

**In vitro diagnostic tests and biosensing devices
for pathogenic bacteria in clinical and environmental settings**

Melania Mesas Gómez

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In vitro diagnostic tests and biosensing
devices for pathogenic bacteria in
clinical and environmental settings

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Resumen

Uno de los principales objetivos en el siglo XXI es la necesidad de pruebas de diagnóstico rápidas eficientes y altamente sensibles, especialmente para patógenos emergentes, enfermedades novedosas y biomarcadores, como una intervención clave para reducir mortalidad y prevenir transmisión. La importancia de las pruebas de diagnóstico rápido se ha destacado aún más con el devenir de la pandemia. Esta tesis doctoral se centra en el diseño y desarrollo de pruebas de diagnóstico *in vitro* y dispositivos biosensores para el diagnóstico y detección de bacterias patógenas tanto en aplicaciones ambientales como clínicas en entornos con recursos limitados, con el objetivo principal de conseguir un dispositivo fácil de usar mediante una mínima intervención del usuario. En esta tesis se han combinado múltiples elementos para ofrecer una solución única y completa para solventar los principales desafíos al detectar bacterias en concentraciones muy bajas y en grandes volúmenes de muestras. Así, se diseñó una estrategia pionera de preconcentración que integra partículas magnéticas y filtración. Esta novedosa técnica mejora significativamente la capacidad de capturar y concentrar bacterias directamente en filtros mediante la separación inmunomagnética para su análisis posterior. La simplificación analítica se aborda combinando la separación inmunomagnética y la marcación en un único paso, con el objetivo de reducir significativamente el tiempo de análisis. Otro objetivo es la incorporación de un cartucho que realizó la actuación magnética y el lavado en un modo de operación sencillo, minimizando la necesidad de intervención del usuario. Esta innovación contribuye a la simplicidad en el uso y la reproducibilidad, eliminando los pasos de pipeteo y lavado. Finalmente, la lectura electroquímica en un dispositivo portátil también es una innovación clave, permitiendo pruebas en el lugar y en el punto de atención. Esta portabilidad representa un cambio fundamental en la aplicación de los biosensores electroquímicos en entornos con recursos limitados. Este proceso simplificado es un avance notable en el campo, que permitirán ahorrar tiempo y recursos valiosos. Finalmente, se explora el uso de teléfonos inteligentes operados con una aplicación para realizar las mediciones, con el objetivo de presentar estrategias que puedan integrarse en el diseño de pruebas de diagnóstico rápido, haciéndolas más accesibles y rentables para entornos con recursos limitados. Todas estas

características podrían tener implicaciones significativas en aplicaciones del mundo real, llevando a una mejora significativa en el límite de detección para diversas aplicaciones, superando los límites regulatorios en la monitorización ambiental para la detección de *Legionella pneumophila*, *E. coli* y micobacterias ambientales, así como en la salud global para la detección de la tuberculosis.

Abstract

The 21st century place crucial emphasis on the need for efficient and highly sensitive rapid diagnostic tests, especially in dealing with emerging pathogens, novel diseases and biomarkers, as a key intervention to reduce mortality and prevent transmission. The significance of rapid diagnostic testing has been further highlighted by the onset of the pandemic. The purpose of this doctoral thesis is to contribute to this field by enhancing the detection of pathogenic bacteria through electrochemical biosensing and *in vitro* diagnostic tests. This involves combining multiple elements to offer a unique and comprehensive solution to the challenges faced in detecting bacteria at very low concentration levels in large volumes of samples. According to this goal, this doctoral thesis introduces a pioneering preconcentration strategy that integrates magnetic particles and filtration. This novel technique significantly improves the ability to capture and concentrate bacteria directly on filters by immunomagnetic separation for downstream analysis. The analytical simplification is addressed by combining the immunomagnetic separation and labeling into a single step, aiming to significantly reduce the analysis time. Another goal is the incorporation of a cartridge to perform magnetic actuation and washing in a user-friendly operation mode, minimizing the need for user intervention. This innovation contributes to the ease of use and reproducibility, eliminating pipetting and washing steps. Finally, the electrochemical readout in a handheld device is also a key innovation, enabling on-site and point-of-care testing. This portability represents a fundamental shift in how electrochemical sensing can be applied in low-resource settings. This streamlined process is a noteworthy advancement in the field, saving valuable time and resources. Finally, the use of smart phones operated with an app to perform the measurements is explored, with the aim of presenting strategies that can be integrated into the design of rapid diagnostic tests, making them more accessible and cost-effective for low-resource settings.

All these features could have significant implications for real-world applications, leading to a significant improvement in the limit of detections for various applications, surpassing regulatory limits in environmental monitoring for the detection of *Legionella pneumophila*, *E. coli*, and environmental mycobacteria and in global health for the detection of tuberculosis.

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Glossary

A.U.	Arbitrary units
Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
AP	Alkaline phosphatase
App	Mobile application
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BIO	Biotin
bp	Base pair
BQ	Benzoquinone
BSA	Bovine serum albumin
CDs	Communicable diseases
CECT	Colección Española de Cultivos Tipo
CFU	Colony forming unit
CV	Cyclic voltammetry
Cy5	Cyanine 5
ddNTPs	Dideoxynucleotides triphosphate
DIG	Digoxigenin
DNA	Acid desoxiribonucleic
dNTPs	Deoxynucleotides triphosphate
dsDNA	Double stranded DNA
ELISA	Enzyme-linked immunosorbent assay
FET	Field-effect transistors
HABA	4'-hydroxyazobenzene-2-carboxylic acid
hCG	Human chorionic gonadotropin
HIV	Human Immunodeficiency Virus
HQ	Hydroquinone
HRP	Horseradish peroxidase
IGRAs	Interferon-gamma release assays
IMS	Immunomagnetic separation
IUPAC	International Union of Pure and Applied Chemistry
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani, Lysogeny broth
LCR	Ligase chain reaction
LFA	Lateral flow assay
LOD	Limit of detection
LOQ	Limit of quantification
Lp1	<i>Legionella pneumophila</i> serogroup 1

LPS	Lipopolysaccharides
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MP	Magnetic particle
MS	Mass spectrometry
MTBC	M. tuberculosis complex
NAAT	Nucleic-acid amplification test
NASBA	Nucleic acid sequence-based amplification
NCDs	Noncommunicable diseases
NGS	Next-generation sequencing
NTM	Non-tuberculous mycobacteria
PBS	Phosphate buffer solution
PBST	Phosphate buffer solution with Tween® detergent
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEM	Pathogenic environmental mycobacteria
POC	Point of care
qPCR	Quantitative polymerase chain reaction
RCA	Rolling circle amplification
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RPM	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SD	Standard deviation
SEM	Scanning electron microscopy
SPE	Screen-printed electrode
ssDNA	Single stranded DNA
Supp. data	Supplementary data
SWV	Square wave voltammetry
TB	Tuberculosis
TMA	Transcription mediated amplification
TMB	3,3',5,5'-Tetramethylbenzidine
TOF	Time of flight
TSA	Trypticase soy agar
TSB	Tryptic Soy Broth
WHO	World Health Organization

CHAPTER 1

Introduction

1. Introduction

1.1. The role of Communicable diseases in Global Health

1.1.1. Definitions and implications

One of the major challenges in the 21st century involves reducing the threat and burden of communicable and noncommunicable diseases. These challenges undermine socioeconomic development worldwide. Depending on their nature of transmission, a classification can be made in noncommunicable diseases (NCDs) and communicable diseases (CDs). Noncommunicable or chronic diseases, which are not transferred between humans or other animals, cost 74% of the deaths globally each year. On the other hand, communicable diseases can disseminate among individuals. They include some of the most lethal infectious diseases, being the ‘big-three’ tuberculosis (TB), Malaria and HIV/AIDS, the most prevalent contributors to mortality in low-income countries¹. Only TB, the deadliest pre-pandemic communicable diseases worldwide, cause around 1.2 million deaths per year².

The classification of the infectious diseases can be established according to clinical and microbiological features, transmission type, or the natural reservoir of the infecting organism³, among other criteria as described in Figure 1.1. Pathogens including viruses (HIV), bacteria (TB), parasites (Malaria), and fungi (histoplasmosis, candidiasis), are the responsible for the most prevalent infectious pre-pandemic diseases². According to the transmission, CDs are classified as direct contact (from person-to-person, animal-to-person, congenital and perinatal infections), or by indirect contact through food or water (food and waterborne infections), inhalation of contaminated air (airborne), or by vectors like as an insect bite (mosquitoes, ticks, among others). Different examples of transmission mechanism are HIV virus, which can be transmitted by biological fluids including blood, semen, vaginal secretions, and maternal milk. Therefore, it can be transmitted by sexual contact, contact with infected blood or during pregnancy⁴. On the other hand, SARS-CoV-2, is primarily transmitted between direct contact of person-to-person through respiratory droplets or droplet nuclei and contacts routes, and

indirect contact with fomites in the immediate environment around the infected person⁵.

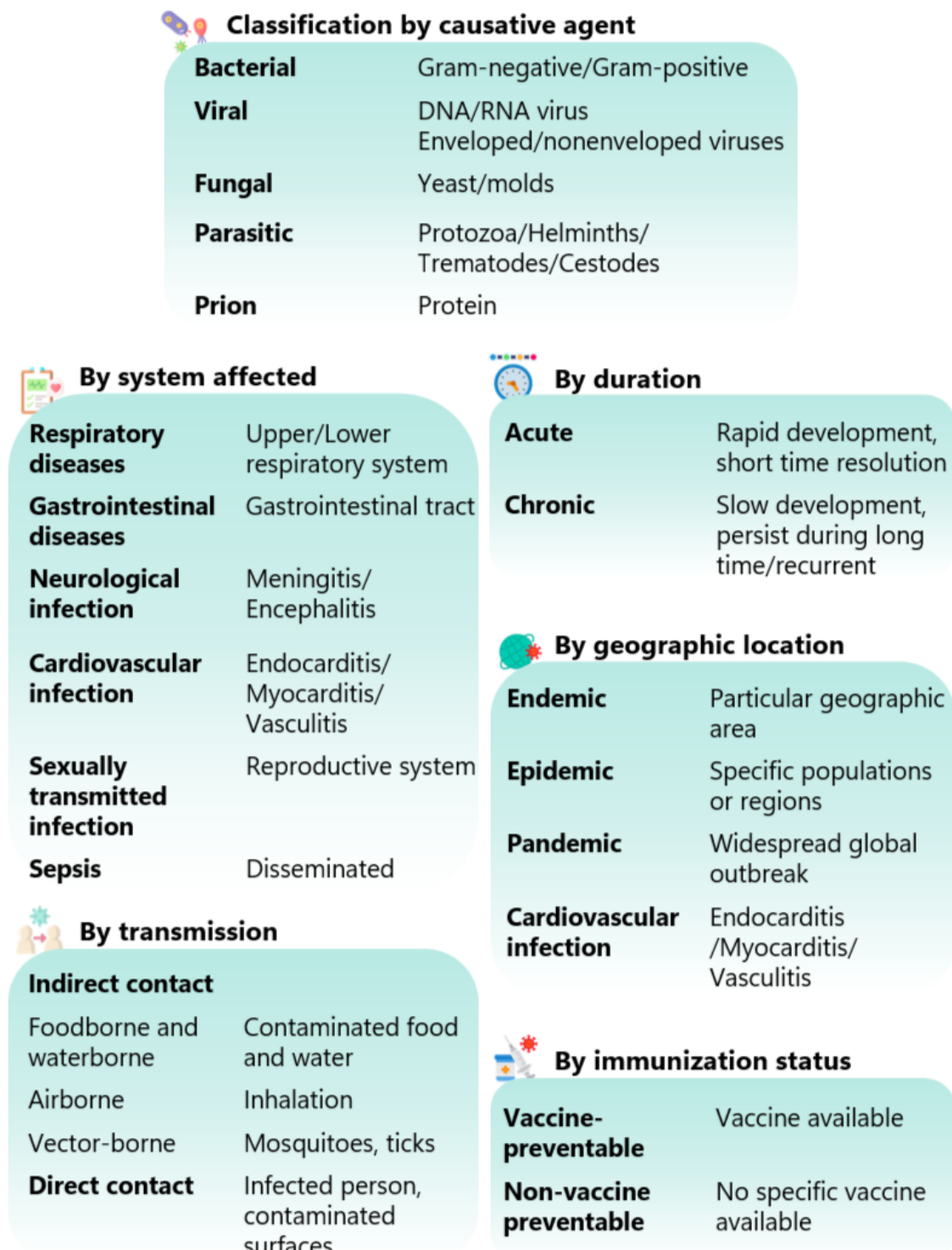


Figure 1.1. Schematic representation of the classification of communicable diseases.

Finally, Dengue virus is a vector-borne disease that are spread to people through the bites of infected *Aedes* species mosquitoes⁶, which also spread Zika virus. Malaria is other example, in this instance transferred by the female *Anopheles* mosquitoes⁷, although the transmission is also possible by contaminated needles, syringes or infected blood transfusion⁸. The histoplasmosis, a systemic mycosis, is contracted by inhalation of the spores of the fungi in microconidia form^{9,10}. The risk of contracting an infectious disease can be reduced by adding more layers and strategies of protection¹¹. Some of the fundamental keys to the prevention of these diseases include, among others, good hygiene practices, food control, vaccines, vector control and educational programs to promote self and community-protection¹². Beside the pandemics, although the global burden of CDs has been reduced in the two last decades, it remains a major challenge in low- and middle-income countries¹³, thus their control and prevention is needed in order to reduce its incidence, prevalence, morbidity, and mortality¹².

Among all the causative agents of CDs, this doctoral thesis will specifically focus on bacterial infections. Bacteria are prokaryotic organism that carry their genetic information in a double-stranded circular DNA. Additionally, certain species may contain small plasmids with extra DNA information. The cell envelope represents a complex multilayered protective structure. Based on their properties, most bacteria can be broadly categorized according to Gram staining¹⁴ in two major general groups: Gram-positive or Gram-negative bacteria. Although mycobacteria are classified as Gram-positive¹⁵, they are referred as acid-fast bacteria from a staining perspective system¹⁶. Figure 1.2 summarizes the schematic representation of the bacteria cell wall and their composition, considering that in this doctoral thesis immunosensing approaches are designed for mycobacteria and Gram-negative bacteria. Three main layers are found in the cell-envelope of the Gram-negative bacteria: the outer membrane that is a lipid bilayer principally composed by lipopolysaccharides (LPS), the peptidoglycan cell wall and the inner plasma membrane. These bacteria are not tinted by the Gram-staining. In contrast, Gram-positive bacteria lack an outer membrane but are surrounded by layers of peptidoglycan, sometimes thicker than in the Gram-negatives and appear tinted by the Gram-staining because of the retention of the colorant by this peptidoglycan layer^{14,17}. Inside the peptidoglycan layer, large anionic polymers of teichoic acids are

found. Mycobacteria, classified as an acid-fast gram-positive bacteria¹⁸, contain an unusual waxy coating of mycolic acids which makes some species of this genus impervious to Gram staining, specifically in the case of the most pathogenic mycobacteria such as *Mycobacterium tuberculosis* (the main cause of TB in humans) and *Mycobacterium leprae* (a cause of leprosy), or some non-tuberculous mycobacteria (NTM), if visible at all, mycobacteria may appear as finely beaded, gram-positive rods with only the beads visible (Gram-positive) and the rest of the organism appearing Gram-negative, or they may appear as negative images (unstained rodlike outlines) in the specimen¹⁹. These species are also called Gram-neutral (neither positive nor negative) or Gram-ghost^{20–22} and it requires special staining techniques for visualization such as the acid-fast staining, like the Ziehl-Neelsen^{23,24} or Kinyoun stain, or auramine O^{25,26}. The composition of the cell wall and their classification is critical in order to perform a precise and effective diagnostic and treatment, and the development of tests and medicine in bacterial diseases.

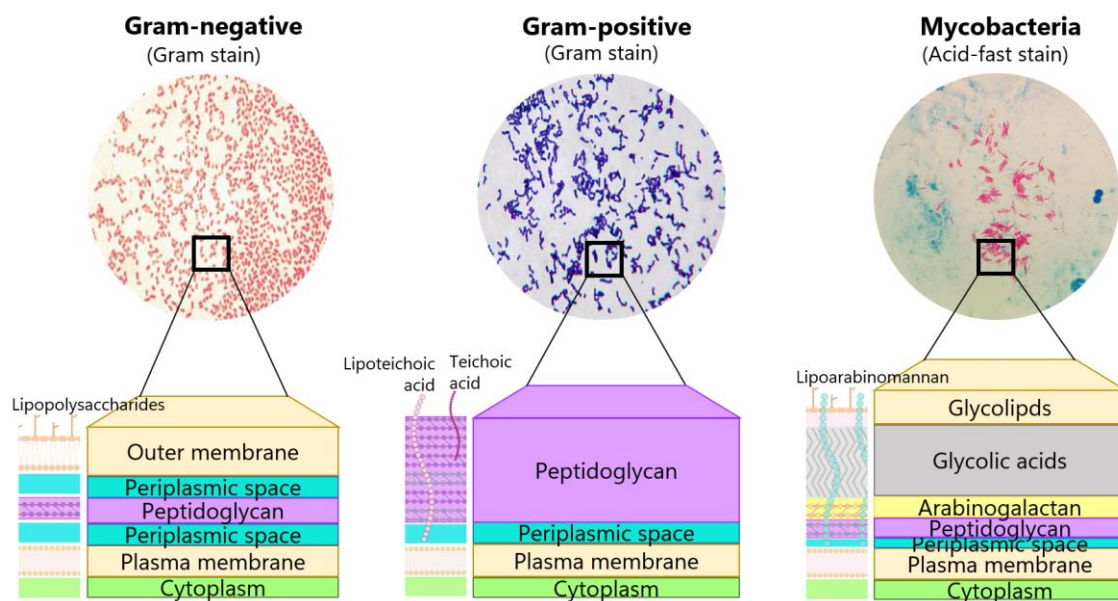


Figure 1.2. Schematic representation of the cell wall of the Gram-negative, Gram-positive bacteria and mycobacteria. The images show the appearance of the cells in the microscope after the Gram and Ziehl-Neelsen stain, for the bacteria and mycobacteria, respectively. The zoom describes the composition of the different layers of the cell wall, depending on their nature. Images of the Gram-negative and Gram-positive bacteria were adapted from reference²⁷.

Although bacteria play an important role in maintaining the environment, a small percentage cause infection and disease²⁸ with a large impact on public health. A recent study highlights the deadly impact of bacterial infections, constituting the 13.6 % of all global deaths in 2019, associated with 33 common bacterial pathogens, excluding those producing TB²⁹. For instance, 55 % of deaths associated with respiratory, bloodstream, and intra-abdominal infections are produced by *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*²⁹. Bacterial infections are, in general, easier to treat than viral due to the wide range of antimicrobial agents²⁸. However, bacterial resistance to antimicrobials is a rapidly growing problem: according to the WHO 2022 Global TB report, the burden of drug-resistance TB increased by 3% between 2020 and 2021³⁰. This rise includes new cases of rifampicin-resistant TB, complicating the management of the disease³¹. Untreated bacterial infections can lead to serious, life-threatening conditions including septicemia or sepsis, that can address to death, increasing the global mortality.

Effective environmental and clinical monitoring, along with early disease diagnosis, plays a critical role in minimizing the risk of pathogen contamination. This process is essential for initiating timely treatment and ensuring a proper follow-up for patients. Environmental monitoring primarily targets potential sources or transmission vehicles, such as air, water, or inanimate surfaces. It facilitates the identification of airborne pathogens such *Legionella*³⁷ (addressed in Chapter 3 of this doctoral thesis), waterborne pathogens such as non-tuberculous mycobacteria (NTM)³⁸ (Chapter 4), or *E. coli* (Chapter 5). Moreover, a single pathogen can be transmitted through different vehicles, such as air, water, or food. Therefore, the selection of sampling and detection methods, including the permitted limits, is established according to the specific application. Environmental Monitoring Programs serve as indispensable tools, particularly in industries like food and pharmaceuticals, among others. These programs adhere to regulations to identify, reduce, or eliminate pathogens, allergens, or other contaminants. Environmental Monitoring Programs rely on valid test methods and procedures, incorporating defined sampling locations and limits of detection. Correction actions are implemented based on these results to ensure the safety and quality of facilities

32–36.

On the other hand, early diagnosis remains very useful for initiating suitable therapy and preventing progression to advanced stages, such as sepsis³⁷ or even death. Additionally, it plays a pivotal role in limiting the spread and an outbreak size of an infection. Clinical monitoring is equally important to control the progression of the infection, ensure an adequate treatment and consider the emergence of antibiotic-resistant organisms. In this context, Rapid Diagnostic Tests (RDTs) emerge as effective tools for the early detection of infectious diseases and patient monitoring due to their rapid and user-friendly nature³⁸. They also hold significance in medical screening, offering diagnostic support in low and middle-income countries owing to their reduced cost³⁹. Over the past decade, there has been a substantial increase in the development of RDTs, and in a way, the global awareness of their value has been highlighted by the coronavirus pandemic⁴⁰.

In conclusion, effectively managing infectious diseases is crucial for minimizing global mortality rates. Addressing infections at the primary care level, followed by referrals to specialized services⁴¹, is vital. A comprehensive patient examination during consultations, coupled with RDTs or laboratory assessments when necessary, forms the cornerstone for infection control at the individual and population levels, while also managing costs. On the other hand, environmental control of pathogens is essential to detect possible outbreaks, prevent the spreading and take disinfection and sanitation measures^{35,42}.

This dissertation explores the technological challenges associated with the advance in RDTs, for the identification of infectious diseases in both clinical diagnostic practices and environmental monitoring. The exploration extends to preconcentration and detection strategies, aiming to improve analytical performance while maintaining a streamlined analytical process. Additionally, the dissertation relies on the utilization of magnetic particles capabilities as a tool for preconcentrating the target from complex clinical and environmental samples. The integration of magnetic particles into various preconcentration strategies and the subsequent readout processes is also thoroughly examined. This study contributes to a deeper understanding of different aspects involved in the development and optimization of RDTs, addressing critical challenges for their implementation in low resource settings.

1.1.2. Technological challenges in the detection of pathogenic bacteria

1.1.2.1. Waterborne bacteria. *E. coli*, *Legionella* and nontuberculous mycobacteria

Waterborne diseases are considered a significant global public health concern, since they contributed to an estimated 2.2 million deaths per year. These diseases have a substantial economic impact and are particularly prevalent in developing countries, where inadequate sanitation systems or contaminated water sources are common. The spreading normally occurs by ingestion of contaminated drinking water, exposure to contaminated sources or indirectly through contaminated food⁴³. As some pathogens occur naturally in waters, climate changes can also affect the quality of water and soils, due to the increase of the temperature in water or an excess of nutrients. Among the pathogenic *E. coli* strains, enterohaemorrhagic *E. coli* O157:H7 is one of the most concerning to the drinking water industry, due to its high pathogenicity to humans, producing diarrhea that can progress to hemorrhagic colitis or hemolytic uremic syndrome⁴⁴. In June 2015, a waterborne outbreak of *E. coli* infection associated with contaminated drinking water occurred in children experienced acute gastrointestinal symptoms at a school camp located in Jeollabuk-do (South Korea)⁴⁵. A similar case was in May 2016, with an outbreak of *E. coli* O157 infection occurred among children in a stream-flowing park in California⁴⁶ (USA) with one reported hospitalization with hemolytic uremic syndrome. One of the most relevant infection outbreaks resulting from waterborne pathogenic bacteria include *Legionella pneumophila*⁴⁷, the responsible of the Legionnaires' disease (Legionellosis) or a mild form the Pontiac fever that is transmitted by the inhalation of contaminated water⁴⁸, including air conditioning cooling towers, hot and cold water systems and artificial water systems. According to the European Centre for Disease Prevention and Control, in 2021 the EU/EEA witnessed the highest annual notification rate of Legionellosis to date, the 75% of all reported cases where in Italy, France, Spain and Germany⁴⁹. Another cluster of pneumonia cases associated with a health clinic was develop in Tucumán (Argentina)⁵⁰. The last reported cases have occurred from august of 2023 in an outbreak of legionellosis in Poland, including 166 positive cases and 23 deaths⁵¹. Nontuberculous mycobacteria (NTM) can be present in both natural and artificial water systems, posing as opportunistic pathogens immunocompromised patients at

risk. These bacteria typically result in respiratory infections, but their potential for dissemination to other parts of the body, along with localized manifestation as skin and soft tissue infection, is also possible⁵² among others. The prevalent mode of transmission often involves inhaling aerosolized droplets of contaminated water. *Mycobacterium avium complex* including the species of *Mycobacterium avium* and *Mycobacterium intracellulare*, stands out as the primary cause of NTM lung disease in the US⁵³. As these bacteria can survive under extreme conditions to chlorine and other chemical disinfectants used in standard drinking water treatments, special attention in monitoring and disinfection protocols is required^{54,55}. Other pathogenic bacteria with high to moderate health significance, which are not addressed in this doctoral thesis, include *Salmonella enterica* serotype *Typhi* (Typhoid fever and serious salmonellosis), *Vibrio cholerae* (gastroenteritis, cholera) and *Pseudomonas aeruginosa* (infections on lungs, urinary tract and kidney)⁵⁶. Effective and sufficient disinfection treatment is crucial, as well as continuous water monitoring to identify potential contamination and assess the disinfection treatment efficacy, since some pathogens, such as mycobacteria, can persist through sanitization processes, creating biofilms in the surfaces of pipes and contributing to outbreaks⁵⁷. The current standard for identifying and detecting pathogens relies on culture-based methods, chosen for their simplicity and cost-effectiveness. Advances in these methods have enhanced media formulations, resulting in more specific, sensitive, and rapid cultures for improved isolation. However, the main drawback remains the time-consuming and labor-intensive nature of these methods. The primary technological challenge for the detection of waterborne pathogens, lies in identifying the bacterial target in large volume of sample. To effectively handle such substantial sample size, preconcentration approaches as filtration, centrifugation, magnetic immunoseparation, culture preenrichment are typically integrated to the detection method. One of the most popular strategy is vacuum filtration using sterile membrane filtration⁵⁸. The filter is then recovered and incubated in a selective medium, allowing bacterial growth. In some instances, the culture method proves ineffective for isolating certain bacteria simultaneously with different species with different growth times, producing an inhibition due to the overgrowth of the other species. Additionally, detecting viable but non-culturable cells presents challenges as can occur, for example, in *E. coli* and *Vibrio cholerae*⁵⁹.

Table 1.1. Summary of commercial tests for the detection of *Legionella* in water samples.

Comercial kit	Type of assay	Assay time	Sample	Preconcentration / preenrichment / amplification	LOD
Legiolert®. IDEXX Laboratories, Inc., Westbrook, USA	Bacterial enzyme detection technology: Legiolert substrate produce a brown color indicator. Quantitative	7 days	100 mL water	culturing	100 mL for potable water: <1 CFU/100 mL 10 mL for potable water: <10 CFU/100 mL 1 mL for nonpotable water: <100 CFU/100 mL
Duopath® Lateral Flow Tests. Merck KGaA, Germany	Immunochromatographic rapid test (lateral flow assay). Qualitative	10 days + 40 min	0.1 to 1 mL water / 10 to 100 mL (if filtering)	culturing / filtering and culturing	1 CFU
Remel Legionella poly-ID test Kit. Remel INC, USA	Immunofluorescence microscopy. Qualitative	2 h	Clinical, culture, environmental water samples	Centrifugation or filtration	<25 bacilli per slide
Hydrosense One Multi. Albagaia, UK Accepta Ltd, UK Insatech A/S, Denmark	Immunochromatographic rapid test (lateral flow assay, LFA). Qualitative	30 min	Water samples from any source	Filtration	1,000 CFU/L and provides an action level indication at 10,000 CFU/L too.
Microproof® Legionella Quantification LyoKit. BIOTECON Diagnostics GmbH, Germany	Real-time PCR. Quantitative	4 h	Clear or turbid water	Filtration / DNA extraction and PCR	3 genomic units
iQ-Check Legionella Real-Time PCR Kit. Bio-Rad Laboratories Inc, USA	Real-time PCR. Quantitative	< 4 h	Water supply (1L) systems, biofilms and aerosol samples.	Filtration / DNA extraction and PCR	5 genomic units
PRImeDETECT™ Legionella spp Detection Kit. Canvax Reagents SL, Spain	Real-time PCR. Quantitative	< 24 h	Environmental and Biological Samples	Filtration / DNA extraction and PCR	Quantitative 2.5 copies
VWR®, Real-time PCR kit for Legionella spp (ISO/TS 12869). VWR International, LLC, USA	Real-time PCR. Quantitative	< 24 h	Drinking water samples and all types of continental waters	Filtration / DNA extraction and PCR	< 10 genomic units
Condagene® Legionella spp. Condalab, Spain	Real-time PCR. Quantitative	< 24 h	Drinking water	DNA extraction and PCR	< 10 genomic units

Lovibond™ Legionella Field Test Kit. Tintometer Lt, UK	Immunochromatographic rapid test (lateral flow assay, LFA). Semiquantitative	25-30 min	Water sample (0.1 mL)	No	100,000 CFU/L
Qualyfast® Legionella qPCR assay. Bioside S.R.L., Italy	Real-time PCR. Quantitative	3 h	Water sample	Filtration / DNA extraction and PCR	5 genomic units
Legipid® Legionella fast detection. Biótica Química SL, Spain	Immunoassay with visual detection. Semiquantitative	50-60 min	Water sample	Filtration / IMS	93 CFU
The Swab Legionella Field Test kit. Insatech A/S, Denmark	Immunochromatographic rapid test (lateral flow assay, LFA). Qualitative	35 min	Biofilm sample	No	200 CFU / swabbed area
Thermo Scientific™ Oxoid™ Legionella Latex Kit. OXOID Ltd, UK	Agglutination test with blue latex particles. Qualitative	10 days	Environmental and clinical samples	Filtration / culturing	1 CFU
Thermo Scientific™ DrySpot™ Legionella Kit. OXOID Ltd, UK	Agglutination test with blue latex particles modified with different serotype antibodies. Qualitative	10 days	Environmental and clinical samples	Filtration / culturing	1 CFU

In fact, other laboratory-based methods such as nucleic acid-based, immunology-based and biosensing approaches are often used as screening techniques in conjunction with culture for confirmation and quantification of bacteria⁶⁰, since they offers better turn-around times for rapid decisions, high specificity and sensitivity. However, some of these methods are still complex, expensive, require benchtop instruments and or qualified personnel⁶¹. Proper assessment of pathogens on water and water quality monitoring are key factors for decision-making regarding infrastructure of water distribution systems, optimal water treatment selection, and preventing waterborne outbreaks⁵⁶. Recently, research focus has shifted towards the development of rapid on-site tests for detecting this bacteria, overcoming the limitations associated with implementing traditional assays ⁶⁰. In the context of the clinical analysis, the criteria for the development of point-of-care tests are included in the acronym ASSURED^{62,63} defined by the World Health Organization (WHO), as described in §1.3.1 later. Although this criterion is not applicable directly or entirety to waterborne pathogens, as far as pathogen analysis or environmental monitoring, it leads to a test design that should consider all these characteristics. Ideally, the test should be simple, robust, with minimal handling, allowing rapid results and on a portable device, to facilitate measurement at the site where the analysis is conducted. Recently, the acronym was expanded to REASSURED, to take into consideration the real-time-connectivity and ease of specimen collection³⁸. An example of rapid tests that meets some of these characteristics are the lateral flow assays (LFA), also described in §1.3.4. A wide range of commercial LFA for the detection and semiquantification of pathogens are available in the market, such as the Singlepath® lateral flow tests for *E. coli* O157, *Salmonella* and *Campylobacter* and Duopath® Lateral Flow for *Legionella*/*Legionella pneumophila* (Merck, Germany). Other rapid tests include PCR kits allowing the detection and quantification of pathogens, such as the iQ-Check Legionella Real-time PCR kits (Bio-Rad Laboratories Inc, USA), SureTect™ *Listeria monocytogenes* PCR Assay (Thermo Fisher Scientific Inc, USA). Other innovative technologies include rapid bioluminogenic tests that emitted light under the presence of a specific microorganisms, like the MicroSnap® adapted for the detection of *Enterobacteriaceae*, *coliforms*, *E. coli*, *Listeria* and *Salmonella*

(Hygiena LLC, USA). More examples of commercial rapid tests or kits for the detection of *Legionella* in water samples are summarized in Table 1.1.

Regarding the detection of pathogens in a clinical sample, the process is typically more challenging, since the specimen must be easy to collect, non-invasive, and representative of the patient. The goal is not only the direct determination of pathogen but also specific biomarkers that enable the diagnosis of the infection. Numerous considerations come into play, ranging from the practical aspects of sample collection to the identification of biomarkers crucial for a comprehensive diagnostic assessment. In any case, the detection limits must be enough to ensure minimal detection since infection can be caused at very low doses. On the other hand, they must also be able to reach the thresholds established in different regulations according to the pathogen, the source of the sample and its final use, for example water for hemodialysis purpose^{64–66}, drinking water for human consumption⁶⁷ or monitoring water in cooling towers or spa pools⁶⁸. The thresholds can also vary depending on local regulations and geographic locations.

In this dissertation, two methods are developed for the detection of *Legionella* (Chapter 3) and NTM (Chapter 4) in water samples, addressing typical drawbacks such as their low concentration in water and the processing of large sample volumes. For this purpose, a preconcentration method that combine filtration and the use of magnetic particles modified with specific antibodies to recover the bacteria from the filters are performed. Afterwards, electrochemical immunosensing is used for the readout, obtaining impressive detection limits that meet the thresholds required by the legislation. On Chapter 5, magnetic particles are directly applied to the sample for the preconcentration of *E.coli*, but in this instance the readout is performed by using optical readout an a smartphone app. These culture-free sensors constitute a rapid, easy and sensitive method, without the need of benchtop instruments, further reducing the cost, resulting in an interesting alternative within water monitoring programs.

1.1.2.2. Airborne bacteria. *Mycobacterium tuberculosis*

Tuberculosis (TB) is considered the oldest communicable diseases and currently ranks as the second leading infectious cause of death, following COVID-19⁶⁹, of 1.6 million people only in 2021³⁰. It is transmitted from person to person by

the inhalation of contaminated airborne droplets. *Mycobacterium tuberculosis* is the primary specie of the *M. tuberculosis* complex (MTBC) affecting the lungs. However, it can also impact other body parts such as the brain⁷⁰, kidney, or spine⁷¹. Despite being preventable and treatable, an estimated quarter of the global population carries the bacterium⁷², with only a small percentage developing active TB disease⁶⁹.

While patients with latent tuberculosis infection cannot transmit the bacteria and remain asymptomatic, those who develop active TB must be treated with properly antibiotics treatment to prevent the transmission and progression of the disease. An inadequate regimen may contribute to the development of drug-resistant TB for first- and second-line drugs. Therefore, proper diagnosis is essential either with molecular or with culture-methods, as a gold-standard has not yet established for drug resistance⁷³. Special attention should be provided to children, pregnant women, and immunocompromised patients such as those infected with HIV, as they are particularly vulnerable.

The most used tests for latent tuberculosis infection are Tuberculin Skin Test and the Interferon Gamma Release Assay (IGRA). However, the Tuberculin Skin Test may present interferences with vaccinated Bacille Calmette-Guerin patients, leading to potential false positive. The use of IGRA is constrained in high burden countries due to the cost, and the text complexity, as the sample have to be previously stimulated. However, these tests are not accurate for the diagnosis of active TB. For active pulmonary TB, chest radiography or chest X-ray, remains the primary method for detecting suspected tuberculosis⁷⁴, especially valuable when pulmonary TB cannot be confirmed by culture⁷⁵. Despite it has high sensitivity, the specificity is poor because many X-ray abnormalities consistent with TB are seen also in other lung pathologies, thus relying only on X-ray for TB diagnosis leads to misdiagnosis^{75,76}. Furthermore, although medical imaging is a valuable tool to aid in screening, confirmation, and documentation of the course of the disease, not all settings have access to this equipment⁷⁷.

Currently, WHO recommends the use of molecular RDTs for the diagnosis in patient with signs and symptoms of TB, such as the GeneXpert® MTB/RIF Ultra (Cepheid, USA) or the recently added Truenat™ MTB, MTB Plus and MTB-RIF Dx (Molbio Diagnostics, India)⁷⁸. These techniques have sufficient specificity and sensitivity according to WHO criteria. GeneXpert® MTB/RIF Ultra, the latest version

of Cepheid cartridge, allows results in approximately 2 h with minimal on-time handling and provides information on resistance to rifampicin, one of the most widely used treatments. The sample, previously inactivated and liquefied, is inserted directly into the cartridge where the processing is automatized (filtration, lysis, DNA extraction and PCR). The module allows the simultaneous processing up to 16 cartridges. However, despite their good sensitivity in smear-positive samples (98%), TB detection is lower in extrapulmonary samples (79%), children (66%), paucibacillary specimens (68%) and HIV-patients (80%)⁷⁹. However, it remains too expensive and requires a constant source of electricity, that is challenging for developing countries or low resource-settings. In this sense, the Truenat™ MTB, MTB Plus and MTB-Rif Dx test, is also a molecular-based assay with similar accuracy compared with GeneXpert but with the advantage of being battery operated and portable, more convenient for low- and middle-income countries. Even though it is not a fully automated system, and the samples must be measured one by one per device. Specifically, Alere Determine TB-LAM Ag test (Abbot Laboratories, US) is recommended for the detection of mycobacterial LAM (see Figure 1.2), which is excreted by the active cells in urine samples, for immunocompromised people such as HIV-positive patients, being the sensitivity improved for TB detection including HIV-negative individuals using the Fujifilm SILVAMP TB LAM (FujiLAM) (Fujifilm, Japan). These are rapid and low cost LFA, with good accuracy in diagnosing TB, with a sensitivity and specificity of 74.2% and 89.3% (FujiLAM) and 53% and 95.6% (AlereLAM)⁸⁰, respectively.

However, the fact remains that in developing countries with the highest incidence of TB, the diagnosis is still relying on sputum smear microscopy using the Ziehl-Neelsen stain or in better cases, the light-emitting diode fluorescent microscopy. These techniques are cost effective, simple, and practical to perform in low-resource laboratories to primary health clinics⁸¹. However, the sensitivity is low, and examination of multiple samples is recommended, which leads to loss of patient follow-up and the risk of transmitting TB due to false negative results. In general, culture based-methods are used to reconfirm smear-positives and to detect multidrug-resistant strains. But not all laboratories can perform this assay, as they do not dispose the appropriate containment and security measures, and although there is a WHO-endorsed mycobacterial growth indicator tube, as BD BACTEC™

MGIT™ automated mycobacterial detection system- 960 system (Becton Dickinson, US), that allows the growing of 960 samples simultaneously and a reduced growth time up to 10-14 days for MTBC recovery with additional 6-10 days for drug sensitivity testing, standard culture requires approximately between 4-8 weeks, and additional 4 weeks for drug sensitivity, so it is a very time consuming method.

Great progress has been made in the development of rapid tests for TB diagnosis, and the validation and assessment of new ones is ongoing. The new tests under development should meet the criteria required by WHO, with good specificity and sensitivity, although these criteria may vary depending on the purpose of the test, e.g. a lower sensitivity may be acceptable if it increases case detection due to its accessibility in developing countries with limited access to traditional tests. On the other hand, specificity may be tolerated if the test is intended to triage patients in the first steps of screening.

The integration of 'omics' techniques including genomic, metabolomic, transcriptomic, and proteomic, enables the identification and characterization of new biomolecules that can serve as biosignatures or biomarkers for TB diagnosis. Current research is directed towards discovering these biomarkers and developing tests based on them, although there are still some limitations to overcome, as the need to test multiple markers in many cases. Moreover, analytical validations should be done in heterogeneous groups of patients, including children, pregnant women, HIV-patients, individuals with low bacillary burden, in addition to confirmed TB and latent TB patients and healthy controls, as cohorts are sometimes limited to a specific population and geographically localized. All this, considering in all instances the minimum criteria established by the WHO to endorse a TB test and in the best case, trying to meet the REASSURED/ASSURED criteria, should be the pillars for the development of a TB rapid diagnostic test.

In this dissertation, the design of a rapid tests for the detection of tuberculosis is addressed in Chapter 7. Upon the double-tagged amplification of the *gyrB* and *IS6110* gene regions with biotin and digoxigenin, a biosensing strategy is performed in only 15 minutes by using streptavidin magnetic particles and HRP-labelled antibodies against digoxigenin. The electrochemical measurements are carried out in a simple and user-friendly device that allows the washing and readout in the novel cartridge using the same buffer. This test intended to be rapid, easy, and

portable in accordance with some of the requirements for the development of a test for TB detection.

1.2. Laboratory-based techniques for the detection of pathogenic bacteria

This section outlines the most classical methods for bacterial identification and determination in the laboratory, which in more of the instances are considered the gold standard, including traditional techniques and more recent approaches that have proven their utility over the years. On the other hand, the next section (§1.3), describes the most popular rapid diagnostic tests and other IVD (in vitro diagnostic test) for on-site testing or that can be performed on low-resource setting facilities.

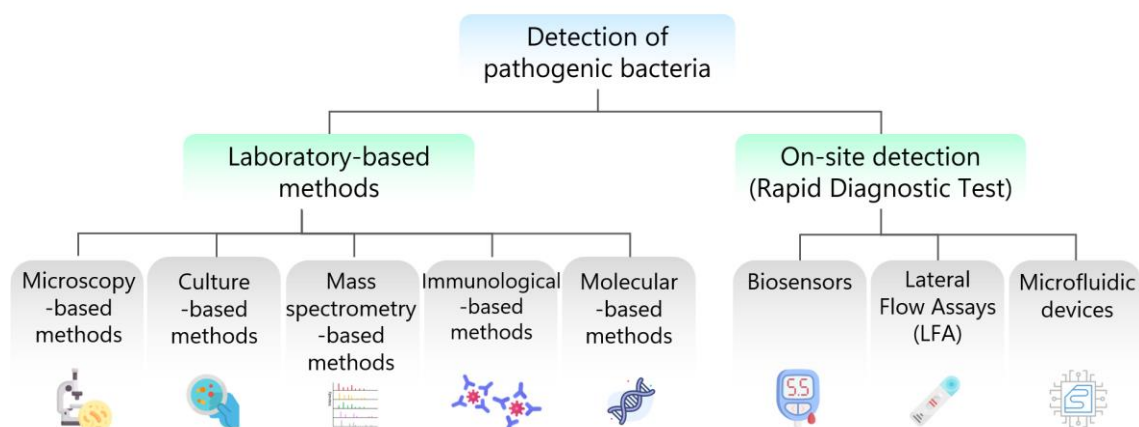


Figure 1.3. Methods for the detection of pathogenic bacteria, classified as laboratory-based methods and on-site detection methods.

In Table 1.2 is shown a summary of different research works for the detection of mycobacteria that combines different traditional methods with more innovative detection strategies described in the next sections, including the features of the electrochemical biosensor developed in this dissertation for the detection of *M. fortuitum* as a model of environmental mycobacteria (Chapter 4).

Table 1.2. An overview of different methods for Non-Tuberculous Mycobacteria (NTM).

