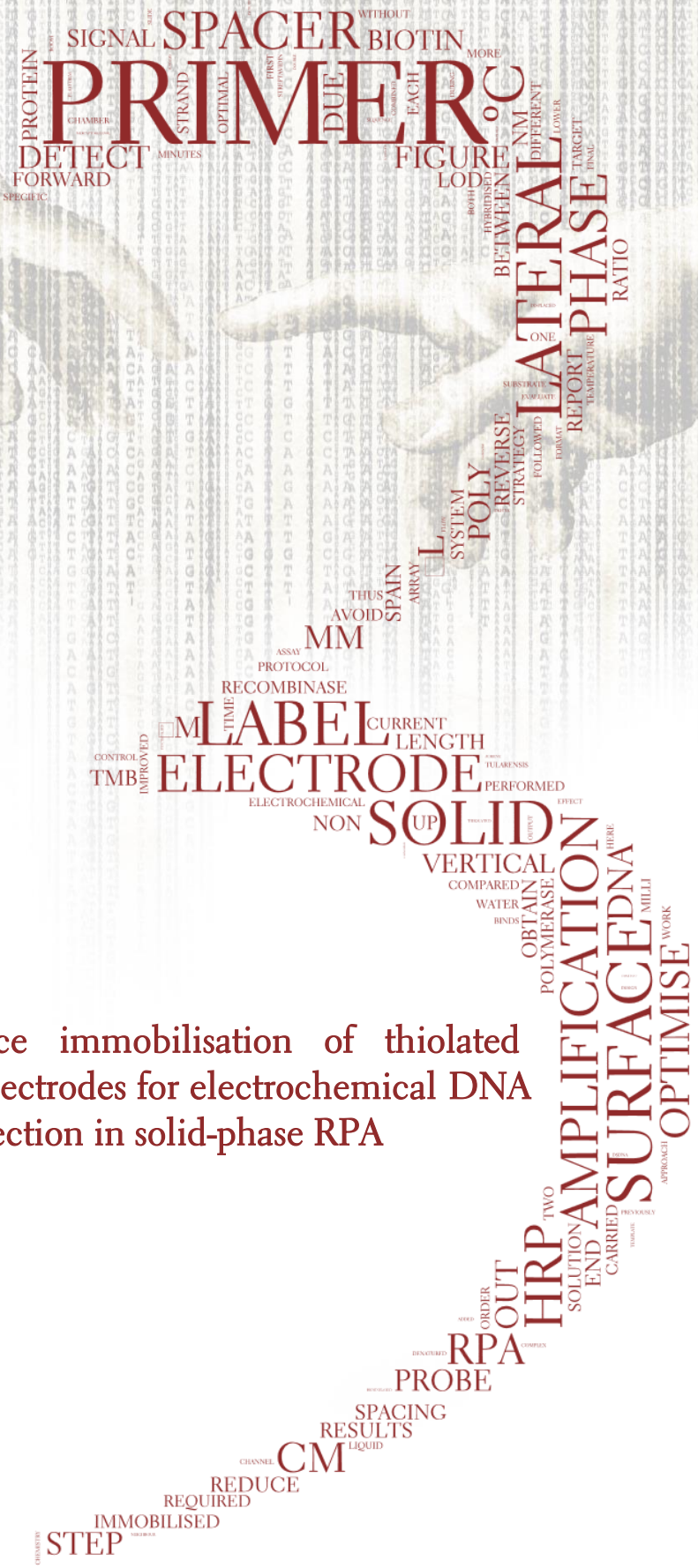
Chapter



Optimised surface immobilisation of thiolated primers in gold electrodes for electrochemical DNA amplification-detection in solid-phase RPA

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Optimised surface immobilisation of thiolated primers in gold electrodes for electrochemical DNA amplification-detection in solid-phase RPA

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3.1 Abstract

Recombinase polymerase amplification (RPA) is an elegant method for the rapid, isothermal amplification of nucleic acids. Previously, we detailed an approach for the electrochemical detection of solid-phase RPA and here we report on the improvement and simplification of this approach, evaluating different surface chemistries and labelling strategies. The surface chemistry was fine-tuned in order to obtain an optimal signal-to-noise ratio, defining the optimal DNA probe density, probe-to-lateral spacer ratio (1:0, 1:1, 1:10 and 1:100), and length of a vertical spacer of the probe as well as investigating the effect of various lateral spacers. The use of different labelling strategies was examined in order to reduce the number of steps required for the analysis, using biotin or horse-radish peroxidase labelled reverse primers. Improvement of the electrode design, amplification temperature used and the use of surface blocking agents was also pursued. The combination of these changes facilitated a significantly more rapid amplification and detection protocol, resulting in a lowered LOD of $1 \cdot 10^{-15}$ M.

3.2 Introduction

Since the introduction of recombinase polymerase amplification,[1] the system has been extensively used in a range of formats due to the simplicity, the ease-of-use and the constant temperature required to achieve amplification, without any need for a first cycle thermal denaturation step. Reported systems include the use fluorescent probes for real-time amplification-detection of either DNA[2-4] or RNA[5-7]. Other common applications are the cost-effective end-point detection system using lateral flow sticks.[8-10] Elegant and simple solutions using DVDs as platforms have been also reported, with the detection been carried out by a lightly modified DVD optical player.[11-13]

In summary, the need for thermal cycling instruments typically used in the polymerase chain reaction is avoided and replaced by three core proteins that operate optimally at 37 °C to 40 °C. The first protein, a recombinase, binds to primers, forming filaments that can then recombine with homologous DNA in a duplex target, forcing displacement of the non-complementary strand and thus provoking the formation of a D-loop. The second protein is a single stranded DNA binding protein, which attaches to the strand of DNA displaced by the primer, preventing the dissociation of the primer and hybridisation of the duplex target. The final core protein is a strand-displacing polymerase that copies the DNA, adding bases onto the 3′ end of the primer, forcing open the DNA double helix as it progresses. When opposing primers are used, exponential amplification occurs. In solid-phase RPA[14] one of the primers is covalently linked to a surface, therefore the elongation of primers and amplification of the target, occurs both in the liquid and the solid-phase simultaneously (Figure 3-1).

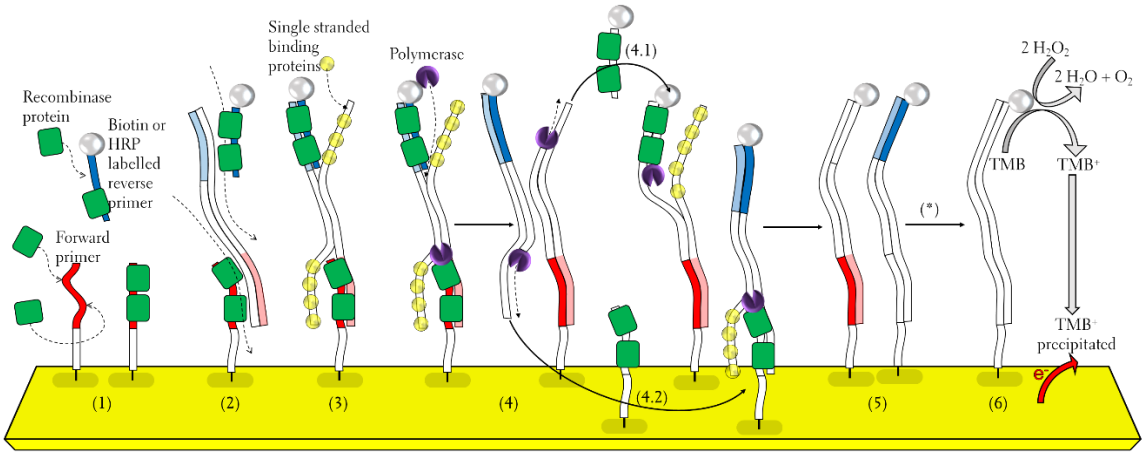

Figure 3-1. Solid-phase RPA with biotin or HRP labelled reverse primers. (1) Recombinase proteins form a complex with forward and reverse primers, (2) scan dsDNA for cognate sites and (3) introduce the primers in the template by a strand-displacement mechanism. (4) The polymerase initiates primer elongation at their 3'-ends and exponential amplification is achieved by cycling of this process either in (4.1) liquid or (4.2) solid phase. (5) Biotin or HRP labelled primers end up ligated with the elongated forward primers in solid-phase. (*) An additional conjugation step of streptavidin-HRP is carried out in case a biotinylated primer was used during the RPA. (6) Chronoamperometry is performed in the presence of TMB/H_2O_2 to detect of precipitated oxydised TMB substrate.

Solid-phase amplification has the inherent advantage that it is highly adaptable to multiplexed amplification, particularly when detection is facilitated by the surface on which amplification takes place *e.g.* electrodes,[14] plastic slides,[15] glass slides,[16] ring resonators,[17-19] or optical discs.[11] Additionally, the spatial separation proffered by solid-phase strategies is that detection can exploit a single reporter for all the sequences, or label-free approaches, need for different labels to differentiate between each target is avoided.

In this communication, we report on the improvement and simplification of an approach we previously described for the electrochemical detection of solid-phase RPA, evaluating different surface chemistries and labelling strategies. In our initial report of electrochemical detection of solid-phase RPA, we used quite a convoluted system requiring post-amplification denaturation of amplified material, and subsequent hybridisation with a biotin labelled probe, followed by addition of SA-HRP, substrate addition and signal detection with inherent washing following each step. Here, we evaluate the use of biotin and horse-radish labelled primers as a means of both simplifying the system and reducing the time required. Furthermore, we explore the importance of a combination of lateral and lateral spacers to assist in the recombinase-primer complex rapidly hybridising to its homologous sequence and initiating amplification, in a further effort to reduce the duration of amplification.

3.3 Materials and methods

RPA kit TwistAmp® Basic was obtained from TwistDx Ltd. (Babraham, United Kingdom). The HRP substrate formulation TMB enhanced one component HRP membrane was purchased from Diarect AG (Germany), and GelRed™ Nucleic Acid Gel Stain from Biotium (Barcelona, Spain). 6 mm thick polymethylmethacrylate (PMMA) was purchased from La Indústria de la Goma (Tarragona, Spain), double-sided medical grade adhesive foil from Adhesive Research (Ireland) and all other chemicals were obtained from Sigma Aldrich S.A. (Barcelona, Spain) and used as received.

Synthetic oligonucleotides designed for the identification of the pathogenic bacteria *Francisella tularensis holarctica*[20] were purchased as lyophilised powder from Biomers.net (Ulm, Germany), reconstituted at 100 µM in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain) and used without further purification (Table 3-1).

Table 3-1. List of oligonucleotide sequences and their respective modifications.

Name	Nucleotide sequence (from 5'-end to 3'-end)
F. tularensis forward primers	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCC (5'-SH-$(CH_2)_6$-T_{15}-, 5'-SH-$(CH_2)_6$-T_{30}- and 5'-SH-$(CH_2)_6$-T_{45}-)
F. tularensis reverse primers	CGCTACAGAAGTTATTACCTTGCTTAACTGTTA (5'- biotin and 5'-HRP)
F. tularensis DNA sequence	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCCAGAAGGTTCTA AGTGCCATGATACAAGCTTCCCAATTACTAAGTATGCTGAGAAGAA CGATAAAACTTGGGCAACTGTAACAGTTAAGCAAGGTAATAACTTC TGTAGCG

All the DNA solutions were aliquot and diluted to convenient concentrations using Milli-Q water unless otherwise stated. Double stranded DNA templates were prepared by mixing equal volumes of complementary strands in PBS buffer, heating to 95 °C for 10 minutes and letting the solutions gently cool to room temperature.

3.4 Experimental

3.4.1 Electrode preparation

The electrochemical platform was designed with a set of six squared shaped electrodes (1 mm^2) made with photolithographic gold on a pre-cleaned 75 mm × 25 mm borosilicate glass slide substrate (Sigma Aldrich, Spain), using four of them as working electrodes, and the remaining two as counter and reference electrodes, respectively. These electrodes were fabricated in a clean room facility by spin-coating a positive photoresist AZ1505 (MicroChemicals GmbH, Germany) at 4000 rpm for 30 sec over a dry glass slide. The platform was then exposed to UV light for 3 sec using a chromium mask in contact mode (LED Paffrath GmbH, Germany). Development of the pattern with a commercial developer AZ 726 was carried out according to the manufacturer's instructions and the slide was introduced into a sputtering chamber (ATC Orion 8-HV, AJA International Inc. USA) and treated with alternate current (AC) O_2:Ar plasma sputtering (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w) for 5 minutes in order to eliminate the presence of any residual non-crossed photoresist. A layer of 20 nm of Ti/TiO_2 was deposited by direct current (DC) sputtering using an increasing oxygen flow rate during the deposition process, going from 5 cm^3/s of O_2 during the first 10 nm to 20 cm^3/s of O_2 the last 5 nm while keeping the Ar flow rate at a constant 5 cm^3/s. This method increased the adherence of the top Au layer on the substrate more than using a bare Ti layer. Finally 100 nm of gold was deposited by AC sputtering (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w). Prior to chemical modification, the electrodes were sonicated 5 minutes in acetone, 5 minutes in isopropanol, rinsed with Milli-Q water, and sonicated for 10 minutes in a mixture containing 3 volumes of KOH 50 mM and 1 volume of 30 % (v/v) H_2O_2. Finally the electrodes were rinsed in Milli-Q water and dried with a N_2 spray gun.

3.4.2 Vertical and lateral probe length spacing optimisation

The electrode arrays were functionalised by co-immobilisation of the forward primer having a poly-15T, a poly-30T or a poly-45T of 2'-deoxythymidine monophosphate nucleotides at the 5'-end of the probe as a vertical spacer with a range of probe-to-lateral spacer ratios: 1:1, 1:10 and 1:100. One microlitre of 2 µM thiolated forward primer of *F. tularensis* in 1 M KH_2PO_4

solution together with either 1-mercapto-6-hexanol, a thiolated poly-15T (with no primer) or a bipodal PEGylated thiol[21] at 2 µM, 20 µM or 200 µM, were dropcast on the electrode surface. Control electrodes without lateral spacer for each of the primers with different length vertical spacers, were also functionalised. Co-immobilisation was carried out in a saturated humidity chamber at room temperature for 20 h. Subsequently, the electrodes were rinsed in milli-Q water and dried in a stream of nitrogen. A medical grade double-sided adhesive and a 3 mm thick PMMA block were aligned and pressure bonded with the functionalised electrodes to produce a fluidic chamber of 10 µL where amplification and detection was carried out. The PMMA gasket and the adhesive were cut and milled using a CO_2 laser marker (Fenix, Synrad, USA) to precisely define channel dimensions, as well as inlets and outlets in the PMMA cover plates. The chamber was then washed with 200 µL of PBS containing 0.05 % (v/v) Tween 20 (PBS-T20), followed by 200 µL of Milli-Q water in order to completely remove any non-specific immobilisation of DNA on the electrode surface. The chamber was dried with a nitrogen beam and kept under vacuum until used (Figure 3-2).

Following optimisation of the vertical length spacer of the primer and the lateral spacing, the preferred conditions were used to further optimise the primer concentration (10, 20 µM) whilst keeping the primer-to-lateral spacer ratio constant. Two electrodes out of the six were always used as negative controls and functionalised with the same immobilisation solution used for the other electrodes but without the *F. tularensis* specific thiolated probe.

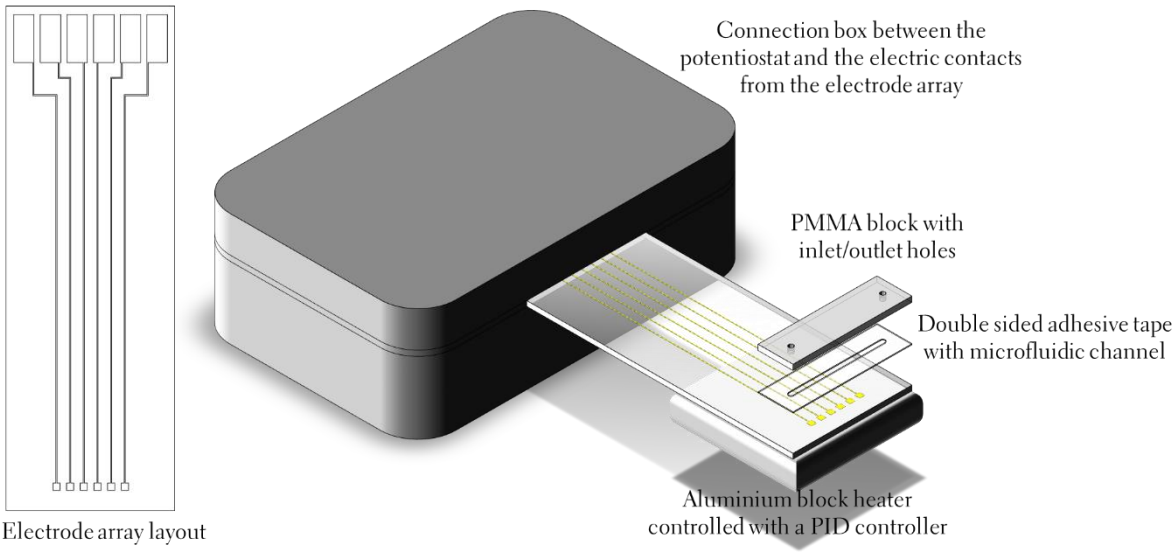

Figure 3-2. Electrode array layout and mounted setup.

3.4.3 Solid-phase recombinase polymerase amplification

The RPA amplification mixture was prepared by mixing 2.4 µL of 10 µM biotinylated / horseradish peroxidase labelled reverse primer and 13.2 µL of 10 nM template dsDNA in 31.9 µL of rehydration buffer. Lyophilised pellets were then added and mixed by pipetting, before finally adding 2.5 µL of magnesium acetate 280 mM to trigger the reaction. The solution was then mixed by pipetting, 10 µL/array injected into each microfluidic channel of the set-up and left to incubate at 37 °C for 1 h.

3.4.4 Electrochemical detection: chronoamperometry

Following completion of amplification, a series of different steps were carried out, each step of which was followed by flushing the microfluidic channels with 800 µL of PBS-T20. In the case of the biotinylated reverse primer, 10 µL of 0.5 nM streptavidin-HRP in PSB was added for 30 minutes, following amplification. The microfluidic channels were then filled with 10 µL of TMB enhanced one component HRP membrane for 5 min prior to carrying out chronoamperometry. Finally, fast chronoamperometry was used to detect the oxidation of precipitated TMB by the HRP label, by applying two consecutive potential steps at 0 V for 0.01 sec and -0.2 V for 0.5 sec to each electrode sequentially, and taking the current readout at the end of the second step. A potentiostat/galvanostat PGSTAT 12 Autolab controlled with the General Purpose Electrochemical System (GPES) was used to make the electrochemical measurements, and a home-made connection box used to plug the electrode arrays and generate electric connection between the potentiostat and the electrode arrays. In both of these strategies, a control measurement to ensure that all the signal readout was due to specific primer-to-target amplification was carried out, where the surface amplified duplex was denatured and then re-hybridised with biotin-labelled reverse primer followed by streptavidin-HRP.

3.4.5 Amplification Temperature

Although the optimal temperature at which the strand-displacing polymerase *Bacillus subtilis* Polymerase I from the RPA enzymatic mixture is around 38 °C, the system has been reported to work in liquid at room temperature but at lower rates of amplification. To this end, the solid-phase RPA strategy has been tested between 35 °C and 42 °C, obtaining lower rates of amplification.[13] Solid-phase amplification (Section 3.4.3) was carried out at 22 °C and 45 °C and the electrochemical response measured to determine the amplification efficiency.

3.4.6 Calibration curve

The functionalisation of electrode arrays was carried out using the optimised protocol, *i.e.* drop-casting 1 µL per electrode of a KH_2PO_4 1 M solution containing the forward primer with a thiolated poly-15T and a primer-to-lateral spacer molar ratio of mercaptohexanol of 1:10 for 20 h in a water saturated chamber. The RPA amplification mixture was prepared as described in Section 3.4.3 with 5 different serial 1/10 dilutions of a *Francisella tularensis* dsDNA 10 nM template, including a blank control assay without template.

3.5 Results and discussion

3.5.1 Vertical and lateral probe length spacing optimisation

Taking into account the heterogeneous format pursued, comprised of the solid-phase (the electrode), the liquid-phase (bulk solution) and the interface between these two phases, (primers attached to the surface of the electrode), we defined different parameters affecting performance, focusing on the optimisation of the interface between bulk solution and the electrode, as this where most of the amplification and detection occurs. First, the recombinase proteins form a complex with the primers and then scan the dsDNA target that is present in the neighbourhood. This initial stage of amplification is critical for minimisation of amplification time. In the set-up used in the work reported here, a simple microfluidic channel defined the boundaries of the amplification chamber. Without applying any positive pressure, the typical flow gradient between the movement of particles in the bulk solution and the surface of the electrodes due to the friction of the liquid on the boundaries of the channel is of minor influence, and the biological material present in the solution only experiences diffusion governed by specific hybridisation mechanisms such as base pairing and formation of complexes between proteins and oligomers.

When considering the most important conditions to be optimised in the interface for improving amplification and detection, we hypothesised that the vertical and lateral probe length spacing were two parameters of importance. A longer vertical spacer between the electrode and the primer would be expected to enhance accessibility between the recombinases, polymerases and ssDNA binding proteins and the immobilised primers. Additionally, lateral spacers could be anticipated to reduce steric hindrance. However, vertically aligned probes (*i.e.* primers) are known to encourage target hybridisation as the negative charge of the backbone cause inter-primer repulsion, resulting in the probes being fully extended from the surface without folding or kinks. Thus, an optimal compromise between lateral primer spacing and primer-to-primer

electrostatic repulsion is required (Figure 3-3). The combination of vertical and lateral spacers was thus evaluated, with the aim of achieving rapid D-loop formation, resulting in reduced time required for amplification.

