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Impedimetric gold nanoparticle lateral flow test for pathogen detection

Grégoire Le Brun

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Dissertation committee:

Prof. Jean-Pierre Raskin (UCLouvain, Belgium), advisor

Prof. Karine Glinel (UCLouvain, Belgium)

Prof. Jacques Mahillon (UCLouvain, Belgium)

Dr. Ignace Adant (UCLouvain, Belgium)

Prof. Marc Debliquy (UMons, Belgium)

Dr. Noan Nivarlet (Unisensor, Belgium)

Dr. Davide Benneventi (Pagora, France)

Prof. Laurent Francis (UCLouvain, Belgium), chair

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Abstract

Climate change and the expanding global population are placing immense pressure on our planet's natural resources and ecosystems. Sustainable resource management is crucial to protect human communities and support necessary economic activities. Specifically, the safety of water resources and food production chains necessitate access to frequent and reliable data on pathogenic infections. Since conventional laboratory-based techniques are limited (too expensive or too slow), the recent pandemics and outbreaks highlighted the need for early diagnostic tools to screen pathogens, combining accurate and rapid detection with simplicity and accessibility.

This thesis first investigates how rapid diagnostic tests, i.e., Lateral Flow Assays (LFA), take advantage of porous paper-like membranes to develop highly specific colorimetric biosensors for the diagnostic of proteins (human immunoglobulins) and critical pathogens (*Bacillus cereus*) at the point-of-care, in a user-friendly and rapid (15 min) format.

The second part of this work tackles the challenges presented by optical LFAs (low sensitivities) by exploring the accommodation of electrical detection techniques on LFAs towards improved performance.

By means of a multidisciplinary approach covering microbiology, nanotechnologies, material science and electrical engineering, this thesis offers a complete characterization of the LFAs in order to support future research and development of LFAs for pathogen diagnostics, and the integration of sensitive and reusable electrical readers on LFAs.

However, the biosensor technologies developed in this thesis are not neutral. Many aspects condition their long-term use, such as the socio-ecological and economic implications of their production, dissemination,

use and end-of-life. Therefore, a systemic approach is proposed early-on in the research process to improve the understanding of the implications of the biosensor design choices on their environmental performances, and orient our decisions towards an appropriate design and manufacturing process for paper-based pathogen biosensors.

List of publications and conferences

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- N. Moreau, T. Pirson, G. Le Brun, T. Delhaye, G. Sandu, A. Paris, D. Bol, J.-P. Raskin, "Could Unsustainable Electronics Support Sustainability?", *Sustainability*, 13, 6541, 2021. doi: 10.3390/su13126541
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List of Acronyms

Ab	Antibody
AP	Absorbent Pad
AuNP	Gold Nanoparticle
BSA	Bovine Serum Albumin
CBD	Cell-Wall Binding Domain
CBM	Carbohydrate Binding Domain
CFP	Cyan Fluorescent Protein
CL	Control Line
CLSM	Confocal Laser Scanning Microscopy
CNPs	Carbon Nanoparticles
CNTs	Carbon Nanotubes
CP	Conjugate Pad
CPB	Conjugate Pad Buffer
CPE	Constant Phase Element
CWDA	Cell Wall Decoration Assay
DI	Deionized water
Dit	Distail-Tail Protein
DL	Double Layer
DLS	Dynamic Light Scattering

DNA	Deoxyribonucleic Acid
DPV	Differential Pulse Voltammetry
EAD	Enzymatic Active Domain
EDX	Energy-Dispersive X-Ray Spectroscopy
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme-Linked Immunoassays
EvoDit	Evolved Distal Tail
FoM	Figure of Merit
GAT	Gold Aggregation Test
GFP	Green Fluorescent Protein
GHG	Greenhouse Gas
H IgG	Human Immunoglobulin G
ICF	Impedance Flow Cytometry
IDE	Interdigital Electrodes
IR	Infrared
LB	Lysogeny Broth
LCA	Life Cycle Assessment
LCI	Life Cycle Inventory
LFA	Lateral Flow Assay
LoC	Lab on a Chip
LoD	Limits of Detection
mAb	Monoclonal Ab
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry
MEMS	Microelectromechanical systems
MIP	Molecular Imprinted Polymers

MNPs	Magnetic Nanoparticles
NC	Nitrocellulose
NP	Nanoparticle
OD	Optical Density
O/N	Grown Overnight
PA	Polyaniline
pAb	Polyclonal Ab
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
pI	Isoelectric Point
PDMS	poly(dimethylsiloxane)
PDP	Product Design Process
PMMA	poly(methyl methacrylate)
PoC	Point-Of-Care
PoN	Point-Of-Need
PSA	Prostate Specific Antigen
PVD	Physical Vapor Deposition
QD	Quantum Dots
RBC	Red Blood Cells
RBP s	Receptor Binding Protein
RH	Relative Humidity
RT	Room Temperature
SELEX	Systematic Evolution of Ligands By Exponential Enrichment
SEM	Scanning Electron Microscopy
sLCA	Simplified Life Cycle Assessment

SNR	Signal-to-Noise Ratio
SP	Sample Pad
SPCE	Screen-Printed Carbon Electrodes
SPGE	Screen-Printed Gold Electrodes
SPR	Surface Plasmon Resonance
T-Dots	Test Dots
TA	Technical Assessment
TEM	Transmission Electron Microscopy
TL	Test Line
TRL	Technology Readiness Level
UCNPs	Upconverting Nanoparticles
UV-Vis	Ultraviolet-Visible Light
μPADs	Microfluidic Paper Analytical Devices
μPEDs	Microfluidic Paper Electrochemical Devices

Introduction

Pathogenic outbreaks are major threats to global health and food safety. The frequency of outbreaks has been steadily increasing [1]. Bacterial infections are the second leading cause of death worldwide [2]. Early diagnosis is critical to identify the responsible pathogen, and thus is a vital step in confining and treating infected people or foodstuffs. There are several approaches for detecting pathogens in samples. However, these conventional methods to identify pathogens require the isolation of a single bacterial colony, obtained after a time-consuming culturing step lasting up to several days. This delay results in a slow decision making process for the management of the infections, and in detrimental impacts on the health-care system or our ecosystems. During critical pathogenic outbreaks, we therefore need faster tools to prevent the spread of contamination, whether to improve the management of our ecosystem resources or to promote food safety.

The need to test the bacteriological quality of our resources in critical situations has led to the development of early diagnostic tools that can be used directly at the Point-of-Need (PoN)¹, i.e. at the place where the sample is taken, saving precious time. When the pathogenic bacteria can be detected by means of biosensors, the detection time can even be reduced to minutes or hours (Figure 1).

Indeed, biosensors are bio-detection platforms, generally taking advantage of micro- and biotechnologies, and integrating all the measurement

¹PoN diagnostics in the healthcare sector is called Point-of-Care (PoC) diagnostics. PoC are the most widely deployed tools, such as PoN is often replaced by PoC in the common language.

systems necessary for generating a signal of the pathogen detection that can be directly read and interpreted by the end-user. This way, they combine selective bio-recognition interfaces and relatively sensitive and fast transducers (e.g. optical or electrical), which makes them true lab on a chip (LoC). Since the goal of the biosensor is to replace the lab, a biosensor must possess several functionalities, including the ability to collect the sample, its handling and treatment, its analysis and the communication of its results in a legible form. Overall, the need for PoN generally requires that these biosensors are easy to use so that they can be appropriated by the general public. The WHO has established a series of guidelines for the development of PoN biosensors: they must be accessible, low-cost, rapid, sensitive, and be able to be performed directly at the site of the test, not in distant laboratories [3].

Therefore, it is anticipated that biosensors could make a substantial impact in fields where traditional detection methods fall short. For instance, they can swiftly diagnose epidemics or food-borne infections, enabling the isolation of the infected and stopping the spread of the disease, as well as identifying the source of the infection. Additionally, biosensors could prove advantageous in settings such as developing countries where access to an analytic laboratory might be challenging, if not impossible.

However, several aspects condition the long-term use of biosensors that go well beyond its technical performances. A critical prerequisite for the possibility of deriving concrete actions from a PoC device is that the data it generates is effectively interpreted and usable by end-users, thus implying the need for reliability of the measurement and confidence in the result, ease of interpretation, communication or reporting to relevant stakeholders [4]. In certain circumstances, particularly in the food chain, data about pathogenic contamination must be generated using certified methods in order to be used in practice. The biosensor technology must therefore be compliant with, for instance, an ISO or AFNOR certification process. Finally, in view of the growing awareness of the global environmental impacts generated by human action, an increasing number of organizations are imposing constraints on the life cycle of products, such as single-use clinical devices among which are included the PoC devices, so that these are manufactured in the most efficient way possible, or that their end-of-life is anticipated.

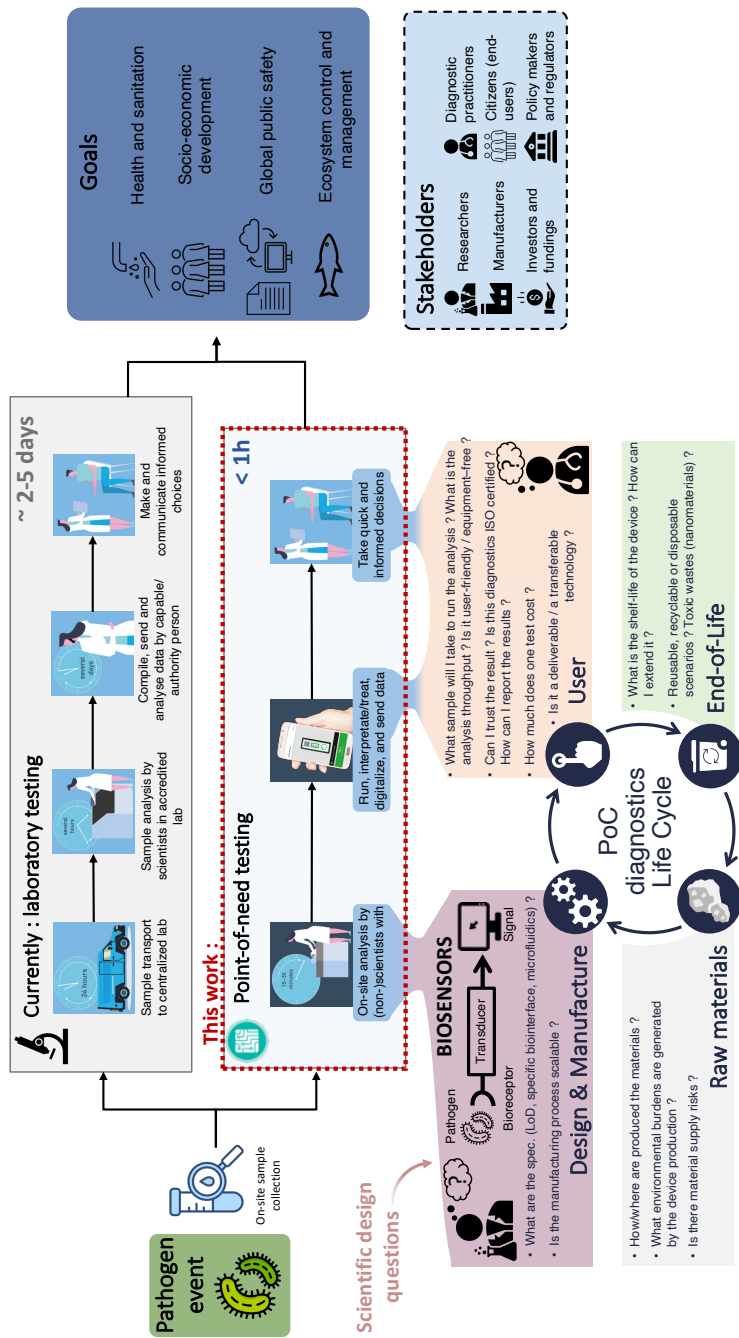


Fig. 1 A system view of the point-of-care (PoC) diagnostics landscape. PoC diagnostics reduces time to reach a final decision as compared to conventional laboratory testing. In the PoC diagnostics paradigm, users and beneficiaries are sometimes the same person. Designer concerns are often mainly focused on the scientific design questions of biosensors, missing decisive questions from other life-cycle stages (e.g. raw materials) and stakeholders (e.g. the end-user) to develop the appropriate technologies, i.e. compliant for their long-term use, dissemination and positive impact in the society.

Thesis Methodology and Objectives The previous observations naturally leads to the **main research question** of this thesis:

What technology is it desirable to use or develop for the early detection of pathogens in critical settings ? How can we engineer appropriate PoC devices for the next pathogenic outbreaks ?

The need to identify the appropriate technology requires studying the systemic impacts of a technology, i.e. taking a holistic view of the research topic. *Systemic* means understanding the entire problem studied and identifying the links and barriers between the factors that make it up. Ashby *et al.* has recently proposed a pedagogical, **5-steps methodology for studying research questions using a systemic approach**, with the aim of developing appropriate or “sustainable” technologies [5] :

- **Step 1 :** Clarify the **research prime objective** : what are the motivations to study the development of novel biosensors for pathogen detection ? What could be the scale of application of new technology compared to the problematic one ?
- **Step 2 :** Identify the **stakeholders** and their concerns. If the concerns are not addressed by the new biosensors, it will face obstacles and may fail to gain acceptance, therefore failing to deliver positive societal value.

Key elements of the two first steps are gathered on Figure 1.

- **Step 3 :** To get further in the identification of the appropriate biosensor, we need **facts** to be discussed and compared between PoC biosensor technologies, and facts need **research**. A systemic approach of a technology necessitates the research about its *design* and *performances*, the *materials* used, alongside its *energy* requirements, the *environmental* impacts generated by the technology, its implications on *society*, its relation with the *economy* and its compliance with *legislation*.

Step 3 covers most of this thesis work. In particular, the researched facts focus on technical specificity of the biosensors. They are expressed in the **three research questions** of this thesis, which are related to the three technical parts of this thesis (see thesis outline). In this thesis, paper-based biosensors, such as colorimetric rapid tests, are selected quite early as promising candidate due to their large scale dissemination, acceptability and rel-

evance for PoC rapid diagnostics. Therefore, this thesis will aim at understanding their working principle, their limitations, their applicability to pathogen detection, and how they could be improved with new electrical transducers :

Research Question 1 *What are the technologies, their performances and research trends for pathogen PoC diagnostics ? How can we guide the design choices towards the most appropriate biosensors, early in the the R&D process, taking into account their technical performances as well as the environmental impacts associated with their material basis and their manufacturing ?*

Research Question 2 *How to develop colorimetric paper-based sensors and what are their intrinsic limits of the performances ? How can we develop colorimetric paper-based sensor for rapid detection of pathogens, with a relevant biointerface ?*

Research Question 3 *What are the strategies available to increase the performances of the paper-based sensors with electrical transducers ? What are the key limiting factors when integrating electrical measurements on paper sensors and LFAs ?*

- **Steps 4 and 5** : consist in the **Synthesis** and **Reflection**, respectively, where we form a balanced judgement about the impacts of the technology relative to the facts we found in step 3, and where we push the reflection on other possible solution to the problematic, if necessary.

Steps 4 and 5 should take the form of a general technical discussion put in perspectives with other facts, in particular coming from environmental impact assessments, to form a **holistic discussion**.

Thesis Outline This thesis is organized in **four parts**, relative to the three research questions, and the holistic discussion. A schematic outline of this thesis is visible on Figure 2.

Part I - A systemic view of PoC pathogen sensors

- **Chapter 1** consists in a state-of-the-art on conventional methods for pathogen detection, describes the different class of biosensors existing for pathogen diagnostics and proposes a comparison based on the performances commonly reported in the literature. In particular, it highlights the lack of consideration for eco-design in the case of pathogen biosensors research and development.
- **Chapter 2** tackles this limitation by proposing a detailed methodological framework to add environmental aspects in the development process of PoC biosensors, supporting the choice of appropriate fabrication techniques and materials. First, the main environmental indicators relative to the materials used in PoC sensors are reviewed and communicated in a convenient way for use. Then, simplified Life-Cycle Analysis are proposed as a way to guide the sensor designer for the choice of manufacturing technologies and materials, with quantified environmental metrics.

The results of the state-of-the-art and environmental analyses conducted in the first part of this thesis suggest suggest prioritizing research on paper-based sensors in this thesis, and more specifically Lateral Flow Assays (LFAs), due to their multiple convenient assets (quick, simple and cheap assays for various diagnostics at the point of care).

Part II - Design of colorimetric LFAs for pathogen detection

- **Chapter 3** provides an in-depth review of the fundamentals about the development of colorimetric LFAs based on gold nanoparticles. This chapter provide the readers with the necessary knowledge to understand the basic principles governing an LFA and to take informed decisions during lateral flow assay design and fabrication. It particularly emphasizes the importance of considering all the interconnected parameters when optimizing LFAs.
- **Chapter 4** proposes a step-by-step example procedure for the design, fabrication and operation of a LFA for the quantitative detection of hu-

man IgG, a small molecule relevant as a first detection use-case due to its simplicity of integration on a colorimetric LFA. The chapter also includes some characterizations of the basic materials that will be further used in this thesis. The results are thoroughly analyzed to gain a comprehensive understanding of the underlying factors contributing to the relatively high detection limits observed in colorimetric LFAs.

- **Chapter 5** leverages the knowledge from chapters 3 and 4 in order to develop a simplified LFA for the detection of whole-cell *Bacillus cereus*. In this chapter, a solution to the limitations of the usual bioreceptors in LFA (antibodies) is proposed by the in depth characterization study for the use of phage-binding proteins as biointerfaces. Their excellent binding properties to bacterial cells and their advantageous properties for coating LFAs membrane and nanoparticles allowed to detect *B. cereus* cells within 15 min in LFA format, which makes them relevant as future bioreceptors in LFAs for pathogen detection.

Part III - Towards electronic paper-based pathogen biosensors

- **Chapter 6** Since colorimetric paper-based sensors have high intrinsic limit of detection, a general design trend was identified in chapters 1 and 2 to integrate sensitive, electrical transducers to the LFA. Here, we propose a preliminary study of how electrodes (integrated or reusable) and non-Faradaic impedance spectroscopy detection mode can be applied to simple piece of nitrocellulose-based membranes towards the detection of pathogens. This chapter sets the basic models for impedance measurements on paper-based sensors, and highlights its limitations.
- **Chapter 7** leverages the knowledge from chapters 3 to 6 in order to investigate a prototype LFA with impedance detection, which final goal aims at detecting the nanoparticles commonly used as colorimetric labels in LFAs. In order to set the basis for future works on the development of electrical LFAs, this chapter proposes an in-depth characterization of the LFA electrical behaviour to assess the inherent complexity associated with integrating electrical detection constraints into the LFA system, which is already limited by its biochemical constraints.

The last section of this thesis consists in a conclusion, highlighting the contribution of each chapter in addressing the original research questions

of the thesis and highlighting the main points of the discussions associated with each chapter. It also investigates the most interesting prospects for future research directions and applications. Then, a broader discussion aims to offer holistic perspectives of PoC diagnostics, in particular by opening up reflection to issues of dissemination, legal framework for use or even industrialization.

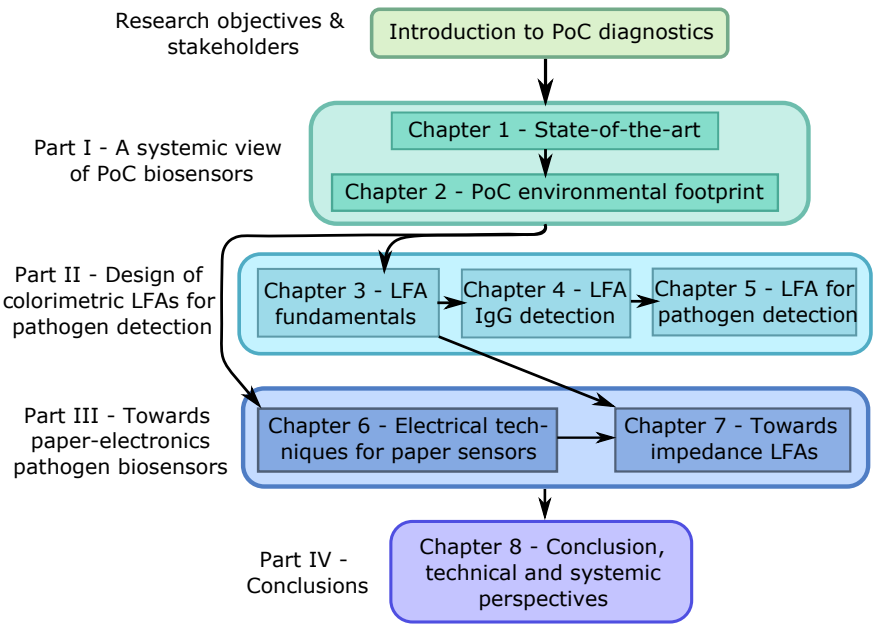


Fig. 2 Schematic thesis outline. A review of the point-of-care pathogen biosensors is performed in **part I**, including their associated environmental impacts. **Parts II** and **III** are the main body of this thesis, presenting the developments and innovations proposed towards the rapid and quantified bacteria detection with paper-based sensors. The conclusion aims to bring together the results of parts I-III, in addition to opening up perspectives for more holistic considerations to discuss the appropriateness of the research carried out with regard to the thesis main research question. Arrows indicate the prerequisites for conducting the research in each chapter. In this configuration, the research methodology implemented during this thesis can be considered *systemic*, according to the 5-steps method proposed by Ashby.

1

State-of-the art of point-of-care pathogen detection

This chapter introduces the concepts of bacterial detection with point-of-care sensors. First, it quickly describes the fundamentals of bacteria cells. Then, we review the conventional detection techniques for pathogens. Finally, we overview the concepts of a biosensor and the different classes of pathogen biosensors, with a special focus on point-of-care biosensors.

1.1 Fundamentals of microbiology

The microscopic agents responsible for many infections are bacteria (Figure 1.1). These microscopic agents can infect their host, i.e. penetrate within the human body, via multiples routes of infection (e.g. inhalation, ingestion, abrasion to the skin, or via insect bite). As bacteria are ubiquitous in nature, such as in the soil, on animals or in water, sources of natural infection are present everywhere. Food and water-borne infections can cause mild to severe gastrointestinal symptoms, as well as life-threatening complications. Typical food-borne pathogens include *Escherichia coli*, *Salmonella*

spp., *Listeria monocytogenes*, *Staphylococcus aureus*, or members of the *Bacillus cereus* group. The potential severity of these infections stems from the low bacterial load required to cause severe illness, necessitating the use of sensitive and accurate detection methods [6].

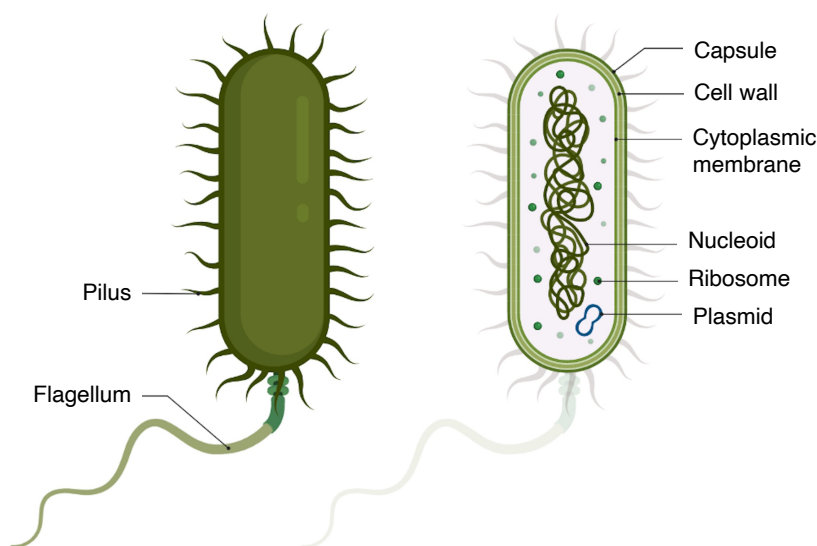


Fig. 1.1 Gram-positive bacterial cell structure. Common features of bacteria include the flagella, to propel the cell through its surroundings, and the pili, that provides points of adhesion to surface of other cells. The size of whole bacterial cell depends on the bacterial strains, but lies in the μm range (0.5 - 5 μm). Drawn with BioRender.

Bacterial cells often are ovoids of a few μm long and a little less in diameter. Some features are common for all bacteria: a cytoplasm, with a nucleoid, ribosomes and often plasmids, a flagella, pili, a cytoplasmic membrane and a cell wall, surrounded by a capsule (Figure 1.1).

The cytoplasm of, for example *E. coli*, contains approximately 15 000 ribosomes, several thousand copies of nearly 1000 different enzymes, a diverse array of metabolites and cofactors, and various inorganic ions. The nucleoid contains a solitary circular DNA molecule, while the cytoplasm contains one or more smaller circular DNA molecule known as plasmids. In nature, certain plasmids confer protection against environmental tox-

ins and antibiotics. In the laboratory, these DNA segments are particularly amenable to experimental manipulation and exceedingly valuable to molecular geneticists. [7]

The layers situated outside the cytoplasmic membrane form the cell wall, which is notably responsible for determining the shape of the bacterial cell, maintaining the integrity of the cell, preventing its lysis and regulating bacterial interactions with its environment and other structures. In addition, the cell wall is also a criterion for bacterial classification: Gram-positive¹ bacteria have a thick cell wall, while Gram-negative bacteria have a thinner cell wall surrounded by an outer membrane (Figure 1.2).

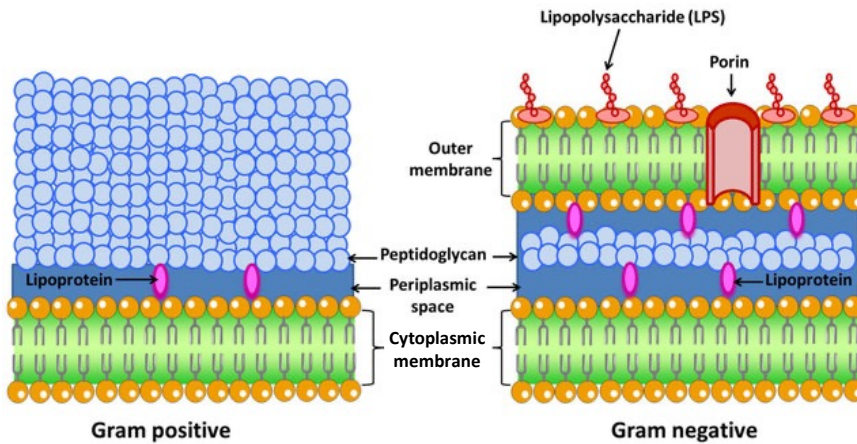


Fig. 1.2 Distinction between gram-positive and gram-negative bacteria. Gram-positive bacteria have a thick cell wall which consists in a thick, rigid layer of peptidoglycan on the outer surface of the cytoplasmic membrane, the latter being made of a thin bilayer of phospholipid molecules. Gram-negative bacteria have a thinner cell wall surrounded by an outer membrane into which are inserted complex lipopolysaccharides (LPS) and proteins called porins, allowing the passage of small molecules. [7]

For Gram-positive bacteria, the cytoplasmic membrane (thin phospholipid bilayer penetrated by proteins) is surrounded by the cell wall made of a thick layer of polymers called the peptidoglycan (Figure 1.3), and a capsule, i.e. a protective solid layer composed of polysaccharide and surface proteins lying outside the peptidoglycan layer (not shown).

¹They are called Gram-positive because they are colored by Gram's stain.

While the main function of peptidoglycan is to maintain cell integrity, it also serves as an attachment site for proteins and polysaccharides, which is of critical importance since it regulates many processes for bacterial recognition and infection. The cell wall polysaccharides, usually covalently bound to the peptidoglycan and closely associated with the cell wall, are considered as ubiquitous components of the cell envelope of gram-positive bacteria and play an important role as mediators of bacterial interactions with their environment. In particular, cell surface components (polysaccharides and proteins) are acting as specific receptors used by bacteriophages, i.e. bacterial viruses, to bind and enter the targeted bacteria [8].

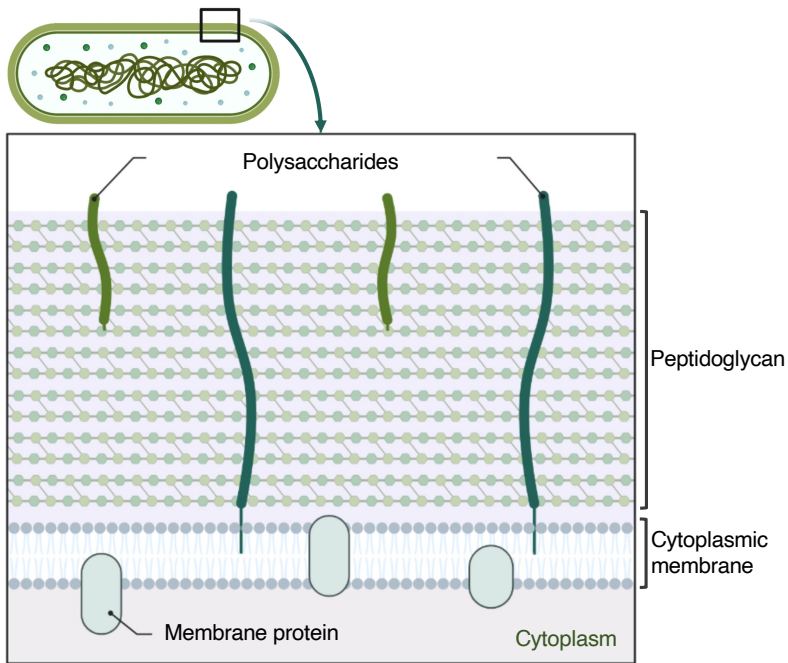


Fig. 1.3 Gram-positive bacterial cell wall structure consists in a thick, rigid layer of peptidoglycan (PG) on the outer surface of the cytoplasmic membrane. A variety of polysaccharides and other complex polymers are interwoven with the peptidoglycan, and surrounding the whole is a porous capsule, a solid layer composed of proteins (not shown). The bacterial cell wall regulates the contact with other cells, including the recognition, attachment and infection of bacteriophages to its bacterial host. Drawn with BioRender.

1.2 Conventional methods for pathogen detection

The prevention and treatment of infectious diseases or pathogenic outbreaks necessitate swift and accurate detection techniques for pathogenic bacteria. Many sectors are reliant on pathogen identification, including the food industry, clinical settings, and water and environmental systems, each dealing with different types of samples (blood, foodstuffs, milk, water, ...). The specific detection levels required for each pathogen highly depends on its matrix. Table 1.1 provides examples of typical tolerated thresholds of pathogens, or infection limits, expressed in Colony Forming Units (CFU), which estimates the number of viable bacterial cells. These thresholds are crucial since they guide the required limits of detection (LoD) of the analytical methods for pathogen detection.

Pathogen	Matrix	Threshold
All	Drinking water	< 1-5 CFU/100 mL
<i>Escherichia coli</i>	Water effluents	10 ³ CFU/100 mL
	Dairy products (milk)	10 ² CFU/mL
<i>Salmonella</i>	Dairy products (cheese)	10 ² -10 ⁵ CFU/g
	Water effluents	10 ³ CFU/100 mL
	Foodstuffs	< 1 CFU/g
<i>Listeria</i>	Foodstuffs	10 ² CFU/g
<i>B. cereus</i>	Dairy products	10 ³ -10 ⁴ CFU/g
<i>S. aureus</i>	Dairy products	10 ⁵ CFU/g

Table 1.1 Detection levels tolerated for diverse pathogens in various matrices. From AFSCA action limits [9], European rules relative to the pathogenic content [10, 11]. According to local legislation, non-conformity to clean water is admitted for enteric contamination in the order 5 CFU/100 mL [12].

Various analytical methods have been devised to detect pathogens in clinical, food, and environmental specimens (see Figure 1.4). Among them, four major methods include culture, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Their benefits and limitations are elucidated below and outlined in Table 1.2.

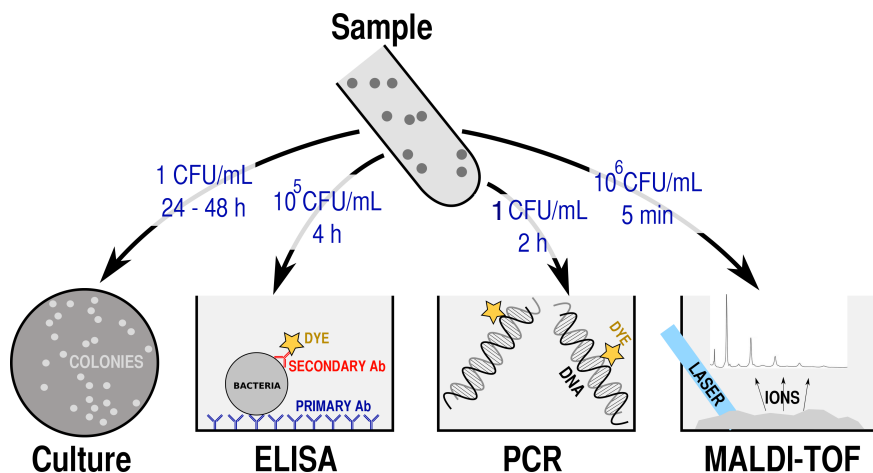


Fig. 1.4 Schematic representation of four conventional techniques for identification of bacterial cells. Reprinted from [13].

The **culture** method, the oldest method used for bacterial identification, is still considered as the gold standard. The process involves transferring microorganisms from their original medium to a liquid or solid culture medium to facilitate their growth (i.e. selective or non-selective growth media such as Lysogeny broth, LB), which can range from a day (for instance, *Staphylococci* and *Escherichia* species) to as long as nine or fifteen days. To identify the bacteria involved, based on a single colony, visual screening allow the colony count on the plates. Despite the prolonged analysis time, this approach offers superior sensitivity, selectivity, and assessment of viability and antibiotic resistance. [14]

The enzyme-linked immunosorbent assay (**ELISA**) uses interactions between antigens on a bacterial surface and specific antibodies, in a sandwich configuration. ELISA procedure involves a bacterial incubation of more than two hours on a surface functionalized with primary antibodies (typically in 96-well plates). Then, the surface is washed and incubated two hours with secondary antibodies labeled with fluorescent probes. The fluorescence or colorimetric level is then measured to quantify the presence of target bacteria. [15]

PCR (polymerase chain reaction) involves amplifying the bacterial DNA, which is present in the cytoplasm and obtained after cell lysis, through nu-

cleic acid amplification. Amplification requires successive cycles and need specific temperatures, for a typical assay duration of 2 hours. Once the DNA concentration in the sample is adequate, bacterial identification is typically performed using gel electrophoresis. Maintaining sample purity is critical in preventing cross-contamination, necessitating qualified personnel [15]. Real-time PCR monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PC, by using non-specific fluorescent dyes that intercalate with any double-stranded DNA. Real-time PCR can be used for quantitative monitoring of the targeted DNA molecule (named quantitative real-time PCR, qPCR) and semi-quantitatively (i.e., above/below a certain amount of DNA molecules).

MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) is a quick and widely adopted technique for the rapid identification of bacteria, albeit at a relatively high cost. It involves comparing the mass fingerprint of ions present in the target bacteria to a reference database, enabling precise identification of bacterial strains in the sample.

	Culture	ELISA	PCR	MALDI-TOF
Complexity	Low	Medium	Very high	Very high
Equip. cost	Low	Medium	Very high	Very High
LoD [CFU/mL]	1	$10^2 - 10^6$	1	10^5
Assay time	> 2 days	> 2h	> 1h	20 min*
Selectivity	Low-Medium	High	High	High

Table 1.2 Conventional laboratory-based methods of pathogen detection, requiring trained operator. (* after sample preparation). Combined from [6, 13].

1.3 Biosensors for pathogen detection

In the previous section, we saw that the bulkiness, the relatively high-costs, the requirements of well-equipped laboratory facilities and the time-constraints involved in the conventional bacteria detection methods are fuelling the interest in the development of simple, portable and low-cost tools suitable for a rapid (~ 1 hour), sensitive, and precise detection of harmful bacteria present in our environment. There is thus an urgent need for analytical platforms that can combine high-sensitivity, accuracy and rapid analysis with simplicity, portability and low cost. Biosensors aim to address these drawbacks as they offer rapid and cost-effective method of bacterial detection, sometimes directly at the point-of-care (PoC) without the need for specialized users [16]. For example, the most prominent biosensor is the electrochemical device for the monitoring and control of diabetes, which has had an extraordinary success [17]. Until recently, it was the only biosensor in this situation along with the pregnancy test based on the lateral flow devices.

As described by Anthony Turner, a pioneer in the field of biosensors since the 1970s [18], a **biosensor** can be defined as "*a compact analytical device incorporating a biological or biologically-derived sensing element (such as an enzyme, antibody, microbe or DNA) either integrated within or intimately associated with a physicochemical transducer*". This broad definition highlights that biosensors can be distinguished on their kind of transducer, recognition element (e.g. bioreceptor), sample handling (microfluidics) and readout interface (Figure 1.5).

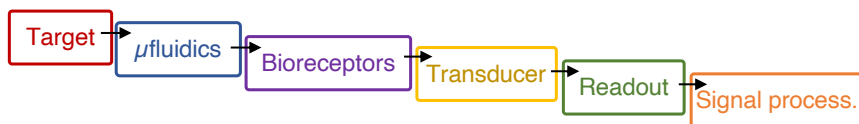


Fig. 1.5 The necessary steps involved in biosensors.

In this section, we present a simplified overview of the vast and relatively prolific field of biosensor research, contributed by scientists coming from various fields (applied physicists, electro- and mechanical engineering, analytical bio-chemistry, applied biotechnologies) [19]. The fact that

biosensor science is **multidisciplinary** in nature gives rise to a somewhat diverse state-of-the-art, often mixing notations and concepts from each field, in such a way that it is complex to get a clear and up-to-date picture of the state-of-the-art of biosensors for a specific application. Numerous good reviews cover the field of (point-of-care) biosensors for pathogen diagnostics [14, 15, 16, 20, 21, 22, 23, 24, 25]. Here, we propose an overview of general principles of biosensors for bacterial detection, i.e. the main support material that composes them and that very often dictates their functionalities, the accessible detection modes, and their preferential application. The detailed description of the physical principles of the biosensors studied in this thesis are distributed within the corresponding chapters.

1.3.1 Functional representation of biosensors

The different building blocks and detection principles recently developed for pathogen biosensors are described hereafter, and related questions and issues are addressed.

A general architecture PoC diagnostics sensor is depicted on Figure 1.6, which describes the sensor in terms of its building, or *functional*, blocks. The functional blocks represent the necessary functionalities for the full operation of a PoC biosensor towards detection of the *target* (e.g. pathogen). This architecture includes the functions of physical support (substrate, supporting all the other functions, especially *microfluidics*), the biointerface - often composed of *bioreceptors*, the *transducer* including the functional materials necessary for detection and any additional reagents added to the system to trigger the transduction mechanism, and the *readout* and *processing* system, i.e. the interface (e.g. microcontroller) that converts the signal emitted by the transducer output into a readable signal. Depending on the system studied, other blocks also exist such as external *pumping* systems for microfluidics sample processing actuation, electrical measurement tools (potentiostats, etc), data *storage* and *communication*, and *power supply* or batteries.

The objective of this classification is to cover and characterize the technical specificities required for each PoC functionalities, and to support qualitative comparison between alternatives offered by the classes of materials used in the PoC test design



Fig. 1.6 Schematic representation of a generic biosensor architecture, describing the sensor in terms of its necessary functional blocks to reach the biosensing functionalities. The required functional blocks include the microfluidics, the transducer, the bioreceptors, the readout and processing system, the data storage capacity and communication, and possible power supply.

1.3.2 Material supports for biosensor applications

Substrate material serves as the device structural basis and used to support the other components and functions. For many of the PoC devices, the biosensor device support is of critical importance, as it is generally directly interfaced with the functionalities needed for sample treatment and handling to the detection area, i.e. the microfluidics. Its structural and mechanical properties have a decisive influence on the applicability of the other functions as well, and related materials and manufacturing techniques. To fulfil its roles, the substrate is usually thicker and larger than the other functional components.

The **microfluidic** functions of a PoC device are generally provided by manufacturing the substrate materials (e.g. to integrate microfluidic channels), or by adding another dedicated component on top of the substrate. Microfluidic has undergone rapid growth with notable impacts to the analytical chemistry community due to a number of capabilities. These include its ability to utilize small amounts of samples and reagents and to perform separation and detection with high resolution and sensitivity, at low cost and rapidly [26]. **Conventional microfluidics** fabrication involves the use of glass [27], silicon [28], and polymers such as poly(methyl methacrylate) (PMMA) or poly(dimethylsiloxane) (PDMS) [29, 30] as well-developed substrates:

- **Silicon** has good mechanical properties (high strength), high thermal conductivity and a low thermal expansion coefficient, which makes it an ideal substrate for the mechanical component of small precision instruments. Most of these microfluidic devices are fabricated using techniques from the microelectronics industry (for example, etching of glass and silicon wafers following UV lithography) [31].
- **PMMA**, thermoplastic, is hard and stiff as polymers go, easy to polish but is sensitive to stress. It shares with glass a certain fragility. It can be blended with several other polymers for improved mechanical properties. PMMA is mainly shaped by casting (using a mold, injection moulding) or low-cost extrusion.
- **PDMS**, or silicone, is a softer polymer, typically used in medical applications.

Paper, for its part, is a very promising substrate material for low-cost microfluidic device for biochemical sensing. Recent studies have demonstrated its interesting properties, including that it is easily printed, coated and impregnated and its cellulose composition which is particularly compatible with biomolecules [32, 33].

Figure 1.7 shows different example of biosensors fabricated from different substrates. Different channel resolutions can be reached, mainly depending upon the substrate material and the fabrication process. Profiting from the microfabrication techniques, the silicon and glass substrates enable the definition of tiny microfluidic channels down to $\sim 1\mu\text{m}$, while paper channels are limited to $> 100\mu\text{m}$. Paper, which is easily patterned into discrete hydrophilic and hydrophobic zones, is able to wick fluids by capillary action without external power sources [34]. However, silicon, glass or hard (PMMA) and soft (PDMS) plastic substrate-based microfluidics usually require an external actuator such as energy-powered pumps. Depending upon the application, more advanced handling operations could be necessary, requiring advanced microfluidics and actuation designs. Finally, the possibility of the transducer manufacturing, requiring the integration of functional materials, is highly dependant on the functional properties of the substrate material (structural, physical, chemical/processability, optical, electro-magnetic, thermal).

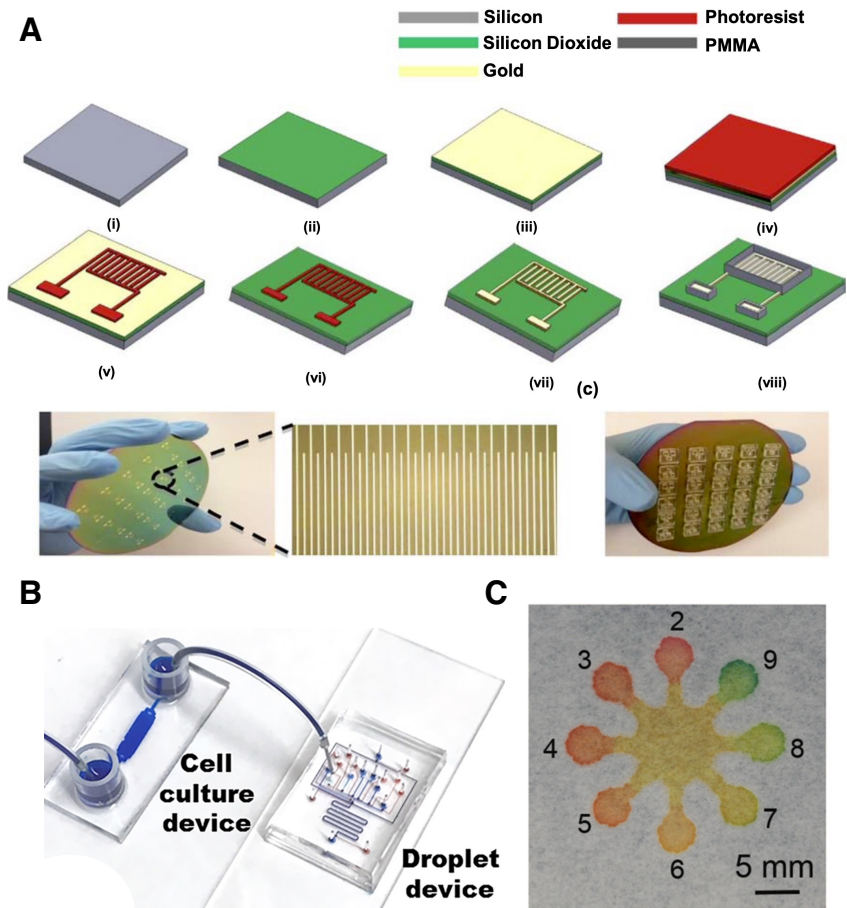


Fig. 1.7 Examples of biosensors fabricated on different substrates. **A.** Microchip fabrication of a silicon-based biosensor. The process flow involves the use of a 4-in. silicon wafer as a substrate. Different process steps (PECVD, e-beam deposition, standard photolithography techniques) are used to pattern interdigitated electrodes. To seal the microfluidics channels, a patterned PMMA cap is attached onto the manufactured wafer. [35] **B.** A plastic-based microfluidic channels on top of a glass substrate, fabricated by multilayer soft lithography [36]. **C.** Patterning of microfluidic channels on a paper substrate, forming 9 reaction zones for colorimetric detection [33].

1.3.3 Biorecognition layer

Biosensors for diagnostics aimed at detection of a certain contaminant need a biorecognition function to specifically isolate the target from the other compounds that could be present in the sample and interfere with the detection. Biorecognition can be implemented through several different pathways, among others the functionalization of the device surface with an immunological layer made of specific **bioreceptors** (e.g. made of antibodies) to capture the antigens at the reaction zone in the device, or the use of specific reagent that will activate only once they come into contact with the target. Immobilization of biomolecules can be achieved using different techniques, but each implies the binding of the biomolecule to functional groups present on the substrate surface. The substrate should therefore be inert against the biomolecules, biocompatible and should contain easily modifiable groups to accommodate the chemistry of biomolecule binding. Many protocols have been developed for the chemical functionalization of silicon, glass and plastics substrates, although stability issues if the bioreceptor layer are still frequently reported. On the contrary, specific types of paper materials and cellulose-derivative, such as the nitrocellulose, are extremely well suited for the biofunctionalization with biomolecules [37].

1.3.4 Transduction principles

The detection of the target in a PoC device happens via the transducer of the device. The **transducer** converts the presence of bacteria within the sensor into a physical signal, typically proportional to their number. Different detection modes are implemented in biosensor devices ; mainly optical and electrical, thermic and acoustic transductions, implying particular transducers that must be interfaced to the device. The electrochemical and optical transducers are the most popular transducers for bacteria sensing.