Method	Type of assay	Assay time	Sample	Preconcentration/ Preenrichment/ Amplification	Specificity	LOD	Ref
Environmental mycobacteria in water supply systems, especially <i>M. kansasii</i>, by culture, subculture and biochemical testing	Standard culturing / Biochemical confirmation test (niacin accumulation, nitrate reduction, catalase, urease, etc)	up to 6 weeks	1 - 10 L water	Filtration /resuspension / centrifugation / culturing	Not tested	Not provided Identification purpose	⁸²
Non-tuberculous mycobacterium strains in environmental samples, especially <i>M. chimaera</i> and <i>M. chelonae</i>	Standard culturing	up to 28 days	1 L water	Filtration /resuspension / culturing Or Filtration /direct culturing of the filters	Not tested	Not provided Recoveries from <i>M. chelonae</i> 130 CFU mL ⁻¹ <i>M. chimaera</i> 230 CFU mL ⁻¹	⁸³
Non-tuberculous mycobacteria in hospital water samples	Standard culturing	up to 2 weeks	50 mL spiked tap water	Filtration /direct culturing of the filters	Tested with <i>M. chimaera</i> , <i>M. smegmatis</i> , <i>M. abscessus</i> , <i>M. wolinskyi</i> , <i>M. chelonae</i> , <i>M. mucogenicum</i> and <i>M. llatzerense</i> <i>Stenotrophomonas maltophilia</i> , <i>Pseudomonas aeruginosa</i> , <i>Sphingomonas paucimobilis</i> ,	3-98 CFU 100 mL ⁻¹	⁸⁴

					<i>Methylobacterium</i> sp., <i>Pseudomonas stutzeri</i>		
NTM in water samples from heater-cooler units and extracorporeal membrane oxygenation machines	Standard culturing	4-8 weeks	100 mL water	Filtration / direct culturing of the filters	<i>Mycobacterium</i> spp. <i>P. aeruginosa</i> , and total viable counts (TVCs)	1 CFU 100 mL ⁻¹	85
Non-tuberculous mycobacteria in hospital water	Standard culturing / qPCR for 16S rRNA gene	<1h (only PCR)	500 mL drinking water	Filtration / direct culturing of the filters Filtration / ultrasonication / vortexing / centrifugation / vortexing / DNA extraction	22 species of <i>Mycobacterium</i> genus 31 non-mycobacterial species	50 fg target DNA in 500 mL (833 CFU 500 mL ⁻¹)	86
NTM in drinking water, especially <i>M. avium</i>, <i>M. intracellulare</i>, and <i>M. avium</i> subsp. <i>paratuberculosis</i>. Other microbes (protozoa, bacteria, mycobacteria) were also tested.	qPCR for <i>IS900</i> / target 251 / 16S rRNA.	Not detailed	4 L treated water	Filtration / DNA extraction / PCR	22 <i>M. avium</i> subsp. <i>Paratuberculosis</i> strains and 10 non- <i>M. avium</i> subsp. <i>paratuberculosis</i> strains	10 copies <i>M. avium</i> and <i>M. intracellulare</i> 1.8 copies <i>M. avium</i> subspecies <i>paratuberculosis</i>	87
<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> in drinking water and biofilms	qPCR for the <i>IS900</i> and target 251 bp	Not detailed	2 L drinking water	Filtration / DNA extraction assisted by beadbeater / PCR	22 <i>M. avium</i> subsp. <i>Paratuberculosis</i> isolates and 10 non- <i>M. avium</i> subsp. <i>Paratuberculosis</i> strains	1.8 copies	88

Mycobacterial species in different sources, including water samples for zebrafish health monitoring	qPCR for <i>16S</i> and <i>18S rRNA</i>	Not detailed	1, 0.5 and 0.15 L natural water	Filtration / DNA extraction by mechanical disruption and One-For-All Vet Kit (Qiagen) / PCR	Tested for <i>M. chelonae</i> , <i>M. fortuitum</i> , <i>M. haemophilum</i> , <i>M. peregrinum</i> , <i>Pseudocapillaria tomentosa</i> , <i>Pseudoloma neurophilia</i>	<10 copies	89
<i>Mycobacterium</i> spp. in natural waters	PCR for <i>16S</i> rRNA and <i>hsp65</i> genes	Not detailed	4 L of natural waters	Filtration / DNA extraction DNeasy Power Soil kit (Qiagen) / PCR	<i>Mycobacterium</i> sp.	Not provided Identification purpose	90
Non-tuberculous mycobacteria in different environmental samples (soil and water sediment samples of rivers, wastewater treatment plants, artificial bathing tanks, among others)	qPCR for <i>16S</i> rRNA and <i>hsp65</i> gene	1h (only PCR)	depends on the matrix	sample preparation depends on the matrix / PCR	Not tested with other species Suitable for detects 37 species of NTM	1.75 x 10 ³ mycobacterial cell	91
<i>Mycobacterium</i> spp. in tap water and lakes	qPCR for <i>atpE</i> gene	Not provided	1 L	Centrifugation (2 h) / DNA extraction QIAamp DNA mini kit (Qiagen) / PCR	31 mycobacteria strains 30 non-mycobacteria strains	6 genome equivalents	92
<i>Mycobacterium ulcerans</i> in different environmental samples	Sequence capture -PCR for <i>IS2404</i> , <i>IS2606</i> and mycobacterial <i>16S</i> rDNA	3h 30 (PCR not included)	2, 1, 0.5 L	Filtration or centrifugation / DNA extraction assisted by mechanical disruption / magnetic separation / PCR	Not tested	Not accurately assessed. ~1 cell 300 mL ⁻¹	93

Label free electrochemical <i>M. simiae</i> DNA genosensor using modified gold electrodes	Standard culturing followed by electrochemical genosensor (DPV)	1 h (Standard culturing and electrode modification not included)	Sputum sample	Standard culturing	Tested with synthetic DNA, DNA from 5 clinical isolates of <i>M. simiae</i> and mismatched DNA Synthetic DNA from 6 non- <i>M. simiae</i> strains	1.40 fM for the synthetic <i>M. simiae</i> DNA	⁹⁴
<i>Bacillus cereus</i> and <i>M. smegmatis</i> based on bacteriophage lysis and electrochemical sensing of beta-glucosidase	Amperometric measurement of enzymatic activity	8 h	2 L (1000, 100 and 10 CFU mL ⁻¹)	Filtration	<i>M. smegmatis</i>	10 CFU mL ⁻¹	⁹⁵
<i>M. fortuitum</i> in hemodialysis water	Magneto-actuated immunoassay with optical readout	3 h	100 mL hemodialysis water	Immunomagnetic separation	Not tested	13 CFU mL ⁻¹	⁹⁶
<i>M. fortuitum</i> in water samples	Paper-based assay (lateral flow assay and vertical flow assay)	1 min vertical flow 15 min LFA (PCR not included)	1 mL water	Double-tagging PCR /immunomagnetic separation	Not tested	10 CFU mL ⁻¹	⁹⁷
<i>M. fortuitum</i> in hemodialysis water	Electrochemical immunosensor	1 h	0.1 L hemodialysis water	Filtration / immunomagnetic separation	<i>M. fortuitum</i> , <i>L. pneumophila</i> , <i>P. aeruginosa</i> , <i>E. coli</i>	5 CFU mL ⁻¹	This work (Chapter 4)

1.2.1. Microscopy-based methods

Microscopic examination is a rapid, low cost and easy-to-perform technique that allows the morphologic observation of many pathogens in samples of different natures (sputum, tissues, water, among many others). Depending on the pathogen, differential staining techniques can be applied as a means of gathering information about bacteria, being the most important the Gram stain, acid-fast staining or trichrome stains. It usually requires high-concentration samples, so it can lead to false negatives if there is not a high bacterial load and is less sensitive than microbiological culture. In addition, it does not allow species identification, therefore is not very specific.

1.2.2. Culture-based methods

Microbiological culture remains the gold-standard in the identification of many pathogens and is a basic diagnostic technique, both for identification and quantification, as well as for the study of drug resistance. It involves the replication of the pathogen in a culture medium, which can be selectively supplemented with various nutrients and specific antibiotics to facilitate its growth and even prevent the growth of other unwanted organisms. Proper conditions have to be selected to grow the cells, such as temperature, presence of oxygen and incubation time. Several bacterial culture formats exist, depending on the downstream applications such as broth culture (liquid medium), agar plates, stab tubes and agar-based dipsticks (solid medium) or collection tubes with liquid or solid medium. It is a cost-effective technique with good specificity and sensitivity. As a drawbacks, culture needs viable cells for replication, since not viable or not-cultivable cells are not detected⁹⁸. The main disadvantage is the turnaround time to obtain confirmatory results and the biological risk involved under inappropriate biosecurity conditions. Innovative strategies are available on the market to make culture easier and more specific, such as the new loops with integrated and stabilized medium to identify the *Legionella anisa* strain in water samples (Culti-Loops™ *Legionella anisa* NCTC 11974™, catalogue no R4601315, Thermofisher) followed by culture in liquid medium if necessary.

1.2.3. Mass spectrometry-based methods

The detection of the specific biomarkers signatures by mass spectrometry to identify bacteria was proposed in 1975⁹⁹. Mass spectrometer consists of three units: an ionizing source, a mass analyzer for separation of ions by their mass-to-charge ratio (m/z) and a detector for monitoring the separated ions. The procedure is much faster than other conventional techniques, as it allows the identification of biomarkers in a few minutes, as 5-30 minutes. In general, a small portion of a bacterial colony is deposited on a conductive metal plate and mixed with a solution of a low mass organic compound, called matrix. Gram-positive bacteria, mycobacteria and other pathogens may require additional pretreatment for lysis¹⁰⁰. After drying, the matrix is crystallized and introduced into the mass spectrometer to be irradiated with a laser beam to produce photon absorption, trigger sublimation and gas phase ionization of the sample. The ionized molecules are accelerated at a fixed potential and separated by their mass-to-charge ratio (m/z) generating a peak spectrum, the peptide mass fingerprinting. The charged analytes may be measured by different types of mass analyzers like quadrupole mass analyzers, ion trap analyzers, time of flight (TOF) analyzers, etc. So, in microbiological applications MALDI-TOF MS are widely used^{101,102}. The m/z ratio of an ion is measured by determining the time of flight required to travel the length of the tube until arrive to the detector. The spectral profiles are compared using a database of spectra that is constructed from reference strains. These peaks can be characteristic of the genus, species and sometimes subspecies¹⁰³. For the microbial identification, the characteristic patterns of highly abundant ribosomal proteins with a mass range m/z of 2–20 kDa is typically used¹⁰⁴, which represents 60-70% of the dry weight of a microbial cell¹⁰¹. The technique has great accuracy and reproducibility. However, as the technique is based on pure colony spectra, samples often need pre-enrichment and culture isolation steps, which ultimately requires more time. Moreover, mass spectrometry instruments are expensive to acquire, operate, and maintain. The complexity of the technology requires trained personnel, which may pose a barrier in resource-limited settings. This also limits MALDI-TOF-MS analysis to culturable pathogens¹⁰⁵. Some commercial automated systems based on MALDI-TOF-MS are the VITEK® MS (bioMérieux, Spain) and the MALDI Biotyper® (Bruker,

USA). Several studies have demonstrated the usefulness of this technique for the identification of yeasts, filamentous fungi, viruses, bacteria and directly from biological samples such as blood, urine or cerebrospinal fluid.

1.2.4. Immunological-based methods

1.2.4.1. Characteristics of antibodies

Immunological-based methods represent a category of techniques primarily relying on the interactions between antigens and antibodies. Antibodies provide the specificity of the assay and can be potentially produced as a reagent towards almost all molecules or biomolecules. Antibodies (Ab), also called immunoglobulins, are glycoproteins produced by the immune system (activated B lymphocytes) of higher organisms in defense against the entry of foreign bodies, which it does not recognize as its own. These foreign materials are antigens (Ag). There are 5 types of immunoglobulins: IgA, IgD, IgE, IgG and IgM, being IgG the most used in immunoassays. IgG antibodies are Y-shaped proteins formed by two main structures: the constant or crystallizable Fc part, recognized by the host as its own, and the antigen-antibody binding fragment Fab part, where the interaction between the antigen and the antibody takes place. These structures consist of four polypeptide chains, two heavy chains and two light chains, linked by disulfide bridges (Figure 1.4, panel B).

One of the most valuable features of Ab in immunological-based methods is their high specificity against different antigens, and the ability to recognize different epitopes. This recognition can be done at any of the four structural levels, from recognition of a sequence of amino acid chains to spatial/steric recognition. The measure of the goodness of fit of the binding between the amino acid residues of the Ab/paratope, and the binding site of the Ag/epitope¹⁰⁶ determines the specificity of the antibody. However, when two different and apparently unrelated Ag share the same epitope or have a high conformational similarity, it can be recognized by the same antibody, presenting cross-reactivity¹⁰⁷ (Figure 1.4, panel C). This can also occur when the same antigen presents more than one structurally different epitope and is recognized by the same Ab¹⁰⁷. The Ab-Ag fitting is constituted by the sum of weak chemical interactions, including electrostatic, hydrophobic, van der Waals and

hydrogen bonds interactions. Individually these forces are weak but the sum of all of them makes the interaction strong, sometimes reaching values near to those of a covalent bond. Thus, the measure of the strength of the fit determines the affinity of the antibody to bind an antigen. Affinity is described as the reversible formation of the Ab-Ag complex, according to the following equation:



Where the affinity constant (K_a) can be determined according to the following equation:

$$K_a = \frac{[\text{Ag-Ab}]}{[\text{Ag}][\text{Ab}]} \quad (\text{Equation 1.2})$$

When Ab and Ag share complementary shapes and physical interactions, the greater the molecular interaction and fit between Ab-Ag, the higher the value of K and the higher the affinity of the antibody to an antigen.

Polyclonal antibodies are those obtained directly from blood derivatives after the immune response following infection/vaccination/immunization with the antigen/immunogen from the animal host. Polyclonal Ab contain a mixture of antibodies with different specificities and affinities against the epitopes of the antigen, when it contains more than one, since they are produced by different B cells. Polyclonal Ab are more economic, faster to produce, and recognized multiple epitopes of the antigen. In contrast, monoclonal antibodies are produced *in vitro* by only one B cell clone. Upon immunization, the B cells are fused with immortalized myeloma cells forming a hybridoma. Further isolation of the clones is performed in order to obtain the desired specific antibody, in its pure state and at high concentration. Monoclonal Ab have high batch-to-batch reproducibility and high specificity against a specific epitope, resulting in low cross-reactivity. Recently, recombinant monoclonal antibody technology has been introduced to the market. Their main advantage is that B cells are not extracted from immunized animals, since are produced from genetically modified cells, from bacterial plasmids or mammalian cells through genetic engineering¹⁰⁸. In addition, the specificity can be modified and optimized, and the monoclonal Ab extracts are even purer.

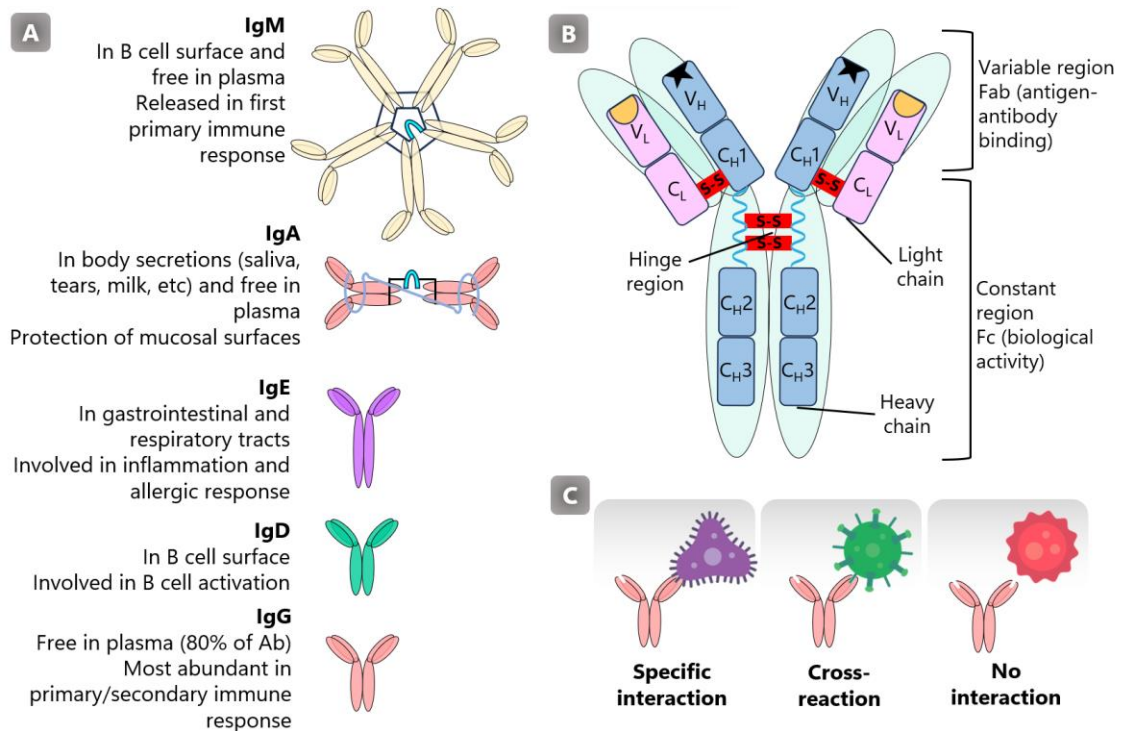


Figure 1.4. Schematic representation of the different classes of immunoglobulins and a summary of their properties. Panel A. Description of the IgM, IgA, IgE, IgD and IgG proteins. Panel B. Schematic representation of the composition of the IgG antibody, including the constant region Fc and the variable region Fab. It is composed by two heavy chains and two light chains that are connected through the hinge region that consisted of disulfide bonds. Each chain contains a constant (C_H : constant heavy chain and C_L : constant light chain) and a variable part (V_H : variable heavy chain and V_L : variable light chain). Panel C. Representation of the antigen-antibody interaction from left to right: specific interaction between the Ab-Ag, cross-reaction when the Ab interacts with a similar epitope of other Ag or other Ag share the same epitope with the target Ag, and no interaction, where there is not Ab-Ag recognition.

1.2.4.2. Immunoassays

Immunoassays offer numerous advantages over traditional techniques, such as lower turnaround times, simplicity, and cost-effectiveness of detection, with improved sensitivity and specificity. These techniques are based on the antigen-antibody reaction described above, to detect the analyte (an antigen or another antibody acting as Ag), involving the labeling of molecules, enzymes, isotopes, or particles, among others, that allow to produce a measurable signal in response to the binding, either qualitative or quantitative.

1.2.4.3. Immunofluorescence assay

Immunofluorescence assay is a microscopic method based on the use of fluorophore labeled antibodies to detect and visualize the antigen via Ag-Ab interactions. Immunofluorescence refers to both immunocytochemistry and immunohistochemistry, where isolated/cultured cells or tissue sections, respectively, are specifically stained by an antibody, in this instance labelled with a fluorophore. During the examination of the samples, the analyte appears colored due to the attached labeled Ab with the fluorophore depending on the wavelength of emission. The basic procedures consist of fixing the sample in a microscope slide, incubate with the fluorophore labeled Ab (via direct or indirect system, as described below) and examine the slide under a fluorescence microscope. Immunofluorescence assay allow the morphological identification, localization of the Ag and studies of tissue invasion¹⁰⁹, and sometimes it can give semiquantitative information. The main advantages are their ease of use and the results obtained within few hours. However, the required equipment, the need for constant power supply and trained personnel hindered the access in low-resource settings. Recently, several low-cost and more affordable smartphone-based microscopy devices have been developed, including smartphone-based epifluorescence microscope¹¹⁰.

1.2.4.4. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was first described in 1971 by Eva Engvall and Peter Perlman¹¹¹. This technique involves the use of a solid support, such as a 96-well polystyrene microtiter plate, to immobilize the reagents and identify the specific reactions based on the Ab-Ag interactions. Usually, enzyme-linked antibodies are used to produce a specific reaction in the solution, to detect and quantify the signal, that is normally directly proportional to the analyte. This detection systems are usually designed to produce a color change that can be measured quantitatively by a spectrophotometer or by naked eye, allowing the development of point-of-care tests too. ELISA may be performed using different formats, according to the analyte and the labelling system, as described in Figure 1.5.

The assay can be performed by immobilizing one of the reagents in the solid support in a sandwich approach, being the antigen (as shown in Figure 1.5, panel A and B), or, more typically, and antibody (as shown in Figure 1.5, panel C and D), followed by a direct or indirect labelling. Moreover, competitive approaches are mandatory (as shown in Figure 1.5, panel D), when the analyte is an hapten, a small organic molecule lower than 6 kDa and displaying only one epitope. Regarding the labelling, it can be classified in direct (as shown in Figure 1.5, panel A and C) or indirect format (as shown in Figure 1.5, panel B and D).

A sandwich approach begins with the coating of an antigen or, more typically, an antibody. The first case is typical when the serological status of an individual (IgG or IgM) is monitored, so the antigen is immobilized on the plate (similar to Figure 1.5, panel B). The second one is the typical format when the target is the antigen (for example, virus, bacteria, protein biomarker, among others), as shown in Figure 1.5, panel C and D. After the immobilization, a blocking step is performed to block the free sites on the solid support and avoid nonspecific adsorption by further reactants. Solutions of proteins as bovine serum albumin (BSA) or casein as well as non-protein polymers as PEG, are typically used for blocking steps. Upon incubation of the sample, the direct, or instead indirect, labelling is performed. In a direct labelling format, a primary antibody is added that can be directly labeled with the enzymatic reporter (examples of direct labelling are shown in Figure 1.5, panel A and C). Instead, the indirect approach involves a non-labelled primary Ab, combined with a labelled secondary Ab. Appropriate saturated substrate/cofactor solution is incubated to develop the enzymatic reaction at maximal velocity (V_{max}), and finally, the stopped solution is added to pause the enzymatic reaction and proceed with the readout. The most typical format to detect bacteria in a sample involves a sandwich approach, either with direct or indirect labelling. The sandwich system starts with the coating of the capture (monoclonal or polyclonal) antibody with specificity towards the bacteria, followed by a blocking step. Afterwards, the incubation with the bacteria is performed. Once is finished, the detector antibody (usually polyclonal) is added to interacts with the bacteria, that can be directly labelled with the reporter in direct sandwich system or not labelled in an indirect sandwich system, forming a sandwich.

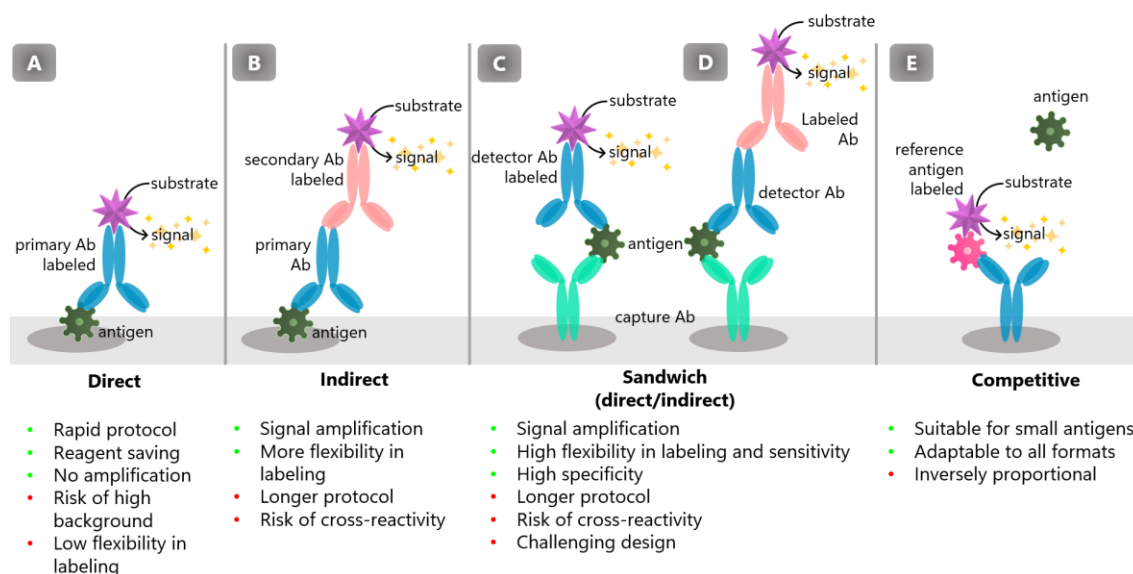


Figure 1.5. Schematic representation of different formats of ELISA. Panel A) Direct ELISA with the antigen immobilized/direct labelling, Panel B) Indirect ELISA with the antigen immobilized/indirect labeling, Panel C) Sandwich format with an antibody immobilized and a direct labeling, panel D) Sandwich format with an antibody immobilized and indirect labeling and panel E) Competitive ELISA using a reference antigen labeled with a reporter enzyme and the target antigen.

The most commonly used enzyme labels includes horseradish peroxidase (HRP) and alkaline phosphatase (AP), but other options as β -galactosidase, acetylcholinesterase, and catalase can be used as well¹¹². Several advantages of using enzyme reporters as HRP or AP includes the long shelf life, the good sensitivity, the versatility of the system and also the availability of ready conjugated HRP or AP-labelled antibodies¹¹². These enzymes are relatively stable when the storage conditions are appropriate. Moreover, the signal output is usually easily detected allowing the determination of very low concentration level of target analyte.

The HRP enzyme are commonly found in the roots of the horseradish¹¹³ and has several native isoforms, being the most abundant and studied the HRP isoform C¹¹⁴. The small size and the high turnover are some advantages in comparison to other enzyme conjugates. The molecular weight is approximately 40 kDa, that is smaller than other reporters, allowing the penetration into sample cells and decreasing the possible interference with the function of the conjugated protein. HRP enzyme is about 3.5 times lower than AP molecular weight (approximately 140 kDa). Then, steric hindrance is lesser too, enhancing the immunologic reactivity

because of their small size, leading to more effective signals. Furthermore, HRP presents a high turnover rate, producing a large amount of reactants in a short period of time at physiological pH¹¹³, in contrast to AP that is optimally active at alkaline pH (8-10)¹¹⁵. Regarding the cost, HRP is less expensive than AP. However, special attention should be considered regarding the nature of the sample. Endogenous peroxidase and AP activity can be found in certain tissues or cells producing nonspecific signals¹¹⁶. Some special inhibitor or blocking solutions are available to avoid the endogenous activity, in the case of HRP and AP, such as 0.3% H₂O₂ in methanol or levamisole¹¹⁷⁻¹¹⁹, respectively. Other considerations should be revised as the presence of certain antibiotics or chemical substances, such as sodium azide, which can perturb the activity of the enzyme. Anyway, a wide range of HRP and AP-conjugated antibodies are commercially available as well as the enzymatic substrates. Thus, the selection of the enzyme label depends on the required level of sensitivity and the equipment available for detection of the signal (e.g. spectrophotometer, fluorometer or luminometer). HRP-conjugated molecules, especially conjugated antibodies, offer most flexibility due to the variety of chromogenic (e.g. TMB, ABTS), fluorescent (e.g. Amplex red) and chemiluminescent (e.g. luminol) substrates¹²⁰. The catalytic activity of the HRP is based on the hemo group as a cofactor. Thus, the enzyme catalyzes the oxidation of a variable range of electron-donating dye substrates, using hydrogen peroxide as an oxidant and principal substrate of the HRP¹¹³. Chromogen substrates form a colored and soluble product that are measurable during the reaction, and in certain instances, a stop solution may be introduced to establish a consistent endpoint for the assay. The product absorbance can be measured with a spectrophotometer or an optical readout device to perform a quantitative detection. However, the color development is also seen by naked eye. For HRP enzyme, TMB (3,3',5,5'-tetramethylbenzidine), OPD (o-phenylenediamine dihydrochloride) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) are typical substrates that produces soluble compounds with maximum peaks at 450, 492 and 416 nm, respectively. In the case of AP, the most used substrate is pNPP (p-Nitrophenyl-phosphate), which the final optical measure is performed at 405 nm.

In the following chapters, TMB is selected as chromogenic substrate for signal detection in ELISA. In the presence of the HRP enzyme, TMB is oxidized by the

H_2O_2 yielding a radical cation that is in equilibrium with a charge transfer complex that appears blue in solution ($\lambda=370$ to 652 nm)¹²¹ and is formed by the diamine and the diimine form of the TMB molecule. When the loss of a second electron is done, it leads to the formation of the diimine yielding a soluble compound, the final oxidized TMB, providing a yellow solution ($\lambda=450$ nm)^{121,122}. The addition of an acid solution, decreases the turnover of the HRP and enhance the absorbance signal at 450 nm, driven the complete oxidation to the final diimine form¹²³ (yellow product). As higher the concentration of the HRP in the sample, higher the signal measured, so the substrates must be added in excess in order to obtain a directly proportional measure of the analyte.

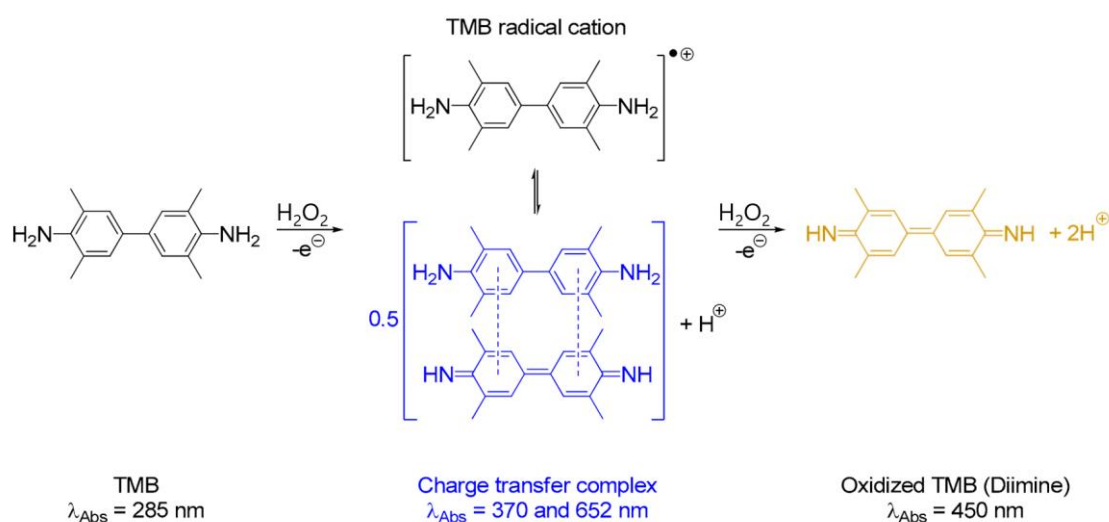


Figure 1.6. Schematic representation of the oxidation of the TMB by the HRP enzyme using H_2O_2 as a substrate. The first electron loss of the TMB produces a TMB radical cation that is in equilibrium with a charge transfer complex that produces a blue color in the solution ($\lambda=652$ nm). When the second electron is performed, the formation of the diimine takes place, producing a yellow color solution ($\lambda=450$ nm). Figure adapted from ¹²¹.

ELISA are widely used in many fields as medical diagnostics, biomedical research, food industry, agriculture, etc. Regarding the detection of pathogens, is a very exploited technique for both the direct detection of pathogens in several biological fluids including serum, urine, saliva among others or for the detection of antibodies or biomarkers produced in response to the exposure of the pathogen. Some advantages are the rapid and high-throughput technique since it allows the detection of multiple samples at a time. The hands-on-time can be reduced, as it can be automated using properly instruments for depositing the reagents in each well,

and for automatic washing. In addition, the specificity and sensitivity are usually very high, although it depends on a good selection of Ab for the target of interest. In fact, this makes the commercial offer of ELISA kits for the detection of pathogens on the market very wide, some examples are the kits for *Salmonella* (Salmonella Antigen ELISA KIT, CD Creative Diagnostics), *E. coli* O157 (TRANSIA® AG EHEC, Merck) and *Chlamydia pneumoniae* (Human Anti-Chlamydia pneumoniae IgM ELISA Kit, Abcam), among many others.

1.2.4.5. Western immunoblotting assay

Western immunoblotting assay, also known as western blot, is a widely used analytical technique to detect and identify specific antigens protein nature from complex sample, tissues or extracts. This assay can be performed more easily with dot blot or, as usual, with electrophoretic separation. Briefly, in a dot blot assay the sample is directly immobilized in a membrane, followed by immunolabeling and signal detection. In the second, the sample is previously separated by size using gel electrophoresis. Then, the sample is transferred to a membrane, for further blocking, incubation with properly labeled antibodies to perform a direct or indirect system, followed by proper readout and visualization. Depending on the label of the antibody and the nature of the readout, the detection can be colorimetric, chemiluminescent, radioactive or fluorescent. It is frequently used for confirmatory medical diagnosis of infectious diseases¹²⁴ such as HIV infection, bovine spongiform, hepatitis C and certain autoimmune disorders, among others. Immunoblotting can detect low levels of the antigen even in a heterogeneous matrix but is not a quantitative technique. Furthermore, it is subjected to the problems that may arise from the antibody-based methods¹²⁵.

1.2.4.6. Immunoprecipitation assay

When soluble antigens are aggregated by the corresponding antibody molecules, it forms immune complexes and when they become large enough to precipitate out of the solution, they become visible¹²⁶ by the formation of insoluble lattices. The immunoprecipitation assay consists in the precipitation of the antigen antibody complex for the semiquantitative detection of a protein antigen, by the formation of insoluble lattices (cross-linkages). The formation of large complexes

resulting in visible precipitates, is done when Ab and Ag are present in optimal proportions, so an excess of any reactant inhibits the formation of the cross-linking. While the precipitation refers to the creation of clearly visible insoluble lattices or cross-linkages, the agglutination refers to the formation of huge solid clumps as a result of antibody-antigen interaction (§1.2.4.1). The differences rely on the size of the antigen.

1.2.4.7. Immunoagglutination assay

The agglutination assay is based on the formation of aggregates or clumps that can be seen by naked eye due to the aggregation of the antigen-antibody complex, after the incubation of the sample with the Ab or an antigenic reactant that may be conjugated with a biological or artificial carrier as particles, commonly of latex, gelatin or other materials. Different reactions of agglutination include active/direct or passive and hemagglutination. In the active format antigens are found naturally on a particle, such direct bacterial agglutination. In passive format, an inert carrier needs to be conjugated, such as latex or carbon particles, so latex agglutination test kits are widely commercialized for the detection of several pathogens. On the other hand, hemagglutination tests involves the interaction between the receptors on the surface of the red blood cells and the proper antigens to agglutinate and form visible, red-colored aggregates¹²⁷. Hemagglutination is observed in presence of bacterial, viral and fungus species, so red blood cells from different hosts are selected depending on the selectivity and the targeted Ag.

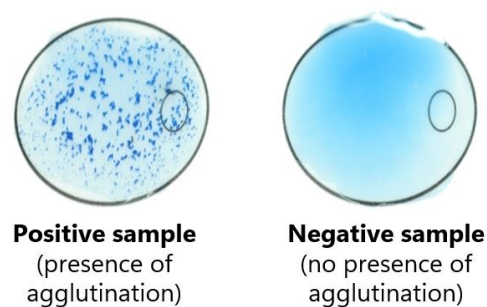


Figure 1.7. Results obtained after performing an agglutination assay.

In general, the immunoagglutination may be less sensitive than immunoprecipitation but is more rapid and easy to interpretate. Besides are easy to

perform as a screening diagnostic method, sometimes the results should be confirmed with another reference technique.

1.2.5. Molecular-based methods

The discovery of the double helix DNA structure by Rosalind Franklin, Watson and Crick in 1953¹²⁸, together with the development of molecular methods has a powerful impact globally, leading in the comprehension of the genetics and the manipulation of the genetic material. The development of molecular techniques has gone from the first DNA sequencing in 1960, to the development of the polymerase chain reaction (PCR) in 1980, to the genetic manipulation of organisms with cloning and genetic modification techniques, to the next-generation sequencing (NGS) at the beginning of the 21st century, allowing massive and high-speed sequencing of DNA fragments.

1.2.5.1. Nucleic-acid amplification test

Nucleic-acid amplification test (NAAT) have been included in the diagnostics of infectious diseases because of their improved sensitivity, specificity, turnaround times and accuracy in comparison with culturing and traditional methods. Furthermore, the identification of difficult-to culture or fastidious pathogens can be performed. NAAT methods are design to amplify the genetic material, a specific DNA or RNA sequence, in a sample, in order to identify and/or determine their presence. Different techniques based on NAAT exists, so the selection is based on the target genetic material or the type of pathogen to detect, among others. In addition to the sample providing the DNA template, a set of primers, nucleotides and a polymerase enzyme are essential to perform the NAAT, typically carried out using a thermocycler.

All the techniques described below fall inside the NAAT tests.

1.2.5.2. Polymerase chain reaction and derived PCR techniques

The conventional Polymerase chain reaction (PCR) allows the rapid replication of millions of copies of a specific segment/gene of the DNA from a sample, according to the primers used. The reaction is done usually in 0.2 mL PCR tubes inside a thermocycler to achieve rapid temperature changes over a given

period of time. The polymerase enzyme is needed to perform the process, according to three steps that are repeated cyclically. In a general protocol, the reaction starts with the denaturation of the double-stranded DNA (dsDNA) of the sample (template), separating each one of the two complementary strands at temperatures around 90 °C - 95°C in order to break the hydrogen bonds between bases. In the next step, the primers are annealed to each of the single-stranded DNA (ssDNA) templates, that contains the target region to amplify, at temperatures near to the melting point (T_m) of the primers. The primer design is critical to PCR success. To increase specificity and avoid primer dimer formation, a length of 18-30 bases is recommended and a similar T_m should be shared with each primer. Then, polymerase start the extension by adding the complementary nucleotides (dNTPs) from the template in the 5' to 3' direction, at the temperature at which the enzyme performs its optimum activity. When the extension is finished, the denaturation step starts again, beginning a new cycle. At this point, the DNA copies is doubled, according to the 2^n formula where n is the total number of cycles, often $n=30$, producing an exponential replication of the DNA. In the last cycle, the temperature is rapidly lowered to 4°C to stop the reaction and storage of the genetic material produced (amplicons).

Polymerase chain reaction (PCR)

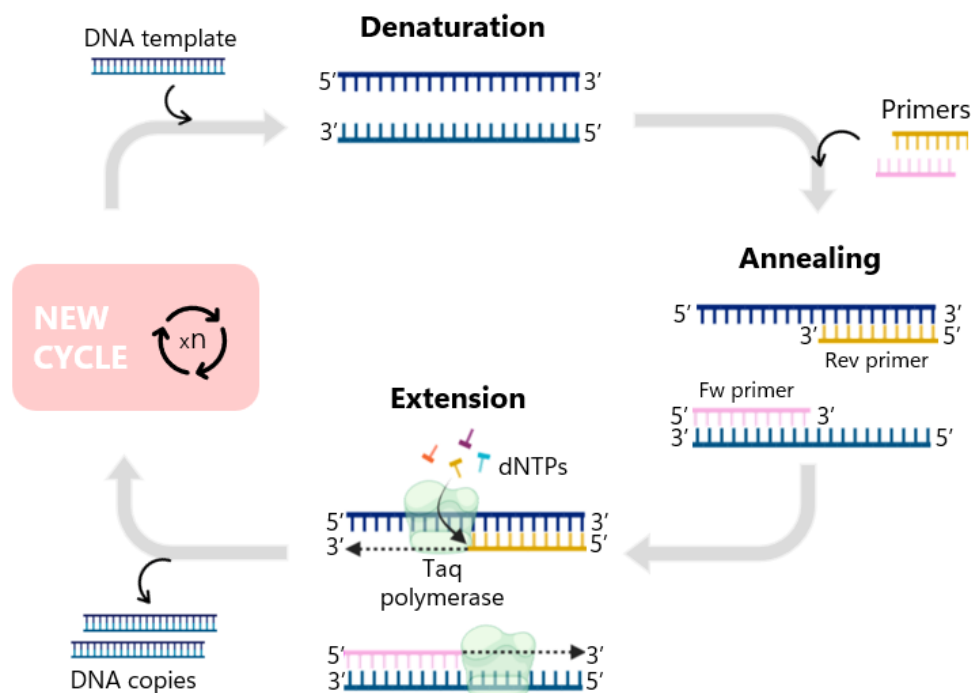


Figure 1.8. Schematic representation of the polymerase chain reaction and its cycles.

To ensure the success of the PCR, the amplicons are usually analyzed in the end-point PCR by gel electrophoresis. A DNA marker and a molecular standard weight ladder is used to identify the amplicons. In the gel the amplicons are separated according to their weight and charge, thus smaller amplicons travel faster than larger ones through the gel. Unlike conventional end-point PCR, the real-time or quantitative PCR (or qPCR) not only amplifies the DNA template, but also allows the real-time monitoring and quantification of the DNA amplification cycle by cycle, without the need for further monitoring of the product by gel electrophoresis. A fluorophore is added to the reaction mix, to quantify the generation of the products after each cycle. The technology can be also expanded to detect, instead of DNA template, RNA or even short sequence of miRNA. When the template is RNA from a virus or a transcript, a previous step of retrotranscription to obtain complementary DNA can be performed, and the technique is called Reverse transcription polymerase chain reaction, RT-PCR. In this case, the use of the enzyme reverse transcriptase is involved to allow the conversion of the RNA to the complementary DNA. The variant that is quantitative is the RT-qPCR. In addition, another popular used variant is the multiplex-PCR, in which the simultaneous amplification of multiple target sequences in a single reaction is performed. It was reported, for instance, for the detection of drug-resistance pathogens¹²⁹. This format allows the simultaneous amplification of different sequences from the DNA template in the same reaction, using different set of primers, allowing the identification of more than one gene. In the nested-PCR, for example, two-set of primers are used with the aim of increase the specificity of the amplification, involving two sequential amplification reactions. The product of the first produced by one set of primers is used as the template for the second PCR using the other set¹³⁰.

1.2.5.3. Isothermal amplification techniques

The need to work at different temperatures for PCR-based methods requires the use of a thermal cycler. This has prompted research efforts to develop isothermal amplification techniques, eliminating the need for thermal cycling and working at constant temperature, simplifying protocols, and making them more accessible in limited-resource settings or for rapid pathogen detection. Both PCR and Isothermal amplification methods require a template, primers, nucleotides and

the properly amplification enzymes. However, these techniques require a rational design of more than one set of primers and the use of more than one enzyme. In addition, the amplified genetic products produced are normally longer in comparison with the PCR, and can be detected with several strategies, including fluorescent labelling, electrophoresis gel, real time detection with molecular beacons, among others.

Two of the best-known techniques are the loop-mediated amplification (LAMP) and the recombinase-mediated amplification (RCA). More than one set of primers are used, in order to amplify a region of the DNA target. Although linear DNA products are produced in both cases, RCA starts with circular DNA as a template, involving the use of the recombinase as an enzyme besides the polymerase and making it a highly intriguing technique for the amplification of DNA molecules that naturally exist in circular form, such as plasmids¹³¹. While RCA produces a linear amplification of the DNA, one of the features of LAMP is its ability to perform an exponential amplification¹³². As a result, large amounts of pyrophosphate ions are produced as a byproduct¹³², increasing the turbidity of the reaction mixture due to the formation of magnesium pyrophosphate¹³². Thus, the amount of precipitate is correlated with the amount of synthesized DNA. Many studies have reported the use of turbidimetry to detect the presence of the target and monitor the reaction, even the use of a brief centrifugation of the mixture can be applied as the precipitate can be observed by naked eye¹³³. Other techniques include the transcription mediated amplification (TMA), which starts with the transcription of the RNA to complementary DNA and, therefore, the amplification of the DNA is produced. Another isothermal strategy, the ligase chain reaction (LCR), involves the use of the ligase enzyme, where the primers are hybridized with the template and then ligated, producing the amplification of a DNA fragment. Finally, one of the most complex processes is the nucleic acid sequence-based amplification (NASBA) that requires the use of three enzymes and two set of primers. Its mechanism is based on, first the elongation of the viral single-stranded RNA of the sample by the reverse transcriptase, creating a complementary DNA copy forming and RNA/DNA hybrid. Second, the RNase H enzyme recognizes the hybrid and hydrolyses the RNA portion leaving the ssDNA. A second primer anneals to this ssDNA and the reverse transcriptase produce the elongation forming a dsDNA with a functional promoter.

Finally, the RNA polymerase recognizes the promoter of the dsDNA and produces multiple copies of RNA transcripts which are anti-sense from the original RNA¹³⁴. It is commonly used in the detection of viral RNA such as HIV. Isothermal methods are a powerful and promising tool as they offer high sensitivities and specificities and the advantage of being adaptable to be performed in laboratory facilities by trained personnel or even in non-health care locations. However, the design of the primers is more complex than the PCR-based methods, and is one of the most important steps, so sometimes requires specialized databases and software for their selection.

1.2.5.4. Next generation sequencing

Sequencing is the process of determining the order of the nucleotides, in the DNA or RNA samples. Sanger sequencing was the firstly DNA sequencing technology developed in 1977 for small genomes and was improved later with the emergence of the shotgun method. This remains the gold-standard for the whole-genome sequence of a bacteria.

Briefly, the original DNA sample is fragmented in smaller DNA fragments and cloned or amplified to obtain a large number of copies of the fragmented DNA template. Then, a cycle sequencing reaction takes place including a denaturation, primer annealing and a primer extension step¹³⁵. Each round of primer extension is terminated by the incorporation of a fluorescent labelled-dideoxynucleotides (ddNTPs). The mixture results in end-labelled extension products of different length which each terminal is related to one ddNTP that is identified by a fluorescent label (one fluorescent label for each ddNTP, hence four fluorescent labels are needed). The different end-labelled products are separated by size, from the smallest to the longest one, and depending on the emission spectra, the ddNTP is identified, giving the correct order of the nucleotide and obtaining the complete sequence. One of the major limitations is that Sanger's sequence allows the identification of one fragment of DNA per reaction, up to a maximum of 700 bases approximately.¹³⁶ So it is a preferred technique for single genes.

However, next-generation sequencing (NGS) are new methodologies that offer several advantages belonging to classical Sanger sequencing, achieving high-throughput, reduced cost and more efficiency and accuracy¹³⁷. NGS allows the

sequencing of millions of fragments of DNA simultaneously and parallelly. In summary, the original sample is fragmented in smaller DNA templates, bound to adaptors and amplified by using PCR to obtain a large number of copies of the fragmented DNA template. Then, the resulted products are immobilized on a solid support, onto beads, microplates or an array for example, to start the sequencing process that depends on the platform used¹³⁶. Generally, the formation of clusters of amplified DNA fragments are performed by adding fluorescently-labelled nucleotides for further read by sequential cycles of ddNTPs incorporation, washing and detection¹³⁶. The analysis is performed using specialized bioinformatic software, for assembling the sequences and compared with data bases to obtain information about the presence of genes, mutations, genetic expression, among several purposes. This technique is preferred for cost-effective sequencing of high amount of genes simultaneously and the rapid turnaround time¹³⁸ for high sample volume. It is also recommended with low amounts of starting material.

NGS holds potential for improving clinical microbiology and public health, because in addition to identifying pathogens more rapidly and precisely than traditional methods, high-throughput technologies and bioinformatics can provide new insights into disease transmission, virulence, antimicrobial resistance, and disease surveillance¹³⁹.

1.3. Rapid test for the detection of pathogenic bacteria

1.3.1. Definition of Rapid Diagnostic Tests

Infectious diseases have a potential impact in low-resource settings and developing countries due to different factors as the lack of access to healthcare, malnutrition, bad hygiene and deficient healthcare systems. Thus, the management and control of communicable diseases present additional challenges in those areas.

Rapid diagnostic tests (RDT) are essential tools in these settings because they allow the rapid identification of the disease, then enabling a rapid decision-making, implementation of control measures, and can be adapted for point-of-care (POC) tests. The desirable properties of the RDTs are defined by WHO under the acronym ASSURED^{63,140}, but recently some properties are being considered for inclusion in the criterion as REASSURED³⁸, and are described here:

- **Affordable.** The final test should be accessible and cheaper than standard methods for low resource settings and limited healthcare system.
- **Sensitive.** The test should be capable of detect the presence of the disease even in the first stage of the infection, avoiding the false negative results and reaching the LOD required.
- **Specific.** The test should be specific enough to detect the target and avoid the false positive results due to other interferences.
- **User-Friendly.** The test should be easy to perform and understand without misleading results, without the minimal training and no advanced technical skills required, facilitating the use to primary care personnel.
- **Rapid and Robust.** The test should provide fast results allowing reliable response to enable on-site detection and facilitate decision making. In POC test, this is a critical point to avoid the loss of patient follow-up, so test should give results at least at the same day in around 20 minutes. Moreover, the test should be resistant to manipulation, transportation and environmental conditions, able to withstand the adverse conditions that can be encountered in low resource settings.
- **Equipment free:** the test should not require special or sophisticated equipment or at least can be operated in small portable devices without the need of a constant source of electricity, using battery or solar powered.
- **Deliverable to end-users:** the test should have the assurance of being delivered and distributed to the end-users in the limited-resource settings, communities, or primary healthcare centers.

With the advancing in technology and connectivity³⁸, two more features are considered in the criterion including:

- **Real time connectivity.** The test should have the possibility to be connected to a smart device (smartphones) or a light source reader, to read the results of the test and manage the clinical information of the patient.
- **Ease of specimen collection and Environmental friendliness.** The test should not require invasive specimen collection and laborious preparation, so the samples should be directly tested using non-invasive samples as blood, urine, sputum, oral tests etc, using easy collection tools as swabs or capillary tubes

from extraction. On the other hand, the environmental impact must be considered, emphasizing the use of recycled materials and avoiding the use of plastic materials when possible. Paper-based tests, as LFA or vertical flow assays, has some advantages related to this point. Also, the disposal of reagents and other electronic tracks and electrodes.

The goal, the analyte and the target community determine the degree of compliance of each of these properties. The speed and simplicity of an RDT often sacrifices sensitivity. But as sensitivity increases, the time required is longer and the complexity of the assay increases. For example, an LFA test can give results in 15-20 minutes, or even less. However, ELISA and PCR assays are more time-consuming, laborious and involve many steps involving pipetting and washings, which makes increased the sensitivity¹⁴¹. But the need of complex equipment and maintenance also increases. Emerging technologies as biosensors, microfluidic platforms and lateral flow assays (LFA) are considered for the design of several RDT. Improvements of LFA techniques are focused to increase sensitivity and reproducibility, and the simultaneous detection of several analytes¹⁴². Microfluidic technology, while promising and some examples are commercially available, is not widely implemented in healthcare systems. Other challenges has to be overcome when design RDT based on biosensors including sample preparation, matrix effects and system integration¹⁴³, so few notable examples exist in the market such as the glucose sensor. All these technologies are described in more detail in the following section.