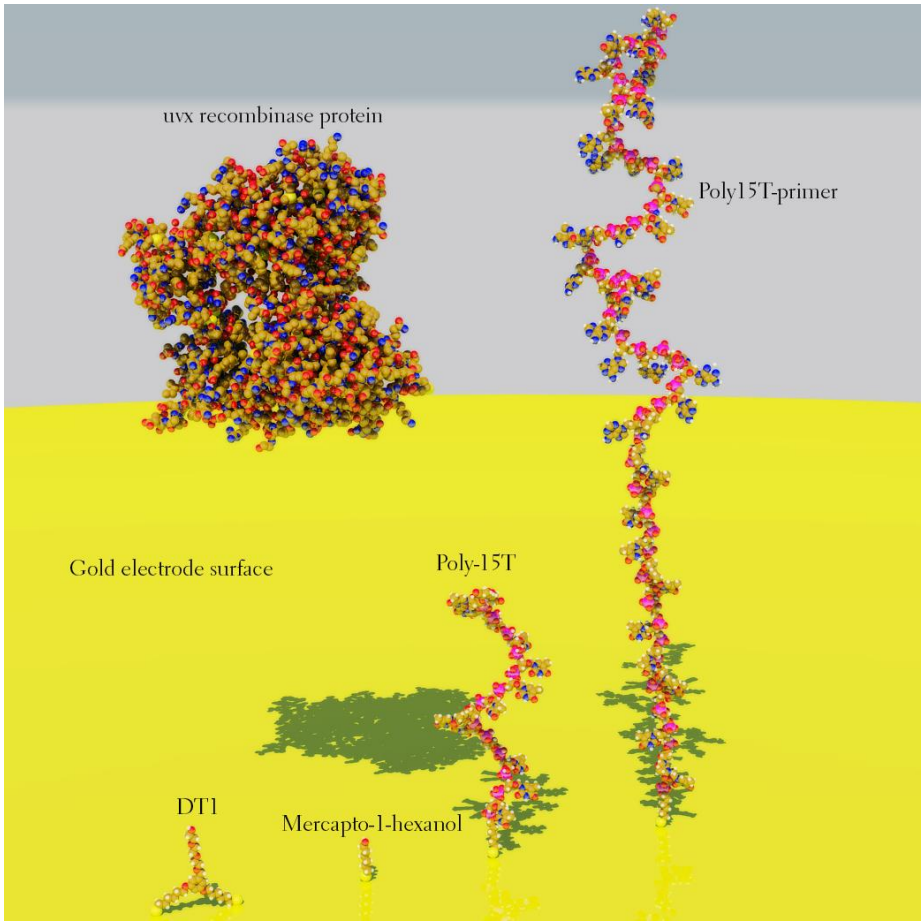

Figure 3-3. Relative size of the back-fillers used *versus* the primer and the uvsx recombinase protein.[22]

Figure 3-4 shows a summary of results obtained after chronoamperometric measurements on arrays using the same amplification conditions, evaluating different spacing configurations (vertical spacer, lateral spacing ratio and lateral spacer type), including controls with no lateral spacing for each vertical spacer used. First, it can be observed that the use of a poly-30T or poly-45T vertical spacer yielded unsatisfactory results when combined with no lateral spacer (Figure 3-4.A), and the same is true when a 1:1 primer-to-lateral spacer ratio was tested in poly-15T (Figure 3-4.B), 10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol (DT1) (Figure 3-4.C) or mercaptohexanol (Figure 3-4.D). This was anticipated and can be explained in terms of undesired interactions between neighbour primers due to the extended length, a trend that is not observed when lateral spacing starts to be effective at higher ratios of 1:10 or 1:100. The shorter vertical spacer appeared to be short enough to avoid neighbour interaction

between primers, even when no lateral spacing was used (Figure 3-4.A). When using DT1 (Figure 3-4.C) or mercaptohexanol (Figure 3-4.D) at 1:100 primer-to-lateral spacer ratios, it is evident that the efficiency of the shortest vertical spacer is diminished due to the fact that the lateral spacer is greater in height than the vertical spacer, thus shadowing the immobilised primer. When using poly-15T as lateral spacer (Figure 3-4.B), the use of poly-30T and poly-45T as vertical spacers gave a poor but comparable yield to the poly-15T, and this can be attributed to be due to the specific nature of the lateral spacer employed. Poly-T is also a negatively charged strand and is expected to be in constant repulsion with the neighbouring strands, which could prevent longer vertical spacers from aggregating or interacting with neighbour primers. However, taking into account the reproducibility of the surfaces generated, the use of either mercaptohexanol or DT1, was preferable. Due to the fact that mercaptohexanol is a more commonly used, more stable and more cost-effective option as compared to DTI, in further experiments it was used at 10:1 ratio with immobilised forward primer. In summary, the enhancement in performance regarding the surface optimisation can be attributed to an improved access to the immobilised primers, which is achieved as a combination of the vertical spacer projecting the primer from the surface, thus facilitating elongation by the polymerase, whilst the lateral spacing avoids undesired interactions between neighbouring primers. Furthermore, vertical and lateral spacing of primers is expected to reduce steric hindrance between the proteins and the surface as well as between proteins during the amplification in solid-phase.

3.5.2 Optimisation of amplification temperature

The solid-phase RPA (SP-RPA) strategy was tested at 45 °C and 22 °C (room temperature) in addition to the optimal working temperature for the polymerase, *i.e.* 37 °C, for comparative purposes. The output current density (j), recorded of the process at 37 °C after 1 h of amplification was (3.5 ± 0.3) $\mu A \cdot mm^{-2}$ (n = 4), while the measurements recorded at 45 °C and 22 °C did not yield a significant output current, (0.7 ± 0.2) $\mu A \cdot mm^{-2}$ (n = 4) and (0.6 ± 0.3) $\mu A \cdot mm^{-2}$ (n = 4) respectively, and is comparable to the output current densities of control electrodes. This means that the amplification efficiency was close to 0 %, although some degree of amplification was expected at least at 22 °C. *Bacillus subtilis* Polymerase I has an optimal activity at 37 °C and besides a reduced activity of the polymerase at non-optimal temperatures, solid-phase RPA strategy adds a singular constraint to the system by anchoring the forward primers on a solid substrate, not only limiting the mobility of the primers, but also requiring the proteins involved to move from the bulk solution to the surface. This effect has been studied by

Santiago-Felipe *et al.*[13] and 5 °C shift from the ideal temperature already yielded a loss in performance of 60 and 40 % compared to a liquid or solid-phase amplification, respectively.

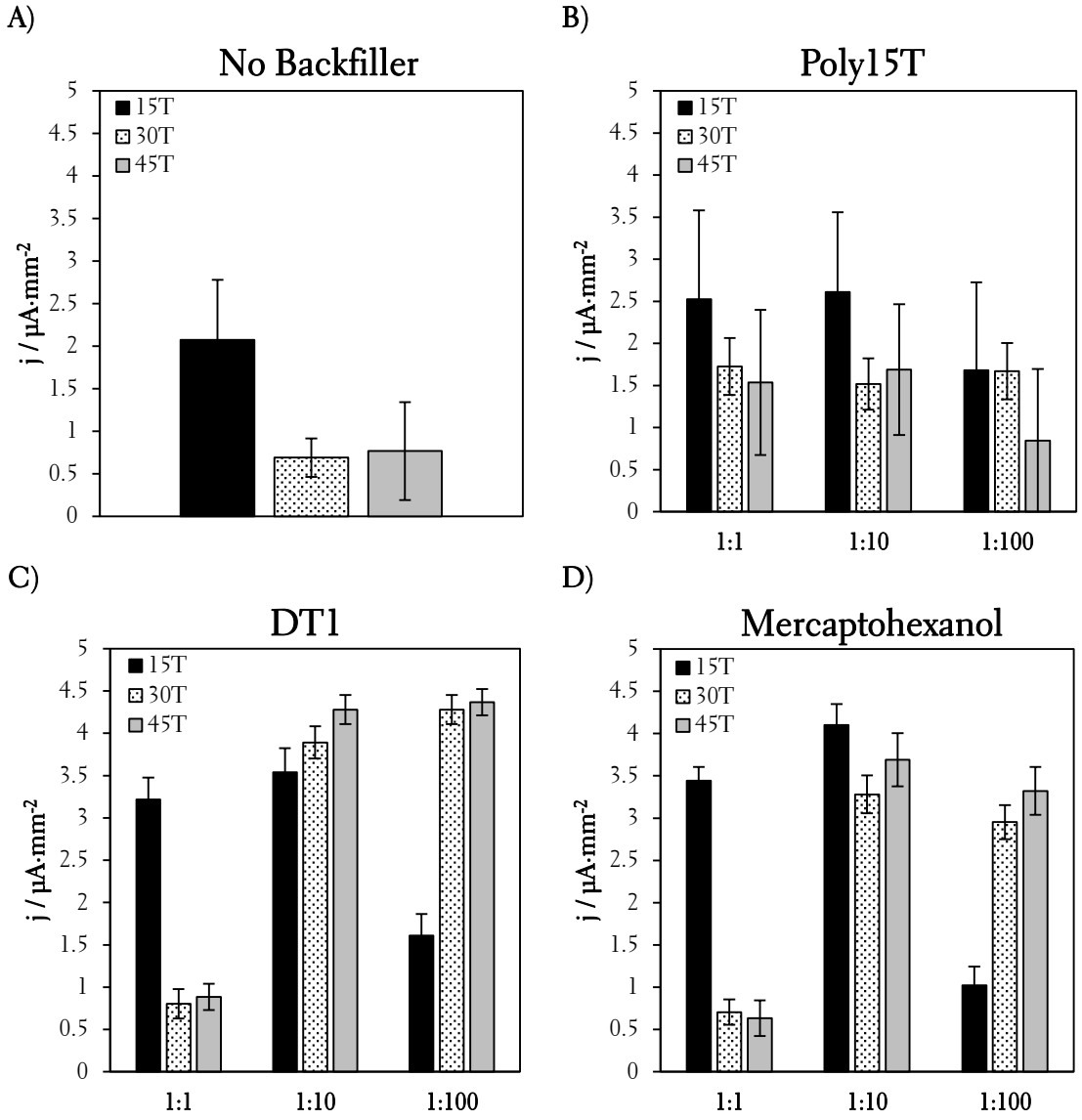

Figure 3-4. Chronoamperometric current outputs for vertical and lateral probe length spacing optimisation using different lateral spacers: A) No lateral spacer, B) Poly-15T, C) DT1 or D) mercaptohexanol.

3.5.3 Labelled reverse primers performance and calibration curve

Solid-phase amplification and detection using either a biotin-labelled reverse primer or a HRP-labelled reverse primer resulted in similar performances. In both cases the chronoamperometric response, in the presence of a TMB/H_2O_2 solution for 5 minutes, was also recorded after denaturation of the surface of the electrodes, showing no signal output, and recorded again after hybridisation with a biotin-labelled reverse primer and complex formation with streptavidin-HRP, showing comparable results to the ones obtained from the beginning

(Figure 3-5). This confirms that the signal obtained is due to solid-phase amplification, and additionally that the signal due to non-specific binding of the HRP-labelled primer on the surface is negligible.

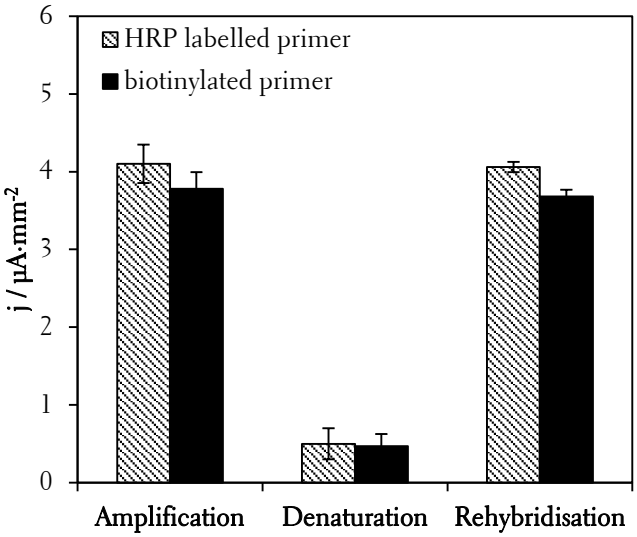

Figure 3-5. Chronoamperometric current recorded in TMB/H_2O_2 substrate after the RPA using biotin or HRP-labelled reverse, after denaturation with NaOH 0.1 M and rehybridisation with a biotin-labelled reverse primer/conjugation with streptavidin-HRP again.

Figure 3-6 shows the calibration curve for the HRP-labelled reverse primer approach for which a LOD of $1.3 \cdot 10^{-15}$ M was obtained within a dynamic range of 5 decades.

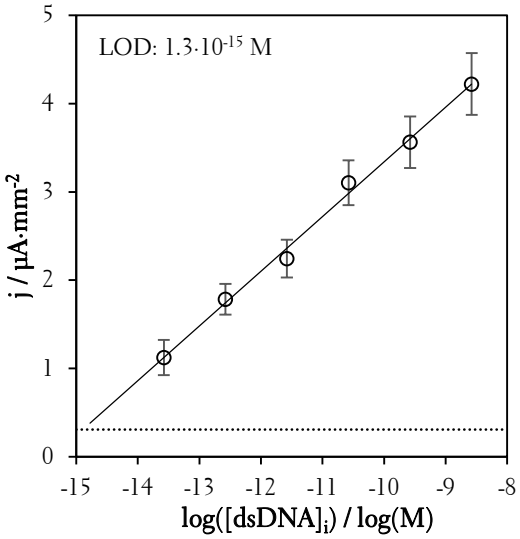

Figure 3-6. Calibration curve of the RPA amplification-detection with an optimised surface using HRP-labelled reverse primers.

The results for the other assays are summarised in Table 3-2 and compared with a previously reported work with a different surface chemistry and a labelling strategy involving regular primers during solid-phase RPA, denaturation, hybridisation with biotinylated primers

and conjugation with streptavidin-HRP. Optimised surfaces, either using HRP-labelled primer or biotinylated reverse primer approach were comparable in performance. Both strategies facilitated a huge improvement in terms of protocol simplicity (avoiding up to 3 or 4 steps) and analysis time required (up to 1.5 h), generating more reproducible results due to the fact that many manual steps are omitted. On the other side, the performance obtained in a non-optimised surface was a much lower efficiency (~60 % less) than the results obtained for the HRP-labelled primer strategy with an optimum surface. The LOD was also much higher than even previously reported, and can be attributed to the non-specific binding of the streptavidin-HRP protein onto the surface, something that was avoided in the previous paper by the use of a back-filler.

Table 3-2. Summary of sensitivity and LOD for different conditions reported.

	SP-RPA†	Biotin-primer	Biotin-primer*	HRP-primer*
Sensitivity ($\mu A \cdot mm^{-2} \cdot M^{-1}$)	0.14	0.26	0.56	0.62
LOD (M)	$3.3 \cdot 10^{-14}$	$1.3 \cdot 10^{-11}$	$2.6 \cdot 10^{-15}$	$1.3 \cdot 10^{-15}$

(*) surface optimised
†Solid-phase recombinase polymerase amplification[14]

3.6 Conclusions

We previously reported the exploitation of solid-phase recombinase polymerase amplification as a DNA detection system. Here, we extended on this work we optimised protocols and pursued alternative strategies in the construction of an electrochemical platform for the detection of DNA, simplifying the protocol and reducing the assay time required. The amplification-detection platform was optimised by addressing various aspects, including fine-tuning of the surface chemistry and optimising the vertical and lateral spacing of the immobilised primers in order to obtain lower LODs and an optimal signal-to-noise ratio. The use of biotin and HRP labelled primers were also evaluated, thus avoiding two or three steps and inherently significantly simplifying the assay. Furthermore, the improvement of the electrode design allowed a higher signal-to-noise ratio, which, in combination with the optimised protocol resulted in a vastly improved amplification-detection protocol, reducing the assay by 1.5 h and obtaining a LOD of $1 \cdot 10^{-15}$ M, an order of magnitude lower than that previously reported.

3.7 References

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Chapter

Electrochemical detection of *Piscirickettsia salmonis* genomic DNA from salmon samples using solid-phase recombinase polymerase amplification

UNIVERSITAT ROVIRA I VIRGILI
DNA BIOSENSORS BASED ON INTEGRATED ISOTHERMAL AMPLIFICATION-DETECTION STRATEGIES
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Electrochemical detection of *Piscirickettsia salmonis* genomic DNA from salmon samples using solid-phase recombinase polymerase amplification

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4.1 Abstract

Solid-phase isothermal recombinase polymerase amplification (RPA) and electrochemical detection of *Piscirickettsia salmonis* in salmon genomic DNA is reported. The electrochemical biosenosr was constructed by surface functionalisation of gold electrodes with a thiolated forward primer specific to the genomic region of interest. Solid-phase RPA and primer elongation was achieved in the presence of the specific target sequence and biotinylated reverse primers. The formation of the subsequent surface-tethered duplex amplicons was electrochemically monitored via addition of SA- HRP. Successful quantitative amplification and detection was achieved in less than 1 h at 37 °C calibrating with PCR amplified genomic DNA standards, achieving a limit of detection of $1 \cdot 10^{-14}$ M ($6 \cdot 10^{4}$ copies in 10 µL). The system was applied to the analysis of real salmon samples.

4.2 Introduction

Piscirickettsia salmonis is a gamma proteobacteria, causative agent of Salmonid Rickettsial Septicaemia (SRS).[1] Related to the genus Coxiella and Francisella, the bacteria was first thought to be exclusively intracellular, but it was then observed to grow in specific culture media.[2, 3] The bacteria was first described in the early 1990s, although the disease had been reported earlier.[4] SRS mainly affects salmonids but related diseases have been found in sea bass.[5] In fish, the bacteria is often found in macrophages where it can replicate and disseminate to the rest of the fish organs. Acute infection provokes necrosis of hematopoietic cells in the kidney and vascular necrosis and coagulation, which produces necrosis of hepatocytes in the liver.[4] The death of the fish can occur only 2 weeks post infection.[6] Similar ricketsial septisemias have been described in Canada,[7] the UK,[8] Australia[9] and Norway.[10] In the

northern hemisphere the disease has rarely caused high mortalities in farmed salmon, however, in Chilean farms mortalities can reach 90 %.[11] In the late 80s SRS killed 1.5 million salmon near market size, and currently causes annual average loses ranging between ten to hundred million US$.[12] *Piscirickettsia salmonis* outbreaks have increased in speed, aggressiveness, frequency, and resistance to antibiotics over time.[12] Several vaccines have been developed, based on bacterines and mixtures of recombinant proteins.[13, 14] Although some of these vaccines have shown protection in controlled assays, regardless of the formulation or administration route, they are largely inefficient in the field, particularly in the long term.[15, 16] Vaccination regularly protects the salmon when transferring from fresh water to the ocean, but the fish are generally unprotected from further infections,[15] most of which occur after the 10th month post transfer, affecting large fish and hence, resulting in great economic losses.[12] *P. salmonis* shows *in vitro* sensitivity to antibiotics such as Clarithromycin, chloramphenicol, erythromycin, gentamicin, oxytetracycline and sarafloxacins.[17] However, and while these antibiotics are usually effective in combating other bacterial diseases in fish, *P. salmonis* is unusually resistant in the field, possibly because once the infection is settled, high amounts of antibiotics are required in order to achieve high enough concentrations within the cells where the bacteria replicates.[18, 19] Added to that, recent strains of *P. salmonis* have shown higher resistance levels than older strains. Hence, over the years the amounts of antibiotic that are used in the farms have increased significantly.[12] SRS was first diagnosed by simple microscopic examination of smeared tissue, and later by immunoassays, including indirect fluorescent antibody technique (IFAT), ELISA and immunohistochemistry.[20, 21] The most commonly used detection technique is PCR, first developed as a nested assay and later as a one-step real time PCR assay.[22, 23] Although nucleic acid amplification tests are a gold standard for molecular diagnosis, these assays require specialised equipment, and thus a detection system that can be used the field is not yet available. This is especially detrimental for the salmon aquaculture system, where the farms are usually far from urban centres. Late detection decreases the effectiveness of treatments and biosafety measures that could be implemented. Therefore, there is a clear need for the development of *in situ* tests for the detection of *P. salmonis* in Chilean salmon farms. The criteria defined by the World Health Organisation for the evaluation of these tests is ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users).[24] The majority of commercial molecular diagnostic devices are shifting from thermal cycling based amplification techniques towards isothermal amplification techniques due to the benefits of reducing instrumental requirements and power consumption, allowing an easier integration

with microfluidic platforms and thus being more prone to be implemented as true portable devices.[25-27] The recombinase polymerase amplification (RPA),[28] achieving very rapid and sensitive amplification at a constant temperature. To date, fluorescence detection methods using EvaGreen binding dye,[29] or sequence-specific fluorophore-quencher probes,[30-51] along with lateral flow strips,[49-57] configure the majority of the reported systems for the detection of nucleic acid sequences from real samples using RPA.

In this study we present the application of an electrochemical platform integrated with solid-phase recombinase polymerase amplification strategy for the detection of *Piscirickettsia salmonis* DNA sequences from real salmon samples. An array of six gold electrodes was fabricated on a glass substrate by photolithography, the forward primer designed for specific amplification of the relevant gene from *P. salmonis* was immobilised on the surface of the electrodes via chemisorption through a thiol moiety. The recombinase polymerase amplification was carried out in a microfluidic chamber using a simple micromachined double adhesive tape, a PMMA block and an in-house constructed proportional-integral-derivative (PID) thermal controller (Figure 4-1).