Optical transducers Optical biosensors are the most widespread biosensors. They measure the light intensity emitted from specific reagents, e.g. chromogenic substrates (peptides that react with proteolytic enzymes under the formation of color), functional nanomaterials (e.g. colorimetric labels such as gold nanoparticles) or chromogenic bacteria themselves, which are integrated to the detection scheme. They emit colour in various forms depending on the emission spectrum and physical principle, that can be visualised by the naked eye or with external optical camera in the visible,

UV or IR spectrum. Optical properties of the materials constituting the PoC are therefore of primary interest to ensure proper signal propagation to the acquisition side, or to monitor the detection process.

Taking advantage of high-resolution camera and microscopy imaging, the optical, fluorescent and colorimetric detection modes were developed with light intensity measurement by means of optical sensor, in which the optical signals are modified by the presence of the pathogens in the system, and are captured by bulk optical attachment setups [38]. Other systems benefits from the nanopatterns enabled by the manufacturing of silicon wafers to detect the bacterial presence through changes in the optical properties of the nanostructured silicon substrates [39]. However, colorimetric detection methods are increasingly benefiting from the huge deployment of image capture devices in the last decades, such as smartphones, and have been much reported for bacterial detection because of its wide potential applications in point-of-care diagnostics [40, 41, 42]. Based on these techniques, the detection of bacteria such as *E. coli* and *Salmonella* has been reported [43] (see Table 1.3 for other examples).

	Target	Substr.	Detection method	LOD CFU.mL ⁻¹	Ref.
Usual substrates	<i>E. coli</i>	Glass	Fluorescence	10	[44]
	<i>S. epi.</i>	Silicon	Capacitive	10 ⁶	[45]
	<i>E. coli</i>	Silicon	Capacitive	10 ²	[46]
Paper substrates	<i>E. coli</i>	Paper	Colorimetric	10 ⁴	[47]
	<i>E. coli</i>	Paper	Colorimetric	10 ²	[48]
	<i>E. coli</i>	Paper	Amperometric	< 10 ²	[49]

Table 1.3 Examples of biosensors for pathogen detection, compared for their target, substrate material, detection method and limit of detection. (Substr.: Substrate; *S. epi.*: *S. epidermidis*)

Electrochemical transducers Electrochemical transducers consist of electrodes applying electrical stimuli to the liquid sample to monitor its electrochemical answer. Electronic detection generally needs interfacing micro-electronic transducers, often made of electrodes or other structure sensitive to electrical properties in the device. The number and types of available fabrication techniques greatly depends upon the substrate material, with highly varying complexity, requirements (energy needs, reagents, emissions) costs and throughput. For example, processing silicon-based PoC could benefits from all the state-of-the-art, subtractive microfabrication

techniques, while implementation of electronics on paper mainly relies on recent developments in additive manufacturing to draw patterns made of conductive nanomaterial-based inks.

Electrochemical measurements (mainly amperometry, potentiometry and impedimetry) have shown broad applications of quantitative detections for important analytes in food safety, environmental and clinical diagnostics, owing to their reliability (precise quantification and low detection limits), and can be integrated and miniaturized to enable portability, at relatively low-costs. Simplified electrochemical measurements carried out with handheld devices provide a promising option for real time detections of pathogens [38]. Table 1.3 refers relevant examples.

1.3.5 Towards PoC paper-based pathogen biosensors

As described above, conventional biosensor systems, although relying on mature manufacturing processes and materials, still require cumbersome components (pumps, sample processors, optical/electrical detectors) and complex setups for operation [34]. This, in addition to bulkiness and overall cost of the readout devices, hinders the development of portable and cost-effective detection.

On the other hand, research on **microfluidic paper-based analytical devices** (μ PADs) has been focused on the development of point-of-care diagnostic devices [50], but applications for environmental monitoring or food analysis have only recently emerged [51]. μ PADs are highly attractive to make diagnostic accessible to scientists as well as for interested unskilled citizen groups [51]. This technology exploits low-cost cellulose materials and their ability to wick fluids to a detection zone by capillary action without the need for external pumping mechanisms [52], enabling highly simple and portable biosensors. One of the most famous μ PADs is the lateral flow assay (LFA), based on colorimetric detections. However, either in academic literature or in the industry, the limit of detection (LOD) of current μ PADs and LFAs designed for bacteria detection is not low enough to ensure, for example, the potability of water for the *E. coli* O157-H7 bacteria (ref. [47] in Table 1.3). However, at the light of the pathogen thresholds reported on Table 1.1, these colorimetric sensors could provide valuable information about the pathogen presence in food samples.

The **microfluidic paper-based electrochemical devices** (μ PEDs) trend is an added value to the μ PADs since it combines the merits of paper as a solid support, to the high sensitivity and selectivity of bioelectrochemical detection [53]. Indeed, most paper-based microfluidic devices rely on optical methods of detection (colorimetric, fluorescence) which can suffer from a lack of quantification. Although colorimetric assays are the best choice for some applications, electrochemical sensing provides a more versatile and quantitative methodology for others [54]. Electronic methods of detection profiting from the paper porosity are attracting increasing interest and novel ways of integrating electronic elements are being explored with printed electronics [31, 55]. μ PEDs present a conceptually different approach to common electrochemical biosensors, combining simple, low-cost and accessible fabrication strategies (e.g. inkjet- and screen-printing using Ag or Au nanoparticle-based conductive inks) on paper substrates with high functionality for the device. As an example, after bacteria concentration through nanoparticle-based magnetic separation, Luo *at al.* obtained sensitivity as low as $\sim 10^2$ CFU/mL for *E. coli* O157:H7 by electrical measurements with two printed electrodes on the paper biosensor surface (ref. [49] in Table 1.3).

1.3.6 Figure of Merit of desirable biosensors

In order to compare different kinds of biosensors, it is crucial to quantify their performances based on various **figures of merit (FoM)**, characterizing many key aspects of biosensing. As a matter of fact, the World Health Organization outlined the **design guidelines** in the research and development for such new point-of-care (PoC) devices. It is known as the **ASSURED** principles : the PoC biosensors must be Affordable, Sensitive, Specific, User friendly, Rapid and Robust, Equipment Free, Deliverable to end users.

Table 1.4 is an attempt to associate quantitative metrics, or FoM, to the ASSURED design guidelines, in order to allow a fair comparison between different kind of biosensors.

Figures of Merit (FoM)	
Affordable	• Affordable to the end-users and the relevant institutions. • Typically 0.5 - 10 €/test (+ need to consider the testing environment and associated necessary costs).
Sensitive	Highly sensitive tests minimize the false negatives. Related to performances of the transducers : • <i>Limit of Detection</i> (LoD), i.e. the minimal detectable concentration of bacterial cells. This critical concentration (CFU/mL) is often computed as² $LOD = \mu_{blank} + 3 \times \sigma_{blank}$ • <i>Sensitivity</i> (S) is the change of the sensor output (e.g. intensity of colorimetric signal, or electrical resistance) per unit variation of the bacterial concentration • <i>Dynamic Range</i> (DR) is the range of detectable analyte concentration from the lowest (LoD) to the highest detectable level, or <i>saturation</i>. Useful for quantification.
Specific	Closely related to <i>binding performances</i> of the bioreceptors. • Ability to have low false positive rates with samples containing for example many background biological components other than the target, and that can interfere with transducer. • <i>Multiplexing</i> is the ability to simultaneously detect several different bacteria within a sample using a single sensor with different functionalizations.
User friendly	Tests should be easy to perform in 2-3 <i>steps</i> (e.g. sample preparation) and require minimal <i>user training</i> with no prior knowledge of diagnostics.
Rapid	• The <i>response time</i> is related to the transducer performances, and is the time needed to obtain a stable biosensor output from its initial state. • The <i>analysis time</i> encompasses all the detection, from sample collection, pre-treatment and response time until results are collected. Should be around 15-60 min.
Robust	• Ability for the bioreceptors to provide adequate binding despite the complex environments (withstand flow, avoid signal drift due to biofouling, retain bioreceptor binding properties). • Also refers to the ability of the test to withstand the supply chain and provide accurate results within its shelf-life. • <i>Reproducibility</i>, quantifying sensor-to-sensor variability.
Equipment Free	Ideally the test do not require any special <i>equipments</i> or facilities, or can be operated in small <i>portable</i> device with limited <i>power</i> requirements.
Deliverable	Refers to organizational logistic structure of procuring, shipping and distributing diagnostic tests even in resource-limited settings

Table 1.4 The ASSURED criteria and related FoM. These FoM will be used in this thesis to compare the relevant biosensors. [3]

1.4 State-of-the art conclusion

In this section, we have provided a brief overview of the current status of point-of-care (PoC) biosensor devices. Based on a representation of a biosensor through the different **functional blocks** that are necessary for a performing diagnostic tools, we discussed the different technologies available for the main sensor functionalities. Recently, the field of POC diagnostics has advanced significantly, with a wealth of tools and information that can be leveraged to develop improved assays. These advancements in the different technologies, from paper-based devices to silicon-based biochips have enabled features like multiplexing, on-device sample processing, real time sensing and the generation of highly sensitive signals to the presence of contaminant in samples. Considering all the attributes of biosensors discussed above, it seems that **both silicon and paper-based sensors offer functionalities that can be of great interest to allow the design of rapid pathogen biosensors at the point-of-care**. While silicon offer the potential for higher-sensitivity assays that provide rich diagnostic information, paper substrates still enable maintaining the affordability and ease of use of traditional lateral flow tests.

First conclusion Since the goal of this thesis is first and foremost to identify the appropriate technology to enable rapid pathogen biosensing in critical situations, the technical aspects raised in this section lead us to **select paper-based biosensors as the priority technology for developing targeted pathogenic biosensors**. While paper-based biosensors have demonstrated advantageous properties and performances for some clinical applications and potential for use in food safety applications, their efficacy has yet to meet the demands of many critical applications such as the detection of waterborne pathogens and various food-borne pathogens. Indeed, it is expected that, when coupled with advanced microelectronics or ubiquitous smartphone-based readers, these assay improvements have the potential to overcome their disadvantages of low sensitivities and high detection limits. This, in turn, could give rise to a new generation of PoC diagnostic

tools, which have the potential to be used in a variety of settings [56].

Since paper-based biosensors have not been much studied in our laboratory at UCLouvain, **our research plan consists in : Part I** (i) first, study and implement the basic principles and manufacture of the most common colorimetric paper-based biosensors, the lateral flow assays (LFAs) and (ii) assess their performances to the detection of whole-cell bacteria. Then, in **Part III** we (iii) detect pathogens on paper with electrical detection methods, and (iv) explore the possibility to integrate reusable electrodes on conventional LFAs for increased performances.

Second conclusion The current state-of-the-art provides information on a large number of technical characteristics of PoC biosensors, i.e. those referred as the *design questions* on Figure 1. However, **performance criteria covering the other stages of a biosensor's life cycle are extremely underreported in the state of the art**. For instance, when environmental impacts related to sensor manufacturing are mentioned (such as *raw materials*, *manufacturing*, and *end-of-life* stages on Figure 1), they are seldom quantified, thus hindering any objective decision-making regarding the choice of the most suitable sensor. Similarly, scientific publications do not consider the possible externalities associated with the use of sensors in field situations (during the *use* phase).

The conclusion drawn is that the present knowledge on sensors solely considers the traditional performance figure of merits, thus hindering a comprehensive decision-making process towards an appropriate biosensor technology. In order to make informed choices, it is crucial to incorporate other aspects, including environmental considerations, in design practices and establish a methodology to implement them. Therefore, in chapter 2, **we propose an evaluation of the environmental impacts of the research conducted in parallel with technical developments, to direct the progress towards solutions that are both technically and environmentally appropriate**. The other economic and social dimensions will be introduced in **Part IV** of this thesis.

2

Eco-design methodology for early-stage research

2.1 Introduction

Since diagnostic devices for emerging disease detection such as LFAs has become commonplace in the clinical field (the focus shifts towards bringing testing as close to the patient as possible, i.e. at the Point-of-Care), the popularity of diagnostic testing has driven a surge in demand for rapid tests in the global market (Figure 2.1 A). Therefore, the number of applications and **the production volumes of PoC diagnostic devices are expected to increase at the global scale**¹. This increase in rapid test uses is also supported by more stringent regulations and quality standards regarding microbiological and chemical contamination in environmental resource and food safety as well (e.g. mycotoxins diagnostics in diaries), requiring a more extensive and frequent monitoring of these criteria. As an example of widespread PoCs, pregnancy rapid tests, which were initially introduced in the 1980s, are now extensively utilized, with approximately 3 million tests sold annually in France. More recently, LFA gained public recognition due to the COVID-19 pandemic. During the crisis, LFA were used for

¹<https://www.maximizemarketresearch.com/market-report/rapid-test-market/544/>

the first time as part of a large-scale testing strategy and billions of tests have been provided worldwide since the start of the pandemic².

As a result, the number of waste associated with diagnostic devices is rapidly increasing, as many of these tests are single-use, with an extremely short lifespan of just a few minutes (Figure 2.1 B). Typically, the percentage of single-use point-of-care (PoC) devices in hospital waste ranges from 5-20% and can account for tens to hundred of kg of wastes every day [57]. The huge number of tests distributed during the pandemic special context was associated with more than 30,000 tones of wastes³. For example, it has been reported that the UK's LFA programme to date has produced enough plastic waste to fill 19 Olympic-sized swimming pools⁴.

Most single-use PoC devices are made of plastic materials derived from non-renewable sources, contributing to the escalating global medical waste problem (Figure 2.1 C). A significant portion of PoC diagnostic waste falls into the category of infectious waste, which requires separate collection and treatment to eliminate the risk of infection. The incineration of infectious waste is the most common method of treatment, resulting in greenhouse gas (GHG) emissions [57]. In low-resource settings without proper treatment facilities, testing devices are often burned on open pits in health facility grounds or at municipal dump sites, releasing toxic pollutants. Additionally, a significant amount of PoC device waste ends up in landfills or municipal water supplies, increasing the risk of people being exposed to hazardous reagents such as nanomaterials or cyanide derivatives used in PCR cartridges [57]. Therefore, while single-use PoC tests have great potential for global health, they also contribute to growing global challenges related to plastic waste, GHG emissions, and human exposure to toxic pollutants.

Beyond healthcare systems, the widespread adoption of PoC device at the global scale could therefore cause significant risk for ecosystems and human health if their entire life cycle is not properly considered. These critical aspects may condition the technology relevance and acceptability in the long term, and the impacts of these technologies must be collectively managed towards robust decisions on their development and implementation.

²<https://www.statista.com/statistics/1028731/covid19-tests-select-countries-worldwide/>

³<https://www.who.int/news/item/01-02-2022-tonnes-of-covid-19-health-care-waste-expose-u>

⁴<https://www.cambridge-design.com/blog/how-to-reduce-the-carbon-footprint-and-plastic-w>

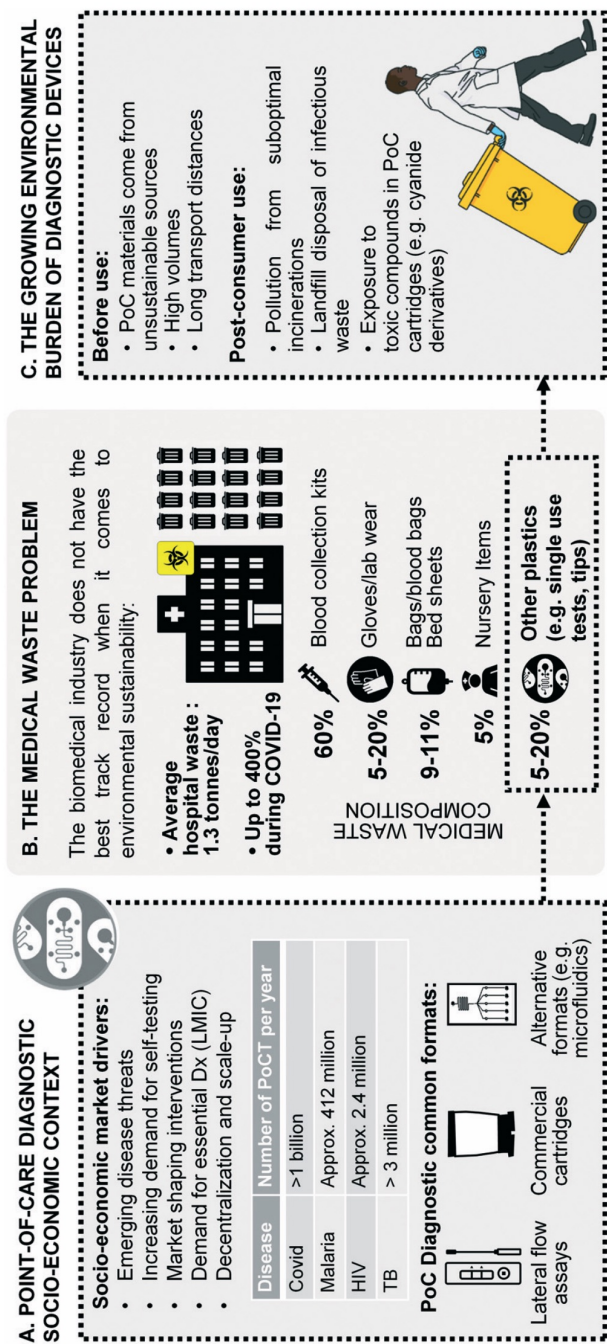


Fig. 2.1 Overview of the challenges and solutions in single-use diagnostic devices. **(A)** The number of PoC diagnostic devices is expected to increase at the global scale. **(B)** The test are mainly made of single-use materials (plastics and cardboard), and toxic chemical reagents. **(C)** The generation of waste from diagnostic devices is now widely recognized and gaining public interest. Reprinted from [57].

Growing public interest in the sustainability of single-use tests, particularly in the amount of plastic needed to meet the demand for rapid COVID tests⁵, highlights the need for responsible and sustainable practices in the PoC sector. However, many stakeholders in the PoC sector have not yet integrated sustainability into their practices. In particular, chapter 1 demonstrated that sustainability metrics are not yet scientifically and systematically considered by researchers and developers in the design of new biosensor platforms. An accessible approach for researchers working on innovative PoC devices is to modify the devices towards greater sustainability [57]. This approach, often referred to as **eco-design**, consists in the development and implementation of environmentally and socially responsible principles in the design of a product to reduce the negatives externalities generated by the design, the manufacturing, the use, and the disposal of these PoC devices.

In light of this definition, implementing eco-design requires integrating a **multi-dimensional impact assessment** (socio-economic, environment and public health) of the innovation early in the research process, prior to its deployment. Its interest lies in (1) informing the designer decisions, by estimating the magnitude and significance of potential impacts of different configurations of a developing innovation and guiding the design towards the most appropriate technology, (2) contribute to the production of a shared knowledge base about technologies and multi-stakeholder multi-dimension impacts evaluation, and (3) prepare the justification of the choices and the potential impacts if the question is put at the public agenda.

The research and development processes are inherently linked to significant **uncertainty** in the design of innovations (e.g., materials to be used, manufacturing techniques, final design). Anticipating impacts in early-stage innovation research is therefore complex, requiring a distinct scientific approach with a **methodology** that uses reliable indicators for fair and comparable assessments, tailored to the needs of developers. Specifically, the methodology to be developed should be able to start with the existing products, and environmental indicators should be able to help improve the technology towards using more sustainable materials and generating

⁵Debate has raged on social media about why the tests need to use so much single-use plastic and how they could be made more 'sustainable' (<https://www.medicaldevice-network.com/features/lfts-plastic-waste-sustainability/>)

less toxic waste. Despite some recent attempts⁶, there is not yet a well-established methodology for conducting a multidimensional impact assessment that is applicable early in the life cycle of an innovation.

Integrating the systematic identification of multi-dimensional impact causes into the research process requires methodologies beyond traditional technical design principles. An appropriate methodology must emphasize skills, tools, and interdisciplinary connections for defining actions within the researcher's boundaries.

2.2 Chapter contributions

This chapter attempts to develop and implemented a methodology to integrate environmental impacts at the early stage in the development of an innovative PoC device.

Given the early stage in the innovation life cycle, this methodology should:

1. Rely on various tools and analytical approaches, such as systemic thinking, to perform and develop a comprehensive understanding of the impact assessments of different device prototypes.
2. Integrates diverse information on economic, social, health, and environmental consequences using a systemic approach.
3. Accommodate evolving knowledge and the continuous influx of information during the uncertain research and development process, facilitating the incorporation of new insights over time.
4. Help to adopt a critical and reflective approach by articulating focus from researchers' objectives to societal implications through iterative exchanges.

⁶For example, Granta Design introduced a methodology of selecting a sustainable design based on material and process trade-offs <https://www.ansys.com/blog/the-impact-of-materials-on-sustainability-part-2>, and the European Commission's science and knowledge service (Joint Research Centre, JRC) published technical reports on, for example, safe and sustainable chemicals and materials by design <https://op.europa.eu/en/publication-detail/-/publication/c3e396d0-0a5e-11ee-b12e-01aa75ed71a1/language-en>.

5. Consider the broader context of the research on low-cost sensors for pathogens diagnostics for environmental monitoring and food safety : users, beneficiaries, producers, and other factors.

To this end, the chapter includes the following sections:

The first section introduces the concept of eco-design and mentions the main tools for comparing technologies based on their environmental impacts. In particular, simplified life cycle analysis is proposed as a suitable tool.

In the second section, we explain an original framework to guide the analysis of the systemic aspects of biosensor technologies ; it relies mainly on life cycle analysis for earliest stages of research.

In the third section, we present the results of combined environmental and technical assessment, that is of importance for the development of biosensors and draw conclusions useful for the research to be pursued in the technical chapters 4, 5, 6 and 7 and, more generally, for the development of PoC devices.

2.3 Eco-design of biosensors

Eco-design refers to the action for designers to concretely implement environmental considerations in the design process. [58, 59]. This is one of the levers that can be employed to mitigate one or several direct environmental impacts of the PoC presented in section 2.1, such as selecting the most environmentally preferable solution among several material and manufacturing options during the PoC design process.

Eco-design can be applied along with **Product Design Process** (PDP), i.e. the process through which a complete biosensor device, made of all its components and sub-components, is designed, developed and optimized in successive steps (see Appendix A for details) to answer the need of an application (Figure 2.2 (a)). Figure 2.2(b) introduces eco-design alongside the traditional PDP by referring to the common actions that aim at minimizing the environmental direct impacts of a product over its whole life cycle, as stated by ISO standards 14006:2020 [58]. This standard fosters the co-optimisation of environmental and technical performance by integrat-

ing environmental aspects into the design process by applying concrete actions such as:

1. **Energy Efficiency** : minimize energy consumption during the device operation and at every stage of production.
2. **Materials Selection**: selecting environmentally friendly and sustainable materials during the manufacturing of PoC device. This involves considering factors such as the use of recycled or biodegradable materials, minimizing the use of hazardous substances and greenhouse gaz emissions, and promoting responsible sourcing practices to reduce the overall ecological footprint.
3. **Durability and Longevity** : promoting high-quality construction, robustness, and include easy disassembly, component replacement or upgradeability
4. **Waste Reduction** : Reduce the amount of raw materials used in the product, eliminate landfill disposal at product end of life and support recycling by following the 10'Rs scale [60] strategies for products and components, including Reduction, Reuse, Repair, Remanufacture and Recycling.
5. **User-Friendly Design** : ease of operation and maintenance procedures, helping maximize the efficiency and effectiveness of the end-user using these devices.

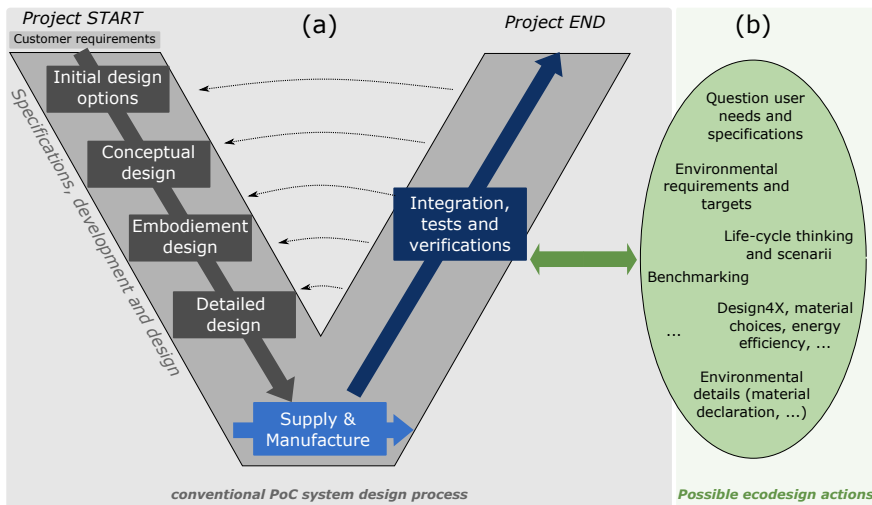


Fig. 2.2 (a) Illustration of the product design process (PDP). Figure adapted from [61]. (b) Generic ecodesign actions alongside the traditional PDP [58].

2.3.1 Eco-design tools

To support the ecodesign throughout the different steps of the PDP, several ecodesign tools can be used. They differ mainly in their recommendation and assessment levels they can provide, as shown in Figure A.1 (Appendices). The tools with high recommendation levels generally required low levels of environmental assessments, therefore missing quantitative data regarding the impacts of the product or service. On the contrary, precise assessment tools usually provide a low level of recommendations as they rather provide specific estimates on the impacts more than recommendations. As a result, much effort is required from the experts to translate the quantitative impact indications provided by the method into clear and useful recommendations for the targeted product or service.

Specifically, **Life-Cycle Assessment (LCA) methodologies** are key tool for ecodesign because (i) they are based on already-existing standardized ISO methodology, (ii) they provide quantitative environmental results over the whole life-cycle of the devices, (iii) they consider several environmental indicators in order to detect impacts transfer, and (iv) they can help improving designs iteratively. The standardized LCA methodology is described in Appendices (Figure A.2). Although LCA tools offer a high level of assessment, they require time, expertise and specific data. Consequently, they usually come together with a non-negligible financial cost. This is why **Simplified LCA (sLCA)** have been developed, where the scope of impact categories is reduced and the study is often limited to the most relevant life cycle phases [62]. They have the advantage of requiring less information, being quick to carry out and easier to interpret, but the results are more limited in the conclusions that can be drawn since the modelling and hypotheses are simplified.

2.4 A methodology for eco-design in early research

Since the figure of merits referring to environmental impacts of the biosensor technologies are very sparse or absent in the scientific literature of biosensors, usual literature review and comparison of different sensors does not support proper eco-design and informed choices between sensor alternatives.

Therefore, a methodological framework is proposed which aims at **initiating environmental eco-design** of biosensors during the **early-design phase of technology**. In practice it should allow to arbitrate between conventional and life-cycle environmental performances, and orient the decision-making process towards the sustainable solutions. In particular, it consists into :

1. Extend the conventional figure of merit for PoC material choice to relevant indicators taking into account the **environmental cost** of the PoC devices.
2. An appropriate eco-design method tailored to PoC devices is needed for early-stage research, where uncertainties exist regarding available data. It combines technical and environmental assessments in the form of simplified LCA studies to support sound design decisions for PoC devices by the co-optimisation of biosensor technical and environmental performances.

2.4.1 Theoretical elements of early-research environmental assessments

The proposed framework was formulated based on a literature review that is described hereafter.

LCA, in most cases, is a decision-making tool in process selection, design, and optimization. However, as a tool designed to quantify the full range of environmental impacts within a system, LCA has traditionally been undertaken retrospectively, using data from existing large scale processes. However, great potential for environmental improvement exists using LCA within the design stage of any innovation where it is estimated that about 80% of all environmental effects associated with a product are determined in the design phase of development [63]. Indeed, multiple research cycles still have to be run through before such technologies can become available at an industrial scale. Integrating LCA early-on in the technology development process can improve the understanding of the implications of design choices on the environmental performance of the technological innovation [64, 65], forming the basis of eco-design. The earlier LCA results can be integrated, the better, since they can prevent environmental burdens whilst a limited amount of investment has been injected

in the device development. Such innovation in an early stage of development, known as emerging technologies, may exist as an industrial pilot, in a lab environment, as a proof-of-concept or even only as a basic design idea, depending on their technology readiness⁷.

A drawback of early research LCA is that only limited data and knowledge on the developing technology is available at this stage. This is also known as the *design paradox* [64]. As such, **carrying out an eco-design of PoC devices in their early stages of development is to create a contradictory position**: too precise an eco-design conducted too early in the development of the technology will be confronted with too much uncertainty about the results that emerge, whereas if one waits too late, the design decisions will already have been made and the eco-design will have no impact. This is probably the main reason for the few LCA study of early research found in the literature, whereas there are plenty of retrospective LCA studies on products, whose actual impact is reporting and not eco-design.

Several authors also discuss options for methodological changes when implementing LCA at early-research stages [66, 67, 68, 69, 70]. They mainly suggest the use of a stepwise LCA procedure in parallel with the development process instead of a retrospective assessment. Use of LCA in this way often entails the assessment of lab or pilot-scale innovation to generate environmental data, which can then be used to optimise the developing product or process. This data may also be used to compare with existing industrial product or processes, to demonstrate or identify the environmental advantages of the innovative process over the existing activities.

In addition to the myriad of methodological challenges that are debated within the LCA community, full-scale LCA is often thought of as too difficult or time consuming to pursue at the research or development stage of an innovation. There are number of methodological and practical difficulties that arise from using LCA at this stage [63], including a lack of reliable data. In fact, it is not relevant to implement a specific and complete LCA at the beginning of the research process, and simplification of LCA cannot

⁷The **technology readiness level (TRL)** expresses a technology degree of maturity to reach the market. It ranges from 0 to 9. The TRL encompasses the different phases of the technology life-cycle : as a basic idea (0), as a proof-of-concept based on preliminary simulations (2-3), in a controlled lab environment (4-5), as an industrial pilot (6-7), or a technology disseminated at mass-scale (9).

be avoided in the development of new products [71]. In this way, studies have been conducted using simplified LCA of emerging products, identifying the simplification options that are still producing reliable results (in particular choices of the life cycle inventory (LCI) model and the result indicator profile) [72].

Early-stage LCAs involve exploring numerous alternative technology choices and prototypes. Emerging technologies and novel products differ significantly from established materials or processes, resulting in varying operational, in-use, and disposal data. LCAs at this stage face challenges due to scale issues and technology uncertainties, making it problematic to determine the functionality for assessment and find appropriate environmental data for modelling the innovations. Published databases generally contain outdated and generic data, which are difficult to update due to intellectual property concerns in industries [73].

Finally, conducting a proper LCA requires to capture the specificity of the field under study. The published works described above are either proposing generic frameworks, or applied in common LCA field (building and chemistry), failing in capturing the specificity of micro- and nanotechnologies which are characterised by a large choice of materials and complex manufacturing process. In fact, there are few or no LCA studies of innovations such as the biosensors studied in this thesis, probably due to the theoretical almost infinite number of design that biosensors (and microsystems in general) can take. A few works have been published, though they are mainly focused on printed electronic systems [74, 75, 76, 77].

There is a need to **understand how to combine the use of LCA and an iterative approach in the case of micro- and nanotechnology innovations** to support the integration of environmental considerations and sustainability into their design. An appropriate methodology for conducting LCA in early research stages must be developed. When information is limited, the simplified LCA offers a solution by providing general guidelines to prioritize the choice of design, materials, and components based on their performance and environmental aspects.

Based on the experience from the collaborative work on LCA for microelectronics carried out during this thesis [73, 78], we propose and implement an eco-design methodology for early-research on PoC biosensors.

The methodology propose to integrate multiple, iterative simplified LCAs whose complexity evolve with the innovation development stage. The specificity of microtechnologies are taken into account by identifying the most important databases of the field. Some of the work described in the next sections have been published [79, 80].

2.4.2 Decision-making framework description

Our framework is divided into 4 successive steps along the development process that are schematically represented on Figure 2.3. It consists in conducting parallel technical assessments (TA) and environmental assessments (sLCA) of different biosensor technology candidates that could meet the need of the application. These assessments, conducted in different ways for the different TRLs, allow the comparison of candidates in the form of a synthesis, closing each stage of the framework. The synthesis allows to avoid making technological choices by default, but rather to identify the preferred options on the basis of objective criteria, including systemic criteria. The assessment methodologies related to the different TRL levels are described in the following sections.

Extending the conventional figure of merit for PoC

Step 0. The project starts with defining the problem statement, or the research question, i.e. the development of a biosensor for a defined application.

Step 1. Then, the start of every research project is generally accompanied with a **technical literature review**, allowing to identify **initial design options** from the literature, by assessing what are the main technical risks, and their associated traditional FoM, typically described along the AS-SURED criteria in section 1.3.6. In our framework, we leverage this initial technical screening of different biosensors by adding a **first qualitative review of the environmental metrics** of the materials and processes that are used in the different design options revealed by the literature review. Since the metrics related to environmental impacts are not typically reported in biosensor reviews, it is necessary to conduct a more thorough literature search in the specialized field of environmental impact assessments. This often involves drawing connections and taking inspiration from environmental studies on topics in other technical areas, as studies on PoC devices and microtechnologies in general are still relatively rare compared to those

on energy systems or the chemical industry [57].

The environmental metrics of interest to be reported actually depends on the application. Interestingly, in the case of PoC biosensors, we can note that authors suggested to follow the REASSURED criteria, including Real-time connectivity and Environmental-friendly guidelines. The latter mainly proposes the substitution of single-use plastics with recycled ones, and care about electronic circuits treatment. However, a deeper dive into the sustainability aspects of the materials used in PoC diagnostics suggest to evaluate at least the metrics reported in Table 2.1.

Environmental & Systemic Figures of Merit (FoM)	
Embodied Impacts	Represents the energy, CO ₂ emissions or water consumption required for primary material production (including extraction, processing and refining) • Embodied energy [MJ/kg] • Carbon footprint, CO_{2,eq} [kg/kg] • Water usage [L/kg] • Energy [MJ/kg], emissions [kg CO_{2,eq}/kg] and water [L/kg] associated to required for manufacturing techniques
Material Sourcing	These metrics also includes economic and geopolitics aspects of material sourcing. Another interesting metric is the main supplier countries. • Abundance in earth's crust (ppm by wt.) • World production (Tonne/year) • Price (€/kg) • Critical material risks (contains > 5wt% critical elements?) : yes/no

Table 2.1 The quantified environmental and systemic FoMs used in this work for the initial technology screening.

In addition, **required initial investment** of the manufacturing techniques required to process the PoC diagnostics is a relevant criteria, since it will influence the technology affordability and/or transferability, as well as reporting of material **durability**, i.e. the tolerance fo the materials towards the environmental conditions. Finally, **End-of-Life** criteria such as material recycling rate, biodegradability or downcycling possibility are of great interest but sometimes face uncertainty/contestability.

The combination of this technical and environmental reviews, in the form of a synthesis (e.g. a table listing the technical and environmental

criteria), should allow the **formulation of initial hypotheses** on the materials, transducers, etc., that would be most appropriate to answer the research question. Completion of this stage requires a **first choice to be made from the design options** available, which is therefore **partly subjective** at this stage. Since there is a lot of uncertainty, both in terms of technical and environmental performance, it is best to **keep several design options in mind**.

Integrate simplified LCA (sLCA) to make informed design decisions

The two next steps deal with the question *How to integrate quantitative assessment, such as sLCA, to guide the choices of technology early in the research process ?*

Step 2. Once the project has just started, a few (2-3) of the biosensor designs are studied more or less in parallel to test the design hypotheses without developing a whole sensor device. At this stage (TRL1), the biosensors are still in the form of **conceptual designs**, meaning they do not need to reach all the functionalities required. Instead, the **early technical assessments** of the conceptual designs involve initial tests of the manufacturing methods and their compatibility with some of the materials selected, the first biointerface developments, first simulations to validate transducer hypotheses and first characterizations.

In parallel to these initial technical assessments, a first simplified life cycle assessment (sLCA), in the form of an **exploratory LCA**, is carried out to obtain an order of magnitude of the environmental impact of the selected conceptual designs at the **technology level**. This stage is mainly useful for getting to grips with simplified life cycle analyses, and consists in assessing the footprint associated with the main materials and manufacturing techniques considered at this stage. In this way, the level of granularity of the study should not be excessive since the process details of each sensor are not yet known. On the other hand, it allows very quickly to identify the big hotspots of the different sensors technologies, which can include: the adequacy of a manufacturing process for a certain design, unexpected impact of a specific material on which the biosensor heavily rely, or some materials or processes for which the data (formulation of chemicals, consumption of machines, ...) are not available in the literature or subject to intellectual property, preventing a proper assessment, etc.

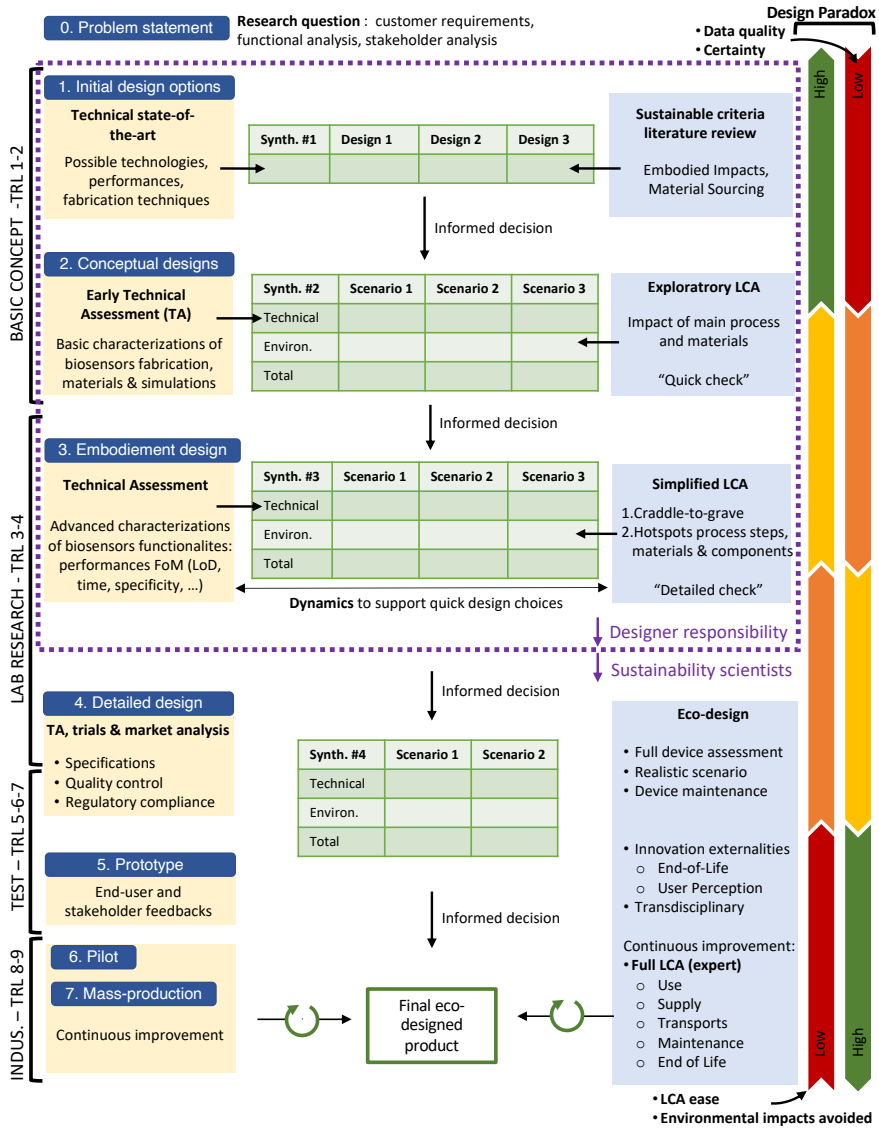


Fig. 2.3 Decision-making framework for the co-integration of technical and environmental performances in the early research of biosensors towards their eco-design. At each TRL stage of the innovation development, the methodology uses suitable tools based on data and knowledge uncertainty. At low TRL levels, simplified environmental studies (sLCA) are used due to high design uncertainty and limited data quality. The methodology includes a synthesis step to compare accessible scenarios at each development stage and make informed design decisions.

The synthesis phase of this step, gathering the findings from the TA and the exploratory LCA, should mainly serve to close the door on less promising concepts, or those with obvious shortcomings, both technical and environmental. It also serves to find, for example, alternative processes before having engaged too long with one specific, inconvenient, process.

Step 3. The first technical results for the different concepts have been achieved, and embodiment designs are being explored, and include some of the critical functions and components required for the biosensors. This way, the **technical assessment** tackles the development of first prototypes at the lab scale that can show the relevance of the initial biodetection concepts in a controlled environment (TRL 3-4). Since the **embodiment designs** already involve some advanced functionalities, it allows to study in deeper details the impacts at the different **process steps level**, particularly for the fabrication processes involving many steps.

The **simplified LCA** in this step takes the form of a cradle-to-gate analysis, meaning it only considers the impact generated by the production of the materials and the manufacturing of the device (see Figure A.2 (a)), also called “pre-consumer waste footprint”. The use phase, associated usage of energy to actuate the PoC device, and all the transports (supply raw materials, to the use location or to the end of life treatment location) are excluded from the scope. Our method considers the calculation of 3 main indicators contributing to environmental impacts: embodied energy, carbon footprint and water consumption.

This study is particularly suited to highlighting hotspots within the manufacturing process, e.g. a production step with a significantly higher impact than another or that can be substituted, the assessment of alternative materials with same functionalities, or already assess the use of certain components, such as the electronic components for sensor readout. In this way, the developments at TRL 3-4 might involve the frequent modifications of some biosensor design parts, and **the sLCA at this stage could be used in a dynamic way to support quick design choices.**

However, according to the fact that possible future states are explored and to the approximations in the data available, it still inherently adds a layer of **uncertainty**. Therefore, the output of sLCA should not be inter-

preted as an absolute result but rather as a comparative indication of what might happen in terms of order of magnitude [64].

From simplified environmental assessments to systemic eco-design

Step 4: Beyond stage 3, eco-design stands out from exploratory and simplified approaches that need to be implemented beforehand. In fact, where sLCA was relevant due to the lack of precision of the data flows (energy, materials, reagents) involved in the development of biosensors, the **design of biosensors at TRL>4 is relatively well established** since they begin to be technically evaluated in increasingly real use-cases (*trials* in the case of medical diagnostics). In this way, **eco-design based on complete LCA of the devices** and its usage framework must be implemented, taking into account as many stages of the life cycle as possible (particularly the influence of transportation, use phase, and end-of-life stages of biosensors) and integrating a greater number of impact indicators, in order to have the most realistic view possible of the impact associated with the deployment of these devices within society. This would allow a fine eco-design of the PoC biosensors.

At this point in the methodological framework, it involves reflection on societal-level impacts, the steps of eco-design must be taken over by **interdisciplinary and transdisciplinary teams of scientists** that conduct systemic analyses and who are accustomed to studying in a cross-cutting way, through articulated design practices, the various externalities related to the manufacture and use of sensors. We are thus at a pivotal moment in the development of the sensors where it is necessary to go beyond the framework of environmental impacts to study technological choices in the light of issues of societal responsibility. Examples of critical reflections are presented in the discussion (section 8.2).

2.5 Results & Discussion

In this section, we apply the framework developed in section 2.4 to the research project of this thesis. Since the technical and environmental assessments have been conducted in parallel during the thesis, this section reflect the chronology of the full PhD project, some of the technical results

described in the following paragraphs are much more detailed in the technical chapters of the thesis.

2.5.1 Step 0 - Project definition

The project statement was defined in this thesis Introduction, i.e. the development of rapid PoC pathogen biosensors for critical situations.

2.5.2 Step 1 - Initial design options

Technical state-of-the-art The state-of-the-art for this project was presented in chapter 1. The three main technologies identified to develop the biosensors are depicted on Figure 2.4. Their respective technical characteristics are reported in section 1.3. It is clear that their respective FoM attributes are not identical, which generally tends to naturally orient them towards different use cases. **Paper sensors** are used for rapid diagnostic or screening where low detection levels are required. In the literature, the term "paper-based biosensors" is often used in a confusing manner to describe a range of PoC sensors made from cellulose or paper-like porous substrates, such as nitrocellulose membranes⁸, or sensors composed of an assembly of these membranes, like lateral flow assays (including glass fiber and a housing plastic cassette in addition to nitrocellulose and cellulose materials). In this chapter, we choose to adopt the same terminology for consistency. **Plastic sensors**, for example based on flexible polymers, are typically developed for wearable biosensors or PoC devices requiring microfluidics instrumentation. In this chapter, it refers to all sensors in which the main functions, particularly microfluidics and the transducer element, are assembled on a main plastic substrate, such as PDMS, polyester, or PET. **Silicon sensors**, on the other hand, offer excellent performance in terms of sensitivity and detection levels, but often require the use of complex and bulky instrumentation, although the trend towards integration allows for increasingly smaller sizes. Nevertheless, they all offer numerous examples of pathogen detection in the literature and are therefore relevant to this study. We refer to all sensors in which the main functions of microfluidics and transductions are based on systems fabricated on silicon substrates as "silicon-based MEMS biosensors".

⁸Even though nitrocellulose has a completely different chemical structure from cellulose, we still use the term "paper-based" to describe sensors made from paper-derivative substrates.

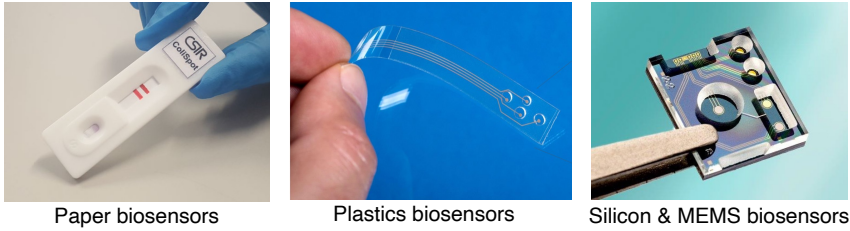


Fig. 2.4 The three main technologies identified from the state-of-the-art as the initial design options to develop PoC biosensors. *Paper biosensors* encompass all sensors in which microfluidic functions are integrated on cellulose substrates or porous paper-based substrates (such as nitrocellulose for LFAs), or sensors that combine these porous supports. *Plastic biosensors* and *silicon biosensors* refer to sensors in which microfluidic functions and the transducer are built on polymer and silicon substrates, respectively. Paper picture from [3], plastic- and silicon-biosensors from [81].

Systemic criteria literature review A deep review of the FoM introduced in Table 2.1, applied to the different materials composing the design options depicted on Figure 2.4, are reported in Figures 2.5, 2.6 and A.3, highlighting the large disparities between materials. The embodied energy, carbon footprint and water are the energy, emissions or water consumed in making one kilogram of material, which include the raw material extraction, purification or processing stages. These figures were compiled based on published data and specialized organization reports [5, 82, 83, 84, 85, 86, 87, 88, 89]. When key data was missing, we have developed our own models for the impact factors of materials (EE, CFP, and Water) by defining the material manufacturing process in a similar manner to what is done in existing databases (e.g., Ansys-Granta Edupack). This approach ensures that the numbers obtained from our models are directly comparable to the EE, CFP, and Water values from these databases. For example, the nitrocellulose embodied energy and carbon footprint have been computed in the frame of Odile Crahay's master thesis, by modelling the whole fabrication process of the nitrocellulose membranes used in PoC devices (see Figure A.4). The computed EE and CFP for nitrocellulose membranes are 91.4 [MJ/kg] and 7.5 [kgCO₂/kg] [90]. The linear evolution of the CFP with the EE in log-scale suggests that the reported carbon footprint are not arising only from the conversion factor [kgCO₂/kWh] expressing the emissions linked to the primary energy generation.