1.3.2. Biosensors

Since the development of the first pHmeter in 1922 by W.S. Hughes, the first ‘true biosensor’ was developed by Leland C. Clark, the father of biosensors, in 1956 for oxygen detection and it was in 1962 when he developed an amperometric enzyme electrode for the detection of glucose. The first commercial biosensor for glucose monitoring was launched in 1975 by Yellow Spring Instruments (YSI). But it was in 1992 with the launch of the Handheld blood biosensor by i-STAT, that a substantial development and progress in biosensors began¹⁴⁴. Nowadays biosensors market is growing each year, and it is expected to increase. While

electrochemical sensors are predicted to maintain 70% of the market, optical biosensors are showing the fastest growing, commercialized for diagnostics, environmental monitoring, biomanufacturing, and food safety¹⁴⁵.

A biosensor is defined by the IUPAC as *A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals*¹⁴⁶, in another words it is an analytical device that convert a biological or chemical reaction response into a measurable signal by the transducer, and it is passed by an electronic circuit to obtain a digital information that is easily interpreted by the end user. A scheme of a biosensor is depicted in Figure 1.9. Biosensors can be classified according to the type of bioreceptor or the kind of transducer, including antibodies, enzymes, aptamers and nucleic acid and biomimetic materials, and on the other hand, electrochemical, optical, thermal-based and mass-based transducer, respectively.

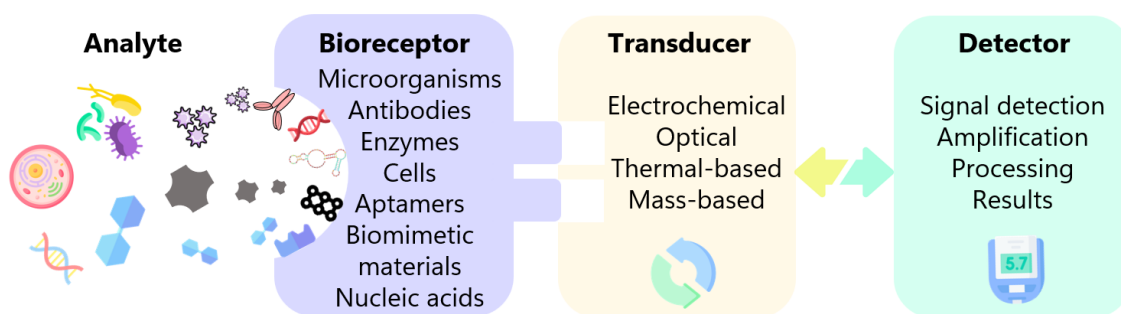


Figure 1.9. Schematic representation of the components of a biosensor including the bioreceptor, the transducer and the detector.

In the next section, electrochemical biosensors are described in more detail.

1.3.2.1. Electrochemical biosensors and classification

Electrochemical biosensor has been extensively applied to pathogen detection¹⁴⁷. They are integrated receptor-transducer devices based on the transduction of the biochemical recognition or reaction into an electric signal, thus using an electrochemical transducer. Depending on the kind of transducer (potentiometric, amperometric, conductometric / impedimetric and ion charge / field effect), the measurement mode change and the classification of electrochemical biosensor can be performed according to the measurement applied.

- Potentiometry

The electric potential is measured resulting from the electrochemical oxidation or reduction of an electroactive specie in the interphase of the electrode-solution in static conditions (no current). The electrode that is modified with a bioreceptor interacts with the target analyte producing a change in the potential, that is proportional to the concentration of the analyte. In this technique, a reference electrode with a well-known and constant potential is needed together with a working electrode where the biorecognition take place. pHmeters are the most popular sensors based on this method.

- Surface charge using field-effect transistors

In Surface charge using field-effect transistors (FET) systems, the bioreceptor are immobilized on the semiconductor path or sensing channels to interact with the target analyte, which are connected to source and drain electrodes¹⁴⁸. Once the biorecognition interaction takes place, the charge distribution changes as well as the surface potential of the semiconductor. This produce changes in the flow of current (conductance) between the source and the drain electrodes that can be measured. Thus, the gate contact modulates the conductance, in response to the potential changes on the gate surface due to the biorecognition. The magnitude of the modulation is directly related to the concentration of the analyte.

- Conductometry

The electrical conductivity is measured between the reference and the working electrode due to the biorecognition reaction. So, the conductivity of the sample change by the production or consumption of charged species during the biological processes¹⁴⁹. Therefore, conductometric biosensors are usually used to monitor metabolic processes in living biological systems¹⁵⁰. In contrast, impedimetric biosensor measure the electrical impedance that is produced when the biorecognition of the analyte and the biorecognition element is done, that is proportional to analyte activity. This biorecognition between the two species

produce a blocking layer in the surface of the electrode increasing the electron transfer resistance¹⁵¹.

- Amperometry

The electric current is measured at constant potential resulting from the electrochemical oxidation or reduction of an electroactive specie¹⁵² in the surface of the working electrode. The concentration of the analyte is directly related with the change of the current. When the measurement is performed during a potential scan is called voltammetry. There are several modalities, including cyclic voltammetry (CV) and square wave voltammetry (SWV). In CV, the potential applied to the electrode is varied linearly in one and the opposite direction, during different cycle that are repeated several times in order to observe reversible redox processes. In SWV a square potential is applied superimposed on a linear background potential¹⁵³. In contrast to CV, SWV is generally more sensitive, with better peaks resolution, which facilitates quantification and identification of the electroactive species. Usually conducting metal such as gold, platinum, silver or carbonaceous material, are used as a transducer. In amperometry, the combination of three electrodes is required, including the working, the reference (normally silver or silver chloride) with a fixed potential and the auxiliary electrode. The simplicity of the transducer allows the fabrication of low-cost and portable devices, which are essential for medical diagnostics and environmental monitoring applications¹⁵¹. In fact, the most popular and commercially available biosensors, the glucometer, is based on amperometry. On the other hand, the significance of enzymes in sensor fabrication is their specificity toward particular molecules, which is the key of selectivity of the biosensor¹⁵⁴. The most used enzymes as a bioreceptor among the great variety are the oxidoreductases and hydrolases. Although the enzymes are influenced by different factors such as the pH, temperature, amount and enzyme loading, the chemical cofactor involved is critical. Therefore, one reason that oxidoreductases are widely spread out than other categories in biosensing is their coenzymes¹⁵⁴, that cause catalytic ability and make detectable signals.

There are two strategies in the electron transfer mediated by enzymes: the direct, when the electric transfer is done by the cofactor or the reaction takes place without a mediator, and the indirect system, where a mediator is involved. Although

the use of a mediator increases the kinetic of the transfer because it is used to transfer the electrons from the working electrode to the enzyme, the direct system is easier and more stable. Some examples inside the oxidoreductase enzymes include oxidase, peroxidase and oxygenases/hydroxylases, such as the glucose oxidase, cytochrome P450 or horseradish peroxidase (HRP). The last one, it is widely used in biochemical assays such as ELISA and biosensing application, due to their facility to produce colorimetric, fluorometric o luminescent signals when the appropriate substrate is selected and the possibility to conjugate it with other molecules, such as the antibodies. Moreover, it is stable, low cost, with a high turnover and smaller than other alternatives, such as the alkaline phosphatase (AP). The electrochemical biosensors described in the next chapters are based on the use of conjugated HRP-antibodies.

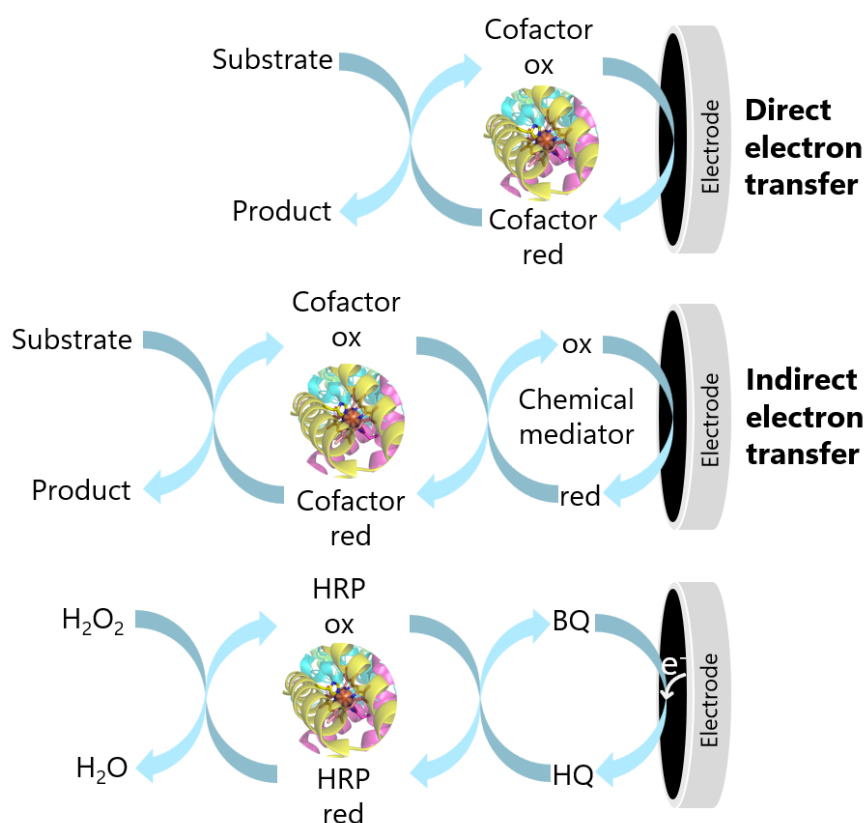


Figure 1.10. Schematic representation of the direct and indirect electron transfer catalyzed by an enzyme. Figure adapted from reference¹⁵⁴. Indirect electron transfer using the HRP enzyme and HQ as a chemical mediator. The HRP catalyzes the oxidation of the HQ to BQ using H_2O_2 as a substrate, that is reduced to H_2O . The current measurement is done on the surface of the electrode where the reduction of the BQ to HQ is performed. The measurement is directly proportional to the concentration of the analyte in the sample.

The HRP contains a heme-cofactor that catalyzes the transfer of two electrons (reduction) to H_2O_2 in the presence of a hydrogen donor, to generate H_2O and an oxidized form. The electrochemical signal is produced by the reduction of this oxidized form. Hydroquinone (HQ) is one of the most used mediators for the HRP enzyme in electrochemical biosensors, thus the application of the HRP with the H_2O_2 and the HQ as a mediator is described in Figure 1.10.

Our group have been working on the development of several biosensing strategies based on amperometric sensors for the detection of bacteria, including the electrochemical detection of specific amplified genes of *Salmonella*, *Listeria* and *E. coli* using silica particles¹⁵⁵, the electrochemical detection of double-tagged amplicons encoding for *Salmonella* and *E. coli* in milk¹⁵⁶ and also of *M. bovis*¹⁵⁷. Also, the direct electrochemical detection of *Salmonella* cells in milk are another example biosensor for the detection of pathogens¹⁵⁸. More information about electrochemical biosensors with different detection strategies of *Legionella* are summarized in Table 1.3, including the biosensor developed in this dissertation that is explained in Chapter 3.

Table 1.3. An overview of electrochemical biosensing approaches for the detection of *Legionella* and other gram-negative bacteria.

Method (genosensing/immunosensing) Bacteria	Electrochemical measurement	Assay time	Preconcentration/ Preenrichment/ Amplification	Specificity	LOD	Ref
Electrochemical genosensor for the detection of <i>Legionella spp.</i> (16S rRNA sequence) that integrates a loop mediated isothermal amplification (LAMP)	Cyclic voltammetry	30 min	YES -Filtration of 250 mL water samples and elution of the DNA - loop mediated isothermal amplification	16 strains of <i>Legionella</i> tested 15 strains of non- <i>Legionella</i> species tested	10 fg of <i>Legionella</i> nucleic acids (NA), corresponding to 2 copies of the bacteria In water samples / 30 CFU 250 mL ⁻¹	159
Electrochemical genosensor for detection of the hybridization and target sequence (<i>mip</i> gene of <i>L. pneumophila</i>), using gold nanoparticles (Au NPs) supported by cysteamine (Cys A) electrodeposited on the surface of gold electrodes	Square wave voltammetry	27 min (electrode preparation NOT INCLUDED)	NO No tested with <i>Legionella</i>	Tested with mismatched DNA	LOQ 1 z-molar of synthetic DNA coding for <i>mip</i> gene	160
Signal-off double probe electrochemical genosensor for the detection of <i>Legionella spp</i> and <i>L. pneumophila</i> by monitoring the DNA hybridization and enzymatic cleavage with the enzyme <i>EcoRI</i> in gold electrodes	Differential pulse voltammetry	50 minutes (PCR NOT INCLUDED)	YES Asymmetric PCR	Tested with mismatched DNA Tested with DNA from <i>E. coli</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>S. penumoniae</i> and <i>S. aureus</i>	1,2 x 10 ⁻⁹ M of synthetic DNA	161
Electrochemical genosensor based on nanoporous alumina membrane for the detection of <i>Legionella spp.</i> (21-mer DNA sequence)	Differential pulse voltammetry	Aprox 45 min (PCR NOT INCLUDED)	YES Asymmetric PCR	Tested with mismatched DNA	3.1 x 10 ⁻¹³ M of synthetic DNA coding for 21-mer gene	162

Electrochemical genosensor based on amplified molecular beacon for the detection of <i>Legionella</i> spp. (21-mer DNA sequence) using thiolated hairpin DNA-ferrocene probes on gold electrodes	Differential pulse voltammetry	45 min aprox (PCR NOT INCLUDED)	YES PCR	Tested with mismatched DNA	2.3×10^{-14} M of synthetic DNA coding for 21-mer gene	¹⁶³
Microfluidic electrochemical genosensor for the detection of target sequence (<i>L. pneumophila</i>) based on two-step sandwich with strep-coated paramagnetic microparticles, a biotinylated capture probe and strep-AP (streptavidin-alkaline phosphatase conjugate)	Amperometry	16 min (PCR NOT INCLUDED)	NO	Tested with mismatched DNA	0.33 nM of synthetic DNA	¹⁶⁴
Electrochemical genosensor for the detection of the target sequence (<i>mip</i> gene sequence) of <i>L. pneumophila</i> based on a multiwall carbon nanotube (MWCNT) electrode	Differential pulse voltammetry	40 min (PCR NOT INCLUDED)	NO	Tested with mismatched DNA	10 pM of synthetic DNA coding for <i>mip</i> gene	¹⁶⁵
Electrochemical immunosensor for the detection of <i>L. pneumophila</i> developing an immunoassay in nitrocellulose filters	Amperometry	2–3 h	YES Filtration of 200 mL	(The antibody used in this paper was previously tested in another work)	4 CFU mL ⁻¹	¹⁶⁶
Electrochemical immunosensor based on a microfluidic assay in water samples for the detection of <i>L. pneumophila</i>	Square wave voltammetry	100-180 min (depending on the detection mode, dynamic or static)	NO	Not tested with other bacteria	10 CFU mL ⁻¹	¹⁶⁷

Electrochemical magneto immunosensor for the detection of <i>L. pneumophila</i> based on disposable core-shell Fe₃O₄@poly(dopamine) magnetic nanoparticles and a sandwich enzyme-linked immunosorbent assay	Amperometry	3 h	YES Filtration of 1 L of sample/ vortexing / centrifugation / Immunomagnetic separation	Tested with <i>E. coli</i> , <i>E. faecalis</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i> , <i>Aeromonas</i> and <i>Salmonella</i>	10 CFU mL ⁻¹	168
Electrochemical immunosensor for the detection of <i>L. pneumophila</i> based on indium-tin oxide (ITO) electrodes	Electrochemical impedance spectroscopy	Not detailed	NO	(The antibody used in this paper was previously tested in another work)	50 CFU mL ⁻¹	169
Electrochemical immunosensor for the detection of PAL (peptidoglycan-associated lipoprotein, immune-dominant component of <i>Legionella</i> antigens) based on a ZnO nanorod (NR) matrix in gold electrodes and a sandwich enzyme-linked immunosorbent assay	Cyclic voltammetry	4 h	NO (The detection is done directly with PAL (peptidoglycan-associated lipoprotein) as an antigen of <i>L. pneumophila</i>)	PAL antigen (peptidoglycan-associated lipoprotein) as an antigen of <i>L. pneumophila</i>	~1 pg mL ⁻¹ of PAL antigen	170
Electrochemical immunosensor for the detection of <i>L. pneumophila</i> based on a gold immunochip using fluorophore-conjugated antibodies	Impedance	1 h	NO	Not tested with other bacteria	2.0 × 10 ² bacteria mL ⁻¹	171
Electrochemical genosensor for the detection of pathogenic bacteria including <i>S. enterica</i>, <i>L. monocytogenes</i> and <i>E. coli</i> using silica particles and a triple-tagging PCR	Amperometry	3 h (PCR INCLUDED)	YES triple-tagging PCR	Not tested with other bacteria	0.04, 0.13 and 0.05 ng mL ⁻¹ for <i>S. enterica</i> , <i>L. monocytogenes</i> and <i>E. coli</i> target DNA respectively	172
Electrochemical genosensor for the detection of <i>E. coli</i> and <i>S. enterica</i> using strepAv-magnetic particles and quadruple-tagging PCR	Amperometry	2 h (PCR INCLUDED)	YES quadruple-tagging PCR	Not tested with other bacteria	0.59 and 0.75 pg μL ⁻¹ for <i>E. coli</i> and <i>S. enterica</i> respectively	173

Electrochemical genosensor for the detection of <i>Salmonella</i> combining phagomagnetic separation, double-tagging PCR and immunomagnetic separation	Amperometry	4 h (PCR INCLUDED)	YES Immunomagnetic separation / double-tagging PCR	Tested with <i>E. coli</i> K12	3 CFU mL ⁻¹	174
Electrochemical genosensor for the detection of <i>M. bovis</i> in milk using double-tagging PCR and streptavidin-magnetic beads	Amperometry	1 h 35 min (PCR NOT INCLUDED)	YES double-tagging PCR	Not tested with other bacteria	10 fmol of target DNA	157
Electrochemical immunosensor for the detection of <i>Salmonella</i> in milk samples using magnetic particles	Amperometry	50 min	YES Immunomagnetic separation / Optional: pre-enrichment of 8 h	Tested with <i>E. coli</i>	1,4 CFU mL ⁻¹ 0,108 CFU mL ⁻¹ (with pre-enrichment)	158
Electrochemical magneto-immunosensor for the detection of <i>L. pneumophila</i> retained in filters	Amperometry	<2.5 h	YES Filtration of 100 and 1000 mL / Immunomagnetic separation of the bacteria retained in filters	Tested with <i>P. aeruginosa</i> , <i>K.pneumoniae</i> , <i>M. fortuitum</i> , <i>Enterobacter spp.</i> , <i>E. coli</i> , <i>S. cholerasuis</i>	2 CFU mL ⁻¹ (100 mL) 0.1 CFU mL ⁻¹ (1000 mL)	This work (Chapter 3)

1.3.3. Microfluidic devices

Microfluidic devices represent the technology of systems that process or manipulate very small amounts of fluids, using channels with very reduced dimensions. So, this miniaturized microscale devices are useful tools for carrying out operations like reactions, separations and detection of various compounds¹⁷⁵, that are based on the use of electrokinetic, capillary, vacuum and other forces to mix and separate liquid samples¹⁷⁶.

Several platforms are based on microfluidics, for instance microreactors, lab-on-a-chip or organ-on-a-chip, but in most of the instances, they are still limited to the research lab, due to the cost of production. The materials and the fabrication techniques are variable depending on the final use, conventional methods include the use of silicon, glass or polymers like polydimethylsiloxane. However, plastic and paper-based fluidics have become extremely popular in recent years due to their cost-effective methods and ease of fabrication in low-resource settings¹⁷⁷. Interestingly, some detection methods have the potential to be integrated in microfluidic platforms like PCR, LAMP and ELISA techniques. Hence, it is a promising approach regarding the miniaturization and the rapid determination of the analyte, thus the integration into portable chips can facilitate the detection of pathogens and diagnosis of diseases in a POC. For example, some studies are based on the design of microfluidic devices for the detection of pathogen antimicrobial susceptibility, that remains important in the management of an infection, with good performance and accuracy in a relative short time. However, additional work is needed to confirm the accuracy with respect to current clinical antimicrobial susceptibility tests¹⁴³. Other examples, include the application of microfluidics for the detection of foodborne pathogens, such as *S. Typhimurium*¹⁷⁸, *Salmonella*¹⁷⁹ or *Campylobacter*¹⁸⁰ based on colorimetric, fluorescence and electrochemical detection, respectively.

However, the low levels of pathogenic bacteria in some matrices and some requirements to process and incubate the sample, difficult the automatic flow in the device. The efficient capture in complex samples for multiplex analysis is another drawback. The integration of modified magnetic particles (MPs) for immunoseparation (IMS) of the bacteria in isolation process can block the

microchannels¹⁸¹ and the surface-to-volume ratios in the microchannels can increase the non-specific adsorption of the reagents¹⁸².

Paper-based microfluidic devices (microPADs) are analytical tools that use paper or porous membranes, including cellulose or nitrocellulose, as a principal substrate for the manipulation and detection of fluids, by capillary action. They are fabricated by patterning paper with hydrophobic barriers to define hydrophilic channels and zones¹⁸³. MicroPADs have several advantages over conventional microfluidic devices such as simpler fabrication, low-cost, easier disposal, and the ability to operate without pumps¹⁸³. These properties make microPADs very attractive for the development of POC diagnostic tests and on-site testing. Other paper-based biosensing technologies most widely used in the development of POC are dipstick and lateral flow assays, that are described in more detail in the next section.

1.3.4. Lateral Flow Assays

Lateral Flow Assays (LFA) are based on the immunochromatographic separation of the analytes and reagents by the migration on a solid support by capillary flow forces, and together with the dipsticks, have dominated rapid diagnostic over the last three decades¹⁸⁴.

The pregnancy test was the most popular LFA per years. Interestingly, the earliest records of a urine-based pregnancy test date back to an ancient Egyptian papyrus in 1350 BC. It explained that the test determined whether it was a boy or a girl depending on whether sprouts grew on one type of seed or another and if there was no growth, the woman was not pregnant¹⁸⁵. In 1963, the theory was tested showing that the 70% of the time, pregnant women promoted the growth and non-pregnant women did not. Much later, scientist recognized a specific hormone in pregnant women: the human chorionic gonadotropin (hCG), thus very useful as biomarker. Various tests were developed for the detection of hCG, such as the A-Z test¹⁸⁶, the hemagglutination inhibition test¹⁸⁷, a radioimmunoassay¹⁸⁸, the Wampole's two-hour pregnancy test (Biocept-G, Wampole Labs., New Jersey) but it was not until the 1977 that the first home commercial test kit for detection of the hCG in urine was launched in USA and approved by the FDA, also known as 'Error Proof Test'. From the 1990s and the beginning of the 21st century, these assays

became popular for the diagnosis of other diseases such as AIDS and was extended to other areas of detection, such as infectious and new emerging diseases or even urine drug screening. Great advances have been made in the development of LFA, including improvements in sensitivity and specificity, adapting it to new biomarkers and analytes of interest. Nowadays, they are fundamental tools in medical diagnostics due to their low cost, ease of use, rapid detection times and in-situ detection.

The introduction of these tests in affected or low-resource communities with high burden of infections, has been shown to improve the management and diagnosis of infections, such as malaria, HIV or COVID-19¹⁸⁹, especially in areas with limited access to specialized instruments, like good quality microscopy services. Recently, the impact of LFA has been evidenced with the COVID-19 pandemic, providing a useful, easy, fast and inexpensive tool, allowing the collection of results at home, in clinics or in pharmacies, encouraging individuals to take greater care and prevention of transmission and facilitating triage.

The LFA are based on the immunochromatography basis for the detection of the presence or the absence of the target analyte in a liquid sample. A typical lateral flow strip consists of overlapping membranes that are mounted on a backing card. The basic structure includes: the sample pad, the conjugation pad, the membrane that includes the test and control lines, and finally the adsorbent pad, all these are immobilized on the backing card. The end in which the sample is applied, is called sample zone or sample pad. This part is impregnated with buffer salts and surfactants in order to facilitate the interaction with the detection molecules, improve the sensitivity and make the sample compatible with the assay. The sample migrates by capillary forces to the conjugation pad, which contains the conjugated biomolecules, or conjugated labels, specific to the target analyte, which will capture the analyte and provide the final signal. The molecules, usually antibodies, may be conjugated with different labels, the most widely used are gold nanoparticles and dye-loaded latex beads¹⁹⁰. Moreover, carbon nanoparticles and nanotubes are interesting materials because of their strong contrast due to the black color¹⁹¹. Quantum-dots are another example in fluorescence-based LFA¹⁹². While the capture of the analyte is performed, the sample migrates to the detection membrane, which they are the test and control lines. Specific molecules are immobilized in each line in

order to recognize the target analyte (test line) and ensure that the test is well-performed indicating a proper reagents flow and binding of unreacted conjugated label (control line). The recognition between the immobilized molecules to the target analyte and the conjugated label complex generate a visible signal. Multiplex detection can be done if more than one test line, apart from the control line, is present in the strip¹⁹³. Finally, the sample flows until the adsorbent pad, to retain the excess of reagents and prevent backflow of the liquid¹⁴². The readout can be assessed simply by naked eye (qualitative and semiquantitative results) or using a reader (quantitative results can be obtained). Commercial strips are usually integrated in a cassette where the sample holder (sample pad) and the detection zone can be easily differentiated.

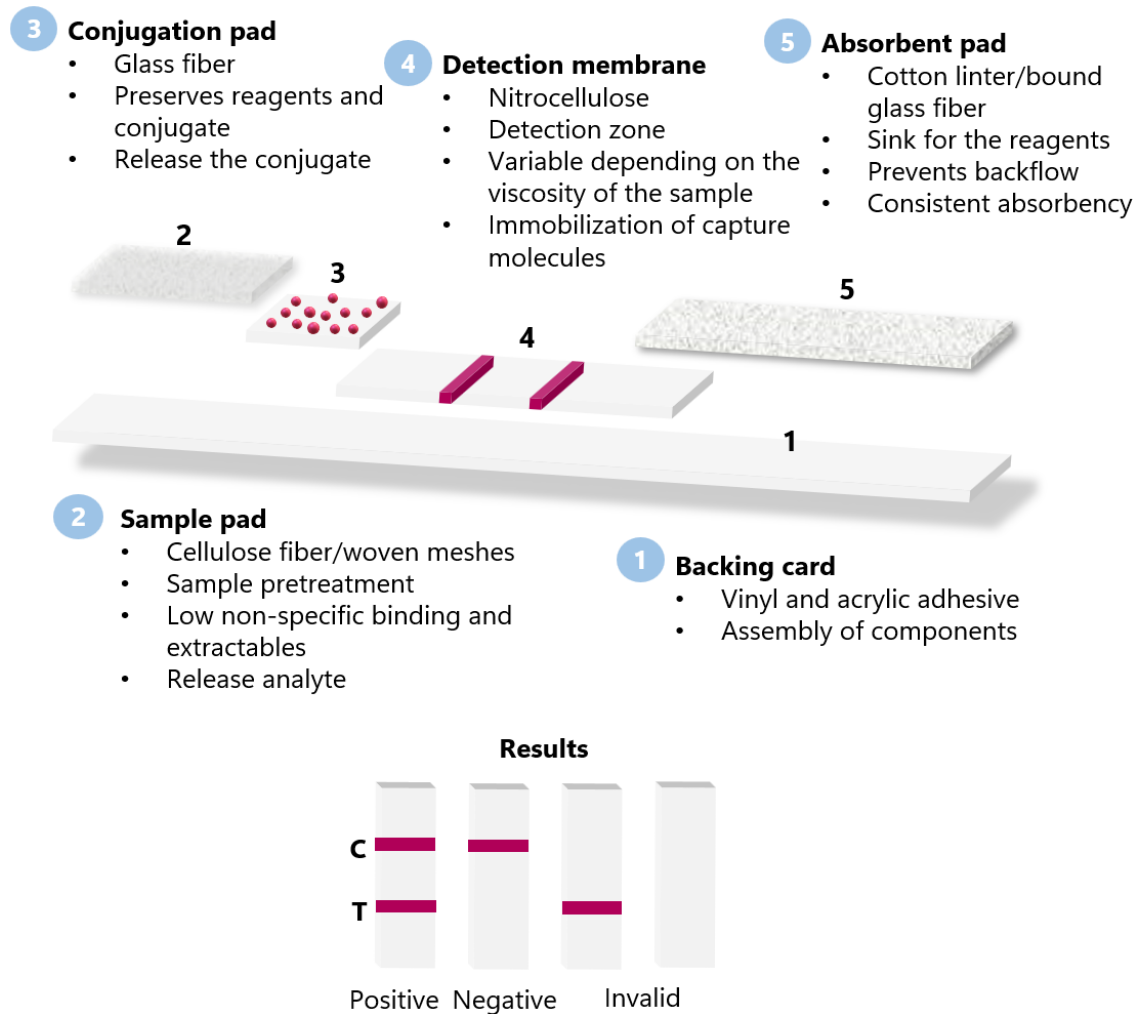


Figure 1.11. Schematic representation of the lateral flow strip and their components.

Currently, there is an important commercial offer available regarding rapid diagnostic LFA for bacteria detection and pathogenic infections, such as the Singlepath® and Duopath® (Merck, Germany) for *Bacillus cereus*, *Campylobacter*, EHEC, *L. pneumophila*, *L. monocytogenes* and *Salmonella*. For instance, LIODetect® TB-ST Tuberculosis Rapid test (Lionex Diagnostics & Therapeutics GmbH, Germany) is a rapid test for the qualitative detection of the antibodies against *M. tuberculosis* in plasma, serum or whole blood intended for the rapid diagnosis of active TB. Another example is the Cer Test Strep A card test (CerTest Biotec S.L., Spain) that provides qualitative detection of *Streptococcus* Group A from throat swabs after colony recovered by solid culture.



Figure 1.12. Commercial lateral flow strip Singlepath® and Duopath® for the detection of different bacteria from Merck (Germany).

Our group has been working in the development of several LFA platforms for the detection of pathogenic bacteria such as *M. fortuitum* in a typical strip but also by a vertical flow format in water samples⁹⁷. Also, the design and comparison of a nucleic acid-LFA for the detection of double-tagged amplicons of *E. coli* comparing the analytical performance of avidin carbon nanoparticles and streptavidin gold nanoparticles as a reporter was reviewed¹⁹¹ but also a multiplex detection of *Salmonella* and *E. coli* O157 in a lateral flow strip was achieved by quadruple-tagging PCR¹⁷³.

Interestingly, in LFA the source of the sample may be different, from a microbial culture, environmental sample (water), a biological sample (saliva, urine, serum, etc) to amplified genetic material or extracted DNA, therefore the nature of the target analyte would vary accordingly. Many of the advantages of LFA have already been discussed. However, the rapid incubation times and the limited volume of sample for the test may result in a reduced sensitivity in comparison to other

rapid tests¹⁴². So, the detection of novel biomarkers in low concentrations in a sample may be a limitation in these assays¹⁹⁴. Sometimes, sample pre-enrichment or amplification is necessary to obtain confirmatory results. Another important factor is the selection of appropriate and compatible membranes to ensure a good flow of reagents through the strip¹⁹⁵.

1.4. Emerging materials and technologies for enhancing rapid test

1.4.1. Magnetic particles

The development of rapid tests is needed in order to improve the early detection of infections, overall, in primary care level and low resource-settings where the burden of infectious disease is higher. The development of these new methods usually needs solid-phase separation techniques in order to improve sensitivity, separation of the target analyte from a complex sample, reduce sample pre-treatment and improve detection times. The integration of magnetic particles (MPs) in biosensing is very promising due to their properties for preconcentrating the target in complex matrices. Coprecipitation, thermal decomposition or microemulsion among others are some methods for the chemical synthesis of MPs¹⁹⁶. The composition of the MPs is generally constituted by the superparamagnetic core, the polymeric shell and the coating that contains different functionalities.

The most widely used MPs are typically composed of magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) but can be tuned with other metals to modify the magnetic properties¹⁹⁷. These properties are essential in preconcentration procedures, due to their ability to capture the target analyte applying an external magnetic field, without applying harmful reactants¹⁹⁸ and keeping the activity of the biological samples, in contrast to other traditional methods such as filtration or centrifugation that can damage the sample¹⁹⁹. The second layer protects the core against oxidation or degradation and provides a useful platform to modify the surface with different ligands²⁰⁰. A polymeric shell of polystyrene, alumina, silica or gold are examples of materials for the shell. The external coating offers chemical and

biochemical functionalities, allowing the modulation of the reactivity and the immobilization of biomolecules on the surface of the MPs, adapting them to different applications and target analyte.

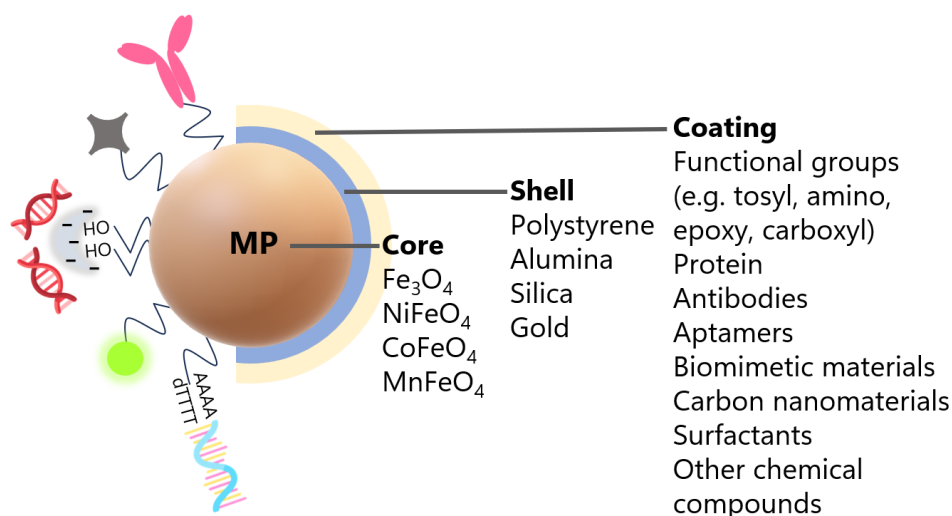


Figure 1.13. Schematic representation of the magnetic particles including the core, the shell and the coating.

In this sense, our group was one of the pioneers in the use of magnetic particles for the isolation of foodborne pathogens^{156,158,174,201–204}, integrating them also in other applications such as infectious diseases^{205–207}, isolation of breast cancer exosomes^{208–210} and environmental mycobacteria in water samples^{96,97}. Additionally, the use of molecularly imprinted polymers-based MPs for biosensing has also been reviewed²¹¹. Apart from their principal use as isolation and preconcentration technique, MPs are successfully exploited as a carrier biomolecule in microfluidics, lateral flow assays and detection in biosensors²¹².

Interestingly, immunomagnetic separation (IMS) based on antibody modified MPs are a powerful tool for the isolation and pre-enrichment of bacteria in complex sample such as clinical or environmental sources. A broad range of commercially MPs are available, with different sizes and chemical functionalities that allows the modification with desired compound. Some examples are the Dynabeads™ M-450 epoxy or tosylactivated (Invitrogen, catalog no. 14011 and 14013, respectively), that are superparamagnetic beads of 4.5 μm diameter size with epoxy or tosyl groups in the surface, for further modification. The latest, they are used in the next Chapters. Moreover, Dynabeads™ M-280 Streptavidin (Invitrogen, catalog no. 11205D) that are 2.8 μm monosized, monodispersed, and

superparamagnetic beads covalently coupled to streptavidin. This streptavidin-MPs (strep-MPs) are used in the last Chapter for the capture of biotin-tagged amplicons. Other commercial MPs are ready for direct use in pathogen capture due to their different coatings, such as the Dynabeads® anti-Listeria and Dynabeads® anti-Salmonella (Invitrogen, catalog no. 71006 and no. 71002, respectively).

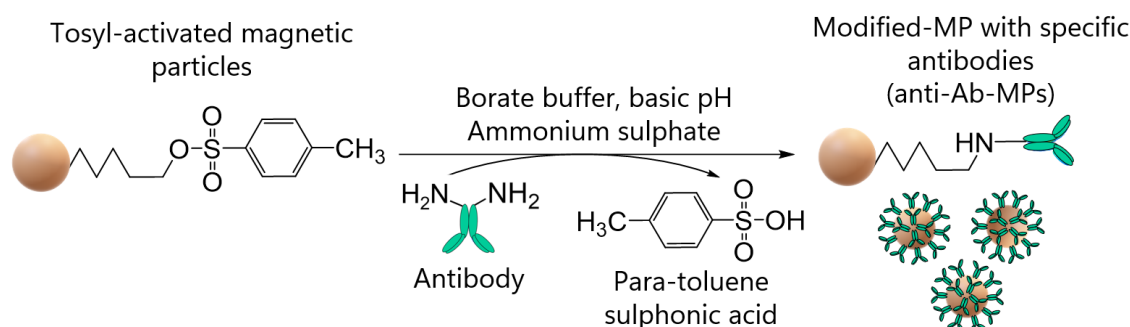


Figure 1.14. Schematic representation of the modification of tosyl-activated magnetic particles with specific antibodies. The modification of the MPs takes place in two steps: i) the nucleophilic reaction with the amino groups of the constant chains of the antibodies acting as a nucleophile and forming a final amide and ii) a blocking step, where the use of another molecule is used to block the unreacted tosyl groups and avoid further unspecific reactions.

1.4.2. Mobile devices and the use of smartphones

Digital health has attracted attention from medical and public health community, most notably in low and middle-income countries, where difficulties to access health care are present²¹³. The use of cost-effective tools, services and communication technologies to improve health and health-care related fields (prevention, diagnosis, treatment, monitoring, management of health-related issues, health literature and education among others) defines the term eHealth. The advances in mobile technology have grown exponentially during the last years until being a powerful medical tool to support health sector at different levels of care. The impact of the use of mobile devices and the large number of users has led to the development of a new sub-concept of eHealth, mobile health or mHealth²¹⁴. WHO refers to mHealth as ‘medical and public health practice supported by mobile devices, such as mobile phones, patient monitoring devices, Personal Digital Assistants, and other wireless devices’²¹⁴, although the concept has been differently defined, so mHealth encompasses the use of mobile computing, medical sensor, and communication technologies for healthcare^{215,216}.

The principal advantages of mHealth involves the improvement in the communication between health professionals and patient and the decision-making of the expert, facilitate the monitoring of the patient and the transmission of the information of the patient, but also the development of applications that encourage the medical education and training²¹⁷. All these advantages may be supported using mobile devices, smartphones and application (apps). This type of technology is very interesting as it brings the development of rapid diagnostic tests closer to reach the REASSURED acronym, especially regarding real time connectivity and equipment free RDT. Smartphones have the opportunity to bring RDT and POC near the patients for portable healthcare monitoring, network connectivity, data collection and transferring, battery-powered and high-resolution cameras²¹⁸, which can work as a transducer or detector. Recently, the development of smartphone-biosensors has increased considerably due to their portability as well as the internal memory and its microprocessor that can easily achieve image acquisition, information processing and quantification²¹⁹, among other properties. Smartphones may be equipped with intrinsic sensors, such as thermometers or optical sensors to sense different type of data. Also, the development of accessories which can be attached intrinsically or extrinsically to the smartphone for analytical biosensing in medical diagnostics has been designed^{220,221}. For instance, smartphones in microbiological applications have also been reviewed. For example, mobile phone cameras can be used as a microscope for morphological examination and quantification of pathogens²²². Sometimes, an objective lens is adapted to the camera lens to enhance the image²²².

On the other hand, colorimetric assays, as is the case of immunological assays or ELISA, can be detected and quantified through the mobile phone operated with an app²²³, avoiding the use of expensive benchtop spectrophotometer, which requires constant electrical current, unlike smartphones.

This doctoral thesis explores the use of a mobile app as an optical readout platform, as an alternative to the traditional spectrophotometer (Chapter 5), Quantitative signal readout in lateral flow assays is also possible with these devices²²⁴. Other strategies combine molecular diagnostics and the use of mobile phones. For example, the SMART-LAMP device is a smartphone-based loop-mediated isothermal amplification biosensor to perform real-time isothermal nucleic acid amplification reactions based on colorimetric measurement, and were

validated in the detection of Schistosomiasis, Strongyloidiasis, and COVID-19 infections, but seems easily adapted to the diagnosis of other infectious diseases²²⁵. Smartphone-based tests are presented as a powerful tool for diagnostics in limited-resource settings. However, some aspects still need to be addressed and improved, which make their implementation more challenging, including the analytical performance (accuracy and sensitivity overall), the multiplex detection and the validation in clinical studies²²⁶.

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CHAPTER 2

Objectives

2. Objectives

The rapid identification of infectious diseases plays a crucial role in early diagnosis and treatment of patients, as well as in improving informed decision-making and implementing actions to prevent transmission. Additionally, monitoring pathogens in the environment is essential to ensure global health and prevent infections through indirect transmission routes. This dissertation aims to design and develop biosensing strategies and *in vitro* diagnostic tests for detecting pathogens in both clinical and environmental samples. Novel routes to enhance sensitivity, preconcentration approaches, simplification strategies and integration of portable devices as readout platforms are explored in communicable infections, specifically targeting legionellosis, mycobacterial infections, and *E. coli*. The overarching objectives are outlined below:

- Define the needs of rapid diagnostic test in low-resource settings for global diseases and the safety of water and food supplies.
- Explore new solid-phase isolation and preconcentration methods for low concentration targets, modelled on contaminating pathogens in the water supply.
- Explore solid-phase separation and preconcentration strategies based on targeted magnetic particles.
- Integrate solid-phase preconcentration strategies into magnetically actuated immunoassays and magnetically actuated biosensors.
- Evaluate the analytical performance of immunoassays and biosensors against global health diseases.
- Develop new strategies of analytical simplification that demand minimal training for end-users and integrate more accessible instruments to economically constrained communities.
- Explores the technology transfer to different targets and biomarkers.
- Disseminate results in conferences and scientific journals.

Chapter 3 addresses the development of an electrochemical biosensing approach integrating in a novel preconcentration system for the detection of *Legionella pneumophila* retained in filters from water samples, being the specific objectives the following:

- Design a novel preconcentration method that combines filtration of high-volume samples and tailored magnetic particles with specific anti-*Legionella* antibodies.
- Study the performance of the filtration and the performance of the immunomagnetic separation to pull-out bacteria from different filtering materials.
- Design a biosensing device combining immunomagnetic separation and the detection with a labelled anti-*Legionella* antibodies with HRP enzymes.
- Evaluate the specificity of the method by challenging the system to detect other bacterial strains.
- Evaluate the analytical performance of the method, including sensitivity and limit of detection of the proposed methodology in water samples.

Chapter 4 addresses the development of an electrochemical biosensor for the direct detection of *Mycobacterium fortuitum* as a model of environmental mycobacteria, integrating the combined preconcentration strategy and a simultaneous incubation with the filter, being the specific objectives the following:

- Integrate the preconcentration strategy that combines the filtration of water samples and the use of modified magnetic particles with specific anti-LAM antibodies.
- Study the performance of the filtrations and immunomagnetic separation and simultaneous incubation by using different materials of the membrane.
- Develop and optimize the experimental conditions for a biosensing approach involving a simultaneous incubation of the filter, with the modified MPs and a conjugated antibody with biotin and streptavidin-HRP.
- Integrate a portable handheld platform for the electrochemical readout.
- Evaluate the specificity of the method by challenging the system to detect other bacterial strains.

- Evaluate the sensitivity and the limit of detection of the proposed methodology in hemodialysis water samples.

Chapter 5 explores the use of the smartphone as a platform readout in different bioanalytical applications, being the specific objectives the following:

- Analyze and compare the results obtained with the smartphone and the spectrophotometer as a gold standard in colorimetric reactions.
- Use different bioanalytical techniques as a model, including the BCA, Bradford, the conventional ELISA and a magneto-actuated immunoassay for the detection of *E. coli*.
- Explore the readout platforms for the detection of immunoglobulins in a veterinary application, based on the design of a conventional ELISA for the detection of mare's immunoglobulins to monitor immunodeficiencies in foals.
- Study the analytical performance comparing the two optical readout platforms, specifically in assays with their maximum absorption is in 450, 562, 595 nm.
- Explore the use of the smartphone and the app as an option of optical readout platform in limited-resource settings.

Chapter 6 is focused on the development of an electrochemical biosensor for the detection of tuberculosis, being the specific objectives the following:

- Develop a biosensing approach combining double-tagged PCR and electrochemical biosensing.
- Optimize the experimental parameters for the double-tagged PCR and evaluate the results by gel-electrophoresis.
- Study the performance of the double-tagged PCR by different set of primers encoding for *gyrB* and *IS6110* genes of *M. tuberculosis* (also present in *M. bovis BCG Pasteur* strain, that is used as an attenuated strain of *M. tuberculosis* for the development of the biosensor)
- Design a rapid electrochemical biosensing approach by integrating the use of double-tagged primers with biotin and digoxigenin labels, streptavidin magnetic particles and HRP labelled anti-digoxigenin antibodies.

- Evaluate the analytical performance of the methodology in spiked samples of *M. bovis BCG Pasteur*.
- Integrate a handheld platform operated by batteries for the electrochemical readout.

CHAPTER 3

*Improved biosensing of legionella by integrating
filtration and immunomagnetic separation of the
bacteria retained in filter*

3. Improved biosensing of *legionella* by integrating filtration and immunomagnetic separation of the bacteria retained in filters

3.1. Abstract

This study presents a novel approach that combines filtration and the direct immunomagnetic separation of the retained bacteria *Legionella* in filters, for further electrochemical immunosensing. This strategy allows for the separation and preconcentration of the waterborne pathogen from high-volume samples, up to 1000 mL. The limit of detection of the electrochemical immunosensor resulted in 100 CFU mL⁻¹ improved to up to 0.1 CFU mL⁻¹ when the preconcentration strategy was applied in 1 L of sample (10³-fold improvement). Remarkably, the immunosensor achieves the limit of detection in less than 2.5 h and simplifying the analytical procedure. This represents the lowest concentration reported to date for electrochemical immunosensing of *Legionella* cells without the need for preenrichment or DNA amplification. Furthermore, the study successfully demonstrates the extraction of bacteria retained on different filtering materials using immunomagnetic separation, highlighting the high efficiency of the magnetic particles to pull out the bacteria directly from solid materials. This promising feature expands the applicability of the method beyond water systems for detecting bacteria retained in air filters of air conditioning units by directly performing the immunomagnetic separation in the filters.