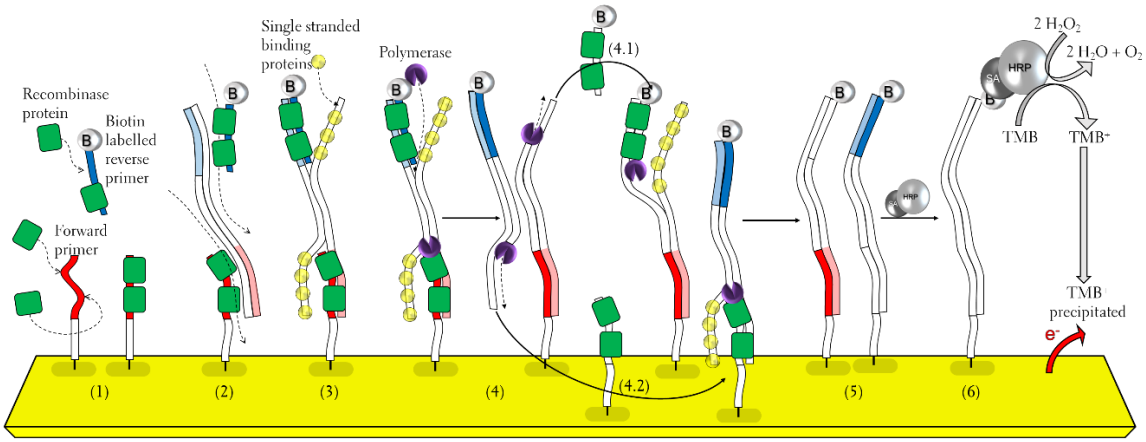

Figure 4-1. Solid-phase RPA with reverse primers. (1) Recombinase proteins form a complex with forward and reverse primers, (2) scan dsDNA for cognate sites and (3) introduce the primers in the template by a strand-displacement mechanism. (4) The polymerase initiates primer elongation at their 3'-ends and exponential amplification is achieved by cycling of this process either in (4.1) liquid or (4.2) solid phase. (5) Biotin labelled primers end up ligated with the elongated forward primers in solid-phase. An additional conjugation step of streptavidin-HRP is carried out and finally (6) chronoamperometry is performed in the presence of TMB/H_2O_2 to detect of precipitated oxydised TMB substrate.

4.3 Materials and Methods

RPA kit TwistAmp® Basic was obtained from TwistDx Ltd. (Babraham, United Kingdom) and provides lyophilised pellets, a rehydration buffer and magnesium acetate. The lyophilisate contains the polymerase system, including dNTPs, adenosine-5'-triphosphate, *Bacillus subtilis*

Polymerase I and components of a bacteriophage derived recombination system consisting of the proteins gp32 (single stranded DNA binding protein), uvsX (recombinase) and the uvsY (recombinase load factor). All PCR reagents were obtained from Life Technologies (Barcelona, Spain). GelRed nucleic acid stain was purchased from Biotium (Hayward, USA) and Certified Low RangeUltra Agarose was supplied by Biorad (Madrid, Spain). The HRP substrate formulation TMB enhanced one component HRP membrane was purchased from Diarect AG (Germany), 6 mm thick polymethylmethacrylate (PMMA) was purchased from La Indústria de la Goma (Tarragona, Spain), Double-sided medical grade adhesive foil from Adhesive Research (Ireland) and all other chemicals including 6-mercapto-1-hexanol and the buffer solutions were obtained from Sigma Aldrich S.A. (Barcelona, Spain) and used as received.

The manufacturer's criteria for RPA-based primer design originally recommended primers of between 30 to 35 nucleotides in length. However, it has recently been demonstrated that primers of just 18 residues also work and regular PCR-based primers can also be used.[58] Sequences must not contain multiple G in the first 3 to 5 base pairs at the 5'-end, keeping the GC content between 30 % to 70 %. Therefore, these considerations were taken into account for designing the primer set for the identification of the pathogenic proteobacteria *Piscirickettsia salmonis*. A specific genomic fragment from *P. salmonis* of 153 bp was selected. This fragment corresponds to a variable area of a GroEl gene[13] which is conserved within the *P. salmonis* species but markedly different compared to other gamaproteobacteria species. In particular, the primer biding areas have no similarity to any other species reported in databases. Synthetic oligonucleotides were purchased as lyophilised powder from Biomers.net (Ulm, Germany), reconstituted in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain) and used without further purification (Table 4-1).

Table 4-1. List of oligonucleotide sequences and their respective modifications.

Name	Nucleotide sequence (from 5'-end to 3'-end)
P. salmonis forward primers (36-mer)	CGACAACGGCGACAGTATTAGCACAAGCAATTATTC (regular and 5'-SH-$(CH_2)_6$-T_{30}-)
P. salmonis reverse primers (33-mer)	CTGTGCACGGTGTAGATAAGTCTTTTAATGCAG (regular and 5'- biotin)
P. salmonis DNA sequence (153-bp)	CGACAACGGCGACAGTATTAGCACAAGCAATTATTCAAGAAGGCG TGAAGTCTGTTGCTGCCGGCATGAACCCAATGGACCTAAAACGCG GCATCGATAAAGCCACTATCGCTGCAGTTGCTGCATTAAAAGACT TATCTACACCGTGCACAG

4.4 Experimental

4.4.1 DNA extraction from samples

DNA was obtained from salmon infected with *P. salmonis* under challenge conditions. Ten days post-infection, DNA from liver samples was extracted using the EZNA DNA isolation kit (Omega Biotek, GA) according to the manufacturer's instructions and subsequently lyophilised. The pellets were reconstituted in 25 µL of PBS to generate stock solutions.

4.4.2 PCR Amplification of extracted genomic DNA from salmon

The amplification of all extracted genomic DNA samples were performed in 100 µL reactions with 200 nM of primers, 5 mM of $MgCl_2$, 0.2 mM of dNTP, 1X Taq buffer (200 mM Tris pH 8.4, 500 mM KCl), and 2.5 units of Taq polymerase. All PCR reactions were heated at 95 °C for 2 minutes, followed by 30 rounds of PCR, with a denaturing step at 95 °C for 30 seconds, annealing step at 58 °C for 30 seconds, and an elongation step at 72 °C for 30 seconds. A final extension step was performed at 72 °C for 5 minutes. PCR amplification was carried out in a DNA Engine Dyad® Peltier Thermal cycler. Double stranded PCR products were analysed using agarose gel electrophoresis. For each sample, 10 µL was run on the gel with 4 µL of loading buffer on a 4 % (w/v) agarose gel stained with GelRed™ nucleic acid stain and visualised with a UV lamp (λ = 254 nm). Fragment sizes were determined by comparison with a 10 bp molecular weight standard and analysed using Image-J software. One of the amplified samples was quantified by UV spectrophotometry with a NanoDrop 2000 at λ = 260 nm in order to be used as a DNA standard. The blank solution contained the same matrix of the samples, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3.

4.4.3 Electrochemical detection of *Piscirickettsia salmonis* genomic DNA by solid-phase RPA

Electrode fabrication

The electrochemical platform was designed with a set of six squared shaped electrodes (1 mm^2) made with photolithographic gold on a pre-cleaned 75 mm × 25 mm borosilicate glass slide substrate (Sigma Aldrich, Spain), using four of them as working electrodes, and the remaining two to be used as a counter and reference electrode respectively. These electrodes were fabricated in a clean room facility by spin-coating a positive photoresist AZ1505 (MicroChemicals GmbH, Germany) at 4000 rpm for 30 sec over a dry glass slide. The platform was then exposed to UV light for 3 sec using a chromium mask in contact mode (LED Paffrath GmbH, Rose FotoMasken, Germany). After the development of the pattern according to the

manufacturer with a commercial developer AZ 726, the slide was introduced in a sputtering chamber (ATC Orion 8-HV, AJA International Inc. USA) and treated with AC O_2:Ar plasma sputtering (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w) for 5 minutes in order to eliminate any presence of residual non-crossed photoresist. A layer of 20 nm of Ti/TiO_2 was deposited by DC sputtering using an increasing oxygen flow rate during the deposition process, going from 5 cm^3/s of O_2 during the first 10 nm to 20 cm^3/s of O_2 the last 5 nm while keeping the Ar flow rate at a constant 5 cm^3/s. This method increased the adherence of the top Au layer on the substrate more than using a bare Ti layer. Finally 100 nm of gold was deposited by AC sputtering (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w). Prior to chemical modification, the electrodes were sonicated for 5 minutes in acetone, 5 minutes in isopropanol, rinsed with Milli-Q water, and finally sonicated for 10 minutes in a mixture containing 3 volumes of KOH 50 mM and 1 volume of H_2O_2 at 30 %. Finally the electrodes were rinsed in Milli-Q water and dried with a N_2 spray gun.

Electrode functionalisation with forward primer

The electrode arrays were functionalised by drop-casting 1 µL of 1M KH_2PO_4 containing 10 µM of thiolated forward primer from *P. salmonis* and 100 µM of 1-mercapto-6-hexanol on each electrode and leaving the electrode arrays overnight (>20 h) in a water saturated atmosphere. Control electrodes were functionalised with the same protocol, with 1M KH_2PO_4 containing only 100 µM of 1-mercapto-6-hexanol. The electrodes were then thoroughly rinsed with Milli-Q water, gently dried with a N_2 spray gun and stored inside an argon filled tube in the fridge until required.

Microfluidic fabrication and mounting

Microfluidics were fabricated using medical grade double-sided adhesive and PMMA cover plates using a CO_2 laser marker (Fenix, Synrad, USA) to precisely define channels dimensions, as well as inlets and outlets in the PMMA cover plates. Functionalised electrodes and adhesive-PMMA assemblies were aligned and pressure bonded to produce a 10 µL microfluidic channel to carry out the RPA and electrochemical measurements (Figure 4-2). Prior to use, the microfluidic channels were flushed with 200 µL of PBS containing 0.05 % (v/v) Tween 20 (PBS-T20), 200 µL of Milli-Q water and dried with a N_2 spray gun.

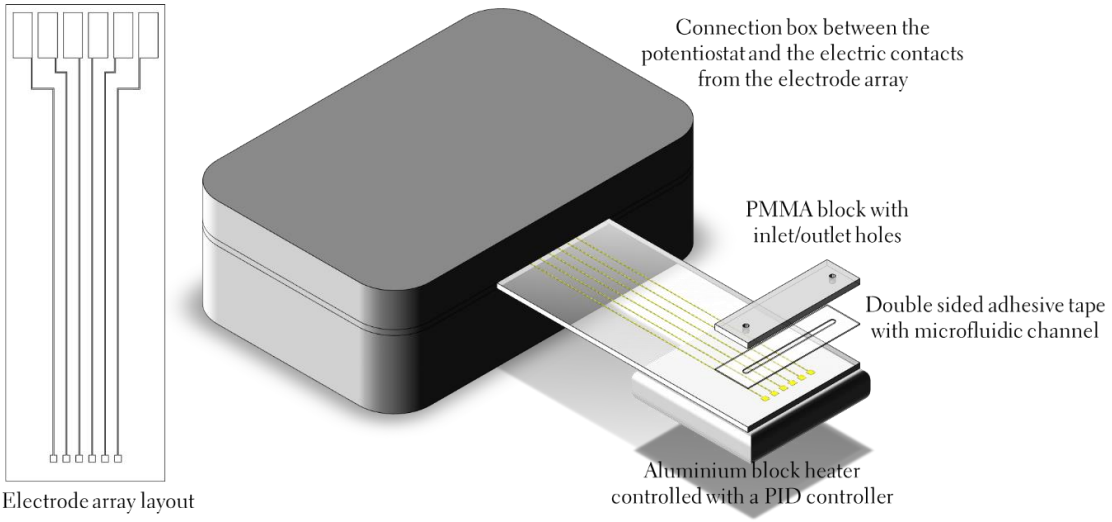

Figure 4-2. Design and representation of the electrode array, the microfluidic channel and the temperature controller.

Solid-phase RPA on electrodes

The RPA amplification mixture was prepared according to the manufacturer, by mixing 2.4 µL of reverse primer 10 µM and 13.2 µL of diluted extracted genomic DNA from salmon or genomic DNA standards in 31.9 µL of rehydration buffer. For the calibration of the sensor, the PCR amplified genomic DNA from salmon, including 8 dilutions, were used as standards in order to construct the calibration curve. Lyophilised pellets were then added and mixed by pipetting. The reaction was finally triggered by addition of 2.5 µL of 280 mM magnesium acetate to a final volume of 50 µL. The solution was then immediately mixed by pipetting and 10 µL injected into the microfluidic channel of the set-up and incubated at 37 °C for 1 h in a water saturated atmosphere.

Electrochemical measurements

All electrochemical measurements, both for the standards and the extracted genomic DNA, were performed with a potentiostat/galvanostat PGSTAT 12 Autolab controlled with the General Purpose Electrochemical System (GPES). To carry out the electrochemical measurements each of the microfluidic channels of the set-up were flushed following completion of the solid-phase RPA with 3 × 200 µL of PBS-T20 to remove all the amplification mixture. Subsequently, each channel was filled with 10 µL of 0.5 nM streptavidin-HRP prepared in PBS and incubated in a humidity box for 30 minutes. Following incubation, the channels were sequentially flushed with 3 × 200 µL of PBS-T20 and 200 µL of PBS. For the electrochemical measurements the microfluidic channels were filled with 10 µL of TMB enhanced one component HRP membrane and allowed to react for 5 min. Finally, the

oxidised/precipitated TMB was detected for each electrode through chronoamperometry by applying two consecutive potential steps, first at 0 V for 0.01 sec and then -0.2 V *vs.* Au for 0.5 sec, and taking the current readout at the end of the second step. The measurements recorded for the genomic DNA standards were used to construct the calibration curve, and later used to calculate the concentration of genomic DNA present in the real samples. Results are represented as mean ± standard deviation from four independent assays. All of the experimental results were analysed by Student's t-test and p-values less than 0.05 were considered statistically significant.

Real-time PCR

For a comparative analysis of the developed method, the extracted genomic DNA fragments were also amplified by real time PCR. 150 ng of each DNA sample was loaded in a PCR master mix containing the dsDNA binding dye Eva-green, and amplified through a standard PCR procedure of 40 cycles with an annealing temperature of 60 °C, and a melting curve analysis from 55 to 95 °C with ramp rate of 0.1 °C/s.

4.5 Results and discussion

4.5.1 Characterisation of extracted genomic DNA from salmon by PCR and AGE

Electrophoretic analysis of PCR amplification of the genomic DNA from eight salmons (7 infected with SRS and 1 not infected), showed bands in all of the seven infected samples (Figure 4-3) with different intensities, of the same length corresponding to the amplified region (153 bp). The bands corresponding to samples 1085 and 1105 were difficult to visualise due to the low concentration of amplicons. The non-template control (NTC) and the non-infected sample (940) showed no bands at the expected region from the selected sequence, and the bands in the lower region of the gel correspond to the primers used during the PCR amplification.

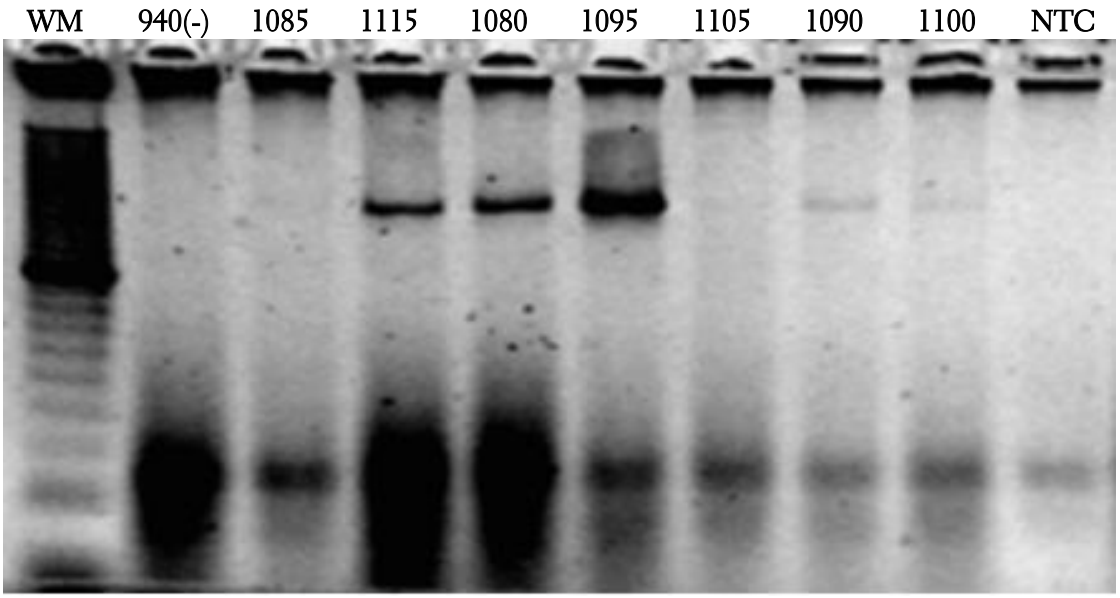

Figure 4-3. Bands corresponding to the PCR products from genomic DNA extracted from salmon livers.

4.5.2 Real-time PCR

Real-time PCR analysis showed positive amplification in all samples (Table 4-2) with threshold cycle (Ct) values lower than 32 and a melting peak at 85 °C. Sample 940 corresponds to a fish not infected with *P. salmonis* (negative control). All other samples are from salmon infected under challenge conditions, 10 days post infection.

Table 4-2. Results of PCR amplification of DNA extracts analysed in this study.

Sample name	Ct
940 (-)	–
1080	28.6
1085	32.3
1090	30.1
1095	26.9
1105	31.6
1110	30.5
1115	29.2

4.5.3 Solid-phase RPA on electrodes

The solid-phase recombinase polymerase amplification on gold electrodes has been demonstrated in a previous work.[59] The calibration curve (Figure 4-4) was constructed using the current density (j) recorded by the reduction of the oxidised TMB when a potential of -0.2 V *vs.* Au was applied for 500 ms in chronoamperometry, the current density having a linear correlation with the amount of starting target concentration of genomic DNA, while the response from the control electrodes was negligible. The assay was completed within 40 minutes and

yielded a LOD of $1 \cdot 10^{-14}$ M, corresponding to $6 \cdot 10^{4}$ starting DNA copies in 10 µL, defined as the concentration of the analyte at the mean blank plus three times the standard deviation of the blank. The sensitivity was 0.86 $\mu A \cdot mm^{-2} \cdot M^{-1}$ with a dynamic range of 5 orders of magnitude.

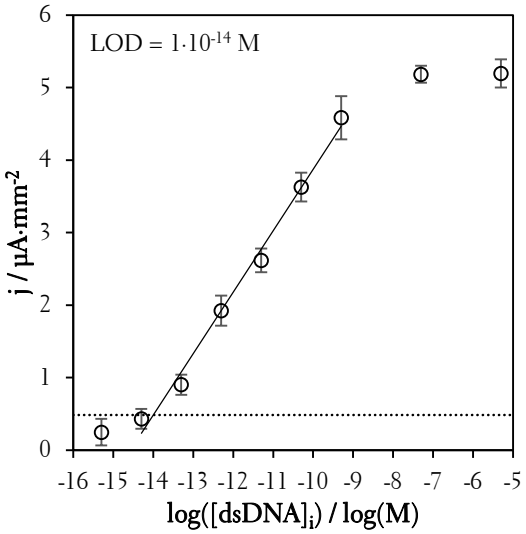

Figure 4-4. Calibration curve using genomic DNA standard dilutions (n = 4).

The diluted genomic DNA samples extracted from eight salmon livers were analysed using the developed platform and the output signals for each sample are represented in Figure 4-5. The signal from the control electrodes at each sample represent the background signal due to non-specific interaction between the streptavidin-HRP and the surface, or signal due to diffusion of oxidised TMB from the specific electrodes towards the controls. The sensitivity of the test was assessed by comparing the electrochemical signal outputs from these samples against the NTC assays and evaluated by the Student's t-test to determine statistical significance or difference. Seven samples, the ones infected by *P. salmonis* showed statistical significance for a significance level of 0.05, thus representing positive results and corroborating the results obtained in real-time PCR (RT-PCR) and gel electrophoresis. Even though the signal output from the samples 1085 and 1105 was low, the system was capable of producing a statistically significant readout, and compared with the gel electrophoresis results, the analytical information was more reliable and easier to interpret.

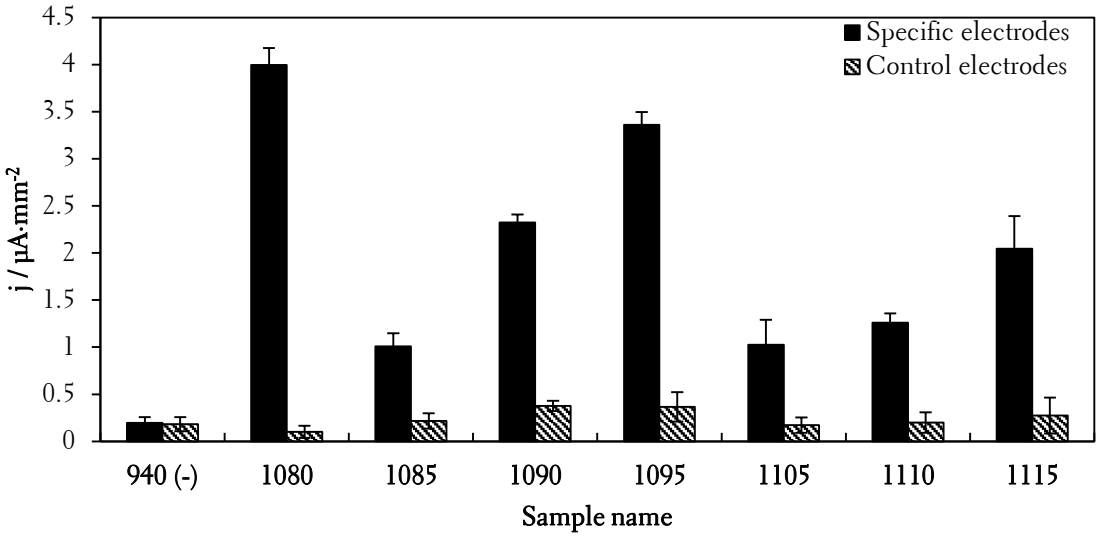

Figure 4-5. Current density signal for each analysed sample.