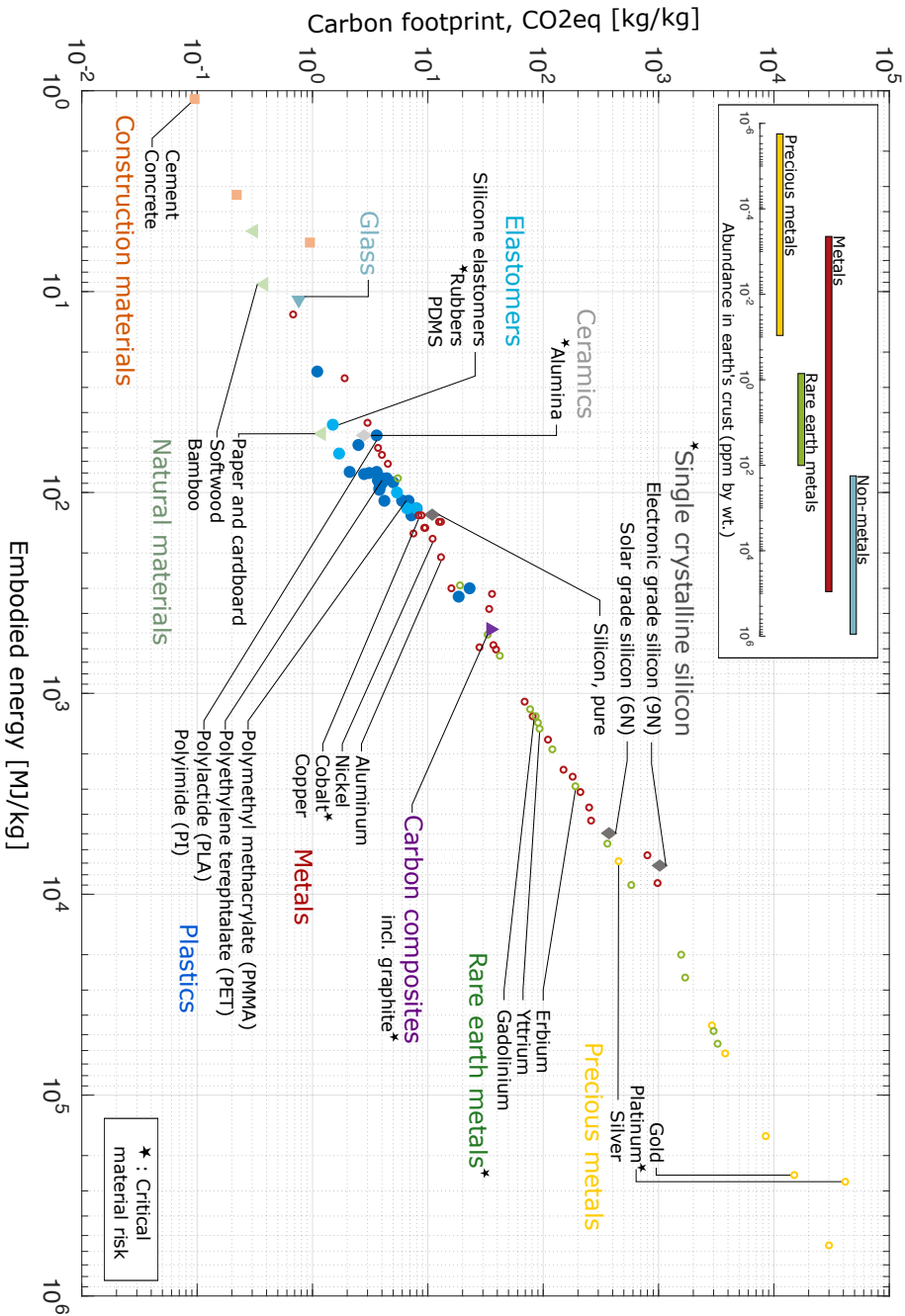


Fig. 2.5 Embodied Energy and Carbon Footprints of classes of materials used in biosensors. Data gathered from several sources.

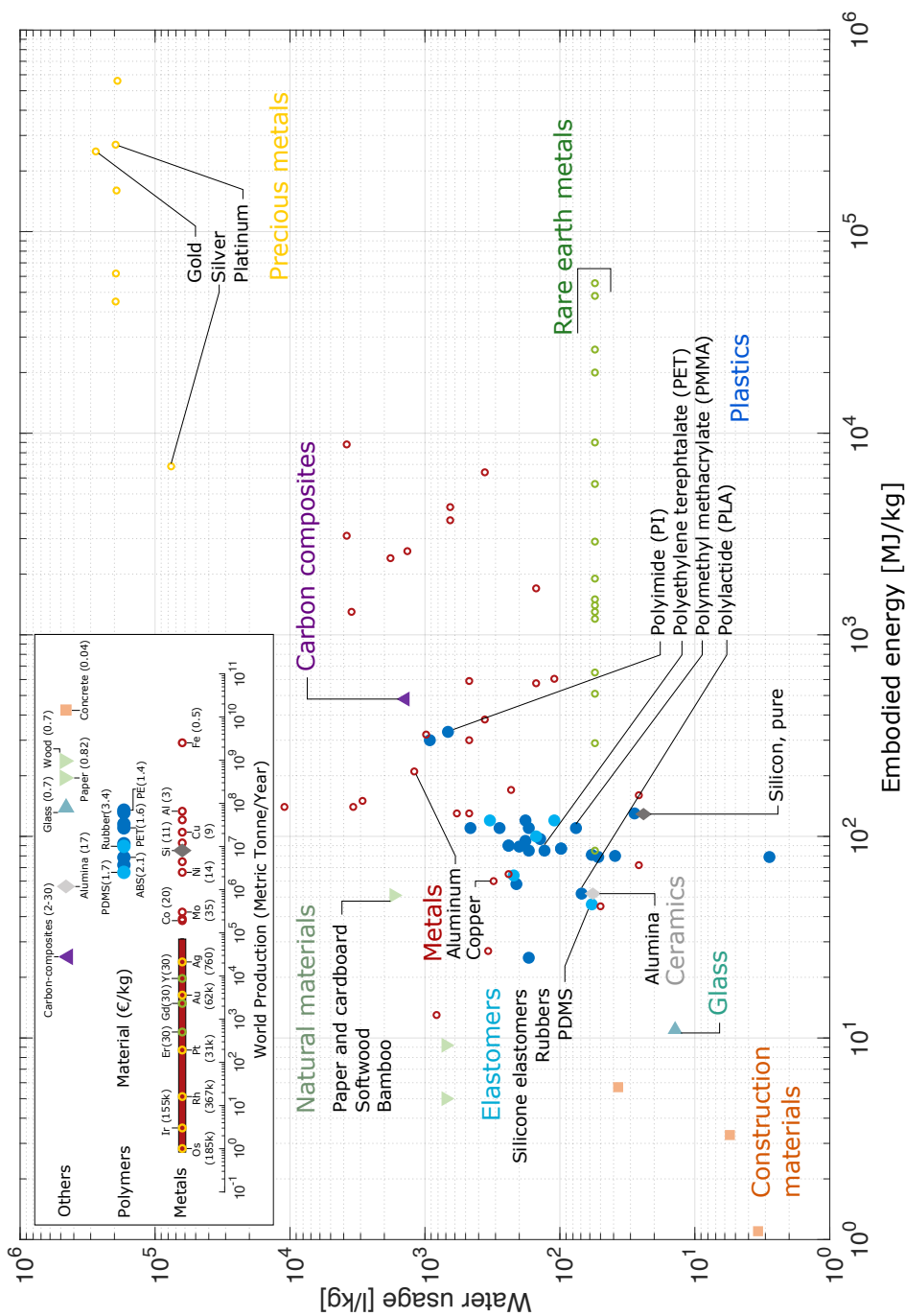


Fig. 2.6 Water consumption per unit material produced [L/kg]. Inset : World production with material price. Data gathered from several sources, in particular [5].

Synthesis n°1 The results from the review performed in step 1 are depicted on Table 2.2. Each biosensor technology has distinct specifications and qualities, primarily resulting from the type of materials composing its main substrate (paper-like membranes, plastics, or silicon), and from the manufacturing techniques and materials compatible with these substrates (introduced in section 1.3), which define the accessible functionalities.

Whereas the highly advantageous characteristics of porosity (and associated spontaneous capillarity) of **paper** and paper-like materials allow for the development of highly user-friendly and cheap sensors without the need for external microfluidic actuation, the limited number of manufacturing techniques (mainly additive fabrication techniques, printing or cutting) and materials (often functional polymeric or nanomaterial-based inks) compatible with these substrates make it challenging to implement sophisticated transduction systems with high performance. However, since the materials and techniques involved are relatively simple and abundant, they are also associated with lower expected environmental impacts and have a connotation of sensors that are democratic and accessible for all.

On the other hand, **plastic-based sensors** are the most widespread devices in the literature due to their convenience in development and handling, thanks to the overall mastery and ease of polymer machining. Notably, the most significant achievements in current microfluidic devices include all sample preparation tools used in biochemistry, ranging from micro-well plates to more complex mixing channels [91]. Given the compatibility of plastic substrates with the integration of a wide range of functional materials, numerous components ranging from electrodes to complex circuits can be directly integrated onto the substrate, offering tremendous opportunities for the development of advanced and multifunctional biosensors. This is well exemplified by the rapidly growing field of flexible electronics, which is expected to revolutionize several domains. However, most of these tools remain single-use plastic devices, and the main issues related to their waste (and the general context of medical wastes) has already been discussed in detail in section 2.1 (see Figure 2.1).

Finally, **silicon-based biosensors** relying on microfabrication techniques receive significant attention in the literature due to the very wide range of functionalities and good performances they can offer, making them highly promising candidates in general. However, a major limitation, similar to

plastic biosensors, is the need to integrate complex, expensive, and often bulky instrumentation to achieve their microfluidic and transduction functions. This often restricts their current role to analytical biosensing tools in laboratory settings and makes it more challenging for them to be used directly at the point-of-care. Furthermore, the entire production chain of these biocapteurs involves materials and manufacturing techniques associated with high embodied energy and emissions. For example, the extraction and refining of metals and semiconductors, including silicon, contribute to significant environmental impacts. Additionally, the operation and maintenance of cleanrooms for fabrication and assembly require substantial energy consumption [92]. Due to their complex and miniaturized integration, which involves multiple layers of materials, the end-of-life management of these microfabricated devices also remains a challenge, particularly with regard to electronic waste (WEEE) which has garnered increasing public attention (just like the issue of medical waste described earlier).

	Paper	Plastics	Silicon
Technical review	+ User friendly	+ Ubiquitous	+ Powerful & versatile
	+ Rapid, passive	+ Processable	+ Functional (fab.)
	+ Proper biofunctionalization	+ Proper handling	+ Integrated
	- Low sensitivities	- Needs microfluidics actuation	- Relatively complex instrumentation
	- High LoDs		
Systemic review	+ Cheap	+ Abundant	- High EE and CFP for most of materials and manufacturing tech.
	+ Relatively abundant materials	- Non-renewable materials mainly	- Supply risks (critical)
	+ Low EE and CFP	- End-of-life emissions	- No End-of-life
	- High water usage		

Table 2.2 Synthesis n°1 relative to the review of the three initial design options for the development of PoC biosensors for pathogen diagnostics. (EE: Embodied Energy, CFP: Carbon Footprint)

Despite the significant advantages of plastic substrates for the development of biosensors, the above-mentioned issues associated with single-use plastic devices prompt us to consider the possibility of implementing alternatives. Therefore, there is likely an interest in exploring other avenues for sensor fabrication. Based on this argument, and because of its need of

complex instrumentation required for microfluidic and electronic component actuation, making it not appropriate with PoC diagnostics biosensor technologies that rely solely on **plastic substrates** to support all critical functions **will not be considered as sensor candidates in the subsequent work**. We emphasize here on the rejection of fully integrated plastic sensors (microfluidics and integrating transduction components and materials using additive printing technologies) that require external actuation for microfluidics and transduction. Plastic is indeed used in many other types of paper-based or silicon-based sensors, for example, as a component of the packaging for protection or general device ergonomics (e.g., for lateral flow assays). However, in those cases, its role is subsidiary and depends solely on the final test design, rather than defining the initial design of critical sensor functions (microfluidics, transduction, biointerfaces) that are of interest at this stage of sensor development.

For their part, silicon-based sensor also requires significant instrumentation, however their high performances and the opportunity of integration and chip reusing (when used under the form of electronic readers) might balance its drawbacks to reach the proper functionalities.

At the lights of their advantages and drawbacks, we identify the most relevant technology options as the paper- and silicon-based technologies, for user-friendliness, and high performances and versatility, respectively. We think that these major advantages would profit to the development of the biosensors in this thesis. However, given the paper-based biosensor low performances, and given the relatively high expected environmental impacts of materials and manufacturing techniques, as well as the limited accessible end-of-life scenarios of silicon-based technology, **we postulate that the appropriate biosensor design might result from the combination of both technologies**, with a special focus on reusing the silicon-based electronics.

It is important to note that the choices made at this stage are difficult, and still based on subjective "semi-quantitative" analysis. Thus, they are still subjected to bias that are strongly influenced by our preconceptions (or our mental representations) of these technologies, influenced by our scientific background, environment or even global public opinion. For example, the advanced silicon-biosensors made from conventional microelectronics facilities are often seen as the "microcircuits of the fluidic world", a "cool" research topic [91], which has led to a surge in academic research over the

last 2-3 decades, even though the number of products actually developed and disseminated as a result of these researches has not yet followed this trend.

2.5.3 Step 2 - Exploration of conceptual designs

The developments from the second step of the framework were conducted during the two first years of this thesis. Some of the results of the early technical assessment and the environmental assessment were published in [80], and [93, 94] presented deeper results on the technical assessments.

Early technical assessment After having identified the complementary performances of both the paper-based and silicon microelectronics biosensors in *synthesis n°1*, we established conceptual pathogen biosensor designs. These designs aimed at combining the advantageous microfluidic properties of paper-like materials (nitrocellulose) with electrodes as transducer, resulting in microfluidics electrical paper-based devices (μ PEDs). From these designs, we proposed two paper-electronics manufacturing processes to reach the necessary electrical functionalities, based on the additive manufacturing (inkjet printing) and conventional microfabrication (sputtering) of conductive materials (carbon nanotubes (CNTs) and aluminum (Al), respectively) onto the paper substrate (Figure 2.7). Early technical characterisations of the designs demonstrated the adequacy of the materials and manufacturing process to integrate suitable conductive tracks for electrodes on paper (see [80] and section 6.4.4 of this thesis). The fabrication and characterization of silicon-based biosensors has already been demonstrated in our lab [13]. Additionally, we also investigated the functionalization of supports with bioreceptors. While our laboratory had already demonstrated that robust functionalization of silicon sensors with bioreceptors can be complex [95], our functionalization tests of nitrocellulose with antibodies (see section 4.2.4 of this thesis) and towards pathogen capture (see section 5.4.2 of this thesis) proved to be extremely positive .

Exploratory Life Cycle Assessment - Methodology In parallel of the early technical assessments, we carried out a prospective environmental impact analysis of the two proposed μ PEDs design strategies to support the material and fabrication process choices, and guide the early researches on μ PEDs. We used a simplified LCA method to evaluate the environmental impacts of the **two different** μ PEDs, and compared the results with a

silicon-based (Si) biosensor originating from traditional microelectronic fabrication techniques.

The **simplified LCA** approach was considered in this work, included (1) setting the study scope (functional unit and system boundaries) by developing the adequate device models, (2) conducting the life cycle inventory analysis to quantify the materials and energy inputs, and (3) identifying environmental impacts through the specified life cycle phases.

We consider the three kinds of devices to have similar functionalities: at this stage, they only consist in the transducer part of the sensor, limited to the fabricated sensitive materials and the substrate (cellulose, nitrocellulose or silicon). As such, the sensing devices modelled here are not fully functional sensors. Each sensing device is assumed single-use because of the complexity linked to the biosensor cleaning procedures [96]. Then, we modelled the sensor material constituents, fabrication processes and energy flows (see Figure 2.8). On this basis, Tables A.1 and A.2 in Appendices present the **life cycle inventory** results for the reference flows, consisting in the material and process quantities required to fabricate 1000 CNT- μ PEDs, Al- μ PEDs and Si-PDMS devices, respectively.

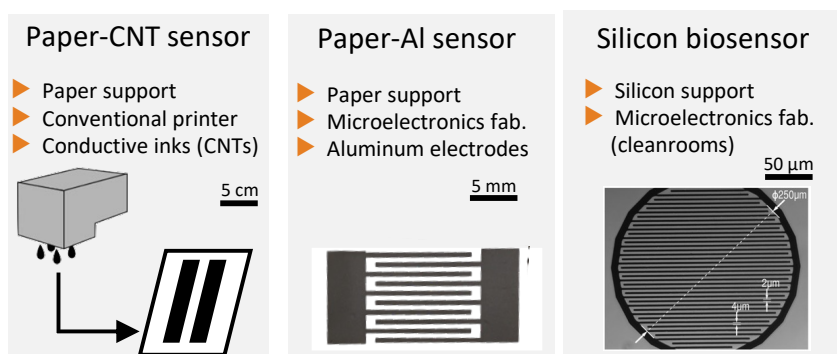


Fig. 2.7 Material and manufacturing strategies for two of the conceptual designs combining paper substrate for microfluidics and biofunctionalization, and the addition of conductive tracks to support the electrical transduction principle, chosen from step 1. Materials, manufacturing and first characterizations of these conceptual design were explored during the first developments of the thesis. In order to benchmark with a highly performing sensors previously developed in our lab, we performed a comparative exploratory LCA of the two conceptual paper-electronics processes, and from the silicon-based microelectronic device [13].

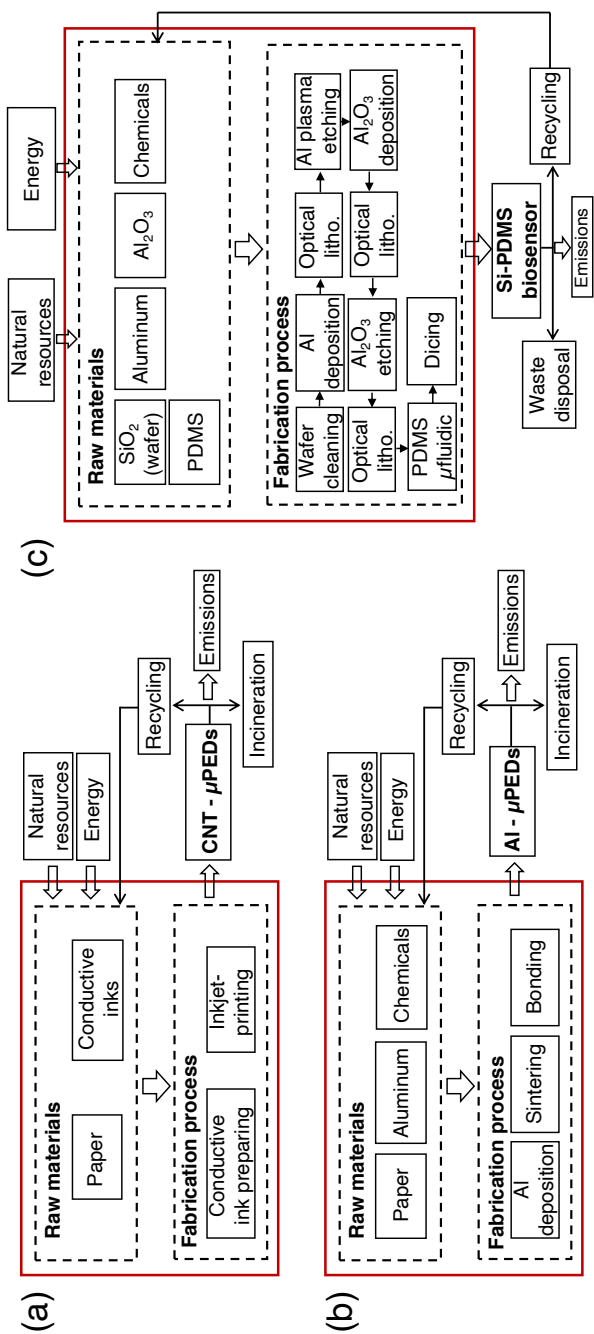


Fig. 2.8 System boundaries (red frames) and life cycle models considered in the simplified LCA study of (a) carbon nanotubes (CNT-μPEDs) and (b) aluminum (Al-μPEDs) paper-based electrochemical device for bacteria detection, and (c) silicon-PDMS based bacteria sensors.

Exploratory Life Cycle Assessment - Results The data collected was then used to determine the **environmental impacts** for such a quantity of product through their embodied energy (EE) and CO₂ footprint (CFP). To this end, we refer to the specific EE and CFP of materials and processes (see Table A.3 in Appendices for details). The EE for each kind of sensor can be expressed as $EE = EE_{materials} + EE_{processes}$ and $CFP = CFP_{materials} + CFP_{processes}$. As reported in Figure 2.9, Al- μ PEDs and Si-PDMS devices have significantly higher environmental impacts than CNT- μ PEDs. Specifically, we see that the processes requiring classical cleanroom facilities show drastically greater energy and CO₂ impacts than inkjet-printing of CNT-inks, *i.e.* $EE_{Al-\mu PED, process} = 4415$ MJ and $EE_{Silicon, process} = 3262$ MJ against $EE_{CNT-\mu PED, process} = 23$ MJ. We observe similar trends for the carbon footprints. This is consistent with the energy-intensive process tools and high number of harmful chemicals required in classical microelectronics processes (*e.g.* successive depositions and etchings) compared to the simplicity of purely additive technique.

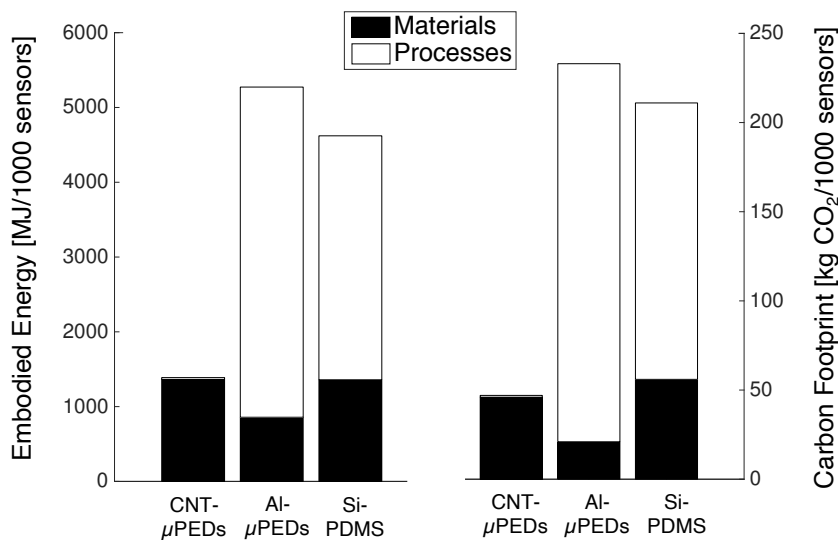


Fig. 2.9 Environmental impacts (embodied energy and carbon footprint) of the materials and manufacturing processes required to produce 1000 CNT- μ PEDs, Al- μ PEDs and Si-PDMS sensors.

The much higher impacts presented by the fabrication process of Al- μ PEDs compared to CNT- μ PEDs is mainly due to the metallization equip-

ment is adapted to μm - and mm -sensor fabrication, not to cm -wide paper-based devices ($\sim 5\text{ cm}$). Hence, a much higher number of runs of the metallization step is then required to fabricate the same number of $\text{Al-}\mu\text{PEDs}$ than Si -based sensors (8 mm in dimensions).

It was also found that the carbon nanotubes synthesis are responsible for most of the EE and CFP involved in the fabrication of $\text{CNT-}\mu\text{PEDs}$. Indeed, The conventional synthesis of carbon nanomaterials by chemical vapor deposition (CVD) has a massive CO_2 -positive footprint, emitting as much as 600 tonnes CO_2 per tonne of carbon product [97]. Substitute nanoparticle-based inks (*e.g.* Ag or more environmental friendly carbon nanomaterials [98]) could be considered to reduce material impacts.

Synthesis n°2 As a general conclusion of the early technical and environmental assessments, we saw that paper-based sensors are promising candidates, and it is worth considering them further as biosensors of interest.

The early assessments of **printed paper-CNTs** transducer showed that additive inkjet-printing of conductive inks seems appropriate for paper-electronics manufacturing given the properties of paper and the size of the resulting transducers, although its capacity to provide with higher production levels should be considered. For example, serigraphy-based printing techniques (screen-printing, flexography) are usual methods for mass production of printed surfaces and could be easily derived to produce μPEDs . However, the electrical characterizations that were performed on the devices are not advanced enough to assert that this technology can effectively be used as a biosensor device for pathogen detection. Further in-depth results are expected to draw a relevant conclusion. The environmental assessment also shown that specific nanomaterial-based inks must be chosen accordingly to their adaptability with the manufacturing process and their environmental impacts. In addition, it is important to mention that the toxicology impacts of nanomaterials on living being should not be underestimated, but rather taken into account, although they may vary depending on the sources [99].

Regarding the **sputtered paper-Al interdigital electrodes**, further characterizations have been conducted, demonstrating the feasibility of detecting bacteria in water. However, at the light of the sLCA results, it is critical to consider the appropriateness of the manufacturing technique with pro-

duction level required and the size of the devices, especially for single-use sensing solutions. In this regard, metallization is not a good candidate as a mean to produce μ PEDs with improved environmental impacts.

Therefore, the fabrication process of μ PEDs can still be optimized by selecting adequate nanomaterials and fabrication methods.

For functional **Si-based devices**, even though the performances in bacterial detection are no longer in question, Figure 2.9 indicates that Si-based transducers would be competitive with the paper-based ones in term of environmental impacts if they could be washed and reused about 5 to 10 times for instance. Interestingly, recent studies have developed protocols for regenerating metallic electrodes ~ 20 times in electrochemical biosensors based on conventional microfabrication [100].

	Printed paper	Sputtered paper	Silicon
Early TA	+ Simple & versatile process + Process suitable with biofunctionalization - Functionalities not yet demonstrated	+ Good electrode materials + Bacterial detection demonstrated - Not convenient with biological process - Not versatile design	+ Controlled, versatile process + Miniaturization + Integrated - Cleanrooms
Exploratory LCA	+ Low impact process & materials - Nanoparticle toxicology - High EE & CFP of nanoparticles	- Highly emitting (and costly) microfab. process not suited at all with paper-devices due to their large size	- High EE and CFP from process (high number steps) and materials • Could be decreased if transducer reused

Table 2.3 Synthesis n°2 relative to the exploration of the conceptual designs. *Printed paper* refers to CNT- μ PEDs while *Sputtered paper* refers to Al- μ PEDs. (EE: Embodied Energy, CFP: Carbon Footprint)

At the light of Table 2.3, we conclude that the advantageous properties of paper and derivatives (nitrocellulose) for immobilization of biomolecules and capture of bacteria is a definitive advantage in the design of a PoC biosensor for pathogen diagnostics. Given the ease of developing biointerfaces on nitrocellulose and the relatively complex interpretation of elec-

trical measurements of bacteria on nitrocellulose membranes, the results of this second stage of conceptual design evaluation have highlighted the clear advantage of colorimetric tests based on nitrocellulose, such as LFAs, as they exploit the best properties of nitrocellulose. Therefore, it seems that the exploration of the design and development of colorimetric lateral flow assays paper-based sensors is justified in parallel with the research path of integrating electrical transducers with the nitrocellulose substrate.

Regarding the paper-based biosensor based on conductive inks (CNTs) and printing technology, it is evident that it offers interesting environmental properties due to low-energy manufacturing processes, despite uncertainties surrounding the actual impacts associated with the nanomaterials used. To assess the true relevance of this technology in the case of PoC biosensors for pathogen detection, further characterizations and technical evaluations would be required. However, these tests demand advanced additive manufacturing infrastructures that are not directly accessible to us at the moment. Therefore, we have decided not to continue with the technical research and development on this topic in this thesis, but rather to follow the state of the art from publications in the scientific literature. Given the increasing interest in the paper-based printed electronics paradigm, driven by environmental constraints, articles regularly investigate the technical performance and environmental aspects of such devices, providing valuable insights.

Furthermore, we have decided to exclude the use of microfabrication techniques such as sputtering on paper-based substrates from further development. Despite being a favoured approach in many recent works⁹ due to the familiarity and general mastery of these manufacturing processes and the excellent performance achieved (e.g., high conductivity), it does not seem realistic to consider large-scale production of paper-based pathogen biosensors due to obvious environmental impacts and colossal financial costs. Moreover, the reliance on advanced microfabrication facilities does not align with the objectives of this thesis, which aim to develop biosensors that are accessible and appropriate (as stated in the desirable figures of merit in section 1.3.6). Therefore, we will focus on alternative manufacturing methods that align better with our goals and prioritize environmental sustainability and cost-effectiveness.

⁹For example, see works pre-published in <https://www.biorxiv.org/content/10.1101/2023.03.09.531916v1> (2023)

Finally, based on the promising results from previous published works within our research group [6, 13] and the electrical characterizations of nitrocellulose membranes using advanced measurement tools, we acknowledge the potential of using advanced electronic transducers, provided that they are not single-use and can be effectively reused. Their ability to yield promising results is valuable. Additionally, the utilization of these transducers, which rely on conventional microfabrication techniques, opens the door to various other detection techniques such as electromagnetism or electrochemistry. Considering their demonstrated performance in other published works, these detection methods and associated transducers have a role to play in the development of biosensors for pathogen diagnostics in applications that require low limits of detection. However, the integration of reuse constraints raises questions as it necessitates designing, from the early-research stages, with considerations for separating the transducer from the contaminated parts of the sensor while maintaining measurement robustness.

2.5.4 Step 3 - Embodiment designs

Based on the results of step 2, the following pathogen biosensor candidates are selected to further evaluate their technical performance. As stated in section 2.4.2, the embodiment designs ($TRL \geq 3$) include some of the critical functions required for a relevant biosensor.

In this way, we propose to investigate 4 types of biosensor devices in step 3 (Figure 2.10). In practice, the **technical assessments of a few selected embodiment designs are conducted in chapters 4, 5, 6 and 7 of this thesis**. Their environmental assessments by means of simplified LCAs are currently performed, in parallel to the technical research process. At present, a synthesis for Step 3 is not feasible. This step requires further in-depth research and the coordinated efforts of multiple sensor designers. As the mentioned designs approach higher TRLs, the research and amount of data required to conduct environmental analyses increase significantly. However, a few key elements and results that have already been obtained are summarized below.

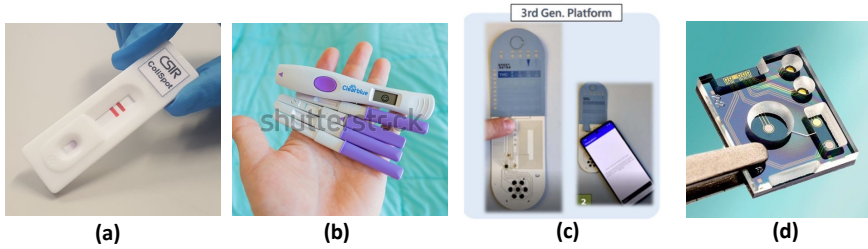


Fig. 2.10 Embodiment designs selected from the previous framework steps. The technical assessment of **(a)** colorimetric paper-sensors (LFA) [3], and **(b)** reusable electronic readers for LFAs are presented in the next chapters of this thesis. **(c)** Printed electronic devices are still being evaluated, in the form of a scientific literature watch, including on their environmental impacts. [101] **(d)** Microfabricated sensors are being explored in other PhD theses in Jean-Pierre Raskin's group. [81]

Leveraging the promising results for pathogen capture, we decide to develop **colorimetric paper-based tests**, such as lateral flow assays (Figure 2.10 (a)), for rapid pathogen diagnostics. The desire to develop LFAs is also supported by the relatively low impact of paper substrates identified in step 2. In this way, simplified LCA were performed in the frame of Odile Crahay [90] and Josquin Vandeputte's [102] master theses, similarly to the exploratory LCA conducted in step 2, with striking results that the plastic housing cassette commonly used accounts for about 90% of the embodied energy and carbon footprint of the whole device : a LFA embodied energy with vs. without cassette : 0.57 MJ/LFA vs 0.034 MJ/LFA, respectively. Regarding carbon Footprint with vs. without cassette : 0.03 kgCO₂/LFA vs. 0.004 kgCO₂/LFA, and for water usage with vs. without cassette: 0.42 L/LFA vs 0.23 L/LFA. This already suggests related eco-design strategies. Indeed, as reported by eco-design studies of LFAs¹⁰, popular idea circulating for LFAs is to minimize the plastic test strip caseworks (without compromising the essential functions of providing a stable platform, and protecting the nitrocellulose test strip). It might be possible to halve the caseworks mass and reduce the overall carbon footprint and plastic waste by 15-20%. This would require significant investment in R&D, production tooling, and regulatory approval hoops to jump through – but could be worthwhile if future demand for tests stays high.

We propose to explore the use of **colorimetric readers** with the devel-

¹⁰<https://www.cambridge-design.com/blog/how-to-reduce-the-carbon-footprint-and-plastic-waste>

oped LFA, i.e. integrate electronic cameras to improve the LFA performances by quantification of the colorimetric signal (Figure 2.10 (b)). The technical assessment of such colorimetry reader is conducted in chapter 4. However, it is now widely acknowledged that colorimetric readers associated with LFAs, particularly when sold on a large scale, generate a significant amount of electronic waste and unnecessary environmental impacts relative to their technical benefits¹¹. Therefore, caution should be exercised in integrating these readers, with a strong emphasis on avoiding single-use devices.

Since we do not have the facilities to explore more in detail the technical feasibility of **inkjet-printed paper-based biosensors**, we identify the closest examples from the literature to pursue the comparative assessment. Recent studies explored in detail the environmental features of paper-based printed electronics [103, 74], and excellent example of technical and environmental assessments of printed paper biosensors include [101] (Figure 2.10 (c)).

Finally, in view of their obvious qualities, we continue to explore the use of **microfabricated electromagnetic biosensors applied to paper sensors** for their single-use functionality (Figure 2.10 (d)). Being aware of the dependence of these technologies on energy-intensive facilities (clean-rooms) and the problematic supply chain of materials they involve (e.g. critical metals), design practices are oriented towards the **reuse of readout systems**. In practice, this research is the subject of other PhD theses in Jean-Pierre Raskin's group. Regarding their environmental assessment feature, we performed a simplified assessment of silicon-based MEMS sensors for co-optimization of sensitivity and environmental performances [78].

¹¹Look at https://www.youtube.com/watch?v=N1x8HnouxXg&t=9s&ab_channel=DeusExSilicium for more details.

2.6 Conclusion

This chapter proposes a methodology for the eco-design of innovative technologies at early-development stage. First, it introduces sustainability metrics such as the environmental impacts of the biosensor materials and fabrication, alongside conventional design figure of merits, in order to account for the environmental footprint of an innovative technology during its early-research stages. Then, it exemplifies the use of simplified life cycle assessment (sLCA) as a tool to quantify the environmental impacts of the developing technology, even at the early-research stages presenting uncertainty about the data availability and quality.

Eco-design results The environmental impacts of the materials and processes (energy consumption and carbon footprint) considered in the different sensor designs studied during this thesis have been analyzed. These environmental indicators are widely used to identify the key drivers of environmental burdens in products and devices life cycles. In addition, technical assessments of the sensor designs were conducted. Findings from the environmental and technical assessments were compared and discussed during *synthesis* steps. Results from these syntheses were leveraged to support the most appropriate choice of sensor technology, in particular for materials and manufacturing processes selection :

1. The exploration of different materials and fabrication techniques for biosensors has highlighted the advantages and limitations of each approach. Paper-based sensors offer simplicity and accessibility, but face challenges in implementing high-performance transduction systems. Plastic-based sensors are widely used due to their convenience in development and manipulation, but their single-use nature raises environmental concerns. Silicon-based sensors exhibit advanced functionalities but require complex instrumentation and often remain limited to laboratory settings.
2. In addition, the integration of microfluidics and transduction el-

ements on plastic substrates shows promise but necessitates external actuation, limiting their potential for point-of-care applications. As a result, we have decided not to continue research in this direction.

3. The use of nitrocellulose in colorimetric tests, such as lateral flow assays, has proven advantageous due to its favourable bio-functionalization properties, technology maturity and accessible development process while offering relatively controlled environmental impacts despite the single-use format. The usefulness of rapid diagnostic tests at the point-of-care has been repeatedly demonstrated, including in the case of large-scale pregnancy tests or rapid tests for COVID-19. However, further characterization and technical evaluations are required to fully assess their suitability for pathogen detection. These reasons served as the driving force for initiating research on these topics in the chapters 4 and 5.
4. Electrical paper-based biosensors utilizing conductive inks, such as carbon nanotube-based inks, have shown environmental advantages in terms of low-energy manufacturing processes. However, the uncertainties surrounding the environmental impacts of nanomaterials used in these sensors call for further investigation. The lack of access to necessary fabrication techniques prevented further research in this direction during this thesis. The continuation of monitoring this research avenue is carried out through literature review.
5. The scalability and production of electrical paper-based biosensors fabricated with microfabrication techniques, such as sputtering, are not considered feasible due to their incompatibility with paper substrates and the associated environmental and cost implications.
6. Advanced electronic transducers hold potential for high performance biosensors, provided they are reusable and designed to separate the transducer from the contaminated parts of the sensor. Such a research direction is initiated in chapters 6 and 7.

Overall, the results of this chapter, based on technical and environmental assessment (through a simplified LCA) conducted in parallel,

allowed to identified the main biosensor designs and research directions studied in this thesis based on both the criteria of technical performance and original results on environmental impacts. It highlights the need for extensive research and collaboration among biosensor scientists to advance further than the step 3 proposed in the framework. The research performed in the next chapters thus intend to pursue the technical assessment of LFAs for pathogen diagnostics and the accommodation of reusable electrical readers on LFAs, at the device level. Environmental assessment are still conducted towards the design of the most appropriate PoC pathogen biosensor. However, the increasing complexity and data requirements necessitate a concerted effort to conduct comprehensive environmental analyses.

Methodological perspectives The application of this methodology aims to anticipate the environmental and societal impacts associated with design decisions for innovative technologies. Considering these impacts and their underlying causes early in the life cycle of innovations entails the responsibility of the designer, but the multidimensional nature of these impacts necessitates a multidisciplinary methodology. To provide a comprehensive approach, the methodology presented in this chapter should incorporate a broader range of indicators, including those assessing the economic feasibility of the proposed biosensor innovations. This is mentioned in steps 4 to 7 of the proposed eco-design methodological framework (see Figure 2.3). Some of these aspects are discussed in the perspectives of this thesis 8.2. This changes the way research and development are organized, as it requires the involvement of stakeholders and information from environmental and socio-economic sciences at an early stage of development. The inherent uncertainty in this complex situation necessitates continuous critical reflection.

When conducted in this manner, multidimensional impact assessment research in academia provides a valuable service to society by going beyond the usual concerns related to industrial knowledge transfer from laboratories or the pursuit of performance improvements typically outlined in technology roadmaps across various sectors. It proposes a different approach by incorporating socio-economic, environmental, and public health impact studies from the outset to guide

decision-making in the early stages of research.

By practising this type of methodology, the university is able to impact its three roles:

1. **Research:** By integrating a multidimensional approach to impact assessment, the university promotes the generation of scientific and technical knowledge that takes into account environmental, social, and economic dimensions. It contributes to the advancement of knowledge and of multidisciplinary research to produce knowledge that is relevant for society.
2. **Education:** By incorporating this methodology into educational programs, the university educates students in a holistic and responsible approach to technological design and development. Students acquire the necessary multidisciplinary skills to better understand how to integrate environmental and social issues into their future professional activities.
3. **Service to society :** By implementing this type of methodology, the university initiates a process and create knowledge that can be transferred to economic actors, policy makers, and society at large. Research findings and developed methodologies can be used to guide decisions and actions aimed at promoting sustainable development and social responsibility beyond the university's wall.

3

Fundamentals on lateral flow assays



Chapter objectives

In chapter 1 of this thesis, we reviewed the biosensors suitable for the rapid detection of pathogens at the point-of-care. Among others, lateral flow assays (LFAs), although presenting some limitations, possess advantageous properties to consider this technology further as an appropriate solution to answer the objectives of this thesis.

In this chapter, we present a non-exhaustive yet detailed overview of the general principles governing an LFA, its capabilities, and its design, development and manufacturing processes. The chapter is structured to present one by one the necessary components, highlighting the key characteristics developers should consider when selecting the most suitable materials and reagents during LFA design and fabrication.

3.1 General principles and components of optical LFAs

The simplicity, rapidity, portability and low costs of lateral-flow assays (LFAs) have made them one of the most used point-of-care (PoC) sensors in various disciplines ranging from clinical diagnostics to environmental, food safety and veterinary analysis [56]. Their success lies in their general design that has remained almost unchanged since their first use as pregnancy tests in 1970s, which partly explained their adoption on an unprecedented scale during the COVID-19 pandemics, and demonstrated their feasibility and acceptability on a global basis. [104]

The most common lateral flow devices are composed of different elements (Figure 3.1 a), each supporting a vital function of a fully functional biosensor (Figure 3.1 b). **A successful test primarily relies on the spontaneous, capillary flow of the sample throughout the strip**, which consists of assembling **four contacting porous components** disposed linearly: (1) the sample pad, (2) the conjugate pad, (3) an assay membrane (e.g. nitrocellulose) with capture agents (e.g. antibodies) immobilized at test and control lines, and (4) an absorbent pad to wick the sample through the device. As discussed further in this chapter, these pads are made of different kind of **papers-like materials**.

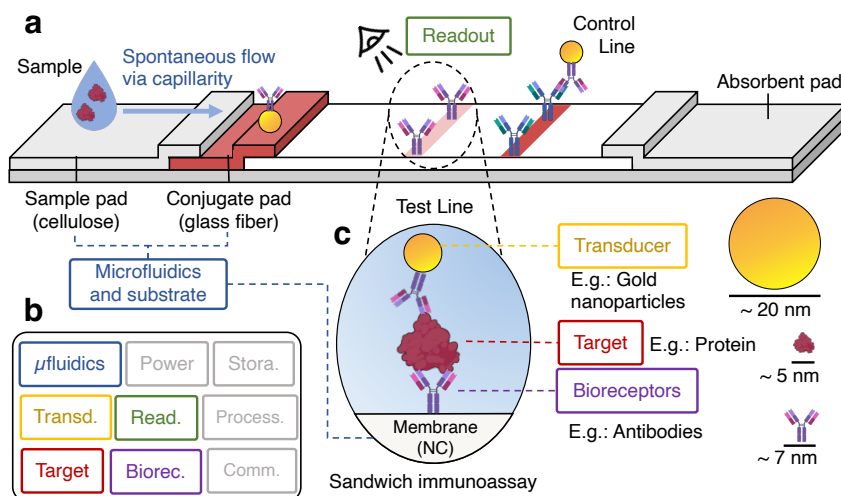


Fig. 3.1 Principle and components of nanoparticle-based lateral flow assays.

From a user perspective, the **operation of a well-designed LFA diagnostic test is strikingly simple**: the user adds a microliter-volume (100–200 μL) of **liquid sample** (e.g., blood, urine, saliva) onto the sample pad, often made of cellulose. Then, the sample passively travels by capillary forces to the conjugate pad (e.g. glass fiber), where previously dried **nanolabels** (e.g. **gold nanoparticles**) conjugated to "**detection**" **bioreceptors** specific to the **target analyte** are resuspended in the sample buffer. As the sample flows, the target analytes are mixed and form complexes with the conjugated nanoparticles. Once the complexes reach the **test line**, which is typically coated, or striped, with a second "**capture**" **bioreceptor**, the analyte is bound in a "**sandwich**" **immunoassay** disposition (Figure 3.1 c). The **accumulation of nanoparticles at the test line generates the signal**, which depends upon the nature of the nanoparticle. In the case of gold, which expressed strong plasmonic absorption, the **accumulation of gold nanoparticles at the test line generates a strong red colour**. Results are typically available in less than **15 minutes** and can be **visually read** (e.g. at the naked eye, or with colorimetric reader) based on the development of colour at the test line. A **control line** binds the excess unbound nanoparticles, verifying that the sample has flowed appropriately along the strip and that detection complex molecules are functional. [105, 56]

To be successful, research, development and innovation of **LFA technology requires an understanding of the multidisciplinary scientific and engineering principles** involved in the LFA assay design. **In this chapter, we provide the basic knowledge to understand the principles governing an LFA and to take informed decisions during LFA design and fabrication.** It should help the reader to elaborate what is the best strategy for making a successful LFAs that rely on nanobiotechnology for the detection of a specific target. In particular, it is important to note that the issues addressed in this chapter must still be considered specifically for each new target of interest as it may present specific LFA design constraints. The end of the chapter is dedicated to the discussion of main research directions to enhance the LFA as PoC diagnostics tools.