Keywords: electrochemical immunosensor, waterborne bacteria, solid-phase separation, magnetic particles, preconcentration strategy

3.2. Introduction

The specie *Legionella pneumophila* is included in the *Legionella* genus of pathogenic gram-negative bacteria and is the most common responsible causing legionellosis, a general term that includes the severe form of pneumonia called Legionnaire's disease discovered first described in 1976 ¹ and a mild febrile illness, the Pontiac fever. Unlike more than 50 species are known for *Legionella*² and at least 15 serogroups are identified for *L. pneumophila*, *Legionella pneumophila* serogroup 1 (Lp1) is the major responsible of the infections in humans³.

Legionella can grow in natural and artificial water systems with little or no circulation^{4,5}. Transmission typically occurs by inhalation of contaminated aerosols or mist, with no person-to-person spread, being the infective dose as low as a single colony. Outbreaks are often associated with poorly maintained artificial water systems, such as cooling towers, evaporative condensers linked to air conditioning and industrial cooling, as well as hot and cold-water systems⁶, among many others sources⁷⁻¹⁰. Hospitals can also be sources of infection¹¹, as well as contaminated drinking water. The prevention of legionellosis relies on implementing control measures to minimize the growth of *Legionella* and the dissemination of aerosols. Besides the good maintenance of devices, regular cleaning, and disinfection⁶, prevention strategies involve periodic on-site testing of potential outbreaks. The specific approaches for *Legionella* infection prevention and monitoring may vary depending on local regulations and geographic locations^{12,13}. Currently, the most widely used methods for detection involve time-consuming culture-based techniques followed by molecular methods. The limit of detection (LOD) for *Legionella* in different sources also vary depending on the regulations established by each country. For instance, the European Technical Guidelines for the Prevention, Control, and Investigation of Infections caused by *Legionella* species recommend testing samples in accredited laboratories with a LOD of 100 CFU L⁻¹ ¹⁴. However, there is a general agreement that corrective actions should be taken when concentrations reach 10,000 CFU L⁻¹ ¹². For instance, cooling towers and spa pools require minor interventions or disinfection, typically at LODs higher than 100 CFU L⁻¹. Moreover, in cooling towers actions need to be taken from 10⁵ to 10⁶ CFU L⁻¹

according to the American Industrial Hygiene Association (AIHA) Guidance on *Legionella* in building water systems¹⁵.

The gold-standard method for the detection of *Legionella* in water samples includes high-volume sample collection of up to 1 L, followed by membrane filtration as a preconcentration step, isolation on culture plates for 10 days, and confirmation using sub-culturing according to ISO 11731. Alternatively, some commercial kits, as Legiolert® test (IDEXX Laboratories, Westbrook, ME, USA) based on enzymatic detection technology, use lower volumes of 1 or 100 mL providing results in 7 days¹⁶. Lateral Flow Tests, such as Duopath® (Merck) or Hydrosense® (Albagaia, Edinburgh, UK) offer rapid visual redouts, but either require several days of enrichment to reach the LOD required by the legislation or yield semiquantitative results. To improve LODs without time-consuming pre-enrichment steps, the only alternative involves DNA amplification rather than bacterial growth. Commercial options for this approach include the microproof® *Legionella* Quantification LyoKit from BIOTECON Diagnostics GmbH (Potsdam, Germany) or the iQ-Check *Legionella* Real-Time PCR Kits from BIO-RAD (Bio-Rad Laboratories Inc, California, USA).

In order to reduce the time of analysis, it becomes imperative that emerging *Legionella* rapid testing technologies eliminate the necessity for pre-enrichment or DNA amplification procedures. As an alternative of amplification procedures, a highly efficient preconcentration strategy that combines filtration with immunomagnetic separation of the bacteria retained in the filters was successfully integrated to an immunosensing device. This novel approach has resulted in a significant improvement in the limit of detection compared to previously reported immunosensing devices¹⁷⁻²⁰. This approach enables the separation and preconcentration of pathogens from large sample volumes, typically 100-1000 mL, where immunomagnetic separation alone would not be feasible. The electrochemical immunosensor developed in this work is optimized and compared with the culturing methods in terms of the analytical features. Additionally, the performance of direct immunomagnetic separation of bacteria retained on various filtering materials is also assessed, highlighting the high efficiency of the magnetic particles to pull out the bacteria from solid materials.

3.3. Experimental section

3.3.1. Instrumentation

A complete filtration system of 25 mm (Product no. 073-0Q7724, Scharlab) was used for filtration. The filters assessed were polycarbonate cyclopore track etched (Catalogue no. 7060-2504, Whatman), nylon (Catalogue no. 7404-002, Whatman), cellulose acetate (Catalogue no. 10404006, Whatman), cellulose nitrate (Catalogue no. 10401106, Whatman) and mixed cellulose ester (Ref. HAWP02500, Merck Millipore Ltd), in all instances 0.45 μm pore size and 25 mm diameter. The magnetic actuation was achieved with the 16-tube magnet (Product no. 12321D, Thermo Fisher Scientific). The electrochemical readout was achieved on carbon screen-printed electrodes (ref. DRP-C110) using a portable bipotentiostat DRP-STAT200 operated by DropView 200 for instrument control and data acquisition (Dropsens, Spain). The scanning electron microscope (SEM) images were taken with the EVO MA-10 (with EDS Detector, Oxford LINCA).

3.3.2. Chemicals and biochemicals

Different set of buffers were used for specific procedures in the experiments, and their composition is described in §3.6.1 (Supp. data). All buffers were prepared from chemicals of analytical grade purchased from Merck and Sigma and using milliQ water. The reagents used for the electrochemical measurement includes hydroquinone (Ref. H9003) and hydrogen peroxide 30% solution (Ref. 31642, Sigma-Aldrich). The anti- *L. pneumophila* monoclonal antibody (G90A) from mouse (Catalogue no. MA5-18213, Invitrogen) was immobilized on tosyl activated magnetic particles (MPs, Dynabeads M-450 Tosylactivated, Product no. 14203, Invitrogen). The tailored modification of the antibody on the MPs is described in detail in §3.6.2 (Supp. data). The anti-Legionella polyclonal antibody labelled with horseradish peroxidase (HRP) enzyme from rabbit was used as a secondary antibody (Catalogue no. PA1-73141, Invitrogen). The strains used were *Legionella pneumophila* (serogroup 1. Philadelphia 1, ATCC 33152), and for the specificity study, *Pseudomonas aeruginosa* (ATCC 15442), *Klebsiella pneumoniae* (Schroeter) Trevisan (ATCC BAA-1705), *Mycobacterium fortuitum* (ATCC 6841), *Enterobacter*

spp (used as control by Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica), *Escherichia coli* (ATCC 10536), and *Salmonella choleraesuis* (ATCC 13311).

3.3.3. *Legionella pneumophila* culture

L. pneumophila strain was grown in selective solid culture plates (*Legionella* MWY Selective Agar, Product no. 10482513, Thermo Scientific) at 37 °C for 72 h. The bacteria were inoculated to distilled water and the solution was adjusted to an OD 0.370 at 600 nm, approximately equivalent to 10^7 CFU mL⁻¹. The concentration of *Legionella* samples was calculated for each experiment by colony counts verification in solid culture, as shown in Figure 3.6 (Supp data).

3.3.4. Evaluation of the immunomagnetic separation by scanning electron microscopy

To assess the performance anti-*Legionella*-MPs for binding *Legionella*, scanning electron microscopy was performed to visualize the interaction between the modified MPs and the bacteria. A volume of 100 µL of anti-*Legionella*-MPs at 10^7 MP mL⁻¹ was incubated with 1 mL of *L. pneumophila* at 10^7 CFU mL⁻¹ approximately, for 1 h at room temperature (RT) with agitation at 750 rpm. Afterwards, the supernatant was discarded, and the sample was washed three times with 1 mL of washing buffer. The sample was resuspended in 4 mL of PBS and it was filtered in a polycarbonate membrane to retain the modified MPs capturing the bacteria. Then, the filter was treated as previously described¹⁷.

3.3.5. Electrochemical magneto immunosensing. Specificity study

The electrochemical magneto immunosensing procedure has been previously developed by our research group in other applications devices^{17,19,20}. Briefly, involves i) the immunomagnetic separation (IMS), ii) the incubation with the label antibody and iii) the electrochemical readout. The optimization of the reagents including anti-*Legionella*-MPs (ranging from 10^4 to 10^7) and anti-*Legionella*-HRP antibody (from 1/250 to 1/2000) are described in §3.6.3 (Supp. data). Briefly, 100

μL of sample containing *Legionella* was incubated with $100\ \mu\text{L}$ of MPs at $10^7\ \text{MP mL}^{-1}$ in a final volume of $1\ \text{mL}$ with PBS for $1\ \text{h}$ under gentle rotation. After a washing step, the sample was incubated with $200\ \mu\text{L}$ of anti-*Legionella*-HRP at a dilution of $1/500$ for $30\ \text{min}$ at $900\ \text{rpm}$ and RT. After washing, the electrochemical readout was performed by resuspending the MPs in $40\ \mu\text{L}$ of ePBS and $18\ \mu\text{L}$ of electrochemical readout substrate solution. After $2\ \text{min}$ of enzymatic reaction, the solution was dropped to the screen-printed electrode and the electrochemical readout was done based on the square wave voltammetry technique (SWV). More details about the SWV measurements are provided in §3.6.5 (Supp. data). The LOD of the electrochemical magneto immunosensing procedure was calculated by processing a calibration plot ranging from 0 to $3.6 \times 10^5\ \text{CFU mL}^{-1}$.

The specificity study was performed by challenging the immunosensor with other waterborne bacteria at high concentration ($10^7/10^8\ \text{CFU mL}^{-1}$) such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Mycobacterium fortuitum*, *Enterobacter spp*, *Escherichia coli* and *Salmonella choleraesuis* in a final volume of $1\ \text{mL}$ and proceeded as above.

3.3.6. Novel preconcentration method. Study of the filtering material

The procedure combines three steps as described in Figure 3.1: i) Filtration of large volumes of sample (typically 100 or $1000\ \text{mL}$), followed by ii) immunomagnetic separation of the bacteria retained in the filter and magnetic actuation, iii) electrochemical immunosensing, as described above.

The experimental details are shown in Figure 3.11 (Supp. data). After filtration from 100 to $1000\ \text{mL}$ sample under vacuum, the $25\ \text{mm}$ diameter filter was placed on a $2.0\ \text{mL}$ tube. $100\ \mu\text{L}$ of anti-*Legionella*-MPs at $10^7\ \text{MP mL}^{-1}$ and $900\ \mu\text{L}$ of PBS were then added to the filter and incubated under gentle rotation at RT for $1\ \text{h}$. Then, the MPs with the captured *Legionella* were recovered under magnetic actuation and washed for $3\ \text{minutes}$ at $900\ \text{rpm}$ and RT. After that, $200\ \mu\text{L}$ of the anti-*Legionella*-HRP antibody was added to the modified MPs and incubated for $30\ \text{min}$ at $900\ \text{rpm}$ and RT. After washing, the electrochemical readout was performed as described above.

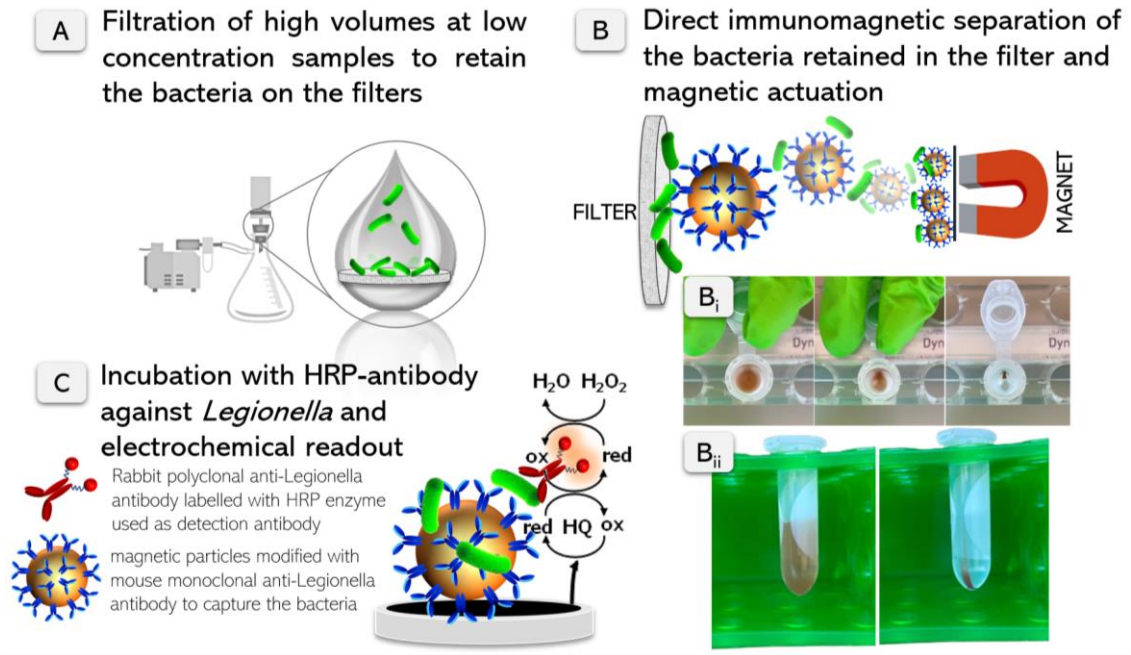


Figure 3.1. Schematic representation of A) filtration, B) immunomagnetic separation of the bacteria retained in the filters and magnetic actuation, followed by C) electrochemical immunosensing for the detection of *Legionella pneumophila* in water samples. In Panel B, experimental details are presented, showing how the filter is positioned on the Eppendorf tube for immunomagnetic separation, along with the evaluation of magnetic actuation efficiency on the filters. The photos in Panel Bi were captured in 1-second frame sequences. More details are presented in §3.6.6 (Supp. data).

In order to validate the approach, different materials were assessed. Polycarbonate (PC), nylon (NY), cellulose acetate (CA), cellulose nitrate (CN), and mixed cellulose ester membranes (MCE) were selected because of their widely use in microbiological filtration techniques with a 0.45 μm porous size and 25 mm diameter. For this study, 100 mL *Legionella* samples at the same concentration ($2 \times 10^3 \text{ CFU mL}^{-1}$) as well as the negative control were filtered using each membrane and submitted to downstream analysis, as above.

3.3.7. Statistical analysis

Statistical analyses were conducted using Prism v 10.0.1 (GraphPad, San Diego, USA). Calibration curves were fitted using nonlinear regression with a four-parameter logistic curve 4PL, defined by the equation:

$$Y = D + (A - D) / [1 + (C / \text{IC50})^{\text{HillSlope}}] \quad (\text{Equation 3.1})$$

Where: Y: Represents the electrochemical signal; D: Stands for the lower asymptote, representing the response at very low concentrations; A: Represents the upper

asymptote, signifying the response at high concentrations; IC50: Denotes the inflection point or concentration at the midpoint of the curve; HillSlope: Signifies the slope parameter.

The Limit of Detection (LOD) was determined by analyzing negative control samples to calculate the mean value and standard deviation (SD). Subsequently, a one-tailed t-test was applied at a 95% confidence level to establish the cut-off value. The LOD was then derived by interpolation of this cut-off value using the 4PL equation, followed by the calculation of the corresponding *Legionella* concentration.

3.3.8. Biosafety considerations

All experiments were performed in a Biosafety Class 2 environment required for the handling of *L. pneumophila*. All biological waste generated from the experiments were disposed in accordance with the local regulations for handling biohazards.

3.4. Results and discussion

3.4.1. Evaluation of the immunomagnetic separation by scanning electron microscopy

Following the incubation of the *Legionella*-containing sample with anti-*Legionella* magnetic particles (anti-*Legionella*-MPs), the sample was observed by scanning electron microscopy (SEM) to confirm the effectiveness of the immunomagnetic separation. In Figure 3.2, it is confirmed that tosyl-activated magnetic particles were coated with anti-*Legionella* monoclonal antibodies to enhance the interaction between the particles and the bacteria. The images clearly demonstrate the successful binding of the modified magnetic particles to the *L. pneumophila* bacteria, resulting in the formation of clusters due to the polyvalency of both the bacteria and the magnetic particles.

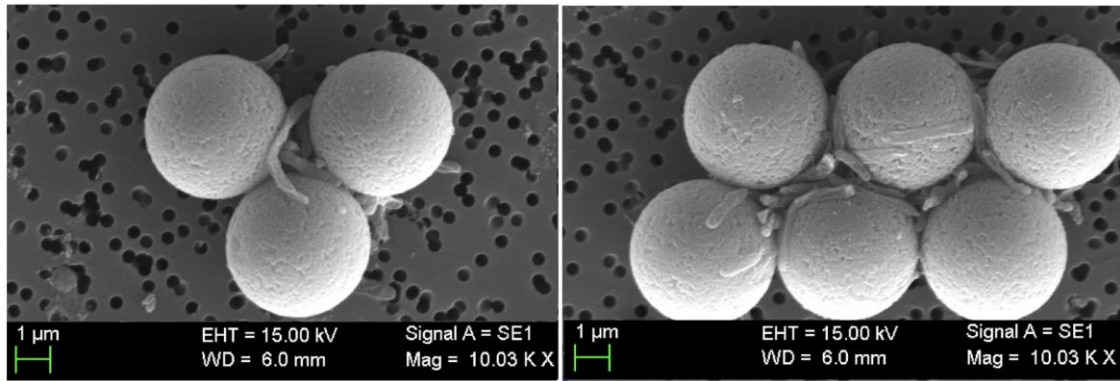


Figure 3.2. Evaluation of the IMS by SEM at a *L. pneumophila* concentration of 10^7 CFU mL⁻¹. The images show the *L. pneumophila* cells attached to the magnetic beads. In all cases, identical acceleration voltage (15 KV) was used.

3.4.2. Electrochemical magneto immunosensing. Specificity study

The conditions for the electrochemical immunosensing were previously optimized as described in § 3.6.5 (Supp. data). From the results, a concentration of 10^7 anti-*Legionella*-MPs mL⁻¹ and anti-*Legionella*-HRP antibody 1/500 was used in all further experiments. Figure 3.9 (Supp. data), shows the calibration plot from 0 to 3.6×10^5 CFU mL⁻¹ for the determination of the *L. pneumophila* with the electrochemical immunosensor without the integration of the novel preconcentration method. The data was fitted with a non-linear regression (Sigmoidal 4PL, GraphPad Prism Software v 10.0.1, $R^2 = 0.9837$) and the LOD was calculated, resulting in a value of 100 CFU mL⁻¹. As expected, this LOD is comparable with a magneto-actuated immunosensor previously reported by our research group but for other bacteria¹⁸⁻²³.

The specificity of the electrochemical biosensor towards a high concentration of other waterborne bacteria was also studied, and the results are shown in Figure 3.3. The signal of the negative control containing 0 CFU mL⁻¹ of *Legionella* (negative control) was found to be comparable with all the interferents bacteria samples ($p < 0.05$), including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Mycobacterium fortuitum*, *Enterobacter spp*, *Escherichia coli*, and *Salmonella choleraesuis*, suggesting a good specificity for the assay.

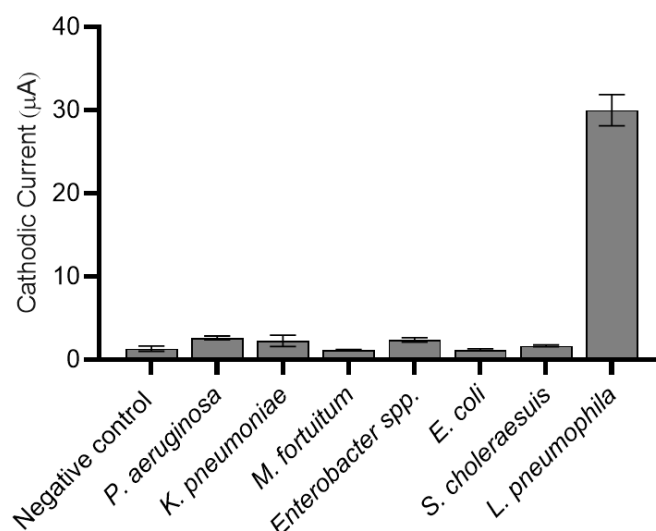


Figure 3.3. Specificity study for *L. pneumophila*, including a negative control, and interferents at high concentration, including *Pseudomonas aeruginosa* (2×10^8 CFU mL⁻¹), *Klebsiella pneumoniae* (2×10^8 CFU mL⁻¹), *Mycobacterium fortuitum* (2×10^5 CFU mL⁻¹), *Enterobacter spp* (2×10^8 CFU mL⁻¹), *Escherichia coli* (2×10^8 CFU mL⁻¹), and *Salmonella choleraesuis* (3×10^8 CFU mL⁻¹). *L. pneumophila* positive control (8×10^4 CFU mL⁻¹) is also included. The concentrations of the viable bacteria were determined by culturing in solid media and CFU counting. The error bars show the standard deviation for n=3.

The electrochemical magneto immunosensor was specifically designed for the determination of *Legionella* by utilizing a monoclonal antibody that targets the lipopolysaccharides of *L. pneumophila* strains. As a result, other bacteria are not detected, ensuring specificity for *Legionella* determination. It is important to note that the immunosensor primarily targets *Legionella pneumophila* serogroup 1, which is known for its high virulence and prevalence in cooling towers and distribution systems, making it the key strain responsible for most infections.

3.4.3. Novel preconcentration method. Study of the filtering material

The LOD achieved by the electrochemical magneto immunosensor (1×10^2 CFU mL⁻¹) are above the limits required by most legislations. Accordingly, and to overcome time-consuming classical preenrichment¹⁸ or DNA amplification¹⁷, a novel preconcentration method is proposed, combining filtration and direct immunomagnetic separation (IMS) of the bacteria retained in the filters, as depicted in Figure 3.1, to match the limits required by the legislation. Selecting the appropriate filter material is crucial to ensure a swift filtration workflow and

minimize nonspecific adsorption of bacteria on the filters. This is essential for subsequent immunomagnetic separation and effective magnetic actuation of the retained bacteria in the filters. Accordingly, a first study was performed with a set of filters of different materials. There is a wide range of commercially available membranes with low retention rates and different properties. Filtering materials commonly used in microbiology were selected in this study, including polycarbonate, nylon, cellulose acetate, cellulose nitrate and mixed cellulose esters, and the results are shown in Figure 3.4. They share a hydrophilic surface to avoid an excessive retention of the bacteria, and thus promoting the capture by the anti-*Legionella*-MPs directly in the filter. Moreover, the hydrophobic nature of the outer surface of *Legionella*^{24,25} makes easier pull-off the trapped bacteria in hydrophilic surfaces.

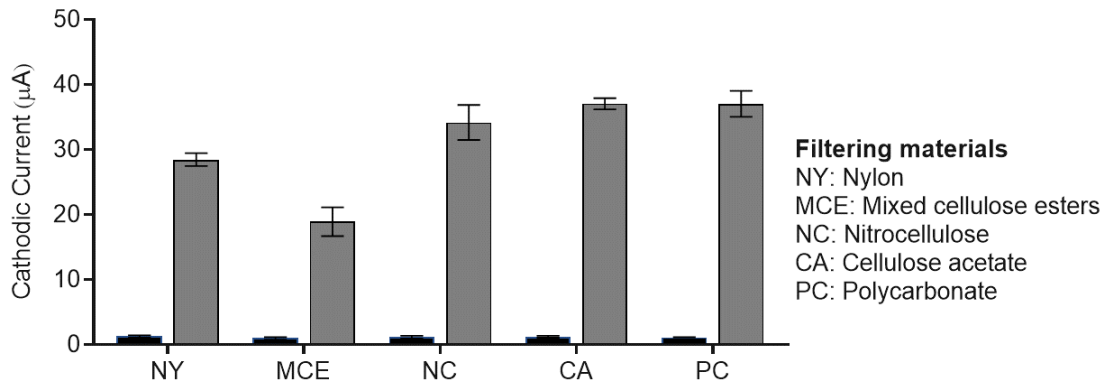


Figure 3.4. Study of the filtering material by the filtration of a 100 mL sample containing *Legionella* (2×10^3 CFU mL⁻¹) as well as the negative controls. All the filters were 0.45 μm pore size and 25 mm of diameter. The filtering materials tested were nylon (NY), mixed cellulose ester (MCE), cellulose nitrate or nitrocellulose (NC), cellulose acetate (CA) and polycarbonate (PC). The concentrations of the viable bacteria were determined by culturing in solid media and CFU counting. The error bars show the standard deviation for n=3.

According to the results shown in Figure 3.4, no significant differences in the current intensities by using polycarbonate (PC), cellulose nitrate and cellulose acetate (CA) filters were obtained ($p > 0.05$). The use of PC membranes in the filtration processes is recommended by some regulations, for instance the standard ISO11731:2017, and several studies support their efficacy as they provide the best recovery rates of this bacterium²⁶. Some of the advantages of PC track-etched membranes are their precision in the cylindrical pores providing a very accurate size cut-off, and their low nonspecific protein binding, similar to the CA membranes,

which also provided good results. In contrast, nylon and mixed cellulose ester materials gave lower intensity currents in comparison with PC as a reference material. The high protein binding capacity of these filters could cause a highly retention of the bacteria in the filter, thus hindering the immunomagnetic capturing and resulting in lower preconcentration yields. Apart from the material, porosity, pore size, and the protein binding capacity, among other features, other variables such as electrostatic charges can also influence in the filtration and the magnetic actuation. Although PC, CA and cellulose nitrate membranes can all be considered as good candidates for the preconcentration strategy, the workflow time was different for each filtering material (Table 3.1, Supp. Data), providing the polycarbonate improved features, such as faster filtration rates, resulting in less than 1 minute for the filtration of 100 mL sample and less than 10 minutes for the filtration of 1 L sample. In the case of the cellulose nitrate and cellulose acetate filters, the workflow time was higher. According to these results, PC filters were selected in all further experiments.

The calibration plot integrating the novel preconcentration method (by filtering 100 mL of sample and IMS), and further electrochemical immunosensor is shown in Figure 3.5 as red dotted line, for the determination of the *L. pneumophila* from 0 to 1×10^4 CFU mL⁻¹. The data was fitted with a non-linear regression (Sigmoidal 4PL, GraphPad Prism Software v 10.0.1, $R^2 = 0.9950$) and the LOD was calculated, resulting in a value of as low as 2 CFU mL⁻¹. Compared with the electrochemical biosensor without the integration of the preconcentration method (Figure 3.5, black solid line), an improving of two orders of magnitude was achieved, from 1×10^2 CFU mL⁻¹ to 2 CFU mL⁻¹. According to some legislations, the approach is useful to detect the threshold of 10,000 CFU L⁻¹ established for corrective actions, especially in cooling towers^{15,27}. However, certain regulations may require even lower LODs. In order to achieve a further improvement in the LOD, integrating the novel preconcentration method (by filtering 1000 mL of sample and IMS), and further electrochemical immunosensor is shown in Figure 3.5 as blue solid line, for the determination of the *L. pneumophila* from 0 to 60 CFU mL⁻¹. The data was fitted with a non-linear regression (Sigmoidal 4PL, GraphPad Prism Software v 10.0.1, $R^2 = 0.9923$) and the LOD was calculated, resulting in a value of as low as 0.1 CFU mL⁻¹ (100 CFU L⁻¹). Interestingly, the filtration and immunomagnetic separation of 1000

mL of sample can avoid the use of time consuming preenrichment steps to reach the limits required by some legislations that establish more restrictive detection limits (from 100 to 1000 CFU L⁻¹). Moreover, the approach is able to clearly detect as low as 0.6 CFU mL⁻¹ with a signal to background ratio of 9.7 (n=3) (Figure 3.5, panel B).

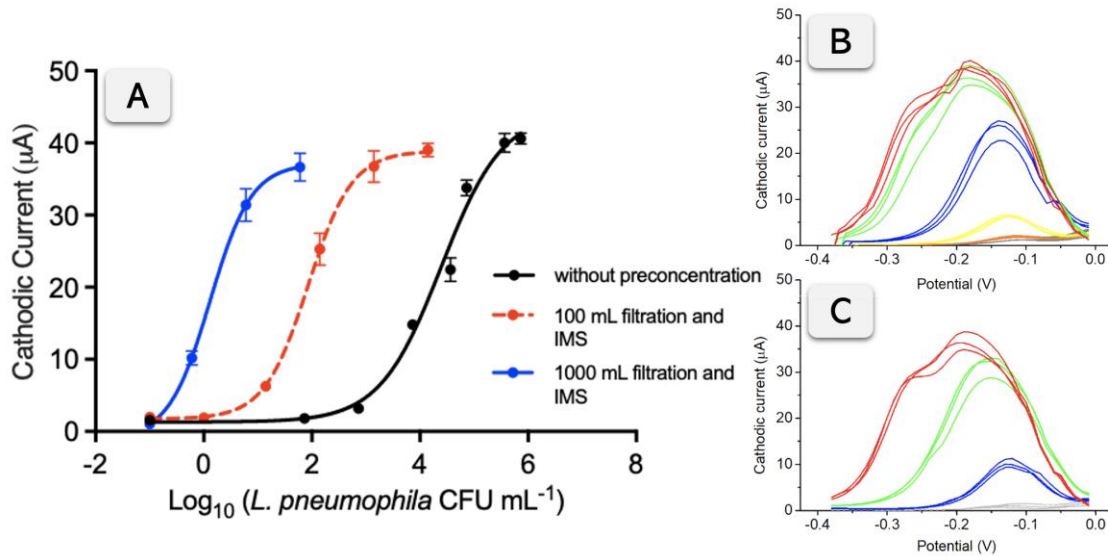


Figure 3.5. Panel A. Calibration plot for the magneto-actuated electrochemical immunosensor in water samples. The black solid line shows the calibration plot ranging from 0 to 7 × 10⁵ CFU mL⁻¹ without the integration of the novel preconcentration method ($R^2 = 0.9886$). The red dotted line shows the calibration plot ranging from 0 to 1 × 10⁴ CFU mL⁻¹ ($R^2 = 0.9950$) with preconcentration by filtering 100 mL samples. Raw data obtained from the SWV measurements is shown in panel B. The blue solid line shows the calibration plot ranging from 0 to 60 CFU mL⁻¹ ($R^2 = 0.9923$) by filtering 1000 mL samples. Raw data obtained from the SWV measurements is shown in panel C. After filtration, IMS and electrochemical immunosensing are performed in both cases. The concentrations of the viable bacteria were determined by culturing in solid media and CFU counting. The error bars show the standard deviation for n=3.

This approach allows the determination of low concentrations in high-volume samples that is one of the bottlenecks in the detection of environmental bacteria, in around 2.5 h. As shown in Figure 3.5, the LOD of the electrochemical immunosensor without filtration (only IMS) resulted in 100 CFU mL⁻¹ improved to up to 0.1 CFU mL⁻¹ when the preconcentration strategy was applied in 1 L of sample (10³ fold improvement). The values of the LODs and the total assay time for the electrochemical immunosensor combining filtration are summarized in Supp. data (Table 3.2). The readout time is also reduced to up to 3 min, based on the incubation of the enzymatic substrates (H₂O₂ and HQ) with a total of 2 min and the

electrochemical measurement of less than 1 min. This represents a 10-fold reduction in time in comparison with an immunoassay with optical readout, where the substrates require a minimum incubation time between 15 and 30 min ²³. In previous work, we explored several strategies to achieve the LOD, such as incorporating a short preincubation step¹⁸ or amplifying the genetic material after the IMS¹⁷. In the present study, we have achieved a remarkable 10³-fold improvement in the LOD, by combining filtration and IMS. Compared with other electrochemical immunosensors and genosensors previously described for *Legionella* or other gram-negative bacteria (and summarized on Table 1.3 in §1), the results confirm the promising features of combining filtration, immunomagnetic separation and electrochemical immunosensing for the ultrasensitive detection of *Legionella* using commercial screen-printed electrodes.

The electrochemical immunosensor described here allowed the determination of very low concentrations of *Legionella* in less than 2.5 h preventing time-consuming preenrichment steps filter treatments or DNA amplification. Further studies will focus on reducing the incubation time and streamlining it into a single step to simplify the analytical procedure.

3.5. Conclusions

A magneto-actuated electrochemical immunosensor is presented in this work capable of detecting and quantifying *Legionella* in contaminated water in less than 2.5 h with an impressive LOD of 0.1 CFU mL⁻¹. The most remarkable aspect of this approach is the elimination of classical preenrichment steps, DNA extraction, and amplification techniques, resulting in the analytical simplification and a significant reduction in the total analysis time. This innovative method combines a preconcentration strategy based on filtration to perform the IMS directly on polycarbonate filters. This allows for the handling of high-volume samples, typically ranging from 100 mL to 1000 mL. The electrochemical readout enables the use of a portable and cost-effective device that is compatible with in-field test. This represents the lowest reported LOD for the electrochemical biosensing of whole *Legionella* bacteria without the need for preenrichment and DNA amplification steps.

The LOD achieved by this method underscores its utility, especially considering that some of the most stringent regulations require a LOD of at least 100 CFU L⁻¹. Another noteworthy fact relies on the analytical simplification. In the gold standard microbiological method, after sample filtration, the retained bacteria were resuspended or extracted from the filter using special buffers or vortexing. In this study, the preconcentration strategy is integrated to the electrochemical biosensing. By filtering high volumes of samples and subsequently employing magnetic actuation to pull out the bacteria directly from the filter, the need for resuspension or additional steps for downstream analysis was eliminated. Another remarkable aspect of this approach is the potential of the magnetic particles of successfully removing the bacteria directly from various filtering materials. This promising characteristic broadens the applicability beyond water systems to include the detection of bacteria retained in air conditioning unit air filters, accomplished through direct immunomagnetic separation within the filters.

3.6. Supplementary data

3.6.1. Chemicals and biochemicals

Buffer and solution composition

The composition of the buffers used in this work are described below:

For covalent immobilization of antibodies on the magnetic particles

- Borate buffer: 100 mmol L⁻¹ H₃BO₃, pH 9.0
- Ammonium sulphate buffer: 3 mol L⁻¹ (NH₄)₂SO₄ dissolved in 100 mmol L⁻¹ H₃BO₃ pH 9.0
- Blocking buffer: 500 mmol L⁻¹ glycine dissolved in PBS pH 8.0
- Washing buffer: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, 0.1% (w/v) BSA
- Storage buffer: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, pH 7.4 plus 0.05% (v/v) Tween 20®, 0.05% (w/v) sodium azide

For sample incubation and washing steps

- PBS: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, pH=7.4

- Washing buffer: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, 0.5% (w/v) Casein

For electrochemical readout

- ePBS: 100 mmol L⁻¹ Na₂HPO₄, 100 mmol L⁻¹ KCl, pH 7.0.
- Electrochemical readout substrate solution: 57 mmol L⁻¹ hydrogen peroxide, 23 mmol L⁻¹ hydroquinone in distilled water

Legionella pneumophila culture

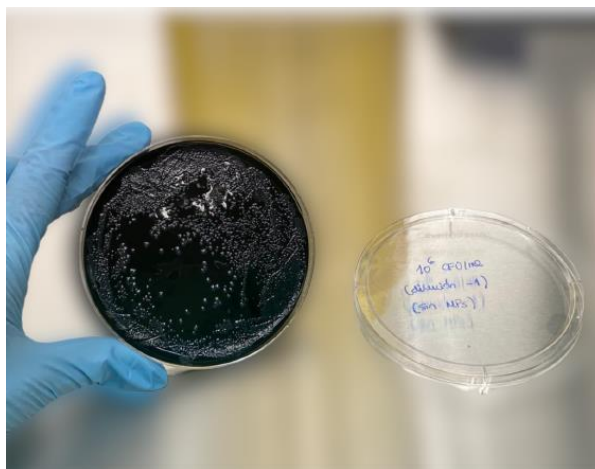


Figure 3.6. *Legionella pneumophila* strain (serogroup 1, Philadelphia 1, ATCC strain n° 33152) was grown in selective solid culture plates (*Legionella* MWY Selective Agar, Product no. 10482513, Thermo Scientific). The concentration of *Legionella* samples was calculated for each experiment by solid culture for colony counts verification.

3.6.2. Covalent immobilization of anti-*Legionella* antibody

Covalent immobilization of anti-*Legionella* antibodies was performed in tosyl activated magnetic particles. The outline of the procedure is schematically represented in Figure 3.7, panel A. Briefly, a volume of 40 µL of MPs was washed twice with 1 mL of borate buffer followed by the addition of 8 µg of antibody and 100 µL of ammonium sulphate buffer performing a total volume of 250 µL.

The MPs were incubated under continuous agitation for a total reaction time of 20 h at 37°C. After incubation, MPs were separated with a magnet and resuspended in 250 µL of 500 mM glycine for 2.5 h at 37°C under continuous agitation to block the unreacted tosyl groups. The prepared anti-*Legionella*-MPs were washed three times with 1 mL of washing buffer and were resuspended in 160

μL of storage buffer at 4°C. The final concentration of the anti-*Legionella* MPs was 1×10^8 MP mL⁻¹. The modified MPs were washed three times with a washing buffer before being used in each experiment.

After the immobilization, the supernatant is collected for the determination of the total amount of antibody immobilized on the magnetic particles by ELISA, providing a coupling efficiency of 95 % (represented in Figure 3.7, panel B).

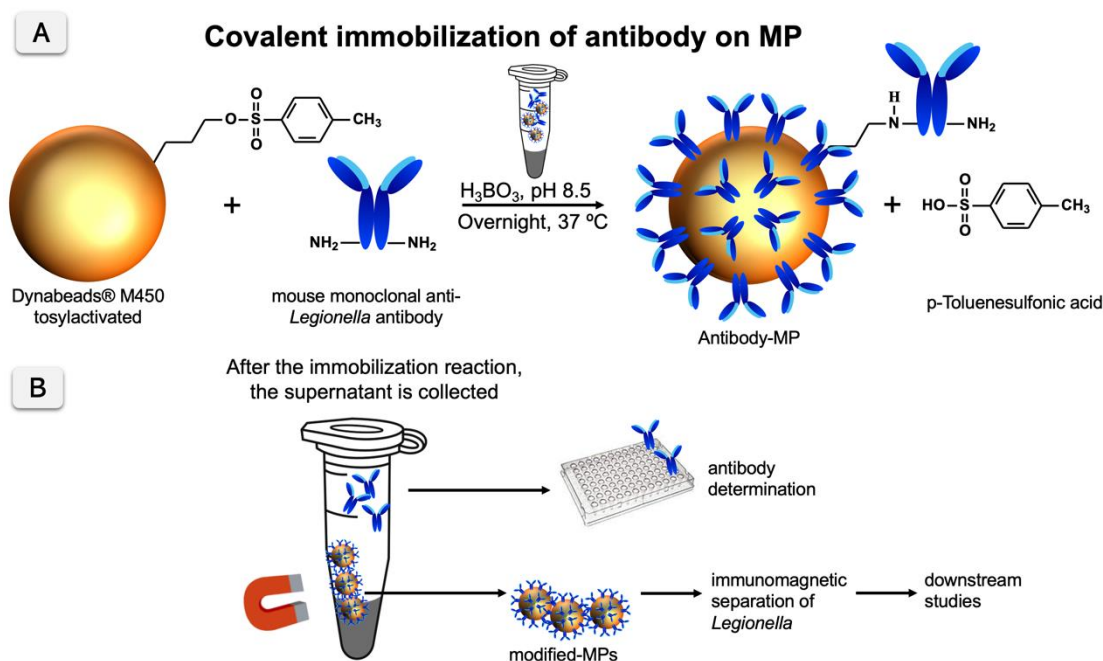


Figure 3.7. Schematic representation of the tailored covalent immobilization of antibodies on magnetic particles, followed by the determination of the coupling efficiency by determining the remaining antibodies on the supernatant by ELISA.

Afterwards, the total immobilization in percentages (coupling efficiency) is calculated based on the following formula, providing a value of 95 %.

$$\text{Immobilization (\%)} = \frac{[\text{ab initial}] - [\text{ab supernatant}]}{[\text{ab initial}]} \quad (\text{Equation 3.2})$$

Magnetic separation by using the magnetic separator is performed after each incubation/washing step to separate the magnetic particles from the supernatant.

3.6.3. Electrochemical magneto immunosensing. Optimization of the reagent concentrations

The reagent concentrations for the magneto-actuated immunosensor were optimized in order to obtain the maximum signal-to-noise ratio. The concentration of the modified magnetic particles with anti-Legionella antibodies and the anti-Legionella-HRP antibody were optimized using an optical readout (magneto-actuated immunoassay).

On the one hand, in a 2.0 mL Eppendorf tube, a volume of 800 μL of PBS, 100 μL of positive or negative controls (1.1×10^6 and 0 CFU mL^{-1} of *L. pneumophila*, respectively) and 100 μL of anti-Legionella-MPs at different concentrations of 10^4 , 10^5 , 10^6 , 10^7 MP mL^{-1} were tested. Then, after 1 h incubation, the supernatant was discarded using a magnet and 1 mL of washing buffer was added to perform one washing at the rotor. Then, the sample was resuspended in 200 μL and it was washed twice in a thermomixer with 200 μL of washing buffer. Afterwards, 200 μL of anti-Legionella-HRP at 1/2000 dilution was incubated for 30 min at the thermomixer for 30 min at 900 rpm and RT. The sample was washed twice again, resuspended in 200 μL and transferred to a magneto-ELISA microplate (polypropylene microplate). One washing was done in the microplate with 200 μL , and 100 μL of the optical substrates TMB/ H_2O_2 were added for 30 minutes in continuous agitation.

Finally, the reaction was stop with 100 μL of H_2SO_4 2M and the absorbance was read at 450 nm to compare the signals (Figure 3.8, Panel A). For the optimization of the anti-Legionella-HRP concentration, the same procedure described above was followed, using 10^7 MP mL^{-1} as modified MPs concentration. Then, the dilutions including 1/250, 1/500, 1/1000 and 1/2000 were tested for the anti-Legionella-HRP antibody (Figure 3.8, panel B). The optimized concentrations for further steps were 10^7 MP mL^{-1} and a dilution of 1/500 for the anti-Legionella-MPs and anti-Legionella-HRP antibodies, respectively.

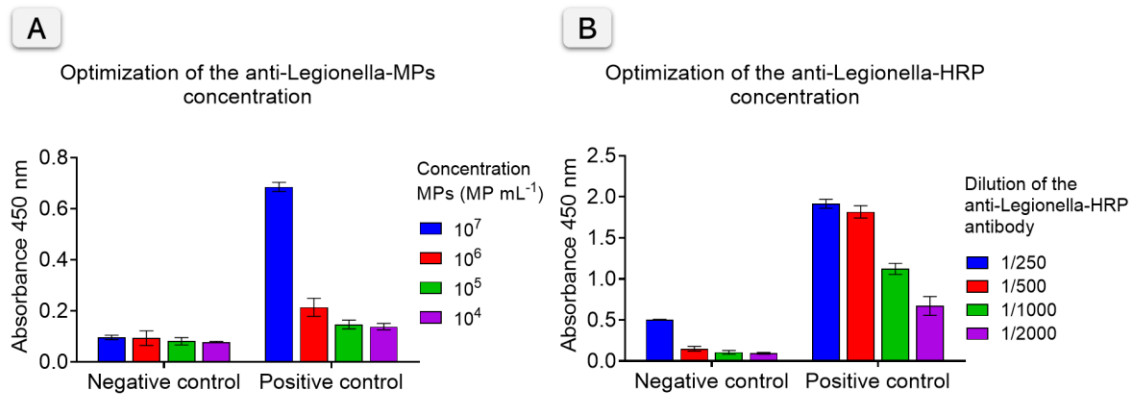


Figure 3.8. Panel A) Bar plot for the optimization of the anti-Legionella-MPs (n=3). *L. pneumophila* 1.1×10^6 CFU mL⁻¹. **Panel B)** Bar plot for the optimization of the anti-Legionella-HRP antibody concentration (n=3). *L. pneumophila* 4.9×10^6 CFU mL⁻¹. In all instances, negative controls were also assessed.

3.6.4. Electrochemical magneto immunosensing for the quantification of *Legionella pneumophila*

The conditions for the electrochemical immunosensing were previously optimized as described in 3.8 (Supp. data). From the results, a concentration of 10^7 anti-Legionella-MPs mL⁻¹ and anti-Legionella-HRP antibody 1/500 was used in all further experiments.

Figure 3.9 shows the calibration plot from 0 to 3.6×10^5 CFU mL⁻¹ for the determination of the *L. pneumophila* with the electrochemical immunosensor without the integration of the novel preconcentration method. The data was fitted with a non-linear regression (Sigmoidal 4PL, GraphPad Prism Software v 10.0.1, $R^2=0.9837$) and the Limit of Detection (LOD) was calculated, resulting in a value of 100 CFU mL⁻¹.

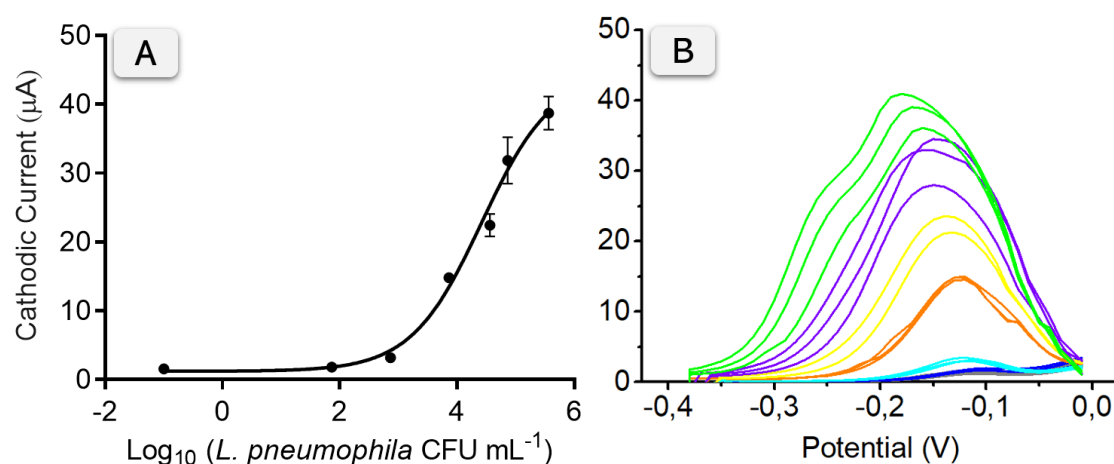


Figure 3.9. Panel A. Calibration plot for the magneto-actuated electrochemical immunosensor in water samples. The black solid line shows the calibration plot ranging from 0 to 3.6×10^5 CFU mL⁻¹ without the integration of the novel preconcentration method ($R^2 = 0.9837$). The concentration of the viable bacteria was estimated by culturing in solid media and further CFU counting. The error bars show the standard deviation ($n=3$). Panel B. Raw data obtained from the SWV measurements for the magneto-actuated electrochemical immunosensor. The potential range was 0 to -0.4 V, with a potential step and amplitude of 10 mV, and frequency of 1 Hz. The maximum current value was used for the calibration plot.