4.6 Conclusions

To date, sensitive RPA-based devices developed to perform nucleic acid amplification tests are mostly either based on lateral flow tests that lack of quantification capabilities, or, alternatively, use fluorescence detection methods that require expensive equipment or rely on even more complex microarray setups to achieve multiplex detection. Thus, the demand for simpler, miniaturised systems and assays for rapid pathogen detection is steadily increasing. Electrochemical biosensors have many advantages over the presented solutions, being less complex and more cost-effective than most optical detection systems and are easier to integrate with microfluidic platforms. Here, we have presented a simple platform that combines the advantages of solid-phase recombinase polymerase amplification with the electrochemical detection of the amplified DNA. As proof-of-concept, the device has been used for the detection of a *Piscirickettsia salmonis* related sequence from genomic DNA extracted from salmon livers in Chilean farms. The assay could be completed in less than 1 h, achieving detection limits of $1 \cdot 10^{-14}$ M ($6 \cdot 10^{4}$ DNA copies in 10 µL) and displaying a good sensitivity in real sample assays.

4.7 References

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Chapter

Solid-phase bridge recombinase polymerase amplification with ferrocene-labelled dNTPs for electrochemical detection

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Solid-phase bridge recombinase polymerase amplification with ferrocene-labelled dNTPs for electrochemical detection

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5.1 Abstract

Solid-phase bridge amplification was achieved by the immobilisation of both primers in gold electrodes while maintaining a convenient primer-to-primer separation with the co-immobilisation of 6-mercapto-1-hexanol in a 1:100 primers-to-spacer molar ratio. Isothermal DNA specific amplification of the primers was generated by the use of recombinase polymerase enzymatic amplification at 37 °C. Bridge formation was achieved by the addition of magnesium and sodium salts at high concentration that allowed to extend the flexibility and the bending limit of dsDNA, thus allowing propagation of the bridge formation between neighbour primers and sustained amplification until saturation of the gold surface was achieved after 3 h. The detection of the amplified primers was electrochemically monitored in differential pulse voltammetry by the oxidation of previously incorporated ferrocene-labelled nucleotide triphosphates during the amplification. The assay yielded successful specific amplification of a 144 bp target with a LOD of $1.3 \cdot 10^{-12}$ M, while non-specific primer controls and non-template assays did not produce statistically significant positive results.

5.2 Introduction

The recombinase polymerase amplification system,[1] has been applied in a wide range of formats due to the simplicity, the ease-of-use and the constant temperature required to achieve amplification, without any need for a first cycle thermal denaturation step. Reported systems include the use fluorescent probes for real-time amplification-detection of either DNA[2-4] or RNA[5-7]. Other common applications are the cost-effective end-point detection system using lateral flow sticks.[8-10] The need for thermal cycling instruments typically used in the polymerase chain reaction is avoided and replaced by three core proteins that operate optimally at 37 °C to 40 °C. The first protein, a recombinase, binds to primers, forming filaments that can then recombine with homologous DNA in a duplex target, forcing displacement of the non-complementary strand and thus provoking the formation of a D-loop. The second protein is a

single stranded DNA binding protein, which attaches to the strand of DNA displaced by the primer, preventing the dissociation of the primer and hybridisation of the duplex target. The final core protein is a strand-displacing polymerase that copies the DNA, adding bases onto the 3′ end of the primer, forcing open the DNA double helix as it progresses. When opposing primers are used, exponential amplification occurs. In solid-phase RPA at least one of the primers is linked to a surface, therefore the amplification may occur both in the liquid and the solid-phase simultaneously or solely in the solid-phase when the two primers are attached. This strategy has been extensively used in DNA amplification for simultaneous analysis and easy isolation of the amplicons.[11-16] Another benefit from solid-phase strategies is that the spatial separation between different targets allow the use of label-free or, at least a single reporter for all the sequences, without the need of use a different label for each target. The solid-phase approach overcomes some limitations present in regular RPA-based analysis due to the fact that it retains the specific sequences on the surface while the random amplified DNA in solution is washed away during the process. Although the random amplification in RPA has been already fixed, the solid-phase strategy offers a more simple way to overcome the present limitation at the cost of an increased reaction time (from typically few minutes to one hour).

The use of additional primers in solution, *i.e.* heminested solid-phase amplification,[17] has proven to boost the amplification efficiency while the primers in the solid-phase are basically used to separate or obtain spatial resolution of different targets[18] and, in biosensors, to enhance the sensitivity by bringing the amplicons near to the sensing area, *i.e.* the transducer. The heminested solid-phase RPA solution[19] combines DNA amplification in bulk solution using one set of primers and solid-phase amplification using other primers that amplify a different region from the target. This strategy overcomes the traditional low efficiencies of solid-phase oriented amplification, and yet has proven to be a fast DNA amplification technique where spatial resolution is necessary for multiplex detection. However, still one of the major problems in DNA amplification comes when multiple targets need to be amplified and detected, so the number of primers required increases and so the chance of cross-hybridisation between primers in a process called primer-dimer formation.[20] Careful primer design involve tedious trial and error assays, increase the cost of the assay significantly and the complexity grows exponentially with the number of sequences required to be analysed simultaneously. This major drawback can cause amplification of undesired genetic material and affect the overall performance of the assay and lead to false positive detections.

When both primers are immobilised and no other primers are present in solution the approach is called bridge amplification and was successfully introduced first by the Illumina system.[21] Therefore, the most important improvement when using the solid-phase bridge amplification is the fact that primer-dimers are completely avoided, thus providing an effective way for multiplex detection with spatial resolution, though generally the amplification efficiency is much lower than other strategies and it is used only for detection. The solid-phase bridge amplification technique present nowadays use cycles of denaturation based on thermal[22] or photo-cleaving of primer containing breaking sites,[21] to unfold the bridge and allow further annealing with neighbour primers. Although this is an excellent strategy for general DNA amplification, DNA detection do not need to produce further amplification once the surface of the biosensor is saturated, moreover the photo-cleaving strategy requires additional extra instrumentation, along with the need of denaturing steps make the system a bad choice for integration in point of care devices.

Here we present an alternative strategy to achieve bridge amplification based on tailoring the surface primer immobilisation to ensure enough spatial separation between primers and the use of sodium and magnesium salts, which at high concentrations (1 M sodium or 10 mM magnesium) have proven to increase the curvature and improve the bending elasticity of dsDNA.[23-27] Electrochemical detection is based on the incorporation of ferrocene-labelled nucleotides triphosphates (Fc-dNTPs) during the amplification that are interrogated by differential pulse voltammetry (DPV) afterwards (Figure 5-1).

5.3 Materials and methods

RPA kit TwistAmp® Basic was obtained from TwistDx Ltd. (Babraham, United Kingdom). The HRP substrate formulation TMB enhanced one component HRP membrane was purchased from Diarect AG (Germany), and GelRed™ Nucleic Acid Gel Stain from Biotium (Barcelona, Spain). 6 mm thick polymethylmethacrylate (PMMA) was purchased from La Indústria de la Goma (Tarragona, Spain), double-sided medical grade adhesive foil from Adhesive Research (Ireland) and all other chemicals were obtained from Sigma Aldrich S.A. (Barcelona, Spain) and used as received.

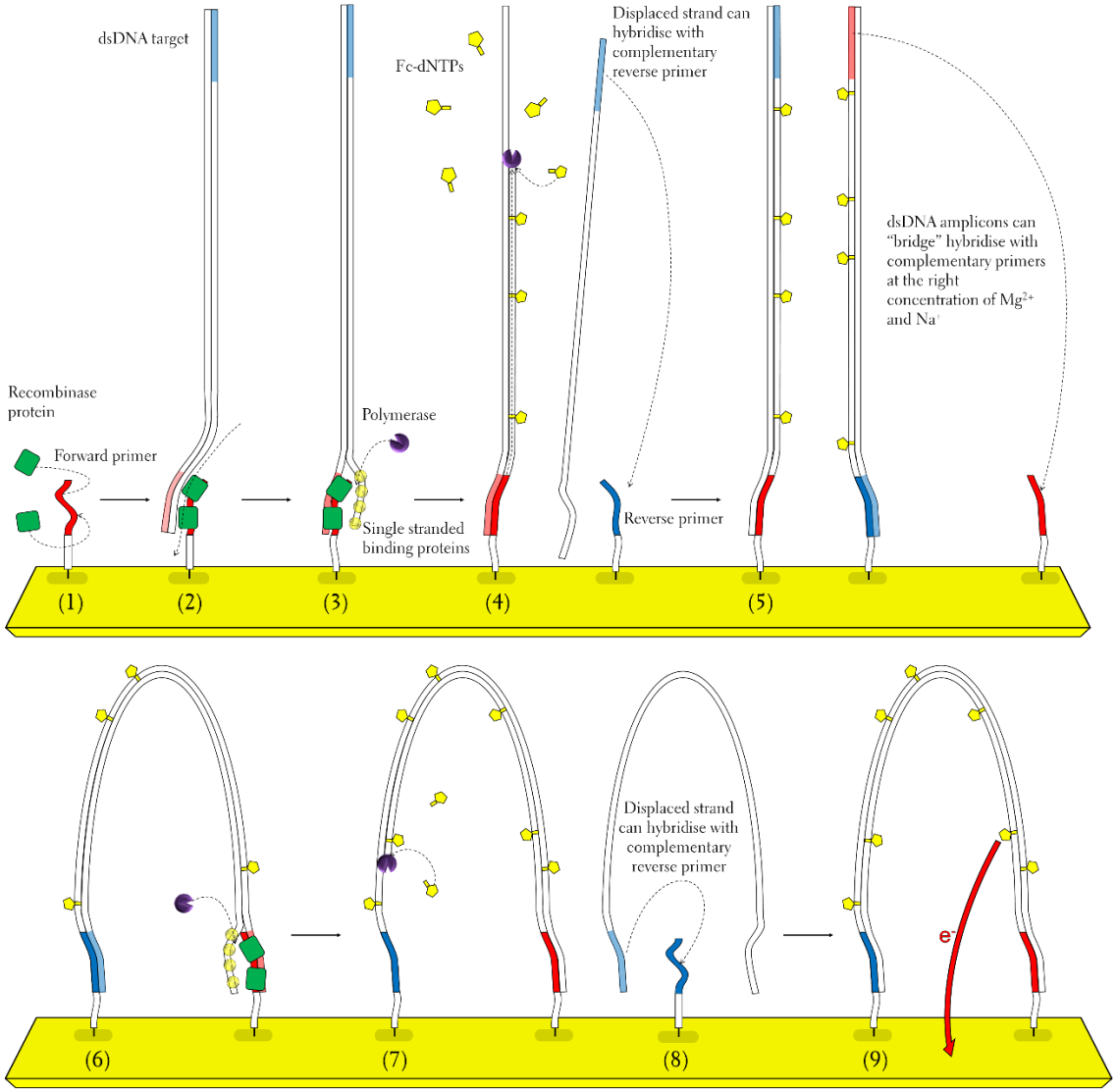

Figure 5-1. Solid-phase "bridge" RPA with incorporation of Fc-dNTPs for electrochemical detection. (1) Recombinase proteins form a complex with forward and reverse primers, (2) scan dsDNA for cognate sites and (3) introduce the primers in the template by a strand-displacement mechanism. (4) The polymerase initiates primer elongation at their 3'-ends and Fc-dNTPs present in solution are incorporated. The displaced strand can hybridise with complementary primers and generate further amplification. (5) The dsDNA amplicons can "bridge" hybridise with complementary primers at the right concentration of Mg^{2+} and Na^{+}. (6) Polymerase can amplify the bridge structure and (7) the displaced strand can allow further amplification in another primer (8). (9) DPV is performed to detect the presence of Fc-dNTPs.

Synthetic oligonucleotides designed for the identification of the pathogenic bacteria *Francisella tularensis holarctica*[28] were purchased as lyophilised powder from Biomers.net (Ulm, Germany), reconstituted at 100 µM in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain) and used without further purification (Table 5-1).

Table 5-1. List of oligonucleotide sequences and their respective modifications.

Name	Nucleotide sequence (from 5'-end to 3'-end)
F. tularensis forward primers	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCC (5'-HRP and 5'-SH-$(CH_2)_6$-T_{15}-)
F. tularensis reverse primers	CGCTACAGAAGTTATTACCTTGCTTAACTGTTA (5'- HRP and 5'-SH-$(CH_2)_6$-T_{15}-)
F. tularensis DNA sequence	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCCAGAAGGTTCTA AGTGCCATGATACAAGCTTCCCAATTACTAAGTATGCTGAGAAGAA CGATAAAACTTGGGCAACTGTAACAGTTAAGCAAGGTAATAACTTC TGTAGCG
Non-complementary primer	CTGTGCACGGTGTAGATAAGTCTTTTAATGCAG (5'-SH-$(CH_2)_6$-T_{15}-)

All the DNA solutions were aliquot and diluted to convenient concentrations using Milli-Q water unless otherwise stated. Double stranded DNA templates were prepared by mixing equal volumes of complementary strands in PBS buffer, heating to 95 °C for 10 minutes and letting the solutions gently cool to room temperature.

5.4 Experimental

5.4.1 Electrode preparation

The electrochemical platform was designed with a set of six squared shaped electrodes (1 mm^2) made with photolithographic gold on a pre-cleaned 75 mm × 25 mm borosilicate glass slide substrate (Sigma Aldrich, Spain), using four of them as working electrodes, and the remaining two as counter and reference electrodes, respectively. These electrodes were fabricated in a clean room facility by spin-coating a positive photoresist AZ1505 (MicroChemicals GmbH, Germany) at 4000 rpm for 30 sec over a dry glass slide. The platform was then exposed to UV light for 3 sec using a chromium mask in contact mode (LED Paffrath GmbH, Germany). Development of the pattern with a commercial developer AZ 726 was carried out according to the manufacturer's instructions and the slide was introduced into a sputtering chamber (ATC Orion 8-HV, AJA International Inc. USA) and treated with AC O_2:Ar plasma sputtering (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w) for 5 minutes in order to eliminate the presence of any residual non-crossed photoresist. A layer of 20 nm of Ti/TiO_2 was deposited by DC sputtering using an increasing oxygen flow rate during the deposition process, going from 5 cm^3/s of O_2 during the first 10 nm to 20 cm^3/s of O_2 the last 5 nm while keeping the Ar flow rate at a constant 5 cm^3/s. This method increased the adherence of the top Au layer on the substrate more than using a bare Ti layer. Finally 100 nm of gold was deposited by AC sputtering (5 cm^3/s of Ar, 5 cm^3/s of O_2, 50 w). Prior to chemical modification, the electrodes were sonicated 5 minutes in acetone, 5 minutes in isopropanol, rinsed with Milli-Q water, and sonicated for 10

minutes in a mixture containing 3 volumes of KOH 50 mM and 1 volume of 30 % (v/v) H_2O_2. Finally the electrodes were rinsed in Milli-Q water and dried with a N_2 spray gun.

The electrode arrays were functionalised by co-immobilisation of the forward and the reverse primers having a poly-15T, a vertical spacing tail consisting of fifteen 2'-deoxythymidine monophosphate nucleotides at the 5'-end of the probe. One microlitre of 1 µM thiolated forward primer and 1 µM of thiolated reverse primer in 1 M KH_2PO_4 solution together with 1-mercapto-6-hexanol at 200 µM, were dropcast on the electrode surface. Two electrodes out of the six were always used as negative controls and functionalised with the same immobilisation solution used for the other electrodes but without the specific thiolated primers. Co-immobilisation was carried out in a saturated humidity chamber at room temperature for 20 h. Subsequently, the electrodes were rinsed in milli-Q water and dried in a stream of nitrogen. A medical grade double-sided adhesive and a 3 mm thick PMMA block were aligned and pressure bonded with the functionalised electrodes to produce a fluidic chamber of 10 µL where amplification and detection was carried out. The PMMA gasket and the adhesive were cut and milled using a CO_2 laser marker (Fenix, Synrad, USA) to precisely define channel dimensions, as well as inlets and outlets in the PMMA cover plates. The chamber was then washed with 200 µL of PBS containing 0.05 % (v/v) Tween 20 (PBS-T20), followed by 200 µL of Milli-Q water in order to completely remove any non-specific immobilisation of DNA on the electrode surface. The chamber was dried with a nitrogen beam and kept under vacuum until used (Figure 5-2).

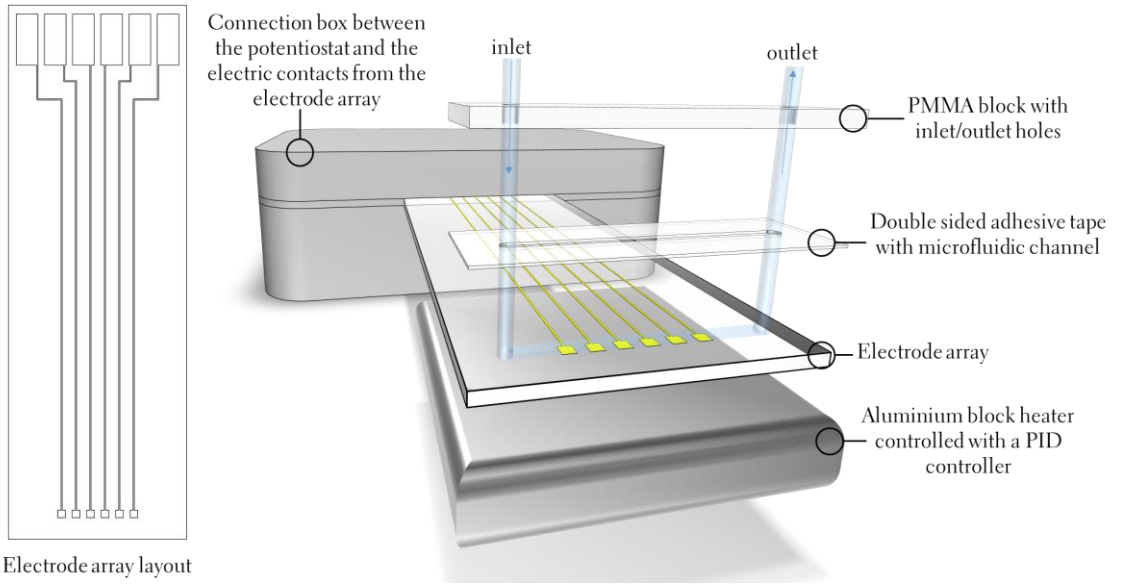

Figure 5-2. Electrode array layout and mounted setup.

5.4.2 Synthesis of ferrocene labelled dNTPs

To a stirring solution of ferrocene carboxylic acid (345 mg, 1.5 mmol) in freshly distilled dichloromethane (DCM) was added N-(3-dimethylaminopropyl) N'-ethylcarbodiimide hydrochloride (288 mg, 1.5 mmol) and N-hydroxy succinimide (NHS) (180 mg, 1.5 mmol). The mixture was left stirring at room temperature for 18 h. Column chromatography in DCM gave a white solid (315 mg, 60 % yield) corresponding to the ferrocene carboxylic N-hydroxysuccinimide ester. Labelled dNTPs were synthesised utilising the NHS esters of the redox labels and subsequent reaction with alkyl amine arm of the dNTPs. To the NHS ester (2.4 µmol, 3 equiv) in DMSO was added 100 µL of Na_2CO_3-$NaHCO_3$ buffer (0.1 M, pH 8.7) followed by addition of 800 nmol of the propargyl amino dNTPs. After sonication for 10 min, the reaction was kept in a thermomixer shaking for 6 h. After the reaction time thin-layer chromatography (TLC) confirmed the complete consumption of the triphosphate. The reaction mixture was kept in the freezer overnight followed by freeze drying. The labelled dNTPs were purified by using preparative TLC with a mixture of solvents $CHCl_3$/CH_3OH : 85/15. Finally, electrospray ionisation mass spectrometry (ESI-MS) confirmed the synthesis of the products. The products were then directly used for SP-RPA experiment.

5.4.3 Solid-phase recombinase polymerase amplification

Lyophilised pellets were reconstituted in 500 µL of rehydration buffer and the dNTPs present in the mixture were filtered by centrifuging the solution in an Amicon® Filter 3K device at 14000 xg at 22 °C for 30 min to obtain a ~50 µL concentrated product. The filtrated volume was discarded and the concentrated product was recovered by inverse centrifugation at 1000 xg for 10 minutes at 22 °C. The RPA amplification mixture was then prepared by mixing 13.2 µL of 10 nM template dsDNA with 6.15 mg of sodium acetate, 1 µL Fc-dGTP, 1 µL Fc-dUTP, 1 µL Fc-dCTP and 1 µL Fc-dATP in the recovered filtrate enzymatic residue. 2.5 µL of magnesium acetate 280 mM was finally added to trigger the reaction, the solution was then briefly mixed by pipetting, and 10 µL/array injected into each microfluidic channel of the set-up and left to incubate at 37 °C for 3 h. The calibration curve of the assay was examined by carrying out assay repetitions at different initial concentrations of target dsDNA.

5.4.4 Optimisation experiments

Different parameters were examined in order to obtain the best analytical performance of the system. To this end, we looked for the improvement of sensitivity by using 1 or 4 ferrocene labelled dNTPs simultaneously, along with the effect of either filtering out or not the regular

dNTPs present in the TwistDX pellet. When the regular dNTPs were replaced by 1 ferrocene-labelled dNTP, the rest of the dNTPs were added to the solution to ensure amplification.

Additionally, the amplification efficiency was also tested out at different concentrations of sodium acetate, as well as the reaction time required to obtain a full surface coverage and the lateral spacing required between primers to successfully achieve solid-phase bridge amplification.