3.2 Fabrication, development and optimisation of LFA

This section provides an overview of the development and optimisation process of a LFA. As introduced, an LFA is made of various materials and reagents that are carefully interconnected to produce a sensor with the aim of being easy to use yet still highly sensitive and specific. The advanta-

geous properties of LFA are obtained by the **linear combination of the four main physical components of the LFA** (sample, conjugate, membrane and absorbent pads), **each supporting one critical function of the LFA**. Figure 3.2 A expresses the major function supported by the different pads : once the sample is dropped on the LFA, it first encounters the **sample pad**, which ensures that the characteristics of the sample match those required for optimal detection (pH, ionic strength, viscosity, purity and concentration of blocking agents). Next, the roles of the **conjugate pad** are to release the labeled detection bioreceptor upon wetting, and to allow for a first analyte-bioreceptor interaction through mixing. Then comes the **membrane** (also called **detection pad**). The roles of the membrane are critical to the test performance : it must be easily coated with the capture bioreceptors forming the two reaction area, i.e. the **test line** and the **control line** and its pore network must conduct the sample through it at a certain speed while allowing for interaction between the different reagents (analyte target, detection and capture bioreceptors) to perform the immunoassay. Finally, the **absorbent pad** should provides enough bed volume for the complete flow of the sample. [106, 104, 107] **In the following sections, each of the components and the effect it has on the LFA will be examined and discussed in detail.**

LFA manufacturing The LFA device simplicity and the dried reagents permits **relatively easy manufacturing and eliminate the need for complex facilities** (e.g. cleanrooms) or storage conditions (e.g. refrigeration). The **manufacturing process** is summarized on Figure 3.2. It mainly consists in **(1) the preparation of the biochemical reagents** (nanoparticles and conjugation with specific bioreceptors), **(2) the coating and drying of the membrane** with capture bioreceptors to create the test and control lines, and with blocking agents, **(3) the deposition and drying of the conjugate nanoparticles** in the conjugate pad for storage, **(4) the assembly of all the pads** via lamination on the supporting (or backing) adhesive card, and **(5) the subsequent cutting** of the cards into individual strips. Packaging step may be added where the individual strips are included into a plastic housing cassette to ensure proper handling and operation of the LFA, and storage into an aluminium pouch to ensure stable storage conditions. **Although LFA operation principle (i.e. immunochromatography) and manufacturing process look simple, their development is far from being straightforward** and the manufacturing process must be highly controlled. LFA technology has historically been associated with an inaccurate reputation, because a few lateral flow tests subjected to variability and poor accuracy in the results have already been adopted on quite a large scale in the past [108]. **Manufacturing tools, materials and processes account**

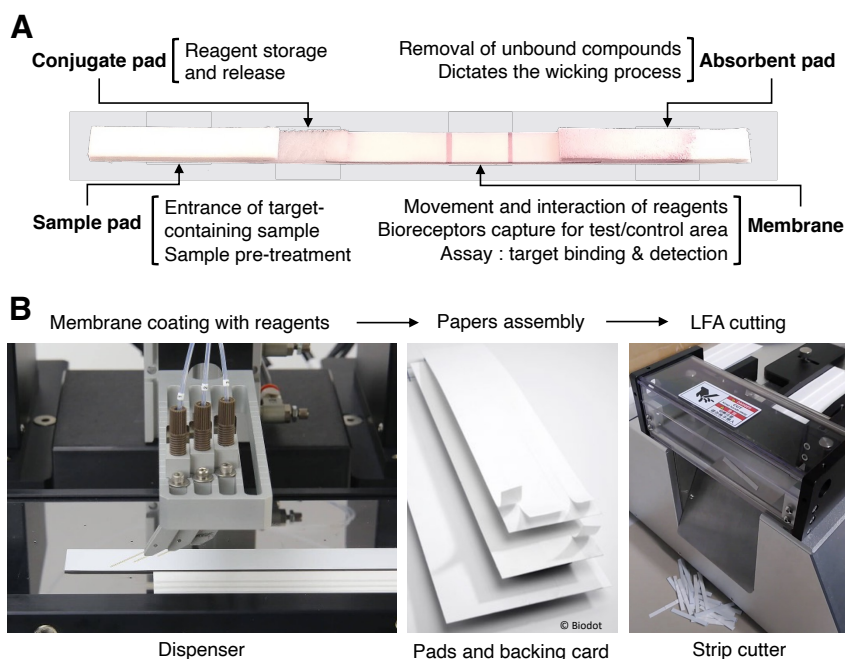


Fig. 3.2 Fabrication of LFAs. **A.** Principal functions of the four main physical components of the LFA. The judicious choice of material for each component, and their careful interconnection is critical to the LFA performances. **B.** Simplified workflow for the fabrication of an LFA. First, the nanoparticles are synthesised and conjugated with the detection bioreceptors (not shown). Then, all the reagents are dispensed on the pads (capture bioreceptors on the assay membrane and conjugated nanoparticles on the conjugate pad) and dried. Next, all the pads are mounted and laminated on a sticky backing card in the correct order to ensure good flow along the strip. The mounted card is finally cutted at the desired lateral flow width.

for a considerable share of the inter-assay variation, mainly because the materials used in the LFA tests have historically been designed for other applications. The requirements for increased LFA performances (reproducibility and sensitivity) is imposing new standards on the manufacturing process [109]. Therefore, proper regulation of both the tests and of manufacturing process is highly important to maintain the quality in order to ensure batch-to-batch reproducibility and strengthen belief in the LFA results. LFA developers and manufacturers must thus critically consider and evaluate the LFA development and manufacturing process to provide the end user with a device with optimal performances, reproducibility and

market readiness. [104]

LFA development process The design requirements - that LFA needs to be simple to use, and that results must be easily readable and interpreted by the user naked-eye - place constraints on the signal intensity at the test line. **The test line intensity must be strong enough and is often the major figure of merit monitored to optimize the LFA parameters.** Test line signal strongly depends on the appropriate LFA functionalities (i.e. fluidic process, bioreceptor capture on the membrane, specific target-bioreceptor reactions) and its multiple parameters and reagents (Figure 3.3 A). Therefore, **designing new LFA applications necessarily involves the simultaneous optimization of its multiple, interconnected parameters**, with the goal to keep a user-friendly operation.

Some of these parameters are straightforward and **predictable**, such as the antigen concentration in the sample which directly correlates with test line intensity, and an appropriate sample preparation could remove unwanted reagents from the sample that could interfere with the test. **Others parameters can be measured with usual analytical tools**, such as the membrane microstructure (pore size), physical properties (surface tension) and surface (bio)chemical properties that influence the proper microfluidics, flow and speed. The same holds for nanoparticle concentration, size, shape and physical properties. However, **other crucial parameters are not intuitively obvious**, such as bioreceptor coverage, the orientation on nanoparticle and membrane surfaces, which influence binding performances, and the prevention of non-specific adsorptions and bioreceptor-target binding affinity in LFA format. Their effects on the test line intensity require time and the combination of different analytical and computational techniques to be estimated. Nevertheless, **Figure 3.3 A can serve as a guide for considering all the factors that are at play** in generating a test line signal, and that must be considered during the LFA development.

Because LFAs and sandwich immunoassays are now a **mature technology**, there are numerous industry-written guides and workshops that discuss best practices for designing and manufacturing LFAs with high sensitivity and specificity, that can be scaled up, and that are appropriate for consumer use [110, 111, 112]. In addition, business-to-business companies such as nanoComposix are providing services to support the LFA development process for specific client demands [113].

LFA optimisation process When developing LFA, it is common to follow an **iterative step-by-step procedure to isolate and optimize the effect**

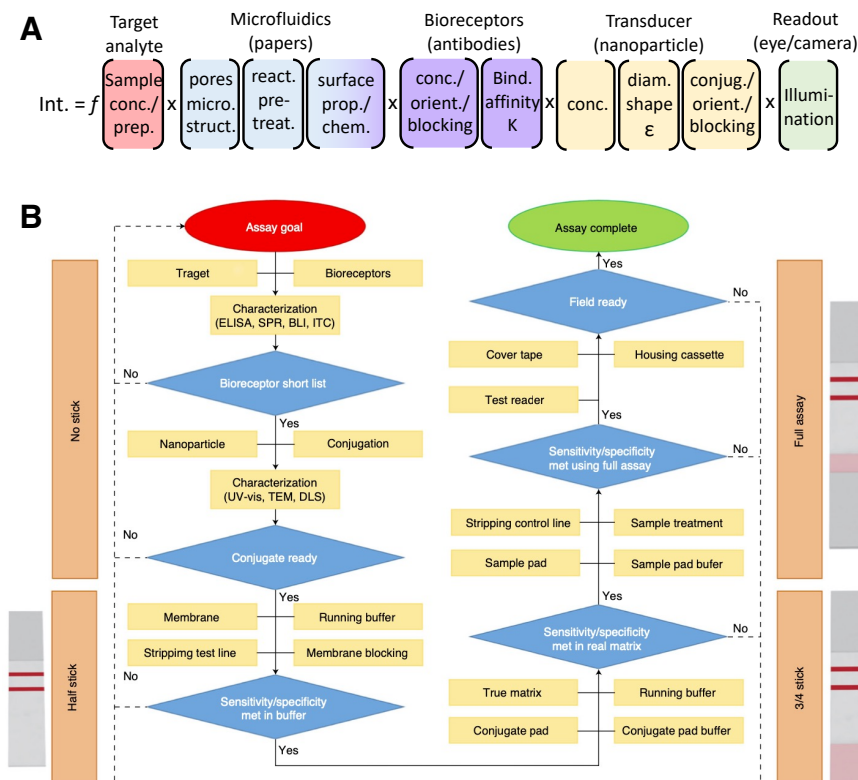


Fig. 3.3 Development and optimisation of LFAs. **A. Test line intensity** is the major parameter to be optimized for performant LFA development, which involves the co-optimization of its multiple, interconnected parameters. While some parameters are predictable and easily optimised, others may require time and advanced analytical techniques. Inspired from [110]. **B.** In order to easily **optimize each of the parameter** and select their appropriate combination during the LFA fabrication process, the ideal optimization route employs a step-by-step procedure. It includes the specific bioreceptors selection, their conjugation to the nanoparticles and finally a three-step LFA stick testing to progressively increase the LFA pads complexity to optimize the effects of the matrices, reagents and pad materials on the LFA operation. Reproduced from [104].

that each component has on the LFA flow and test result. More generally, the optimization of an LFA relies on an iterative approach of trial and error, depicted on Figure 3.3 B, as described by Hsieh et al [114] and reported by Parolo *et al.* [104], which explain that the developer first defines requirement of the desired LFA application (analytical performances and

usability). On this basis, there is the first screening of bioreceptors to assess the binding properties of the bioreceptor to the analyte in solution. The resulting bioreceptors are then conjugated to the nanoparticle labels. The conjugation and the stability of the conjugate should be optimized. The next steps consists in assessing the specific capture and detection of the analyte in the lateral flow assay format. To increase the complexity of the LFA gradually, the developer first uses the half stick format (made of membrane and absorbent pad only) to evaluate the combinations of membranes, blocking agents, running buffers, test and control line coating conditions and conjugates. If the original goals are met, developers may use the 3/4 stick format to further evaluate the optimal preparation of the conjugate pad as well (type, drying, buffers and release). Finally, the sample pad is incorporated to evaluate the overall sensing performance with real sample matrix. Although this approach for LFA optimization heavily relies on an iterative trial and error procedure, leading to a repetition of the same optimization steps until specifications are met, it is still time- and cost-efficient [115, 109].

Characterization techniques for LFA development The proper development of an LFA involves the **characterisation of materials at different scales, from nanomaterials and interactions at bio-interfaces to macroscopic flow properties at the device scale**. Due to their tiny dimensions, characterization of nanobiomaterials is not straightforward, and requires a “puzzle” of different nano- and bio-technologies that need to be compared in order to draw thorough conclusions:

- The **screening of bioreceptors** uses standard biotechnology techniques such as ELISA, or fluorescence microscopy techniques, which mainly provide information on the quality of the interaction between a bioreceptor and its target, as well as on the specificity of the interactions. These techniques can be qualitative or quantitative.
- The **nanoparticle morphological characterizations**, their conjugation to bioreceptors, their optimization and stability are characterized using spectroscopy and microscopy methods, such as ultraviolet-visible (UV-Vis) light, transmission electron microscopy (TEM) or dynamic light scattering (DLS).
- The **characterization at the bio-interfaces** benefits from conventional fluorescence microscopy and 3-D microscopies such as confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). SEM is often coupled with energy-dispersive X-ray spectroscopy (EDX) to assess the surface physico-chemical properties (composition) of the nanoparticles.

- Finally, the **assessment of the lateral flow assay** global performances mainly relies on the visual, colorimetric behaviour of the test strips, either to analyse the proper flow in the LFA or to assess the test and control line signal intensities arising from nanoparticle accumulation. This is commonly performed by the naked eye or using adequate scanners and cameras. The chapter 6 and 7 of this thesis also explore the possibility to couple colorimetric evaluation of the LFA behaviour with electrical techniques, to gain insights for example in the charge transport within the flow.

Some of these techniques are used in chapter 4 to exemplify how to set up an LFA from the selection of bioreceptors to the design of the final device via an optimisation of each of the steps. Other advanced techniques for the characterisation of biointerfaces are explored in chapter 5. For an exhaustive review of characterization techniques involved in LFA research and development, the reader is referred to the work of Hsieh *et al.* [114].

Major limitations in LFA development The simplicity of LFA operation makes them appear deceptively straightforward to develop and optimise. At the cross-road between biology, chemistry, nano-physics and engineering, the science of LFAs involves a highly multidisciplinary background, requiring adaptation to the specific research and development methods (materials and processes) and languages of each domain. In particular, the presence of **two biological–material biointerfaces**, one for the nanoparticle and another for the membrane substrate, in the LFA creates numerous challenges. Despite their importance, interface issues are often overlooked and underappreciated due to their complex, multidisciplinary nature. Therefore, solving interface issues can be tedious and/or viewed as having limited innovative aspects, and for this reason they are often not published in the literature. Anyway, many outstanding issues still remain unsolved for LFAs, and as de Puig *et al.* points out, in biological fields that intersect with nanotechnology and engineering, **understanding and overcoming issues at the interface between the different fields has facilitated the success of innovative applications** [110]. In the upcoming sections, special importance will be given to understanding the interfaces required in LFAs and how they influence the performance of LFAs.

3.3 Review of optical LFA for environment and food safety

Figure B.1 in Appendix depicts the dynamics of the research conducted on LFAs for the main fields of LFA applications. Clinical diagnostics is still the main field of interest for LFA research, probably related to the amount of funding granted for these applications. However, food safety has shown strong increase of interest for LFA PoC diagnostics in the last decade. Table 3.1 presents several optical LFAs developed for the detection of pathogens in the environmental and food safety fields, highlighting their main design parameters and performances.

As depicted in Table 3.1, nowadays, several optical LFAs for pathogen detection are already published in the literature. However, a relatively small proportion of these tests has been able to reach the market so far. In general, it is important to note that gold nanoparticle colorimetric LFAs currently represent more than 80% of published studies in the literature or on the market, highlighting the continued prevalence of these nanoparticle labels in the field of LFAs to this day [116]. LFA specifically targeting pathogens usually rely on antibodies (either monoclonal or polyclonal) due to the widespread use of antibodies in immuno-diagnostics and their maturity. Although presenting some drawbacks (see section 3.4), they allow to reach appropriate sensitivities and selectivities of the assays.

Typically, these Ab–AuNP-based LFAs present limit of detection (LOD) is between 10^5 and 10^6 CFU mL⁻¹ without any additional sample preparation step. However, LOD can be further improved by using highly sensitive fluorescent reporters or nanoparticles (e.g. CdTe quantum dots), employing magnetic nanoparticles for bacteria pre-concentration, or adding incubation steps to let the bacteria grow and increase their numbers. Nonetheless, the aforementioned methods either require additional steps or expensive detection systems (i.e. fluorescent readers) and long processing time to achieve greater LOD, which is far from ideal for the end-user. Interestingly, most of these LFA aim to target pathogenic strains of (e.g. *E. coli* or *Salmonella*) in food samples, but few of them do it for environmental sample analysis [47].

Table 3.1 Overview of relevant optical LFA applications for contaminants detection in the environmental and food safety fields, in particular for pathogen detection, with their main design parameters and performances. (Note: Gray lines are commercially available LFA. Aqu. buf.: aqueous buffer. Fluo. Si.: Fluorescent silica nanoparticles. CdTe : Cadmium Telluride quantum dots. Bact.: Bacteriophage. CBD : Cell-wall Binding Domain. Dit : Distal-tail protein. ⊗: not available.)

Target	Princ.	NP type	Biorecept.	Time	LoD	Preparation	Ref.
Human IgG	Color.	AuNPs	pAbs	15 min	28 ng/ml	Full LFA	Us
<i>B. cereus</i>	Color.	AuNPs	Ab	15min	1×10 ⁵ CFU/ml	-	[117]
<i>B. cereus</i>	Color.	AuNPs	Bact. CBD	17min	1×10 ⁴ CFU/ml	Half-stick, 2-steps, AP replac.	[118]
<i>B. cereus</i>	Color.	AuNPs	Bact. CBD & Dit	15 min	1×10 ⁶ CFU/ml	Half-stick	Us
<i>Campyl. jejuni</i>	Color.	AuNPs	Ab	4h	1×10 ² CFU/ml	Incub.	[119]
<i>Campyl. coli</i>	Fluo.	Fluo. Si.	Ab	1h	3.2×10 ³ CFU/ml	Possible incub.	[120]
<i>Edwardsiella</i>	Color.	AuNPs	Ab	6 min	1×10 ⁶ CFU/ml	Possible incub.	[121]
<i>E. coli</i>	Color.	AuNPs	Ab	10 min	1×10 ⁶ CFU/ml	Possible incub.	[122]
<i>E. coli</i> O157:H7	Color.	AuNPs	Ab	15min	1.87×10 ⁴ CFU/ml	-	[117]
<i>Pseudo. aerugin.</i>	Color.	AuNPs	Ab	20min	2.41×10 ⁴ CFU/ml	-	[123]
<i>Shigella boydii</i>	Color.	AuNPs	Ab	10 min	1×10 ⁶ CFU/ml	-	[122]
<i>Strept. agalact.</i>	Color.	AuNPs	Ab	10min	1.5×10 ⁴ CFU/ml	-	[124]
<i>Staph. aureus</i>	Color.	AuNPs	Ab	15min	1×10 ⁶ CFU/ml	-	[117]
<i>S. Typhimurium</i>	Color.	AuNPs	Ab	15min	1.47×10 ⁴ CFU/ml	-	[117]
<i>Vibrio cholerae</i>	Fluo.	CdTe	Ab	15 min	1×10 ³ CFU/ml	-	[125]
<i>S. pneumo.</i>	Color.	AuNPs	⊗	15 min	5×10 ⁴ CFU/ml	-	Abbott
<i>Legionella</i>	Color.	AuNPs	⊗	20 min	1×10 ⁶ CFU/ml	-	Oxoid
<i>E. coli</i> O157:H7	Color.	AuNPs	⊗	15 min	1×10 ⁴ CFU/ml	Enrich.	CORIS

3.4 Bioreceptors for biointerfaces in LFA

When designing a biosensor, the choice of the proper bioreceptor remains a key element as it drives the specificity and sensitivity of the assay. [126] **Effective bioreceptors in LFA need three main characteristics:** (i) stability (i.e. preservation of its structure and functionality in the various LFA buffers and after drying and rewetting), (ii) fast association kinetics (to bind in the flow), and (iii) strong binding affinity for the target molecule (to withstand the flow that remains for minutes).

The **binding affinity and kinetics** of the reversible bioreceptor interaction with its target is physically described by a **dissociation rate constant**, K_D [M], which relates the concentrations of the bioreceptor protein (P) and its target ligand (L) to the amount of complex bioreceptor-target formed (or dissociated) (PL), in the solution:

$$[P][L] = K_D[PL] \quad (3.1)$$

K_D thus serves as a quantitative metric to compare affinity properties of receptors. A lower value of K_D corresponds to a higher affinity of ligand for the protein¹. Affinity binding properties are often assessed in conventional immunoassays (e.g. ELISA). However, the operating principle of LFA significantly differs as it requires the binding between target, nanolabels and bioreceptors in a few seconds and under a flow. Actual affinity binding properties of bioreceptors can thus significantly differ in LFAs, and must be assessed during the LFA development. [104]

In LFAs, two bioreceptors are involved : the **capture bioreceptors** which act as **test line**, and the **detection bioreceptors** that are conjugated to the **nanoparticles**. In addition to the intrinsic binding performances, crucial design aspects must be considered for the choice of the bioreceptors during the development of a LFA sandwich assay :

- The detection bioreceptor should not interfere with the binding of the test-line capture bioreceptor to the target.
- The conjugation and immobilization chemistries must be considered separately for the detection and capture bioreceptors. To be properly conjugated to a chosen nanoparticle, the bioreceptor must possess

¹ K_D is equivalent to the molar concentration of ligand at which half of the available ligand-binding sites are occupied. The more tightly a protein binds a ligand, the lower the concentration of ligand required for half the binding sites to be occupied, and thus the lower the value of K_D .

specific functional groups, depending upon the type of conjugation. On the other hand, the capture bioreceptor must tolerate immobilisation on the assay membrane without unfolding of its structure and the loss of its functionality.

Biorecept.	Advantages	Disadvantages
pAbs	Multiple binding site, relatively cost-effective	Low specificity, cross-reactivity, high batch-to-batch variability
mAbs	High specificity, low batch-to-batch variability	Expensive, long development process
Whole-phages	Highly specific, stability under various conditions	Use of whole phages as sensing elements can result in a decrease in signal intensity due to wrong orientation
Aptamers	Cheaper than antibodies, ability to recognize any type of target analyte, high stability and batch-to-batch reproducibility, <i>in vitro</i> generation	Binding activity is highly dependent on the ionic strength of the buffer and the presence of interfering molecules in the buffer
Phage proteins	High specificity and stability, low costs, recombinant protein production (without animals)	Not yet often used in biosensing applications, mainly because only a few of them are well characterized.

Table 3.2 Overview of common bioreceptors used in LFAs. The use of Abs for protein detection with colorimetric LFA is explored in this chapter, whilst the use of phages (or bacteriophages) proteins is explored for the LFA detection of whole-cell bacteria. (*Biorecept.:* Bioreceptors. *pAbs:* polyclonal antibodies. *mAbs* : monoclonal antibodies.)

Different bioreceptors are available for the detection and capture of targets in LFA (Table 3.2) :

Antibodies with regard to the most common targets detected by LFA (section 3.3), antibodies (Abs), or Immunoglobulins (Igs), are the **more common bioreceptors applied in LFA**. Antibodies are host proteins produced by the immune system to neutralize and eliminate pathogens. These “Y shape” proteins possess an antigen-binding region that recognizes and binds to antigenic agents through a specific recognition domain called epitope (Figure 3.4). Therefore, a properly immobilized Ab can easily interact with antigens on microbial surfaces and bind with its target. The chemical functionalization of the Abs to substrates and labels is well-documented, leading to ease and versatility of integration into different sensor systems.

However, despite their ubiquitous applicability in LFA and other biotechnology fields, **Abs present some limitations** due to their production process, which is still mainly dependent upon animal host infection and bleeding. They can suffer from variability (batch-to-batch variation), low specificity, relatively high costs and need for ethical approval. Antibody generation using animal immunization raises scientific and ethical concerns [127]. Also, generating, selecting Abs, and their mass production requires 16–24 months, which is much longer than the critical initial period when an outbreak is emerging.

Certain antibodies can recognise several different epitopes on an antigenic target : they are called **polyclonal** Abs (pAbs). In contrast, **monoclonal** antibodies (mAbs) bind to a unique specific epitope. During the testing of a sandwich assay using a monoclonal antibody and a polyclonal antibody, the monoclonal antibody should be the detection bioreceptor as it binds to just one epitope of the analyte, while the polyclonal antibody should be the test-line capture bioreceptor (the binding to multiple epitopes maximizes the chances of the sandwich formation, and do not interfere with the epitope available to the mAb). [126]

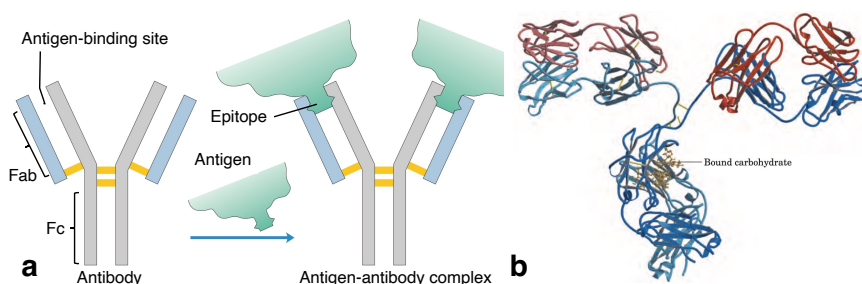


Fig. 3.4 Binding of antibody to target antigen. **a.** **Immunoglobulin G (IgG)** is the major class of antibody molecule of the organism's ongoing immunity to antigens, and therefore one of the most abundant proteins in the blood serum. The **Fab** region allows formation of the antigen-binding complex, made of the variable domains of the protein, while the **Fc** remains constant. **b.** A ribbon model representation of a complete IgG molecule. Conformation flexibility may be important to the function of the IgG. Reproduced from [7].

Phages Bacteriophages, or phages, are viruses that infect and replicate only within bacteria. Due to their concomitant evolution with bacteria, bacteriophages possess highly specific mechanisms to recognize, adsorb and subsequently infect their bacterial host for propagation, and are stable (i.e. ability to recognize their targets on a wide range of pH and tem-

peratures), making them particularly suitable for biotherapeutics [128] or bacterial binding and detection [129, 130, 131]. Bacteriophages are usually made of two distinct parts, the head (capsid) containing phage DNA and the tail responsible for bacterial recognition, adsorption and infection (Figure 3.5 a). Phages use specific binding proteins localized at their tail (3.5 b)) for the recognition of their bacterial hosts through binding of bacterial receptors localized at the host surface. The **binding properties of bacteriophages** have shown potential for many biosensor applications, and different works have exemplified the use of whole phages as bioreceptors in various biosensors, including immunomagnetic separation [132], electrochemical sensors [133, 134] and LFA [135]. However, in view of the bacteriophage inadequate morphology, size (tenths of nm) and location of the bacteriophage binding proteins, **wrong orientation on the sensor surface significantly reduces its binding capabilities to the bacterial target**, and, in turn, the sensor sensitivity. Obtaining a correct orientation of the phages is still one of the technical barrier to their dissemination in biosensor applications. [126]

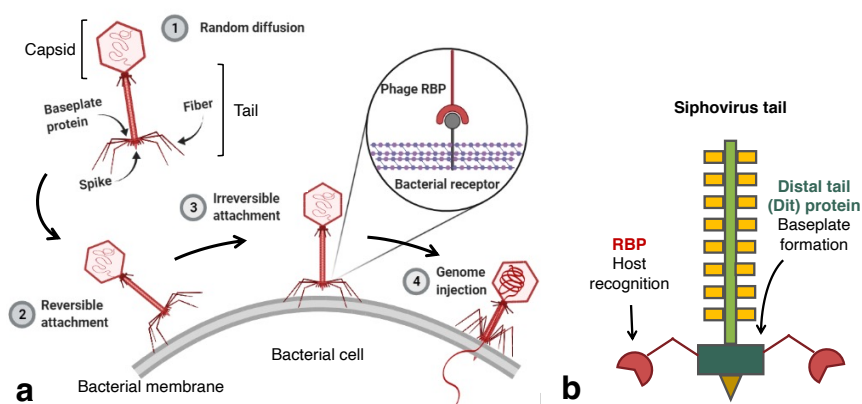


Fig. 3.5 Phage adsorption process. **a.** To survive, bacteriophages need to infect and kill bacteria in a multi-step cycle that begins with bacteria recognition, attachment and genome injection. The phage tail actively participates in the phage cycle and involves different proteins, such as the interaction between Receptor Binding Proteins (RBP), located at the distal part of the tail, and receptors at the surface of the bacterial cell envelope (inset). **b.** Advances in cloning and gene expression techniques allowed the understanding the molecular roles of the phage binding proteins such as RBD and Dit. They can thus be produced individually as recombinant proteins and used as bioreceptors in biosensors. Reproduced from Audrey Leprince's PhD thesis (2022).

Aptamers Also called “chemical Ab”, they have been investigated since their discovery at the beginning of the 1990’s as bioreceptors for LFA to address the restrictions from the Abs. They have several clear advantages over Ab, including **high stability**, **in vitro generation** via the SELEX process ², ease in functionalization, and **capacity for large-scale synthesis**. Aptamers are single-stranded DNA molecules that fold in a complex 2D or 3D structure, bind to a target with high affinity and selectivity, and have already been generated for many targets (hormones; drugs; heavy metals; proteins; toxins; enzymes; cancer cells; and bacterial, viral, and fungal pathogens) in different biosensor configurations, especially electrochemical sensing [136]. Aptamer-based LFA tests already took advantages of aptamers as bioreceptors; however, they do not solve the lack of sensitivity of the LFA and their binding activity is highly dependent on the ionic strength of the buffer and the presence of certain cations. [137, 138, 139, 140]

Phage proteins With recent progresses in the bacteriophage research field, many proteins involved in the phage lytic cycle (recognition and binding process, phage exit from host) have been isolated and their role identified [141, 8], **empowering their use as bioreceptors instead of their parental phages**. Examples includes the use of the receptor binding protein (RBPs) [142, 143], the cell-wall binding domain (CBD) of the endolysin [144, 118], or the distal tail protein (Dit) [145, 146] (3.5 b). The genes of the proteins can be cloned and modified (by genetic engineering) to produce protein variants. Their sequences can then be inserted and produced in other organisms such as bacteria, which is called **recombinant protein** production, to achieve high protein production yield at low cost, and without having to use animals [147]. Extensive characterizations have shown that phage proteins could display broader host activity spectrum than their parental phages, the latter being often so specific that they only target a subset of one bacterial species [148]. Studies also shown that **the use of engineered phage proteins enhanced** six times the **capture efficiency of pathogens** compared to the wild type (WT) protein or the entire phage [146], making them promising bioreceptor in sensors. The use of phage proteins as bioreceptors in LFA is the topic of chapter 5.

Other available bioreceptors, including *Molecular Imprinted Polymers (MIP)* or *peptides*, are extensively covered in reviews [126].

²“Systematic evolution of ligands by exponential enrichment”

3.5 Nanoparticle labels for optical detection

Nanostructured materials such as nanoparticles (NPs) express **unique optical, electronic and chemical properties**. The property of individual NP can be readily tuned by varying their surrounding bio-chemical environment. The binding event between the NP and an analyte can enhance or alter its physicochemical properties, such as its interaction with light, conductivity or redox behavior, that in turn can generate a detectable response signal, and provide the opportunity to use them as **suitable transducer or labels in LFA tests**. In addition, when concentrated at the TL and CL, **their accumulation enables to measure a sufficiently large signal** that will confirm the presence (or not) of the target in the test. Depending on the NP materials and properties, **this signal can be colorimetric, fluorescent, electrical, magnetic or thermal**. Because **NPs are key to assay performances** (sensitivity and LoD), many new nanomaterials with highly interesting properties to improve LFA performance were developed in recent years [149, 150]. Table 3.3 summarises the main classes of NPs proposed for optical LFA [104, 151, 152, 153]. NPs associated with other transduction mechanisms such as electrical or magnetism are introduced in chapter 7.

In this section we present a nanoparticles for colorimetric LFA and in particular the gold nanoparticles (AuNPs) that are still the most widely used in LFA (88% of recently developed LFAs [116]).

3.5.1 AuNP principles

Colloidal metal particles, in particular gold, have found many biological applications, first as specific probes in light and electron microscopy back in the 1950s, and more recently, since the 1980s, in membrane-based immunochromatographic tests such as LFA [154]. Indeed, gold nanoparticles possess distinct physical and chemical attributes that make them excellent labels for nanobiosensors :

1. AuNPs can be **synthesized** in a straightforward manner, at lower costs, large-scale, with high stability and reproducibility, and can be made in convenient well-defined though tuneable size (in the nanometer range) and shapes (colloid, rods, stars, ...).
2. They possess **unique optoelectronic properties**, such as a strong colorimetric response (generation of an intense red color visible to the naked-eye), tunable with their morphology.
3. They provide high surface-to-volume ratio with **excellent biocompatibility**.

Label	Size	Advantages	Disadvantages
COLORIMETRIC DETECTION			
AuNP	10-100 [nm]	Strong signal, naked-eye detection, fast qualitative response, easy synthesis, highly biocompatible, simple conjugation methods, stable in time, tunable size and shape	Only qualitative (quantification requires extra hardware), poor sensitivity & LoD if no enhancement method
CNPs CNTs	10-250 [nm]	Qualitative naked-eye detection, high signal-to-noise ratio, cheaper, stable	Only qualitative (quantification requires extra hardware), Weaker signal than AuNPs, unspecific adsorptions
Latex beads	200-500 [nm]	Qualitative naked-eye detection, resistant to chemical and physical damage, cheaper, multiple colors	Quantitative detection requires extra hardware, less sensitive, Weaker signal than AuNP, more complex synthesis and conjugation, high amount of dye needed
MNPs	10-800 [nm]	Enable colorimetric & magnetic signal, enable sample pre-treatment & pre-concentration	More complex synthesis and conjugation
FLUORESCENCE DETECTION			
QDs	1-10	Strong fluorescent intensity, good sensitivity and LoD, multiple colors available	Requires extra hardware (UV light and reader), higher toxicity, complex synth. and conjug.
UCNPs	10-80	Strong visible fluorescent signal, no UV source needed, no membrane autofluorescence, enable low target conc. detection	Requires extra bulky and expansive hardware (NIR laser), more expensive than QDs (they are made of rare materials)

Table 3.3 Overview of common labels used in optical LFAs. Owing to their advantageous properties, gold nanoparticles (AuNPs) are the most widely used detection labels in LFAs. Carbon nanoparticles (CNPs) and nanotubes (CNTs), latex beads, and magnetic nanoparticles or superparamagnetic beads (MNPs) are alternatives. Quantum dots (QDs) and Upconverting nanoparticles (UCNPs) have been proposed to increase LFA sensitivities thanks to stronger, fluorescent signal emission.

4. AuNPs are **suitable for biofunctionalization** and can easily be rendered immuno-specific by **adsorption** of protein bioreceptors, as found

by Faulk and Taylor in 1971, for selective binding and detection of molecules and biological targets. [155, 154]

These attributes make gold nanoparticles ideal for qualitative, naked-eye detection, and have conducted them to be the **most widely used detection labels in optical LFAs**. Other nanomaterials, such as carbon nanoparticles (CNPs) and quantum dots (QDs, e.g. CdSe nanoparticles) or upconverting nanoparticles (UCNPs, e.g., Eu-based nanoparticles), also presenting respectively advantageous colorimetric and fluorescence capabilities, are becoming popular in research papers to improve sensitivity and enable quantification, but not in the same way (yet) than AuNPs mainly because large-scale production of these novel nanomaterials and their use in commercial tests could still be challenging or at higher costs (integration with specific readers) [156].

Physics of AuNPs AuNPs exhibiting an **intense red color** can be explained with quantum physics through **surface plasmon resonance (SPR)**. When the AuNPs are irradiated with light, as their size is much smaller than the incident wavelength, the conduction electrons of the nanoparticle surface oscillate in a collective fashion, which is known as SPR. This results in a strong SPR absorbance in the visible spectroscopy region, and scattering of light. The absorption peak and color of AuNPs depend heavily on the nanoparticle size, shape and coating (**20 nm AuNPs strongly absorb 520 nm wavelength light** and scatter red light). [157, 158, 159]

Synthesis of AuNPs Gold colloids are produced through the chemical citrate reduction of a gold precursor (tetrachloroauric acid, HAuCl_4) in aqueous solution, a method introduced by **Turkevich** in 1951 [160]. In this method, illustrated in Figure 3.6 A, sodium **citrate** (NaCt) acts as both reducing and stabilizing agent during the synthesis: first, citrate anions reduce gold ions to atoms and stabilize colloidal AuNPs formed from clustered atoms, and second, the steric repulsion between the negatively charged $\text{H}_2\text{Citrate}^-$ anions adsorbed onto the AuNP surface contribute to particle stability and **confers to the AuNP a negatively charged outer surface** [161]. It is possible to control AuNPs size and shape by varying the feed ratio of gold salt to sodium citrate. Turkevich's method typically provides spherical AuNPs of 10–100 nm in diameter. For higher AuNP sizes, the particles become less spherical, the size distribution becomes broader, and the results are less reproducible. Today, the protocol has been modified and improved, focusing on control of their size, shape, solubility, stability, and functionality.

A comprehensive overview of the nanoparticles used in LFA (especially AuNPs and MNPs), their synthesis, their characterization and their properties was performed during Josquin Vandeputte's master thesis [102].

3.5.2 AuNP characterizations

AuNPs are commonly characterized for size, aggregation and quality using a range of different yet complementary techniques: UV-visible (UV-Vis) absorption spectroscopy, transmission electron microscopy (TEM) and dynamic light scattering (DLS).

TEM images allow to study the **morphology** of gold nanoparticles by direct visualization, such as the black spheres observed on Figure 3.6 B. Important morphological parameters of the gold nanoparticle solutions include their aggregation, their sizes and their polydispersity index (PDI). PDI is defined as the standard deviation (σ) of the particle diameter distribution divided by the mean particle diameter d : $PDI = (\sigma/2d)^2$. PDI is used to estimate the average uniformity of a particle solution, and larger PDI values correspond to a larger size distribution in the particle sample. PDI can also indicate nanoparticle aggregation along with the consistency and efficiency of particle surface modifications throughout the particle sample. A sample is considered monodisperse when the PDI value is less than 0.1. From TEM micrographs, image software analysis can be performed to study the size distribution of the AuNPs (Figure 3.6 C). For their part, DLS measurements result in the hydrodynamic diameter of the gold colloids in the solution, and also bring useful information about their size distribution and number density.

To study their **optical properties**, the UV-vis curve of the AuNP solution can be recorded (Figure 3.6 D), showing the characteristic shape of AuNP absorbance spectra. Three main characteristics are observed: (i) non-zero absorption towards UV wavelengths, (ii) the characteristic **absorption peak**, or plasmonic peak, at 520 nm, (iii) near-zero absorption from 580 nm to the infrared rays, which accounts for the red color of AuNPs. The height of the absorption peak at 520 nm, i.e. the absorbance at 520 nm A_{520} is commonly used to compare the different synthesized solution. The absorbance is often referred as the **optical density (OD)** of the AuNP solution, and can be used interchangeably. The width of the absorption peak is characteristic of the AuNP dispersion in the solution (partially aggregated AuNP have a wider plasmonic peak) and the color of the AuNP solution (wider peak indicates changes from red to blue, while monodispersed AuNPs maintain the original red color).

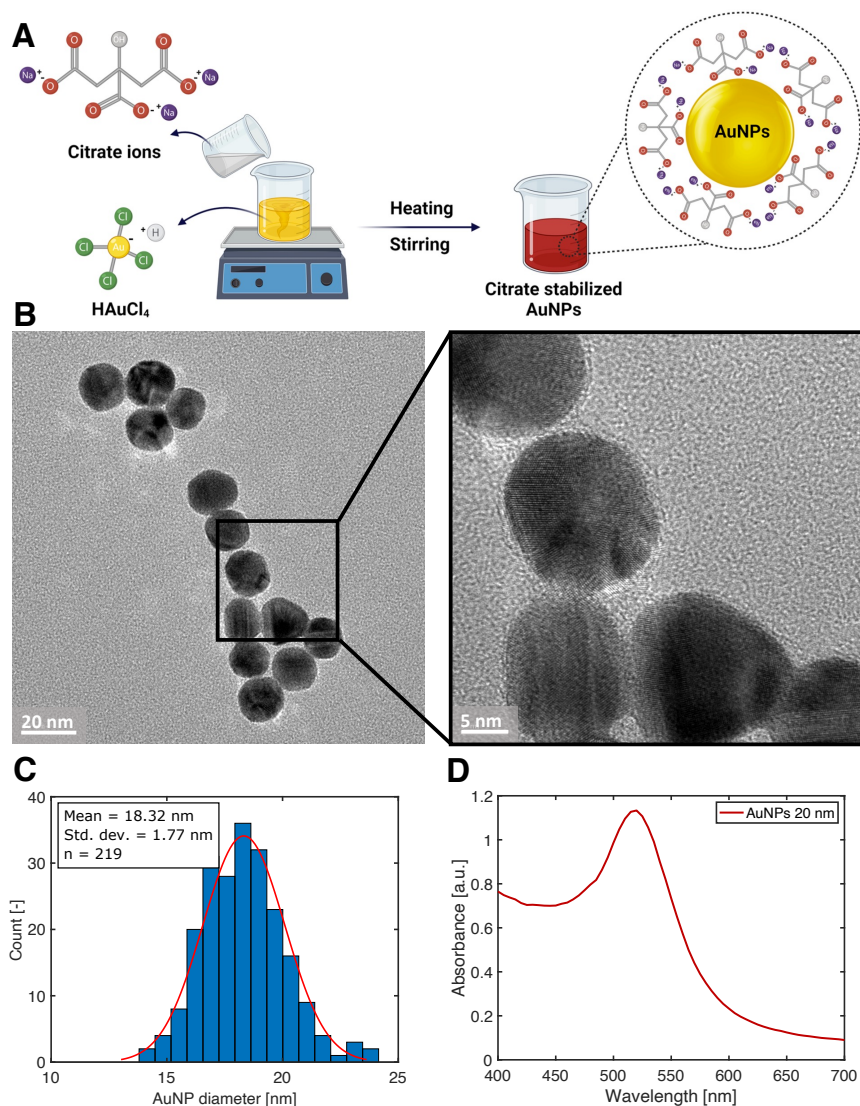


Fig. 3.6 Synthesis and characterizations of gold nanoparticles. **A.** The synthesis of gold nanoparticles via the Turkevich method implies the reduction of the gold salt with citrate anions, that stabilizes the gold colloid formation by forming a coating layer. It provides negative charge to the AuNP surface. Reprinted from [162]. **B.** Common characterizations include TEM images of the AuNPs allow to study their morphology (shape, curvature), and **C.** size distribution. **D.** The optical properties of the 18 nm AuNPs synthesized in this thesis are studied using UV-vis spectra, where the observed absorbance peak at 520 nm means a strong red colour emission from the AuNP solution.

In addition to the morphological and optical properties, the **concentration** of the synthesized AuNP solution can be derived directly from UV-Vis Spectra. Haiss *et al.* have studied the correlation dependence of the **number density of the AuNP** in solution N [#/mL] with their size and absorption spectra [163]. Since the diameter d of the AuNP has been determined by TEM images, then N can be determined from the absorbance³ at 450 nm (A_{450}), expressing a linear increase of the number density of AuNP with A_{450} and a more complex, inverse correlation with the particle diameter d :

$$N = \frac{A_{450} \times 10^{14}}{d^2 \left[-0.295 + 1.36 \exp \left(- \left(\frac{d-96.8}{78.2} \right)^2 \right) \right]} \quad (3.2)$$

3.5.3 AuNP conjugation to detection bioreceptors

Directly binding of bioreceptors to AuNPs to form the detection biointerface can be achieved through quick protein adsorption onto citrate-capped AuNPs, creating the AuNP conjugates [164]. Here, we **describe the basis of the preparation and optimization of AuNP conjugates**. In this chapter we study the antibody conjugation to AuNP via adsorption. Phage protein adsorption to AuNP is studied in detail in chapter 5.

The **adsorption of proteins to gold nanoparticles** is a **three-step process** based on a combination of **three separate but dependent phenomena** (Figure 3.7 A-B):

1. **ionic interaction** between the negatively charged nanoparticle and the positively charged sites of the available protein amino acids (Figure 3.7 A);
2. **hydrophobic attraction** between the protein and the metal surface;
3. **dative binding** between the metal and the conducting electrons of nitrogen and sulphur atoms of the protein (Figure 3.7 B).

This last binding process can only occur in proteins containing surface **cysteine residues**, since these residues can irreversibly oxidize on the AuNP surface : cysteine binds to the gold surface through chemisorption

³The use of A_{450} in the formula is justified by the fact that, for decreasing particle size, the absorbance at surface plasmon resonance wavelength (typically 520 nm for 20 nm AuNP) is increasingly damped relative to the absorbance at other wavelengths (typically A_{450} remains quasi-constant for AuNP of different sizes and can serve as a reference value). Therefore, the ratio of the absorbance at different wavelengths was used to build the formula [163].

via the sulfur atom presents in its thiol group⁴. For example, antibodies possess thiol groups in cysteine amino acids located in the Fc constant region [165]. Although Au-thiolate bond formation is one of the most commonly used methods of biosensor surface functionalization (e.g. Au electrodes in electrochemical sensors and AuNP in LFAs), making it one of the most important chemistries in biosensor fabrication, it is still one of the least understood chemistries currently in use. [166, 167, 168]

The **protein adsorption process on gold** is typically done **spontaneously**, in solution, over a couple of hours. It generally results in **randomly oriented molecules** on the AuNP surface, with an uncontrolled surface repartition. **Proper immobilization of bioreceptors on interfaces is critical** for the system as proper bioreceptor-antigen binding activity governs the LFA sensitivity, dynamic range and reproducibility [126]. Therefore, the manufacture of poor-quality and poorly characterised bioconjugates remains a major issue that can result in the under performance of the LFA. An appropriate conjugation process thus requires that proteins adsorb strongly to colloidal gold to form stable AuNP conjugates, while the conjugate still retains the good bioreceptor binding properties.

AuNP conjugate optimisations To prepare good quality and stable AuNP conjugates, several parameters should be considered, including the isoelectric point (pI) of the protein, the pH of the reaction, the concentration of protein loaded onto the colloids, and the stability of the colloids :

- Generally, most proteins can be adsorbed onto the metal surface in an optimal manner using buffer systems close to their **pI** value for optimal protonation. Away from this pH, the adsorption decreases.
- The **concentration of the bioreceptor** loaded on the AuNPs can be optimised in a process called the **gold aggregation test (GAT)**, where the addition of salt (NaCl) to AuNPs coated with different bioreceptor concentrations affects the colloidal stability of the AuNPs, leading to aggregation⁵. Once the AuNP are sufficiently coated with bioreceptor proteins, aggregation cannot happen and they remain monodispersed even at high salt concentrations. This analysis allows

⁴The reaction implies the concomitant loss of the -SH proton.

⁵Adding salt to the AuNP solution conducts to the formation of a double-layer on the surface of each particle. The first layer consists of ions absorbed onto the surface of a particle while the second one is composed of counter ions dissolved in solution which start to surround the particles attracted by its surface charge. This second layer shields the original charge of the particle, reduces the electrostatic repulsion forces acting between particles and leads to AuNP aggregation. [169, 170]

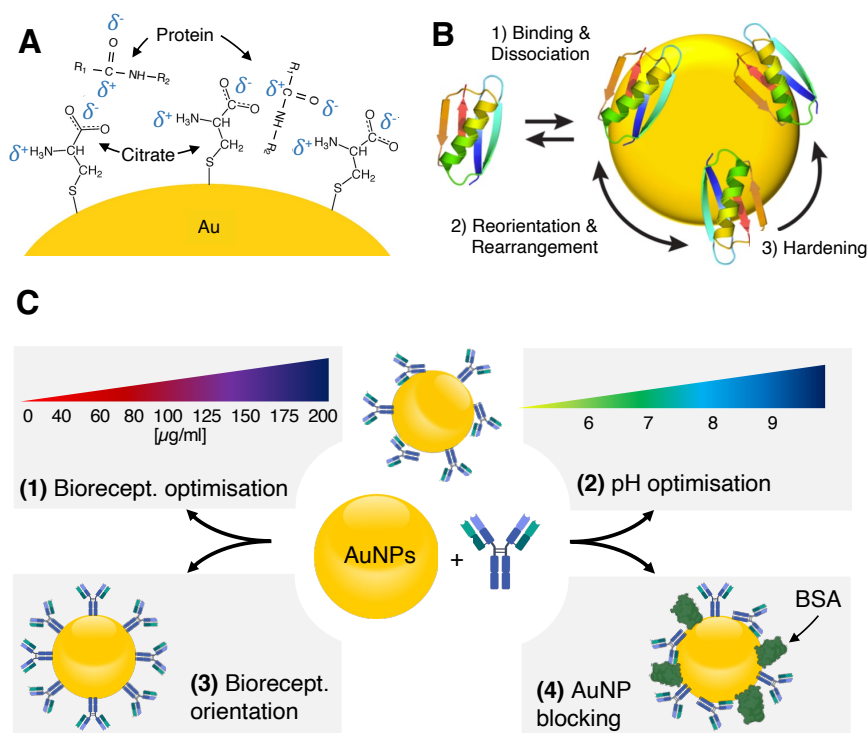


Fig. 3.7 Conjugation of protein bioreceptors to AuNPs through adsorption is a passive three-steps process. It starts with **A**, a first reversible binding (and dissociation) through electrostatic interactions between the negatively charged citrate layer and the protein that are positively charged at neutral or physiological pH, followed by **B**, closer interactions that reorient the protein on the AuNP surface and ends up with a strong, dative binding between the protein cysteine and the AuNP atoms. **C**, To ensure stable AuNP conjugates, the passive adsorption process conditions must be optimised during a gold aggregation test (1-2). The AuNP affinity binding performance can be improved by orientation of the bioreceptors on the AuNPs (3, optional), and the use of blocking biomolecules to reduce non-specific interactions (4).

for estimation of the minimum bioreceptor concentration to give stable conjugates (Figure 3.7 C).