3.6.5. Electrochemical readout

The electrochemical readout was based on square wave voltammetry using carbon screen-printed electrodes (ref. DRP-C110, DropSens, Spain). The electrochemical cell consists of a working and auxiliary electrode made of carbon, while the reference electrode is made of silver, and a permanent neodymium magnet was placed above the electrode (Figure 3.10, panel A). These electrodes are of small dimensions (L 33 x W 10 x H 0.5 mm) and allow working both with sample microvolumes or even in solution. The electrodes were connected to the boxed connector for SPE (Ref. DSC) which operates as an interface between the electrodes and the portable bipotentiostat (DRP-STAT200, DropSens, Spain). The square wave voltammetry (SWV) measurements were performed in a laptop computer in which the portable bipotentiostat was connected by a universal USB port. The anti-*Legionella* antibodies labeled with the HRP enzyme, were used as electrochemical reporters in the presence of hydrogen peroxide (H_2O_2) as a substrate of HRP and hydroquinone (HQ) as a mediator. HRP catalyze the transfer of two electrons from HQ to H_2O_2 to generate water and benzoquinone (BQ), the oxidized form of HQ. First,

the HRP is oxidized catalyzing the reduction of H_2O_2 to H_2O , then the HRP is reduced again by oxidizing HQ to BQ. Thus, the final readout at the surface of the electrode is based on the reduction of the BQ to HQ (Figure 3.10, panel B). Thus, since the enzyme works at saturated substrate conditions, HRP works at maximum speed and turn-over range, providing a directly proportional signal to the sample (hence the higher the number of bacteria with anti-Legionella-HRP, the higher the signal).

The SWV measurements were performed with a total of 62 μL of sample. The sample performed by the modified MPs capturing the bacteria and the HRP enzyme labelled antibody was resuspended in 40 μL of ePBS, and 18 μL of the electrochemical readout substrate solution containing hydrogen peroxide and hydroquinone was added. After the homogenization, the enzymatic reaction was done in 2 min and the solution was transferred to the surface of the screen-printed electrode. The potential range used was from 0 to -0.4 V, with a potential step and amplitude of 10 mV, and frequency of 1 Hz. The data were recorded and processed using DropView200 software.

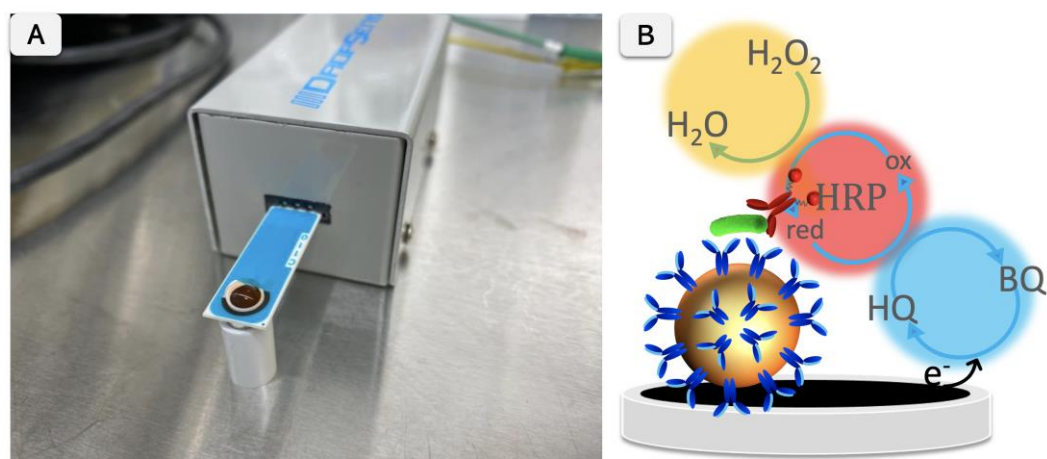


Figure 3.10. Schematic representation of the electrochemical set-up and the HRP enzymatic reaction on the electrode surface, to achieve the electrochemical readout.

3.6.6. Novel preconcentration method. Study of the filtering material

Briefly, the procedure combines three steps as described in Figure 3.1 and 3.11: i) Filtration of large volumes of sample (typically 100 or 1000 mL), followed by ii) immunomagnetic separation of the bacteria retained in the filter and magnetic actuation, iii) electrochemical immunosensing, as described above.

In detail, following the filtration from 100 to 1000 mL sample under vacuum (Figure 3.11, panel A), the 25 mm diameter filter was placed on a 2.0 mL tube (panel B). 100 μL of anti-*Legionella*-MPs at 10^7 MP mL^{-1} and 900 μL of PBS were then added to the filter (panel C) and incubated under gentle rotation at RT for 1 h (panel D). Then, the MPs with the captured *Legionella* were recovered under magnetic actuation by means of a permanent magnet (panels F to I) and washed for 3 minutes at 900 rpm and RT. After that, 200 μL of the anti-*Legionella*-HRP antibody was added to the modified MPs and incubated for 30 min at 900 rpm and room temperature. After washing, the electrochemical readout was performed as described above.

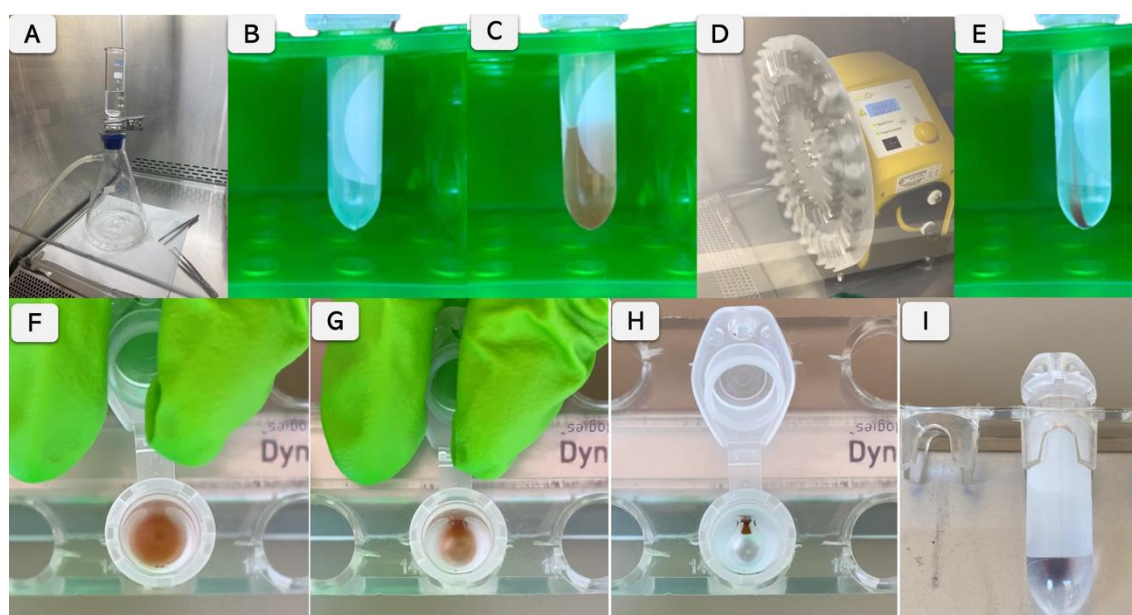


Figure 3.11. Schematic representation of A) Filtration, B to D) immunomagnetic separation of the bacteria retained in the filters, showing how the filter is positioned on the Eppendorf tube for immunomagnetic separation (B and C) under rotation (D) and (F to I) magnetic actuation. The photos (F to I) were captured in 1-second frame sequences.

The workflow time was calculated for each filtering material and the values are described in Table 3.1. The LOD of the electrochemical immunosensor without the integration of the novel preconcentration method, and the electrochemical immunosensor with the integration of filtration of 100 and 1000 mL and IMS are summarized in Table 3.2.

Table 3.1. Workflow time by using different filtering materials: nylon (NY), mixed cellulose ester (MCE), cellulose nitrate (NC), cellulose acetate (CA) and polycarbonate (PC), by the filtration of 50 mL sample and time required for 100 mL samples.

FILTERING MATERIAL	Nylon	Mixed cellulose ester	Cellulose nitrate	Cellulose acetate	Polycarbonate
Workflow time (50 mL sample)	74 s	23 s	38 s	45 s	22 s
Approximate filtration time (100 mL sample)	>2 min	< 1 min	> 1 min	> 1 min	< 1 min

3.6.7. Summary of the analytical performance

The LOD of the electrochemical immunosensor without the integration of the novel preconcentration method, and the electrochemical immunosensor with the integration of filtration of 100 and 1000 mL and IMS are summarized in Table 3.2.

Table 3.2. Summary of the results obtained for the electrochemical immunosensor including sample volume processed, LOD and number of replicates of a zero calibrator or blank sample used to calculate the LOD, and the total assay time.

	Without preconcentration strategy	With preconcentration strategy	
Sample volume (mL)	0.1	100	1000
LOD (CFU mL⁻¹) / number of negative sample (replicates)	1 x 10 ² / n=8	2 / n=11	0.1 / n=3
Total assay time	~2h 15 min	~2h 17 min	~2h 26 min

3.7. References

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CHAPTER 4

Mycobacterium detection method combining
filtration, immunomagnetic separation, and
electrochemical readout in a portable biosensing
device

4. *Mycobacterium* detection method combining filtration, immunomagnetic separation, and electrochemical readout in a portable biosensing device

4.1. Abstract

This study addresses a straightforward and highly sensitive approach for detecting *Mycobacterium fortuitum*. The method involves a combination of filtration and direct immunomagnetic separation to isolate the bacteria retained on filters for further electrochemical magneto immunosensing in a handheld device. Unlike conventional methods involving pre-enrichment by culturing, this approach employs a simplified preconcentration technique, which includes filtration of large water samples, up to 100 mL, followed by a one-step process of immunomagnetic separation and labeling. After a 60-min, during which the filter with the retained bacteria and all the reagents (including modified magnetic particles and enzymatic conjugates) are incubated, the resulting product is directly drop into a cartridge, capable of performing magnetic actuation and washing. The electrochemical readout is carried out on a portable battery-operated device within 30 sec. Remarkably, the immunosensor demonstrates an outstanding limit of detection of 5 CFU mL⁻¹ in hemodialysis water processing 100 mL of sample. This achievement is remarkable considering the short and simplified analytical procedure, compared to the traditional isolation and culturing of mycobacteria, which typically takes 2 weeks.

Keywords: preconcentration strategy, immunomagnetic separation, *Mycobacterium fortuitum*, PEM, hemodialysis water, handheld device

4.2. Introduction

The genus *Mycobacterium* contains more than 170 species of non-tuberculous mycobacteria (NTM) that are commonly referred to as environmental mycobacteria^{1,2}. Unlike *Mycobacterium tuberculosis complex* are the major responsible of infections, opportunistic NTM infections are increasingly prevalent worldwide^{3,4}, particularly among individuals with compromised immune systems or underlying lung diseases. Pathogenic environmental mycobacteria (PEM) frequently cause infections, often transmitted through water⁵⁻⁸, via ingestion and inhalation, particularly from aerosols^{7,9}. Rapidly growing PEM, including *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium mucogenicum* and *Mycobacterium fortuitum* can eventually progress to disseminated bacteremia, meningitis, or other syndromes^{7,10}. Rapidly growing PEM is also responsible for health care-related outbreaks by contamination of potable water. An example is infections in hemodialysis patients, highlighting the need to identify reservoirs and minimize proliferation to prevent outbreaks^{6,11}. Traditional detection of PEM in water relies on culturing⁷. Rapidly growing PEM takes 7 days to produce observable colonies, whereas slowly growing PEM need several additional days. Before culturing, water samples are decontaminated with alkali or acid solutions to eliminate other bacteria and fungi that could mask the PEM colonies, which can also have an impact on mycobacterial viability. Traditional culture-based PEM detection is time-consuming, labor-intensive, and potentially inefficient due to loss or underestimation of PEM. Nowadays, molecular techniques play an important role for detecting mycobacterial species in water samples. These methods involve identifying distinct mycobacterial DNA sequences, often employing culture enrichment or direct DNA isolation, followed by PCR amplification. Given the typically low concentration of mycobacterial DNA in environmental samples, PCR amplification is commonly employed^{7,12,13}. Predominant reports on *Mycobacterium* detection are mostly focused on *M. tuberculosis complex*. While reports have increased due to the emergence of NTM infections, research on PEM detection remains limited. Given PEM's significant role in environmental systems and potential health risks, there is a need for a straightforward, rapid, and user-friendly PEM monitoring approach, especially in low-resource settings. In this work, an

electrochemical magneto-immunosensor is introduced for quantifying *M. fortuitum*, serving as a PEM model in this study. According to the low concentration of this bacteria in water, the collection of 100 up to 1000 mL of sample is typical for further filtration or centrifugation, as a preconcentration step⁷. A preconcentration strategy relying on the combination of filtration and immunomagnetic separation (IMS) is used in this procedure to pull out the bacteria directly from the filter. Moreover, and in order to simplify the analytical procedure, the filter is incubated at the same time with the magnetic particles modified with anti-lipoarabinomannan antibodies and the enzymatic conjugate, performing a one-step incubation in only 60 min. Furthermore, the electrochemical detection takes place in an easy-to-use and handheld device with a novel cartridge, in which the magnetic actuation is performed, while the excess of sample and reagents are removed.

4.3. Experimental section

4.3.1. Instrumentation

The electrochemical measurements were performed on carbon screen-printed electrodes (ref. DRP-110) in a disposable cartridge (Figure 4.1, panel C) for magneto-actuation, washing and reading (BioEcllosion SL, Spain). The cartridge can be either inserted to a handheld device operated by batteries (BioEcllosion, Spain) which includes the external magnet, for the quantitative amperometric readout directly on the display in 30 sec, or in a bipotentiostat DRP-STAT200 operated by DropView 2.2 for instrument control and data acquisition (Dropsens, Spain). A complete filtration system of 25 mm (Product no. 073-0Q7724, Scharlab) was used to assess polycarbonate track-etched membranes (Catalogue no. 10417106, Whatman), nylon (Catalogue no. 7404-004, Whatman), cellulose acetate (Catalogue no. 10404006, Whatman), cellulose nitrate (Catalogue no. 10401106, Whatman) and mixed cellulose ester (Ref. HAWP02500, Merck Millipore Ltd), in all instances 0.45 μm pore size and 25 mm diameter. The cyclopore polycarbonate track-etched membranes of 0.2 μm pore size were used as a SEM support (Catalogue no. 10417606, Whatman). The SEM images were taken with the scanning electron microscope EVO MA-10 (with EDS Detector, Oxford LINCA). The confocal images were collected on the microscope Leica, TCS SP5 (Leica Microsystems, Germany).

4.3.2. Chemicals and biochemicals

Different set of buffers were used for specific procedures in the experiments, and their composition is described in §4.6.1 (Supp. Data). All buffers were prepared from analytical grade purchased from Merck and Sigma and using milliQ water. The monoclonal anti-LAM antibody from rabbit (Catalogue no. MA533311, ThermoFisher Scientific) was immobilized on tosyl activated magnetic particles (Dynabeads™ M-450 Tosylactivated, Catalogue no. 14013, Invitrogen). The anti-*Mycobacterium* polyclonal antibody (anti-TBS) from rabbit (Catalogue no. PAB29798, Abnova) was biotinylated with EZ-Link NHS-Biotin Kit (Catalogue no. 20217, ThermoFisher Scientific) following the manufacturer's protocol, as detailed in §4.6.4 (Supp. Data), providing a biotinylation degree of 6 biotin per IgG molecules (anti-TBS-BIO). The excess of biotin tag was removed by desalting using the Zeba™ spin and desalting columns of 7K cut-off (Catalogue no. 89882, Pierce Rockford, IL). An enzymatic conjugate of anti-TBS-BIO and streptavidin-HRP (Catalogue no. 11089153001, Roche) was used for labelling, and previously prepared by incubation for 45 min at 37°C.

4.3.3. Culturing *Mycobacterium fortuitum* and other strains

The strain of *Mycobacterium fortuitum* ATCC 6841 was growth in Tryptic Soy Broth (TSB) medium supplemented with 0.015% (v/v) of tyloxapol at 37 °C for 72 h in continuous agitation, until reaching an OD of 1.000 AU at 550 nm. The liquid culture was aliquoted and preserved supplemented with 20% (v/v) of glycerol at -40 °C. The concentration of the stock was calculated by CFU counting in Tryptic Soy Agar (TSA) plates, after the incubation for 5-7 days at 37 °C. The concentration of viable cells was quantified for each experiment by CFU counting, as shown in Figure 4.5 (Supp data). The strains for the specificity study were: *E. coli* (CECT 405, ATCC 10536), *L. Pneumophila* (serogroup 1, Philadelphia 1, ATCC 33152), *P. Aeruginosa* (CECT116, ATCC 15542), and *M. fortuitum*. The concentrations of the viable bacteria were determined in all the experiments by the gold standard culturing method in solid media and CFU counting, as detailed in §4.6.2 (Supp. Data).

4.3.4. Covalent immobilization of anti-LAM antibodies

The tailored modification of the MPs with anti-LAM antibodies was performed on tosyl activated magnetic particles as reported previously¹¹, following by blocking the unreacted tosyl groups with glycine buffer. The outline of the procedure is schematically represented in Figure 4.6 (Supp. data). The coupling efficiency was determined as 98.3 %, as detailed in Figure 4.7 (Supp. Data). The final concentration of the anti-LAM-MPs was 1×10^8 MP mL⁻¹ and were previously washed three times with washing buffer before used in each experiment.

4.3.5. Evaluation of the immunomagnetic separation of *M. fortuitum* by scanning electron and confocal microscopy. Specificity study

To assess the performance anti-LAM-MPs for binding *M. fortuitum*, scanning electron microscopy was performed. A volume of 100 µL of anti-LAM-MPs at 10^7 MP mL⁻¹ was incubated with 500 µL of *M. fortuitum* at 2.2×10^8 CFU mL⁻¹ for 1 h at 750 rpm. Afterwards, the sample was filtered in a polycarbonate membrane. Then, the filter was treated as previously described¹⁴. In order to visualize the interaction in a sandwich format of *M. fortuitum* with both anti-LAM-MPs and anti-TBS-BIO antibody, confocal fluorescence microscopy was performed. To provide blue fluorescence to the bacteria, *M. fortuitum* 2.2×10^8 CFU mL⁻¹ was incubated with 5 µL of Hoechst for 30 min at 750 rpm. To provide green fluorescence to the MPs, 100 µL of anti-LAM-MPs at 10^7 MP mL⁻¹ was preincubated with an anti-rabbit-ALEXA488 for 30 min. The anti-LAM-MPs were then incubated with 100 µL of *M. fortuitum* for 1 h at 750 rpm, and then, with 200 µL conjugate streptavidin-Cy5/anti-TBS-BIO (80 µg mL⁻¹) for 1 h. Confocal images were thereafter collected with the laser line of Hoechst (350-461 nm excitation, 461 nm emission), anti-rabbit Alexa Fluor® 488 (anti-rabbit-ALEXA488) (495 nm excitation, 518 nm emission) and streptavidin-Cy5 (600-650 nm excitation, 670 nm emission).

The specificity study was performed by magneto-actuated immunoassay as described in §4.6.5 (Supp Data) at the optimized concentration of reagents, as shown in Figure 4.8 (Supp Data). In this study, bacteria commonly found in contaminated water were included at a high concentration range: *E. coli* 3.6×10^8

CFU mL⁻¹, *L. Pneumophila* 7.2 x 10⁷ CFU mL⁻¹, *P. Aeruginosa* 4.1 x 10⁸ CFU mL⁻¹, and *M. fortuitum* 2.1 x 10⁵ CFU mL⁻¹. The bacteria grown in solid plates were inoculated in water, adjusting the absorbance to 0.200 AU at 550 nm.

4.3.6. Electrochemical magneto immunosensing for the quantification of *M. fortuitum*

The electrochemical magneto immunosensing procedure involves i) the immunomagnetic separation of *M. fortuitum* with the anti-LAM-MPs and incubation with the enzymatic conjugate anti-TBS-BIO/strep-HRP in a one-step incubation and ii) the electrochemical readout in 30 s. Briefly, 100 µL of sample containing *M. fortuitum* was incubated with anti-LAM-MPs at 10⁷ MP mL⁻¹ and enzymatic conjugate (40 µg mL⁻¹ anti-TBS-BIO and 5 U mL⁻¹ strep-HRP), in a volume of 1 mL in with PBST for 60 min under gentle rotation. The product is introduced in the cartridge in the “OPEN position”, in which the magnetic actuation is performed, while the excess of sample and reagents are removed. Three drops (150 µL) of the cartridge solution are added. The cartridge is then put in “CLOSE position”, and three drops (150 µL) added again for the further amperometric measurement under enzyme saturation conditions at -0.15 V and 30 s. All the cartridge operation as well as the electrochemical readout were performed following the product specifications provided by the manufacturer. More details about the amperometric measurements are provided in §4.6.8 (Supp. Data). The LOD of the electrochemical magneto immunosensor was calculated by processing a calibration plot ranging from 0 to 2.2 x 10⁶ CFU mL⁻¹.

4.3.7. Novel preconcentration method. Filtration, immunomagnetic separation and electrochemical immunosensing in handheld device. Study of the filtering material

A preconcentration strategy is integrated to the electrochemical immunosensing to improve the LOD, by filtering high volume of samples and further immunomagnetic separation to pull-out the bacteria from the filter for downstream analysis. Briefly, the procedure combines three steps as described in Figure 4.1 and

Figure 4.12 (Supp. data): i) Filtration of large volumes (typically 100 mL) of sample followed by ii) the immunomagnetic separation of *M. fortuitum* retained in the filters with the anti-LAM-MPs and incubation with the enzymatic conjugate anti-TBS-BIO/strep-HRP in a one-step incubation and ii) the electrochemical readout in 30 sec.

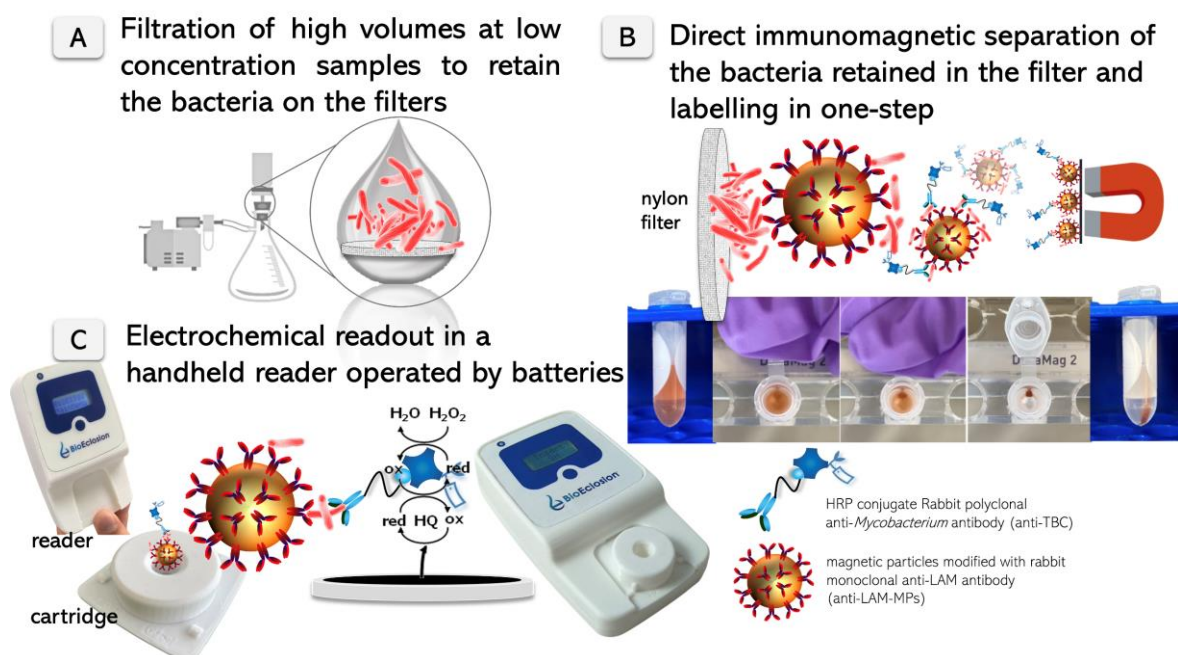


Figure 4.1. Schematic representation of A) filtration, B) immunomagnetic separation of the bacteria retained in the filters and labelling in one-step, followed by C) electrochemical readout for the detection of *M. fortuitum* in hemodialysis water performed in a cartridge and handheld device. In Panel B, experimental details are presented, showing how the filter is positioned on the Eppendorf tube for immunomagnetic separation. The photos in Panel B were captured in 1-second frame sequences. More details are presented in §4.6.9 (Supp. Data).

The experimental details are provided in §4.6.9 (Supp. data) and the Figure therein. After filtration from 100 mL sample under vacuum, the 25 mm diameter filter was placed on a 2.0 mL tube. 100 μ L of anti-LAM-MPs at 10^7 MP mL⁻¹, 200 μ L of the enzymatic conjugate anti-TBS-BIO/streptavidin-HRP were added and PBST buffer to complete a final volume of 1 mL, to fully cover the filter and to ensure complete interaction of the MPs with the bacteria retained in the filters. All reagents were used in the optimized concentrations (as described in §4.6.7 Supp. data), and incubated under gentle rotation at RT for 60 min. Then, the MPs labelled complexes with the captured bacteria were recovered under magnetic actuation and

resuspended in 200 μL of washing buffer for further transfer to the cartridge. Then, the electrochemical readout was performed as described in §4.6.8 (Supp. data).

In order to validate the approach, different materials were assessed. Polycarbonate (PC), nylon (NY), cellulose acetate (CA), cellulose nitrate (CN), and mixed cellulose ester membranes (MCE) were selected because of their widely use in microbiological filtration techniques with a 0.45 μm porous size and 25 mm diameter. For this study, 100 mL *M. fortuitum* samples at the same concentration ($2.4 \times 10^3 \text{ CFU mL}^{-1}$) as well as the negative control were filtered using each membrane and submitted to downstream analysis, in this instance magneto-actuated immunoassays described in §4.6.5 and §4.6.6 (Supp. Data).

4.3.8. Electrochemical magneto immunosensing for the quantification of *M. fortuitum* in hemodialysis water samples

M. fortuitum is one of the most common mycobacteria in contaminated hemodialysis waters¹⁵ which can put immunocompromised patients at risk. Hence, it is important to continuously monitor water supplies in hemodialysis centers, to minimize the exposure of renal transplantation and immunocompromised individuals to contaminated sources. In fact, a magneto-actuated immunoassay for the detection of *M. fortuitum* in hemodialysis water samples was reported previously by our group¹¹. Therefore, a calibration curve with different concentrations of the bacteria in hemodialysis water samples was performed and the LOD was calculated.

4.3.9. Biosafety considerations

All experiments were performed in a Biosafety Class 2 environment required for the handling of *M. fortuitum*, *P. aeruginosa* and *L. pneumophila*. All biological waste generated from the experiments were disposed in accordance with the local regulations for handling biohazards.

4.3.10. Statistical analysis

The statistical analyses were performed using Prism v 10.0.1 (GraphPad, San Diego, USA). The calibration curves were adjusted to a non-linear regression fitting,

using the four-parameter logistic curve, abbreviated 4PL. The limit of detection was calculated considering the number of negative controls for each curve, using the one-tailed test with a significance level of $p > 0.05$ (t Student, $p > 0.05$).

4.4. Results and discussion

4.4.1. Evaluation of the immunomagnetic separation of *M. fortuitum* by scanning electron and confocal microscopy. Specificity study

Following the incubation of the *Mycobacterium*-containing sample with anti-LAM-MPs, the sample was observed by scanning electron microscopy (SEM) to confirm the effectiveness of the immunomagnetic separation. In Figure 4.2, it is confirmed that tosyl-activated magnetic particles were coated with anti-LAM monoclonal antibodies to enhance the interaction between the particles and the bacteria. Figure 4.2, panel A clearly demonstrate the successful binding of the modified magnetic particles to the *M. fortuitum* bacteria, resulting in the formation of clusters due to the polyvalency of both the bacteria and the magnetic particles. Large corded bacterial aggregates are also observed in Figure 4.2, panel B, since they are known to form clumps associated with biofilm formation in the natural environment¹⁶. In order to visualize not only the ability of the anti-LAM-MPs in capturing mycobacteria, but also anti-TBS-BIO to detect them in a sandwich-like format, confocal fluorescence microscopy was performed using streptavidin-Cy5 for labelling (emitting far-red-fluorescence at 670 nm). According to Figure 4.2, panel C, anti-LAM-MPs (incubated with ant-rabbit-ALEXA488 antibodies emitting green at 518 nm) are capable of capturing aggregates of mycobacterium which appears in blue due to the Hoechst staining of the DNA at 461 nm recovered by red due to anti-TBS-BIO/streptavidin-Cy5 conjugate. The monoclonal anti-LAM antibody was selected for capturing in the MPs considering that the lipoarabinomannan is a lipoglycan and major virulence factor in the bacteria genus *Mycobacterium*. It is thus expected that other bacteria will not be detected, as demonstrated in Figure 4.2, panel D, when challenged with a high concentration of gram-negative bacteria. The signal of the negative control containing 0 CFU mL⁻¹ of *M. fortuitum* was found to be

comparable with *E. coli*, *L. Pneumophila*, *P. Aeruginosa* ($p < 0.05$), suggesting a good specificity for the assay.

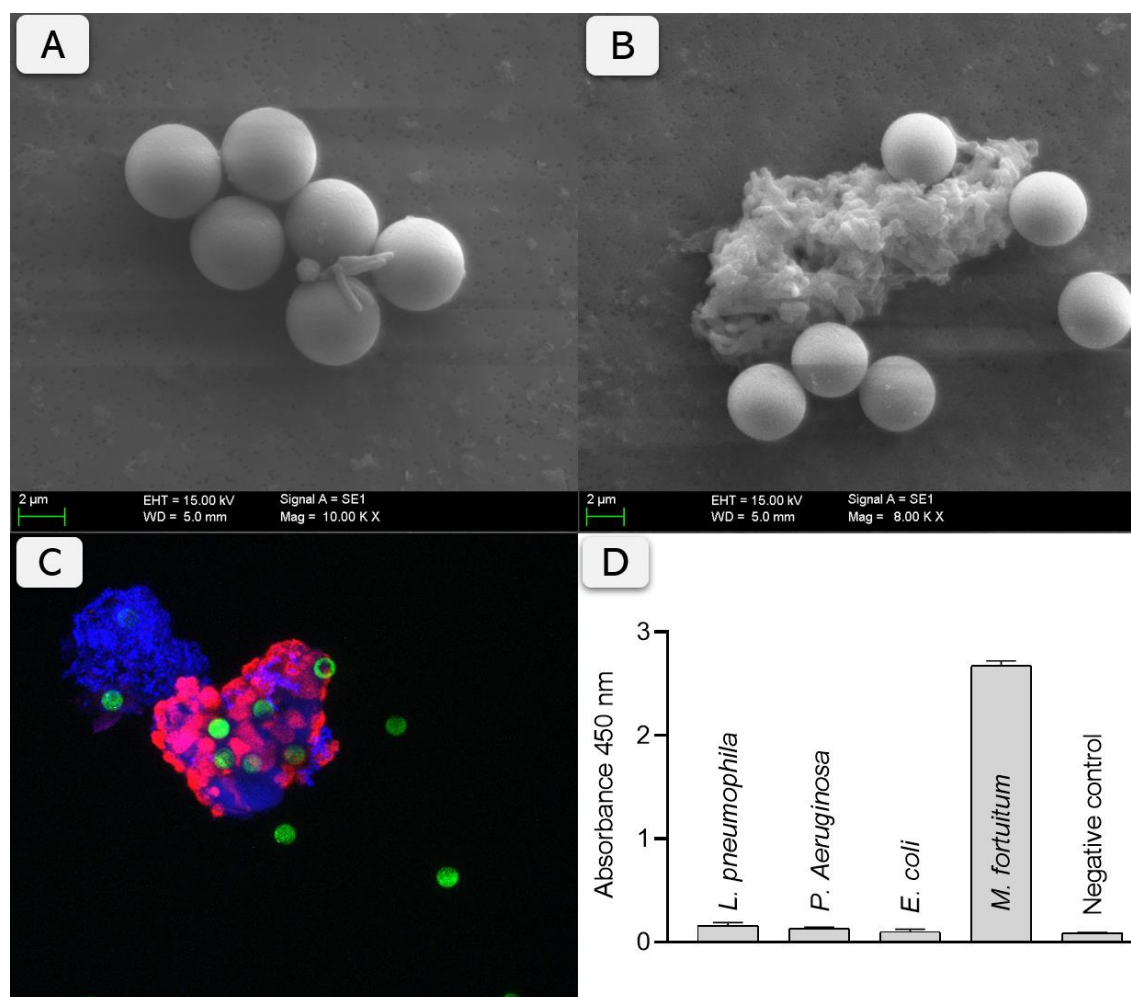


Figure 4.2. Panel A and B. Evaluation of the IMS by SEM at a *M. fortuitum* concentration of 2.2×10^8 CFU mL⁻¹. In all cases, identical acceleration voltage (15 KV) was used. Panel C. Confocal microscopy study of the sandwich-like format. Anti-LAM-MPs are shown in green, capturing a cluster of *M. fortuitum* that was stained in blue by Hoechst, and superimposed with the specific red fluorescence from anti-TBS-BIO/ streptavidin-Cy5 conjugates. Panel D. Specificity study for *M. fortuitum* 2.1×10^5 CFU mL⁻¹, *E. coli* at 3.6×10^8 CFU mL⁻¹, *P. Aeruginosa* at 4.1×10^8 CFU mL⁻¹ and *L. pneumophila* 7.2×10^7 CFU mL⁻¹. The error bars show the standard deviation for n=3.

4.4.2. Electrochemical magneto immunosensing for the quantification of *Mycobacterium fortuitum*

The primary objective of this study is to achieve analytical simplification, aimed at minimizing user intervention while maintaining analytical performance. The optimized assay conditions were thoroughly tested in §4.6.6 and §4.6.7 (Supp.

Data). This work is also focused on detecting *M. fortuitum* through an amperometric readout using a user-friendly handheld electrochemical device with an integrated internal potentiostat. The disposable cartridge encompasses both the microfluidic system and the electrode, enabling magnetic actuation and efficient removal of excess sample and reagents. Notably, the same solution serves a dual role in both washing and catalyzing the reaction through the HRP enzyme (referred to as the cartridge solution). As a result, the necessity for employing diverse buffers is eliminated, leading to reduced time in a simpler approach for the end-user. Additionally, the cartridge design allows for the use of a small solution volume, up to 300 μL . Figure 4.3, Panel A shows the calibration plot from 0 to 2.2×10^6 CFU mL^{-1} for the detection of the *M. fortuitum* with the electrochemical immunosensor without the integration of the novel preconcentration method. The data was fitted with a non-linear regression (Sigmoidal 4PL, GraphPad Prism Software v 10.0.1, $R^2 = 0.9987$) and the limit of detection were calculated, resulting in a value of 4.3×10^3 CFU mL^{-1} , by processing 0.1 mL of sample.

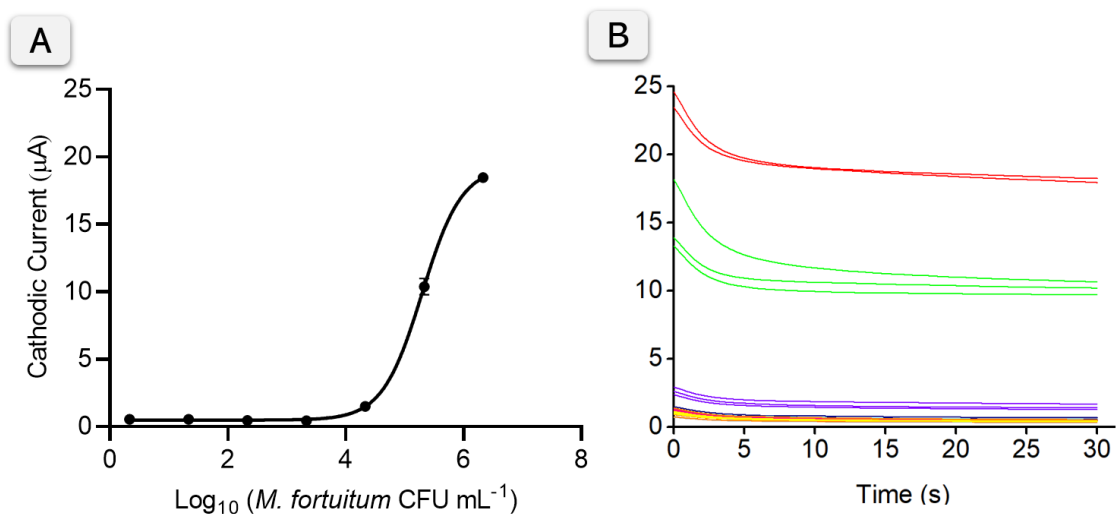


Figure 4.3. Panel A. Calibration plot for the for the detection of *M. fortuitum* 0 to 2.2×10^6 CFU mL^{-1} with the magneto-actuated electrochemical immunosensor in water samples, without the integration of the novel preconcentration method ($R^2 = 0.9987$). ($n=3$). Panel B. Raw data obtained from the amperometric measurements, performed at applied potential = -0.150 V (vs. Ag/AgCl). The current value at the steady current (30 s) was used for the calibration plot.

This LOD is comparable with a magneto-actuated immunosensor previously reported by our research group but for *Salmonella*, in which a LOD of 7.5×10^3 CFU mL^{-1} in milk was achieved in 50 min without any pretreatment¹⁷. In

order to further improve the LODs, a new approach to handle higher volume of sample, combining filtration and IMS, was presented in the next section.

4.4.3. Filtration, magnetic actuation and electrochemical immunosensing. Study of the filtering material

The LOD achieved by the electrochemical magneto immunosensor (4.3×10^3 CFU mL⁻¹) by processing 0.1 mL of sample are above the limits required by most legislations. In order to improve the LODs, a new approach to handle higher volume of sample (up to 100 mL), combining filtration and direct immunomagnetic separation (IMS) of the bacteria retained in the filters, is proposed in this work, as depicted in Figure 4.1. Selecting the appropriate filter material is crucial to ensure a swift filtration workflow, a good removing of the bacteria retained in the filters by the MPS, while minimizing nonspecific adsorption of all the reagents (including the enzymatic conjugate) during the one-step incubation on the filters.

A collection of filter materials frequently employed in microbiology, such as polycarbonate, nylon, cellulose acetate, cellulose nitrate, and mixed cellulose esters, was chosen. These materials share a hydrophilic surface to prevent excessive retention and thereby facilitate immunomagnetic separation and further labelling. The results obtained from filtering 100 mL water containing *M. fortuitum* and negative controls were compared for each material, as illustrated in Figure 4.4 panel A. While the negative controls yielded similar results across all materials, nylon emerged as the choice due to its significant difference in signal compared to other materials. In this study, nylon filters exhibited the highest signal intensity, promoting interaction among different biorecognition elements during the one-step incubation and yielding optimal recovery rates for the bacteria. The calibration plot integrating the novel preconcentration method in a one-step incubation (by filtering 100 mL of hemodialysis water), and further electrochemical immunosensor is shown in Figure 4.4, panel B as green line, for the detection of the *M. fortuitum* from 0 to 8.7×10^4 CFU mL⁻¹. The data was fitted with a non-linear regression (Sigmoidal 4PL, GraphPad Prism Software v 10.0.1, $R^2 = 0.9906$) and the LOD was calculated ($n=9$), resulting in a value of as low as 5 CFU mL⁻¹. Compared with the electrochemical biosensor without the integration of the preconcentration method

(Figure 4.4, blue solid line), an impressive 10^3 fold improvement was achieved, from 4.8×10^3 CFU mL⁻¹ to 5 CFU mL⁻¹. Moreover, the LODs for the magneto-actuated electrochemical immunosensor in water (Figure 4.3) and hemodialysis water was found to be the same, 4.8×10^3 (n=8).

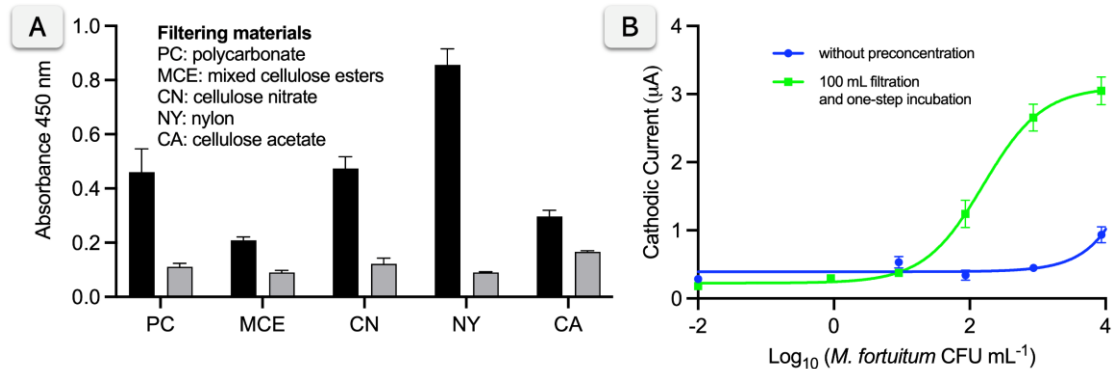


Figure 4.4. Panel A. Study of the filtering material by the filtration of 100 mL samples containing *M. fortuitum* ($2.4 \cdot 10^2$ CFU mL⁻¹) as well as the negative controls (0 CFU mL⁻¹). All the filters were 0.45 μm pore size and 25 mm of diameter. The filtering materials were polycarbonate (PC), mixed cellulose ester (MCE), cellulose nitrate (CN), nylon (NY) and cellulose acetate (CA). Panel B) Calibration plot for the magneto-actuated electrochemical immunosensor in hemodialysis samples. The blue solid line shows the calibration plot ranging from 0 to 8.7×10^4 CFU mL⁻¹ without the integration of the novel preconcentration method ($R^2 = 0.9967$). The green solid line shows the calibration plot ranging from 0 to 8.7×10^4 CFU mL⁻¹ ($R^2 = 0.9906$) with preconcentration by filtering 100 mL hemodialysis samples with nylon membranes. In all cases, a one one-step incubation of the reagents and the electrochemical readout in the cartridge with the handheld device was performed. The error bars show the standard deviation for n=3.

This approach allows the detection of low concentrations in high-volume samples that is one of the bottlenecks in the detection of environmental bacteria, in around 60 min (including filtration, which takes only 2 min). A similar LOD (13 CFU mL⁻¹) was previously reported by our research group in a magneto-ELISA, but in 3 h ¹¹. However, the new method offers several key advantages over to time-consuming and labor-intensive ELISAs. It streamlines the process by integrating immunomagnetic separation and labeling into a single step, significantly reducing the analysis time and minimizing washing steps. User intervention as pipetting is minimized through a user-friendly cartridge design, enhancing accessibility and reproducibility. The incorporation of handheld electrochemical readout makes on-site testing possible, offering portability and immediacy, crucial for point-of-care

and field-based applications. The readout time is further reduced through several steps: the direct introduction of the modified-MPs particle conjugate into the cartridge, the subsequent washing, the addition of enzymatic substrates (using the same solution) within the cartridge, and the subsequent electrochemical measurement, accomplished in less than 30 seconds. This represents a 10-fold reduction in time in comparison with an immunoassay with optical readout, where the substrates require a minimum incubation time between 15 and 30 minutes¹¹. The Table 1.2 (§1) provides a more comprehensive overview of the proposed method in relation to other methods including standard culturing and PCR, enhancing the main features. The preconcentration strategy not only eliminates preenrichment and DNA extraction or amplification techniques but also enables detection of intact mycobacteria in a streamline process. In earlier studies, we explored several strategies to improve LOD, such as incorporating a short preenrichment step or amplifying the genetic material after the IMS. In the current study, we have achieved a remarkable 10^3 -fold improvement in the LOD, reducing it from 4.8×10^3 CFU mL⁻¹ to 5 CFU mL⁻¹.

The Advancement Medical Instrumentation (AAMI)¹⁸, the European Pharmacopeia (ISO13959, 2014)¹⁹ or the Japanese Society for Dialysis Therapy (JSDT)²⁰ establish as maximum level of heterotrophic bacteria 100 CFU mL⁻¹ of total viable counts in conventional hemodialysis water. On the other hand, the Centers for Medicare and Medicaid Services (CMS), Department of Health and Human Services set the regulations in 200 CFU mL⁻¹ as a maximum allowable limit, while urgent action should be taken from 50 CFU mL⁻¹ ²¹. These values underscore the effectiveness of the method as a monitoring tool for conventional dialysis water and dialysis fluids. Notably, the calculated limit of detection is as low as 5 CFU mL⁻¹, and a remarkable signal-to-background ratio of 6.8 is achieved for 86 CFU mL⁻¹. This is achieved using a portable device powered by batteries in only 1 h and requiring minimal intervention from the end-user.

4.5. Conclusions

The presence of non-tuberculous mycobacteria in water distribution systems constitutes a critical global health concern, owing to its link with an increased

incidence of NTM-associated pulmonary diseases and related complications. Instances of these bacteria being detected in drinking water and hemodialysis systems were reported several times^{8,22–25}. The innovative aspects of the strategy presented here for *M. fortuitum* integrates key components aiming to achieve simplification and portability while maintaining outstanding analytical performance. In conclusion, the method offers a preconcentration strategy that combines magnetic particles and filtration, facilitating efficient capture and concentration of bacteria via immunomagnetic separation. In the standard microbiological method, after sample filtration, the retained bacteria are resuspended or extracted from the filter using special buffers, vortexing, centrifugation or ultrasonication. In this study, the preconcentration strategy is integrated to the electrochemical biosensing. By filtering high volumes of samples and subsequently employing magnetic actuation to pull out the bacteria directly from the filter, the need for resuspension or additional steps for downstream analysis was eliminated. Other further source of analytical simplification relies on the integration of immunomagnetic separation and labeling into a single step in only 60 minutes, significantly reducing analysis time in contrast to the conventional two-week timeframe needed for traditional isolation and culturing methods. The user-friendly cartridge design minimizes user intervention, enhancing ease of use and reproducibility. Moreover, the handheld electrochemical readout powered by batteries enables on-site and point-of-care testing, marking a significant advance in electrochemical biosensing applications.