5.4.5 Electrochemical detection

A potentiostat/galvanostat PGSTAT 12 Autolab controlled with the General Purpose Electrochemical System (GPES) was used to make the electrochemical measurements, and a home-made connection box used to plug the electrode arrays and generate electric connection between the potentiostat and the electrode arrays. After the bridge RPA amplification the microfluidic channels were cleaned with 200 µL with PBS-T20 to remove the excess of ferrocene labelled dNTPs in solution and then the electrochemical measurements were carried out directly in the same buffer by differential pulsed voltammetry (0 to 0.6 V (*vs.* Ag/AgCl). Modulation time: 0.003 sec, interval time: 0.2 sec, step potential: 0.01 V, modulation amplitude: 0.1 V).

After the measurements, the amplified primers were unfold by denaturing with a NaOH 0.1 M solution, following with the hybridisation of the immobilised primers with both HRP-labelled reverse and forward primers at a concentration of 50 nM for 30 min to test out that the amplified regions were sequence specific. Chronoamperometry was used to detect the oxidation of precipitated TMB by the HRP label, by applying two consecutive potential steps at 0 V for 0.01 sec and -0.2 V for 0.5 sec to each electrode sequentially, and taking the current readout at the end of the second step.

5.5 Results and discussion

The electrochemical monitoring of the RPA by differential pulsed voltammetry of Fc-dNTPs present in the amplification mixture reported specific bands at 0.43 V *vs.* Ag/AgCl with areas as large as 230 nC, whereas the controls were giving areas of 30 nC. The current from the control electrodes was produced by the residual labelled dNTPs left on the surface of the blank electrodes after the PBS-T20 cleaning due to non-specific binding. However, the specific signal was still clearly and statistically distinguishable from the blank electrodes. Therefore, the Fc-dNTPs at the given concentration were effectively introduced in the elongated forward primer during the recombinase mediated solid-phase amplification without producing false

positives due to the amount of non-specific binding. This strategy allowed us to skip assay time compared to previously reported electrochemical detection methods in RPA.[29]

The performance for the different optimisation assays are listed in (Figure 5-3). The best conditions in terms of signal output, for a given concentration, were found when the regular dNTPs were removed and replaced by Fc-dNTPs, improving the yield barely 2 to 6 times compared to the signal outputs by adding only one Fc-dNTP at a time. Although it is hardly expected that amplification might occur in a scenario where all the dNTPs are labelled due to steric hindrance effects between base pairs, the fact that filtration step do not remove all the regular dNTPs would allow amplification take place when Fc- dNTPs might not be incorporated. It is observed that the dispersion of the ferrocene oxidation signal in the assays where the regular dNTPs have been removed is clearly thinner than the assays where regular dNTPs were not removed. This effect can be explained by the fact that in this situation regular dNTPs and Fc-dNTPs are in a competitive fashion to be introduced during the solid-phase amplification, therefore the total incorporation of Fc-dNTP may change drastically from one electrode to another. This introduces a factor of uncertainty at the number of Fc-dNTPs that finally end up in the elongated primers and so the signal output obtained during the electrochemical measurements. It is also expected that this dispersion might be further improved if the amplification mixture could be prepared in a way that the reagents can be added separately, thus the concentration of regular dNTPs well known.

The necessary time required for the bridge assay to complete surface saturation was examined by stopping the amplification reaction of different arrays at different stages (Figure 5-3.B). After 1 h, the amplification yield achieved in the surface of the electrodes reached 1/6 of the total saturation signal obtained typically after 3 h, when completion of the assay was typically achieved. The amplification time is much slower than those reported in liquid-phase, heminested strategies, of one primer in solution and the other nested to the sensors. Also the amplification yield through the time appears to be linear instead of exponential, and this phenomena can be explained due to the fact that the bridge amplification strategy entrails much more impediments that liquid-phase amplification. Higher steric hindrance of the proteins and the surface, more limited mobility and availability of the primers to be elongated and the probabilistic mechanism required for the elongated dsDNA to bend towards the surface and find an additional primer to further expand the amplification.

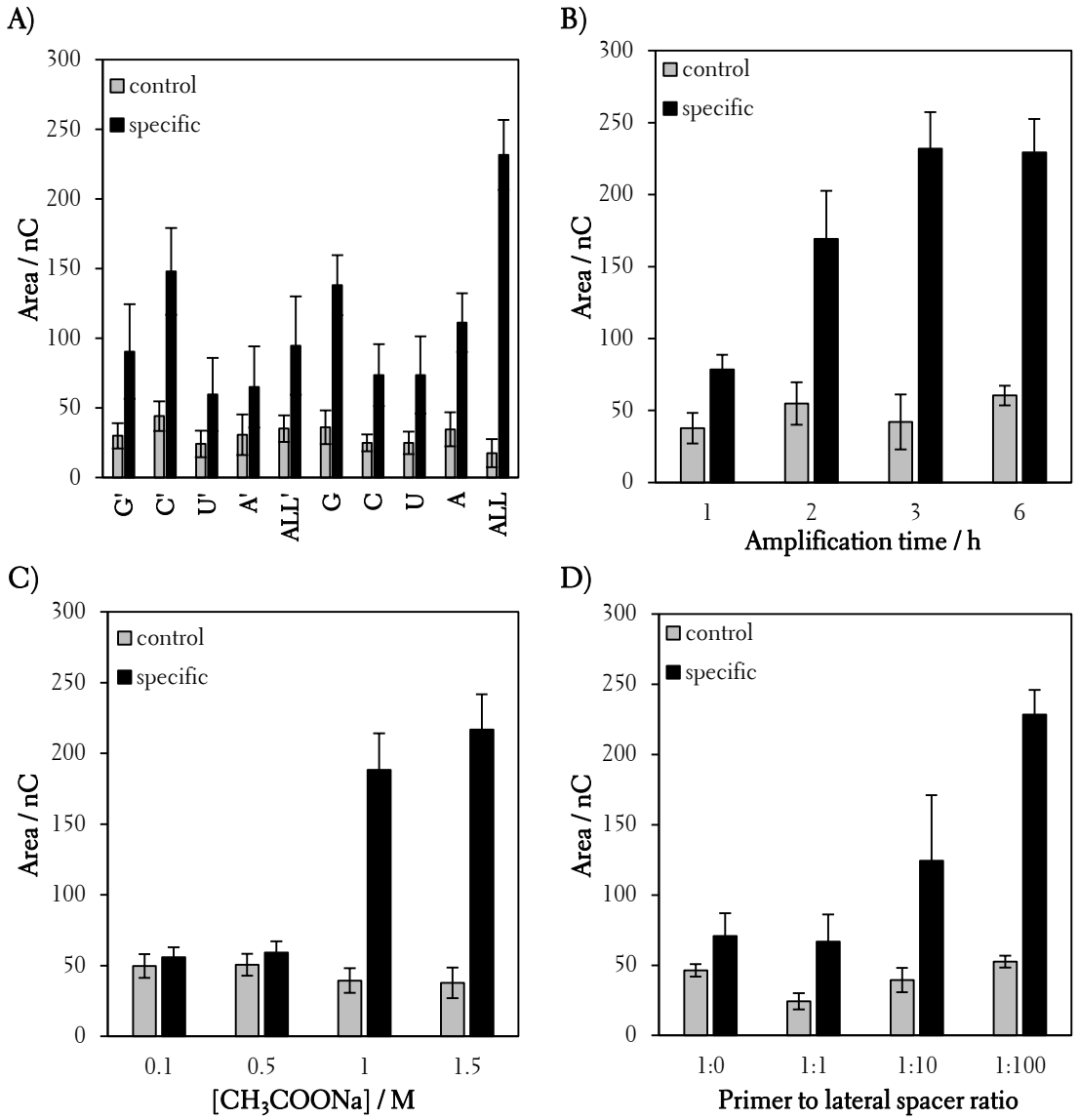

Figure 5-3. Optimisation experiments, regarding A) amplification time, B) concentration of sodium acetate, C) Primer to lateral spacer ratio and D) labelling of one or multiple dNTPs with the presence (indicated with ') or absence of their standard nucleotide counterparts.

The concentration of sodium acetate proved to be a critical factor to achieve bridge amplification, and concentrations below 1 M were unable to generate enough DNA bending to self-sustain the amplification rate, thus not yielding any amplification even after 6 h (Figure 5-3.C). Although the concentration of magnesium and the bending effect on dsDNA could have been tested, this ion is correlated to the rate of reaction: a higher concentration increases the amplification rate, but an excess amount causes non-specific amplification.[30] Therefore we did not want to further push the limit of the system by increasing the concentration of magnesium, and only the amount added to initially trigger the reaction was used.

Different ratios of the lateral spacer were checked in order to obtain the right surface tailoring, *i.e.* the sufficient spacing between forward and reverse primers to allow the formation of dsDNA bridge structure and ensure a self-sustained amplification rate (Figure 5-3.D). The spacing was obtained by increasing the concentration of 6-mercapto-1-hexanol while keeping the concentration of the primers constant. No spacing (1:1:0 molar ratio) and a 1:1:2 ratio yielded an amplification that was statistically distinguishable from the controls, we thought this signal was due to the initial solid-phase amplification phase in which target DNA is amplified on the surface of the electrodes but bridge structures is seldom generated or not generated at all. Although a spacing generated by a 1:1:20 ratio provided larger bands, the variance between electrodes was quite high probably due to the low chance of bridge formation and only ensuing extension towards the nearest neighbours, not producing enough saturation until a 1:1:200 molar ratio was tested.

The areas from the specific Fc-dNTPs bands obtained by differential pulsed voltammetry method were proportional to, respectively, the amount Fc-dNTP present in the amplified region of the surface tethered primers. And therefore, the amount of label was also proportional to the starting target concentration of dsDNA for each assay. The strategy represents a huge improvement in terms of protocol simplicity, due to the fact that detection can be performed immediately after the amplification without the need of adding any extra hybridisation or conjugation step nor any substrate or reagent like TMB/H_2O_2. The amplification time required to achieve surface saturation, 3 hours, was longer than previously published methods due to the fact that pure solid-phase amplification is less efficient. Non-specific primers were used for control assays yielding no significant difference between the specific and the control electrodes (Figure 5-4.A). Specificity of the amplified sequence on the primers was examined by denaturing the surface, thus producing the unfolding of the bridge formation, and hybridisation the end of the amplified primers with HRP-labelled reverse and forward primers. The amount of hybridised HRP-primers was determined by chronoamperometry in the presence of TMB/H_2O_2, producing a negative current proportional to the amount of oxidised TMB in solution (represented as positive current) (Figure 5-4.B).

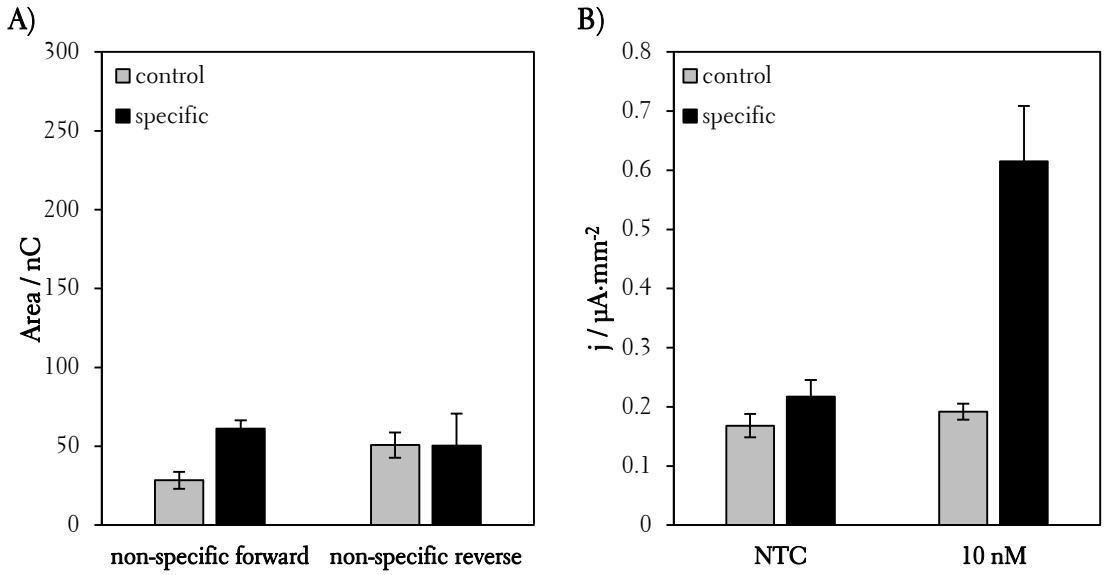

Figure 5-4. Control assays to check A) non-specific primers in DPV and B) the specificity of the amplified solid-phase immobilised primers by hybridisation with specific HRP-labelled primers after the amplification. Current output recorded after 5 minutes in TMB/H_2O_2 substrate.

The calibration curve of the Fc-dNTP strategy is shown in Figure 5-5. In terms of LOD, sensitivity and reproducibility, this strategy did not show as good results as previously reported strategies using labelled-primers. The non-template control is represented in the calibration curve as the mean value of the blank plus three times the standard deviation of the blank. The fact that DPV is a very sensitive technique in terms of signal-to-noise ratio gave very low control signals, and still an acceptable LOD of $1.3 \cdot 10^{-12}$ M.

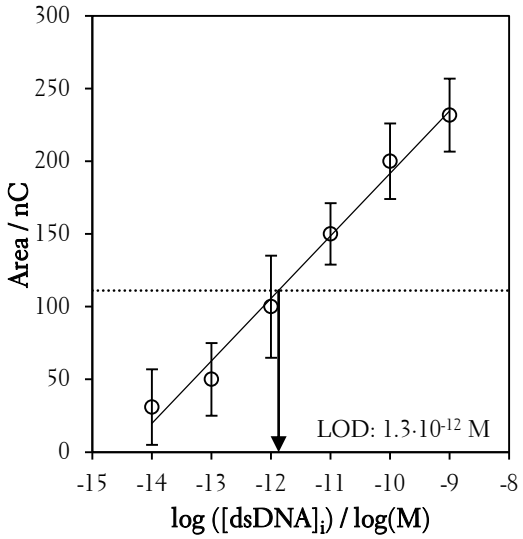

Figure 5-5. Calibration curve of the solid-phase bridge RPA with Fc-dNTPs.

5.6 Conclusions

Solid-phase bridge recombinase polymerase amplification of a dsDNA target have been achieved on the surface of gold electrodes. Both forward and reverse primers were immobilised by thiol chemistry co-immobilisation of the primers and 6-mercapto-1-hexanol, using a primer-to-spacer molar ratio of 1:1:200, and a concentration of sodium acetate of 1.5 M to allow enough bending flexibility to the 144-bp long dsDNA target to ensure the sustained amplification in neighbour primers. Detection of the amplified material was carried out by incorporation of Fc-dNTPs during the amplification and end-point differential pulse voltammetry measurements following the complete electrode saturation after 3 h. The performance of the assay in terms of limit of detection was $1.3 \cdot 10^{-12}$ M. This strategy represents a simple and generic electrochemical option to amplify and detect multiple sequences simultaneously due to the fact that primers are anchored and spatially separated and primer-dimer formations are not allowed.

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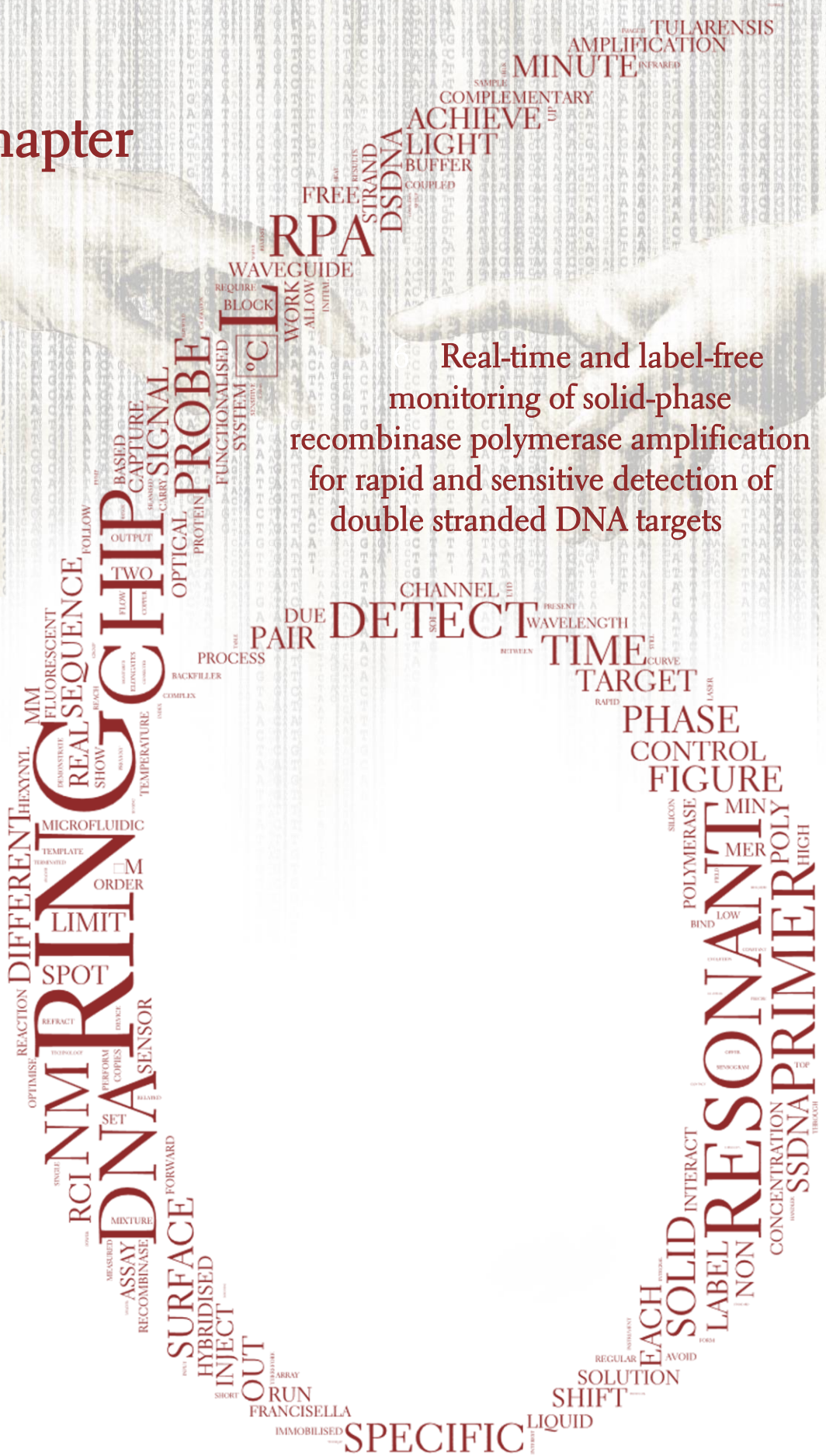
DNA BIOSENSORS BASED ON INTEGRATED ISOTHERMAL AMPLIFICATION-DETECTION STRATEGIES
Jonathan Sabaté Del Río

Chapter

Real-time and label-free monitoring of solid-phase recombinase polymerase amplification for rapid and sensitive detection of double stranded DNA targets



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Real-time and label-free monitoring of solid-phase recombinase polymerase amplification for rapid and sensitive detection of double stranded DNA targets

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6.1 Abstract

In this work we present the use of a silicon-on-insulator (SOI) chip featuring an array of 64 optical ring resonators used as refractive index sensors for real-time and label-free DNA detection. Single ring functionalisation was achieved using a click reaction after precise nanolitre spotting of specific hexynyl-terminated DNA capture probes to link to an azido-silanised chip surface. To demonstrate detectability using the ring resonators and to optimise conditions for solid-phase amplification, hybridisation between short 25-mer ssDNA fragments and a complementary capture probe immobilised on the surface of the ring resonators was carried out and detected through the shift in the resonant wavelength. Using the optimised conditions demonstrated via the solid-phase hybridisation, a 144-bp dsDNA was then detected directly using recombinase and polymerase proteins through on-chip target amplification and solid-phase elongation of immobilised forward primers on specific rings, at a constant temperature of 37 °C and in less than 60 minutes, achieving a limit of detection of $7.8 \cdot 10^{-13}$ M ($6 \cdot 10^{5}$ copies in 50 µL). The use of an automatic liquid handler injection instrument connected to an integrated resealable chip interface (RCI) allowed programmable multiple injection protocols. Air plugs between different solutions were introduced to prevent intermixing and a proportional-integral-derivative (PID) temperature controller minimised temperature based drifts.

6.2 Introduction

Light-based sensors offer a myriad of interesting and powerful sensing capabilities both in quantitative and qualitative terms. Depending on the integration or detection scheme, optical sensors often show additional benefits including rapid analysis, high sensitivity, high specificity due to specific light-matter interaction, very low interaction with the sample and capability to detect multiple analytes at once.[1, 2] Optical sensors based on microcavities[3] offer the possibility to perform simple, real-time and label-free detection and are of great interest due to the simplification of the overall bioanalytical assay.[4] For biosensing, especially planar waveguide-based optical resonators have garnered increasing attention as fabrication of these devices is compatible with standard lithographic processing techniques, therefore facilitating mass production. The latter also allows for an excellent integration with microfluidic systems, thus having the potential for achieving functional and compact devices, critical in the field of optofluidics.[5] Planar optical resonators can be presented in different sizes, shapes and configurations (number of waveguides and resonators coupled) depending on the use they are required for, but always follow a closed loop geometry typically in the form of a ring, disc or toroid coupled to other waveguides that act either as light inputs and/or outputs. For the sake of simplicity, the illustration used to represent the working principle of a planar optical resonator is the configuration used in this work: a ring with a bus waveguide (input/output) and a drop waveguide (output) (Figure 6-1).