- The **pH of the conjugation** is also optimized during the GAT : performing a GAT experiment for three different pH (e.g. pH 7-8-9), one can study the condition for which there is the smallest aggregation amongst the AuNP conjugate solutions, which indicates the optimal

pH for bioreceptor adsorption process (Figure 3.7 C).

The conjugation of nanoparticles, their subsequent immunoactivity and stability can be monitored using UV/Vis absorption, fluorescence microscopy, TEM, DLS, activity-based measurements including ELISA, and of course LFA testing. There are illustrated in chapter 4 and chapter 5.

In addition, interfacing nanomaterials in complex biological environments is challenging and can result in undesirable side effects such as non-specific adsorption, surface fouling, protein denaturation, and steric hindrance (Figure 3.10) [110]. The **adequate conjugation of the AuNP bio-interfaces** must address these **interface challenges** :

- Bioreceptor binding affinity to the target can be reduced because of its attachment to a nanoparticle. Bioreceptor **denaturation** can occur because of perturbing effects of the NP and its surface coating ligand, preventing proper interaction and binding. Most often, this is manifested by a change in K_D .
- **Random orientation** of the bioreceptor on the NP surface can potentially obscure its binding epitopes for the target, and lower or prevent the target binding [171]. Engineering a specific orientation of the bioreceptor immobilization could ensure that they remain functional and their binding epitopes accessible (Figure 3.7 C) [164]. For example, [172] have found that oriented immobilization of IgG on NPs allows better recognition of analytes in LFA format, especially in the case of small size analytes. [173]
- Finally, the interaction of the conjugated AuNP with its environment can also lead to **non-specific adsorptions** of proteins, molecules in presence, or onto the membrane matrix. To prevent or decrease non-specific adsorption, it is common to add blocking agents to cover remaining site of the AuNP free of ligands, such as bovine serum albumin (BSA) (Figure 3.7 C). However, the surface blocking ligands added onto the NP surface can sterically obscure the binding epitopes of the bioreceptors. It is thus critical to know the sizes of the bioreceptors and choose the blocking agents accordingly. Except the BSA (66 kDa), other smaller molecules can be used to block the conjugated gold nanoparticles, such as thiol functionalized PEG molecules (SH-PEG, a few kDa in size), of other smaller proteins: caseins (25kDa) and ovalbumins (45kDa).

3.6 Nitrocellulose membranes in LFA

3.6.1 Overview of nitrocellulose as substrate assay

Nitrocellulose (NC) membranes have been manufactured for filtration purposes for decades. In the 1970s, molecular biologists discovered the utility of **nitrocellulose membranes as a substrate for biomolecule detection by immobilization** (e.g. the “Western blot” for protein transfer to nitrocellulose), highlighting the unique ability of NC to interact with three of the most important classes of biomolecules (proteins, DNA, and RNA) [56]. Building on this experience, LFAs were patented and introduced in the 1980s [109]. NC is the most common membrane used in lateral flow assays. In this section, we present general considerations regarding NC as the assay membrane in LFA and we study the antibody adsorption on the membrane. Phage protein binding to NC membranes is studied in chapter 5.

Also known as cellulose nitrate, NC is created commercially by the reaction of cellulose (purified from plants, commonly wood pulp and cotton) with nitric acid, replacing the cellulose hydroxyl groups (OH) with nitrate groups (NO_3^-) [174]. In terms of surface properties, NC is naturally hydrophobic. **Membranes are made hydrophilic** by the addition of rewetting agents, i.e. a **surfactant**, during the membrane production process by the manufacturer [109].

In the perspective of LFAs, **critical properties of the membrane** include surface chemistry, which influences the immobilization of bioreceptors, morphology (pore size distribution and porosity), which enables proper fluid wicking, and optical properties since colorimetric detection is the preferred option for this type of device. The ubiquitous use of NC in LFAs can therefore be found in its **extremely functional and suitable properties as an analytical membrane** : relatively low-cost compared to other microfluidics substrates, true capillary flow with different wicking rates available, high protein-binding capacity, relative ease of handling, and presenting an ideal white background for colorimetry. Although it still has some drawbacks (variable performance reproducibility between lots or with environmental conditions such as relative humidity, shelf-life issues), a limited number of alternative to NC membranes has been proposed [109].

3.6.2 Nitrocellulose membrane properties for LFA development

In general, the NC membranes consist of a matrix of randomly oriented fibres that are bonded together to form a tortuous maze of highly porous channels. **NC membranes are available with different microstructure properties to allow optimisation during the LFA development**, i.e. varying pore sizes, wicking rates and surfactant contents. Selecting a membrane with appropriate pore sizes is really important in order to reach an homogeneous flow of the complex analyte-gold conjugates, and especially true when dealing with large analytes such as whole-cells bacteria. [111] Membrane choice related to its pore size and wicking rate is discussed in detail in section 3.8.

In this thesis, **we characterized and used three different NC membranes** from Sartorius (Unisart CN95, CN140 and CN180) to optimise the tests. SEM was used to visualise the surface morphology of the membranes. Figure 3.8 shows SEM micrographs and the pore size distribution of the three NC membranes, obtained by the image analysis (ImageJ software). Among the three membranes, CN95 membranes (Figure 3.8 A) have the largest range in pore sizes that were scattered randomly within the polymer matrix, with a broad distribution from about 5 μm to 20 μm , with the mean pore size at $11.56 \pm 6.64 \mu\text{m}$. Compared to CN95, the CN140 membrane has a smaller pore range from about 1 μm to 12 μm , with a mean pore size of $7.54 \pm 6.71 \mu\text{m}$ (Figure 3.8 B). The pore size of the CN180 membrane was more evenly distributed, with the smallest range of membrane pores (Figure 3.8 C) and the mean pore size of $5.06 \pm 3.56 \mu\text{m}$. The smaller pores are expected to offer good accessibility and large surface areas for bioreceptor adsorption. However, in order to make bacterial cells migrating in the membrane, in view of the pore size distribution and membrane morphologies, the CN95 and CN140 membranes have the highest potential. Complete characterization of NC membranes used in this thesis is depicted on Table 3.5.

The void volume of a membrane is approximately 80% of the entire membrane volume, and there is no significant difference in the void volume between CN 95 and CN 180, as referred by Sartorius.

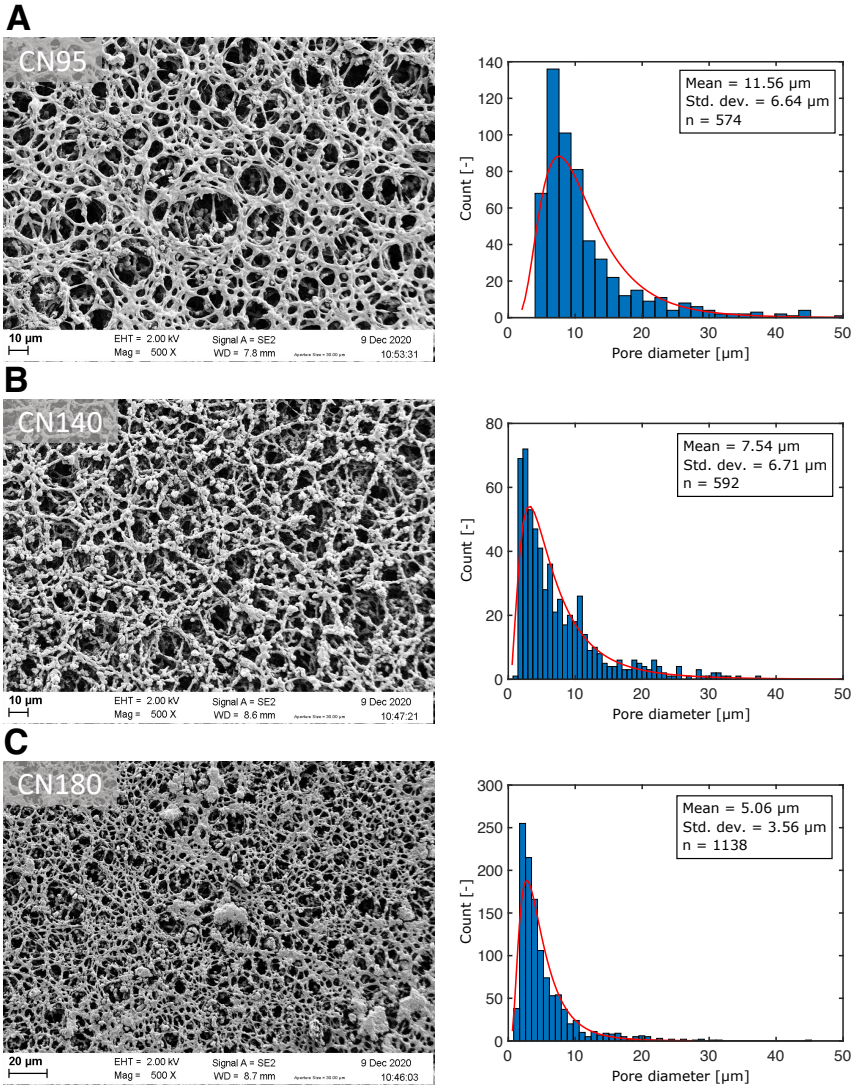


Fig. 3.8 Pore size distribution and morphology of nitrocellulose membranes used in this thesis, from Sartorius: **A.** CN95 **B.** CN140 **C.** CN180. Pore size distributions follow a lognormal distribution, obtained by SEM micrographs analysis (ImageJ).

3.6.3 Capture biointerfaces on the NC membrane

Proteins are the most common bioreceptors applied onto the membrane surface to form the capture biointerface in LFA, test and control lines. The protein-binding capacity of a membrane, loading capacity and the kinetics of the protein-binding process are the parameters that determine the suitability of a protein for the membrane. As already introduced for the detection biointerface, consistent immobilization of bioreceptors to the test and control lines is critical for the bioreceptor to maintain its original binding functionality, which influences the sensitivity of the diagnostic tests.

The adsorption of proteins bioreceptors to the porous nitrocellulose membrane While it is well-established that the proteins (e.g. Abs or phages proteins) will adsorb to porous NC membranes after their deposition, the process is not fully understood yet. However, known principles should be considered when trying to achieve protein immobilization at a test line, or to prevent non-specific adsorption elsewhere. **Proteins bind to NC through adsorption, governed by a complex interplay of non-covalent interactions** between the two, including :

- **Electrostatic interactions** between the dipoles of the NC nitrate groups (all of the NC membrane surfaces exhibit a negative charge) and dipoles in the protein, giving rise to an electrostatic affinity (Figure 3.9). It is expected to be the major factor that dominates the adsorption behaviour [175] ;
- **Hydrophobic forces** from interaction between the carbon-containing nitrocellulose and the hydrophobic portion of the protein ;
- **Hydrogen** and Van der Waals forces.

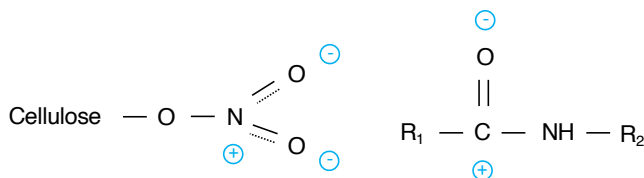


Fig. 3.9 Regarding the physiochemical properties of both NC membranes (left) and proteins (right), the strong dipole of the nitrate group in the NC membrane would interact with the strong dipole of the peptide bonds within the protein, such that the electrostatic interaction is the main factor that dominates protein adsorption on NC.

The commonly accepted **model** for thermodynamically driven adsorption of protein to NC is that **proteins are initially attracted to the mem-**

brane surface by electrostatic attraction. Long-term bonding is then accompanied by a combination of hydrophobic and hydrogen bonds. [56]

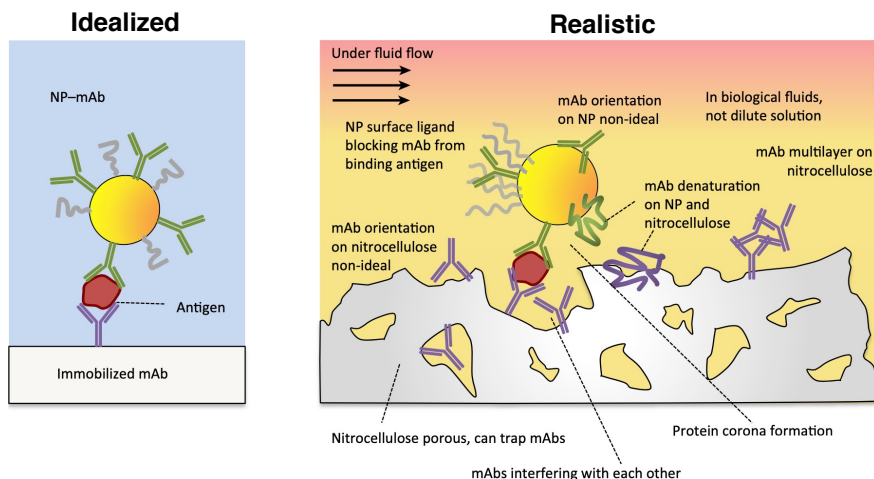


Fig. 3.10 Idealized versus realistic capture and detection biointerfaces in LFA. Both the detection and the capture biointerfaces present challenges including re-sorption, non-specific adsorption, wrong orientation and loss of bioreceptor binding functions that may ultimately affect the LFA performances (sensitivity and specificity). Their careful control and prevention (e.g. with blocking agents) helps to reduce these unwanted effects. Reproduced from [110]. (mAbs : antibody, NP: nanoparticle)

Just as for the detection biointerface on AuNP, interfacing bioreceptors in NC membranes can result in **challenges at the capture biointerface** (Figure 3.10). These issues are even more acute in LFAs as there are many different types of inorganic and bio-interfaces, often of a complex nature. Some of them are summarized hereafter [110, 109] :

- The **pH of the medium** could affect the adsorption process on the NC. Above their pI, the protein molecules demonstrates the highest inter-molecular electrostatic interactions. These repulsive forces tend to unfold the proteins, which modifies its binding process. Protein unfolding also decreases the compactness of its structure, tending to decrease its loading capacity on the membrane.[175]
- The LFA, subjected to a **fluid flow velocity**, render protein adsorption a kinetic, two-way process, such that the kinetics of protein adsorption and desorption to the membrane must also be considered.

- In addition, the **presence of agents** in the bioreceptor solution such as salts and detergents may interfere with the protein adsorption, influence stability or promote desorption of the bioreceptors when the sample wicks through the test line. To maximize adsorption, binding proteins should be applied to the NC free of other salts than the buffer itself, surfactants, and sugars [109].
- Finally, **real samples** are more complicated than pure solutions. Laboratory testing is often performed using controlled saline buffers, not nearly as complex as samples obtained in the field that can contain interfering agents. These factors can contribute to variability at the capture biointerface. Sample preparation can greatly influence assay functionality and accuracy, although minimization of processing steps is more convenient.

Binding affinity of immobilized capture bioreceptors NC is a much more complex substrate compared to the flat glass, polystyrene, and metal surfaces that are typically used for ELISA or other assays. The binding behaviour of bound bioreceptor to its target can thus differ significantly. It can lose binding affinity or denature due to NC effects. Many proteins lose immunological activity after passively binding to membrane surface due to inability to bind covalently or directionally to nitrocellulose. Also, pores can trap biomolecules and decrease target binding efficiency. Finally, the surfactant of a chosen NC membrane, used as rewetting agent, has to show compatibility with the bioreceptor protein since not every protein is compatible with every surfactant. Indeed, to properly bind to the NC membrane, the bioreceptor protein has to show higher affinity for the nitrocellulose than the surfactant does with the aim of coming into close proximity to the NC surface. Therefore, **it is mandatory to assess the bioreceptor binding performances to the target in the LFA format.** [109, 176]

Blocking Typically **many of these adsorption issues are mitigated by membrane blocking.** The main purpose of blocking a NC membrane is **to prevent non-specific binding**, i.e. the binding of targets and labelled conjugate to the membrane at areas other than the test and control lines. In addition, blocking also stabilizes the test and control line bioreceptors, controls and stabilizes the flow-rates and hydration characteristics. Blocking is typically performed via adsorption of non-specific proteins such as albumin (e.g. bovine serum albumin, **BSA**) or casein [109].

Test and control lines deposition To lay down the test and control line proteins on the membrane, the membrane is functionalized, or **striped**,

with proteins in solution in particular pattern suitable with the LFA format (line or dot) using either contact or non-contact dispensing systems: **bio-spotters**. Piezoelectric, non-contact dispensing bio-spotters provide the best solution for quantitatively dispensing proteins onto nitrocellulose, while continuous, contact spotters allows the easiest and fastest deposition of protein lines, but can mechanically damage the sensitive NC surface [109]. The main parameters influencing the striping process are the bioreceptor concentration (typically ~ 1 mg/mL), the flow rate and speed (defining the quantity of bioreceptor solution dispensed on the membrane, typically ~ 1 μ L/cm for the flow rate and 20 mm/s for the position, and the TL position and size (see section 3.8).

Drying After dispensing the test and control lines, the **NC membranes are dried to remove surface fluids and end up with a stable coating of immuno-active bioreceptors**. The drying process enables the proper binding of the bioreceptors to the NC membrane through the process described above (electrostatic, then hydrophobic interactions, and finally the protein reorganization, defining whether or not the protein will remain active). It is thus critical to the final performance of the test. [109]

3.7 Sample, conjugate and absorbents pads

Conjugate pad The conjugate pad (CP) performs multiple functions: (i) storing the dried conjugated nanoparticles for detection, (ii) releasing them upon wetting by the sample and (iii) providing the first interaction between the labeled bioreceptor and the target, and (iv) consistent transfer of the labels and test sample onto the membrane (which ultimately determine how much analyte can be measured at the test line). Given its key role in the LFA, **preparation of the conjugate pad is one of the most critical stages during fabrication of the device**. It is achieved in a similar way than the test line striping, except that the conjugated AuNPs are instead deposited on the CP with a sprayer. In particular, obtaining a consistent drying, with the correct degree of moisture removal is critical to achieving stability of the dried conjugate and bioreceptors, facilitating their even resuspension when contacted with the sample. CP materials are selected according to the following criteria: low non-specific binding, high porosity for consistent flow characteristics and bed volume, and consistent mechanical properties regarding compressibility or material release, and are most commonly glass fibers [109, 104, 111]. Their key properties are summarized in Table 3.4.

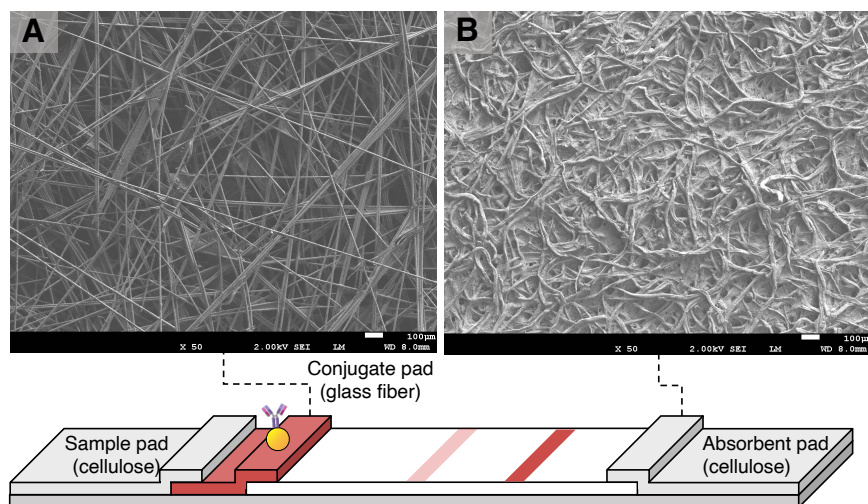


Fig. 3.11 Morphology of **A.** glass-fiber conjugate pads and **B.** cellulosic sample and absorbent pads. SEM was used to visualise glass fiber diagnostics pad and cellulose fiber sample pad (SureWick, Millipore, Burlington).

Sample pad The sample pad (SP) is used to perform multiple tasks as well, mainly to **promote the even and controlled distribution of the sample onto the conjugate pad**. It controls the rate at which liquid enters the conjugate pad, preventing flooding of the device, and enhancing the ability of the sample to solubilize the detector conjugated labels. When impregnated with proteins, viscosity enhancers and buffer salts, the sample pad is also used to standardize the sample buffer conditions in order to prevent non-specific binding of AuNPs and analytes downstream and favours the binding of immunocomplex formation at the test line.

Sample pads are most commonly made of **cellulose** (Figure 3.11 B) due to their advantageous properties (Table 3.4). When using cellulose filters, care must be taken to ensure sufficient and consistent contact with the underlying CP material [111]. SPs are sometimes made of glass fibers, which have opposite structural properties to cellulose but still enable even distribution of the sample over the conjugate pad. In this thesis, LFAs developed in chapter 4 use SP made of cellulose, while LFAs developed in 7 use glass-fibers.

Absorbent pad Absorbent pads are placed at the distal end of the test strip (Figure 3.11). The primary function of the absorbent pad is to **control or increase the total volume of sample that enters the test strip**. This increased volume can be used to wash unbound detector particles away from

the test and control lines, thereby lowering the background and enhancing assay sensitivity [111]. If the strip design does not include an absorbent pad, the volume of sample analyzed in the strip is determined solely by the bed volume of the membrane, which is only a few μL , see section 4.2.4. Dimensions of the SP should be based on the volume of liquid that must pass through the membrane, for example if a washing step is required with extra buffer. Absorbent pads are made of high bed volume materials, such as the **cellulose** used for the sample pad.

Material	Description	Advantages	Disadvantages
Glass fibers	Mainly CP, 100-500 μm thick, web of fibers, large pores	Good hold-up volumes, low non-specific binding	Poor tensile properties, difficult to slit and web handle
Cellulose	Mainly SP and AP, 300-1000 μm thick, compact fibers of consistent density, large pores, cheap	Very low non-specific binding, uniform, good bed volume, tolerance to chemicals present in the SP buffer	Sometimes too high hold-up volumes ($> 50 \mu\text{L}/\text{cm}^2$), weak when wet

Table 3.4 Overview of common materials used for conjugate pads (mainly glass fibers) and sample and absorbent pads (mostly cellulose).

Finally, the **assembly** of the different pads and membrane on the strip is the last step before storing. It implies :

- **Lamination** : the different materials are stuck to an adhesive plastic backing surface, forming the laminate (Figure 3.2 B).
- **Cutting** : cut the final test strip from the laminate, most commonly with guillotine cutter (Figure 3.2 B).
- **Cassette Assembly** : typical LFA test strips are placed in a plastic housing cassette to prevent material damages and end-user handling errors to facilitate result reproducibility.
- **Packaging** : packaging the cassette in a foil pouch with a desiccant for humidity control in the range of 20% RH (relative humidity).

3.8 Paper-based fluidics in LFA

Since the functionality of the LFA test depends directly on the spontaneous liquid flow through each porous materials and the test strip as a whole, it is important to understand its microfluidics behaviour and how to optimize flow within the strip. [109]

The **passive fluid transport** in paper is a spontaneous process enabled by capillary action. Capillary flow, or wicking, within the paper matrix is a result of the interplay between cohesive and adhesive forces between the liquid and the paper surface. **Cohesive forces**, such as surface tension, arise due to intermolecular attraction between fluid molecules at the liquid-air interface, while **adhesive forces** (i.e., van der Waals force) result from intermolecular attraction at the liquid-fiber interface. If the replacement of the liquid-air interface by the liquid-paper fiber interface induces energy reduction in the system, i.e. **if the adhesive forces between the liquid and the paper outweigh the cohesive intramolecular forces within the liquid**, then the liquid is said to be wetting on the paper ⁶ and a positive **capillary force** and related pressure drives the liquid movement towards remaining dry paper fibers (Figure 3.12 A). The surface properties of the paper dictate adhesion and, consequently, the degree of wetting. Physically, this adhesion is quantified by the fluid contact angle at the liquid-fiber interface and the surface tensions at the interfaces. [177] The Lucas-Washburn equation, which balances capillary force and the viscous resistance to fluid propagation (drag) is typically used to model fluid transport in paper :

$$l(t) = \sqrt{\frac{\gamma R_c \cos \theta}{2\mu}} t \quad (3.3)$$

where $l(t)$ is the distance travelled by the fluid front under capillary pressure, or wetting length, γ is the liquid-air surface tension, R_c is the effective pore radius of the paper matrix, θ is the liquid-fiber contact angle, μ is the fluid viscosity, and t is the time (Figure 3.12 A). Although some of its underlying assumption are highly simplifying, equation 3.3 enables a simple but predictive model for the fluid transport in paper strips⁷, notably highlighting that the **liquid front speed** (capillary flow rate) is **proportional to the radius of the pores** and **decays exponentially as the water front moves** along the membrane. The flow rate is thus constantly

⁶or the surface is said hydrophilic in the case of water

⁷Theory behind spontaneous capillarity in paper, alternative models taking into consideration geometry and design, and experimental validations in different relative humidity conditions were extensively studied in the frame of Odile Crahay's and Ramy Moumneh's master theses [90, 178].

changing (which makes it difficult to measure) and flow in LFAs can be described by **four flow regimes** [179]. In practice, lateral flow membranes are assessed and compared on the basis of **capillary flow time** (or wicking time), which is the time required for water to travel up and completely fill a 4-cm long strip of membrane. Capillary flow time [s/4 cm] is thus inversely related to capillary flow rate. Example of the capillary flow times for the membranes analyzed in section 3.6.2 are summarized in Table 3.5. [109, 177, 180]

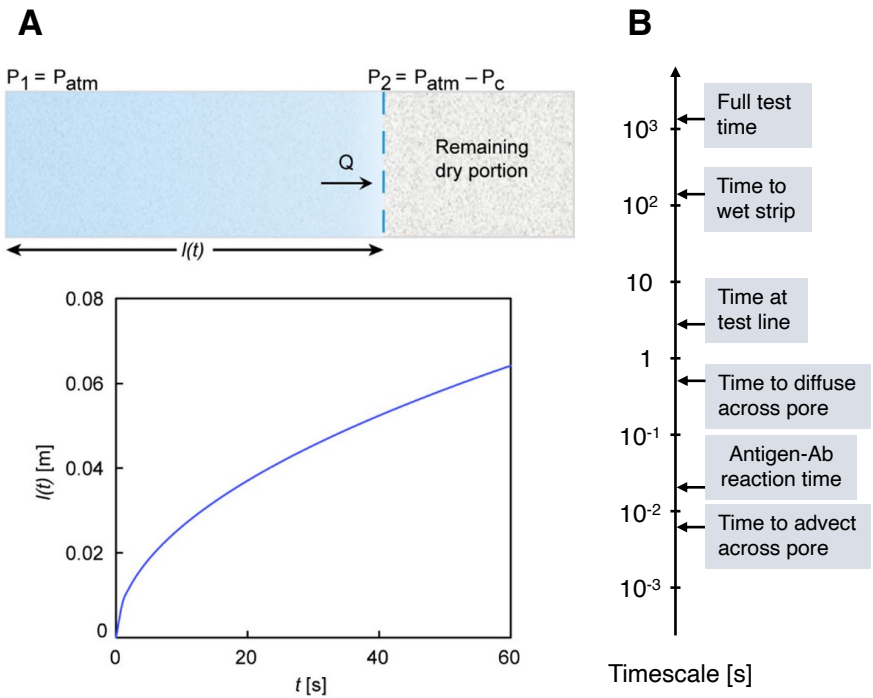


Fig. 3.12 Fluid transport in paper and time-scales in LFAs. **A.** The Lucas-Washburn equation 3.3 is used to model fluid transport in paper strips. The liquid front $l(t)$ is proportional to the wicking time, but the liquid front speed decreases over time. (P_c is the capillary pressure). Reproduced from [177]. **B.** Depiction of the various time scales within a lateral flow assay, moving top to down from the macro- to microscale. Strikingly, the advection time across the NC pores is lower than time required to established a bioreceptor-target binding. Therefore, correct positioning of the test line on the LFA is critical to have proper flow speed at TL to allow the binding. Inspired from [179].

The **capillary flow rate** is the membrane’s most critical performance

parameter in LFA as it determines how rapidly a test strip runs and how sensitive the detection system will be. Indeed, the success of LFA depends on the capabilities for analytes and bioreceptors to bind each other under a flow which aims at mixing and transporting them to the test line, making it a **dynamic system**. However, bioreceptor-target binding depends on the finite time period that the components are sufficiently close at the molecular level to bind to one another. The assay sensitivity is thus generally inversely proportional to the flow rate, which modulates the time available for reaction, i.e. the *effective concentrations* of bioreceptors and target in equation 3.1 and in turn the number of bound complex.

The exponential decay of the flow rate along the membrane also means the **test line location has an impact on the sensitivity** : the further it is placed from the the conjugate pad, the more time is allowed for the labelled detection bioreceptors and the target to interact, thus increasing the number of possible immune complexes to be captured onto the test line [104, 111]. Typically, the test line is dispensed 12–13 mm from the NC membrane origin, and the control line 4–5 mm further [181].

Unisart	CN95	CN140	CN140	CN180
Backing	yes	no	yes	yes
Thickness [μm]	240-270	120-160	225-255	225-255
Pore size [μm]	11.56 ± 6.64	7.54 ± 6.71	7.54 ± 6.71	5.06 ± 3.56
Capillary speed [s/4cm]	65-115	90-150	95-155	135-175

Table 3.5 Specifications and characteristics of the Unisart nitrocellulose membranes used in this thesis (Backing : 100 μm polyester, clear). Capillary speed and thickness from [182]. Pore sizes from section 3.6.

Finally, **mass transport** of the various components and particles in LFA is governed by **flow (advection), diffusion, and dispersion**⁸ owing to membrane porosity, but is typically flow-dominated during the first minutes of the test. **Mass transport timescales** must be taken into account (Figure 3.12 B), e.g. in multiple-steps LFA where diffusion can occur. In particular, the transport of charges in paper-based microfluidics is studied in chapter 7. In some situations, the need of a stationary flow rate for **continuous mass transport across the LFA** may lead to modifications of the absorbent pad geometry in order to increase the unwetted volume, compensating for increase of the viscous forces [183, 184]. Also, it has been shown that modifying the classical LFA architecture by increasing sample, conjugate

⁸Molecular diffusion is the process of molecular transport associated with the stochastic movement of molecules due to a concentration gradient. Mechanical dispersion is the process of mechanical mixing that takes place in porous media as a result of the movement of fluid through the pore space.

and absorbent pads width may lead to higher LFA sensitivity thanks to its higher potential of label concentration at the TL [104].

3.9 Colorimetric assay evaluation

The binding and accumulation of nanoparticles at the test line (TL) and control line (CL) generates the colorimetric signal. The appearance of signal in the TL and CL indicates the presence of the target analyte in the sample, while an only colored CL indicates its absence. The absence of visible TL and CL makes the LFA unreliable and is often indication of a malfunction of the strip, either binding affinity or microfluidics issues. Successful optical detection in LFA depends on the **signal intensity of the AuNPs accumulated in the NC membrane**. The signal intensity of accumulated AuNPs at the TL directly correlates with the target concentration in the sample. Micrographs showing the accumulation of different sizes of AuNP at the TL are presented on Figure 3.13.

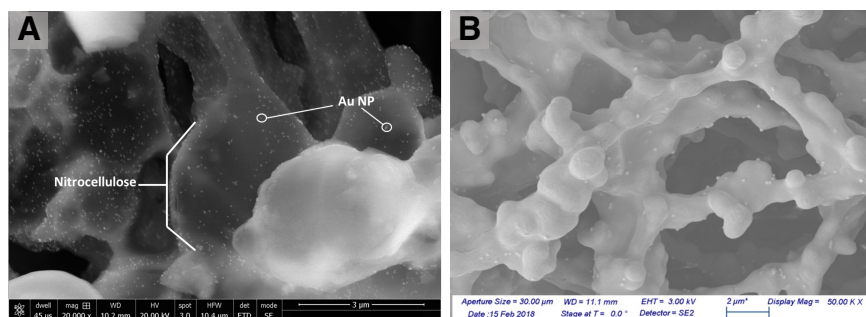


Fig. 3.13 SEM images of AuNP accumulation at the test line for two different AuNP sizes : 40-nm AuNP (left) and 150 nm gold nanoshells (white dots on the NC matrix, right). These micrographs illustrate the disparate length scales between AuNPs and nitrocellulose pore sizes. Left picture from [114], right from [185].

The binding process to form an immunosandwich at the TL is limited by target-bioreceptor binding kinetics rather than by the flow mass transport (Figure 3.12 B). The LFA microfluidics makes the target and conjugated AuNPs flow evenly throughout the entire thickness of the membrane, in order to interact with capture bioreceptors. As shown in section 4.2.4, even though capture bioreceptors may be more concentrated on the side of the membrane to which it is applied (or concentrated due to the drying process) in some situations, in most of the cases no discernible gradi-

ent of capture bioreceptors is observed in the nitrocellulose. Consequently, AuNPs are captured and distributed evenly through the thickness of the membrane. However, **due to the absorption spectra of the nitrocellulose [186], any AuNP bound deeper than $\sim 10\ \mu\text{m}$ from the surface becomes invisible to the user** (Figure 3.14), as its color is masked by the membrane's opacity, and the amount of visible signal is thus inversely proportional to membrane thickness.

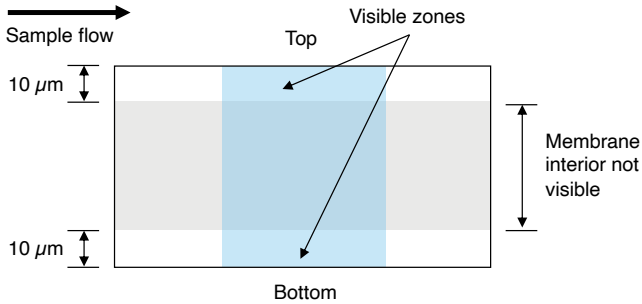


Fig. 3.14 Schematic of the test line cross-section. Due to the opaque optical properties of the nitrocellulose, only the AuNPs situated approximately in the first $10\ \mu\text{m}$ are visually perceived. Inspired from [111].

Most **LFA are read by visual inspection** after 5-30 minutes (Figure ??), in a qualitative or semi-quantitative way. However, quantitative readout data can also be captured from optical LFAs [104, 105]:

- **Qualitative detection** The presence of the TL and/or CL is qualitatively analysed by the naked eye, leading to a yes/no answer. This intuitive analysis is the reason for the popularity of the LFA, such as pregnancy tests.
- **Semi-quantitative detection** The intensity of the TL is still read by the naked eye, but is then approximately compared to a calibrated colorimetric ladder indicating the level of contamination (similar to pH or alkalinity paper-strips test for water), which is useful for the detection of a defined target concentration threshold (e.g. *E. coli* in water).
- **Quantitative detection** The development of LFA optical readers and the ubiquity of smartphones has facilitated the quantitative analysis of LFAs by image analysis. However, to achieve quantification, the fabrication process must provide highly reproducible strips to avoid batch-to-batch deviations, and an accurate calibration curve must be used as reference. The illumination is critical when reading an LFA as it could influence the analysed color intensity.

In chapters 4 and 5, we study the colorimetric detection of human IgG and whole-cells pathogens, respectively.

3.10 Summary of chapter contributions

In this chapter, we carried out an overview of the scientific basis of colorimetric LFA technology, from design to development and fabrication, following a breakdown according to its basic functions as a biosensor (substrate, microfluidics, bioreceptors, transducer and visual readout).

First, we reviewed the design process of a LFA, emphasising on the strong interdisciplinary background required (engineering, material sciences, biology and chemistry) to properly develop and optimize a lateral flow test for a specific application.

Then, for each function, we identified the principal properties of the materials or the main design parameters that will be investigated throughout the following chapters of this thesis : the lateral flow immunoassay format, functional gold nanoparticles (morphology, optical properties and number calibration), nitrocellulose substrate (morphology and pore size distribution), the different paper-like materials composing the LFA (sample, conjugate and absorbent pads), and the bioreceptors (antibodies, bacteriophage proteins) to functionalize the nitrocellulose membrane. The ability to reach the LFA performances (sensitivity and specificity) results from the co-optimisation of these multiple, interconnected parameters.

4

LFA design for quantitative human IgG detection



Chapter objectives

In order to support and exemplify the theory and methodological approaches introduced for each LFA functionality in chapter 3, here we present and discuss the step-by-step implementation in our lab of a LFA based on gold nanoparticles for the colorimetric detection of a protein (human immunoglobulin, H IgG). This model target is chosen for the ease of exploring and experimenting with the LFA development process.

This chapter presents most of the basic characterization methods for LFAs. The detailed assessment of the results of the human IgG LFA allows to identify the main limitation of colorimetric LFAs in a quantitative manner, and to propose ways to increase their sensitivity performances. The findings from this chapter will then be applied in chapter 5 to explore the development of a rapid test for pathogen detection, and chapters 6 & 7 to explore new transduction mechanisms on LFAs.

4.1 Materials and Methods

The choice of the **parameters** that guide the **LFA design** are described in Table 4.1 and on Figure 4.1:

Parameters	Description
Target	H IgG proteins. Size ~150 kDa, appropriate for sandwich immunoassay
Targeted conc.	0 - 1000 ng/mL as relevant thresholds from literature ¹
Detection biore.	Anti-human IgG (whole molecule) polyclonal antibody produced in goat
TL bioreceptors	Same as detection bioreceptor (valid because pAbs)
CL bioreceptors	Chicken anti-goat IgG (to capture the detection bioreceptor)
Label NPs	Homemade 20 nm AuNPs for colorimetric detection
Assay mem- brane	Here, the membrane choice was not optimized: the wider pores were chosen to ensure proper flow and mixing (Sartorius CN95)
SP, AP, CP	SP/AP are made of cellulose, and the CP from glass fibers. The materials used are specifically developed for rapid diagnostics applications from Millipore (SureWick, presented in section 3.7).
TL/CL location	Generic from literature to ensure proper binding time, i.e. TL located 12mm from membrane bottom, TL/CL separated by 5mm (section 3.8)
Evaluation	Qualitative and quantitative detections are performed

Table 4.1 Specifications used for the design of AuNP-based LFA for human IgG detection.

We will not describe here the whole development process from half-stick LFA to full-stick LFA that was followed during the development process (half-stick actually consists in the perform of certain process steps only). Rather, we focus on the **full-stick LFA**. The followed **LFA development process** is described in Table 4.2 :

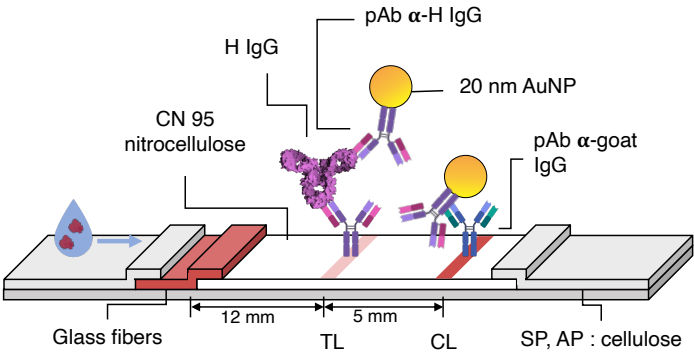


Fig. 4.1 Design specifications of an AuNP-based LFA for H IgG detection.

Process step	Description
AuNP synthesis & charac.	AuNP synthesis and characterizations (morphology and optical properties) are reported in section 3.5.2.
AuNP conjug. & optim.	As advised in section 3.5.3, a gold aggregation test (GAT) is performed in order to optimise the pH and the Abs concentration. A blocking step with BSA and no specific orientation of the Abs are considered.
TL/CL coating	Bioreceptor coating on NC & AuNP deposition on CP : use of a contact biospotter from BioDot, followed by drying. Blocking of the membranes with BSA.
Assembly	Lamination on backing card by hand.
Cutting	3-mm width, with automatic guillotine cutter.
Packaging	No housing cassette. No storage as tests were performed directly after process fabrication.
LFA operation	Drop-casting $\sim 70\mu\text{L}$ of sample solution onto the SP, dropwise. After 15min, TL and CL stabilizes. Triplicate experiments were performed.
Readout & re- sults analysis	By eye for qualitative detection. The strips were scanned with a professional scanner for the quantitative analysis. Analysis of the TL and CL intensity is performed with a software (ImageJ).

Table 4.2 Process steps and parameters for LFA H IgG fabrication and operation.

The protocols that were followed to perform the LFA development process steps, optimizations, and result analyses are reported in Appendices.

4.2

Results

4.2.1 AuNP synthesis

The synthesis of "homemade" 20 nm-size AuNP solutions was performed with the Turkevich method, as reported in section 3.5.1 (Figure 3.6 A).

4.2.2 AuNP characterizations

The **size and morphology** of the "homemade" AuNPs synthesized in this chapter were characterized by TEM (LEO 922 OMEGA Energy Filter TEM, 120 kV, and HR-TEM from ICN2, Barcelona). The black spheres indicate the AuNPs with spherical shapes (Figure 3.6 B). Different sizes in the range of 15 to 25 nm are observed. Although presenting some aggregation, the nanoparticles are overall well dispersed, and have monodispersed sizes. From TEM micrographs, image software analysis were performed to study the size distribution of the AuNPs (Figure 3.6 C), showing a mean AuNP diameter of 18.3 ± 1.7 nm. To study their **optical properties**, the UV-vis curve of the AuNP was recorded (Figure 3.6 D), showing the character-

istic shape of AuNP absorbance spectra. Three main characteristics are observed: (i) non-zero absorption towards UV wavelengths, (ii) the characteristic **absorption peak**, or plasmonic peak, at 520 nm, (iii) near-zero absorption from 580 nm to the infrared rays, which accounts for the red color of AuNPs. The height of the absorption peak at 520 nm, i.e. the absorbance at 520 nm A_{520} is commonly used to compare the different synthesized solution. The absorbance is often referred as the **optical density (OD)** of the AuNP solution, and can be used interchangeably. The width of the absorption peak is characteristic of the AuNP dispersion in the solution (partially aggregated AuNP have a wider plasmonic peak) and the color of the AuNP solution (wider peak indicates changes from red to blue, while monodispersed AuNPs maintain the original red color).

The **concentrations** of the different AuNP solutions used in this thesis ("homemade" AuNPs synthesized in this chapter, and "commercial" AuNPs used in chapter 5) were then studied. Table 4.3 refers the number density of the homemade 18 nm AuNPs found with equation 3.2. Our experimental AuNP number density, 1.04×10^{12} [AuNP/mL] was compared with the reference tables published by the BBI company. The slightly higher density of our solution could be explained by the smaller size of our AuNPs than theirs, as well as a higher optical density for ours. Our AuNP number density was also compared to the one computed by Khlebtsov *et al.* for 16 and 21 nm-sized AuNPs, which are 0.47 and 1.11×10^{12} [AuNP/mL], respectively [187]. The slight difference between our 18 nm and their 16 nm AuNP densities could result from small differences in the synthesis process conditions. All together, these results remain quite in the same range of magnitude, though.

In order to **calibrate the number density of the AuNP solutions** that we will use in chapter 5, which were provided by the Unisensor company, we recorded UV-Vis spectra for different dilutions in milliQ water of AuNPs (Figure 4.2). The **decreasing absorbance peak qualitatively correlates well with the dilution factor of the solution**. Original AuNP solution OD was referred by Unisensor to 8, and the mean diameter was referred to 40 nm. In section 5.4.3, we further characterized the AuNP size, obtaining a mean diameter of approximately 42 nm. From the nanoparticle diameter and solution absorbances, Table 4.3 reveals a **linear dependence of the computed number density of AuNPs with the solution dilution**. Again, AuNP solution from Unisensor shown lower density number than that referred by BBI, though it remains in the same order of magnitude. It could be explained by a slightly higher nanoparticle size for Unisensor AuNPs than for BBI ones.

AuNPs origin	Diameter [nm]	OD 520 nm	A ₄₅₀ 450 nm	Density from eq. 3.2 [# /mL]	Density from BBI [# /mL]
Home-made	18	1.13	0.7	1.04×10^{12}	7.00×10^{11} (20nm, OD1)
Uni., original batch	42	8	6.62*	-	9.00×10^{11} (40nm, OD10)
Uni., dil. 2x	42	4	2.03	2.14×10^{11}	4.50×10^{11} (40nm, OD5)
Uni., dil. 4x	42	2.08	1.02	1.08×10^{11}	
Uni., dil. 8x	42	1.06	0.55	5.84×10^{10}	9.00×10^{10} (40nm, OD1)
Uni., dil. 10x	42	0.89	0.47	4.93×10^{10}	
Uni., dil. 20x	42	0.44	0.25	2.61×10^{10}	

Table 4.3 Determination of AuNP concentration from absorbance UV-Vis spectra at 450 nm A_{450} on Figures 3.4 D and 4.2, and comparison with reference tables published by BBI company. A_{520} is here referred to the AuNP OD, and is commonly used for easy comparison of AuNP solutions with each other. (A : Absorbance, OD: optical density, Uni.: Unisensor, dil.: diluted, * : this A_{450} is expected to be a measurement error linked with the spectrophotometer, see Appendix 3.1 for details.)

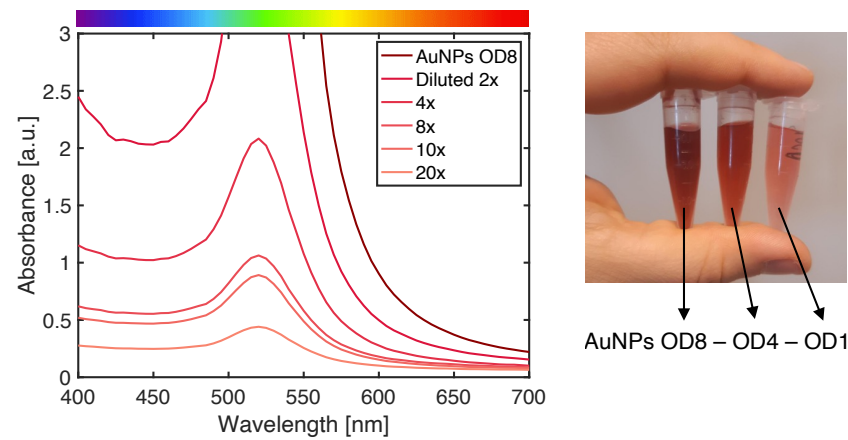


Fig. 4.2 Calibration of 40 nm AuNP number in solutions. AuNPs provided by Unisensor. UV-Vis spectra of dilutions of 40 nm AuNPs provided by Unisensor were recorded in order to determine their respective AuNP density number with respect to their OD reported in Table 4.3.