4.6. Supplementary material

4.6.1. Chemicals and biochemicals

The composition of the buffers used in this work are described below:

For covalent immobilization of antibodies on the magnetic particles

- Borate buffer: 100 mmol L⁻¹ H₃BO₃, pH 9.0
- Ammonium sulphate buffer: 3 mol L⁻¹ (NH₄)₂SO₄ dissolved in 100 mmol L⁻¹ H₃BO₃ pH 9.0
- Blocking buffer: 500 mmol L⁻¹ glycine dissolved in PBS pH 8.0
- Washing buffer: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, 0.1% (w/v) BSA

- Storage buffer: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, pH 7.4 plus 0.05% (v/v) Tween 20®, 0.02% (w/v) sodium azide

For sample incubation and washing

- PBST: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, 0.015% (v/v) tyloxapol, pH 7.4
- Washing buffer: 10 mmol L⁻¹ Na₂HPO₄, 0.137 mol L⁻¹ NaCl, 0.2% (w/v) Bovine serum albumin (BSA), 0.015% (v/v) tyloxapol, pH 7.4

For electrochemical readout

The reagents used for the electrochemical measurement includes hydroquinone and hydrogen peroxide 30% (v/v) solution (Catalogue no. H9003 and 31642 from Merck, respectively).

- ePBS: 100 mmol L⁻¹ Na₂HPO₄, 100 mmol L⁻¹ KCl, pH 7.0,
- Cartridge solution: ePBS, 2 mmol L⁻¹ Hydroquinone, 4 mmol L⁻¹ H₂O₂ (30% (v/v))

For optical readout

The reagents used for the optical measurement of the enzymatic reaction includes hydrogen peroxide and TMB (3,3',5,5'- tetramethylbenzidine) solutions (TMB Substrate Kit, Ref. 34021, Thermo Scientific Pierce). Also, a 2.0 M solution of sulfuric acid was prepared and used to stop the TMB reaction in the magneto-actuated immunoassay.

4.6.2. *Mycobacterium fortuitum* culture

The strain of *Mycobacterium fortuitum* ATCC 6841 was growth in Tryptic Soy Broth (TSB) medium supplemented with 0.015% (v/v) of tyloxapol at 37 °C for 72 h in continuous agitation, until reaching an OD of 1.000 AU at 550 nm. The liquid culture was aliquoted and preserved supplemented with 20% (v/v) of glycerol at -40 °C. The concentration of the stock was calculated by CFU counting in Tryptic Soy Agar (TSA) plates, after the incubation for 5-7 days at 37 °C. The concentration of viable cells was quantified for each experiment by CFU counting, as shown in Figure 4.5.

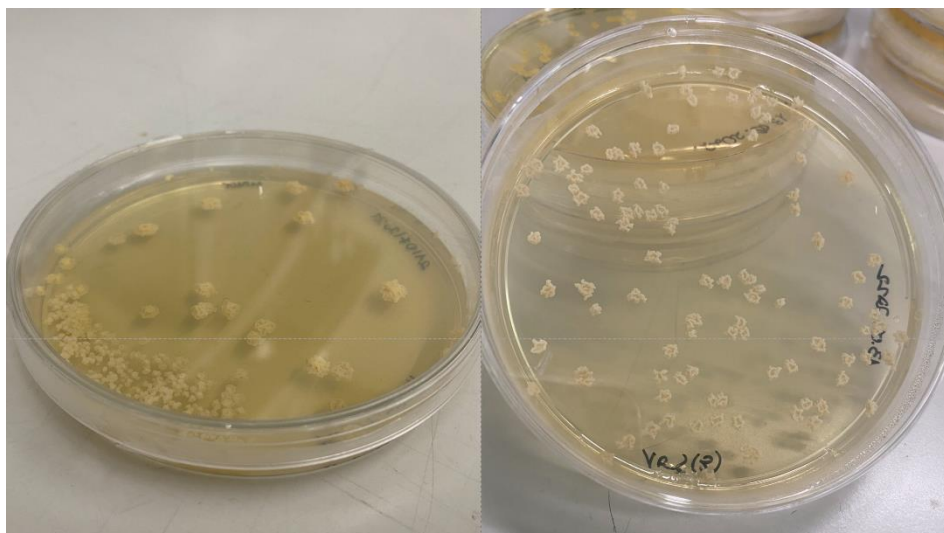


Figure 4.5. *Mycobacterium fortuitum* ATCC 6841 was grown in Tryptic Soy Agar (TSA) plates. The concentration of *M. fortuitum* was calculated for each experiment by solid culture colony counts.

4.6.3. Quantification of the level of biotin incorporation to anti-TBS antibodies

After completing the biotinylation procedure to attach biotin molecules to the anti-TBS antibodies, the extent of biotin incorporation was determined using the manufacturer's recommended protocol. Following the biotinylation step, the protein was purified using desalting columns to remove any remaining unreacted NHS-Biotin molecules. As outlined in the protocol, 180 μ L of the HABA/Avidin solution was added to a well in a microplate, and the absorbance was measured at 500 nm (HABA\Avidin). Then, 20 μ L of anti-TBS-BIO antibodies were added to the well and mixed using an orbital mixer. The absorbance at 500 nm was measured once a stable value had been attained for at least 15 seconds, and the reading of the complex HABA/Avidin/Biotinylated sample was recorded again. The following values were obtained in this instance:

$$A_{500} \text{ HABA\Avidin} = 0.5916$$

$$A_{500} \text{ HABA\Avidin\Biotin Sample} = 0.5092$$

After the calculation, the value obtained for the biotinylation of anti-TBS antibodies was found to be 5.91 biotin molecules per IgG molecule.

4.6.4. Covalent immobilization of anti-LAM antibodies

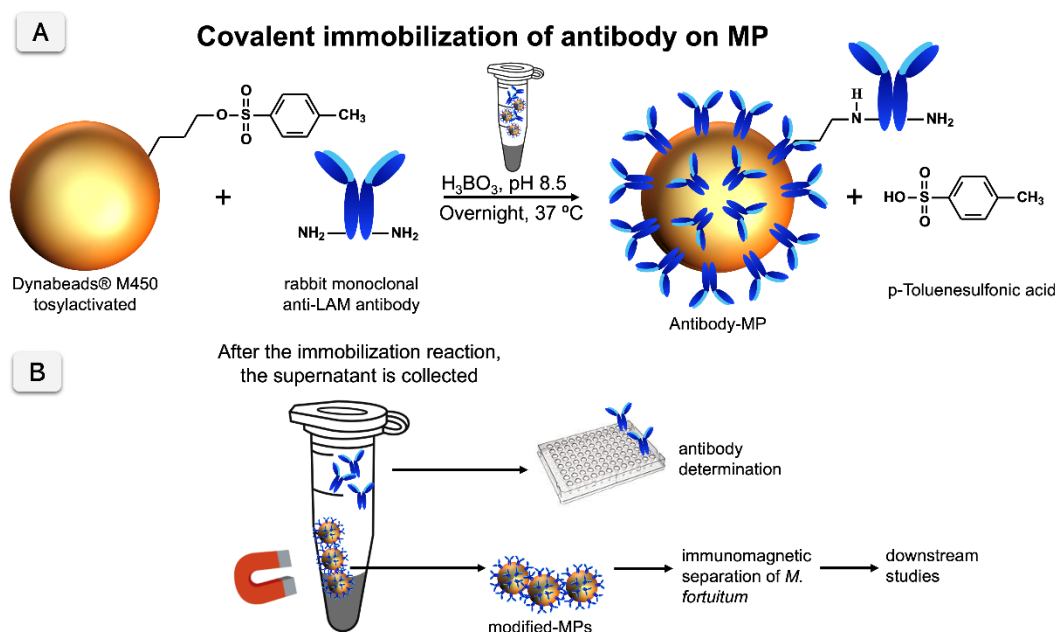


Figure 4.6. Schematic representation of the tailored covalent immobilization of antibodies on magnetic particles, followed by the determination of the coupling efficiency by determining the remaining antibodies on the supernatant by ELISA.

Briefly, a volume of 40 μL of MPs was washed twice with 1 mL of borate buffer followed by the addition of 8 μg of antibody and 100 μL of ammonium sulphate buffer performing a total volume of 250 μL with borate buffer. MPs were incubated under continuous agitation for a total reaction time of 20 h at 37°C. After the incubation time, MPs were separated with a magnet and the supernatant was stored for subsequent quantification of remaining antibodies, as detailed in Figure 4.6, panel B (Supp. Data). The coupling efficiency was determined as 98.3 %. Then, they were washed twice with washing buffer and resuspended in 250 μL of 500 mM glycine for 4 h at 37°C under continuous agitation to block the unreacted tosyl groups. The prepared anti-LAM-MPs were washed three times and were resuspended in 160 μL of storage buffer to store them at 4°C. The final concentration of the anti-LAM-MPs was $1 \times 10^8 \text{ MP mL}^{-1}$ and were previously washed three times with washing buffer before used in each experiment.

Using direct adsorption ELISA, the concentration of the unbound antibodies following immobilization was determined by preparing a calibration curve using the same anti-LAM antibody immobilized in the magnetic particles. So, the initial and

final quantity of anti-LAM antibodies determined by the calibration curve in Fig. 4.7, were compared to calculate the percentage of antibody covalently coupled or the immobilization efficiency in the MPs, and it was found to be 98.3% according to Equation 4.1.

Initial concentration of anti-LAM antibodies in the immobilization procedure, $C_0 = 32.0 \mu\text{g mL}^{-1}$

Final concentration of anti-LAM antibodies in the immobilization procedure, $C_F = 5.57 \times 10^{-1} \mu\text{g mL}^{-1}$

$$\% \text{ of immobilization efficiency} = \frac{C_0 - C_F}{C_0} \times 100 \quad (\text{Equation 4.1})$$

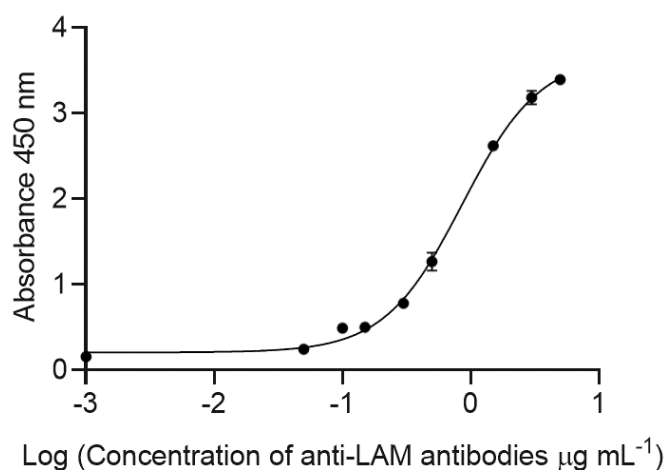


Figure 4.7. Calibration plot for anti-LAM antibodies ($n=3$). The supernatant was quantified using direct adsorption ELISA to calculate the unbound antibodies and calculate the % of coupling efficiency.

4.6.5. Magneto-actuated immunoassay for the quantification of *Mycobacterium fortuitum*. Optimization of the reagent concentration

Magneto-actuated immunoassay for optimization of reagents was performed in polypropylene 96-well microtiter plates (Ref. no 153364, Corning) while the optical readout in polystyrene 96-well microtiter plates (Ref. no. 260836, Thermo Fisher Scientific). The magnetic actuation was achieved with a 96-well plate magnet (Product no. 21358, Thermo Fisher Scientific) and the 16-tube magnet (Product no. 12321D, Thermo Fisher Scientific). The optical readout was performed on the

spectrophotometer Infinite® M200 PRO with Magellan v4.0 software, and the absorbance measurement in all cases done at 450 nm.

The reagent concentrations for the magneto-actuated immunosensor were optimized to achieve the highest signal-to-noise ratio. Initially, the concentration of the modified magnetic particles with anti-LAM antibodies and enzymatic conjugate was optimized using an optical readout (magneto-actuated immunoassay). 100 μL of anti-LAM-MPs at different concentrations (1×10^5 , 5×10^5 , 1×10^6 , 5×10^6 MP mL⁻¹) were tested in combination with 100 or 200 μL of enzymatic conjugate anti-TBS-BIO ($20 \mu\text{g mL}^{-1}$) and streptavidin-HRP (2.5 U mL^{-1}). Then 100 μL of positive or negative control (2.4×10^5 and 0 CFU mL⁻¹ of *M. fortuitum*, respectively) were added to a final volume of 1000 μL with PBST in 2.0 mL eppendorf tube. After 60 min of incubation, the samples were washed with washing buffer and then transferred to a microplate. A volume of 100 μL of substrates of TMB (3,3',5,5'-Tetramethylbenzidine) and H₂O₂ were added to the wells to drive the catalytic reaction and after 30 min, 100 μL of H₂SO₄ 2.0 M was added to stop the reaction. The addition of H₂SO₄ serves to lower the pH abruptly, effectively stopping the enzymatic reaction by denaturing the enzyme, stabilizing the color developed at a specific time point and allowing for more accurate measurements without further changes in color development. The absorbance was read at 450 nm and the signals were compared, as shown in fig. 4.8.

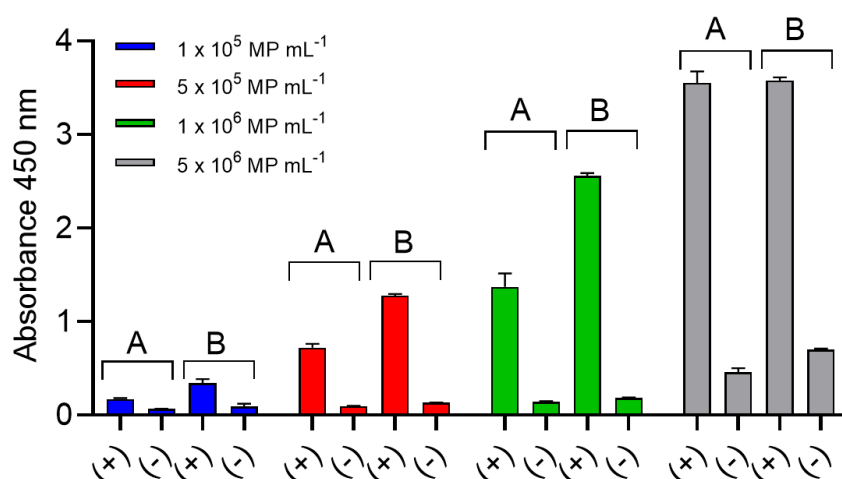


Figure 4.8. Optimization plot for the concentration of increasing amount of anti-LAM-MPs (from 1×10^5 to 5×10^6 MP mL⁻¹), as well as A) 100 μL or B) 200 μL of the enzymatic conjugate anti-TBS-BIO ($20 \mu\text{g mL}^{-1}$) and streptavidin-HRP (2.5 U mL^{-1}). The positive control was performed at 2.4×10^5 CFU mL⁻¹ *M. fortuitum*. In all the cases, the negative control at the same condition was processed. (n=3).

The final concentrations used for the magneto-actuated immunoassay were: 100 μL of 1×10^6 MP mL^{-1} of anti-LAM-MPs and 200 μL of the conjugated anti-TBS-BIO ($20 \mu\text{g mL}^{-1}$) and streptavidin-HRP (2.5 U mL^{-1}).

4.6.6. Study of the analytical simplification and the incubation in one single-step

With the aim of reducing the number of steps and minimizing pipetting, two incubation strategies were compared (see Table 4.1): the step-by-step method and the one single-step incubation approach. The incubation time for the one single-step was also studied at 15, 30, 45 and 60 min incubation at *M. fortuitum* concentration ranging from 0 to 1.3×10^6 CFU mL^{-1} . In all cases, the signals were obtained by optical readout using the optimized concentrations for the magneto-actuated immunoassay and processing 100 μL of sample, as described in §4.6.5 (Supp. data).

While the step-by-step method took 90 min including the washing steps, the one single-step incubation was conducted in 60-min. In addition to time savings, the outcomes presented in Figure 4.9, Panel A, highlight the advantages of employing the one single-step incubation method. Moreover, the results showed an enhanced signal when utilizing the one single-step strategy rather than the step-by-step. Furthermore, the highest signal was achieved within 60 min of incubation, contrasting with 15, 30, and 45 min as shown in Figure 4.9, Panel B. Further experiments were conducted with 60 min incubation, although it is important to highlight the good performance of the method even with 15 min incubation.

Table 4.1. Summary of the experimental procedure features for analytical simplification.

Time	Step-by-step incubation	One single-step incubation
60 min	MPs + bacteria (IMS)	MPs + bacteria (IMS) + anti-TBS-BIO & streptavidin-HRP
10 min	2 washings steps	-
30 min	Anti-TBS-BIO/streptavidin-HRP	-
30 min / 2 min	Optical/electrochemical readout	Optical/electrochemical readout

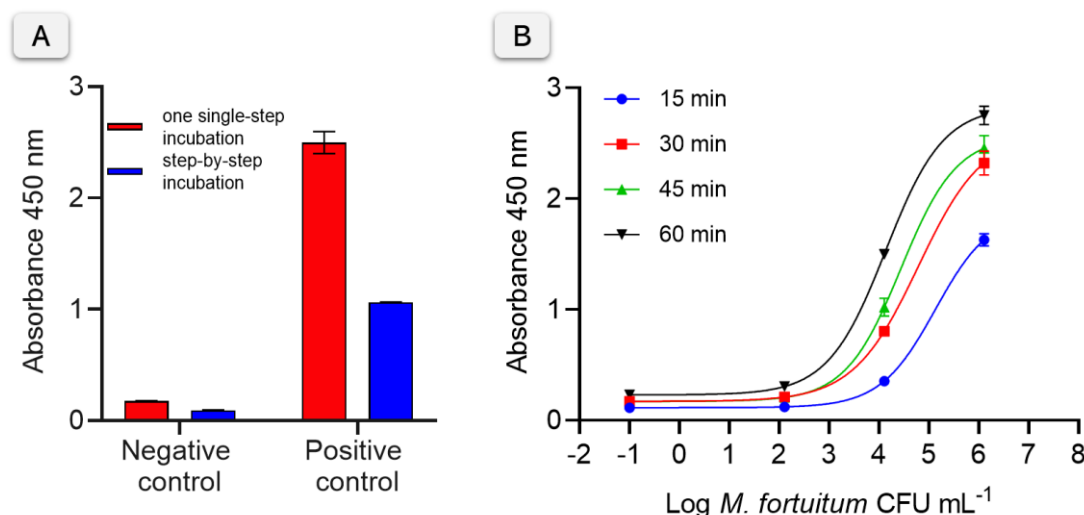


Figure 4.9. Panel A. Comparative bar plot for the one single-step incubation and step-by-step incubation for detecting *M. fortuitum* 1.6×10^5 CFU mL⁻¹. Panel B. Calibration plot for the one single-step at different incubation time at concentrations of *M. fortuitum* ranging from 0 to 1.3×10^6 CFU mL⁻¹. In all cases, the negative control was also tested. The *M. fortuitum* concentration was determined by CFU counting in solid culture. (n=3).

4.6.7. Electrochemical magneto immunosensing for the quantification of *M. fortuitum*. Optimization of the reagent concentration

Different concentrations of the enzymatic conjugate anti-TBS-BIO/streptavidin HRP were tested using 1×10^7 MP mL⁻¹ anti-LAM-MPs. 200 μ L of enzymatic conjugate anti-TBS-BIO/streptavidin-HRP at different concentrations (20 μ g mL⁻¹ with 2.5 U mL⁻¹, 40 μ g mL⁻¹ with 5 U mL⁻¹ and 60 μ g mL⁻¹ with 7.5 U mL⁻¹, of anti-TBS-BIO and strep-HRP respectively) were tested with 100 μ L of anti-LAM-MPs at 1×10^7 MP mL⁻¹. Then 100 μ L of positive or negative control (4.2×10^5 and 0 CFU mL⁻¹ of *M. fortuitum*, respectively) were added until reach a total volume of 1000 μ L with PBST in a 2.0 mL eppendorf tube. After 60 min of incubation, the samples were resuspended in 200 μ L and transferred to the cartridge. The supernatant was discarded, and a washing was done with 3 drops (150 μ L) of ePBS MIX. The solution was removed, and 3 drops (150 μ L) added again for the further amperometric measurement under enzyme saturation conditions at -0.15V and 30 s. The results were shown in in fig. 4.10 panel A.

After the optimization of the conjugated anti-TBS-BIO and strep-HRP, different concentrations of anti-LAM-MPs were tested at 1×10^5 , 5×10^5 , 1×10^6 , $5 \times$

10^6 and 1×10^7 MP mL⁻¹, following the procedure described before. The results were shown in Figure 4.10, panel B.

The final concentrations used for the electrochemical magneto-immunosensor were: 100 μ L of 1×10^7 MP mL⁻¹ of anti-LAM-MPs and 200 μ L of the conjugated anti-TBS-BIO (40 μ g mL⁻¹) and streptavidin-HRP (5 U mL⁻¹).

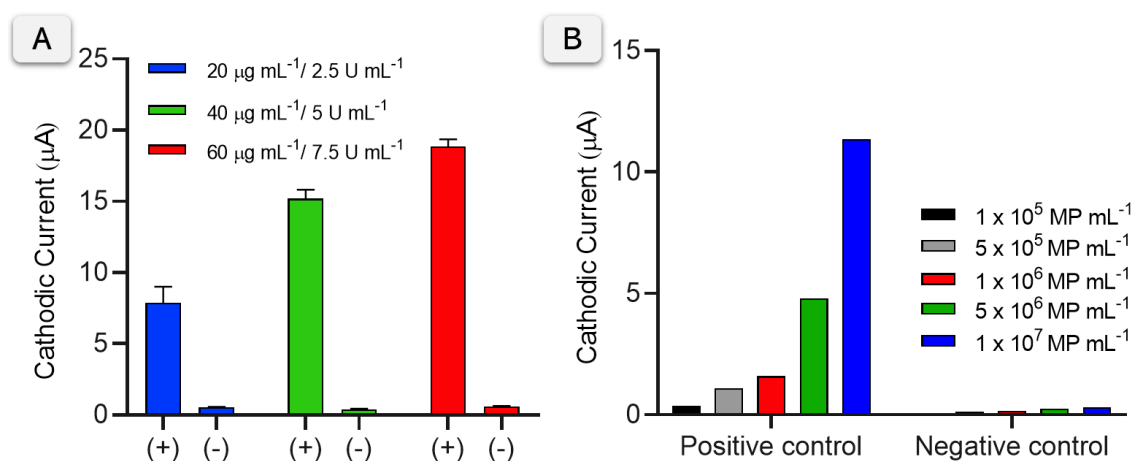


Figure 4.10. Panel A. Optimization plot for the concentrations of the enzymatic conjugate anti-TBS-BIO/strep-HRP for detecting *M. fortuitum* 1.6×10^5 CFU mL⁻¹ (n=3). Panel B. Optimization plot for the concentration of anti-LAM-MPs concentration for detecting 6.9×10^5 CFU mL⁻¹ of *M. fortuitum*. In all cases, the negative control was also tested. The *M. fortuitum* concentration was determined by CFU counting in solid culture.

4.6.8. Amperometric readout

The conjugated of anti-TBS-BIO antibodies with streptavidin labelled with the HRP enzyme, was used as electrochemical reporter in the presence of hydrogen peroxide (H₂O₂) as a substrate of HRP and hydroquinone (HQ) as a mediator.

HRP catalyze the transfer of two electrons from HQ to H₂O₂ to generate water and benzoquinone (BQ), the oxidized form of HQ. First, the HRP is oxidized catalyzing the reduction of H₂O₂ to H₂O, then the HRP is reduced again by oxidizing HQ to BQ. Thus, the final readout at the surface of the electrode is based on the reduction of the BQ to HQ (Figure 3.10, panel B). Thus, since the enzyme works at saturated substrate conditions, HRP works at maximum speed and turn-over range, providing a directly proportional signal to the sample (hence the higher the number of bacteria with anti-TBS-BIO and strep-HRP, the higher the signal).

Once the simultaneous incubation was finished, the sample performed by the anti-LAM-MPs capturing the bacteria and the conjugated anti-TBS-BIO and strep-HRP was recovered in a 2.0 mL tube with a total of 200 μ L of washing buffer and directly transferred to the cartridge (BioEcllosion SL). After few seconds in open position, the supernatant is eliminated, and 3 drops of cartridge solution was added. The cartridge is then put in close position and 3 drops of cartridge solution were added again to instantly start the measurement. The total volume of the cartridge solution containing the HQ and H₂O₂ in excess to perform the electrochemical readout was approximately equivalent to 50 μ L. The cathodic current was measured with the potential set at -0.15 V upon 30 s.

4.6.9. Novel preconcentration method. Filtration, immunomagnetic separation and electrochemical immunosensing. Study of the filtering material

A preconcentration strategy is integrated to the electrochemical immunosensing to improve the LOD, by filtering high volume of samples and further immunomagnetic separation to pull-out the bacteria from the filter for downstream analysis. Briefly, the procedure combines three steps as described in Figure 4.1: i) Filtration of large volumes (typically 100 mL) of sample followed by ii) the immunomagnetic separation of *M. fortuitum* retained in the filters with the anti-LAM-MPs and incubation with the enzymatic conjugate anti-TBS-BIO/strep-HRP in a one-step incubation and ii) the electrochemical readout in 30 sec.

The experimental details are shown in Figure 4.12 (Supp. Data). After filtration from 100 mL sample under vacuum (panel A), the 25 mm diameter filter was placed on a 2.0 mL tube (panel B). 100 μ L of anti-LAM-MPs at 10^7 MP mL⁻¹, 200 μ L of the enzymatic conjugate anti-TBS-BIO/streptavidin-HRP and 700 μ L of PBST were added to the filter (panel C) in the optimized concentrations (as described in §4.6.7), and incubated under gentle rotation at RT for 60 min (panel D). Then, the MPs labelled complexes with the captured bacteria were recovered under magnetic actuation and resuspended in 200 μ L of washing buffer for further transfer to the cartridge (panel E to I). Then, the electrochemical readout was performed as described above in §4.6.8.

In order to validate the approach, different materials were assessed. Polycarbonate (PC), nylon (NY), cellulose acetate (CA), cellulose nitrate (CN), and mixed cellulose ester membranes (MCE) were selected because of their widely use in microbiological filtration techniques with a 0.45 µm porous size and 25 mm diameter. For this study, 100 mL *M. fortuitum* samples at the same concentration (2.4×10^3 CFU mL⁻¹) as well as the negative control were filtered using each membrane and submitted to downstream analysis, in this instance magneto-actuated immunoassays described in §4.6.5 and §4.6.6 (Supp. Data).

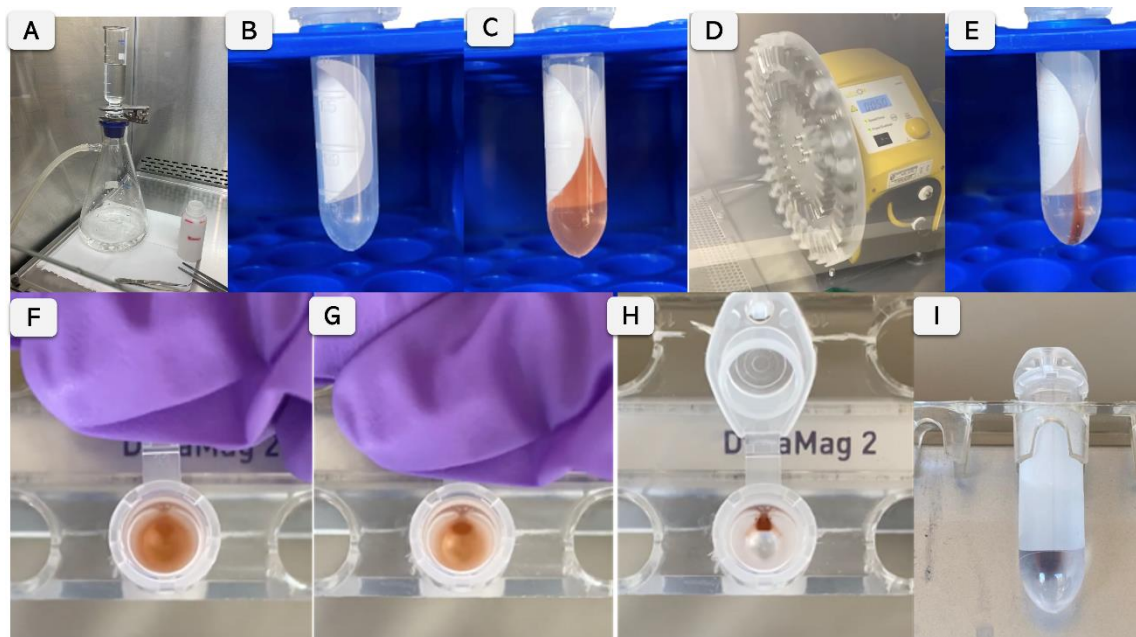


Figure 4.12. Schematic representation of A) Filtration, B to D) immunomagnetic separation of the bacteria retained in the filters, showing how the filter is positioned on the Eppendorf tube for immunomagnetic separation (B and C) under rotation (D) and (F to I) magnetic actuation. The photos (F to I) were captured in 1-second frame sequences.

4.7. References

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CHAPTER 5

*Study of the analytical performance of smartphone
for immunoassay readout in human and veterinary
health applications*

5. Study of the analytical performance of smartphone for immunoassay readout in human and veterinary health applications

5.1. Abstract

In recent years, significant progress has been reported in using smartphones devices to achieve optical readouts in medical devices. The integration of high-resolution cameras in mobile devices enables the capture of images in bioassays for further processing. These images can be further analyzed using specialized apps to extract relevant information, such as the presence of specific biomarkers. This approach offers several noteworthy advantages, including wide availability, cost-effectiveness, portability, compared to traditional and expensive benchtop instruments. Moreover, mobile devices facilitate on-site remote monitoring and data sharing, thereby enabling experts to make timely decisions. The mobile phone as reading platform combined with a mobile application for treatment imaging processing, is explored in this work. This technology is assessed through a series of protein quantification techniques and immunoassays, which produce colored solutions with maximum absorption peaks at distinct wavelengths. In addition to BCA and Bradford methods for protein quantification, conventional immunoassay for the detection of mare's antibodies in colostrum samples to control and monitoring immunodeficiencies in foals are assessed. The mobile phone is also used, as a reading platform for magneto-actuated immunoassays designed for the detection and quantification of bacteria. In all instances, a benchtop spectrophotometer was used as a reference instrument to acquire absorbance at 595, 562 nm and 450 nm. The results were then compared with the mobile phone for acquiring color intensities (purple, blue and yellow, respectively) showing very promising correlations and obtaining similar Limit of Detection for the unknown samples.

Keywords: electrochemical immunosensor, waterborne bacteria, mare antibodies, solid-phase separation, magnetic particles, preconcentration strategy

5.2. Introduction

Technological innovation is influencing many aspects of society and science, enhancing, and accelerating processes with the aim of improving the quality of life. Some advances in mobile digital devices have evolved into smaller, more portable devices that can connect to several type of networks, with long-lasting batteries integrated, such as the smartphones. The healthcare sector has also experienced the impact of this technological revolution, with trends such mobile health or mHealth. The World Health Organization (WHO) defines mHealth as ‘medical and public health practice supported by mobile devices, such as mobile phones, patient monitoring devices, personal digital assistants, and other wireless devices’^{1,2}. Ease of monitoring, data collection or mobile telemedicine are included among several advantages of mHealth^{1,2}. One particularly interesting aspect is the potential for achieving global health coverage, especially in low- and middle-income countries with hardly accessible areas and limited medical services³⁻⁵. Some studies suggest that mHealth improves the communication and the long-term follow-up, allowing the decrease in travel time and offering new forms of cost-effective healthcare⁶. Mobile devices have evolved into versatile and powerful tools for diagnosis and healthcare, enhancing efficiency, accessibility, connectivity, and precision of medical services.

Significant progress has been made in integrating mobile devices into portable tools with applications that extend beyond biomedical analysis and medical diagnosis. They are now also used in environmental monitoring and food safety⁷⁻¹⁰. The integration of smartphone-based sensors is a growing trend due to the versatility they offer. This versatility ranges from the use of specialized apps to plug-and-play sensors and more sophisticated commercial kits.

For instance, several mobile applications allow users to photograph suspicious lesions and obtaining a first evaluation using the phone camera¹¹⁻¹⁴. On the other hand, several on-site smartphone-based sensors for the measurement of air pollutants and the monitoring of air quality have been developed. All these

approaches use the phone camera as a reader, for example to determine airborne redox-actives compounds¹⁵, volatile organic compounds¹⁶, or 2,4,6-trinitrotoluene¹⁷. Several options of water quality monitoring are described also in the literature, as the determination of heavy metals in drinking water¹⁸, mapping of nitrite contamination¹⁹ or the colorimetric detection of bisphenol-A²⁰.

The integration of high-resolution cameras enables mobile devices to function as optical reading tools in bioanalytical techniques. By taking a photo of the bioassays, the captured images can be further analyzed using specialized software or applications to extract relevant information, such as analyte concentration or the presence of specific biomarkers.

In this study, the use of a mobile phone as an optical platform, in combination with a mobile application for processing signals in various bioassays, is demonstrated. The results obtained through the mobile phone are also compared to those obtained using a spectrophotometer, considered the gold standard. The selected applications are known for generating colored solutions with maximum absorbance peaks at various wavelengths, such as 450, 562, and 595 nm, leading to solutions of different colors. These solutions are then examined using both readout platforms. Additionally, in all instances, 96-well microplates serve as the assay platform for subsequent absorbance reading and imaging with the mobile phone to capture color intensities. For this purpose, the bicinchoninic acid (BCA) (562 nm) and Bradford (595 nm) assays were performed as a model of protein quantification assays for its wide use in biochemical fields, including an enzyme-linked immunosorbent assay (ELISA) (450 nm) designed for the quantification of mare's antibodies in colostrum samples for control the immunodeficiency in foals. Furthermore, the mobile phone is used as a reader platform in magneto-actuated immunoassays (450 nm) for the detection and quantification of bacteria based on the use of modified magnetic particles and a simultaneous incubation. Besides the comparison of the calibration curves obtained by each reading platform, unknown samples are analyzed to obtain the concentration using each platform and the correlation is studied. The comparison of the detection limits (LOD) calculated for each reading platform is very promising, highlighting the potential of using a mobile phone as a reading platform. Interestingly, besides the techniques employed in this

study, other methods can be employed with the mobile phone and the application, particularly those designed for 96-well microplates that yield colored solutions.

5.3. Experimental section

5.3.1. Instrumentation

The optical readout was performed with the spectrophotometer Infinite® M200 PRO using Magellan v4.0 software. The photos of the microplates were taken with the mobile phone iPhone 11 Pro (Apple, Ref. A2215) for further processing using the app Spotxel® Reader software (Sicasys Software GmbH). The images were taken using a 64 LED light box with USB power supply to control the lightning conditions with a distance from the mobile camera to the plate of 23 cm, applying the white lights (T~6500K) with a 14 W power.

5.3.2. Chemicals and biochemicals

The reagents used for protein quantification, conventional ELISA and magneto-ELISA are detailed listed in §5.6.1.2 (Supp. Data). Different set of buffers were used for each experiment, and their composition is also described in §5.6.1.3 (Supp. Data). All buffers were prepared from chemicals of analytical grade purchased from Merck and Sigma and using milliQ water.

5.3.3. Bicinchoninic Acid and Bradford Assays for protein quantification

A calibration curve was prepared for the quantification of protein samples based on the Bicinchoninic Acid (BCA) and the Bradford method, starting from a known concentration standard of BSA as a protein model, from 0 to 2000 $\mu\text{g mL}^{-1}$. In addition, 48 samples of unknown concentration of BSA were quantified and interpolated in the curves obtained by each method. The detailed protocol and fundamentals for BCA is described in Figure 5.4 (Supp. data). The optical readout was performed at 562 nm with the spectrophotometer. Images were also taken with the mobile phone to quantify the purple color intensity with the mobile app. The detailed protocol and fundamentals for Bradford assay is described in Figure 5.5

(Supp. data). In this instance, the optical readout was performed at 595 nm with the spectrophotometer and the images were quantified by the blue color intensity with the mobile app.

5.3.4. Enzyme-linked immunosorbent assay for quantification of IgG in colostrum from mares

The detection of IgG in colostrum samples was used as model for enzyme-linked immunosorbent assay (ELISA). More details about the ELISA protocol are described in 5.6 (Supp. Data). A calibration curve from a colostrum pool with a known concentration of antibodies was performed in a range of 0 to 8.41 $\mu\text{g mL}^{-1}$. In addition, 43 colostrum unknown samples were quantified and interpolated in the curve. The signals obtained were quantified by measuring the absorbance at 450 nm with the spectrophotometer and photos of the microplate were taken with the mobile phone to quantify the yellow color intensity with the mobile app.

5.3.5. Magneto-actuated immunoassay for the detection of *E. coli*

Escherichia coli (CECT 405, ATCC 10536) was used as a model of gram-negative bacteria. The bacteria were grown in a liquid culture at 37 °C for 24 h for *E. coli* until reaching an OD of 1.000 AU at 550 nm. The bacteria were inoculated in PBS for the preparation of the samples and the concentration was calculated by colony counts verification in solid culture. The quantification of bacteria in liquid samples using a magneto-actuated immunoassay was used as a model in order to evaluate the versatility of the mobile phone as a portable optical reader. A simultaneous magneto-ELISA for the detection of *E. coli* was performed and the details of the procedure are described in 5.6.5 (Supp. Data). Briefly, one-step incubation of all the reagents, including antiEcoli-MPs and the enzymatic conjugate antiEcoli-HRP antibodies and 100 μL of *E. coli* samples was performed in agitation at RT for 1 h, followed by washing and the enzymatic reaction. The readout is performed either by reading the absorbance at 450 nm in the spectrophotometer and by taking an image of the microplate to quantify the yellow color intensity with

the mobile app. A calibration curve was performed from 0 to 3×10^8 CFU mL⁻¹ and 17 unknown samples were also analyzed.

5.3.6. Reproducibility study of the optical readout for the individual wells in a 96-well microplate over preconcentration method. Study of the filtering material

In order to study the reproducibility of the readout, the same solution was dispensed at the same volume in each of the wells of a 96-well microplate, in order to evaluate possible effects when taking and processing the image with the mobile application, including distance of the microplate and the incident light, position of the well within the microplate, light intensity, reproducibility of the microplate, among others factors. For that, two readout platforms were studied by preparing two bulk solutions (purple color/ λ 562 nm and yellow color/ λ 450 nm). To achieve that, a known BSA sample of 1 mg mL⁻¹ was incubated with the BCA reagent at 37°C for 30 min. Finally, 200 μ L of the same solution was dispensed in each of the 96 well of the microplate for further readout at 562 nm and purple color intensity with the mobile app.

On the other hand, HRP at 0.02 U mL⁻¹ was incubated with TMB/H₂O₂ (1:1) substrates for 10 min and the reaction was stop with acid. Finally, 200 μ L of the same solution was dispensed in each of the 96 wells of the microplate for further readout at 450 nm and yellow color intensity with the mobile app.

5.3.7. Image treatment and statistical analysis

For each of the representative colors (purple, blue and yellow), images of the microplates were taken with a light box, specially to preserve the light conditions as described in §5.6.6 (Supp. Data). The distance between the microplate and the mobile phone camera was kept constant at 23 cm, and the same lighting conditions were used, including white light (6500K) at 14W. The images were stored with a resolution of 12 megapixels and taken with the default zoom x1 by the mobile phone. The camera flash was disabled, and the focus used was automatic. The images were

stored in the internal memory and later processed with the Spotxel® Reader mobile application, as described in §5.6.6 (Supp. Data).

Briefly, the image to be processed was selected and upload and the 96-well virtual microplate was aligned with the image. The grid's distortion factor was applied, and the measuring color was selected depending on the assay. Finally, the signals were quantified to obtain the relative signal value in color. The obtained data was further analyzed with Prism v 10.0.1 (GraphPad, San Diego, USA). The readout color mode selected in the app was yellow for the immunoassays, purple for the BCA test, or blue for Bradford tests. The absorbance was also measured in the spectrophotometer corresponding to the wavelengths of maximum absorption for each assay, 450, 562, and 595 nm, respectively.

The statistical analyses were performed using Prism v 10.0.1 (GraphPad, San Diego, USA). To compare the data obtained by both readout platforms, the values were previously normalized from 0 to 100, for the minimum to the maximum value. The calibration curves were adjusted to a non-linear regression fitting, using the four-parameter logistic curve, abbreviated 4PL. Each concentration was analyzed per triplicate. The limit of detection (LOD) was calculated as considering the number of negative controls for each curve, using the one-tailed test with a significance level of $p > 0.05$ (Student's t, $p > 0.05$). The limit of quantification (LOQ) was determined by calculating the blank signal as $10\sigma_{blank} + \bar{x}_{blank}$ and interpolating the value into the curve. For the analysis of the unknown samples, the concentrations obtained from each readout method were plotted on a linear regression graph to calculate the correlation coefficient (R^2). The x-axis represented the data from the gold standard spectrophotometric method, while the y-axis represented the data from the mobile phone and app platform.

5.3.8. Biosafety considerations

The experiments were performed according to Biosafety Class 1 required for the handling of *E. coli*. All biological waste generated from the experiments were disposed in accordance with the local regulations for handling biohazards.

5.4. Results and discussion

5.4.1. Study of the mobile phone as readout platform in Bicinchoninic Acid and Bradford Assays for protein quantification

Bicinchoninic acid (BCA) assay (or Smith assay) for total protein quantification relies on the formation of a Cu^{2+} -protein complex in a basic media, followed by the reduction of the Cu^{2+} to Cu^+ .²¹ The Cu^{2+} that is reduced is proportional to the protein in the solution. Basically, two molecules of BCA chelate to each Cu^+ ion causing a change of color from green to purple with a strong absorbance at 562 nm, as shown in Figure 5.1, panel A and Figure 5.4 (Supp. data). The bicinchoninic Cu^+ complex is influenced by the number of peptide bonds, and the presence of amino acids as cysteine, cystine, tyrosine, and tryptophan side chains²².

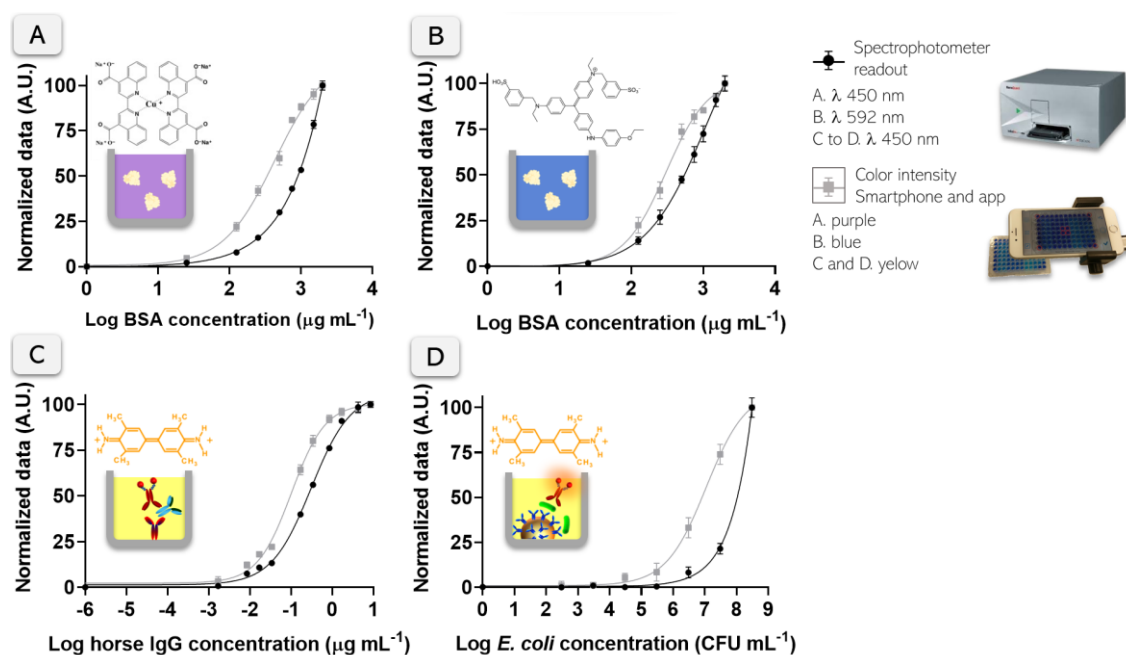


Figure 5.1. Calibration plots and fundamentals for each determination performed in 96-well microplates with the mobile phone and app platform (gray squares) compared with the gold standard spectrophotometer (black circles). Panel A. Bicinchoninic Acid for protein quantification (purple color / $\lambda = 562$ nm, respectively). Panel B. Bradford assay for protein quantification (blue color / $\lambda = 595$ nm, respectively). Panel C. Enzyme-linked immunosorbent assay for quantification of IgG in colostrum from mares. Panel D. Magneto-actuated immunoassay for the detection of *E. coli*. The readout in Panels C and D was performed in yellow color and $\lambda = 450$ nm. In all cases, $n=3$.

The assay was developed in 96-well microplates and the readout was achieved at 562 nm with the spectrophotometer and with the mobile phone in the purple color intensity mode of the mobile app, and the results are shown in Figure 5.1, panel A.

The Bradford assay is a rapid and sensitive method for measuring the concentrations of proteins. It is based on the shift in absorbance maximum of the anionic form of Coomassie Brilliant Blue G-250 dye from 465 to 595 nm following binding to arginine and lysine residues of proteins in solution²³, as shown in Figure 5.5 (Supp. data). The assay was developed in 96-well microplates and the readout was achieved at 595 nm with the spectrophotometer and with the mobile phone in the blue color intensity mode of the mobile app, and the results are shown in Figure 5.1, panel B.

In both instances, it is observed that the calibration plot obtained with the mobile phone and app platform saturate at lower concentration, which indicates a lower dynamic range. However, the signals obtained at lower concentration ranges were comparatively higher with the mobile phone than the spectrophotometer, reaching an order of magnitude less for the LOQ, especially in the case of BCA. The LOD calculated for each readout system were found to be the same order of magnitude and very similar among them for both wavelengths and colors, obtaining values as 12.3 and 22.8 $\mu\text{g mL}^{-1}$ for the BCA assay ($\lambda = 562$ nm, purple color) and, 36.7 and 45.4 $\mu\text{g mL}^{-1}$ for the Bradford assay ($\lambda = 595$ nm, blue color), for the spectrophotometer and the mobile phone, respectively.

All calculated values related with the analytical performance of both methods are comparatively summarized in Table 5.1.

The analysis of the results for the Bradford assay exhibited a good correlation of 0.8845, and up to 0.9527 for the BCA assay, as shown in Figure 5.2, panel B and A, respectively.

Table 5.1. Summary of the calculated values from the data analysis of the calibration curves obtained by the spectrophotometer and the mobile phone for each technique. The calculated parameters were the R square for the adjustment of the four-parameter logistic curve fitting, limit of detection (LOD), limit of quantification (LOQ) and the correlation of the unknown samples analyzed by each reading platform.