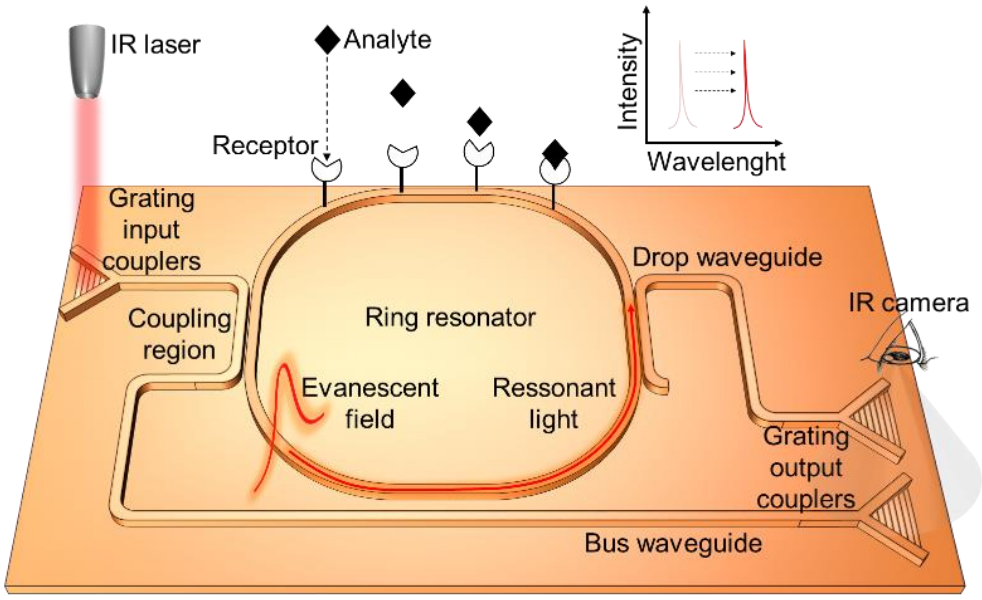

Figure 6-1. Schematic representation of the working principle of a ring resonator as a biochemical transducer and the corresponding spectrum evolution with two waveguides. The ring resonator configuration illustrated here, with a bus and drop waveguides, is also the configuration used in this work.

Silicon optical ring resonators exploit the well-established Si microfabrication processes[6, 7] and the convenience of infrared (IR) light to travel through silicon due to its transparency at the lower energy region of the infrared portion of the spectrum. The IR light does not refract out of the ring due to the difference between the refractive index (RI) of the silicon and the surrounding medium, leading to a total internal reflection effect and thus allowing silicon features to conduct the light as waveguides. Although the light is mostly confined in the waveguide core, a fraction of this light extends out as an evanescent field and can be transmitted (coupled) from a waveguide into another waveguide (here a ring) in close contact. The particular scenario when light is transmitted into a ring-shaped structure is interesting as the light can perform 10^4 to 10^8 loops inside the ring (depending on the quality factor of the ring) before it couples out again or is lost. Only specific wavelengths will constructively interfere after a roundtrip in the ring, and hence be at resonance. The evanescent field also penetrates several tens of nanometres into the surrounding medium (*e.g.*, liquid, gas, and polymer coatings) and interacts with the analytes near the resonator surface. Biomolecule binding events on the surface of the microring increase the effective RI and a shift in the resonant wavelength can be measured, which can be exploited as a transduction mechanism for sensing applications.[8-10]

Ring resonators as transducers are much more compact than other designs such as Mach Zehnder waveguide interferometers, which require long interferometer arms. In combination with the real-time, label-free detection mechanism this results in a compact sensing platform where the sample volume required and assay time are potentially reduced. A broad variety of analytes have been detected using ring resonators, including proteins,[11, 12] cells,[13] gases,[14, 15] viruses,[16, 17] or nucleic acids.[18, 19] The use of diverse receptors such as aptamers,[11] antibodies[20] or phages[17] clearly demonstrate that ring resonators are a powerful and versatile tool for label-free and multiplex analyte biosensing.

In the work described herein, we describe the fabrication of a refractive index sensor based on SOI microring resonator array chips in combination with the use of solid-phase recombinase polymerase amplification (RPA) for the direct detection of dsDNA sequences. RPA is an isothermal DNA amplification technique that, unlike polymerase chain reaction, does not rely on the use of thermal cycling to achieve denaturation of the target and annealing with the primers. Instead, RPA uses an enzymatic mixture of single stranded binding proteins and DNA recombination proteins, thus elongation is achieved at constant and low temperature. Recombinase proteins assemble with ssDNA present in solution, *e.g.* primers, to form a stable protein-DNA complex. This complex is highly efficient at scanning dsDNA, *i.e.* the target, to

identify homologous sequences to the ssDNA. When a homologous sequence in the target dsDNA is found, the recombinase proteins facilitate the introduction of the ssDNA into the complementary dsDNA by a strand-inversion mechanism, thus forming a D-loop structure.[21, 22] Primers are introduced at the cognate site of the template, leaving the 3'-end of the sequence accessible to a strand displacing DNA polymerase. The polymerase elongates the primer according to the template sequence and the amplification is rapidly accomplished by the cyclic repetition of this process.[23] In asymmetric solid-phase RPA[24] one of the primers is covalently linked to a surface, therefore the elongation of primers, thus amplification of the target, occurs both in the liquid and the solid-phase simultaneously (Figure 6-2).

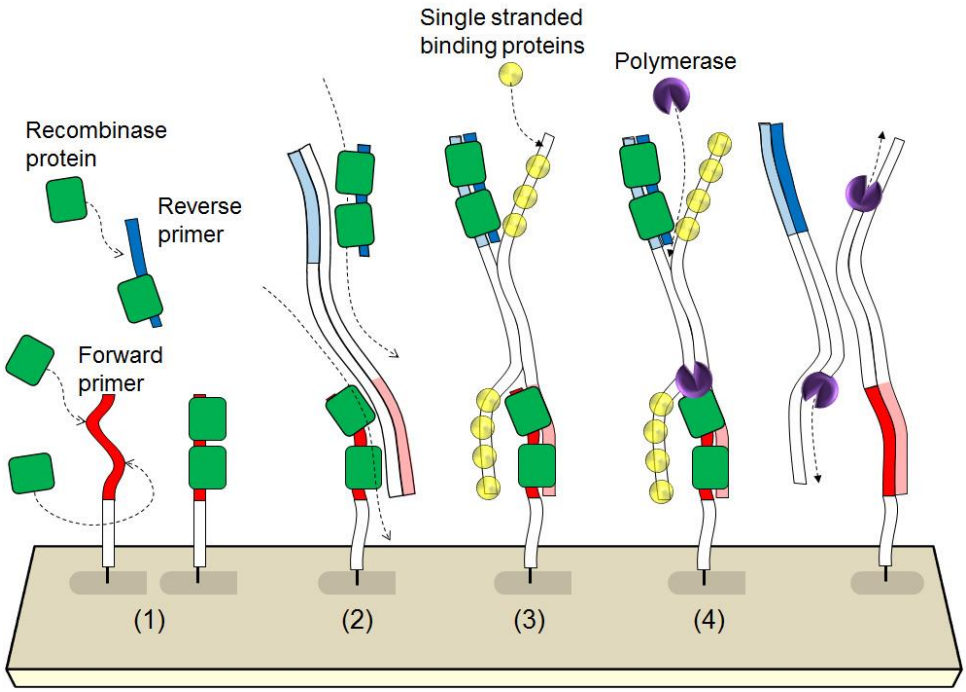

Figure 6-2. Schematic of solid-phase Recombinase polymerase DNA amplification (RPA). (1) Recombinase proteins form a complex with forward and reverse primers, and then (2) scan dsDNA for cognate sites and (3) introduce the primers in the template by a strand-displacement mechanism. (4) The polymerase initiates primer elongation at their 3'-ends and exponential amplification is achieved by cycling of this process.

If the primer is immobilised into a specific region of a sensitive surface, like a ring resonator, then a label-free, real-time and multiplex[25] monitoring of the surface amplification is possible. Solid-phase RPA permits the amplification-detection of dsDNA directly, avoiding further denaturation and hybridisation of the amplification products, speeding up the analysis time and reducing the overall consumption of reagents. Herein we emphasise the use of the ring resonators in combination with RPA to exploit these features without falling behind in performance, granting a simple assay and demonstrate rapid (<60 minutes) and sensitive

amplification-detection of nucleic acid targets (10^4 copies of dsDNA/µL). As a proof of concept, we applied this technique for the detection of a dsDNA sequence related to a *Francisella tularensis* gene, describing the integration of the sensing chip with all the required components to automate the assay, *e.g.* instruments, chip interfaces, devices, software, microfluidics, heating system and optical alignment. For comparative purposes, we also show experimental results using the ring resonator setup for the detection of a ssDNA target through hybridisation with an immobilised capture probe.

6.3 Materials and methods

6.3.1 Chip design and fabrication

The fabrication of the high-contrast waveguides was achieved as previously reported.[26] Briefly, SOI wafers with a silicon thickness of 220 nm and a buried oxide layer of 2 µm were used. After coating the wafer with photoresist and a pre-bake step, the wafers were illuminated (ASML deep UV stepper at 193 nm), followed by a post-exposure bake. The patterns in the photoresist were then transferred to the underlying SOI using a low pressure, high density inductively coupled plasma reactive ion etching (ICP-RIE) dry etch. Following resist removal, 5 nm of thermal oxide was grown. An array of 8 × 8 rings with a Q factor up to 12700 was used in the work reported here. Grating-based fiber couplers were used for the in-coupling and out-coupling of light (Figure 6-3).

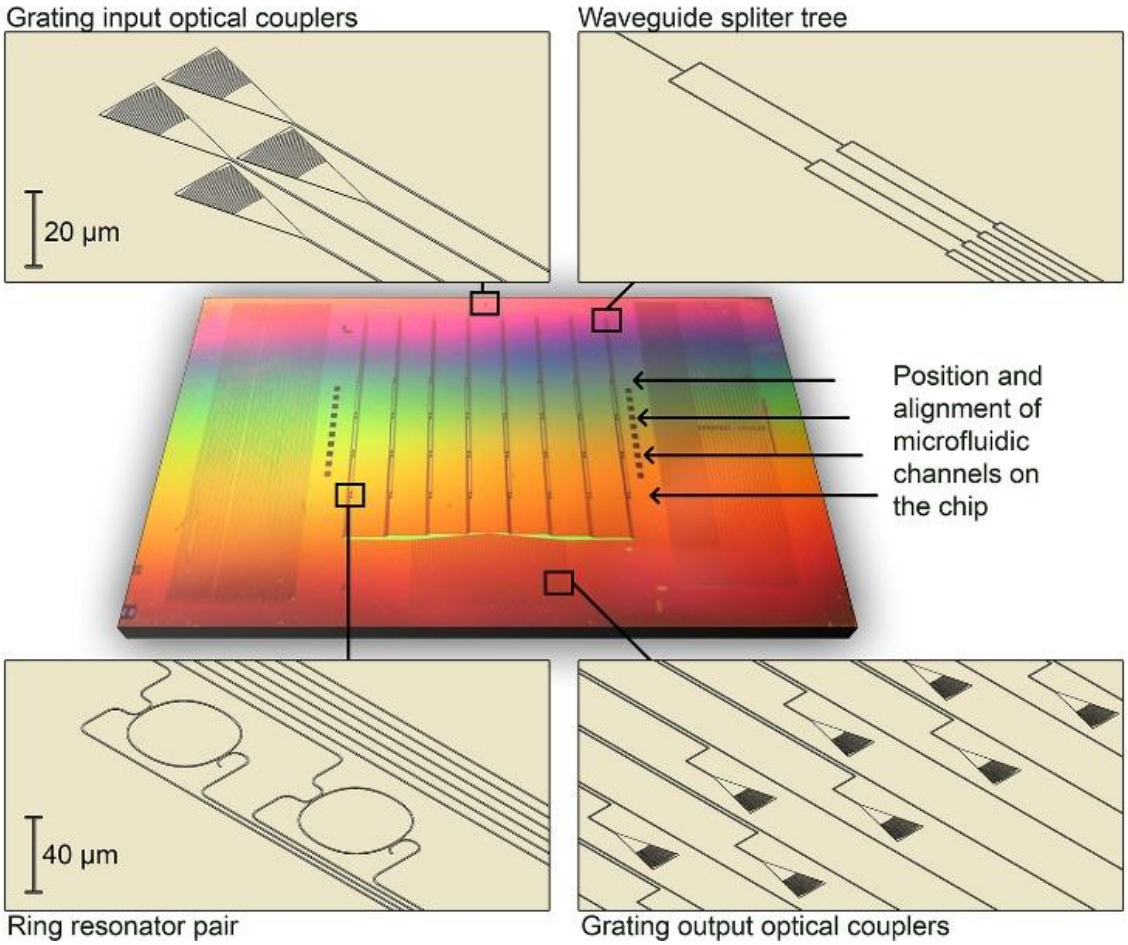

Figure 6-3. Ring resonator array chip overview. Light source is collected at the grating input couplers, directed through the waveguides towards the ring resonators array (8 columns of 4 pairs) and finally the resonant wavelength shift measured at the grating output couplers.

6.3.2 Experimental Set-up

The setup was composed of optical and hydraulic components, namely an infrared tuneable semiconductor laser TSL-510 (Santec Europe Limited, U. K.) and a short wave infrared InGaAs camera Xeva-Lin-1.7 (Xenics, Belgium), the main optical instruments for the measurements. The liquid handler GX-271 used for sample injection into the RCI comprised a sample tray and two needles held by a programmable robotic arm connected to four syringe pumps GILSON 402 and two VALVEMATE® II Valve Actuators (Gilson International B. V., The Netherlands). A syringe pump LEGATO 111 (KD Scientific, Massachusetts) was used to continuously flow running buffer over the set-up. A high-power thermoelectric PID temperature controller 3700 series (Temp. Sensor Control Accuracy ± 0.0001 °C) (NEWPORT Electronics Ltd., U. K.) was connected to an aluminium block beneath the RCI for temperature control of the chip (Figure 6-4).

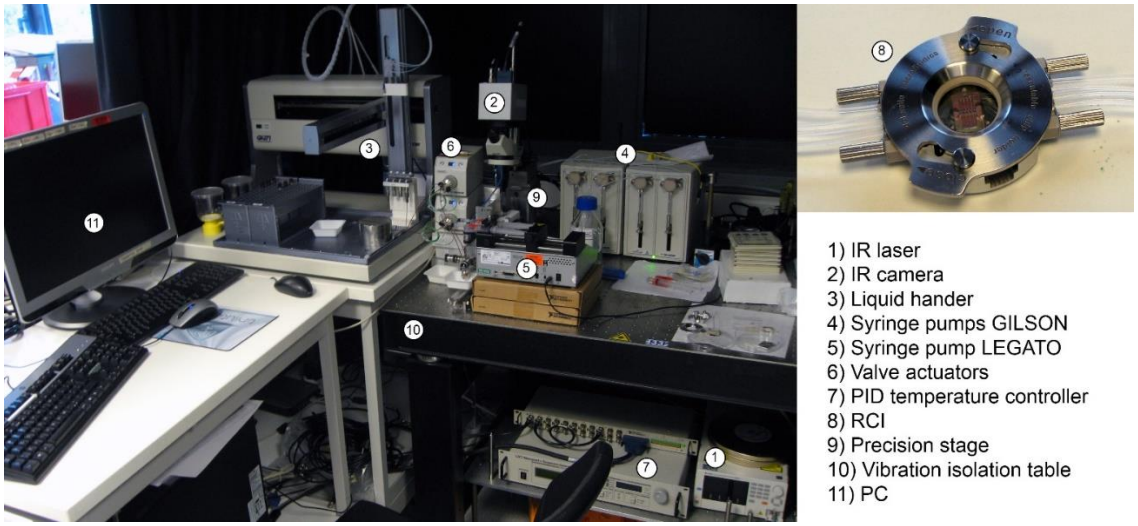

Figure 6-4. Photograph of the setup with all the hydraulic and optical components.

The configuration of the liquid handler and the 6 port 2 way valves allowed the use of a protocol to inject samples into the RCI using air gaps to prevent reverse diffusion from hydraulic liquid of the syringe pumps to the running buffer. The injection protocol was programmed to prevent air gaps reaching the sensors. A commercial RCI (The Dolomite Centre Ltd., U. K.) was used for all the measurements, consisting of an assembly formed by a poly(methyl methacrylate) (PMMA) gasket patterned with a 4 microfluidic channels (100 µm height and 200 µm width each channel) in polydimethylsiloxane (PDMS), an aluminium seat for heat transfer to the chip, all embedded in a metallic holder and tight with a steel clamp on top to seal and close the system when mounted (Figure 6-5).

The RCI with the temperature controller, the IR camera and the IR laser were mounted on an ULTRAlign Precision Fiber Alignment Stage M561-D Metric (NEWPORT Electronics Ltd., U. K.) fixed on a high performance vibration isolation laboratory table 63-560 (Applied Laser Technology, The Netherlands) to ensure a correct and stable alignment of all the optical instruments with the chip. All the instruments were controlled from a PC running Windows 7® using specific software for each instrument. Trilution LH® was used for controlling the liquid handler and the valve actuators. PyMeasure, a program written in Python® and developed by Wim Bogaerts *et al.* (University of Ghent, INTEC Photonics Research Group), was used for controlling the infrared camera, the thermal controller and the infrared laser. IGOR (WaveMetrics, Inc., Oregon) was used for data treatment and analysis.

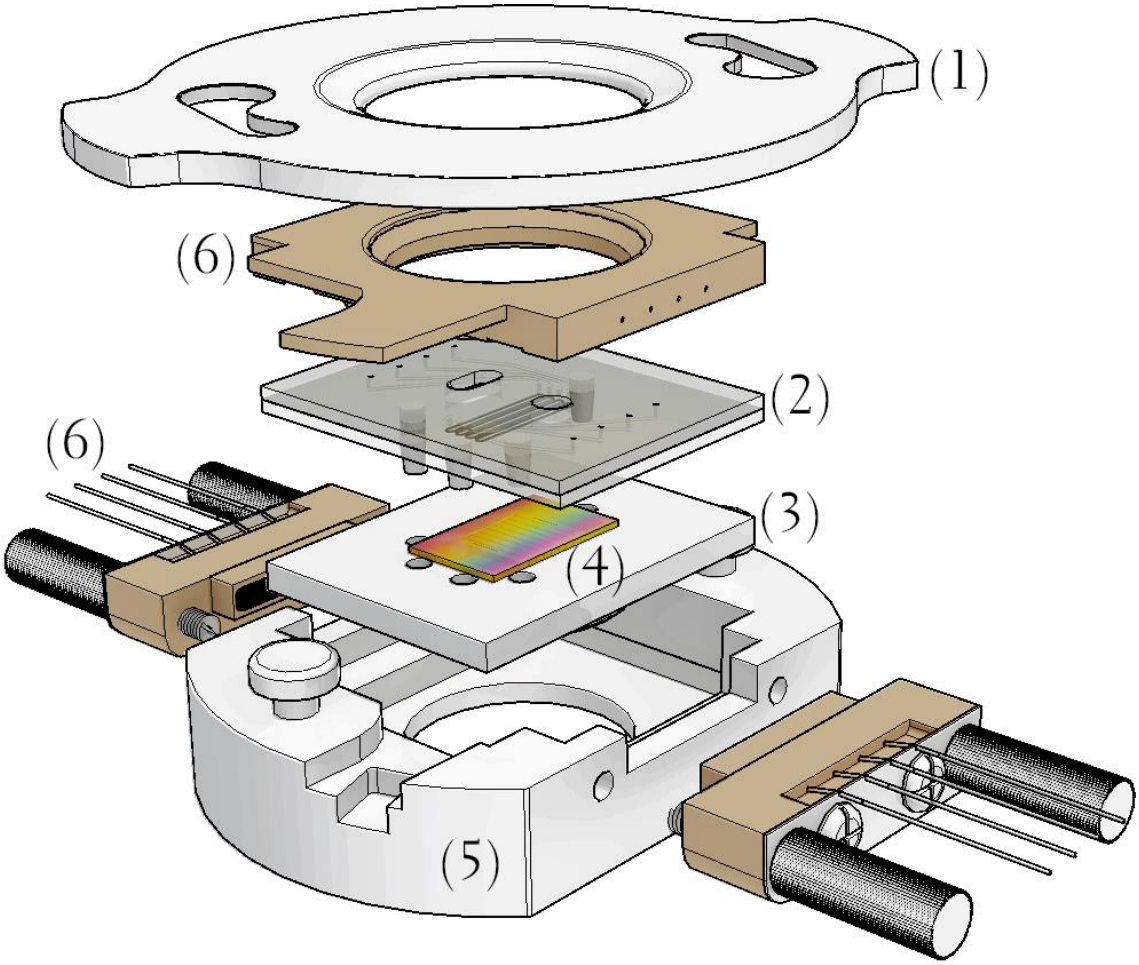

Figure 6-5. Illustration of the Dolomite RCI[27] disassembled in a (1) top metallic clamp, (2) PMMA gasket patterned with a 4-channel PDMS microfluidic unit, (3) aluminium seat for heat transfer to the chip, (4) ring resonator chip, (5) metallic holder, (6) connectors and tubing.

6.3.3 DNA sequences

Synthetic oligonucleotides designed for the identification of the pathogenic bacteria *Francisella tularensis holarctica*[28] and other necessary oligonucleotides to carry out the DNA assays were purchased as lyophilised powder (Biomers, Germany) as listed in Table 6-1. All the DNA solutions were reconstituted in high purity deionised water (18 MΩ). Short dsDNA templates of *Francisella tularensis* were prepared by mixing equal volumes of complementary strands in appropriate buffer solution, heating at 95 °C for 10 minutes followed by gentle cooling to room temperature (*i.e.* 22 °C).

Table 6-1. List of oligonucleotide sequences and their respective modifications.