4.2.3 AuNP conjugation with anti-H IgG antibodies

The optimal pH to perform the functionalization and the minimum antibody concentration to fully cover the nanoparticles of AuNPs with the detection bioreceptor were determined via a **gold aggregation test (GAT)**. Figure 4.3 depicts the results of a gold aggregation test for 20-nm diameter AuNPs and anti-human IgG Abs.

The aggregation rate was qualitatively assessed by the naked eye. The color gradient after addition of salt to the different anti-human IgG concentrations incubated with AuNPs indicates the aggregation for AuNP coated with a too low Ab concentration. Fully aggregated AuNPs induced a color change in the suspension from red to transparent (due to their precipitation), visible in Figures 4.3 a-c (photos of the resulting GAT) for the 3 pH conditions, while properly conjugated AuNPs maintained their original red color.

The aggregation of the AuNPs was quantitatively measured using UV-visible spectra (Figures 4.3 d-f). The AuNPs that aggregated during the GAT showed a lower and wider absorbance peak shifted to the right. For stable AuNP conjugates properly covered by the Abs, the **peak position was slightly shifted to higher wavelengths (in this case, 528 nm)**. This shift is likely due to the different optoelectronic properties of the conjugates (AuNPs + antibody) compared to that of bare AuNPs. The binding of biomolecules to the gold surface changes the electron field and thereby the absorbance maximum [188, 189] compared to that of bare 20-nm-sized AuNPs (maximum absorbance peak at 520 nm).

Further analysis (Figures 4.3 g-i) indicates that **optimal conditions for the conjugation** are reached for pH 8 and anti-H IgG Abs concentration value of 100 $\mu\text{g/mL}$. The bar charts of Figures 4.3 g-i, relative to the photos in a-c and the spectra in d-f, obtained by subtracting the absorbance value at 520 nm from the one at 580 nm for each concentration (i.e. on each recorded curve). This analysis allows for estimation of the minimum antibody concentration to give stable conjugates. The higher the calculated value the narrower the peak, indicating more monodispersed AuNPs.

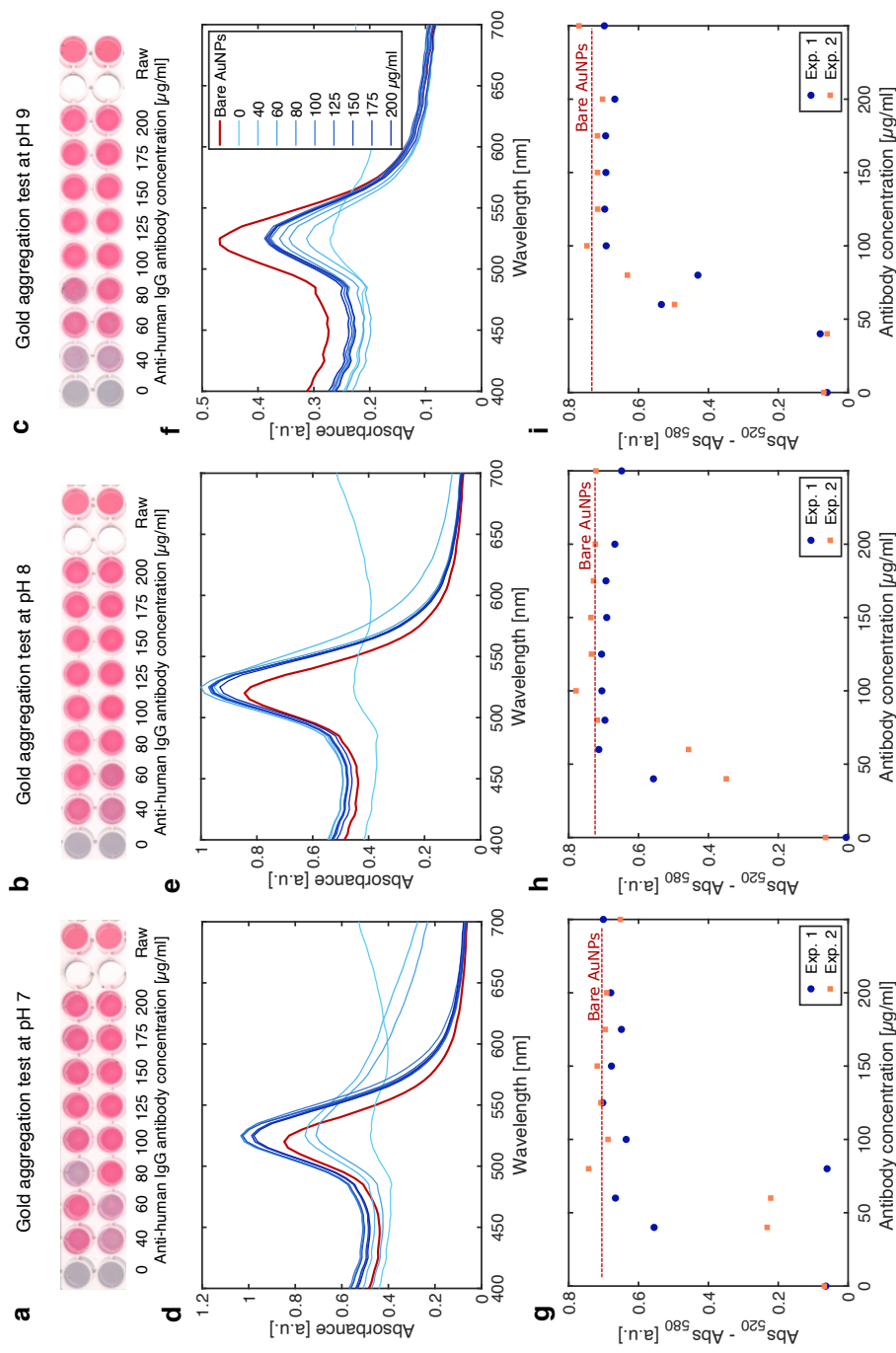


Fig. 4.3 Gold aggregation test for 20-nm AuNPs and anti-human IgG Abs. (Abs, absorbance; a.u., arbitrary units.)

4.2.4 Study of the antibody coating of NC membranes

In order to assess the proper coating of antibodies inside nitrocellulose membranes to form the test and control lines, the functionalization of unbacked nitrocellulose membranes (Unisart CN140, Sartorius, Göttingen, Germany) with antibodies was studied by epifluorescent microscopy. Top and cross-section views of the functionalized membranes were observed in order to reveal the distribution of the bioreceptors in the membrane after coating. The coating was performed by drop-casting 0.1 mg/mL GFP-tagged antibody dispersed in aqueous solution (Goat anti-Human IgG Alexa Fluor 488, ThermoFisher) to cover 1 cm² NC piece samples. After washing and drying steps, microtomy was used to produce extremely thin sections of the functionalized NC membranes (Figure C.1). Fluorescence images (Olympus IX71) of the functionalized unbacked NC top surface and cross-section were recorded.

Antibody coating of the NC membrane was found to be complete and homogeneous on the top surface area (Figure 4.4 A) and on the whole membrane thickness (Figure 4.4 B and Figure C.2), on the contrary of the study of Mujawar *et al.* where non-homogeneous distribution of the deposited antibodies was observed in Unisart membranes by CLSM, resulting in doughnut-shaped spots [190]. The automatic deposition by a non-contact inkjet-printer (~ 250 pL droplet volume) used in their study, compared to ours (drop-casting ~ 50 μ L), could explain this difference. Indeed, for a piece of NC membrane that measures 1 cm $\times$ 1 cm $\times$ 0.014 cm (i.e., 140 μ m thick) has a volume of 0.014 cm³ (14 μ L). With 80% porosity, the volume of air comprising the pores is 11.2 μ L (0.8×14 μ L). The volume of air is also referred to as the membrane's **bed volume** or void volume. The bed volume of a membrane or other porous material can be used to calculate the total volume of liquid reagent required to wet it out. In our study the antibody volume used for coating was thus enough to cover the entire NC inner area, while it was probably not the case in the study of Mujawar *et al.* where the tiny Ab solution migrated and dried in a circular fashion (the well known **coffee ring** effect in paper-microfluidics) on the NC membrane quickly after deposition.

Therefore, if one wants to functionalize the entire thickness of a NC membrane with bioreceptors, the deposited volume must be adjusted accordingly (e.g. by slowing down the biospotter speed in the case of test line printing).

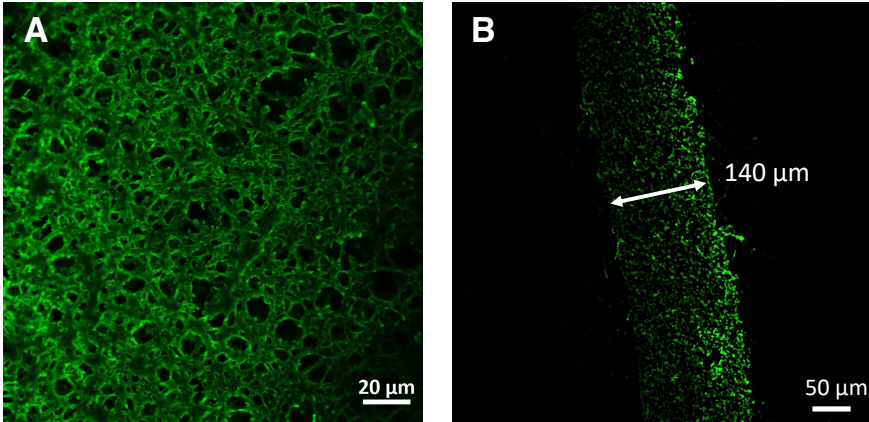


Fig. 4.4 Top and side-view of CN140 unbacked nitrocellulose membrane functionalized with GFP-tagged antibodies and observed under epifluorescent microscopy. The functionalization process, through Ab adsorption, permits an relatively homogeneous coating over the surface and membrane thickness.

4.2.5 LFA qualitative evaluation

In order to proceed to the test, 70 μL of dilutions of human IgG in PBS buffer (1, 3, 10, 30, 100, 300, 1000 ng/mL) were drop-casted on the sample pad. After 15 min, stabilization of the TL and CL intensities can be assessed at the naked-eye. The TL color intensity increases with the human IgG concentration in the sample (Figure 4.5). The presence of a CL indicates that the LFA reaction (passive migration of the sample along the strip) occurred correctly. It can be seen that the colour intensity of the CL was lower in the case of higher concentrations when compared to strips that used low target concentrations, since high target analyte concentrations (in a sandwich assay) may lead to a dramatic depletion in the concentration of AuNPs after the test line.

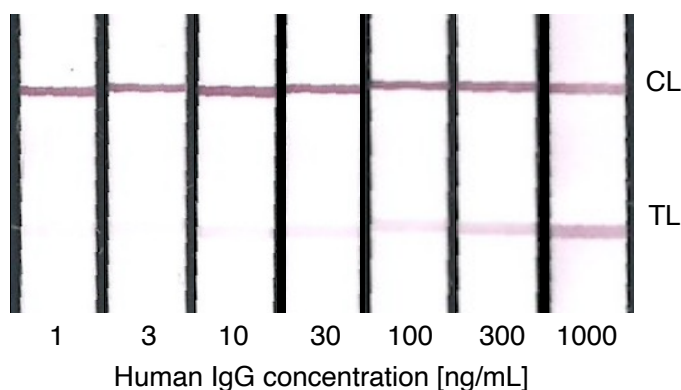


Fig. 4.5 Qualitative evaluation of H IgG detection with regards to its concentration. At the naked-eye, the TL color intensity increases with the H IgG concentration in the sample.

4.2.6 Quantitative evaluation and calibration curve

After the qualitative evaluation, the strips were scanned, the peak colour intensity (in gray scale) of the test line was calculated with an image processing software, and plotted versus the target H IgG concentrations (Figure 4.6). The peak intensity was obtained by subtracting the peak value of the test line from the background value for each LFA, and the mean and standard deviation ($n = 3$) were computed for each concentration.

Then, in order to obtain quantitative evaluation from LFAs and to determine the limits of detection (LoD) and the dynamic range of the LFA, we performed a data curve fitting to generate a **calibration curve** for H

IgG detection between the signal intensities and the human IgG concentrations. For assays that employ protein-protein binding interactions or study protein binding kinetics, a **sigmoidal relationship** is commonly observed as shape of the binding curve. These curves are effectively described by a **four-parameter logistic curve** (a sigmoidal curve) representative of the Hill equation [104, 7, 191]:

$$y = \text{start} + (\text{end} - \text{start}) \times \frac{x^n}{x^n + k^n} \quad (4.1)$$

with y the signal intensity from the TL (i.e. fraction of target-binding sites of the test line antibodies occupied), x the ligand concentration (i.e. human IgG concentration in the sample), k corresponding to K_D , and with the *start* and *end* values corresponding to the non-responsive and saturated signals, respectively.

The **Hill model** reflects the underlying binding kinetics of cooperative ligand binding to multiple-subunits proteins, which is representative of our system since antibodies possess two epitopes 3.4. Hill model and its match with a four-parameter logistic curve is further described in Appendices 3.3.

From the fit, we extracted the value of the affinity constant $K_D = 148 \pm 38$ [ng/mL] for the capture bioreceptor-human IgG interaction. In molar terms, it translates to $0.98 \text{ nM} \approx 10^{-9} \text{ [M]}$ (Appendices 3.3.1), which lies well within the range of affinity constants that are reported in the literature for IgG protein interactions [192]. The fitted value of K_D confirm us that the binding affinity of the bioreceptor is not affected by its attachment in the nitrocellulose membrane. This supports the use of this antibody as bioreceptor in the LFA format for H IgG detection. Also, the degree of cooperation n fitted from the calibration curve ($n = 1.56 \pm 0.35$) confirms that the binding of one molecule of IgG facilitates the binding of the others (the measured n will always be less than the actual number of bioreceptor-target binding sites), see Appendices C for details.

Then, we calculate the **limit of detection (LoD)**, equal to the mean $\mu_{\text{blank}} + 3 \times \sigma_{\text{blank}}$ its standard deviation (blank not shown on Figure 4.6), which corresponds to $\sim 28 \text{ ng/mL}$. The **dynamic range (DR)** of our LFA test is from ~ 34 to 400 ng/mL (computed as between 10% and 90% of the maximum signal, therefore expressed in *normalized signal change*). Our LoD and DR lie in the same order of magnitude as reported by [156], but are slightly higher than another study [104].

This latest results tends to indicate that our LFA could still be optimized

in order to reach a lower limit of detection. In fact, for analytes of this size, such as many protein markers, the LoD of published LFA could reach ~ 1 ng/mL or slightly lower concentrations (see [187, 104, 156, 176] among others).

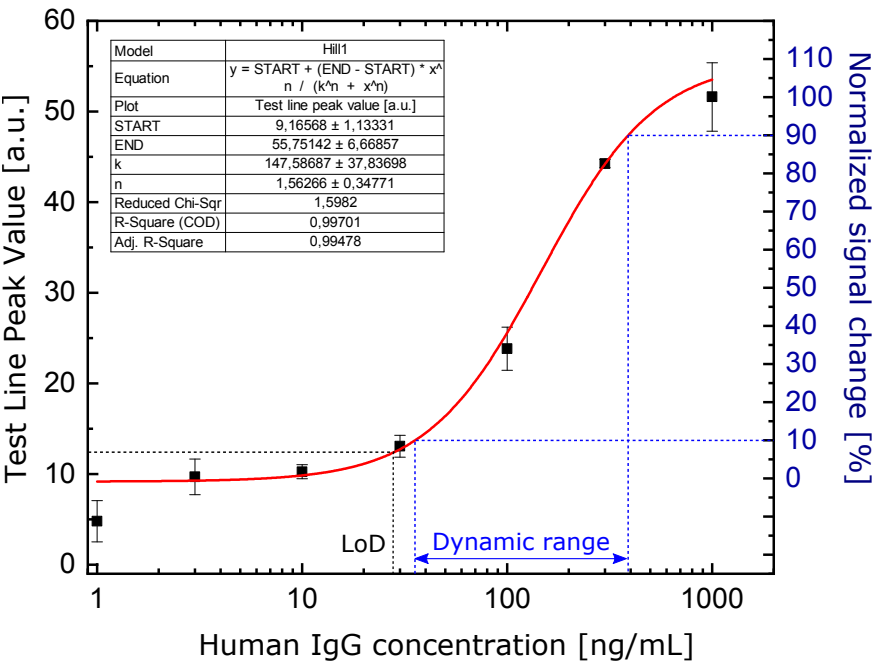


Fig. 4.6 Calibration curve for the quantitative detection of H IgG. Black dots are mean value $\pm$ standard deviation (n=3).

One reason for our higher LoD and DR with respect to other works could be the fact that, in order for a four-parameter logistic curve to reach best data fit, we need to use a range of target concentrations that generates both non-responsive and saturated signals. We could therefore extend the range to lower and concentrations to reach a better match (and expecting a shift of the calibration curve towards lower concentrations). However, our blank value 6.45 ± 2.26 [a.u.] already indicates that we are close to non-responsive signals.

4.2.7 Sensor response calibration in terms of AuNP number

For various reasons, it is **interesting to quantify the number of nanoparticles that are bound to the TL, and how they correlate to the sensor con-**

centration thresholds identified in the previous section. It can serve to better calibrate the number of AuNPs that are conjugated and deposited in the conjugate pad in order to properly adjust the sensor sensitivity. Estimation of relationships between the number of analyte molecules and the number of AuNPs needed to reach low detection levels is also relevant in order to design experimental strategies to improve the LFA sensitivity. Finally, it is also particularly interesting to know the typical number of AuNP bound at the TL when considering other transduction principles of the AuNP, such as magnetism or electro-chemical detections, for simulation purposes or prospective comparison of the sensor sensitivities.

Estimation of AuNP number bound at the test line considering ideal interactions In Appendices 3.3.2, we computed that **each LFA strip contains about 6.25×10^{10} AuNPs** available for binding to the target. Then, we computed the number of H IgG molecules that are injected in each LFA, depending on the sample concentration in H IgG. When compared to the H IgG concentrations of interest (Table 4.4), we see that at the top part of the sensor DR, close to the saturation, the number of H IgG molecules exceeds the number of AuNP in the sample, which in turn results in the sensor saturation. On the opposite, at the bottom of the calibration curve, we observe that the number of H IgG in the LFA reaches $\sim 10\%$ of the total AuNP number.

H IgG [ng/mL]	H IgG [#LFA]	Max number of AuNP bound at TL
1000 (satur.)	2.81×10^{11}	6.25×10^{10}
400 (high DR)	1.12×10^{11}	6.25×10^{10}
34 (low DR)	9.56×10^9	9.56×10^9
28 (LoD)	7.9×10^9	7.9×10^9
1 (non-respons.)	2.81×10^8	2.81×10^8

Table 4.4 Sensor response calibration in terms of AuNP number by comparison of the H IgG molecule numbers in the sample used to the AuNP number in each LFA strip. Ideal interactions considered. Number of H IgG molecule in the sample and the number of AuNP/strip are computed in Appendices 3.3.2. (satur. : saturation, non-respons.: non-responsive)

To take conclusions about the number of AuNP bound at the TL depending on the H IgG concentration, **we need to hypothesise the number of H IgG that bound to a AuNP, and then their fraction that successfully bound to the TL.** The maximal theoretical sensitivity, or the minimal theoretical LOD, of an ideal colorimetric LFA can be evaluated **assuming that one analyte molecule corresponds to one AuNP captured in the test**

zone. Using this assumption, we can only express the maximum possible number of AuNP bound at the TL that are contributing to the signal as an ideal, upper limit. It is unlikely for two AuNPs to bind to a single H IgG molecule regarding their size difference, as here the target and the antibodies are in the same nm-size range (Figure 3.1). In addition, in our system, these IgG molecule would anyway not contribute to the signal since they would have no more free epitope to bind at the TL.

If we suppose that each H IgG molecule binds to one nanoparticle, **the highest signal intensity** would arise if all the AuNP would bind the H IgG in the assay and then at the TL, i.e. **about 6.25×10^{10} AuNPs**. In this case, this is limited by the AuNP number in the solution. On the contrary, in the ideal situation, **the lowest number of AuNP that we are able to detect with our colorimetric sensor (i.e. the detection limit) is therefore at most about 7.9×10^9 AuNPs**.

Taking into account non-idealities of real systems Considering the imperfect mixing between the H IgG and AuNPs, it turns out that many of the AuNP are actually not attached to the H IgG during the LFA assay, even when they are not in excess in the system.

At **high H IgG concentrations**, e.g. 400-1000 [ng/mL], the observation of a CL indicates that some free AuNPs are remaining in the sample and not bound to H IgG or captured at TL. **The actual number of AuNPs contributing to the TL signal is thus lower than 6.25×10^{10} AuNPs**. However, a clue that the number of AuNPs that are bound to H IgG is quite substantial at these concentrations comes from the fact that the intensity of the CL starts to decrease seriously, see Figure 4.5). Indeed, the significant depletion in the unbound AuNPs that can travel across the TL to reach the CL suggests that a large fraction is bound at the TL.

At **lower H IgG concentrations**, where the number of AuNPs is in excess compared to the number of H IgG molecules, **the fraction of AuNP bound to the TL is highly dependent upon H IgG binding at the test line** and their interactions, which mainly drives the AuNP accumulation at the TL. However, in an LFA experiment, not all analyte molecules interact with the AuNP-antibody conjugate, and some of them pass through the membrane in a free form, therefore reducing the number of possibly accumulated AuNPs. Even though it is very unlikely that two AuNPs can bind to a single H IgG analyte, the opposite effect would in fact tend to occur when using antibodies with a high affinity for the analyte : a single AuNP can bind several or even hundreds of analyte molecules. Evidently,

such an unwanted effect would decrease the assay sensitivity by reducing the total number of AuNP accumulated at the TL for a given H IgG in the sample.

Proper estimations of the number of AuNPs bond at the TL should also **consider the non-specific interactions** that can happen between AuNPs not bound to the IgG and the TL bioreceptors, although the blocking of our LFA NC membrane and our AuNP conjugates with BSA should significantly lower the non-specific interations. In addition, the AuNP conjugation tends to create some small-size aggregates of a few number of AuNP (see observation made in section 5.4.3), such that one label attached to one H IgG molecule could actually consist in several AuNPs.

Quantitatively accounting for non-idealities in this exercise is challenging. However, based on the previously stated ideal estimation, we can hypothesize that **the maximum number of captured AuNPs at the TL at the limit of detection (LOD) for H IgG is about 10^9 AuNPs.**

Going further: refining the estimation Going into more details, we can **quantify the AuNP number density per surface area of the TL**. For a TL of approximately 0.5-1 mm-width (typical width of the TL, arising from the spreading of the capture bioreceptor solution after its deposition on the NC membrane with the biospotter), and 3-mm size (width of the strip), our range of AuNP number density lies between $\sim 1 \times 10^8$ and $\sim 2 \times 10^{10}$ [AuNP/mm² TL]. This is ~ 10 - $100 \times$ higher than the AuNP number density reported by Klhebtsov *et al.* for AuNP of similar size ($\sim 1 \times 10^7$ to $\sim 2 \times 10^8$ [AuNP/mm² TL]), however obtained with smaller volumes (~ 10 - $20 \times$) [187]. Nevertheless, their results support our discussion about the number of AuNP bound to the TL : for different protein/antibody couples, they computed that in practice, around the LoD, an order of magnitude of $\sim 10^2$ target molecules was needed to deliver one AuNP at the TL. This confirm that the *Max number of AuNP bound at TL* reported in Table 4.4 are indeed highly idealized, upper limits for AuNP concentration at TL, and that the reality is probably minimum 10-100 times lower because of the non-idealities of such a complex nano-biological system.

It follows that **we can refine our first hypothesis** such that **the LoD of the colorimetric human IgG LFA expressed in terms of AuNP number captured at the TL probably lies in the range of maximum 10^7 - 10^8 AuNP bound at the TL**. Since lower LoDs of LFA for protein detection have been reported (see above), the lower bound could probably be as low as 10^6 AuNPs accumulated at the TL.

Strategies to increase AuNP-based LFA sensitivity

In view of the non-idealities discussed in this section, in order **to decrease the LoD** of AuNP-based LFA, **two main options** are identified to **increase the sensitivity of the sensor at low H IgG concentrations**, i.e. either

1. Increase the number or the contrast of AuNPs that bind at the TL for low human IgG concentrations, or
2. Implement a transducer that is more sensitive than image analysis to the number AuNPs at the TL at the LoD concentration.

Regarding the **first option**, a possible solution is to preincubate the analyte with conjugated AuNPs before the LFA is run in order to decrease the probability of unbound AuNP and IgG analyte in the flow, or include an **additional run of the conjugates** through the membrane after a conventional LFIA has been finished [187]. However, this would add unwanted process step execution. Similarly, Panferov *et al.* recently proposed to use dielectrophoretic assembly of antibody-antigen complexes in the LFA instead of relying only on passive capillarity, which shows a 10-fold improvement in the LoD of H IgG as compared to conventional LFA as presented in this chapter, reaching a LoD of 0.6 ng/mL [193].

Also, in order to avoid situations where a single AuNP binds many of the H IgG, which reduces the assay sensitivity, we could imagine **low antibody loading on the AuNP surface**. This way, it would increase the number of AuNP bound to H IgG. Of course, reducing the Ab coating of the AuNP might lead to less probable binding to H IgG in the LFA flow, such that it would be necessary to optimise the coating with respect to the TL intensity [104]. It would also require a greater care of AuNP blocking to avoid non-specific interactions.

Finally, **increasing the diameter d of the nanoparticle** (e.g. up to 60 or 100 nm) would allow to increase the signal per AuNP and thus the color density generated by the TL via an increased surface nanoparticle density [194]. Therefore the LOD decreases, and scales like $\sim d^{-3.1}$. However, scaling of the AuNP also increases the degree of receptor interaction, i.e. the AuNP will capture more analytes (which scales like $\sim d^2$, decreasing the sensitivity, leading to a global rule of LoD for colorimetric LFA in $\sim d^{-1}$. This latter capture effect may be avoided with less antibody coating on the AuNP. [187]

Regarding the **second option**, it could be of interest to integrate **external transducers more sensitive to the AuNP presence at the TL**, for example by being sensitive to the AuNP that are accumulated deeper than 10 μm in the NC at the TL (Figure 3.14), and thus non-visible at the naked eye or with conventional camera [195, 149].

4.3 Summary of chapter contributions

In this chapter, we exemplified the design, fabrication and optimization of a colorimetric LFA based on gold nanoparticles for the detection of a simple model protein, human IgG, yet relevant because of the ubiquitous detection of proteins and *relatively* small sized analytes in LFA diagnostics.

The statistical analysis carried out for the colorimetric detection of H IgG led us to derive the limit of detection and dynamic ranges of the LFA, which shows a LoD of around 28 ng/mL and a dynamic range that encompass 40-400 ng/mL. This is still 1 order of magnitude higher than other LFAs published in the literature, suggesting optimization of our LFA is still possible.

In addition, we also converted this LoD in terms of the number of AuNP accumulated at the TL. The derivation of this limit of detection in terms of the number of AuNP is of fundamental importance in the understanding of the LFA limitations : the lowest number of detectable analytes that is visible with a colorimetric LFA is limited by the possibility of accumulated AuNP to be visible in the NC, which depends primarily on the binding mechanism between the target and the labels, and the AuNP properties, such as its size.

For 20 nm AuNP, it is therefore seen that the lowest number of detectable AuNP accumulated at the TL is about $10^6 - 10^8$ at most. Because of the unidealities in analyte-AuNP binding in the complex environment of an LFA, one AuNP will never be attached to only 1 molecule to be detected. Instead, experiment showed that tens to hundreds of molecules are actually required to bind one AuNP, such that **the lowest number of molecule that can be detected in LFA sandwich immunoassay format is in the range of 10^8 - 10^{10} molecules, i.e. approximately in the ng/mL concentration range for proteins of the size of human IgG.**

Because many bioreceptor-target systems used in sandwich immunoassay behave just as protein-ligand interactions (e.g. IgG detection with antibodies), it means that **this range of LoD could be extended to many systems that target such kind of analyte^a.** This is

confirmed by the reviews on the topic [195]. However, in more complex biological systems however, such as bacterial cell immunodetection, this rule is probably not valid anymore since the interactions are of a different nature and the scale that are at play are completely different (a bacterial cell sizes in the μm range, i.e. 10^2 - 10^3 times larger than a protein).

^aThis is valid for AuNP-based sandwich immunoassay. The LoD of competitive AuNP-based LFAs, or other optical LFAs using fluorescent quantum dots or other nanoparticles, could significantly vary.

5

Phage-protein based LFA for *Bacillus cereus* detection

Chapter objectives

In chapters 3 and 4, the LFA principles, advantages and major limitations for the colorimetric detection of small sized analytes (proteins) were studied. In this chapter, we explore how these findings could be applied to the detection of pathogens, a much more complex biological system, with colorimetric LFAs. A complete step-by-step development of colorimetric LFAs for the detection of *Bacillus* pathogens is proposed, from the selection and characterization of new, innovative, bioreceptors (phage proteins) to the implementation of the detection of *Bacillus* cells in simplified LFAs devices.

A manuscript gathering the collaborative research conducted in this chapter is in preparation : G. Le Brun, M. Nuytten, A. Leprince, M.-C. Eloy, D. Magnin, K. Glinel, A. Gillis, J. Mahillon, J.-P. Raskin. Engineering bacteriophage-binding proteins for highly specific detection of *Bacillus* cells with gold-nanoparticle lateral flow assay, *in preparation*. Audrey Leprince and Manon Nuytten, from the Laboratory of Food and Environmental Microbiology (Earth and Life Institute, UCLouvain), identified, produced and characterized the phage proteins, and performed the bioinformatic analyses.

5.1 Introduction and motivation

The detection and identification of bacterial pathogens in food and the environment have become of primary importance for health and ecosystem safety reasons [2]. The *Bacillus cereus* group includes several species known for their pathogenic potential including the causative agent of anthrax: *B. anthracis*. Also, some *B. cereus* strains are commonly recognized as food poisoning agents representing a serious threat in the food safety field as their toxins were the most frequently reported cause of food poisoning outbreaks in 2020 at the EU level (EFSA & ECDC microbiol.spec.GPP3-0032-20). Conventional, standardized detection methods for *B. cereus* in food and environmental samples, such as agar plate-based counting (ISO 7932), require long incubation periods (up to 72h for the whole detection process) and costly laboratory settings [196]. Since more than two decades, the emergence of nanobiotechnologies, molecular chemistry processes and functional nanomaterials have enabled the development of point-of-care diagnostic biosensors to address these drawbacks, including LFAs.

As introduced in section 3.4, antibodies are still the gold standard bioreceptors for LFAs. Their limitations (variability in specificity and stability, high prices due to dependency and high pressure on the suppliers, unethical production process) especially highlighted during the COVID-19 pandemics, call for the urgent need to find alternatives to the Abs when it comes to the development of new diagnostics tests.

In this chapter, we propose to replace antibodies with the specific binding proteins used by the bacteriophages for their adsorption on the surface of bacterial cells. These affinity proteins can be engineered (customized from their genetic sequences) and produced recombinantly, allowing for a thorough investigation of the suitability of the engineered proteins as bioreceptors for diagnostic purposes using LFAs. Their microstructural characterization and engineering has been largely facilitated by the emergence of accurate bioinformatics tools including AlphaFold2, the DeepMind's machine-learning protein structure prediction program that has largely revolutionized structural biology [197].

In LFA, the most common immobilization method has always been direct physical adsorption of the affinity bioreceptors to the nitrocellulose (NC) assay membrane and strong adsorption to gold nanoparticles (AuNP) detection labels, due to the suitability of antibodies (advantageous surface charge and cysteine residues). However, the phage protein binding prop-

erties were not assessed in detail to form LFA biointerfaces, or under LFA working conditions. This lack of experimental data and understanding of interactions occurring at the interface level reduces the ease of design of phage protein-based LFAs, such that developers still uses phage proteins as "black boxes" in biointerfaces. Since the performance of LFAs critically depends on the immobilization of its bioreceptors to the NC membrane and the AuNPs [175], studies drawn attention towards the importance of comprehensive characterisation of bioreceptors both before and during their implementation into LFAs to understand whether the selected bioreceptors exhibit the required binding properties (stability, strong binding affinity and fast binding kinetics). [191, 176].

The aim of this work is to **investigate and validate the use of phage proteins as credible alternatives to antibodies for the capture and detection of whole-cell *Bacillus* in LFA formats.**

5.2 Concept

In this work, we used two recently identified phage binding proteins as specific bioreceptors for LFAs: the cell wall binding domain (CBD) of an endolysin (PlyB221) and an evolved distal tail (EvoDit-Gp28), encoded respectively by Deep-Blue [148] and Deep-Purple [141] bacteriophages targeting *B. cereus* group. The phage proteins were selected based on their host spectrum, their size and their binding efficiency on the bacterial cells. Keeping in mind the specificities of the LFA settings, PlyB221 and EvoDit-Gp28 were implemented into LFA capture and detection biointerfaces, respectively. To assess their suitability as bioreceptors in LFAs, the need for a thorough assessment of their binding properties motivated the use of a panel of computational and experimental nanobiotechnology characterization techniques, both at the microstructure and the biointerface levels. A step-by-step approach was followed (Figure 5.1) :

1. First, activity and microstructural computational analysis of PlyB221 and EvoDit-Gp28 were performed to predict the protein molecular structures and their binding orientation on nitrocellulose and gold nanoparticles, respectively (Figure 5.1 a).
2. Then, we developed protocols for the optimal implementation of the capture and detection biointerfaces. Their implementation was complemented by detailed characterizations. Immobilization of PlyB221 on the NC membrane was assessed by fluorescence microscopy (CLSM) in order to confirm preservation of binding functionalities through

the specific capture of *Bacillus* cells (Figure 5.1 b). The proper conjugation of EvoDit-Gp28 to AuNPs, and the conjugate stability was evaluated with UV-vis, DLS and TEM. Binding functionality and specificity of the protein-AuNP conjugates onto *Bacillus* whole-cells in controlled buffers was also quantified by SEM (Figure 5.1 b).

3. Finally, we demonstrate the concept of direct and specific detection of bacterial cells in controlled buffer in a simplified LFA format. A half-stick assay based on gold nanoparticles for the colorimetric *B. cereus* detection was used to assess the specificity of the protein biointerfaces and to confirm their compatibility with the conditions encountered in a LFA (Figure 5.1 c).

In particular, we designed the phage-protein biointerfaces in order to maintain the ease of use of the LFA, i.e. one of its bigger requirement to reach practical uses. Indeed, Kong *et al.* already set up a prototype lateral flow device based on the use of a single phage binding protein, an engineered CBD of endolysins encoded by bacteriophage targeting *B. cereus*. Although their research is promising as they reached functionality of the CBD-based LFA, the quality of the biointerfaces made of the CBD were poorly assessed (based on hypotheses only) and the use of a single phage-protein did not allow them to develop a convenient LFA : operating the device involves two separated steps and the replacement of the absorbent pad (AP) in a vertical scheme, thus preventing a user-friendly operating. Since not all the functionalities of Ab-based LFAs were substituted by their work, further research was needed before claiming achievement of a fully functional LFA based on phage proteins.

To the best of our knowledge, our approach of assessing and combining multiple recombinant phage proteins as specific bioreceptors in AuNP and NC-based LFAs have not been designed and demonstrated, particularly for the phage distal tail (Dit) proteins which have never been used as LFA bioreceptor. Our prototype lateral flow assay demonstrated the utility of using two recombinant phage proteins to keep a user-friendly LFA format suitable for the point-of-care, with minimal sample preparation and operations steps, thus overcoming the limitations of reported phage protein-based LFAs. The approach described herein and our results are therefore expected to expand the use of bacteriophage proteins for advantageous biointerfaces in LFAs and paper-based diagnostics towards the specific detection of other bacterial cells.

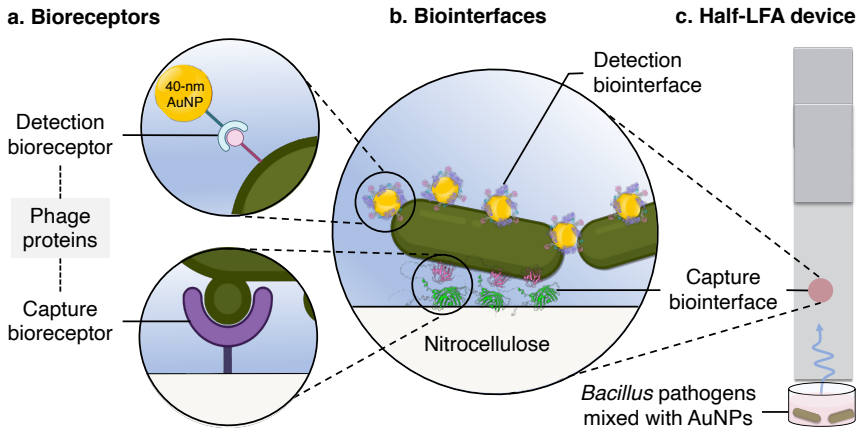


Fig. 5.1 Schematic of the chapter contributions. This study proposes the integration of two phage binding proteins as bioreceptors for AuNP-based LFA, and investigates their binding properties at the **a.** molecular-, **b.** biointerface-, and **c.** device-level.

5.3 Material and Methods

All the materials and methods are in the Appendices D.

5.4 Results

5.4.1 Phage proteins displaying binding properties

Investigation of engineered PlyB221 properties as capture receptor

PlyB221 is the endolysin encoded by the *B. cereus* Deep-Blue phage (*Herelleviridae* family). Its peptidoglycan-degrading activity, involved in phage-mediated lysis of the bacterial host at the end of the phage replication cycle, has been confirmed and characterized in a previous work [148]. PlyB221 was shown to display a broad activity spectrum (see Appendices Figure D.2), targeting all the tested strains of the *B. cereus* group. PlyB221 turned out to be active in a pH range from 6 to 9 and to withstand up to 200 mM of NaCl without loss of activity. The binding activity of its Cell Wall Binding Domain (CBD), the domain of the protein responsible of its specificity, was also confirmed through Cell Wall Decoration Assay (CWDA), fusing a

green fluorescent protein (GFP)-tagged CBD adsorbed onto the bacteria.

Further *in silico* analysis, including protein structure prediction using the machine learning method AlphaFold2 were performed (Figure 5.2). PlyB221 CBD consists of two tandem SH3 domains (Figure 5.2 A-C). The two tandem SH3 domains of PlyB221 CBD exhibit 76% amino acid sequence identity, and the predicted structure of the PlyB221 SH3 domains is very similar to that of another endolysin encoded by a phage targeting *B. cereus* (Figure 5.2 D). The CBD binds to the peptidoglycan (PG) at the bacterial cell surface via the tandem SH3 domains conferring the host specificity to the endolysin [198]. The whole structure of PlyB221 was predicted by AlphaFold2 with high confidence (Figure D.3).

In this work, the **capture agent used in the test line of the LFA is 6His::GFP::PlyB221 CBD** (named PlyB221 hereafter; Molecular weight (MW): 20,5kDa; isoelectric point (pI): 5,79), which is basically **the CBD of PlyB221, N-terminally fused to a fluorescent GFP, and displaying a hexa-histidine tag** used for purification of the recombinant protein by affinity techniques. The protein adsorption on NC membrane depends on various physiochemical interactions between the two partners including electrostatic attraction, van der Waals interactions, hydrophobic interactions and hydrogen bonding (section 3.6.3) [199].

Figure 5.2 E highlights the **positive charges of the protein purification tag** (displaying a hexa-histidine tag) that **are likely to interact with the strong dipole (-) of the nitrate group of the NC membrane (electrostatic affinity)**. Indeed, the purification tag contains *ca.* 2 times more positive residues than the GFP and *ca.* 3 times more positive residues than the CBD. Notably, at the pH used in the LFA, the side chains of Arginine and Lysine are predicted to be positively charged. Figure 5.2 F shows a uniform distribution of hydrophobic and hydrophilic residues between domains which is therefore not likely to play a role in preferential binding of the protein to the NC (*ca.* 85% of residues are hydrophobic or hydrophilic residues in each domain).

Surface EvoDit-Gp28 cysteine residue binds to AuNP

The *B. cereus* Deep-Purple phage (*Siphoviridae* family) encodes an EvoDit-like (evolved distal tail) protein termed EvoDit-Gp28 [141]. This protein turned out to bind to numerous *B. cereus* strains. EvoDit proteins are documented as accessory proteins, assisting phage adsorption (phage binding to bacteria followed by viral DNA injection into the host). Indeed, the reversible binding of EvoDit proteins are thought to allow the phage to be in close proximity to the bacterial surface to facilitate the search for the spe-

cific receptors that will be irreversibly linked by the phage receptor binding protein (RBP).

Further bioinformatic analyses of EvoDit-Gp28 confirmed the presence of a Carbohydrate Binding domain (CBM), comprising long stretches and folds, which binds the bacterial cell surface (Figure 5.3 A-C). The predicted structure of the EvoDit-Gp28 CBM fold is very similar to the CBM2 fold of *Lactobacillus casei* phage J-1 (Figure 5.3 D) [200]. The whole structure of EvoDit-Gp28 was predicted by AlphaFold2 with high confidence (Figure D.4).

In this work, the **AuNP labels were conjugated with the 6His::EvoDit-Gp28 protein**, named **EvoDit-Gp28** hereafter. As introduced in section 3.5.3, the orientation adopted by proteins on AuNP surfaces determines its activity and interactions with bacteria or other living organisms. The adsorption of proteins to AuNP is a three-step non-covalent process based on a combination of three separate but dependent phenomena: (a) ionic interaction between the negatively charged nanoparticle and the positively charged sites on the protein; (b) hydrophobic attraction between the protein and the metal surface; (c) dative binding between the metal and protein cysteine residues in proteins, as it was shown that cysteine has the highest affinity towards AuNP and can irreversibly oxidize on the AuNP surface [166, 167].

As shown in Figure 5.3 E-F, the 6His purification tag of EvoDit-Gp28 is predicted to be the part of the protein harbouring the most positive charges (bottom-right part in Figure 5.3 E) whereas the galectin region contains the most hydrophobic residues. Figure 5.3 G highlights the three cysteine residues comprised in EvoDit-Gp28. Recently, Xu et al. (2022) have developed a model enabling high-throughput predictions of protein behaviour on gold nanoparticles considering both residues affinity scale and surface accessibility [201]. This algorithm was applied to EvoDit-Gp28 and **predicted an attachment of the protein to the AuNPs by the Cys residue of the 6His purification tag**, located away from the bacteria binding surface of the CBM, therefore leaving its access sterically unaffected (Figure 5.3 H).

The binding of the proteins used in this work (PlyB221 and EvoDit-Gp28) to overnight *B. cereus* culture was confirmed through cell wall decoration assays (Figures 5.4 A and 5.5 A). To further assess the specificity of the binding events observed between the proteins and *B. cereus* cultures, controls were performed by cell wall decoration assays with *E. coli* ATCC 8739 strains (insensitive to the parental phages), showing no binding to

the bacterial surfaces (Figures 5.4 B and 5.5 B). Finally, the purity of the recombinant proteins was evaluated by SDS-Page (Figure D.5).

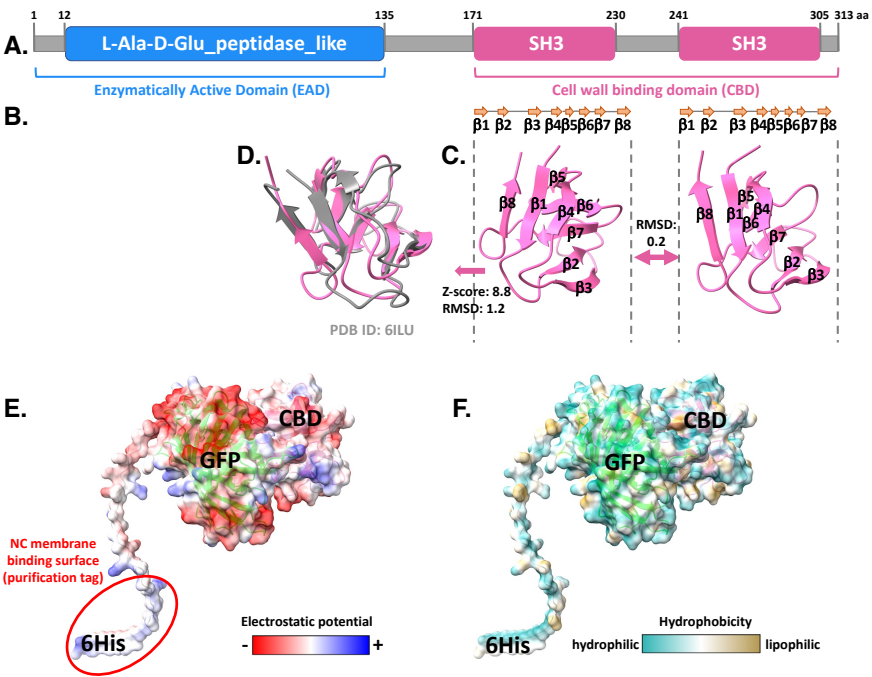


Fig. 5.2 *In silico* analysis of PlyB221. **A.** PlyB221 two domain organization. The enzymatic active domain (EAD) domain is responsible for the bacterial cell lysis, while the CBD domain (made of two SH3 domains) is responsible for the adsorption on the cell surface. **B.** Predicted PlyB221 CBD secondary structure. **C.** Ribbon representations of SH3 domains predicted structures. The domain boundaries predicted by AlphaFold2 are indicated with dotted lines. **D.** Superposition of PlyB221 SH3 (pink) and LysPBC5 SH3 (grey) domains. LysPBC5 belongs to another bacteriophage targeting *B. cereus*). **E.** Electrostatic surface potential distribution of the PlyB221 CBD protein used as capture bioreceptor in our study. PlyB221 is fused to a GFP and a His-tag for the protein purification. Blue and red indicate positive and negative charge potential, respectively. **F.** Hydrophobicity coloring. Turquoise indicates hydrophilic residues and beige lipophilic residues. Figures were generated with ChimeraX [Pettersen et al., 2021].