	Absorbance / Spectrophotometer	Color intensity /Mobile phone
BCA Assay	562 nm	Purple color
R square	0.9988	0.9944
LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	12.3 (n=8)	22.8 (n=8)
LOQ ($\mu\text{g}\cdot\text{mL}^{-1}$)	50.3	116
Correlation in calculated unknown samples (n=47)	0.9527	
Bradford assay	595 nm	Blue
R square	0.9940	0.9905
LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	36.7 (n=6)	45.4 (n=6)
LOQ ($\mu\text{g}\cdot\text{mL}^{-1}$)	138	136
Correlation in calculated unknown samples (n=40)	0.8845	
Immunoassays	450 nm	Yellow
	ELISA for quantification of IgG in colostrum from mares	
R square	0.9978	0.9958
LOD ($\text{ng}\cdot\text{mL}^{-1}$)	1.14 (n=9)	1.72 (n=9)
LOQ ($\text{ng}\cdot\text{mL}^{-1}$)	26.7	27.3
Correlation in calculated unknown samples (n=40)	0.8945	
	Magneto-ELISA for the detection of <i>E. coli</i>	
R square	0.9942	0.9937
LOD ($\text{CFU}\cdot\text{mL}^{-1}$)	$2.0\cdot 10^4$ (n=6)	$2.9\cdot 10^4$ (n=6)
LOQ ($\text{CFU}\cdot\text{mL}^{-1}$)	$1.2\cdot 10^6$	$7.7\cdot 10^5$
Correlation in calculated unknown samples (N=14)	0.9144	

5.4.2. Study of the mobile phone as readout platform in the enzyme-linked immunosorbent assay for quantification of IgG in colostrum from mares

Horseradish peroxidase (HRP) is a widely employed enzyme label in immunoassays, since it offers a range of significant advantages. These include a high turnover rate and the ability to amplify signals, increasing the sensitivity. Moreover, HRP have a long shelf life, ensuring the stability of labeled reagents. Its versatility is

another key asset, as HRP readily conjugates with many proteins, enabling diverse immunoassay formats. The enzyme compatibility with standard laboratory equipment enhances accessibility and simplifies implementation even in low-resource setting. Additionally, HRP displays low levels of nonspecific adsorption. In this instance, an immunoassay model with TMB substrate solutions, for the quantification of mare antibodies in colostrum samples was used for the monitoring of foals, based on HRP-labelled antibodies and TMB-H₂O₂ substrate, producing a yellow solution with a maximum absorbance peak at λ 450 nm. An effective transfer of antibodies from the mare through colostrum plays a pivotal role in providing the foal with sufficient passive immunity and protection against viruses and bacteria, reducing the risk of developing serious medical conditions^{24,25}. Hence, monitoring, and quantifying antibodies in colostrum are crucial for ensuring the health of foals.

In Figure 5.1, panel C it was remarkable the similar fitting of both calibration curves for the conventional immunoassay. Similar to the curves obtained by BCA and Bradford methods, the curve obtained using the mobile phone saturates before that of the spectrophotometer, slightly reducing the dynamic range. Both LOD and LOQ were very similar for the two readout platforms, obtaining values as 1.14 and 1.72 for the LOD and 26.7 and 27.3 ng mL⁻¹ for the LOQ, obtained by the spectrophotometer and the mobile phone, respectively. The analysis of the unknown samples for the ELISA also exhibited a good correlation between both reading platforms of 0.8954 (Table 5.1 and Figure 5.2, panel C).

5.4.3. Study of the mobile phone as readout platform in magneto-actuated immunoassay for the detection of *E. coli*

In this work, the ability of the mobile phone as readout platform was also evaluated based on a magneto-actuated immunoassays for the detection and quantification of *E. coli*, producing a yellow solution with maximum absorbance at 450 nm, as in the case of conventional immunoassay. The results showed a similar pattern in the calibration plots after the fitting (Figure 5.1, panel D), although the dynamic range with the mobile phone was found to be lower as the saturation of curve was reached at a lower concentration in comparison with the spectrophotometer, as described above. But as in the previous examples, at lower

concentration ranges, the signals were significantly higher for the curves obtained with the mobile phone compared to the spectrophotometer. Accordingly, the LOQ for the magneto-actuated immunoassay was found to be lower with the mobile phone readout (Table 5.1). The LOD, however, we found to be comparable between those calculated with the mobile phone and the spectrophotometer. In addition, the analysis of the samples of unknown concentration showed a good correlation between both readout platforms of 0.9144 (Figure 5.2, panel D).

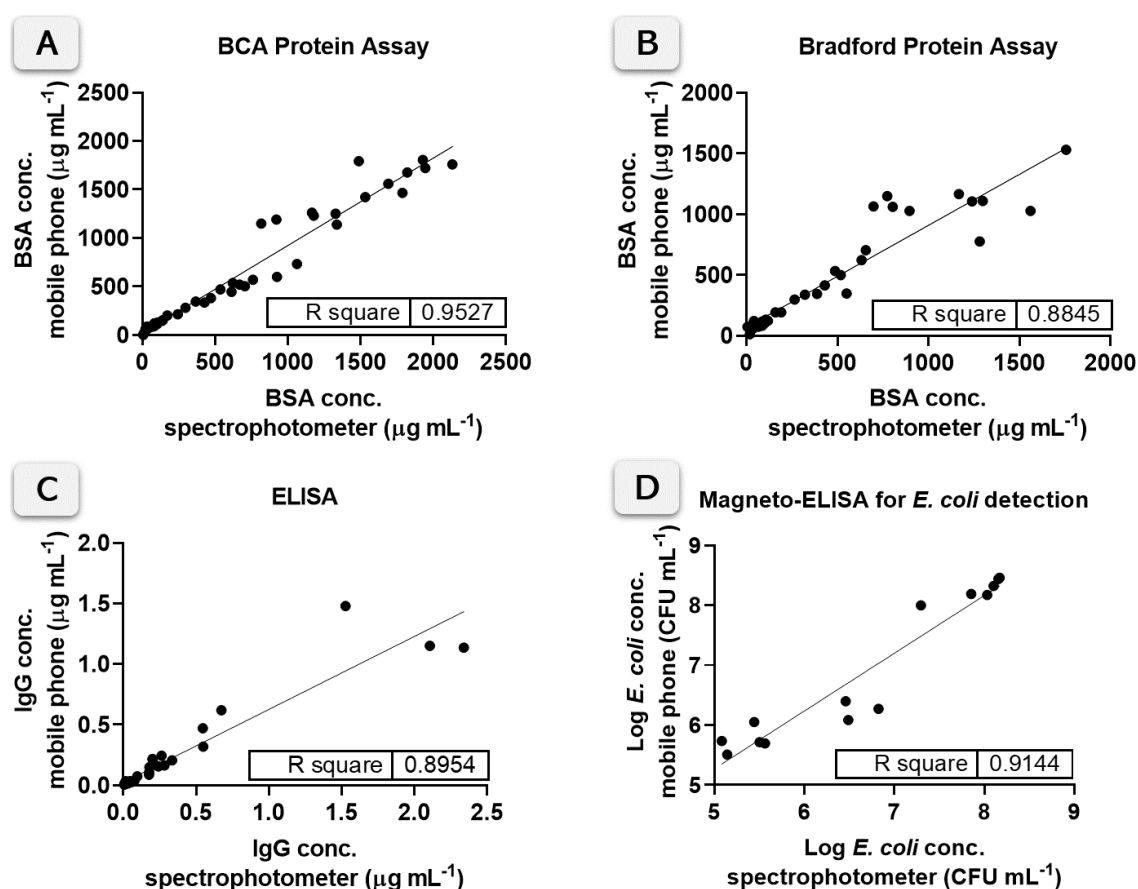


Figure 5.2. Correlation plots for each determination performed in 96-well microplates with the mobile phone and app platform (y-axis) compared with the gold standard spectrophotometer (x-axis). Panel A. Bicinchoninic Acid for protein quantification (purple color / $\lambda = 562$ nm, respectively). Panel B. Bradford assay for protein quantification (blue color / $\lambda = 595$ nm, respectively). Panel C. Enzyme-linked immunosorbent assay for quantification of IgG in colostrum from mares. Panel D. Magneto-actuated immunoassay for the detection of *E. coli*. The redout in Panels C and D was performed in yellow color and $\lambda = 450$ nm.

5.4.4. Reproducibility study of the optical readout for the individual wells in a 96-well microplate

In this work, the reproducibility of the optical readout for the individual wells in a 96-well microplate was studied at 450 (yellow) and 562 (purple) by preparing a bulk large volume solution and by dispensing the same solution on each well. In all instances, the signals were normalized in order to maximize the small differences of the plates. As the solution is the same in all cases, the differences of signals in the wells can be attributed to other systematic errors such as light conditions, microplate geometry, image acquiring or data processing among others. As shown in Figure 5.3 panel B and E, a similar circle-shaped pattern in the signal can be observed in the center of the microplates when analyzed with the mobile phone. This pattern was much more noticeable in the case of the purple color intensity (panel E) but was also slightly observed in the case of the yellow color intensity (panel B). This pattern of signals was not observed in the absorbance measurements neither for the assay in yellow color and $\lambda=450$ nm, nor with the purple color and $\lambda=562$ nm, respectively, represented in Figure 5.3, panel A and D, respectively. The signal difference in these last cases seems to be random, without specific pattern in the variation of the absorbance values. As described, the images of the microplates were taken in controlled lighting conditions, under a LED light circular-shaped. However, it must be noted that the variability between signals both in absorbance and color intensity were found to be very small, although they were magnified by normalization in Figure 5.3 to identify possible patterns. Accordingly, 0 and 100% were attributed to the minimum and maximum value obtained, respectively. In addition, as has been verified in the previous sections, the use of the mobile phone as an optical readout platform resulted very promising, obtaining calibration curves with similar fittings to those obtained with the spectrophotometer, just like the calculated LOD and LOQ.

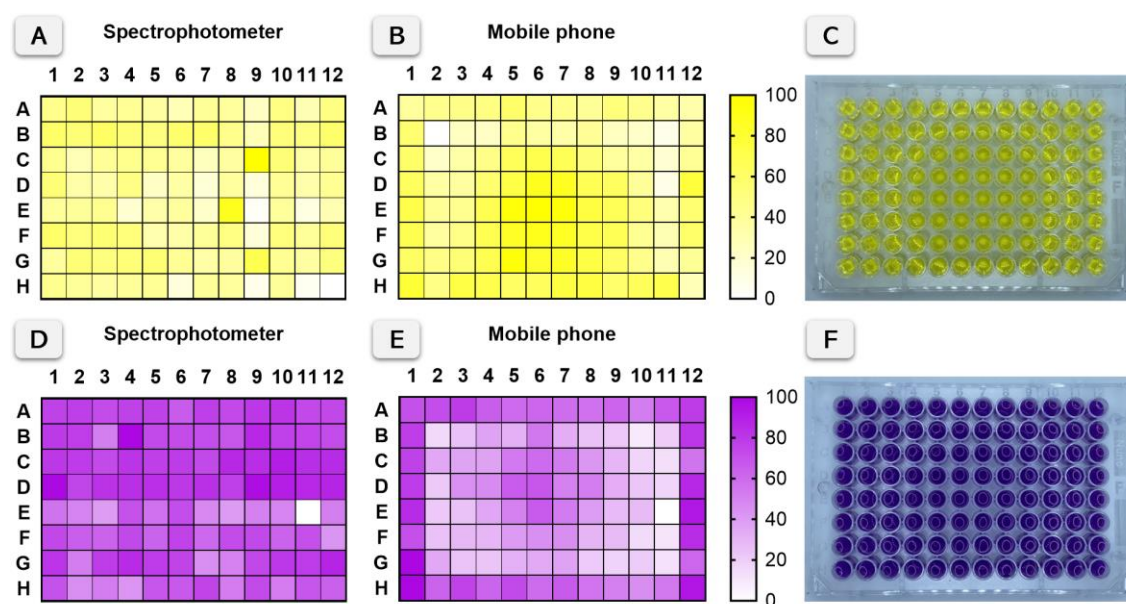


Figure 5.3. Schematic heat-map of 96-well microplates representation of the results obtained for the reproducibility test after data normalization of the absorbance with the spectrophotometer and the color intensity by the mobile phone. The panels show the results obtained for panel A) absorbance measurement at 450 nm, B) intensity of yellow color signal and C) image of the 96-well plate with the yellow solution. On the other hand, the results obtained for panel D) absorbance measurement at 562 nm, E) intensity of purple color signal and F) image of the 96-well plate with the purple solution.

5.5. Conclusions

Portability, connectivity, easy-to-use and affordability are just some of the advantages that make the use of mobile devices increasingly integrated as portable analysis tools, whether in several applications in medicine, food safety or environmental monitoring.

In this work, we have studied the use of the smartphone as an optical readout platform in techniques widely used in biochemistry, such as the BCA and Bradford protein quantification assays, also in an ELISA developed with the aim of quantifying mare antibodies in a colostrum samples for monitoring immunodeficiencies in foals, as well as in a magneto-actuated immunoassay for the detection of bacteria, as a model. For this, we used a free mobile app, which allows the direct processing, or the post upload of the microplate photos and we compared the results with the spectrophotometer, such as the gold-standard.

According to the results, the use of the mobile phone and the app was very promising, with LOD of the same order of magnitude for both readout platforms. The calculated LOQ were also similar, and again, in several cases smaller than those calculated with the spectrophotometer. The correlation between both readout platforms, calculated from the concentration of the unknown samples, exceeded values from 0.8845, observing higher deviations especially in samples of higher concentration. Moreover, a critical factor is to control the lighting conditions for taking the images, since, as verified in the reproducibility test, the incidence of external light can influence the final results.

Interestingly, these were just some examples of assays that could be processed with the mobile phone and the app. The app has different measurement color options, as well as the gray scale, making versatile this type of app and allowing to be adapted to other colorimetric tests, thus minimizing the costs associated with benchtop traditional instruments²⁶ among others.

5.6. Supplementary data

5.6.1. Instrumentations, materials, chemicals and biochemicals

5.6.1.1. Instrumentation and materials

The optical readout was performed with the spectrophotometer Infinite® M200 PRO using Magellan v4.0 software. The photos of the microplates were taken with the mobile phone iPhone 11 Pro (Apple, Ref. A2215) for further processing using the app Spotxcel® Reader software (Sicasys Software GmbH). The images were taken using a 64 LED light box with USB power supply to control the lightning conditions with a distance from the mobile camera to the plate of 23 cm, applying the white lights (T~6500K) with a 14 W power. BCA and Bradford assays were performed in polystyrene 96-well microplates (Thermo Fisher Scientific, Ref. 260836). ELISA was performed in Nunc™ MaxiSorp™ (Thermo Fisher Scientific, Ref.442404). The magneto-actuated immunoassays were performed in polypropylene 96-well microtiter plates (Ref. no 153364, Corning) while the optical readout in the polystyrene 96-well microtiter plates described before. The magnetic actuation was achieved with a 96-well plate magnet (Product no. 21358, Thermo Fisher Scientific).

5.6.1.2. Chemicals and biochemicals

The protein quantification tests were performed with the kit ThermoScientific™ Pierce™ BCA Protein Assay (ThermoFisher Scientific, Ref. 23227) and the kit Pierce™ Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific, Ref. 23200), using BSA standards (ThermoFisher Scientific, Ref. 23209). ELISAs used the following antibodies: anti-horse IgG (Fc specific) produced in rabbit (Ref. SAB3700150, Sigma-Aldrich) as a capture antibody and anti-Horse IgG H&L labelled with horseradish peroxidase (HRP) produced in rabbit (Ref. ab102405, ABCAM) for labelling. The sample used was a colostrum pool quantified by radial immunodiffusion with a total value of 16820 mg dL⁻¹ horse antibodies. M450 tosyl activated magnetic particles (Dynabeads™ M-450 Tosylactivated, Catalogue no. 14013, Invitrogen) were used for immobilizing specific antibodies to capture the bacteria: polyclonal anti-E. coli antibodies (Ref. ab137967, abcam) from rabbit. The rabbit polyclonal anti-Ecoli labelled with HRP (Ref. ab20425, abcam) was used as a secondary antibody. The HRP enzyme (Ref. 77332, Roche) was used to prepare the solutions for reproducibility assay. For all immunoassays, TMB Substrate Kit (Ref. 34021, Thermo Scientific Pierce) were used for the enzymatic reaction and color development, and a 2.0 M solution of sulfuric acid was used to stop the TMB reaction. The bacterial culture reagents were purchased from Sigma, including Luria Bertani (LB) medium (Ref. L3522) and LB-agar plates (Ref. L3147-250G) for *E. coli* growing.

5.6.1.3. Buffer and solution composition

All buffers were prepared using milliQ water with reagents of analytical grade. The composition of the buffers used in this work are described below:

Enzyme-linked immunosorbent assay for quantification of IgG in colostrum from mares

- Bicarbonate/carbonate coating buffer (100 mM): 3.03 g L⁻¹ Na₂CO₃, 6.0 g L⁻¹ NaHCO₃, pH 9.6
- Phosphate buffer solution (PBS): 100 mM Na₂HPO₄, 137 mM of NaCl, pH=7.4
- Washing buffer (PBS/BSA 0.1%): PBS with 0.1% (w/v) Bovine serum albumin (BSA)
- Blocking buffer (PBS/BSA 2%): PBS with 2% (w/v) Bovine serum albumin (BSA)

- Substrates solution: TMB/H₂O₂ 1:1 (v/v)
- Stop solution: H₂SO₄ 2.0 M

Magneto-actuated immunoassay for the detection of *E. coli*

- Phosphate buffer solution (PBS): 100 mM Na₂HPO₄, 137 mM of NaCl, pH=7.4
- Washing buffer (PBST/0.1% BSA): PBS with 0.05% (v/v) Tween 20®, 0.1% (w/v) Bovine serum albumin (BSA)
- Substrates solution: TMB/H₂O₂ 1:1 (v/v)
- Stop solution: H₂SO₄ 2.0 M

5.6.2. Procedure for the Bicinchoninic Acid for protein quantification

A calibration curve was prepared for the quantification of protein samples based on the Bicinchoninic Acid (BCA), starting from a known concentration standard of BSA as a protein model, from 0 to 2000 µg mL⁻¹. In addition, 48 samples of unknown concentration of BSA were quantified and interpolated in the calibration plot. For the BCA method, the microplate procedure recommended by the kit manufacturer was as follows. Briefly, 25 µL of each standard or unknown sample were incubated with 175 µL of the BCA reagent at 37°C for 30 minutes. Then, the plate was cooled to RT and the signals obtained were quantified in the spectrophotometer at 562 nm. Images were taken with the mobile phone to quantify the purple color intensity with the mobile app.

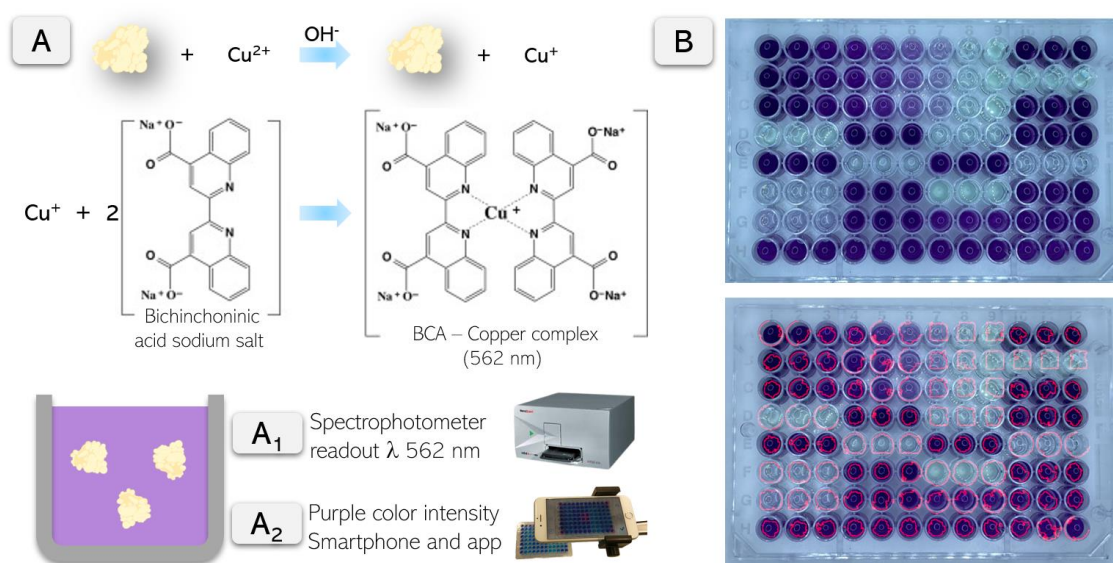


Figure 5.4. Outline of the BCA assay method for protein quantification.

5.6.3. Procedure for the Bradford assay for protein quantification

A calibration curve was prepared for the quantification of protein samples based on the Bradford method, starting from a known concentration standard of BSA as a protein model, from 0 to 2000 $\mu\text{g mL}^{-1}$. In addition, 48 samples of unknown concentration of BSA were quantified and interpolated in the calibration plot. The protocol used for the quantification of proteins with the Bradford method was based on an incubation of 5 μL of each standard or unknown sample with 195 μL of the Bradford reagent at RT for 10 minutes. Finally, the quantification of the signals obtained was carried out by measuring the absorbance at 595 nm with the spectrophotometer. Images were also taken with the mobile phone to quantify the blue color intensity with the mobile app.

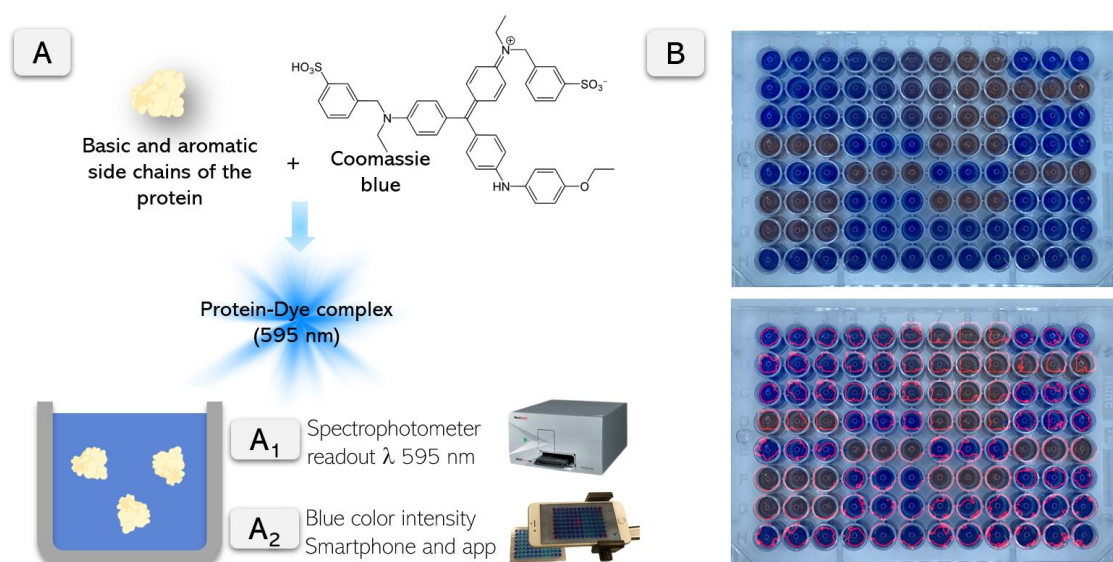


Figure 5.5. Outline of the Bradford assay method for protein quantification.

5.6.4. Electrochemical readout

In the assay, both standards of the curve and the unknown samples were processed by triplicate. The protocol was optimized previously and described as follows: i) Incubation with the capture antibody. 100 μL of anti-horse IgG antibodies at 5 $\mu\text{g mL}^{-1}$ diluted in carbonate/bicarbonate buffer were incubated overnight at 4°C. Then, a washing step was performed removing the supernatant of the microplate and adding 200 μL of PBS/BSA 0.1%. The washing solution was

removed, and the step was repeated for a total of two washings steps. ii) Blocking of the microplate. 200 μL of PBS/BSA 2% were incubated at 37°C for 1 h. Two washing steps were performed. iii) Incubation with the standard of the curve and the unknown samples. 100 μL of the sample were added for 30 minutes at 37°C. Two washing steps were performed. iv) Incubation with the labelled antibody. 100 μL of the anti-horse IgG labelled with HRP enzyme at 0.05 $\mu\text{g mL}^{-1}$ were incubated for 20 minutes at RT. Three washing steps were performed. v) Enzymatic reaction. 100 μL of TMB/ H_2O_2 (1:1) solution were incubated for 15-30 minutes at RT. The reaction was stop by adding 100 μL of H_2SO_4 2 M solution. vi) Optical readout. The absorbance was measured at 450 nm by the spectrophotometer. Images of the microplate were taken with the mobile phone to quantify the yellow color intensity with the mobile app.

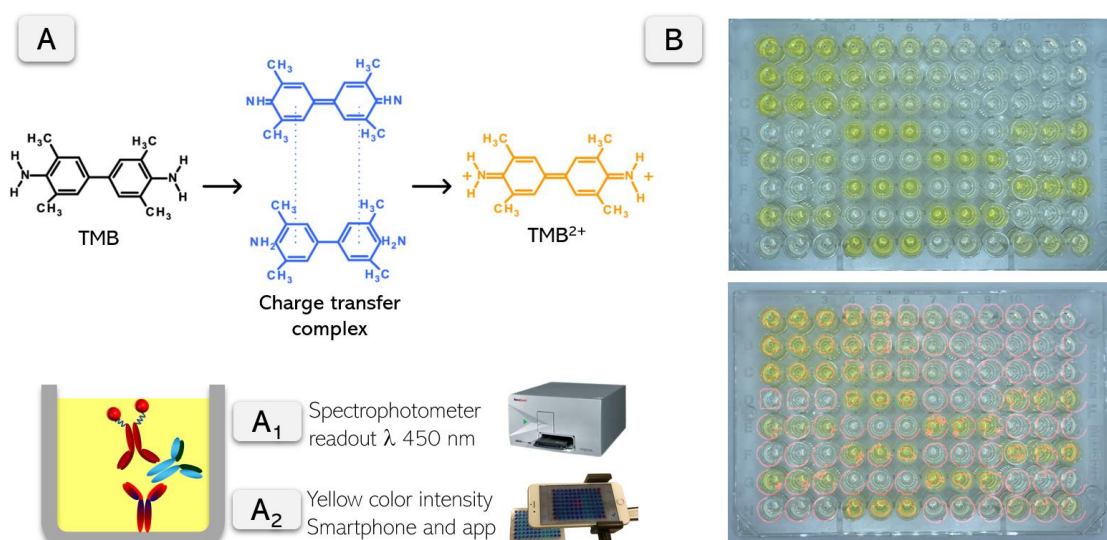


Figure 5.6. Outline of the Enzyme-linked immunosorbent assay for quantification of IgG in colostrum from mares.

5.6.5. Procedure for the magneto-actuated immunoassay for the detection of *E. coli*

The assay was performed at RT in polypropylene microplates. Both the standards of the curve and the unknown samples were processed by triplicate. The protocol was optimized previously and described as follows: i) One-step incubation. 50 μL of antiEcoli-MPs at 10^7 MP mL^{-1} , 100 μL of sample and 50 μL of anti-Ecoli

antibodies labelled with HRP enzyme were incubated simultaneously in continuous agitation for 1 h. Then, the supernatant was removed using a magnet and 200 μL of PBST/0.1% BSA was added for 3 minutes in continuous agitation. Then, the supernatant was removed again. The washing step was done per triplicate. ii) Enzymatic reaction. 100 μL of TMB/ H_2O_2 (1:1) solution were added and incubated for 30 minutes in continuous agitation. The reaction was stop by adding 100 μL of H_2SO_4 2 M solution. iii) Optical readout. The absorbance was measured at 450 nm by the spectrophotometer. Images of the microplate were taken with the mobile phone to quantify the yellow color intensity with the mobile app.

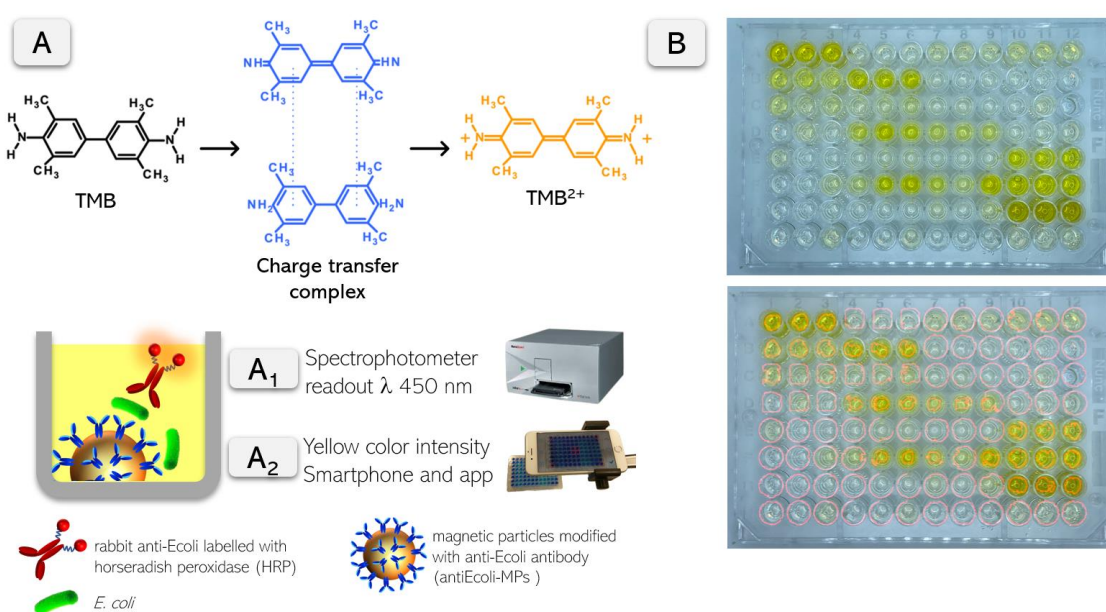


Figure 5.7. Outline of the magneto-actuated immunoassay for the detection and quantification of *E. coli*.

5.6.6. Image treatment

Once the experiments were performed, the images of 96-well microplates were taken and stored in the internal memory of the smartphone for further processing with the mobile app Spotxel® Reader. Please note that the images can be processed directly, taking the photo from the app, without the need to store and process them later. The procedure for image analysis was as described in Figure 5.8: A) Image capture. The 96-well microplate was introduced into the light box in order to preserve the light conditions and then, the mobile phone was placed in the top of the box to take the photo of the microplate. The photo was stored. B) Grid size

alignment. The image was upload in the app and the virtual 96-well microplate scheme was aligned and adjusted to the photo, so the wells in the photo must coincide with the wells of the virtual plate. C) Correction distortion factor. The correction distortion factor was applied in order to avoid the unexpected artifacts produced by the lens distortion of the camera and the different angles in the wells. D) Color selection. The measurement color was selected according to the assay: ELISA and magneto-ELISA (yellow), BCA (purple) and Bradford (blue). E) Signal detection and quantification. The work area of each well was identified by the app and the color signal calculated. F) Visualization of the results. The assay setup showed the quantification values of each well depending on the intensity of the color measurement.

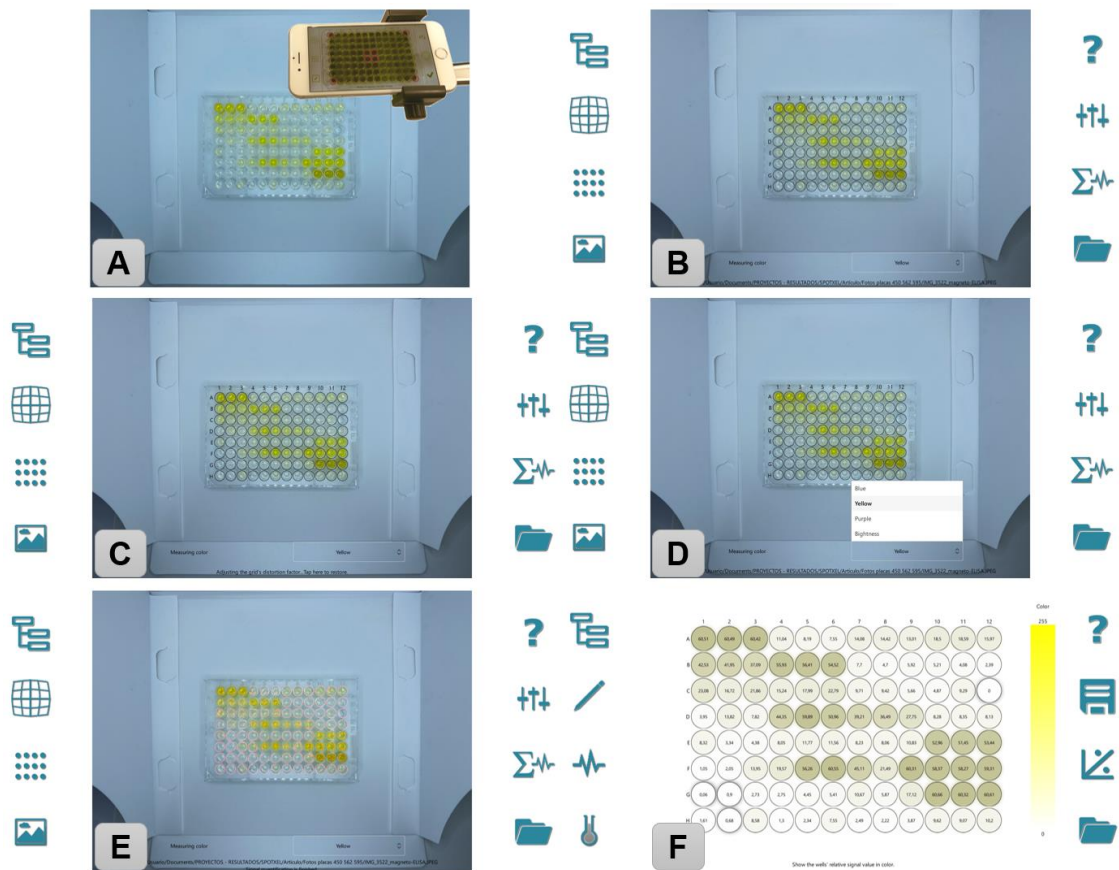


Figure 5.8. Schematic representation of the image capture and data collection of the plate assay with the app Spotxcel® Reader: A) Image capture with the smartphone in the light box, B) Alignment of the virtual microplate to the image, C) Apply distortion factor correction in order to avoid the effects produced by the camera lens' distortion and the view angles to the wells, D) Selection of the measurement color (Blue, yellow, purple or brightness), E) Signal detection after applying the distortion factor correction and adjust the grid size, F) Signal quantification results graphically reported in the virtual microplate. Figure adapted from <https://www.sicasys.de/spotxcel-reader/>.

- **Photos of the microplates obtained for each experiment**

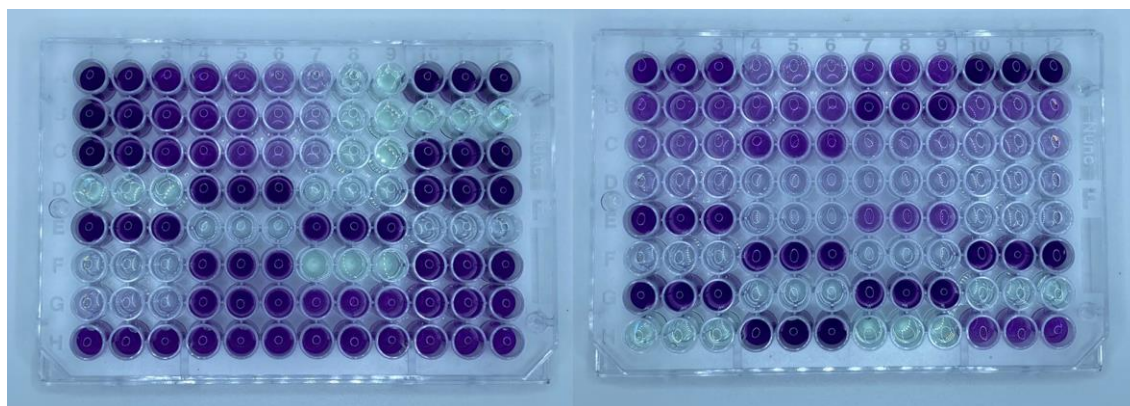


Figure 5.9. Photos taken of the microplates for the BCA assay.

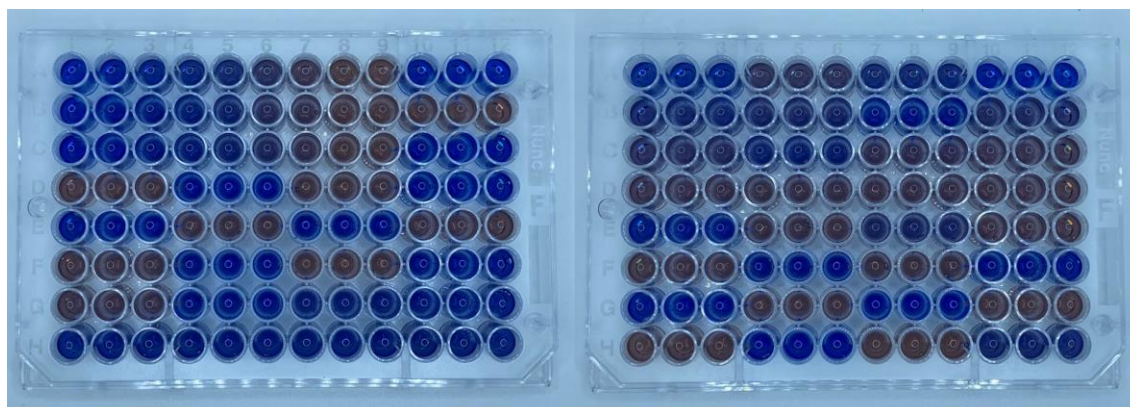


Figure 5.10. Photos taken of the microplates for the Bradford assay.

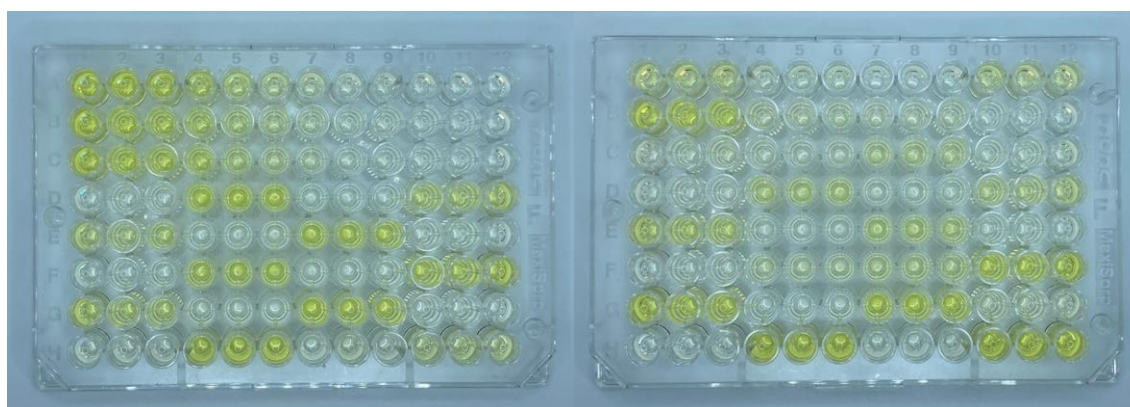


Figure 5.11. Photos taken of the microplates for the ELISA.

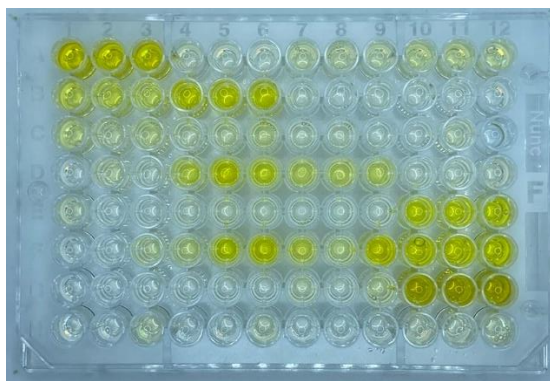


Figure 5.12. Photos taken of the microplates for the magneto-ELISA for the detection of *E. coli*.

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CHAPTER 6

*Rapid test for the amplification and
electrochemical genosensing of Mycobacterium
tuberculosis*

6. Rapid test for the amplification and electrochemical genosensing of *Mycobacterium tuberculosis*

6.1. Abstract

Tuberculosis remains one of the most causes of death in lower and middle-income countries ranking above HIV, being *Mycobacterium tuberculosis* the major causative. It was calculated 1.3 million deaths caused by tuberculosis and estimated 10.6 million people fell ill globally only in 2022. Drug-resistant tuberculosis is also a bottleneck for the diagnosis and treatment of the disease. Several rapid tests exist and are endorsed by WHO, presenting in general high accuracy and sensitivity, but some limitations are still present overall for limited-resource settings. Some limitations are related to the cost and the infrastructure needed to develop some of these tests. This work addresses the development of a rapid and portable molecular test for the screen-out of tuberculosis. Such a test can be used both in low resource settings where microbiological cultures are not available, and in high-income countries to dramatically improve turnaround times for results and reduce costs. This approach can generate billions of tagged DNA copies from a single target molecule, increasing the sensitivity, which can be rapidly detected with the RDT platform to provide the results of infection within half an hour and in low resource settings. The pandemic highlighted the role of PCR as a dominant technology for a massive laboratory testing to confirm the diagnosis of an infection disease. The diagnostic test is rapid and inexpensive, requiring minimal handling, and can be easily introduced into the general practitioner's armory for ambulatory screening of infection. Furthermore, although the first application that is envisaged for is targeted to tuberculosis infection, this approach will be also transferable to the diagnosis at community and primary-care level of other infectious diseases related with global health, thus increasing its valorization.

Keywords: double-tagged amplification, double-tagged genosensor, *Mycobacterium tuberculosis*, handheld device

6.2. Introduction

Tuberculosis (TB) remains one of the oldest diseases in the world accounted for millions of deaths annually. In 2022, it ranked as the second most deadly infectious disease due to the emergence of COVID-19, although for many years it was considered the first^{1,2}. Fortunately, advancements in research and the implementation of TB programs focusing on prevention and treatment lead to decrease the incidence of TB cases over the years. However, the 8.7% reduction in TB cases from 2015 to 2022, is still far from the WHO's End TB strategy goal of a 50% reduction by 2025¹. The disruptions caused by the COVID-19 pandemic resulted in a decrease in reported cases and services provided, hindering access to diagnosis and treatment and leading to underdiagnosis of the disease³⁻⁵. Despite these challenges, the reported TB cases in 2022 reached 7.5 million, the highest number since the first global monitoring was started in 1995 by WHO¹. Drug-resistant TB implies an additional challenge, as accurate diagnosis and appropriate treatment are essential for successfully overcoming the disease⁶. The costs faced by TB patients are very high, being a significant economical barrier to accessing and completing TB treatment¹, as mentioned in the WHO Tuberculosis Report 2023. Among the different priorities to overcome TB, the development of rapid diagnostic tests (RDT) for accurate TB detection at the point-of-care¹ is one of the most challenging. TB diagnostic tests have expanded considerably and are essentials for the early detection and the initiation of treatment⁷. Several formats of RDT include molecular tests (such as nucleic-acid amplification tests for TB, HIV-patients and drug-resistant TB), interferon-gamma release assays (IGRAs)⁸ and biomarkers-assays for detection of the infection, and line-probe assays^{1,3,7}, among others. Preferably, these tests should also perform the simultaneous detection of resistance to conventional treatments, at least the first-line drugs, such as rifampicin and isoniazid antibiotics. However, it should be noted that those patients diagnosed with TB using culture, rapid molecular tests recommended by WHO or smear microscopy techniques are considered "bacteriologically confirmed" cases of TB; patients

diagnosed in the absence of microbiological detection are classified as “clinically diagnosed” cases of TB. A proportion of clinically diagnosed cases result in incorrect TB diagnoses¹. So, it is important to scale up the WHO-endorsed molecular rapid tests and detect drug-resistant TB cases through bacteriological confirmation.

Microbiological culture remains the gold standard, despite their specificity, sensitivity, contamination rates and detection times are variable depending on the media used⁹, it is a restricted technique in laboratories with sub-optimal facilities. Although newer technologies and improved media have emerged allowing detection of *M. tuberculosis* in about 12-14 days, the main drawback is the time required to obtain confirmatory results¹⁰. The sputum smear microscopy is a rapid and inexpensive technique most used in limited-resource settings¹¹. However, the success of the technique is operator-dependent leading to a variable range of specificities and sensitivities. In addition, specificity is often lower than other techniques, as strains of other mycobacteria may be recognized in the sample, leading to misdiagnosis or mistreatment. Several methods have replaced traditional Ziehl-Neelsen staining in order to improve efficacy, including the use of mercury vapour fluorescence and light-emitting diode microscopy^{9,11,12}.

Due to specificity and sensitivity issues, WHO recommends the use of molecular tests in TB suspect patients⁷. For example, the next generation the GeneXpert® MTB/RIF Ultra (Cepheid, USA) is a molecular test that has substantially improved the diagnosis of TB and TB resistant to rifampicin compared to traditional techniques¹³, although it requires adequate facilities and access to constant current. The instrument may be too expensive for low-resource settings. The Truenat™ MTB, MTB Plus and MTB-RIF Dx (Molbio Diagnostics, India) is better option for more limited settings^{14,15}. Sensitivity of these tests may be reduced in specific populations¹⁶ such as children¹⁷, HIV patients¹⁸ and extrapulmonary cases of TB. Other options, including line-probe assays such as Alere Determine TB LAM Ag (Abbott, USA) or Fujifilm SILVAMP TB LAM (Fujifilm, Japan) are interesting alternatives¹⁹, especially in HIV populations²⁰. Therefore, portable, user-friendly, and accurate technologies need to be developed for TB screening especially in vulnerable communities or to be adapted to point-of-care (POC) settings¹⁰. In this work, a rapid test for TB detection based on the double amplification of mycobacterial nucleic acids followed by electrochemical biosensing, with as low as

15 min incubation, is proposed. This methodology has been developed using two sets of primers encoding for the regions of *gyrB* and *IS6110* genes. The results obtained are very promising, especially when amplification with *gyrB* primers is used, leading a limit of detection (LOD) comparable to some tests endorsed by WHO (e.g. the GeneXpert® MTB/RIF). Besides the methodology still has some limitations, it is a promising method as the electrochemical readout is developed in a fully portable device, battery-operated, easy to use and rapid, and can be adapted in a primary healthcare setting.

6.3. Experimental section

6.3.1. Instrumentation

PCR tests were performed using the Veriti™ 96-Well thermal cycler (Applied Biosystems™, USA). The electrophoresis was done in a Mini-Sub Cell GT horizontal electrophoresis system and PowerPac Basic power supply (Ref. 1640300, Bio-Rad Laboratories). The photos of the electrophoresis gels were taken using the Gel Documentation System, GenoSens 2100 Touch (Clinx Science Instruments Co., Ltd, China). The electrochemical readout was achieved with a cartridge which allows magnetic actuation and washing, and a portable electrochemical device operated with batteries (BioEcllosion SL, Spain). The samples were also measured on carbon screen-printed electrodes (ref. DRP-C110) using a portable bipotentiostat DRP-STAT200 operated by DropView 200 for instrument control and data acquisition (Dropsens, Spain).