Name	Nucleotide sequence (from 5'-end to 3'-end)
F. tularensis forward primer	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCC (5'-hexynyl-T_{30}-)
Non-complementary forward primer	GAGCAACTAGGGATAGGCCTTAGTGAGGCACGAT (5'-hexynyl-T_{30}-)
F. tularensis reverse primers	CGCTACAGAAGTTATTACCTTGCTTAACTGTTA (regular and 5'- biotin)
F. tularensis DNA sequence	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCCAGAAGGTTCTAA GTGCCATGATACAAGCTTCCCAATTACTAAGTATGCTGAGAAGAAC GATAAAACTTGGGCAACTGTAACAGTTAAGCAAGGTAATAACTTCTG TAGCG
F. tularensis DNA complementary sequence	CGCTACAGAAGTTATTACCTTGCTTAACTGTTACAGTTGCCCAAGT TTTATCGTTCTTCTCAGCATACTTAGTAATTGGGAAGCTTGTATCA TGGCACTTAGAACCTTCTGGAGCCTGCCATTGTAATCTTACACTT CCTTGTG (regular and 5'-Atto 488)
Backfiller (poly-30T)	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT (5'-hexynyl)
Capture probe	TTCACAGGTACTGGATTTGATTGTG (5'-hexynyl)
Complementary sequence to capture probe	CACAATCAAATCCAGTACCTGTGAA
Non-specific DNA target	TTTTTTACGTGACAATGTAGTTGCG

6.4 Experimental

6.4.1 Surface activation, functionalisation and optimisation

Prior to use, chips were rinsed with acetone and isopropanol to remove the protective resist. A cleaning process of ultraviolet light and ozone exposure for 16 minutes was carried out for every chip in an ultraviolet ozone (UVO) cleaner 144AX-220 (Jelight, Inc., California). After cleaning, the ring resonator chips were activated by silanisation of the surface in a liquid-phase process.[29] The chips were transferred to a controlled N_2 atmosphere (glove box) and immersed in solution of toluene and N,N-diisopropylethylamine containing 2 % of 11-azidoundecyltriethoxysilane (ABCR, Germany). After overnight incubation, the substrates were rinsed with toluene and dried with N_2. Finally, the substrates were baked for 1 h at 110 °C. As a quality test, control blank SiO_2 wafer pieces were silanised simultaneously in the same chamber. These control wafer surfaces were characterised by contact angle measurements (Abtronix B.V., The Netherlands) after silanisation, producing homogeneous surfaces with consistent contact angles of 72.7 ° ± 0.9 °, and demonstrating evidence of a highly reproducible silanisation protocol.

DNA functionalisation of the silanised chips was achieved via copper-catalysed azide-alkyne cycloaddition reaction.[30, 31] In other words, a click chemistry reaction between the azido modified sensor surface and the hexynyl-terminated single stranded DNA probes, in the presence

of a copper (I) salt as a catalyst, was used to achieve DNA probe immobilisation. Sodium L-ascorbate was used to prevent the oxidation of copper(I), therefore maintaining a necessary concentration of catalytically active copper and eliminating the need for inert atmospheres.[32] Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) (TBTA) was used as a copper(I) stabilising agent to avoid DNA strand scission.[33] Right before use, a fresh coupling solution was made by mixing 20 µL of 2 mM TBTA (in DMSO), 20 µL of 2 mM tetrakis(acetonitrile)copper(I) hexafluorophosphate (in DMSO), 20 µL of 2.6 mM sodium L-ascorbate and 27 µL of 50 µM hexynyl terminated specific DNA and 3 µL of 50 µM hexynyl terminated backfiller DNA. The co-immobilisation of probe and backfiller was optimised in order to obtain the best results in terms of performance and reliability, using different probe-to-backfiller ratios (1:0, 1:1, 1:10 and 1:100) and measuring the resonant wavelength shift of the hybridisation event with a complementary sequence.

Each pair of ring resonators was independently functionalised with this DNA coupling solution using a SynQuad-02 (Cartesian Technologies Europe, Ltd., U. K.) automatic non-contact nanolitre dispensing machine to precisely deposit small drops of the functionalisation solution on each pair. The spotter consists of a hollowed ceramic needle connected to a solenoid pump and syringe pump, held in a mobile programmable robotic arm for precise liquid dispensing. In order to achieve the necessary spotting reproducibility, a Teflon block was machined to allocate the chips in known coordinates during the spotting process. Additionally, a monochrome camera with focus lenses DCC 1545M (Thorlabs, Ltd., U. K.) was mounted on the robotic arm to track and record the spotting process in real-time as a means of quality control. Chips identified as such with dispensing errors were omitted for further analysis. Following spotting, the sensors were kept in the darkness for an hour to allow the reaction to take place, and then rinsed thoroughly with DMSO and deionised water. Subsequently, the rest of the surface was blocked by covering the chip with 200 µL of a solution of hexynyl terminated poly-30T DNA probes used as a lateral spacer and to prevent non-specific interaction. Again the reaction with the poly-30T probes was allowed to take place for an hour protected from light, followed by a thorough rinsing with DMSO and deionised water. The efficiency of the probe immobilisation and blocking step was examined by hybridising with a complementary sequence labelled with a fluorescent marker Atto 488 and imaging the spots by fluorescence microscopy afterwards.

6.4.2 Optimisation of operational parameters by detection of hybridisation of 25-mer single stranded DNA with complementary immobilised capture probe

In all the ring resonator experiments the microfluidic channels were purged before the assays with sodium dodecyl sulphate 0.5 % (w/v) for 10 minutes at a flow rate of 5 µL/min and then priming with glycine at pH 9.5 for 10 minutes at a flow rate of 5 µL/min in order to clean all components. 1 M NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 10 mM tris(hydroxymethyl)aminomethane HCl (TRIS) at pH 7.2 was used as running buffer at a constant flow of 1 µL/min, a flow slow enough to allow the fluid to reach the desired temperature inside the RCI and avoid temperature dependent signal response or bias. The thermostat from the temperature controller was set to 20 °C during the experiments and kept constant to avoid temperature-based drifts in the output signals from the ring resonators. The infrared laser was set to scan at 5 mW in a window range span from 1530 to 1536 nm and at a scanning speed of 1 nm/s whilst keeping the gain settings from the infrared camera constant.

As shown in Figure 6-6, four channels on top of the chip address 8 pairs ring resonators. Per channel, 6 pairs of rings were functionalised with capture probe, while the other 2 pairs were used as negative control rings (functionalised only with poly-30T backfiller). Injections of a solution prepared in running buffer containing a series of different ssDNA concentrations of 25-mer specific complementary sequence capture probe (500 nM, 250 nM, 125 nM, 62.5 nM, 31.3 nM, 15.6 nM, 7.8 nM and 500 nM of unrelated DNA, as a non-specific target) were performed. This series of injections were run each for 30 minutes to allow the target to interact with specific captures probes, then followed by a 10 minutes time period of pure running buffer in order to spot a possible signal decay due to nonspecific binding on the surface of the ring resonators, and finally two consecutive pulses of 2 mM HCl to regenerate the capture probes.

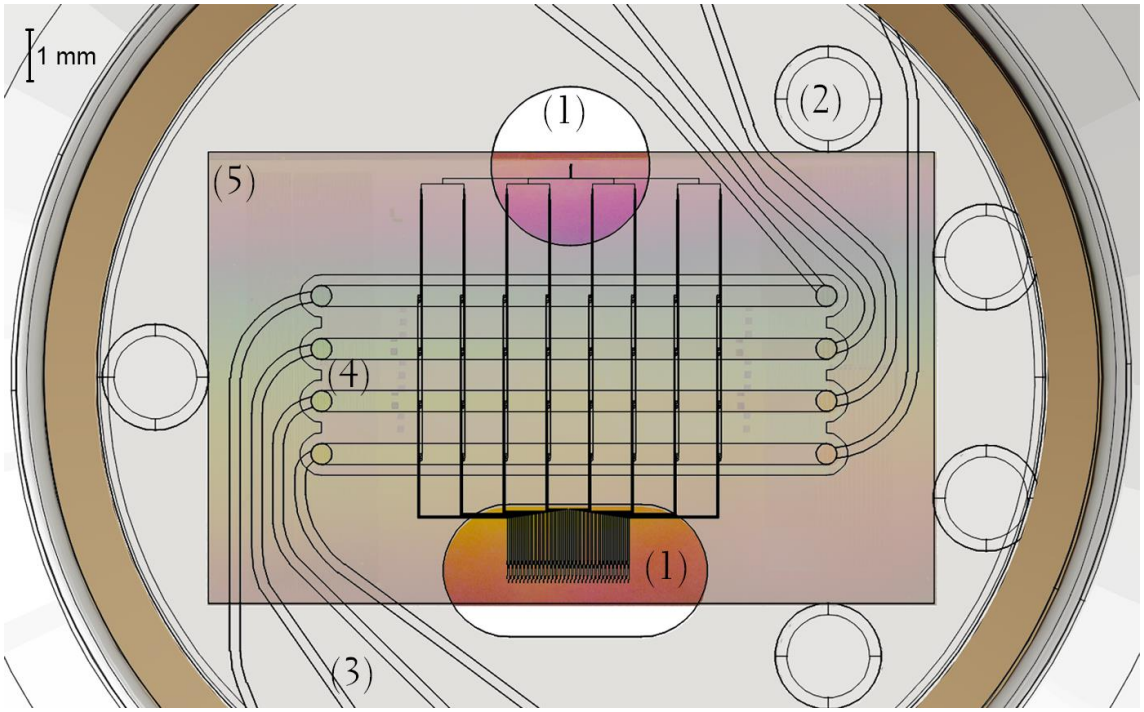

Figure 6-6. Top view of the RCI. The PMMA gasket has (1) two holes for the laser input and the output reading, (2) five alignment pins to fix the chip in position, (3) four channels patterned inside the PMMA and connected to (4) PDMS channels in contact with the chip, each one feeding 8 pairs of rings. (5) Ring resonator chip

6.4.3 SP-RPA/detection of *F. tularensis* related dsDNA

Solid-phase RPA on the ring resonators was carried out using the conditions described in Section 6.4.2, with the exception that the assays were carried out at 37 °C to ensure optimal DNA amplification.

As shown in Figure 6-6, four channels on top of the chip address 8 pairs ring resonators. Per channel, 6 pairs of rings were functionalised with *Francisella tularensis* forward primer, while the other 2 pairs were used as blank control rings or were functionalised with negative control forward primer. In each microfluidic channel a different starting DNA concentration was tested, injecting in each case 50 µL of a recombinase polymerase amplification reaction mixture prepared accordingly to the manufacturer (TwistDX), *i.e.* a solution containing 2.4 µL of *Francisella tularensis* reverse primer 10 µM, 29.5 µL of rehydration buffer provided in the kit, 13.2 µL of *Francisella tularensis* template (with concentrations ranging from 10^{-9} M to 10^{-13} M including a negative control without template), 2.5 µL of magnesium acetate 280 mM, 2.4 µL of Milli-Q water and a supplied lyophilised pellet containing the recombinase enzymatic mixture necessary for the reaction. After the injection of the recombinase polymerase amplification mixture the assay was monitored for 40 minutes.

Fluorescence microscopy imaging was carried out on the surface of the chips following the solid-phase amplification to independently confirm the RPA process. The microfluidic channels were flushed with HCl 2 mM for 5 minutes at 5 µL/min in order to clean the surface of the chip and denature the complementary DNA from the elongated primers. Subsequently, running buffer was flowed through the setup for 10 minutes and a solution of 100 nM 5'-end biotinylated reverse primer from *Francisella tularensis*, prepared in the same buffer, was then injected for 30 minutes for hybridisation. Next, a 1:100 PBS diluted stock of Cy®5-Streptavidin solution (Life Technologies Europe B.V., Belgium) was flushed over the sensors for 30 minutes, again protected from light. Finally, the specific and control rings were examined under a fluorescence microscope at 650 nm.

6.5 Results and discussion

6.5.1 Surface optimisation

The effect of different probe densities was studied by using different probe-to-backfiller ratio, being 1:10 the optimal to achieve the best resonant wavelength shift during hybridisation with a complementary DNA strand at 125 nM (Figure 6-7).

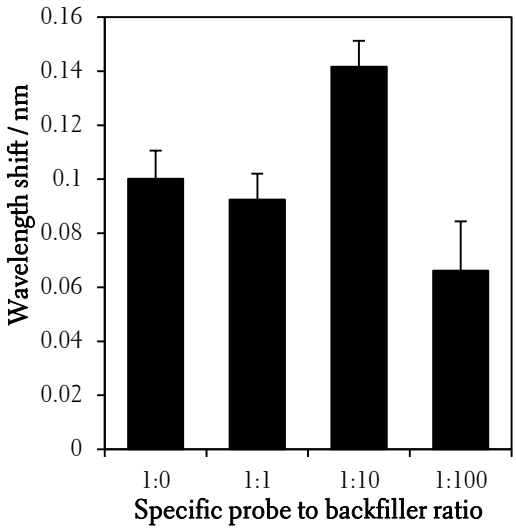

Figure 6-7. Wavelength shift after hybridisation with a complementary ssDNA at 125 nM for different surface probe densities using different probe-to-backfiller ratios (1:0, 1:1, 1:10 and 1:100).

It is expected that a less packed surface probe could enhance the hybridisation efficiency at the surface inasmuch as steric hindrance is reduced. The use of a poly-30T sequence as a surface blocking agent after the spotting was imaged with fluorescence microscopy, revealing well-defined bright spots with dark boundaries for the chips blocked (Figure 6-8.A). In contrast, the chips that were not blocked with a poly-30T sequence, revealed a background fluorescence

20 times higher caused by non-specific interaction from the labelled complementary strand on the rest of the surface (Figure 6-8.B).

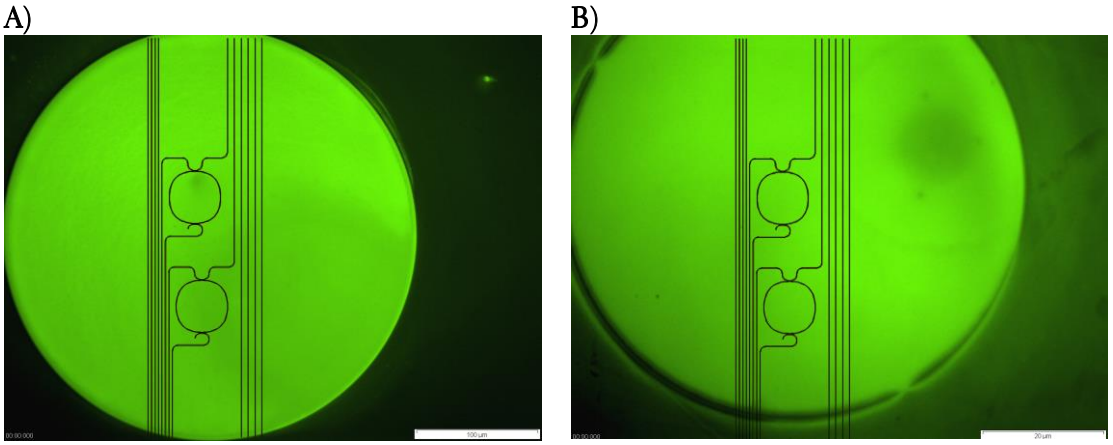

Figure 6-8. A) Fluorescence images of a ring resonator pair functionalised with a probe and hybridised with a specific fluorescent labelled complementary target having the background blocked with a poly-30T sequence as a backfiller. Since the brightness of the spots made impossible to distinguish the ring resonator profiles in the picture, we have superimposed the profiles (black lines) to illustrate the relative position and size. B) Non-blocked background.

6.5.2 Optimisation of operational parameters by detection of hybridisation of 25-mer ssDNA with complementary immobilised capture probe

The sensogram from Figure 6-9 show the evolution of the wavelength shift for two ring resonators, a specific ring functionalised with a capture probe (in blue), and a control ring functionalised with a poly-30T backfiller (in red).

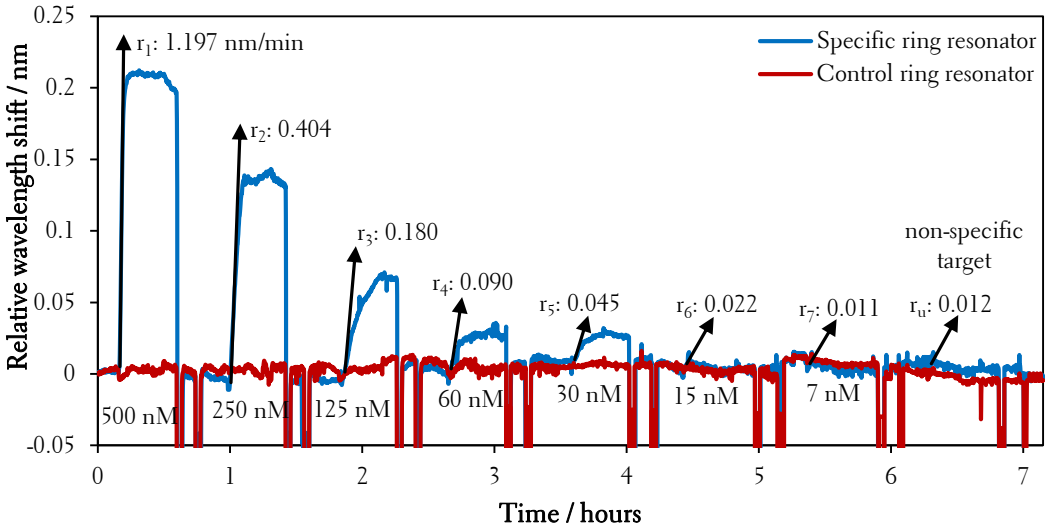

Figure 6-9. Sensogram for the hybridisation detection of 25-mer ssDNA at different concentrations. The initial transient slope for each interaction was represented with an arrow and the magnitude in nm·min^{-1}. The signal drift, 0.005 nm in 7 hours, was corrected from the sensogram.

For the sake of simplicity only the evolution of these two representative rings is displayed, although the measurements from all the rings from the channel were taken into account to calculate the calibration curve and limit of detection. As the shift in the resonant wavelength is related to a change in the refractive index, a specific hybridisation interaction is occurring between the short 25-mer ssDNA target and the capture probes from the specific ring resonator, given that the control ring showed no binding interaction. The injection of a non-specific ssDNA target did not lead to an increase in signal for the specific ring resonator, nor for the control ring resonator; therefore the small fluctuations observed in the control ring can be attributed to background noise. After the injection of different concentrations of target, no significant loss of signal was observed for this sequence at these concentrations, meaning that the running buffer did not drag any DNA that could be non-specifically bind on the surface of the rings. The total shift for each specific interaction did not show linear correlation with the concentration of ssDNA target, so instead the calibration curve was built taking into account the initial transient slope for each hybridisation event.[34] The calibration curve (Figure 6-10.A) was built with the initial transient slope from the sensogram (Figure 6-9) *versus* the initial target ssDNA concentration. The dynamic range spanned five orders of magnitude, and the limit of detection, defined as the concentration of the analyte at the mean blank plus three times the standard deviation of the blank, was calculated to be $20 \cdot 10^{-9}$ M (n = 12).

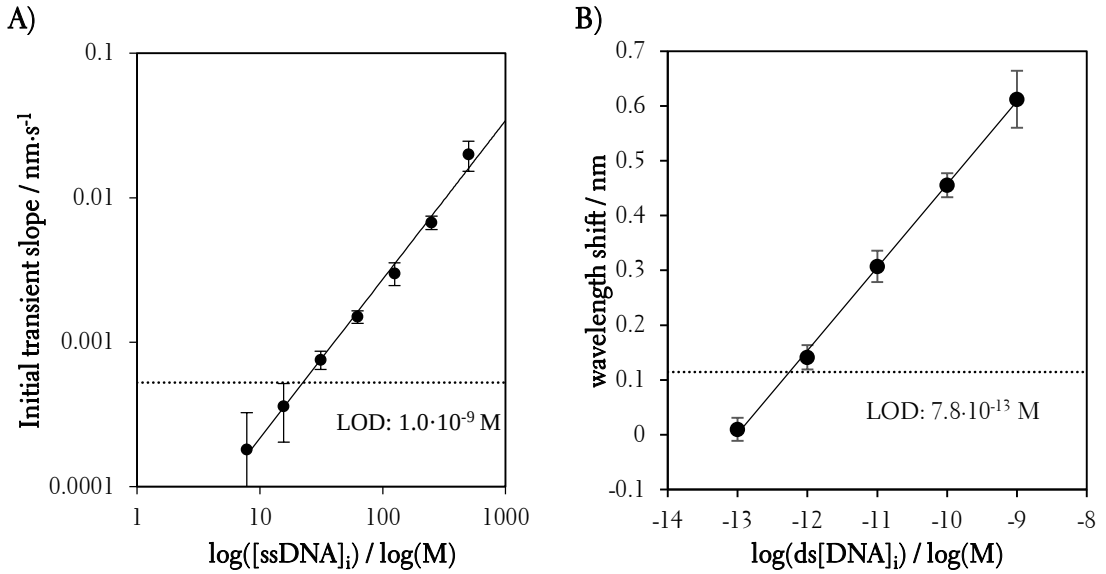

Figure 6-10. A) Calibration curve for the detection of 25-mer ssDNA sequence. B) Calibration curve for the amplification-detection of RPA of *F. tularensis* related dsDNA sequence.

6.5.3 SP-RPA/detection of *F. tularensis* related dsDNA

The sensogram in Figure 6-11 shows the evolution of resonant wavelength for ring resonators over time and at different initial concentration of dsDNA of *Francisella tularensis*, ranging from 1 nM to 0.001 nM. The resulting signal for each concentration (and each microfluidic channel) is derived from the difference between the signal from the rings functionalised with specific forward primers and the average signal from the rings functionalised only with poly-30T (n = 4) from the same microfluidic channel.