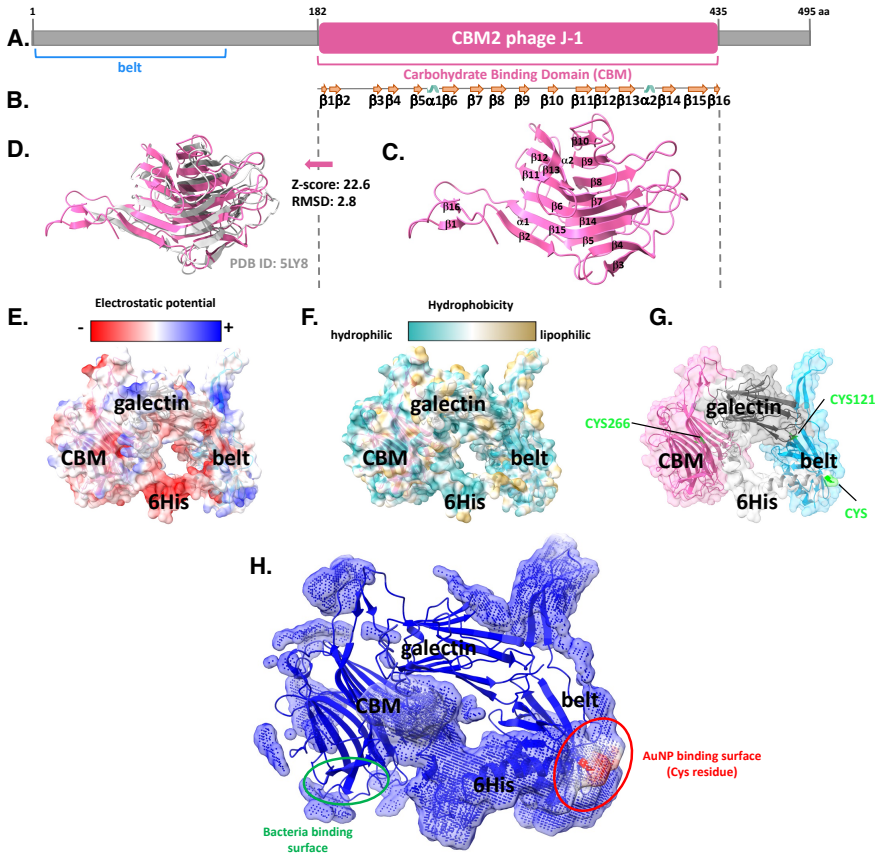


Fig. 5.3 *In silico* analysis of EvoDit-Gp28. **A.** EvoDit-Gp28 domain organization. **B.** Predicted EvoDit-Gp28 CBM secondary structure. **C.** Ribbon representations of CBM domain predicted structures. The domain boundaries predicted by AlphaFold2 are indicated with dotted lines. **D.** Superposition of EvoDit-Gp28 CBM (pink) and J-1 EvoDit CBM2 (grey) domains. **E.** Electrostatic surface potential distribution of 6His::EvoDit-Gp28. Blue and red indicate positive and negative charge potential, respectively. **F.** 6His::EvoDit-Gp28 hydrophobicity coloring. Turquoise indicates hydrophilic residues and beige lipophilic residues. **G.** Surface and ribbon representations of 6His::EvoDit-Gp28 with cysteine residues highlighted in green. **H.** Ribbon representation of the prediction of preferred bound surface of 6His::EvoDit-Gp28 on AuNP taking both the affinity scale and the surface accessibility into account using blue to red scale (red highlighting the most preferred bound surface) [201]. The accessible area of the protein calculation with NACCESS stand-alone program is depicted by surface representation (Ding et al., 2006). Figures were generated with ChimeraX (Pettersen et al., 2021).

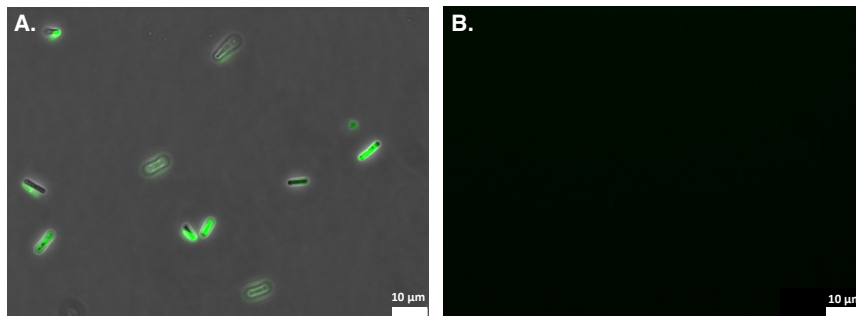


Fig. 5.4 Cell wall binding assay of Deep-Blue PlyB221 to (A.) *B. cereus* ATCC 10987 and (B.) *E. coli* ATCC 8739 (control). The CBD of PlyB221 was fused to a GFP tag to assess their binding capacities. Overnight cultures were incubated with *ca.* 50 μ g of proteins and observed using epifluorescent microscope. Fluorescent revelation of bacterial shapes confirms the binding of GFP-fused PlyB221 onto *B. cereus* bacterial surfaces, but not onto *E. coli*, suggesting that the binding specificity of the phages proteins is retained when produced as recombinant proteins. The overlays between the contrast phase and fluorescent images are shown in Figure A (ImageJ treatment).

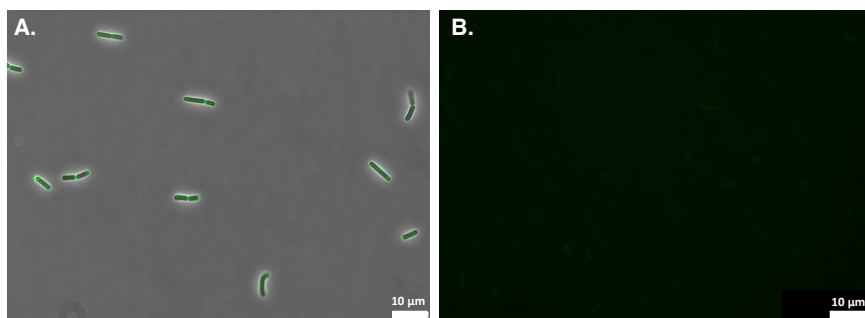


Fig. 5.5 Cell wall binding assay of Deep-Purple EvoDit-Gp28 to (A.) *B. cereus* ATCC 10987 and (B.) *E. coli* 10 β (control). EvoDit-Gp28 was fused to a GFP tag to assess their binding capacities. Overnight cultures were incubated with *ca.* 50 μ g of proteins and observed using epifluorescent microscope. Fluorescent revelation of *B. cereus* bacterial shapes confirms the binding of EvoDit-Gp28 onto bacterial surfaces. The cell wall binding assay of EvoDit-Gp28 to *E. coli* shows negligible green fluorescent background, therefore confirming the insensitivity of this strain to the phage proteins. The overlays between the contrast phase and fluorescent images are shown in Figure A (ImageJ treatment).

5.4.2 PlyB221 coating on NC membrane as specific capture biointerface

Immobilised PlyB221 shows advantageous orientation on NC membranes

A consistent immobilization of PlyB221 onto the NC membrane is critical for the bioreceptor to maintain its original binding functionality, which influences the binding performances of the test line of the resulting LFA device, and consequently its sensitivity. Our previous bioinformatic analyses strongly suggest an attachment of PlyB221 CBD to the NC membrane through its purification tag, mainly through electrostatic attraction between the positively charged residues of the purification tag to the negatively charged NC membrane.

Regarding the preferential orientation of the CBD, i.e., the epitope capable of binding to the bacteria, it is important to note that the strong negative charges on the fused GFP could be responsible of electrostatic repulsion within the whole protein, promoting the unfolding of its different domains. The GFP could therefore likely play the role of a "spacer" between the purification tag attached to the NC membrane and the CBD, **allowing reduced steric hindrance of the CBD, and good orientation for binding to bacterial cells** (Figure 5.6).

Similarly to the methodology used in section 4.2.4, we validated the nitrocellulose membrane homogeneous functionalization with GFP-fused PlyB221 proteins by fluorescence images in the membrane. By means of confocal laser fluorescence microscopy (CLSM) experiments, we explored the in-depth functionalization of the NC. Figure 5.7 A shows the characteristic porous microstructure within the CN95 nitrocellulose membrane revealed by the GFP-fused PlyB221. This confirms the SEM membrane images depicted on Figure 3.8 A. The size of *Bacillus* cells, about $0.5\text{-}1.0\ \mu\text{m} \times 2\text{-}5\ \mu\text{m}$ [202], and their tendency to form aggregates [203], justifies the choice of the NC with large pore diameter since clogging and retention to the membrane should be avoided.

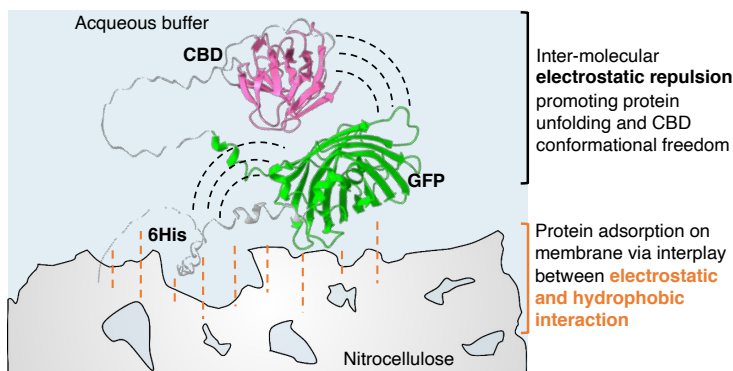


Fig. 5.6 Preferred orientation of PlyB221 after immobilization on NC membranes. Bioinformatic analyses suggest that the adsorption of the protein onto NC is thermodynamically driven by the electrostatic attraction of the positively charged 6His and negatively charged NC membrane. The GFP, strongly negatively charged could act as a spacer, allowing good orientation and high accessibility of the protein binding domain (CBD) to the bacterial cells.

Binding properties of PlyB221 are retained after immobilisation

Then, in order to validate the preservation of PlyB221 binding functionalities after immobilization, CLSM was used to visualise the capture of *Bacillus* whole-cells within NC membranes. *Bacillus* suspension containing 10^7 CFU.mL⁻¹ cells expressing the cyan fluorescent protein (CFP) were incubated on previously functionalized NC membranes with PlyB221 proteins, washed with PBS and observed with CLSM laser settings such as to capture both the wavelength emissions from the GFP-fused PlyB221 and CFP-fused retained bacterial cells.

The visualisation of *Bacillus* cells in the pores, both within (Figure 5.7 B) and with 3D CLSM images (Figure 5.7 C) of the NC membranes suggests **strong affinity and robust capture of *B. cereus* cells by the PlyB221 biointerface**. 3D CLSM images of the membrane were also acquired without bacteria incubation (Appendices Figure D.6) to serve as control. *Time series* images were also acquired over 60 seconds in order to assess the dynamic of the bacterial cell capture on the NC membrane (data not shown). Although some bacteria may disappear from the microscope focus following the natural convection of the buffer in the sample, the capture of most bacteria was stable over time. SEM images of immobilized and fixed bacterial cells as a comparative visualisation technique confirmed the results of fluorescent CLSM images (Figure 5.7 D).

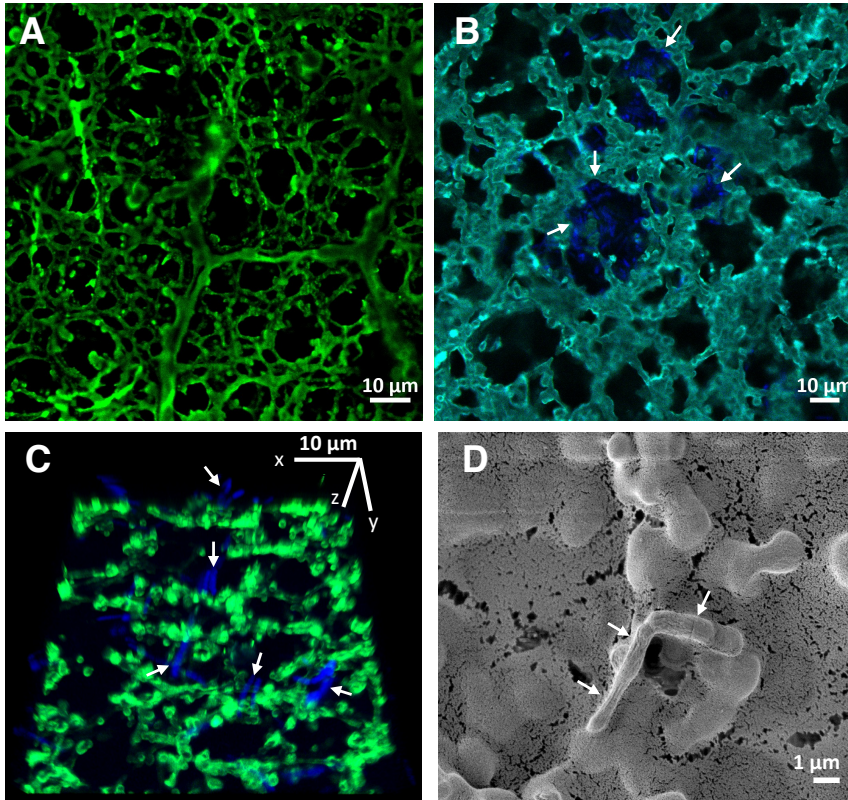


Fig. 5.7 Confocal laser scanning microscopy (CLSM) images of immobilized PlyB221 binding proteins on the nitrocellulose membrane (green or turquoise) and captured *Bacillus* whole-cells in the pores (blue). **A.** CLSM image of a functionalized membrane without bacterial cells (negative control). **B.** CLSM image of captured bacteria within the NC membrane (5 - 10 μm deep). **C.** 3D image of the functionalized NC membrane surface presenting captured bacterial cells, built from Z-stack CLSM images on 15 μm height. **D.** SEM images of functionalized NC membrane incubated with *Bacillus* cells. The compacted aspect of the NC results from the glutaraldehyde fixing. In all the images, the bacterial cells captured at the surface of the membrane pores are represented by white arrows.

5.4.3 AuNP-EvoDit-Gp28 conjugates as specific detection biointerface

As AuNPs are the transducers of colorimetric LFAs, their morphology (shape, size and size distribution) and optical properties are crucial to achieve the sensitivity and reproducibility of the visual signal required by LFAs. In addition, the immobilization of the detection bioreceptor, EvoDit-Gp28, on the AuNPs must be carefully optimised to retain the AuNP properties and the binding functionality of the EvoDit with *Bacillus* cells. Finally, the binding performance of the AuNP conjugates must be validated to ensure their implementation in LFAs.

Based on previous works for the LFA detection of whole-cell bacteria [47], 40 nm round-shaped AuNPs were selected as colorimetric labels to build the detection biointerface. Their morphological properties were characterized with TEM and DLS, while optical properties were characterized with UV-vis. TEM images of the bare AuNPs confirmed their homogeneous and highly spherical shape (Figure 5.8 A), and further TEM image analysis show an average diameter of 42.5 ± 3.5 nm (Figure 5.8 B). DLS experiments were also conducted on the bare AuNPs, confirming an average hydrodynamic radius of roughly 18 nm (Figure 5.8 D). The UV-vis spectrum of the 40 nm AuNPs shows a sharp absorbance peak around 522 nm due to the SPR of the nanoparticles, meaning they emit a deeply red colour (Figure 5.8 E and inset).

AuNP conjugation to EvoDit-Gp28

Next, we conjugated the AuNPs with the EvoDit-Gp28 binding proteins. As introduced in section 3.5.3, electrostatic interactions dominate the early stages of protein binding onto the AuNP surface, i.e., in the seconds to minutes range. Then, reorganization of the protein layer occurs to maximize the number of favorable interactions. In the third step, a dative binding, i.e. the formation of a strong thiol-gold bond between the protein cysteine residue and the AuNP, definitively anchors the protein on the AuNP. Bioinformatic studies identified the Cys residue of the EvoDit-Gp28, located in its 6His-tag region (Figure 5.3 G), concerned by the binding to the AuNP. This ensures an ideal orientation of the EvoDit-Gp28 on the AuNP surface since the CBM, responsible of the binding to the bacterial cell, is exposed radially to the AuNP towards the outside of the surface, thus maximizing binding probabilities. The conjugation process followed is depicted on Figure 5.9.

The optimisation (pH and protein concentration) of the AuNP conjugation with EvoDit-Gp28 binding proteins was performed via a gold aggregation test (Appendices Figure D.7). Most stable conjugates for the lowest

protein concentration was obtained at pH 8 with EvoDit-Gp28 concentration of 80 $\mu\text{g}/\text{mL}$. In LFA, the properties of the conjugated gold labels are critical for a performing assay. We characterized the AuNP conjugates to evaluate the conjugation process. TEM images showed blurred corona (~ 10 nm thickness) around the AuNPs, which has already been previously reported as successive protein layers adsorbed on the AuNPs (Figure 5.8 B) [204]. The TEM images also showed repetitive occurrence of small aggregates (2-10) of AuNPs, suggesting small aggregation during the conjugation process. This was supported by DLS experiments showing a slightly higher hydrodynamic radius (around 42 nm) for the conjugated AuNPs, associated with a broader range of small aggregate sizes (Figure 5.8 D). Similar aggregation has already been reported for 40 nm AuNPs but other bioreceptors (antibodies) [47]. The AuNPs-EvoDit-Gp28 shows a peak at 528 nm, i.e. a peak shift of 6 nm from the bare AuNP solution (Figure 5.8 E and inset). This shift reflects the binding of the proteins onto the AuNP surface.

In addition, in order to prevent the non-specific adsorption of the AuNP conjugates during the LFA operation, which could decrease the LFA sensitivity and specificity, the AuNPs were consequently blocked with a BSA layer (Figure 5.9). The adsorption of the BSA onto the AuNP surface was characterized by UV-vis, showing a similar absorbance peak location than conjugated AuNPs with phage proteins only (Figure 5.8 E and inset).

Finally, stability of the AuNP conjugates blocked with BSA was assessed over one week by UV-vis. The absorbance peaks remained unchanged (Figure 5.8 F), confirming good stability of the conjugate particles. We did not test the stability over a longer period of time since the AuNP conjugates were stored at most a few days before subsequent use in LFA detection experiments.

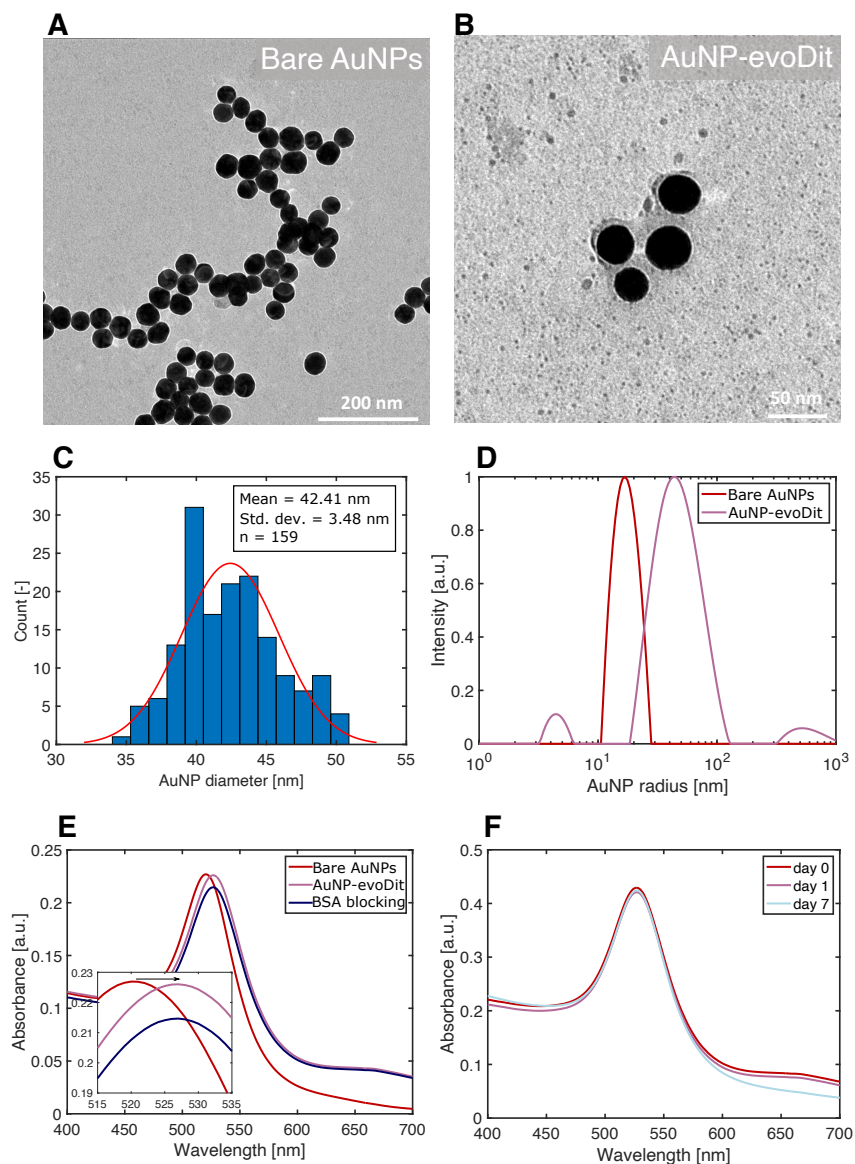


Fig. 5.8 Characterization of AuNP-EvoDit-Gp28 conjugates. **A.** TEM images of the bare and **B.** conjugated 40 nm AuNPs with EvoDit-Gp28. **C.** Size histogram of the bare AuNPs shown in **A**, obtained from TEM image treatment (ImageJ). **D.** Size distribution by intensity of the hydrodynamic radii of bare and conjugated AuNPs measured by DLS. **E.** UV-vis spectra of bare AuNPs, AuNPs conjugated with EvoDit-Gp28 and AuNPs blocked after conjugation. The slight shift of the absorbance peak from 522 nm to 528 nm reflects the immobilization of the proteins onto the AuNP surface (inset). **F.** Stability study of the AuNPs conjugates over one week.

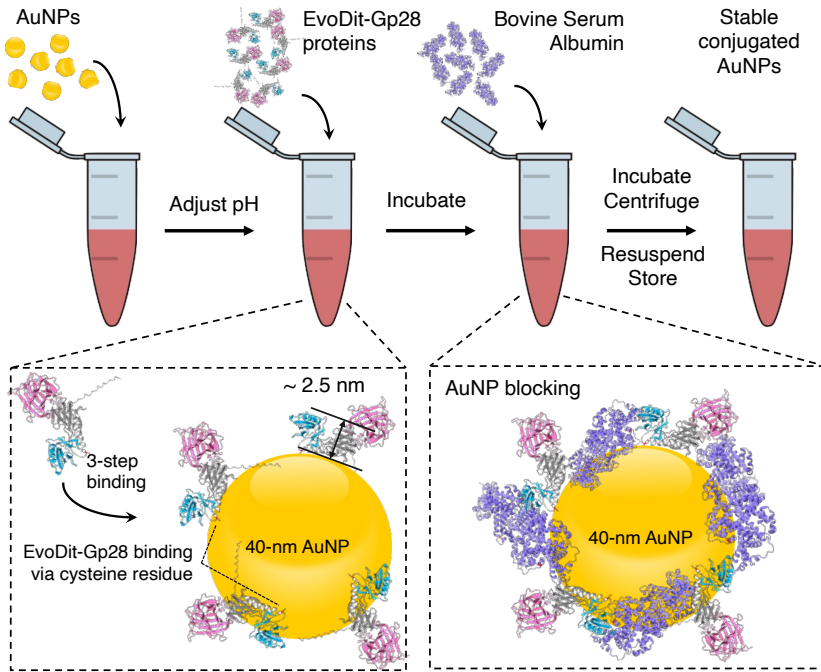


Fig. 5.9 Conjugation process of AuNPs with EvoDit-Gp28. Bioinformatics studies showed that adsorption of the EvoDit-Gp28 onto the AuNPs occurs through the most available Cysteine group of the protein. This ensures an oriented immobilization of the protein, with the CBM (rose domain, responsible of bacterial binding) oriented outwards. The process conditions (pH and protein concentration) were optimized, and bovine serum albumin (BSA) was added as blocking agent to prevent non-specific binding in the LFA.

AuNP labelling of B. cereus cells

Following successful AuNP bioconjugation to EvoDit-Gp28, we study the extent of AuNP-labelling of the bacterial cells at the microscopic (biointerface) level. This section has three objectives :

1. to assess the functionality of the immobilized protein on the AuNP, i.e. examine the performance of the interaction;
2. to evaluate the specificity of AuNP binding onto the bacterial surface;
3. to characterize the number of AuNPs bound to the bacteria cells.

While the first and second reasons are evident, the third relates to the need to quantify the consistency of the AuNP adsorption onto the *Bacillus* cells. As a reminder, in section 4.2.7 (chapter 4), we investigated the LFA quantitative response calibration in terms of AuNP number for a simple and fairly predictable protein antigen-antibody, allowing to derive the fundamentals detection limits of the system in terms of AuNP number. In order to extend this methodology to the detection of pathogens, we need to study and quantify the binding process of AuNP to the bacterial cells.

AuNP interaction with bacterial cell surface To examine the interaction between EvoDit-Gp28 immobilized on AuNPs and bacteria in aqueous solutions, conjugates AuNPs were incubated with bacterial suspensions. The successful bacteria labelling with the AuNPs was confirmed by scanning transmission electron microscopy (STEM) images of the incubated suspensions, showing an interaction between the AuNPs and the bacterial surface (Appendices Figure D.8). However, bacterial residues on the pictures complicates the image analysis process.

In order to provide a better contrast between the cell surface and the AuNP, and allow the AuNP quantification, **the bacteria-AuNPs complexes were fixed with glutaraldehyde in solution**, which also implies several washing steps. Figure 5.10 A shows unlabelled bacteria, exhibiting a rod shape. Bacteria are retaining their size and shapes after fixation. When in presence of AuNP conjugates, the STEM pictures of fixed complexes clearly show an in-homogeneous **covering of the AuNPs on the bacterial surface** (Figure 5.10 B and inset), with a high contrast. Since the bacteria labelled with AuNPs where washed several time in buffers during the fixation process, Figure 5.10 B shows that washings did not desorb the AuNPs from the cell surface, indicating a strong interaction on the surface of the bacteria. TEM images of AuNP binding onto bacterial cells were also previously reported observed, with a lesser contrast though [205].

A relatively random arrangement of AuNP binding on the bacterial surface is observed, where we could have expected a distribution of AuNPs more concentrated at the poles of the bacteria for example, which often have more receptors accessible to bind antibody-conjugated nanolabels, as shown by Russo *et al.* [206]. It is thus difficult to raise conclusion about the surface repartition of the bacterial bioreceptors involved in EvoDit-Gp28 binding.

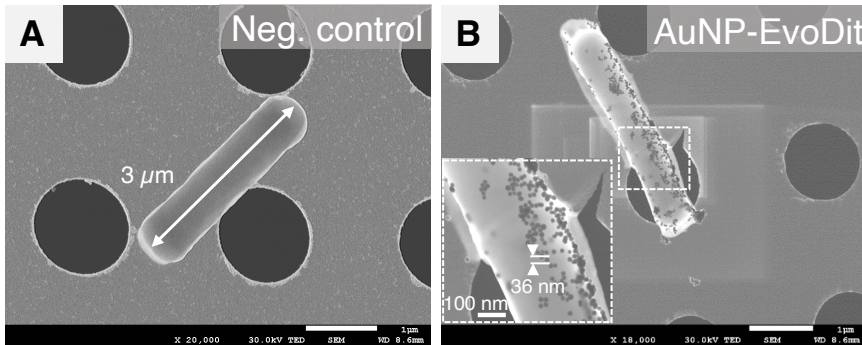


Fig. 5.10 STEM images of glutaraldehyde-fixed bacterial suspensions. **A.** Unlabelled bacteria, exhibiting a rod shape and sizes varying from 1 to 3 μm . **B.** When incubated with AuNP conjugates, the STEM pictures of fixed bacteria highlight the AuNPs (black spheres) strongly bound to the bacterial surface since the interaction has withstood many washings. Use of glutaraldehyde fixation significantly increases the contrast between bacterial cells and AuNP conjugates.

AuNP labelling specificity Then, the specificity of the AuNP-labelling onto *Bacillus* pathogens was assessed by **comparing the extend of the AuNP binding onto bacterial surfaces of different *Bacillus* strains**. 10^7 CFU.mL^{-1} *Bacillus thuringiensis* (*B.t.*) AW43 cells, sensitive to the EvoDit-Gp28 (see Figure D.2) were incubated with AuNP conjugates at DO9 to serve as reference. To prove the specificity, we incubated AuNP conjugates with *Bacillus weihenstephanensis* (*B.w.*) KBA4, insensitive to EvoDit-Gp28 (Figure D.2), in the same proportions. To study the extend of possible non-specific adsorption of AuNPs on the pathogens, we incubated *B.t.* AW43 with AuNPs coated only with BSA, which shows no specific interaction with the bacterial cell surface receptors since it is used as blocking agent. Finally, a negative control was included without AuNP conjugates. STEM images of the fixed bacteria-AuNP complexes were recorded.

Figure 5.11 depicts **qualitative observation and quantitative analysis of AuNP-labelling on the bacterial strains**. After recording STEM im-

ages, image analysis (ImageJ) was performed on the high contrast images to quantitatively measure the surface coverage of bacterial cells by AuNPs. STEM images with reference *B.t. AW43* show **significant binding between of the AuNP conjugate** on the cell surface (Figure 5.11 C). Adhesion efficiency is confirmed by statistical image analysis, showing 7.85 ± 3.05 % bact. surface coverage, or $0.13 \pm 0.07 \mu\text{m}^2/\text{bact.}$ (Figure 5.10 D), with some variation between bacteria.

Negative control pictures are not shown, but are highly similar to Figure 5.10 A. The tiny AuNP coverage of the bacterial surface measured with the image analysis algorithm for negative control (0.19 ± 0.23 % bact. surface coverage, or $1.3 \times 10^{-3} \pm 1.7 \times 10^{-3} \mu\text{m}^2/\text{bact.}$, Figure 5.11 D) relates to measurement errors due to the bacteria varying shapes, sometimes including black area from outside the bacterial surface.

As depicted on Figure 5.11 A and D, the incubation of *B.t. AW43* **suspensions with BSA-capped AuNPs** significantly reduces the overall binding (1.14 ± 1.69 % bact. surface coverage, or $2.4 \times 10^{-2} \pm 4.6 \times 10^{-2} \mu\text{m}^2/\text{bact.}$) as compared to *B.t. AW43*. The relatively high variability computed (presence of several outliers) and the image observation of a few yet relatively large AuNP-BSA covered areas on the bacterial surface (black spots on Figure 5.11 A) could be explained by medium-large size aggregates arising from the uncontrolled BSA coating, facilitating non-specific adsorption onto bacterial cells due to their size.

Finally, the incubation of **insensitive *B.w. KBA4*** with AuNPs significantly reduces the binding proportion as compared to sensitive bacteria (0.49 ± 0.47 % bact. surface coverage, or $9.1 \times 10^{-3} \pm 1 \times 10^{-2} \mu\text{m}^2/\text{bact.}$), confirming the specificity of the conjugates (Figures 5.11 B and D).

To assess the **significance of the AuNP binding** between the different controls and the reference, we performed a two-sample t-test on the % bacterial surface covered by the AuNPs. The statistical test confirmed that the means of the AuNP binding coverage for *B.t. AW43* do differ significantly from the means of the control conditions (p-value $< 10^{-4}$ for each).

Taken together, **our results suggest good binding functionalities of the AuNP-EvoDit-Gp28**, including sufficient EvoDit-Gp28 immobilization orientation and preservation of their specificity. The slight adsorption of BSA-capped AuNPs onto bacterial surfaces however raises attention towards the possibility of non-specific adsorption, especially when considering the complex and uncontrolled environment of the LFA final device.

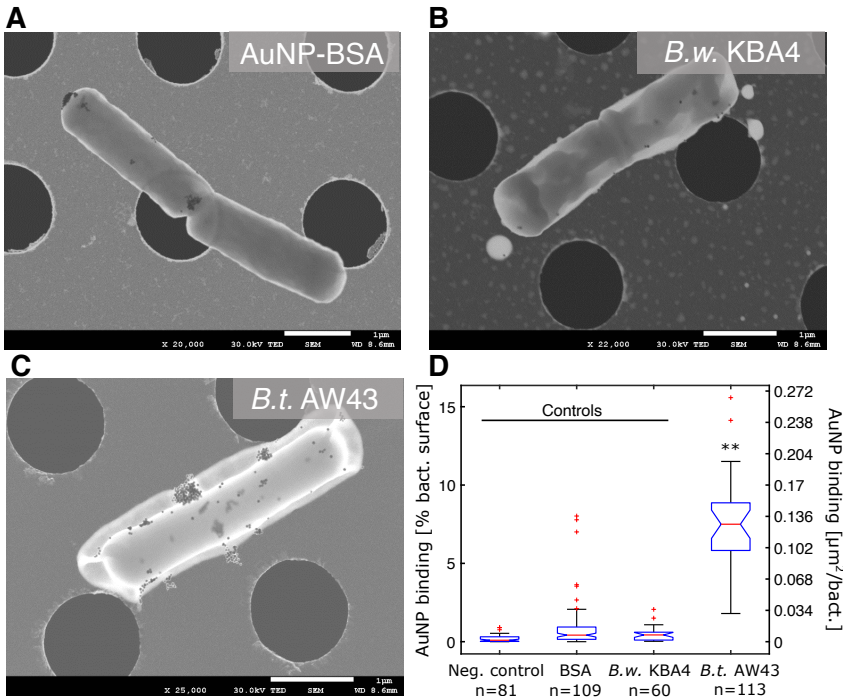


Fig. 5.11 STEM images to assess the specificity of the AuNP labelling onto bacterial cells. Control conditions, including **A.** BSA-capped AuNP (without phage protein bioreceptor) and **B.** *B.w.* KBA4 bacteria (insensitive to EvoDit-Gp28) were compared to **C.** reference *B.t.* AW43 strain, sensitive to EvoDit-Gp28. **D.** The quantification of the AuNP-labelling on the bacterial cells indicates significant reduction of the AuNP binding events for the control conditions as compared to the reference strain, suggesting binding functionality (strong adsorption and specificity) of the prepared AuNP-EvoDit-Gp28 conjugates. Two separate experiments showing similar differences were performed, and the data presented are means of n bacterial cells. Significant shifts (** $p < 0.001$) are indicated. (Neg. control : negative control, i.e. observation of reference strain bacteria without AuNPs)

AuNP labelling quantification In chapter 4, we proposed a quantitative view of the AuNP number accumulation in the LFA and the correlation with LFA signal intensity for a model antigen-antibody system with a simple interaction pattern (maximum two binding sites for the labels on the protein due to a limited number of epitopes and its small size). However, in more complex systems such as adsorption of AuNP coated with phage proteins on large bacterial cells, which are $\sim 10^2$ - $10^3 \times$ larger than a protein, the rules derived in chapter 4 are probably not valid anymore since

the interactions are of a different nature.

Indeed, bacteria likely have a large number of specific receptors accessible for phage protein binding on their cell wall, allowing the binding of many nanoparticles. To predict the amount of AuNP required to sufficiently cover the bacteria and allow for the emission of light intensity, this section **calibrates the bacterial surface area covered for different concentrations of AuNP conjugates**, or different AuNP Optical Densities (OD, i.e. the measured absorbance of the AuNP conjugate at 520 nm).

STEM was used to further characterize the complexes that formed between the bacteria and different concentrations of AuNPs. In order to mimic similar amounts of AuNPs and sample volume used in application, e.g. in a 5mm wide LFA, we incubated 150 μL of overnight bacterial suspensions with 35 μL of AuNP conjugates :

- The **choice of overnight bacterial suspensions** at 10^7 CFU.mL⁻¹ is justified by the relevance of studying the binding of AuNPs for the extreme case where bacteria would be the most numerous in the sample.
- The **choice of the AuNP OD** used for this study was based on previous experience and published works. The use of AuNPs at OD4 was reported by Anfossi *et al.* [207] for toxin detection and by Bergua *et al.* [47] for pathogen detection using 40 nm AuNP-based LFAs. Therefore, we selected OD 1, 4 and 9 as a range to calibrate the bacterial surface coverage.

As qualitatively shown on Figures 5.12 A-C, under these three conditions, different amounts of adsorbed AuNPs were exhibited on the bacterial cell surfaces. Again, quantitative image analysis was performed to quantify the bacterial coverage by AuNP, expressed in [% bacterial surface covered] and [$\mu\text{m}^2/\text{bact.}$], depicted on the box-plot (Figure 5.12 D) and reported on Table 5.1 for each AuNP OD used. We then computed the number of AuNPs captured by each bacteria, and thus the proportion of the initial AuNP solution that effectively bound on the bacterial cells of the sample, for each OD (see Appendices D section 4.6.2 for the computations).

The bacterial surface coverage with AuNP determined in this section is used to discuss the **binding efficiency** between AuNPs and bacteria. Indeed, the number of AuNP bound per bacteria gives interesting insights into the minimal number of bacteria required to generates colour at the TL. While we have seen in the previous chapter that many proteins are needed to transport a single AuNP to the TL and generate signal (in the order of magnitude of 10^2 molecules), the dynamics are completely differ-

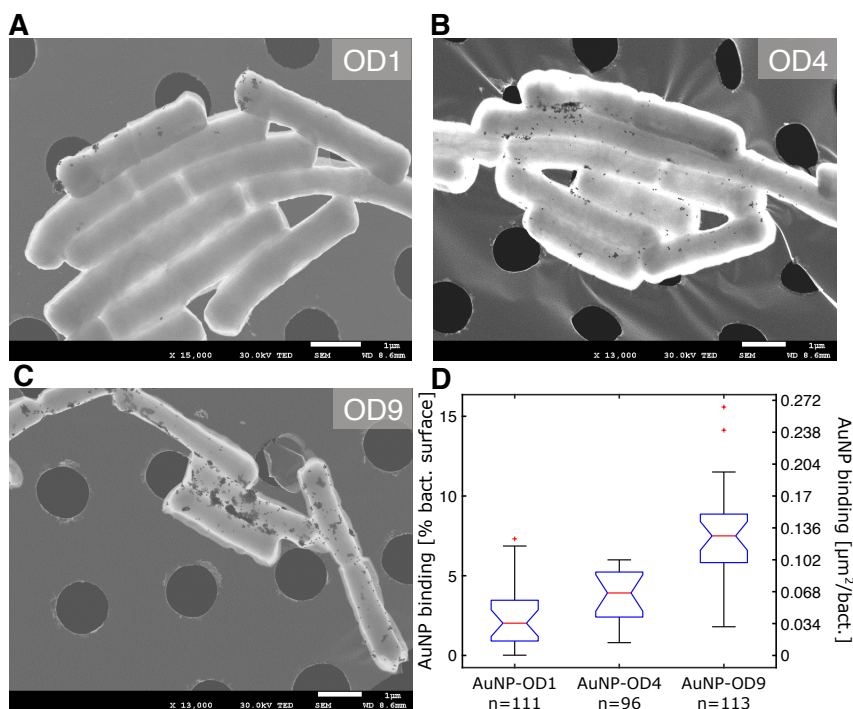


Fig. 5.12 Calibration of AuNP binding to *Bacillus* cells qualitatively observed by STEM images for different concentration of AuNPs : **A.** OD 1, **B.** OD 4 and **C.** OD 9. Quantitative image processing permits the quantification of the AuNP labelling of bacterial cells. Quantitative results are reported in Table 5.1. (OD: optical density. $n > 100$ for each condition, from two independent experiments)

ent in the case of pathogen-based LFAs. The results of our study show that a pathogen can transport up to $\sim 10^2$ AuNPs if the initial concentration of AuNP conjugate incubated with the bacteria is sufficient. Thus, a consequent number of AuNPs accumulated at the TL will be achieved with a much smaller number of pathogen targets in the sample than was required for the proteins (we estimated that 10^8 - 10^{10} protein analytes were required in the sample to generate a visible signal via AuNP accumulation at the TL). Given the different interaction dynamics of small proteins and pathogens with their nanolabels, **it can be reasonably be assumed that the intrinsic detection limit of optical LFAs for whole-cell pathogen detection will fall in the range 10^4 - 10^6 bacterial cells per mL.** This is indeed a good picture of the LoD referred in the literature for whole-cell pathogen detection with AuNP-based LFAs, without any visual signal enhancement

strategy, summarized by Bergua *et al.* [47] and in Table 3.1.

Finally, the proportion of bound AuNP can be used to assess the ideal number of AuNP to be considered during the design of the LFA to achieve consistent binding to the bacterial cell targets. According to Figure 5.12 and Table 5.1, it seems that AuNP conjugates at OD 9 is appropriate in order to significantly cover the bacterial surface, and that the AuNPs must be added in excess to obtain a relatively similar ratio of adsorbed AuNPs to bacteria for every detection experiment [205].

AuNP OD	AuNP number [# /sample]	Bact. cover. [% bact. surf.]	Bact. cover. [μm^2 /bact.]	Numb. AuNP [# /bact]	AuNP bound [%]
1	3.15×10^9	2.54 ± 2.18	0.044 ± 0.04	35 ± 32	1.67
4	1.4×10^{10}	3.92 ± 1.67	0.066 ± 0.03	53 ± 25	0.38
9	3.15×10^{10}	7.82 ± 3.05	0.13 ± 0.072	103 ± 58	0.17

Table 5.1 Calibration of AuNP binding to bacterial cells, corresponding to the results depicted on Figure 5.12. The number of AuNP bound to the bacteria was computed from the bacteria surface area covered by the AuNPs. By knowing the initial bacterial cells and AuNP numbers in the incubated samples, the proportion of initial AuNP solution bound to the bacteria surface was expressed, highlighting a low efficiency binding ($\leq 1\%$), probably due to the size difference between the bacterial cells and the AuNP labels. Computations are reported in Appendices 4.6.2. (OD: optical density, Bact. cover.: Bacteria surface coverage)

5.4.4 Proof-of-concept of *Bacillus* whole-cell detection in LFA format

Finally, in order to check the compatibility of the phage protein biointerfaces and binding performances with the condition encountered in a LFA (i.e., under a constant flow within a nitrocellulose membrane), we used the half-stick assay format to perform the detection of whole-cell.

Establishment of a half-stick LFA

The use of dotted half-sticks rather than full LFA is recommended in our case as they are fast to prepare, they offer versatility in determining the most appropriate detection protocol and smaller reagent amounts are required (taking into account that the phage proteins were expressed and purified in our lab facilities) [176]. The half-stick consisted in a nitrocellulose membrane assembled with cellulose sample pads (Figure 5.13 A):

- As reported by Bergua *et al.*, due to their large sizes, the proper flow of whole-cell bacteria is challenging and must be optimized by selecting the **appropriate nitrocellulose membrane**. To this end, we selected two nitrocellulose (NC) membranes based on their mean pore size and

pore sizes distribution that could allow the flow of large bacterial cells (CN95 and CN140, see section 3.6.2). NC membranes were adhered to laminated cards .

- Absorbent pad (AP) made of cellulose membrane was assembled on the laminated card, overlapping 2 mm with the NC membrane. Regarding the vertical disposition of the half-stick, in order to provide enough bed volume and prevent a quick decrease in the necessary flow to drive the bacterial cells to the test area, we assembled two consecutive pieces of AP in a row at the end of the strip. Then the card was cut into 5 mm-wide lateral flow strips.
- The test line, or testing area on the NC membrane was formed by manually dropping 0.3 μL of GFP-PlyB221 solutions at 1 mg.mL^{-1} onto nitro-cellulose half-sticks as **Test Dots (T-Dot)**, and then dried for 1h at 37°C. This avoids the use of test line dispensers which consume significantly more capture receptor solution.
- Since the objective of the half-stick study is to assess the binding properties of the developed phage binding-protein biointerfaces in a LFA format, we did not include a proper control area made of specific bioreceptor for the capture of the detection bioreceptors. Rather, **to evaluate the binding performances** we compare the signal intensity of the T-Dot with the intensity of a downstream circular area of the size of the T-Dot, which is considered as background signal intensity from the membrane. The background signal intensity is thus subtracted from the measured T-Dot signal in order to obtain the *T-Dot Peak Value* used to compare the different LFA detection conditions.
- The **detection protocol** consisted in incubating 150 μL of the bacterial suspension of interest with 35 μL of AuNP-EvoDit-Gp28 (prepared as in section 5.4.3) in a 96-well plate during 5 min to allow for binding of AuNP conjugates on the bacterial cells. Then, the half-stick dotted strip was immersed vertically in the well. After 15 min, the T-Dot color intensity was stable, the half-stick were scanned with a professional reader and the images analysed with ImageJ.

Half-LFA optimization

We optimized the half-stick assay in terms of the NC membrane for appropriate flow by testing the half-stick performance for different *Bacillus* strains sensitive to the phage-binding proteins, *Bacillus thuringiensis* (B.t. AW43) and two *Bacillus cereus* strains (B.c. ATCC10987 and B.c. VD021). Figure 5.13 B depicts the T-Dot intensity values resulting from the half-stick assay for the CN140 and CN95 NC membranes, in a relative scale with respect to the T-Dot intensity value of the CN95 membrane. Indeed, CN95

membrane provides the highest sensitivity for the detection of 10^6 and 10^7 CFU.mL⁻¹ bacterial suspension, for the three tested *Bacillus* strains. CN140 membranes were showing retained red color at the bottom of the half-stick, suggesting partial clogging of the pores due to bacterial cells. In general, many strips showed partial clogging of the pores at the bottom of the stick when testing the 10^7 CFU.mL⁻¹ bacterial concentration. Therefore, the degree of overlapping between the conjugate pad and the NC membrane will be critical for an optimal flow of the bacteria and the AuNPs between the pads, and must therefore be considered in the full LFA format.

Sensitivity estimation

The half-stick assays, i.e. a prototype lateral flow assay using the phage binding proteins, shows the ability to significantly detect 10^6 and 10^7 CFU.mL⁻¹ bacterial suspension in 15 min (Figure 5.13 C). The blank signal corresponds to the T-Dot intensity signal for half-stick assessed without bacterial cells in the tested sample. Although *B.t.* AW43 shows slightly lower T-Dot intensity, the successful detection of the three tested *Bacillus* strains proved the versatility of the prototype LFA for the rapid detection of whole-cell *Bacillus* pathogens in suspension. Since the quantification of such whole-cell pathogen AuNP-based LFA should fall around 10^4 - 10^5 CFU.mL⁻¹, we should test lower concentrations of bacterial suspension to confirm our hypothesis and determine the LoD of our prototype LFA.

Half-LFA specificity towards B. cereus

Finally, we assessed the specificity of the half-stick detection towards *Bacillus* cells. To this end, we selected different bacterial species, *Bacillus* pathogens from the same genus but non-specific, i.e. *Bacillus subtilis* (*B.s.* Bs168), and pathogens from other genus and therefore non-specific as well the phage binding proteins, i.e. *Staphylococcus epidermidis* (*S.e.* ATCC12228), showing strong reduction of the binding at the T-Dot, and hence its signal intensity. However, signal is not always as low as the blank (negative control), meaning that non-specific binding occurs between the AuNP conjugate, the control strains and the PlyB221 capture bioreceptors. In order to study the non-specific interactions in the LFA format, we also tested the detection of all the strains at 10^7 CFU.mL⁻¹ with BSA-coated AuNPs. The signal intensities are reduced as compared to the detection of the sensitive *Bacillus* strains since the BSA is insensitive to the bacteria, but the residual signal indicates that non-specific adsorption still occurs, supporting the results in Figure 5.11 showing a few but significant non-specific adsorption of BSA-coated AuNP on the bacterial surfaces. This should be further optimised by membrane blocking with BSA, for example.