6.3.2. Chemicals and biochemicals

The magnetic particles streptavidin Dynabeads™ M-280 (Ref. 11205D, Invitrogen) and anti-digoxigenin-POD (Ref. 11207733910, Roche) were used a detection system for the amplicons. Different set of buffers were used for specific procedures in the experiments, and their composition is described in §6.6.1 (Supp. Data). The reagents used for the electrochemical measurement includes hydroquinone and hydrogen peroxide 30% (v/v) solution (Catalogue no. H9003 and 31642 from Merck, respectively). The PCR was performed by using the Ready Mix

Taq PCR Reaction Mix with $MgCl_2$, as a cofactor of the enzyme (Ref. P4600, Sigma-Aldrich). For gel electrophoresis, Sybr Safe DNA gel stain (Ref. S33102, Invitrogen) and GeneRuler 100 bp DNA Ladder (Ref. SM0241, ThermoFisher) as a molecular weight marker were used. *Mycobacterium bovis BCG-Pasteur* strain was grown in DIFCO™ Middlebrook 7H9 Broth (Ref. 271310, Becton Dickinson) supplemented with Tween® 80 (Ref. P4780, Sigma-Aldrich) 0.05% (v/v), ADC and glycerol at 0.08% (v/v) for 2-3 weeks at 37°C in 25 cm² cell culture flask. For solid culture, DIFCO™ Middlebrook 7H10 Agar (Ref. 262710, Becton Dickinson) supplemented with glycerol 0.5% (v/v) and OADC was used.

6.3.4. DNA extraction

The extraction protocol was adapted from the GenoType MTBDR plus and MGIT culture (Hain Lifescience GmbH, Germany), and it was optimized as described in 6.6.2.1 (Supp. Data). Briefly, 1 mL of *M. bovis BCG-Pasteur* culture was transferred into 2.0 mL tube and centrifuged at 10,000 g for 15 min. The supernatant was discarded, and the pellet was resuspended in 150 µL of water and mixed with vortex. Then, the sample was incubated at 95°C for 20 min and centrifugated at maximum speed for 5 min. Finally, the supernatant was transferred to a new tube and stored at -20°C.

6.3.5. Oligonucleotides sequences

The oligonucleotides sequences were obtained from Sigma-Aldrich. These primers were selected for the amplification of *IS6110* (245 bp)²¹, *rpoB* (351 bp)²², and *gyrB* (483 bp)²² genes. It was verified with BLAST that the selected sequences were present in both *Mycobacterium bovis BCG-Pasteur* and *Mycobacterium tuberculosis* strains in order to be able to use them later in TB samples. Each set of primer was double tagged in 5' end with biotin (BIO) and digoxigenin (DIG), as summarized in Table 6.1.

Table 6.1. Information about the primers selected for the amplification of the *IS6110* and *gyrB* genes.

Gene	Primer Sequence (5'-3')	Type	5'-Labels	Size (bp)
<i>IS6110</i>	CCGTAGGCGTCGGTGACAAA	Forward	Biotin	245
	CGTGAGGGCATCGAGGTGGC	Reverse	Digoxigenin	
<i>gyrB</i>	ACATCAACCGCACCAAGAACGC	Forward	Biotin	483
	GTGCCTTACGTGCCGCGATACG	Reverse	Digoxigenin	
<i>rpoB</i>	CGACCACTTCGGCAACCG	Forward	Biotin	351
	TCGATCGGGCACATCCGG	Reverse	Digoxigenin	

6.3.6. Double-tagging polymerase chain reaction

The double-tagging polymerase chain reaction (PCR) was achieved by two set of primers labelled with BIO-Fw and DIG-Rev for the amplification of the *IS6110* and *gyrB* genes. Briefly, 1.5 µL of chromosomal DNA was added along with 10 pmol of the forward and reverse primers and 0.75 U of Taq polymerase, for a total reaction mixture of 25 µL. The reaction mixture was exposed to an initial step at 94 °C for 5 min followed by 30 cycles of 94 °C at 30 s, 62 °C for 30 s, 72 °C for 45 s, and a last step of 5 min at 72 °C, as shown in Figure 6.1, panel B. Negative controls were performed except adding DNA. Further details about double-tagging PCR procedure are also described in §6.6.2.1 (Supp. Data). The resulting amplicons were analyzed with conventional agarose gel electrophoresis.

6.3.7. Electrochemical magneto-genosensing

With the aim of developing a test for the detection of tuberculosis, an electrochemical magneto-genosensor was developed in this work, based on a cartridge and a handheld device for the readout. The procedure consists of the following steps, as it is described in Figure 6.1: i) sample collection and DNA extraction, ii) double-tagging PCR using two set of primers coding for the *IS6110* and *gyrB* genes and iii) the electrochemical genosensing of the amplified amplicons, in a portable and easy-to-use device. In order to optimize the procedure, an attenuated strain of *Mycobacterium bovis BCG-Pasteur* was used, and it was verified by BLAST

that the sequences were present in both genomes of *M. bovis BCG-Pasteur* and *M. tuberculosis*, one of the major causes of tuberculosis disease in humans.

Regarding the electrochemical genosensing, streptavidin magnetic particles (strep-MPs) and anti-digoxigenin antibodies labelled with the horseradish peroxidase (HRP) enzyme (anti-DIG-HRP) were used to be attached in the biotin and digoxigenin ends of the amplicons, respectively. More information about the optimization of the reagents was described in §6.6.2.2 (Supp. Data). In a 1.5 mL tube, 10 μL of amplified sample diluted 1/10, were incubated simultaneously with 10 μL of strep-MPs diluted $\frac{1}{2}$ from the original stock and 100 μL of anti-DIG-HRP at 500 mU mL^{-1} , for only 15 minutes at room temperature. Afterwards, the sample was directly transferred into the sample holder of the cartridge for washing and the electrochemical readout by adding the substrate to perform the amperometric measurement in the surface of the electrodes. Both washing and measurement were performed with the same buffer to simplify the procedure.

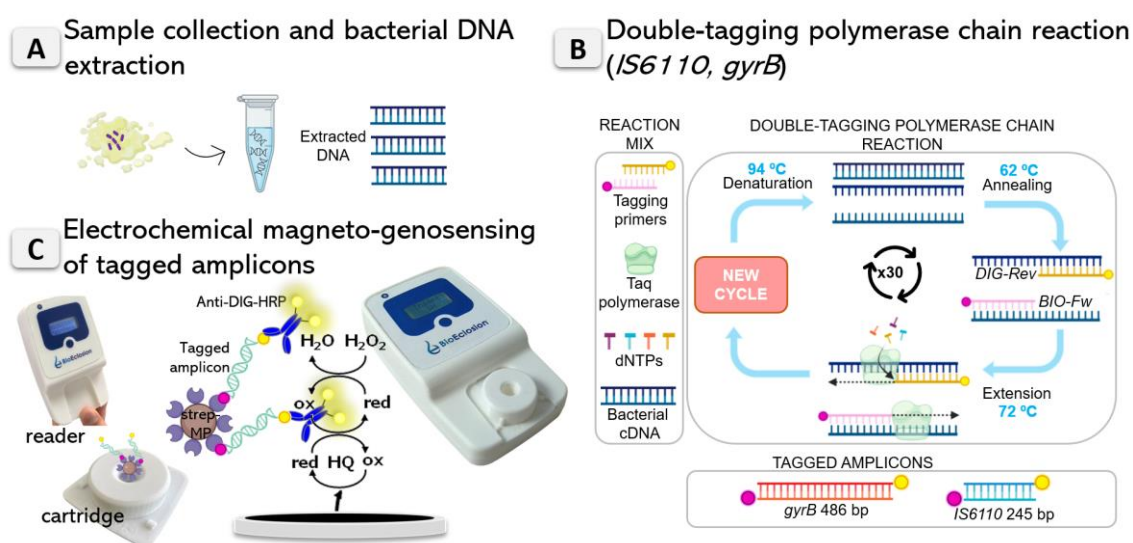


Figure 6.1. Schematic representation of the test for the detection of tuberculosis. The procedure includes: A) sample collection and bacterial DNA extraction, followed by B) the double-tagging PCR using the two set of primers coding for *IS6110* and *gyrB* genes, and finally C) the electrochemical magneto-genosensing using strep-MPs and anti-DIG-HRP antibodies for only 15 min. The electrochemical readout was performed at -0.12 V for 30 s, after one washing with the ePBS MIX.

In order to study the analytical performance of the procedure for each set of primers, a calibration curve of samples of *M. bovis BCG-Pasteur* ranging from 0 to 2.6

$\times 10^7$ CFU mL⁻¹ was processed. The DNA extraction of 1 mL of each sample was performed, followed by the double-tagging PCR with 1.5 μ L of DNA, using the two set of primers in parallel (Table 6.1). The electrochemical genosensing was carried out with the resulting amplicons and in addition, they were quantified by Qubit to know the final concentration of the amplicons obtained. Furthermore, the amperometric measurements were done using a cartridge prototype.

6.3.8. Comparison of the detection of mycobacteria using the handheld device

Four samples of *M. bovis BCG-Pasteur* at different concentration of 2.6×10^7 , 2.6×10^5 and 2.6×10^3 CFU mL⁻¹, including a negative control (0 CFU mL⁻¹), were processed following the procedure described before (Figure 6.1). The samples were amplified using the set of primers coding for the *IS6110* gene in the double-tagging PCR, for further electrochemical genosensing using the hand-held portable device, to check the robustness of their measurements. These same samples were also measured using the cartridge adapter connected with the Dropsens potentiostat, as a reference method. All samples were tested in triplicate on each readout device.

6.3.9. Biosafety considerations

The experiments regarding the bacterial culture were performed in a Biosafety Class 2 environment required for the handling of *M. bovis BCG-Pasteur*. All biological waste generated from the experiments were disposed in accordance with the local regulations for handling biohazards.

6.4. Results and discussion

6.4.1. Double-tagging polymerase chain reaction

The performance of the DNA extraction on the amplification under different temperatures are shown in 6.6.2.1 (Supp. Data). Although all set of primers for the amplification of *IS6110* (245 bp), *rpoB* (351 bp), and *gyrB* (483 bp) genes showed good amplification performance at the optimized conditions (as shown in Figure

6.5), *rpoB* products showed also high background signal in preliminary experiments due to primer dimers formation. Accordingly, further experiments were thus performed with *IS6110* (245 bp) and *gyrB* (483 bp). The end-point amplicons obtained according to the procedure described in Figure 6.1 with each set of primers, from the *M. bovis BCG-Pasteur* culture samples ranging from 0 to 2.6×10^7 CFU mL⁻¹, were first studied by agarose gel electrophoresis as shown in Figure 6.2. The amplicons coding *IS6110* and *gyrB* genes, were shown in panel A and B, respectively.

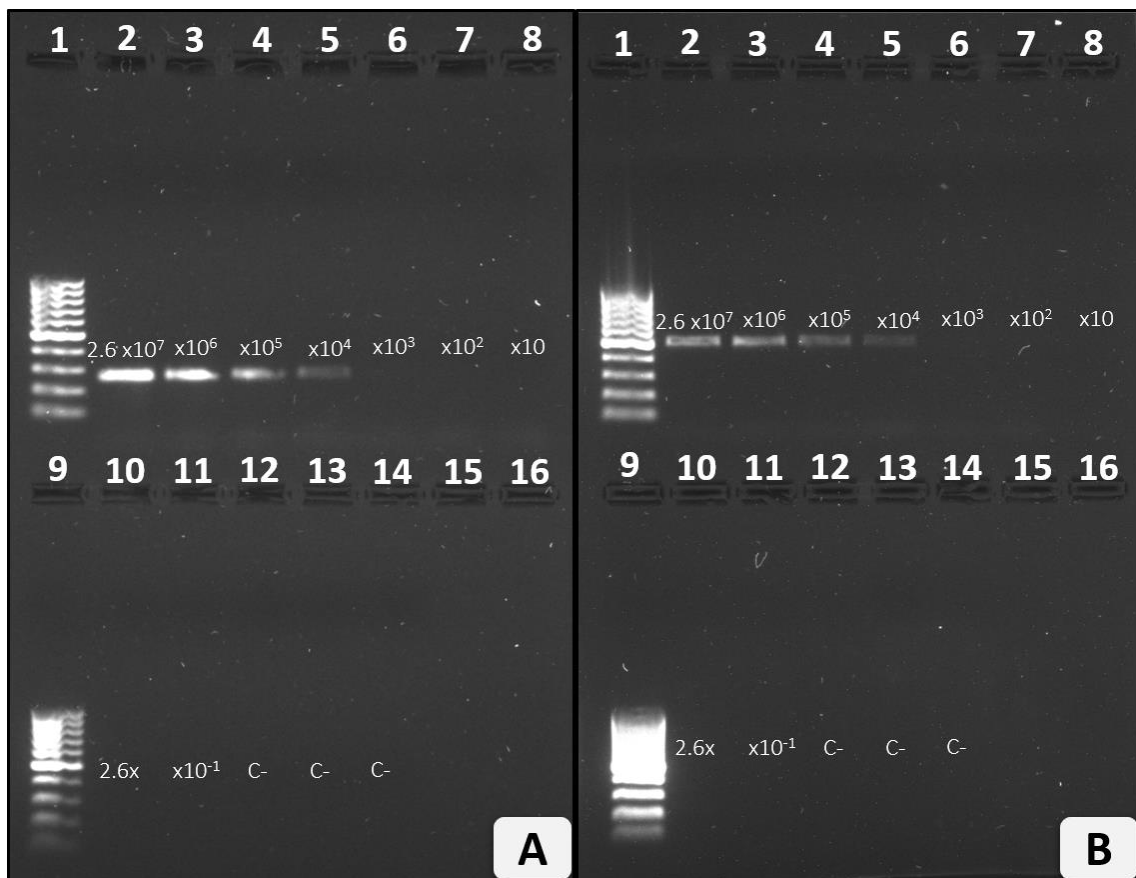


Figure 6.2. Results of the agarose gel electrophoresis of the amplicons obtained by the double-tagging PCR from samples of *M. bovis BCG-Pasteur* culture ranging from 2.6×10^7 to 0 CFU mL⁻¹ using the selected primers coding for *IS6110* (Panel A) and *gyrB* (Panel B). In all panels the order of the lanes was the same, and corresponds to the amplicons obtained through the samples with the following concentrations, described from lane 1 to lane 11: gener ruler, 2.6×10^7 , 2.6×10^6 , 2.6×10^5 , 2.6×10^4 , 2.6×10^3 , 2.6×10^2 , 2.6×10 , gene ruler, 2.6, 2.6×10^{-1} ; and from lane 12 to 14: all negative controls, containing no DNA (0 CFU mL⁻¹).

In all cases, it was observed the greater the concentration of bacteria, more intense was found the band of the gel. The bands of higher intensity (lane 2, 3, 4, 5)

correspond to the concentrations of 2.6×10^7 , 2.6×10^6 , 2.6×10^5 and 2.6×10^4 CFU mL⁻¹ of bacteria, respectively. In addition, a single DNA band was obtained in positive samples according to the size of the amplicon for each set of primers (*IS6110* 245 bp and *gyrB* 483 bp). Negative controls containing no template were also included in lane 12 to 14, in panels A, and B, regarding each set of primers. The absence of signal in the negative controls suggested no undesirable amplification products. The presence of undesired amplification products can be detected in the negative control, including primer secondary structures which can affect the annealing, leading to poor or no yield of the desired product, and also, such undesirable products carrying tags leading to false positive results²³.

6.4.2. Electrochemical magneto-genosensing

To check the analytical performance of the electrochemical magneto-genosensor for the detection of tuberculosis, a calibration plot with attenuated samples of *M. bovis BCG-Pasteur* strain at different concentrations ranging from 0 to 2.6×10^7 were analyzed according to the procedure described above (Figure 6.1). The results for the detection of the amplicons obtained after the double-tagging PCR with each set of primers *IS6110* (blue), and *gyrB* (red) were shown in Figure 6.3.

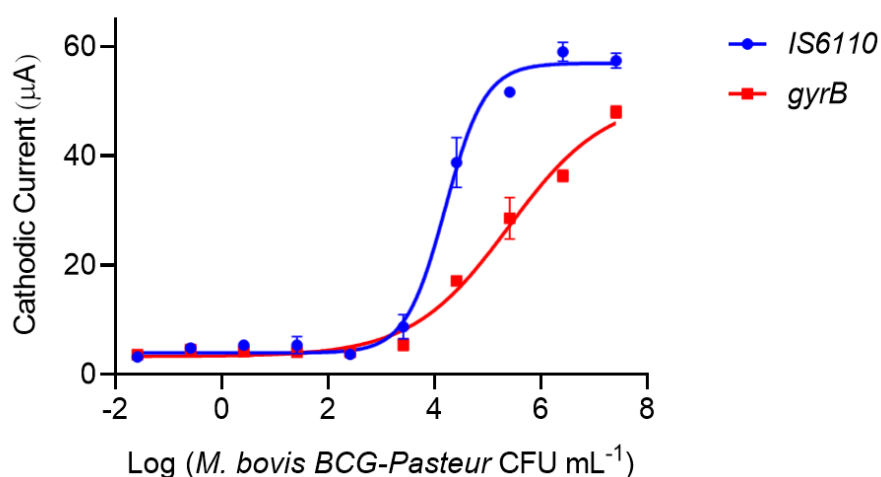


Figure 6.3. Calibration curve of the electrochemical magneto-genosensing of the amplicons obtained by the primers coding for *IS6110* (blue) and *gyrB* (red) by processing samples of *M. bovis BCG-Pasteur* at different concentrations ranging from 0 to 2.6×10^7 CFU mL⁻¹. The error bars show the standard deviation for n=3.

The data were analyzed using a nonlinear regression, the four-parameter logistic curve (Sigmoidal 4-PL, GraphPad Prism Software, v.8.0) with values of $R^2=0.9909$ and $R^2=0.9815$, employing the *IS6110* and *gyrB* amplicons, respectively. The limit of detection (LOD) was calculated for each calibration curve by processing of the negative controls ($n=20$) leading a promising LOD as low as 117 CFU mL^{-1} using the primers coding the *gyrB* gene. The LOD using the set of primers of *IS6110* was 518 CFU mL^{-1} . According to the electrophoresis gel (Figure 6.2), no signal appeared in the negative control, suggesting no primer dimers formation for the *IS6110* and *gyrB* genes, thus the increase of the signal occurs according to the increase of the concentration of bacteria in the samples, as expected. The raw data of the amperometric measurements was detailed in Figure 6.7 (Supp Data).

6.4.3. Comparison of the detection of mycobacteria using the handheld device

The electrochemical genosensing of samples of *M. bovis BCG-Pasteur* at different concentrations ranging from 0 to $2.6 \times 10^7 \text{ CFU mL}^{-1}$ using the hand-held device proposed in this work to perform the electrochemical measurements according to the procedure described above (Figure 6.1) were compared with a reference reader device, and the results were shown in Figure 6.4. The current intensity results were comparable, and there were no significant differences in lower concentration ranges and negative controls. This leads to a very similar LOD between the two reader devices, and in addition there would be no difference in the lowest concentration range that are the most interesting in this type of test, being able to detect the minimum threshold.

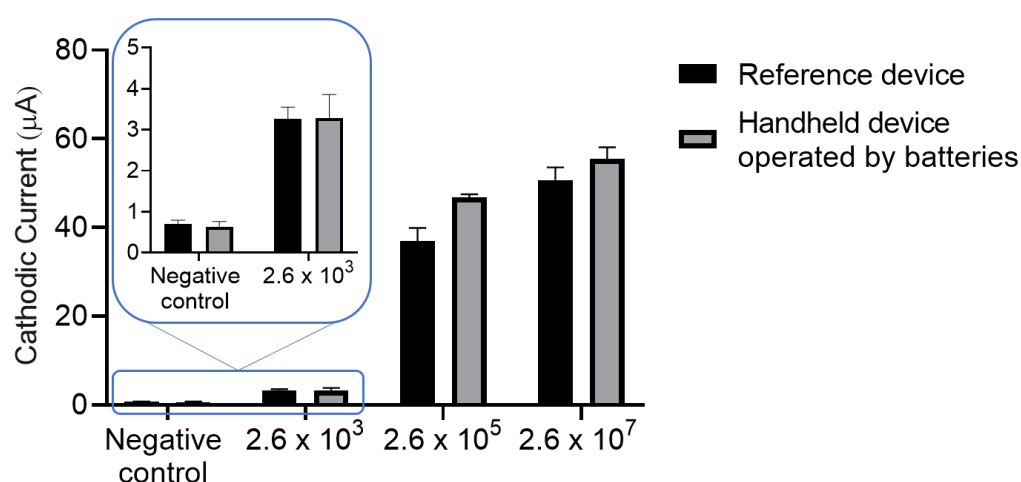


Figure 6.4. Comparison of the electrochemical measurements obtained for the electrochemical magneto-genosensing of samples at different concentrations of *M. bovis BCG-Pasteur* ranging from 2.6×10^7 to 0 CFU mL⁻¹, using the handheld device and a reference reader. The error bars show the standard deviation for n=3.

6.5. Conclusions

In this study, an electrochemical magneto-genosensor was introduced with the aim of detecting tuberculosis through a rapid and a simple test using a handheld device that facilitates the electrochemical readout, making it simpler and portable for the end-users. Among some of the advantages of the test, it stands out that it allows obtaining results on the same day as the sample collection, since it was performed in a total of 2 h 15 min, and in addition, the use of the portable reader avoids the costs related to traditional benchtop equipment. Interestingly, the double-tagging PCR could also be integrated into a portable battery-powered thermal cycler, in order to maximize the portability and simplicity of the method. In this instance, the RDT relies on two modules, being the first a hand-held thermocycler operated by batteries to perform double-tagging end-point PCR in combination with an RDT platform including the disposable cartridge and the reader. Although the LOD obtained with the test vary depending on the primer set used, an impressive LOD of 117 CFU mL⁻¹ was calculated when the set of primers coding for the *gyrB* gene was used, comparable to the LOD of 114 CFU mL⁻¹ reported for one of the most used tests for TB detection, GeneXpert® MTB/RIF (Cepheid). In fact, the first step of the procedure which includes sampling and DNA extraction,

could be optimized to avoid the use of a centrifuge for DNA extraction, employing the use of magnetic particles to preconcentrate the bacteria combined with a lysis buffer for DNA extraction for the ready use in the PCR. The obtained LOD of 117 CFU mL⁻¹ when the *gyrB* primer was used is a promising finding, which could signify the emergence of a highly competitive rapid test when improved.

6.6. Supplementary data

6.6.1. Buffer and solution composition

The composition of the buffers used in this work are described below:

For the electrochemical-magneto genosensing

- For the anti-DIG-HRP dilution: Phosphate buffer solution (PBS): 100 mM Na₂HPO₄, 137 mM of NaCl, pH=7.4
- For washing and resuspension of streptavidin magnetic particles: Binding and washing buffer (B&W): 10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH=7.5
- For the dilution of amplicons sample: Ultrapure water (Millipore System, resistivity 18.2 MΩ cm)

For electrochemical readout

- ePBS: 100 mmol L⁻¹ Na₂HPO₄, 100 mmol L⁻¹ KCl, pH 7.0,
- ePBS MIX: ePBS plus 2 mmol L⁻¹ Hydroquinone, 4 mmol L⁻¹ H₂O₂ (30% (v/v))

6.6.2. Optimization of the rapid test for the amplification and electrochemical genosensing of mycobacteria

6.6.2.1. DNA extraction and double-tagging polymerase chain reaction

The extraction protocol was adapted from the GenoType MTBDRplus and MGIT culture (Hain Lifescience GmbH, Germany) with the aim of performing a simple, fast and efficient extraction minimizing the total number of steps. However, different conditions for DNA extraction were tested and these samples were

amplified with the two sets of primers to check their influence on the final extraction protocol. Table 6.2 summarized the conditions tested to obtain four DNA samples following the extended protocol, omitting or including steps 6 and 7. The extensive DNA extraction protocol was as follows:

1. Transfer 1mL of sample to an eppendorf tube; 2. Centrifuge at 10,000 *g* for 15 min; 3. Discard supernatant; 4. Resuspend pellet with 300 µl of water and mix with vortex; 5. Incubate at 95°C for 20 min; 6. (*Optional*) Incubate in an ultrasonic bath for 15 min; 7. (*Optional*) Add 30 µL of sodium acetate and mix; 8. Centrifuge at maximum speed for 5 min; 9. Transfer supernatant to a new tube and store DNA at -20°C.

Table 6.2. Summary of the conditions tested for the optimization of DNA extraction protocol.

Sample of <i>M. bovis</i> BCG-Pasteur				
Number of protocol	1	2	3	4
Step 6				
Ultrasonic pretreatment		NO		YES
Step 7				
Sodium acetate adding	NO	YES	NO	YES

The resulting amplicons were analyzed with conventional agarose gel electrophoresis on 0.8 % agarose gels containing TBE 0.5 % buffer and Sybr Safe DNA gel stain and using a 250 bp ladder as a molecular weight marker. Figure 6.5 shows the electrophoresis gels obtained with the different protocols. As observed, the amplification obtained with protocol 2 and 4, in which step 7 was included in the DNA extraction, were the ones with the lower signal intensity, resulting in a lower amplification efficiency. However, where protocol 1 and protocol 3 were used, in which step 7 was not included, the signals were more intense leading to a higher PCR yield product. According to the results, further experiments were performed without including step 6 and 7, since the PCR product yield was clearly lower when NaAc was added in the DNA extraction protocol and no improvement was observed when the ultrasonication treatment, step 6, was included, thus leading to a simplification in the analytical procedure.

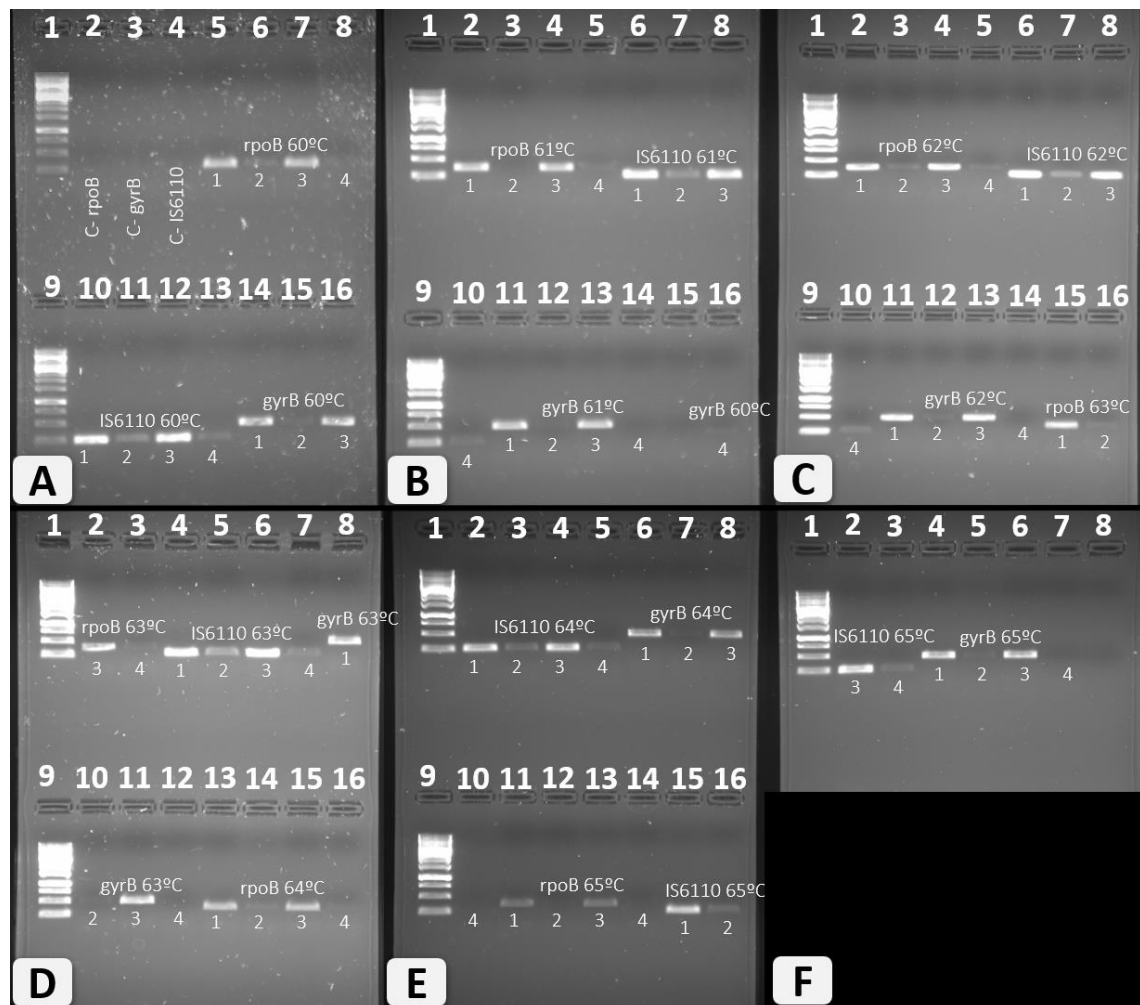


Figure 6.5. Results of the agarose gel electrophoresis of the amplicons obtained by the PCR from a sample of *M. bovis BCG-Pasteur* culture using the non-labeled primers coding for *IS6110*, *gyrB* and *rpoB*. In the panels were analyzed the samples of amplicons providing from protocols 1 to 4, represented as 1, 2, 3 and 4 according to the procedure followed for obtain the bacterial DNA. All these samples were tested with each set of primers with different annealing temperature ranging from 60 to 65°C. Panel A) Negative controls setting 60°C of annealing temperature, lane 2, 3 and 4 for *rpoB*, *gyrB* and *IS6110* primers, respectively. Positive controls for 60°C using *rpoB* in lane 5, 6, 7, 8, *IS6110* in lane 10, 11, 12, 13 and *gyrB* in lane 14, 15, 16. Panel B) Positive controls for 61°C using *rpoB* in lane 2, 3, 4, 5, *IS6110* in lane 6, 7, 8, 10 and *gyrB* in lane 11, 12, 13, 14. Positive control for 60°C using *gyrB* for protocol 4 in lane 16. Panel C) Positive controls for 62°C using *rpoB* in lane 2, 3, 4, 5, *IS6110* in lane 6, 7, 8, 10 and *gyrB* in lane 11, 12, 13, 14. Positive controls for 63°C using *rpoB* in lane 15 and 16. Panel D) Positive controls for 63°C using *rpoB* in lane 2, 3, *IS6110* in lane 4, 5, 6, 7 and *gyrB* in lane 8, 10, 11, 12. Positive controls for 64°C using *rpoB* in lane 13, 14, 15, 16. Panel E) Positive controls for 64°C using *IS6110* in lane 2, 3, 4, 5, *gyrB* in lane 6, 7, 8, 10. Positive controls for 65°C using *rpoB* in lane 11, 12, 13, 14 and *IS6110* in lane 15 and 16. Panel F) Positive controls for 65°C using *IS6110* in lane 2, 3 and *gyrB* in lane 4, 5, 6, 7.

In addition, the primers selected for the amplification of *IS6110* and *gyrB* genes were previously tested with no tags (BIO/DIG), using the sequences described in Table 6.1, to verify their efficiency in DNA amplification. Since the sequences contain a high guanine and cytosine (GC) content, characteristic of mycobacteria, the required annealing temperature is typically somewhat higher. For this reason, an optimization of the annealing temperature was carried out ranging from 60 to 65°C with no tagged primers. Figure 6.5 shows successful amplification of the previously extracted DNA was observed using the two set of primers even if the annealing temperature was modified between 60-65°C.

Finally, for the double-tagging PCR for the detection of tuberculosis, 62°C was selected as the annealing temperature, ensuring high efficiency in the PCR, and with the objective of minimizing the lack of specificity or the production of undesired secondary structures. The final double-tagging PCR used in this work, consisted of an initial step at 94 °C for 5 min followed by 30 cycles of 94 °C at 30 s, 62 °C for 30 s, 72 °C for 45 s, and a last step of 5 min at 72 °C.

6.6.2.2. Electrochemical magneto-genosensing

The reagent concentrations for the electrochemical magneto-genosensor were previously optimized in order to obtain the maximum signal to noise ratio. Firstly, the concentration of the streptavidin magnetic particles (strep-MPs) was optimized. A volume of 10 µL of strep-MPs at different dilutions from the stock of 1/2, 1/10, 1/20 (approx. 3.25×10^8 , 6.5×10^7 , 3.25×10^7 MP mL⁻¹) were tested in combination with 100 µL of anti-digoxigenin-HRP (anti-DIG-HRP) antibodies at 1000 mU mL⁻¹. Then 10 µL of positive controls containing double-tagged amplicons (BIO/DIG) and negative controls were incubated for 15 min at RT, performing a total volume of 120 µL of sample. After the incubation time, the sample was transferred to the cartridge to perform the electrochemical readout: the supernatant was discarded, and one washing was done with 3 drops of ePBS MIX. The solution was removed, and 3 drops were added again for the further amperometric measurement under enzyme saturation conditions at -0.12V and 30 s. Secondly, the concentration of the anti-DIG-HRP antibody was optimized. A concentration of 250, 500, 1000, 2000 mU mL⁻¹ of anti-DIG-HRP was tested in combination with 10 µL of strep-MPs at 1/2 dilution from the stock and 10 µL of sample, including positive and negative

controls. Then, the electrochemical magneto-genosensing and the measurements were done as described before. In all cases, amplicons obtained from the *IS6110* tagged primers were used as a model for the optimizations and were tested by triplicate ($n=3$). Finally, the $\frac{1}{2}$ dilution from the stock for the strep-MPs and 500 mU mL^{-1} for the anti-DIG-HRP antibody were selected for further experiments according to the results described in Figure 6.6, panel A and B.

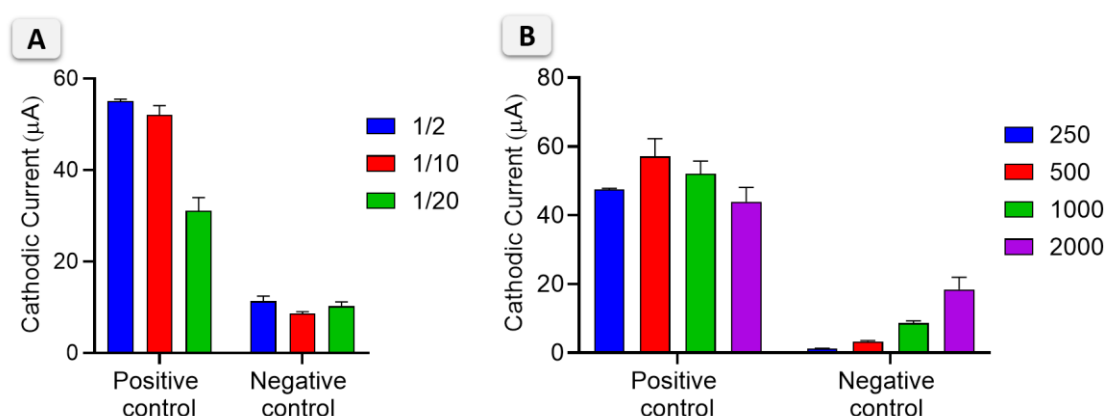


Figure 6.6. Plot for the optimization of the reagent concentrations for the electrochemical magneto-genosensor. Panel A shows the optimization of the strep-MPs concentration, while Panel B, the optimization of the anti-DIG-HRP concentration. Positive and negative controls (from 0 to 2.6×10^7 CFU mL^{-1} of *M. bovis BCG-Pasteur*, respectively) ($n=3$).

6.6.2.3. Amperometric measurements

The final readout at the surface of the electrode is based on the reduction of the BQ to HQ according to previous chapters (Figure 3.10, panel B). Briefly, once the incubation of the tagged amplicons with the strep-MPs and anti-digoxigenin-HRP antibodies was performed, the sample was directly transferred to the cartridge. The supernatant was eliminated, and a washing step was done, for further proceed with the measurement. The cathodic current was measured with the potential set at -0.15 V upon 30 s. The difference on cathodic current was measured with the potential set at -0.12 V, with an interval of 0.5 s for a total time of 30 s. The raw data of each calibration curve for the electrochemical magneto-genosensor are presented below (Figure 6.7).

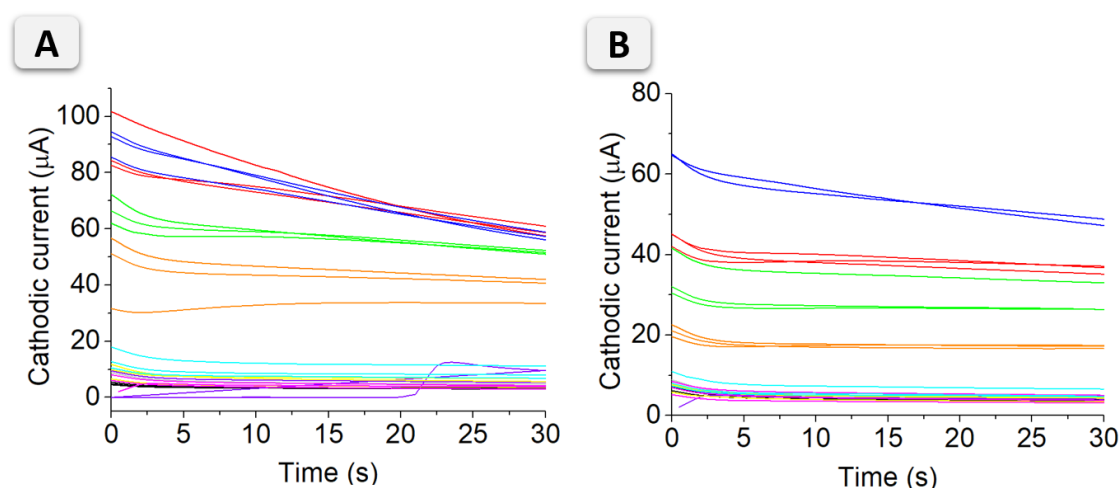


Figure 6.7. Raw data obtained from the amperometric measurements for the electrochemical magneto-genosensor in *M. bovis BCG-Pasteur* samples, ranging from 0 to 2.6×10^7 CFU mL⁻¹ including the replicates of each sample and negative controls (n=3) and using the two set of primers coding for *IS6110* (panel A), and *gyrB* (Panel B) genes. The potential was set at -0.12 V, with an interval of 0.5 s for a total time of 30 s.

6.7. References

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CHAPTER 7

Final remarks

7. Final Remarks

The design of rapid diagnostic test for the detection of pathogens is a challenging goal, involving the integration of several fields such as analytical chemistry, microbiology, biochemistry, molecular biology and material sciences. Enhancing rapid tests for the detection of infectious diseases and emerging pathogens is imperative in the 21st century, particularly for countries with higher incidence rates, often coincident with less developed countries. Among the main characteristics that must be considered for designing a rapid test, analytical features play a pivotal role, influencing the accuracy, sensitivity, specificity, and turnaround time of the test. It is also essential to consider the end-users, ensuring that the test can be executed under appropriate training of the personnel and as the needs vary depending on the intended use. A bedside or home test will not have the same requirements as one for a primary point-of-care or a more advanced hospital setting. The associated instrumentation also depends on the settings, the training of the final user, the analytical performance needed for the application, among other relevant technological factors. Additionally, the complexity of target, their structure, variability, metabolic processes, and biomarkers involved in infection processes must be also considered. It is important that science move forward to the finding of new biomarkers allowing specific and early detection of infections in a rapid and feasible manner. Lastly, environmental monitoring are key elements in preventing and detecting potential infection outbreaks. Effective detection methods are crucial for timely identification. Legislation for pathogen control in water and food can vary greatly depending on the geographic location and the assessment source. Corrective actions are also variable. Therefore, moving towards a consensus that provides global security and finding methods that offer reliable and safe results is crucial.

This doctoral thesis aims to contribute to the research and development of rapid tests for the detection of communicable diseases in clinical or environmental settings, focusing on improving solid-phase preconcentration procedures, and exploring alternatives to traditional benchtop instruments by the integration of portable platforms for the readout of electrochemical biosensors.

As a general remark, all Chapters provide detection strategies based on the use of modified magnetic particles, enhancing the preconcentration and isolation of the studied pathogen or captured target. Except for Chapter 6, where commercial streptavidin magnetic particles are used, Chapters 3, 4 and 5 use tosyl-activated magnetic particles, which allowed for a simple and efficient tailored modification protocol. In addition, the MPs were the solid support for all biosensing strategies. The concentration of bacteria in each Chapter was determined from microbiological culture as a reference technique.

In Chapter 3, an electrochemical biosensor for the detection and quantification of *L. pneumophila* in water samples is developed. For this purpose, a preconcentration method combining filtration of large volumes of water and immunomagnetic separation with specific antibodies against lipopolysaccharides found in the membrane of *L. pneumophila* bacteria was designed. First, the water samples were filtered, and the membrane was directly incubated with the modified-MPs to capture the trapped bacteria. Next, the particles were separated from the sample by an external magnetic field, and then incubated with HRP-labelled anti-*Legionella* antibodies to obtain the immunological sandwich. The use of the combined preconcentration method significantly improved the LOD by two orders of magnitude, in contrast to only applying immunomagnetic separation. Several membrane materials were tested to obtain the minimum retention of bacteria on the filter and to ensure efficient IMS, with polycarbonate membranes giving the best results, in agreement with previous reports. To test the specificity of the method, the system was challenged for the detection of other bacterial species in water, and it was found that even at high concentrations, the signals were comparable to those obtained by the negative controls, suggesting a good specificity. In addition, SEM microscopy showed that the anti-*Legionella*-MPs were able to capture *Legionella* bacteria. The biosensor presented LODs of up to 100 CFU L⁻¹ (or 0.1 CFU mL⁻¹), which is within the most restrictive detection limits described by some legislations, especially in the monitoring of pathogen levels in water in industrial environments.

In Chapter 4, an electrochemical biosensor was developed for the detection and quantification of *Mycobacterium fortuitum*, as a model of environmental mycobacteria in hemodialysis water samples. In contrast to Chapter 3, the combined

preconcentration method was also applied, including filtration and IMS using modified particles with antibodies specific for the lipoarabinomannan (anti-LAM) groups present on the membrane of the mycobacterial genus, but in this case, the filter was incubated simultaneously with the anti-LAM-MPs and the detector antibodies conjugated with biotin and HRP-labelled streptavidin, simplifying even further the analytical procedure. For this work, although other filter materials were also studied, nylon membranes were selected as they provided the lowest retention rates and improved measurement results. The simultaneous incubation was only 1 h followed by the electrochemical readout. Interestingly, a portable measurement platform powered by rechargeable battery was used, which, once the sample is incubated, allows for washing and electrochemical readout, after addition of the corresponding substrates, performing a user-friendly methodology. The biosensor was able to detect up to 5 CFU mL⁻¹ of mycobacteria in hemodialysis water samples, an improvement of three orders of magnitude when only the IMS is applied instead of the full preconcentration method. The specificity of the sandwich biosensing system was also studied by processing samples with other bacterial species, obtaining comparable measurements with the negative controls, suggesting good specificity. Moreover, the biosensing system was also studied by SEM and TEM microscopy, where we observed efficient capture of the mycobacteria and successful interaction with the detector antibody labelled with biotin and strep-Cy5.

In Chapter 5, the use of the smartphone and a mobile app as an optical readout platform in different bioanalytical techniques, such as the Bradford and BCA method, conventional ELISA for the detection of antibodies and a magneto-actuated immunoassay for the detection of coliform bacteria was explored. These techniques were selected because of their wide use in bioanalytical analysis and because the reactions involved in each technique provide solutions with absorbance maxima at different wavelengths including 450 nm (yellow, immunoassays), 562 nm (purple, BCA method) and 595 nm (blue, Bradford method). The results obtained were very promising, as the LODs and LOQs calculated, through the calibration curves obtained with the readout instruments, were very similar compared to the spectrophotometer as a reference instrument. The mobile application can be used with any mobile phone that contains a camera, allowing the direct results or further processing of an image. The use of mobile devices can be a useful tool instead of

conventional benchtop instruments, which are more expensive and require the use of qualified personnel. For this study, a LED light box was used to take images of the microplates for each assay for further processing, ensuring reproducibility. Although the light source was found to influence the processing of the signals obtained by the app, this was not a limitation because it did not result in significant errors.

In Chapter 6, an electrochemical biosensor for the detection of tuberculosis was presented using *M. bovis BCG-Pasteur* as an attenuated strain in order to develop and optimize the different parameters of the biosensor. The methodology consisted of i) DNA extraction and amplification of the bacterial genetic material using biotin and digoxigenin double-tagged primers encoding regions of the *gyrB* and *IS6110* genes and ii) the biosensing strategy based on a rapid and simultaneous incubation of only 15 minutes, even without continuous shaking, using commercial streptavidin magnetic particles and HRP-labelled anti-digoxigenin antibodies, to measure directly in a portable and easy-to-use device. Both the experimental parameters for the PCR and the biosensing strategy were optimized, the results confirmed by gel electrophoresis and mycobacterial detection curves at different concentrations performed, respectively. The LODs obtained for each primer set used, *gyrB* and *IS6110*, were 117 and 518 CFU mL⁻¹, respectively. The LOD for the *gyrB* amplification and detection is comparable to the LOD of 114 CFU mL⁻¹ reached by the GeneXpert® MTB/RIF (Cepheid), that is endorsed by WHO. The LOD, especially when using the *IS6110* primer set, should be optimized, as some more recent techniques have LOD of up to 16 CFU mL⁻¹, as is the case of the most recent version of GeneXpert® MTB/RIF Ultra (Cepheid). The method should be tested with other bacterial species to check the specificity of the PCR primers and tested on clinical samples to check the efficiency of the test on samples with high complex matrixes. Future perspectives are also focused on performing the double-tagged PCR on a portable thermocycler operated by batteries. Although some improvements remain to be applied, the proposed biosensor is a promising strategy.

Finally, the biosensing strategies studied, along with the integration of portable and user-friendly readout platforms, have opened the way for further research in the group, including the investigation of novel biomarkers for risk

stratification of fever in children under 5 years of age, such as the sTREM-1 and Ang-2, for further biosensing. Furthermore, the detection of Alzheimer's biomarkers in exosomes based on similar biosensing strategies is also being studied. In addition, the detection of other bacterial pathogens such as *Streptococcus B* in vaginal samples from pregnant women is also being explored using this type of technology. As discussed previously, rapid diagnostic tests for both communicable and non-communicable diseases have seen significant growth in recent years and remain a crucial focus of research to improve diagnostic capacity, reduce mortality, and enhance quality of life for all communities.