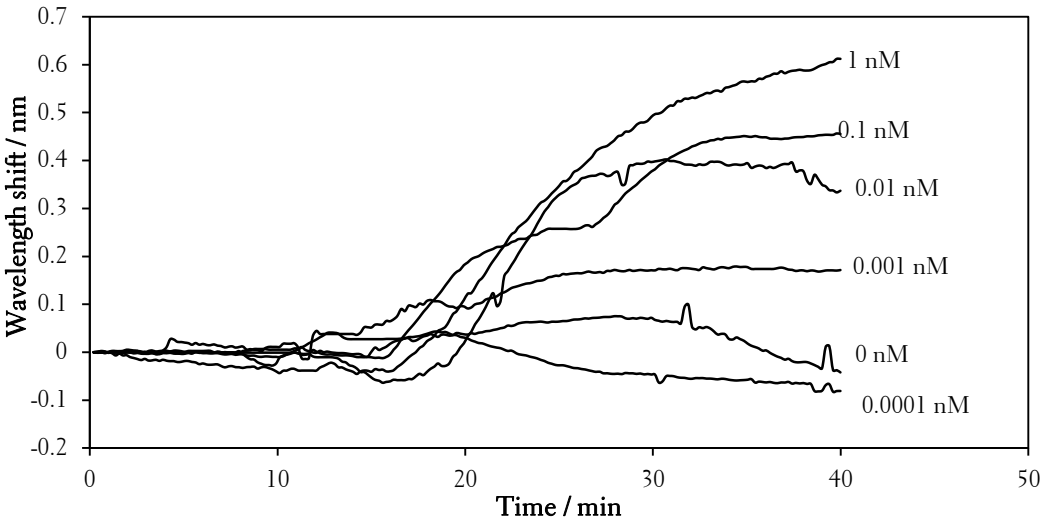

Figure 6-11. Sensogram for the RPA *F. tularensis* detection at different starting concentrations of target. The signals are normalised to the rings with poly-30T.

The signal from the rings functionalised with poly-30T was used to normalise the specific output signal and reduce interferences produced by external factors. At minute 0 the RPA mixture is injected by the liquid handler in the injection ports, taking 10 minutes to reach the RCI and the chip. A rapid wavelength shift can be observed at minute 15, with each initial target concentration requiring a different interval of time to reach a stationary regime or plateau, which is usually achieved within 30 to 40 minutes after the first interaction of the RPA mixture with the chip. The assay yielded a resonant wavelength shift for the specific amplification with negligible response for the controls, indicative of successful on-chip solid-phase amplification-detection. The calibration curve (Figure 6-10.B) was built using the signals obtained from the sensogram (Figure 6-11) for different starting concentrations of dsDNA target from *Francisella tularensis*. Some of the signals for the lowest concentrations (*e.g.* 0 nM and 0.0001 nM) shown in Figure 6-11 display erratic evolution over time due to the fact that only the signal from one ring resonator is displayed, though all the signals for each concentration (n = 12) were taken into account to build the calibration curve, and the error is displayed in the standard deviation of each point, not being the signal from 0.0001 nM statistically distinguishable from the negative

control (0 nM). The dynamic range spanned five orders of magnitude, and the limit of detection, defined as the concentration of the analyte at the mean blank plus three times the standard deviation of the blank, was calculated to be $7.8 \cdot 10^{-13}$ M ($6 \cdot 10^{5}$ copies in 50 µL of amplification mixture). The limit of detection presented in this work offers a lower detection threshold than most of the available techniques (Table 6-2), exhibiting a comparable but slightly better performance (a 100-times lower LOD) than the first reported ring resonator device using RPA for the detection of genetic alteration in cancers,[35] but not as low as the limit of detection reported using a microfluidic lab-on-a-foil system (<10 copies). Although this system offers an incredible limit of detection and configures an interesting self-sufficient DNA detection platform, still requires complex and expensive engineered primers, with the probe molecule requiring up to three functional groups: the fluorophore fluorescein, a quencher and an abasic site mimic of tetrahydrofuran.[36] Therefore, we believe the lower sensitivity of our system still pays off in order to avoid the listed drawbacks, due to the fact that the LOD achieved is still suitable for the detection of biomarkers of interest in point-of-care (POC) fields.

Table 6-2. Comparison of methods.

	Standard RPA	Results reported in this paper	Isothermal solid-phase Amplification-detection (ISAD)[35]	Real-time RPA[36]	PCR	Real-time PCR
Limit of detection	50 pg · µL^{-1}	2 fg · µL^{-1}	500 fg · µL^{-1}	<10 copies	50 pg · µL^{-1}	5 pg · µL^{-1}
Amplification time	40-50 min	40 min	20-30 min	<20 min	2-3 h	1-2 h
Detection method	EtBr or fluorescence dye	Label-free	Label-free	Fluorescence dye	Ethidium Bromide	Fluorescence dye
Primers	Regular primers	Regular primers	Regular primers	Complex and expensive	Regular primers	Regular primers
Multiplex	Yes	Possible	Yes	Possible	Yes	Yes

Amplification of genetic material and elongation of surface tethered primers was imaged by fluorescence microscopy with the use of a Cy5-labelled *Francisella tularensis* reverse primer, revealing fluorescence in the region of the specifically functionalised ring resonators with *Francisella tularensis* forward primer DNA sequences (Figure 6-12.A), whereas the control rings remained dark (Figure 6-12.B). The results obtained demonstrate that the specific sequences amplified in solid-phase retain the complete DNA sequence from the template used, whereas the control sequences show no evidence of specific amplification.

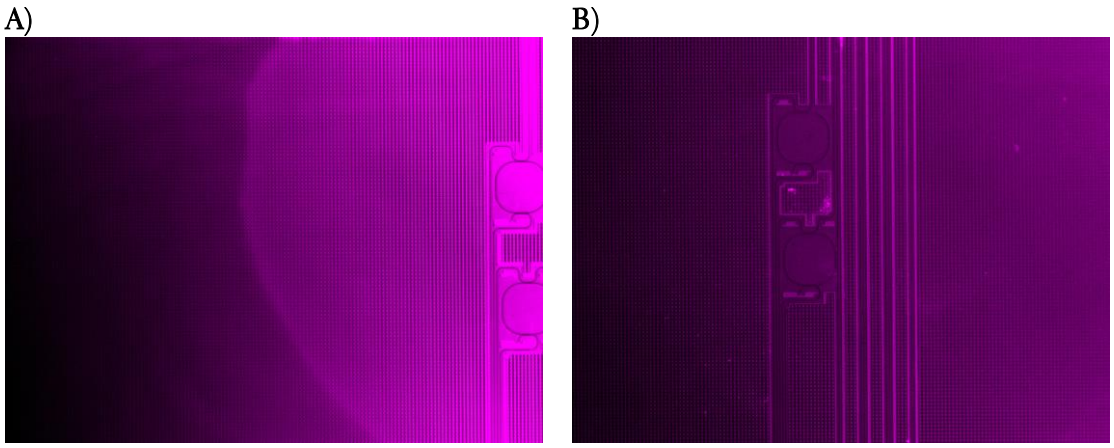

Figure 6-12. A) Fluorescence imaging of ring resonators amplified with RPA and specific *Francisella tularensis* primers and B) control ring resonators with non-specific primers.

6.6 Conclusions

In this work we present a robust and automatised optical detection platform for dsDNA targets, emphasising the benefits of conjugating the sensitivity, label-free and real-time monitoring from ring resonators with the rapid, still specific and simple amplification-detection assay of solid-phase RPA. Although this strategy has been previously reported, to the best of our knowledge we are the first to have successfully achieved and shown experimental data of real-time monitoring of solid-phase RPA in ring resonators. The strength of this approach is also in its simplicity, using regular unmodified primers, avoiding the need for tetrahydrofuran-modified primers as well as avoiding the complexity of design associated with thermal-cycling based amplification in portable systems: liquid evaporation, heat dissipation, time and power consumption, slow amplifications, etc. Detectability with the present platform was demonstrated with short ssDNA targets (25-mer) and low concentrations could be detected, reaching a limit of detection of $20 \cdot 10^{-9}$ M. Subsequently direct solid-phase recombinase polymerase amplification of a 144-bp target was successfully achieved reaching an excellent limit of detection of $7.8 \cdot 10^{-13}$ M ($6 \cdot 10^{5}$ copies in 50 µL). In order to produce a useful POC device we know some aspects still need to be addressed, therefore future work will focus on exploiting the multiplexing capabilities of the system, the detection of real samples as well as a true miniaturisation of the setup.

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Chapter

Conclusions

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7.1 General conclusions

First of all we have demonstrated the success of the isothermal solid-phase RPA concept as a DNA amplification method combined with detection to enhance DNA biosensing sensitivity. The general objective of this doctoral thesis was the development of a detection platform by exploiting a combined strategy of isothermal solid-phase amplification technique with genosensor detection for a simple, sensitive and rapid analysis of genetic material. Typically LOD of $1.3 \cdot 10^{-15}$ M within a dynamic range of 5 decades were obtained in less than 1 h. Further optimisation allowed us to improve the analytical parameters of the platform in terms of LOD, signal-to-noise ratio and steps required. Moreover, our solid-phase RPA approach overcomes not only the limitations present in regular PCR amplification techniques, but also many of the regular RPA-based published methods, due to the simplicity and genericity of the system.

The concept has been applied for the electrochemical detection of genomic DNA from pathogens in the analysis of real samples, and the device has proven its robustness when successfully amplified and detected genomic DNA of *Francisella tularensis* and *Piscirickettsia salmonis* from extracted from real samples.

The DNA amplification in solid-phase bridge structures have been achieved by exploiting the knowledge of surface primer immobilisation and pushing the bending limits of dsDNA in a way that opens possibilities for exploring the selectivity of solid-phase bridge recombinase polymerase amplification to perform multiplex analysis of different nucleic acid targets simultaneously due to the fact that the system avoids the formation of primer-dimer formations.

Detection of the amplified material has been carried out by hybridisation with labelled primers, incorporation of Fc-dNTPs during the amplification and also in real time using ring resonators. The main added value of this thesis is that it presents a flexible solution for detecting DNA with biosensors, exploiting a general concept of solid-phase amplification and detection, thus integrating two nucleic acid tests, PCR and microarrays, in one single device.

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APPENDICES


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MEAN
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PRODUCE
BARCELONA
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RECEPTOR
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DESCRIBE
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Appendix 1. List of figures

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Appendix 3. Glossary

Abbreviation	Definition
μ-TAS	Micro total analysis
A	Adenine
AC	Alternate current
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users
ATP	Adenosine triphosphate
Bio-MEMs	Bio-microelectromechanical systems
bp	Base pair
C	Cytosine
cDNA	Complementary DNA strand
Ct	Threshold cycle
dATP	Deoxyadenosine triphosphate
DC	Direct current
DCM	Dichloromethane
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribose nucleoside triphosphate, also known as nucleotide
DPV	Differential pulse voltammetry
dsDNA	Double stranded DNA
DSP	3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester
DT1	10-(3,5-Bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
DVD	Digital versatile disc
EDTA	Ethylenediaminetetraacetic acid
EFP	Exonuclease fluorescent probe
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
ESI-MS	Electrospray ionisation mass spectrometry

Fc-	Ferrocene labelled
G	Guanine
Gp32	Single stranded DNA binding protein
GPES	General purpose electrochemical system
H	Hypoxanthine
HDA	Helicase-dependent amplification
HRP	Horseradish peroxidase
ICP-RIE	High density inductively coupled plasma reactive ion etching
IFAT	Indirect fluorescent antibody technique
IR	Infrared
ISAD	Isothermal solid-phase amplification-detection
IUPAC	International Union of Applied Chemistry
j	Current density ($\mu A \cdot mm^{-2}$)
LAMP	Loop-mediated isothermal amplification
LOC	Lab on a chip
LOD	Limit of detection
mer	From Greek *meros*, "part". The length of an oligonucleotide
mRNA	Messenger RNA
NAT	Nucleic acid test
NHS	N-hydroxy succinimide
NTC	Non-template control
PBS	Phosphate-buffered saline
PBS-T20	Phosphate-buffered saline containing 0.05 % (v/v) Tween 20
PC	Personal computer
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEG	Polyethilene glycol
PID	Proportional-integral-derivative
PMMA	Polymethylmethacrylate
POC	Point of care
Poly-15T	A single stranded DNA sequence with 15 deoxythymidine monophosphate
Poly-30T	A single stranded DNA sequence with 30 deoxythymidine monophosphate
Poly-45T	A single stranded DNA sequence with 45 deoxythymidine monophosphate
RCA	Rolling circle amplification

RCI	Resealable chip interface
RecA	Homology protein recombinase A
RI	Refractive index
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT-PCR	Real time polymerase chain reaction
SA-HRP	Streptavidin conjugated with horseradish peroxidase
SAM	Self-assembled monolayer
SDA	Strand displacement amplification
SNP	Single nucleotide polymorphisms
SOI	Silicon-on-insulator
SPR	Surface plasmon resonance
SP-RPA	Solid-phare recombinase polymerase amplification
SRS	Salmonid Rickettsial Septicaemia
SSB	Single stranded DNA binding protein
ssDNA	Single stranded DNA
T	Thymine
TBS	Tris-buffered saline
TBTA	Tris[(1 benzyl 1H 1,2,3 triazol 4 yl)methyl]amine
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
TRIS	Tris(hydroxymethyl)aminomethane
U	Uracyl
UV	Ultraviolet
UVO	Ultraviolet ozone
uvsX	Recombinase protein
uvsY	Recombinase load factor

Appendix 4. Quality parameters of biosensors

As a broad variety of recognition methods are utilised in chemical sensors, a general description of the recognition process is necessary to further describe the quality parameters involved in such process. The recognition processes may be defined according to the following reaction scheme in which A is the analyte, R is the recognition receptor and P is a product of the analyte-receptor interaction:

$$A + R \rightleftharpoons P \quad [1]$$

The double arrow indicates that the recognition process is a reversible process at equilibrium. Reversibility of the recognition process arises from the fact that the product P involves non-covalent chemical bonds, such as ionic bonds, hydrogen bonds and van der Waals interactions.

The following parameters are statistical measures that define the performance of chemical and biochemical sensors:[1]

Affinity

Affinity describes the appetence for the analyte to interact with the receptor. The affinity can be indicated, for a given recognition process (Equation 1) in a reversible reaction, as the equilibrium constant. The equilibrium constant K_A which is defined as:

$$K_A = \frac{C_P}{C_A \cdot C_R} \quad [2]$$

where symbols C represent concentrations of the species indicated by subscripts. Great affinity results in a high value of the equilibrium constant.

Accuracy and Error

The accuracy represents the correctness of the sensor's output signal in comparison to the real value of the measured property. To assess the accuracy the sensor must be benchmarked against a standard measurement system with superior accuracy. Error is the difference between the actual value of the measured property and the output value generated by the sensor.

Precision

Precision represents the ability of the sensor to deliver the same output signal when repetitively measuring the same property under the same conditions.

Repeatability

Repeatability is the sensor's capacity to produce a constant output signal for successive measurements after a given period of time while keeping the operating and environmental conditions constant.

Reproducibility

Reproducibility is the sensor's aptitude to generate the same output signal after measurement conditions that do not affect the measured property have been altered, for instance when the readings are performed by different operators or at different laboratories.

Stability and drift

Stability is the sensor's ability to produce the same output signal when measuring the same property over a period of time. Drift is observed when there is a gradual change in the sensor's output while the measured property remains constant.

Calibration curve

The calibration curve is the relationship between the concentration of the analyte, X_a, and the signal output, Y_a generated by the sensor when calibrated against a known concentration. Therefore, if the relationship is linear, can be described as:

$$Y_a = A \cdot X_a + B \quad [3]$$

Where A is the slope of the calibration curve and B the intercept.

Sensitivity, noise and resolution

Sensitivity is the ratio of the incremental change in the sensor's output to the incremental change of the measured property in input, also known as the slope of the calibration curve, A, from Equation 3. Noise is the fluctuations in the output signal of the sensor when the measured property is not changing over time. Resolution is the smallest detectable change in analyte concentration, is dependent of the sensitivity and is highly limited by the noise in the signal.

Selectivity and Specificity

According to IUPAC, selectivity and specificity are two terms that qualitatively express the extent to which other substances interfere with the determination of a substance according to a given procedure, and specificity is considered to be the ultimate of selectivity, which means that no interferences are supposed to occur.[2]

However, selectivity is the recommended term in analytical chemistry because can be graded and to avoid confusion, the use of the term "specificity" for the same concept is to be discouraged.[3] Methods may show good selectivity, or high, excellent or extreme but even low selectivity. Specificity is, in the opinion of many analytical chemists, something exceptional as there are, in fact, very few methods that are specific by responding to only one analyte.[4] Selectivity towards a given analyte can be explained by comparing the affinity towards the analyte of interest *versus* the affinity towards the interferents.

Limit of detection and quantification

The limit of detection LOD is the minimum concentration of analyte that still provides a detectable signal output, y_c, and is significantly distinguishable from the absence of substance, *i.e.* the mean average blank signal, $\bar{y}_b$. The limit of detection in terms of signal output, y_{LOD}, can be defined as:

$$y_{LOD} = \bar{y}_b + k \cdot s_b \quad [4]$$

Where s_b is the standard deviation of the blank measurements and k is a numerical factor chosen according to the confidence level desired. The LOD in terms of concentration, X_{LOD}, can be expressed by using Equation 3 and Equation 4:

$$X_{LOD} = \frac{\bar{y}_b + k \cdot s_b - B}{A} \quad [5]$$

The value of k allows, with more or less confidence, to determine whether the signal output is statistically distinguishable from the absence of substance or not. For many years it has been used the $k = 3$ criteria,[5] by which the possibility to generate a false positive error, *i.e.* the possibility of a blank measurement to be interpreted erroneously as the presence of analyte is 0.13 %. The limit of quantification (LOQ) is defined as the mean average blank signal plus ten times the standard deviation of the blank, and represents an arbitrary threshold to define where quantification of analyte is acceptable. A graphical visualisation from both LOD and LOQ is represented in Figure A-1.

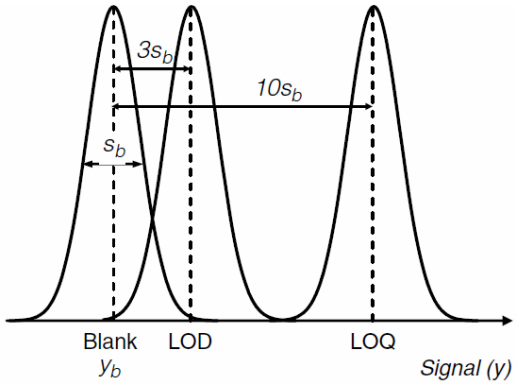

Figure A-1. Grafical definition of the limit of detection (LOD) and limit of quantification (LOQ). Curves represent the normal (Gaussian) distribution of errors.

Dynamic range

The dynamic range is the span range of concentrations between the LOD and the concentration at which the signal output deviates significantly from the assumed calibration function.[6]

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Appendix 5. Short CV

Papers directly resulting from the doctoral thesis

Published

- Real-time and label-free ring-resonator monitoring of solid-phase recombinase polymerase amplification. Biosensors & Bioelectronics 11/2015. 6.45 Impact Factor.
- Electrochemical detection of Francisella tularensis genomic DNA using solid-phase recombinase polymerase amplification. Biosensors & Bioelectronics 11/2013. 6.45 Impact Factor.

Submitted or in preparation

- Optimised surface immobilisation of thiolated primers in gold electrodes for electrochemical DNA amplification-detection in solid-phase RPA.
- Solid-phase bridge recombinase polymerase amplification with ferrocene-labelled dNTPs for electrochemical detection
- Isothermal solid-phase amplification system for detection of *Yersinia pestis.*
- Electrochemical detection of *Piscirickettsia salmonis* genomic DNA from salmon samples using solid-phase recombinase polymerase amplification

Collaborations

- Rubber-based substrates modified with carbon nanotubes inks to build flexible electrochemical sensors. Analytica chimica acta 05/2014. 4.52 Impact Factor.
- Electro-catalytically active Au@Pt nanoparticles for Hydrogen evolution reaction: An insight into Tryptophan mediated supramolecular interface towards a universal core-shell synthesis approach. RSC Advances 09/2014. 3.84 Impact Factor.

Oral communications

- "19th Transfrontier Meeting of Sensors and Biosensors (TMSB-2014)". September 25-26, 2014. Hotel Campus UAB (Universitat Autònoma de Barcelona), Bellaterra, Barcelona, Spain. Oral presentation: "Solid-phase recombinase polymerase amplification as a DNA detection tool".
- "11th Workshop on Biosensors and Bioanalytical Microtechniques in Environmental, Food and Clinical Analysis (BBMEC)". September 26 – 30, 2015. University of Regensburg Campus, Regensburg, Germany. Oral presentation: "Monitoring of solid-phase recombinase polymerase amplification as a DNA detection tool".

Posters

- "V Workshop en Nanociencia y Nanotecnología Analíticas". 21-23 September 2011 Toledo, Spain. Poster: "Rubber Based Substrates for Flexible Electrochemical Sensors".
- "III International Workshop on Analytical Miniaturization and Nanotecnologies" 11-12 June 2012 Barcelona, Spain. Poster: "Nanogaps for labeless DNA detection".
- "XVII Trobada Transfronterera de sensors i biosensors". 20-21 September 2012 Tarragona, Spain. Poster: "Nanogaps for labeless DNA detection".
- BCNano'12 Workshop Series. 1st Worshop: Advanced course on Electrical Nanocharacterization by AFM (C-AFM) (Inside the topic Chemistry/Physics on the nanoscale). 24-25 April 2012 Nanometric Techniques Unit (Scientific and Technological Centers of the UB), Barcelona, Spain.
- Doctoral day "12th Doctoral day" meeting in the doctoral program in Chemical, Environmental and Process Engineering and in Nanosciencie, Materials and Chemical

Engineering". Poster: Recombinase polymerase amplification in ring resonators for real-time and label-free detection of dsDNA. (Poster contest winner, awarded with the first prize 500€). May 20, 2015. University Rovira i Virgili, Tarragona, Spain.

- "11th Workshop on Biosensors and Bioanalytical Microtechniques in Environmental, Food and Clinical Analysis (BBMEC)". September 26 – 30, 2015. University of Regensburg Campus, Regensburg, Germany. Poster: Recombinase polymerase amplification in ring resonators for real-time and label-free detection of dsDNA. (Poster contest winner awarded with the fifth prize 150€).

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