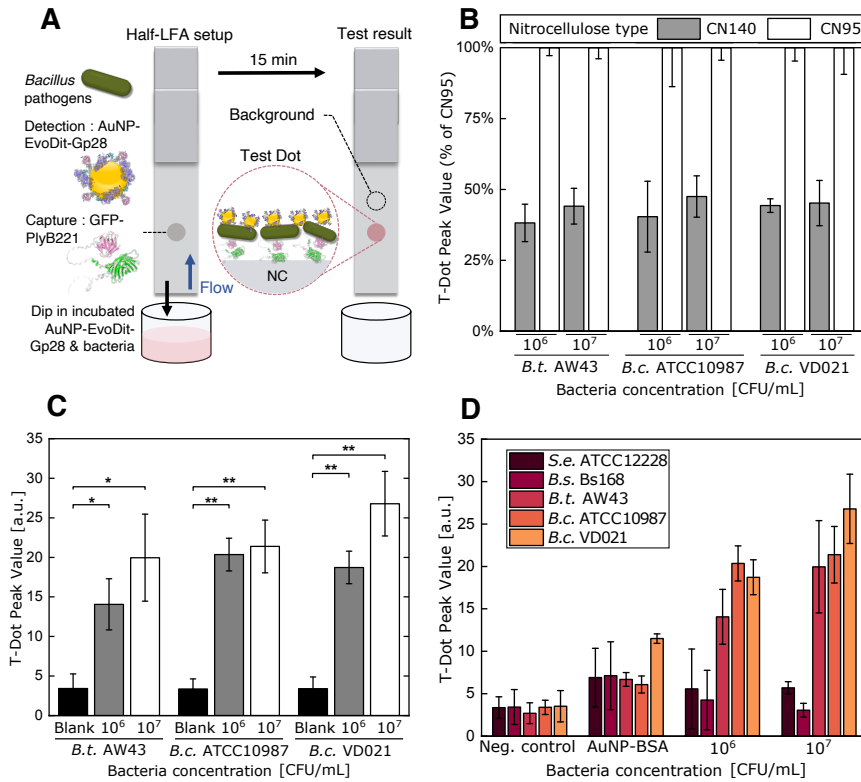


Fig. 5.13 Half-stick testing for phage-protein binding assessment in LFA format. **A.** Schematic representation of the test dot based on half-stick LFA : PlyB221 protein was manually dropped on the NC membrane as capture bioreceptor at the test dot location. Half-stick testing was performed by dipping the strip in the sample previously incubated with the AuNP detection conjugate. **B.** Half-stick testing with CN140 and CN95 NC membrane reveals that CN95 is more appropriate to whole-cell bacteria flow as its T-Dot intensity is twice as strong as that obtained with the CN140 membrane, most probably due to its larger pores. **C.** The detection of 10^6 and 10^7 CFU.mL⁻¹ suspension of the three *Bacillus* strains (*B.t.* AW43, *B.c.* ATCC10987 and *B.c.* VD021) with respect to blank (no bacteria, neg. control) confirmed the good binding properties of the phage proteins in a prototype LFA format. Significant results (* $p \leq 0.05$, ** $p \leq 0.01$) are indicated. **D.** Different, non-sensitive to phage proteins, bacterial species were tested for the specificity validation of the half-sticks. In addition, AuNPs coated with BSA were tested to evaluate the non-specific adsorptions. Although signal intensities were significantly reduced for the controls, they remained a bit higher than the negative controls, indicating remaining non-specific adsorption of the pathogens in the strips. All the conditions were tested with triplicate half-sticks ($n = 3$). Experiments B and (C-D) were performed using different batches of half-sticks.

5.5 Discussion

Recombinant phage affinity proteins represent a useful class of affinity proteins that have thus been far under-utilized in paper-based diagnostics. Phage affinity proteins can be expressed recombinantly in bacterial cells (produced by self-replication in bacterial cells, followed by purification), making them a simpler, lower-cost and more sustainable alternative to the animal-based production of antibodies customarily used in lateral flow-based diagnostics [191]. In addition, their detailed characterization showed that they present high affinity to the bacterial cells and could display broader host activity spectrum than their parental phages, the latter being often so specific that they only target a subset of one bacterial species [148]. Phage proteins are therefore expected to be promising bioreceptor alternative to antibodies for use in LFA formats.

Here, we have leveraged the customizability of recombinant affinity proteins isolated and characterized encoded by bacteriophage targeting *B. cereus* cells to develop biointerfaces made of these endolysin CBD and evolved distal tail (EvoDit) proteins onto nitrocellulose and gold nanoparticles, respectively, for the detection of whole-cell *B. cereus* in colorimetric lateral flow assays. The proteins used in this study are encoded by two bacteriophages, Deep-Blue and Deep-Purple, whose infection activity was proved by activity and binding experiments against members of the *B. cereus* group.

First, we used recently developed bioinformatic algorithms in order to predict the microstructure of the phage protein and their preferential orientation on the biointerfaces. In particular, it demonstrated the oriented affinity for the EvoDit protein to bind on gold nanoparticles.

Then, we developed and assessed the capture and detection biointerfaces on the nitrocellulose membranes and AuNPs, respectively. In particular, we performed a quantitative analysis of the number of AuNP that could bind to each bacterial cell, in order to derive a model of the AuNP conjugate binding affinity to the cells.

In presence of AuNP conjugate in excess, we showed that ~ 100 AuNP could bind to each bacterial cell. However, since every bacteria expresses its specific binding receptor in a different way on its cell membrane, and probably in a different proportion, care must be taken about the generalization of these results to others systems or even to other *Bacillus* strains.

Ideally, the quantitative relationships outlined in this chapter on the binding of AuNPs to bacteria must be reconsidered for each new system studied.

Nevertheless, based on this number of AuNP bound to each bacterial cell, and the minimal number of accumulated AuNP required at the TL to show colorimetric signal found in chapter 4, we derived the theoretical limit of detection of whole-cell pathogen detection in AuNP based LFA, ranging from 10^4 - 10^6 bacterial cells per mL.

As mean of comparison, the rapid SARS-CoV-2 antigen test can detect virus at the levels equivalent to approximately 10^5 to 10^6 SARS-Cov-2 viral genome copies per millilitre, i.e. 10^5 to 10^6 virus particle per mL [208]. Viruses are $\sim 10 \times$ larger than a protein (between 50 and 140 nm for SARS-CoV-2 virus), or $\sim 10 \times$ smaller than the bacterial cells studied in this chapter. Their binding process to specific AuNPs should therefore be different than nm-sized proteins or μm -bacteria. In this case, we can assume that several AuNPs could bind to a single virus, explaining that the detection of viruses requires a smaller number of molecules than proteins, and hence show lower LoD than proteins in terms of molecule number. However, due to their smaller size, the number of AuNP bound to the viruses should be significantly lower than than of whole bacterial cells. This could explain the 10 - $10^2 \times$ lower LoD (in molar terms) for bacteria than the LoD reported for viruses.

Finally, as introduced in section 3.4, effective bioreceptors in LFA need three main characteristics: stability, a fast association kinetics and strong binding affinity for the target molecule. While the stability of phage binding-proteins has been shown by previous studies [148, 209], and the strong binding affinity in the form of LFA biointerfaces has been studied and demonstrated experimentally in this chapter, the association kinetics for the target cell surface receptors needs to be determined to fully characterize the binding properties of the phage proteins as bioreceptors in LFAs. To support our experimental results on the phage protein binding properties and justify their use as biointerfaces, their binding affinity constant K_D could be determined. Although this requires advanced biotechnology techniques, and this characterization is not fully within the scope of this thesis, a literature analysis of the affinity constant of phage binding-proteins has been performed in order to allow quantified comparison with other affinity systems (Figure 5.14). In particular, the K_D of several phage endolysin CBD have been reported by Schmelcher *et al.* [210, 211] and Kong *et al.* [130], indicating that CBDs feature and strong binding affinity

(K_D values of pico- to nanomolar range) to bacteria, in addition to their high specificity. Figure 5.14 shows that K_D values for CBD lie in the range of K_D values typically reported for antigen-antibody system, the gold standard in affinity immunoassays. **This, in addition to the results reported in this chapter, suggests that the phage binding proteins are indeed very promising alternative candidates to antibodies for bioreceptors in LFAs.** No K_D values for distal proteins such as the EvoDit have been reported so far in the literature. This could be a research direction of great interest for the microbiologists involved in bacteriophage binding protein identification and characterization.

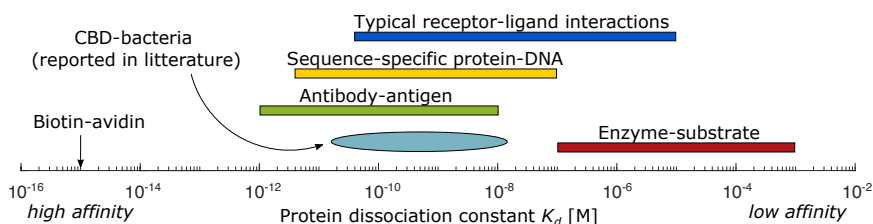


Fig. 5.14 Binding affinity constants of major protein-ligand complexes. The K_D of several phage endolysin CBD was reported in the literature, indicates strong binding affinity to bacteria. K_D values for CBD lie in the range of K_D values typically reported for antibody, which are currently the mainstream bioreceptor technology (K_D for IgG antibody extracted from LFA detection experiments in chapter 4 is $\sim 10^{-9}$ [M]), suggesting their use as performing bioreceptors in LFAs. Partly inspired from [7].

5.6 Conclusion

Taken all together, the results presented in this study demonstrated the potential of recombinant affinity phage proteins as capture and detection biointerfaces in LFAs for whole-cell pathogen detection. The use of two different phage proteins allowed to overcome the limitations displayed by the other study using phage proteins within LFA [118], which had a much less suitable mode of operation for PoC detection due to the use of the same CBD as capture and detection bioreceptor. While the advantageous binding properties of the phage-proteins were thoroughly characterized and demonstrated at the molecular-, biointerface- and LFA device-level, their performance in terms of LFA LoD still need to be validated against traditional antibody-based methods. This way, the next work should focus on the determination of the assay ultimate LoD towards *B. cereus* detection.

Because recombinant phage proteins can be produced in large quantities and show very good binding affinity to their host, the results of this study strongly support their large usage as bioreceptors instead of the currently used antibodies in pathogen LFAs.

6

Impedance detection of pathogens in nitrocellulose



Chapter objectives

In chapter 5, we introduced the design of paper-based colorimetric LFA for pathogens detection. Our results showed that the intrinsic limit of detection of AuNP-based LFAs for whole-cell pathogen detection are constrained to $\sim 10^5$ CFU.mL⁻¹. This limits their practical uses in many application, such as pathogen diagnostics of water environments that need to detect much lower thresholds. In order to decrease the paper-based sensor limit of detection, a higher sensitivity to the targeted pathogen is required. Possible solutions highlighted in the previous chapters include either time-consuming sample pre-enrichment steps, or the use of more sensitive transduction mechanisms than colorimetry, such as electrical transducers that have shown excellent sensitivities to their environment [13]. **The purpose of this chapter is to initiate the accommodation of electrical transducers to the complex and highly inhomogeneous medium of porous paper substrates towards the detection of pathogens.**

This chapter is partly adapted from [94], a journal publication resulting from a solid collaboration with Margo Hauwaert in the context of her master thesis [212].

6.1 Abstract

Paper substrates are promising for development of cost-effective and efficient point-of-care bio-sensors, essential for public healthcare and environmental diagnostics in emergency situations. Most paper-based biosensors rely on the natural capillarity of paper to perform qualitative or semi-quantitative colorimetric detections. To achieve quantification and better sensitivity, technologies combining paper-based substrates and electrical detection are being developed. In this work, we demonstrate the potential of electrical measurements by means of a simple, parallel-plate electrode setup towards the detection of whole-cell bacteria captured in nitrocellulose (NC) membranes. Unlike current electrical sensors, which are mostly integrated, this plug & play system has reusable electrodes and enables simple and fast bacterial detection through impedance measurements. The characterized NC membrane was subjected to (i) a biofunctionalization, (ii) different saline solutions modelling relevant water samples, and (iii) bacterial suspensions of different concentrations. Bacterial detection was achieved in low conductivity buffers through both resistive and capacitive changes in the sensed medium. To capture *Bacillus thuringiensis* cells, the model microorganism used in this work, the endolysin cell wall binding domain (CBD) of Deep-Blue, a bacteriophage targeting this bacterium, was integrated into the membranes as recognition bio-interface. This experimental proof-of-concept illustrates the electrical detection of $\sim 10^7$ CFU.mL⁻¹ bacteria in low-salinity buffers within 5 min, using a very simple setup. This offers perspectives for affordable pathogen sensors that can easily be reconfigured for different bacteria. Water quality testing is a particularly interesting application since it requires frequent testing, especially in emergency situations.

Keywords paper-based sensors; nitrocellulose; impedance measurements; dielectric properties; parallel-plate electrodes; interdigital electrodes; endolysins; *Bacillus thuringiensis*.

6.2 Introduction

The benefits of paper, or paper-derivative, such as capillarity, ease of bioreceptor functionalization, low price, straightforward handling and manufacturing made it a valuable platform for biodetection assays as presented in chapter 3 for the LFA. In order to enable new functionalities (sam-

ple pre-treatment, multi-reactions or multi-detection schemes), the highly simple structure of the **LFA (1-dimensional fluid transport) have recently evolved into microfluidic paper analytical devices (μ PADs)**, introduced by Whitesides in the 2000's [213], presenting more complex structures enabling 2- or even 3-dimensional fluid transport.

The opportunities offered by the μ PADs functionalities include the integration of new transducers to complement the conventional colorimetry, such as electrodes, enabling the design of new microfluidic paper electrochemical devices (μ PEDs) that are performing electrochemical detection of bio-analytes on paper substrates.

Conventional electrical biosensors rely on the monitoring of changes in material electrical properties when bacteria bind in close proximity from the surface of, e.g., interdigital electrodes (IDE) designed on a solid substrate (e.g. silicon, glass, plastics). These biosensor benefit from advanced microelectronic manufacturing and materials, enabling the manufacture of electrodes on a microscopic scale, i.e. in the order of magnitude of the size of pathogens, making it possible to be sensitive to these contaminants [13, 214, 215]. The signal response, often proportional to the number of bacteria, is used as an electrical fingerprint of the sample to provide fast, precise and quantified information about the bacteria presence in water. Impedimetric pathogen sensors based on microelectronics technologies can reach LoD as low as 10^2 CFU.mL⁻¹ in short time period (e.g. 20 min) for label-free detection of whole-cell pathogens. For a comprehensive review of microtechnology-based impedimetric pathogen biosensors, see [13].

However, particular mechanisms of electron transfer between electrodes and specific bacterial cells, as well as within the cells, are still under fundamental studies in bioelectrochemistry [216]. Further, the production and usage of electrical biosensors face significant obstacles. Grafting a biorecognition layer, e.g., bacteriophages, phage-proteins or antibodies, on the surface of conventional surface-based electrical biosensors faces problems such as reproducibility, uniformity and stability over time [95]. The functionalization protocol needs to be adapted to every surface material and grafting molecule. In addition, the capture percentage of bacteria by the biorecognition layer is relatively low since only bacteria in close vicinity to the surface bind to the specific receptors. Many of the target pathogens are thus flowing over the electrode without binding, decreasing the sensor sensitivity. Finally, conventional surface-based electrical biosensors utilize gold electrodes functionalized with bioreceptors/antibodies using classical thiol chemistry, introduced in section 3.4 for the coating of nm-sized AuNPs [217, 218]. However, insufficient chemical stability of thiolates on

large planar surface area is one of the most serious problems for their applications in ambient and aqueous environments [219].

Despite the aforementioned drawbacks of both individual sensing technologies, studies have shown that the **integration of highly sensitive electronic transducers with μ PADs**¹ is an attractive approach to circumvent these drawbacks and capitalize on the advantages of paper substrates and electrical biosensors [54]. Given the favorable electrostatic properties of nitrocellulose, bioreceptors can be readily immobilized through the whole pore volume of the paper membrane, thus drastically increasing the number of interactions with targeted pathogens. Unlike surface-based methods, electrical measurements taking advantage from paper-porosity thus allow to quantify the number of bioreceptor-bacteria conjugates in the whole tested sample volume. However, the **development of electrical paper-based sensors remains very challenging** for two main reasons :

1. Due to significantly different properties of paper-based and more conventional substrates for electronic circuits, innovative design methods are needed. Alternative manufacturing techniques, e.g., conductive ink-based inkjet- or screen-printing to manufacture sensors such as IDE, are being explored towards integrated sensing devices [31, 55, 220]. However, integrating electronics on paper substrates shows difficulty of implementation because of the inhomogeneous nature of the paper, resulting in low electrode resolutions ($\sim$ hundreds of μm) and high electrical resistances [221] with respect to the classical design of microelectrodes suitable for bacteria sensing ($\sim$ a few μm finger gaps) [13].
2. Prior works that attempted to accommodate electrical bacteria detection on common μ PADs were mostly based on direct charge transfer measurements [222, 49]. However, these require cumbersome redox probes. Furthermore, electrical measurements are influenced by the ionic strength of the analyzed solution. Direct current measurements are particularly affected by this ionic background noise, since they only measure the solution resistance, inversely proportional to the number of ions. When dealing with aqueous samples presenting varying electrical conductivities, it is therefore challenging to calibrate the sensor and to differentiate the electrical response of target compounds from the electrical background signal.

Contributions In this chapter, we address the aforementioned challenges by emphasizing **three contributions towards paper-based electrical biosens-**

¹This way, μ PADs become microfluidic paper electrochemical devices (μ PEDs)

ing for simple and rapid bacteria detection in water :

1. First, we propose a paper-based electrical sensor design profiting from the porosity of the NC membrane to capture whole-cell bacterial suspensions with a high efficiency into the membrane pores. The membrane is functionalized with the endolysin CBD (PlyB221) introduced in chapter 5. *Bacillus thuringiensis* [148] were used as target pathogen in this work. This sensor design ensures high efficiency binding and immobilizing capacity towards this specific bacteria, enabling further volume-based electrical measurements.
2. Second, we demonstrate the relevance of the simple parallel-plate setup presented in [93] to accomodate electrical detection on paper-based substrates. This common material dielectric measurement system can be judiciously used as sensor to perform electrical measurements on NC membranes inserted in between the electrodes. This plug-and-play setup eliminates the unpractical need to integrate electronics components directly onto the paper matrix.
3. Third, **non-Faradaic impedance sensing** is proposed to monitor the porous NC membrane permittivity changes caused by various electrolyte solutions. Since no electron transfer occurs at the electrode surface, changes in electrical properties are mainly observed through **volumic impedance properties**. By measuring the later with an AC signal, both resistive and capacitive properties are estimated at high frequencies, which enables the label-free and non-intrusive detection of bacteria immobilized by specific bioreceptors. Indeed, their presence in the membrane affects both the global conductivity and permittivity [13].

This chapter is organized as follows :

1. We begin by **introducing the principles of the electrical impedance spectroscopy (EIS)** used in the chapters 6 and 7.
2. Then, we electrically **characterized the biointerface** formed by the phage proteins on the nitrocellulose membrane.
3. In order to characterize the system behavior and ionic noise for aqueous solutions with different ionic strengths, we then analyzed and calibrated the **sensor response and resolution to dilutions of saline solutions**, used as models of real aqueous samples. An equivalent electrical model of the sensing system was developed to quantify the impact of ionic concentration on the total measured impedance.
4. Then, the **sensing principle was validated in the presence of bacterial cells**. A proof of concept of the simple and rapid (< 5 min)

parallel-plate biosensor was demonstrated by detecting *B. thuringiensis* cells in low-conductive buffers.

5. Finally, the **bacterial detection results** with the plug & play parallel-plate setup were compared with a planar fringing field electrodes system, composed of IDE directly applied on a single side of the NC membrane. The sensing principles of both parallel-plate and IDE devices were modeled and analyzed using small-signal electrical equivalent circuits, highlighting the **contribution of ions in both bacterial detection mechanism**. Their potentials, advantages and limitations are also discussed.

6.3 Impedance spectroscopy for pathogen biosensing

The theory elements of this section are gathered from several sources, which can be consulted for a deeper understanding of the complex impedance behaviour or for numerous examples of applications for pathogen biosensing [223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233], and from recent works in our laboratory [13, 212].

Impedance spectroscopy is a powerful technique to investigate the electrical properties of materials, or the features of surface-modified electrodes. In impedance-based detection systems, a small-amplitude perturbing sinusoidal (AC) voltage signal of amplitude V_0 is applied over a range of frequencies via one electrode to the electrochemical cell, comprising the electrodes and the system under test, and the resulting AC current response is measured using a second electrode and characterized by an amplitude I_0 and phase θ with respect to the excitation signal. The complex impedance $Z(\omega)$ is calculated as the ratio between the complex system voltage, $V(j\omega)$, and the complex current, $I(j\omega)$, the latter being generated by a impedance analyzer (or frequency response analyzer) during the experiment :

$$Z(j\omega) = \frac{V(j\omega)}{I(j\omega)} = \frac{V_0 \cdot e^{j\omega t}}{I_0 \cdot e^{j\omega t + \theta(\omega)}} \quad (6.1)$$

with $\omega = 2\pi f$ the angular frequency, or pulsation, and have units of rad.s^{-1} and f the excitation frequency in Hz.

In biosensing applications, the derived impedance is representative of the electrical signature of the sample at a certain time, and its dynamic. For example, minute changes of the complex impedance $Z(\omega)$ are monitored when biological species bind between the electrodes. Two transduc-

tion mechanisms using impedance are distinguished: (i) **electrochemical impedance spectroscopy**, which utilizes electrochemical reactions from redox probes (electroactive species such as $[\text{Fe}(\text{CN})_6]^{3-/4-}$) to quantify the electron transfer through the charge transfer resistance R_{ct} (**Faradaic**) and (ii) **electric impedance spectroscopy**, which involves no electron transfer between the two electrodes (**non-Faradaic**). For EIS involving electrochemical reactions, it is critical to control the electrolyte potential to have reproducible results. In that case, the impedance is measured between a counter (CE) and a working electrode (WE), while a reference electrode (RE) maintains a constant potential in the electrolyte thanks to a potentiostat. In the case of non-faradaic electrodes, the solution potential does not necessarily need to be controlled since no electrochemical reactions occur. As a result, a two electrode system is sufficient to probe the system impedance [13].

In impedance sensing, two graphical representations of the complex impedance $Z(\omega)$ are typically used and come from two possible decompositions of $Z(\omega)$:

$$Z(\omega) = |Z(\omega)| \cdot e^{j\theta_Z(\omega)} \quad (6.2)$$

$$= \text{Re}(Z(\omega)) + j\text{Im}(Z(\omega)) \quad (6.3)$$

The complex impedance can be presented as the sum of the real, $\text{Re}(Z(\omega))$, and imaginary, $\text{Im}(Z(\omega))$ components that originate mainly from the resistance and capacitance of the cell, respectively. Based on equation 6.2, **Bode plots** represent the impedance modulus $|Z(\omega)|$ and phase $\theta_Z(\omega)$ in function of the frequency f . The **Nyquist plot**, also called the Cole-Cole plot, is based on equation 6.3 and represents the opposite imaginary part $-\text{Im}(Z(\omega))$ of $Z(\omega)$ as a function of its real part $\text{Re}(Z(\omega))$.

For the purpose of illustrating typical curves observed during impedance measurements, which are often directly displayed as Bode and Nyquist plots, we consider a parallel RC circuit for its relevance in dielectric material characterization and modelisation, including fibrous and porous materials [234]. The capacitor accounts for the dielectric constant of the material, which relates to the electromagnetic energy stored in the material due to polarization of its dipoles, while the resistor accounts for the dielectric loss². Note that biomatter is considered a dielectric substance, exhibiting conductivity on application of an electric field. The impedance of a parallel RC circuit is

²Dielectric losses account for loss of electromagnetic energy in a dielectric attributed to the polarization and conduction currents [226].

$$Z(\omega) = \frac{R}{1 + j\omega RC} \quad (6.4)$$

$$|Z(\omega)| = \sqrt{\frac{R^2}{1 + \omega^2 R^2 C^2}} \quad (6.5)$$

$$\Phi(Z(\omega)) = \text{atan}(-\omega RC) \quad (6.6)$$

Observing equation 6.4 and Figure 6.1, we see that when the frequency is very high, the total impedance of a parallel RC-circuit tends towards a closed circuit, which means that the circuit behaves more like a capacitor at infinite frequency. On the other hand, when frequency tends to 0, the total impedance tends towards R , independent from the frequency. The impedance phase corresponding to a resistive behavior is 0° while the phase corresponding to a capacitive behavior is -90° . The phase analysis remains essential to attest the dominant behavior. Equation 6.6 indicates the crucial value of $\omega = 1/RC$, called the **relaxation angular frequency** or cutoff frequency, which corresponds to the frequency at which the maximum of the imaginary part occurs³, i.e. at the transition between both resistive and capacitive behaviors.

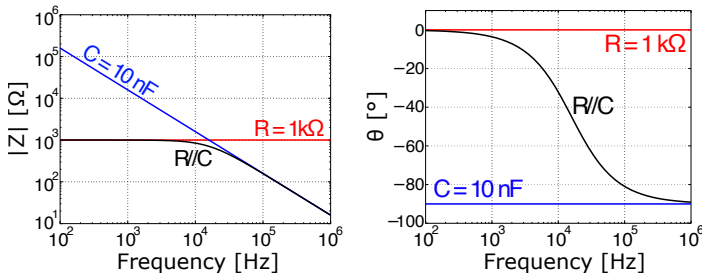


Fig. 6.1 Representation of the complex impedance $Z(\omega)$ for a $1\text{-k}\Omega$ resistance R , a 10-nF capacitance C and the parallel association of both using Bode plots of the impedance modulus $Z(\omega)$ and phase θ_Z versus the applied frequency f . Reproduced from [13].

Electrochemical transformations occurring at the electrode/electrolyte interface can be understood and modelled by extracting components of the electronic equivalent circuits that correspond to the experimental impedance spectra. As several distinct configuration may result in the same impedance, the equivalent circuit needs to make physical sense [212]. For impedance

³Also seen as the dielectric loss factor reaches a maximum.

measurements, a general electronic equivalent circuit, the **Randles model** (Figure 6.2 A) is very often used to model interfacial phenomena for a metal (i.e. the electrode) immersed in a liquid (e.g. the buffer containing the target bioanalytes). When solid materials are immersed in electrolytes, charge redistribution occurs at the interface, giving rise to an electrical double layer. The Randles model includes the ohmic resistance of the electrolyte solution, R_s , the double layer capacitance, C_{dl} . Related to electrochemical reactions, the Warburg impedance, Z_W , characterizes the ion diffusion from the bulk electrolyte to the electrode interface through the double layer, and the electron charge transfer resistance at the electrode-solution interface R_{ct} that exists if a redox probe is present in the electrolyte solution, i.e. in the case of a Faradaic impedance sensor.

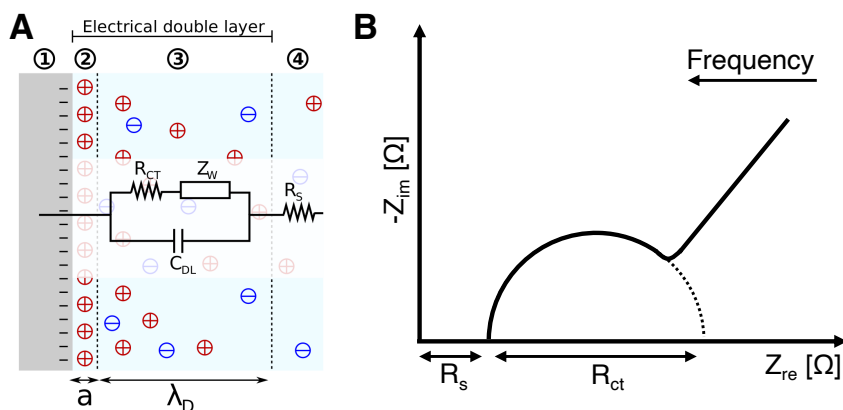


Fig. 6.2 **A.** Schematic representation and Randles equivalent electrical model for a solid-electrolyte interface. When a metal is immersed in an electrolyte, charges redistribute to form an electrical double layer, with different charge distributions. R_{ct} is the charge transfer resistance, Z_w the Warburg impedance, C_{dl} the double layer capacitance and R_s the electrolyte resistance. Reproduced from [13]. **B.** Schematic Faradaic impedance spectra presented as a Nyquist plot for a system where the impedance is controlled by diffusion of the redox probe at low frequencies (Z_w dominant) and by the interfacial electron transfer at high frequencies (R_{ct}). The circle is representative of the pure RC parallel circuit, illustrating the interplay between the capacitive and resistive behaviours, while the off-centre is due to the addition of R_s in serie.

The resistance, capacitance and Warburg impedance are the basic circuit elements that are commonly used to describe impedance behavior of an electrochemical cell. Sometimes, particularly when the electrode surface is rough [224], the electronic properties of the interface cannot be de-

scribed sufficiently well with a purely capacitive element, and a constant phase element (CPE) is introduced to better represent the real phenomena, which is a mathematical construction to describe the observed data. For detailed explanations, modelling and mathematical expressions of the Warburg impedance and CPE, refer to [212].

Physically, R_s and Z_W represent bulk properties of the electrolyte solution and the diffusion of redox probes in solution, respectively. Therefore, these parameters are not affected by chemical transformations occurring at the electrode surface, but rather sensitive to dielectric changes in a volume lying between two planar electrodes (e.g. patterned at the top and bottom of a microfluidic channel [13]). This is mainly used to detect large cells (e.g. bacteria) suspended in the electrolyte, including bacterial counting such as impedance flow cytometry. When used with conventional microfluidic channels (e.g. plastics), its main disadvantage is the inability to selectively distinguish one bacteria from another since there is no specific bioreceptor for their capture. On the other hand, R_{ct} and C_{dl} depend on the insulating and dielectric features at the electrode/electrolyte interface (e.g. binding of biomolecules on the electrode surface) and are the main parameters used to analyze the response of a surface-sensing impedance biosensor. Surface-based method uses functionalized planar electrodes atop of a solid substrate where bacteria progressively accumulates. The main disadvantage of this method is that many cells flowing atop the sensor are lost, reducing the sensor sensitivity.

The parallel elements (C_{dl} and $Z_W + R_{ct}$) of the equivalent circuit are introduced since the total current through the working interface is the sum of distinct contributions from the charge transfer (Faradaic process) and the interfacial double-layer charging. Since all of the current must pass through the uncompensated resistance of the electrolyte solution, R_s , is inserted as a series element in the circuit [224].

In the Nyquist plot (Figure 6.2 B), the spectral information is not explicit. The semicircle is swept through in counterclockwise direction as the frequency increases. Both graphs offer valuable information for understanding the spectral behavior of a measured sample. Although the spectral information provided by Bode plots is more consistent for direct interpretation of the EIS experiment results, Nyquist plots better represent time constants and are suitable for visualization of impedance detection relying on the change of frequency-independent parameters such as the charge transfer resistance R_{ct} in Faradaic EIS [235].

6.4 Materials and Methods

6.4.1 Materials

Nitrocellulose membranes on a polyester backing (UniSart Lateral Flow CN95 Backed, 20 μm nominal pore size referred by the supplier - 12 μm mean pore size found in section 3.6) were purchased from Sartorius (Germany). Phosphate buffered saline (PBS 10mM, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4) and sodium chloride solution (NaCl, 1 M) were purchased from Sigma-Aldrich (USA). Deionized (DI) water was produced in our facilities (conductivity $\sigma = 6.6 \times 10^{-6}$ S/m).

6.4.2 Biological procedures

Bacterial Strains and Growth Conditions

B. thuringiensis GBJ002 expressing the Cyan Fluorescent Protein (CFP) was used as proof-of-concept for this study. Bacteria were plated on Lysogeny Broth (LB)-agar plate containing kanamycin (50 $\mu\text{g}/\text{ml}$) and acid nalidixic (25 $\mu\text{g}/\text{ml}$) and grown overnight (O/N) at 30°C. Next, an individual colony was used to inoculate 5 mL of LB supplemented with antibiotics and the suspension was incubated O/N at 30°C with agitation (120 rpm). Then, the culture was washed twice with an appropriate buffer (water or diluted PBS) to remove residual LB (centrifugation at $10000 \times g$ for 5 min at room temperature (RT)). The pellet was finally re-suspended in the appropriate buffer yielding a bacterial suspension of ca. 10^8 CFU mL^{-1} .

Phage Endolysins Expression and Purification

A detailed description of the expression and purification of the CBD of PlyB221 endolysin, encoded by phage Deep-Blue can be found in [148]. The CBD was fused to a Green Fluorescent Protein (GFP) for fluorescence assays. The protein concentration was adjusted to 1 mg/mL.

Preparation and Characterization of the CBD Biointerface for Specific Bacteria Capture

A protocol for optimal biofunctionalization of the NC membrane pieces with endolysin CBD based on simple physisorption was developed (Figure 6.3). Membranes were prepared by individually depositing and shaking 50 μL of 1 mg/mL solution of purified CBD on the NC. Membranes were then dried in an oven for 60 min at 37°C followed by desiccation for 30 min at RT to fix the proteins to the membrane. Following desiccation,

membranes were washed twice during 2 min with deionized (DI) water to remove proteins in excess. Membranes were wiped, dried, and finally stored in a desiccation chamber at RT. Effective binding of specific bacteria to the biointerface was assessed by depositing a droplet of *B. thuringiensis* suspension on the biofunctionalized membranes followed by a washing step (Figure 6.3). Bacteria immobilization in the membrane depth was shown in chapter 5 (Figure 5.7).

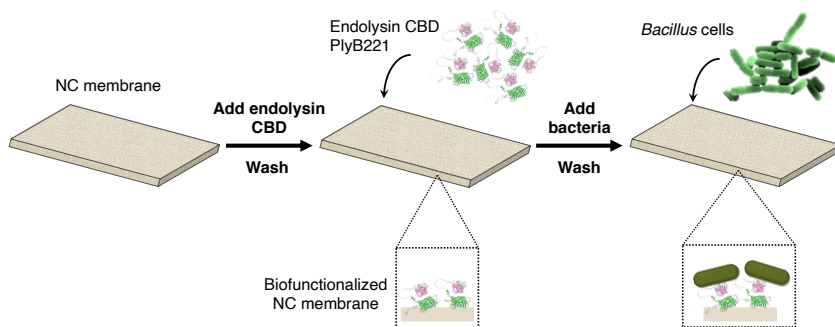


Fig. 6.3 Protocol of the NC membrane biofunctionalization with an endolysin cell-wall binding domain (CBD), PlyB221 and capture of *B. thuringiensis* cells. (1) The deposition of the phage endolysin CBD in the NC membrane is followed by drying, washing and desiccator steps. (2) The bacteria are then deposited on the membrane and specifically captured by the CBD.

6.4.3 Parallel-Plate Setup

Impedance Sensing

Figure 6.4A shows a schematic of the experimental system for the simple parallel-plate measurements. The nitrocellulose membranes were held between the two parallel-plates of the dielectric test fixture (16451B, Agilent, Santa Clara, CA, USA), connected to an impedance analyzer (LCR 4284A, Agilent, USA). The impedance spectroscopy measurements were carried out with the LCR, remotely controlled by a computer through the Labview software (Labview National Instrument, Austin, Texas, USA) to perform an automatic sweep from 1 kHz to 1 MHz, at voltage amplitude of 200 mV. Before impedance measurement, an open-circuit calibration was performed. The impedance data were extracted in a magnitude-phase data-structure. The ZVIEW software (Scribner Associates Inc, USA) is used to fit the electrical equivalent model of the parallel-plate sensing system, as presented in Figure 6.4A, and extract the model parameter values from the measurements. The material real and imaginary dielectric constant are de-

rived for a given membrane thickness by measuring its capacitance and dissipation factor. An electrical model is used to differentiate the actual dielectric properties of the NC from those of the polyester backing (Figure 6.4A).

Sensor Modelling

An analytical model of the NC membranes soaked with biological solutions and inserted in between the two parallel-plate electrodes is established to provide a physical understanding of the system. In presence of an electrolyte, the equivalent electrical model considers a double layer capacitance (C_{dl}). The equivalent circuit also considers the NC membrane, modelled through the parallel association of its capacitive (C_{NC}) and conductive (R_{NC}) properties, as well as the polyester backing, modelled using its capacitive properties only ($C_{Backing}$), since its dielectric losses are considered to be negligible in the studied frequency range. The value of the polymer backing capacitance (~ 100 pF) is determined experimentally through measurement of its permittivity by means of dielectric measurements (Section 6.4.3 Impedance Sensing). C_{NC} and R_{NC} , representing the electrical properties of the NC membranes saturated with saline solutions or bacterial suspensions, strongly depend on the ionic concentration in the solution. The double layer capacitance, representing the interfacial properties is given by [13]:

$$C_{dl} = \frac{\epsilon_0 \epsilon_{r,sol}}{\sqrt{\frac{\epsilon_0 \epsilon_{r,sol} k_B T}{2q^2 N_{av} c_{ions} 10^3}}} A_e \quad (6.7)$$

with C_{dl} the double layer capacitance, k_B the Boltzmann constant, c_{ions} molar ionic concentration of the solution in which the double layer occurs, $\epsilon_{r,sol}$ the relative permittivity of this solution and A_e the surface of the parallel-plate electrode. The values for C_{dl} at different ionic concentrations were simulated using COMSOL Multiphysics v.5.4 (COMSOL AB, Stockholm, Sweden) based on equation 6.7, and lie in the 10 – 100 μ F range. Regarding the values of the other parameters, their influence can therefore be neglected at the considered frequencies between 1 kHz and 1 MHz.

The equivalent model has three cut-off frequencies:

$$f_1 = \frac{C_{dl} + C_{Backing}}{2\pi \cdot R_{NC} \cdot [C_{dl}C_{Backing} + C_{NC}(C_{dl} + C_{Backing})]} \quad (6.8)$$

$$f_2 = \frac{C_{dl} + C_{Backing}}{2\pi \cdot C_{dl}C_{Backing}} \quad (6.9)$$

$$f_3 = \frac{1}{2\pi \cdot R_{NC}C_{NC}} \quad (6.10)$$

6.4.4 IDE Setup

Interdigital Electrode Design and Fabrication on NC Membranes

The deposition of IDE on nitrocellulose substrate was conducted using a physical vapor deposition (PVD) e-gun evaporation technique. Gold IDE were deposited by applying patterned nickel masks on top of the nitrocellulose membrane. This deposition technique is more cumbersome than screen-printing and inkjet printing techniques, which are usually used for deposition of electrodes on paper [236]. However, these have the disadvantage of not allowing precise electrode deposition on chemically untreated nitrocellulose. Hence, PVD is chosen because it allows for precise deposition without inducing variability through additional treatment. The IDE finger width and interdigit gap are 200 μm , which enables the detection of dielectric properties over the whole NC membrane depth ($\sim 140 \mu\text{m}$) [237].

IDE Impedance Sensing

Figure 6.4B shows a schematic of the experimental system for IDE measurements. The IDE were connected through toothless crocodile clips to an impedance analyzer (LCR 4284A, Agilent, USA) through BNC connectors. The impedance spectroscopy measurements were carried out with the LCR, remotely controlled by a computer through the Labview software (Labview National Instrument, Austin, Texas, USA) to perform an automatic sweep from 1 kHz to 1 MHz, at voltage amplitude of 20 mV. Before impedance measurement, an open-circuit calibration was performed without any electrical contacts between the crocodile clips. The impedance data were extracted in a magnitude-phase data-structure.

IDE Sensor Modelling

The equivalent circuit of the IDE in Figure 6.4B incorporates the surfacic phenomenon of double layer capacitance through C_{dl} and the volumic phenomena through C_{air} , R_{air} , C_{NC} , and R_{NC} , corresponding to the upper air layer and lower nitrocellulose layer, respectively. Given the width of

IDE fingers, the backing is not taken into account (Section 6.4.4 Impedance Sensing). The double layer capacitance for IDE electrodes in contact with a given solution is extended from equation 6.7 to

$$C_{dl} = \frac{\epsilon_0 \epsilon_{r,sol}}{\sqrt{\frac{\epsilon_0 \epsilon_{r,sol} k_B T}{2q^2 N_{av} c_{ions} 10^3}}} A_e (N - 1) \quad (6.11)$$

with A_e the surface per electrode finger and N the number of fingers. The equivalent resistances and capacitances of the nitrocellulose and air volume depend upon the cell constant, which is determined experimentally and incorporates the geometric properties of the IDE [238], and ϵ_r and σ the relative permittivity and conductivity of the sensed nitrocellulose and air volumes, respectively. Hence, it does not vary with frequency nor with the electrical properties of the material.

6.4.5 Sensing of Saline Solutions as Models for Relevant Water Samples

Prior to electrical measurements, sodium chloride solutions of various concentrations (c_{NaCl}), 10^{-5} , 10^{-4} , 5×10^{-4} , 10^{-3} , 5×10^{-3} , 10^{-2} , 10^{-1} mol.L $^{-1}$ [M], were prepared by dilutions of a 1 M NaCl solution in DI water, in order to model the electrical properties of different types of relevant water samples and biological buffers regarding the applications targeted in this study (Table 6.1). Regarding the impedance measurements considered in this study, the parameters of interest to be modelled are the mean ionic strength of the liquid (through adjustment of c_{NaCl}), and hence its dielectric properties (electrical conductivity and permittivity). Then, 50 μ L of the diluted sodium chloride solutions were deposited on NC membranes, placed in between the parallel-plate electrodes, and reference dielectric or impedance measurements were performed within 5 min. The parallel-plates were wiped between each measurement to remove remaining water and salt on the electrodes.

Modelled solutions	c_{NaCl} [M]	$\epsilon_{r,sol}$ [/]	σ_{sol} [S/m]
PBS:1000	1.6×10^{-4}	~ 80	1.8×10^{-3}
Drinking/surface water	$\sim 10^{-3}$	~ 80	$10^{-1} - 10^{-2}$
PBS/highly saline water	1.5×10^{-1}	~ 70	1 - 5

Table 6.1 Modelling of the electrical properties at 20°C of real aqueous samples and biological buffers using saline solutions at different concentrations. Mean concentrations and therefore dielectric properties are considered as the physico-chemical content of the water samples can vary from place to place [239, 240].

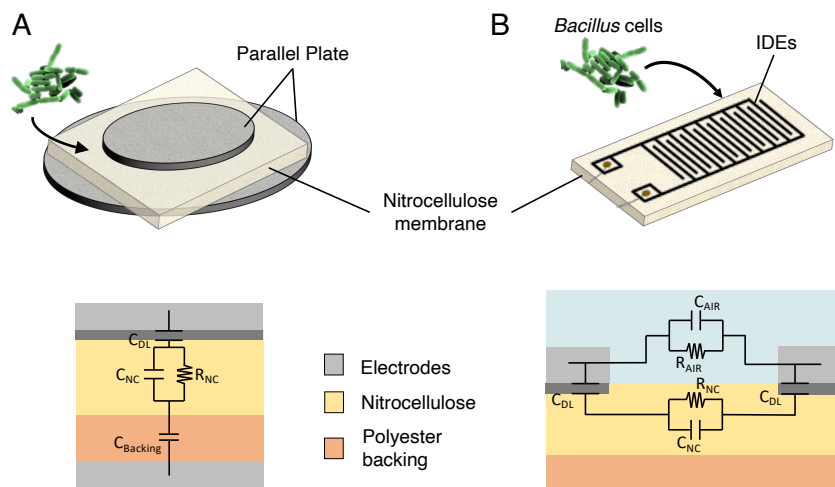


Fig. 6.4 Experimental setups and corresponding electrical models investigated towards bacterial detection in this work. **(A)** Parallel-plate probes are a common material dielectric measurement system. The bacterial sample is deposited on the NC membrane and captured at the test zone. A simple electrical model is proposed to consider the dielectric effect of the polyester backing supporting the NC and the electrical double layer that arises from charge redistribution at the interface between the electrolyte and the probe. **(B)** Interdigital electrodes are generally used as sensors to monitor impedance changes at the proximity of the metallic fingers, here deposited on NC membrane. The bacterial samples are dropped on top of the IDE-NC sensor. The model does not include the polyester backing as its impact on the impedance seen by the IDE is negligible due to its depth.

6.4.6 Bacteria Detection in Physiological Buffers

Before depositing bacterial suspensions, 50 μL of PBS buffer diluted 1000 $\times$ (PBS :1000) in DI water was deposited on a previously biofunctionalized membrane, and reference dielectric or impedance measurements were performed within 5 min with parallel-plates or IDE, respectively. PBS:1000 was chosen as biological buffer because largest detection sensitivities were shown to be achieved with low-salt buffer solutions [45, 13], and such low-salt buffers have electrical properties similar to surface water samples (Table 6.1). Five min is a time limit before which it is assumed that the wet impedance has not changed by more than 5% due to drying. Then, suspensions of 10^8 , 10^7 and 10^6 CFU mL^{-1} of stationary-state *B. thuringiensis* resuspended in PBS 1:1000 were deposited (50 μL) on top of the membrane sample with a micropipette (Figure 6.4A and 6.4B) and spread within the

membrane due to capillarity, and impedance or dielectric measurements were performed. In order to observe the global sensitivity of the setup, including both sensitivities of the impedance modulus and phase to bacteria presence, the sensitivities (S) in the explored frequency range were computed as the amplitude of the differences in complex impedance measured with and without bacterial cells, in percent:

$$S = \left| \frac{Z_{Bact} - Z_{PBS}}{Z_{PBS}} \right| \quad (6.12)$$

6.5 Results

6.5.1 Characterization of Dry and Biofunctionalized Nitrocellulose

In order to characterize the impact of the biofunctionalization on the membrane electrical properties, we performed dielectric measurements with the parallel-plate electrodes on raw and CBD-biofunctionalized NC membranes, under dry conditions. The measured permittivity of a raw nitrocellulose membrane drops from 1.52 to 1.5 between 1 kHz and 100 kHz (Figure 6.5). The biofunctionalization is observed to cause a permittivity reduction of approximately 1-3%.

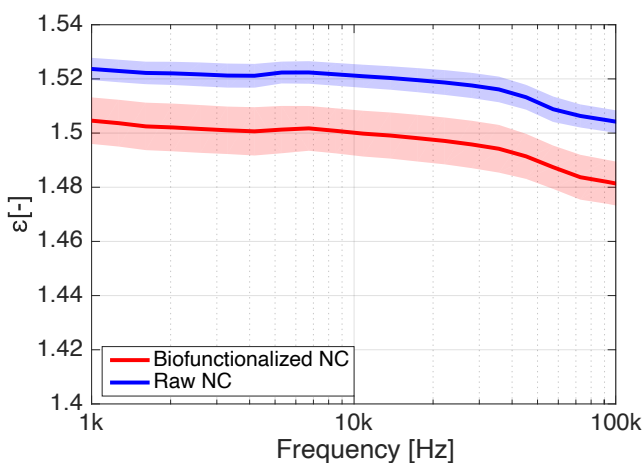


Fig. 6.5 Relative permittivity of raw and CBD-biofunctionalized nitrocellulose membranes over 1 kHz - 100 kHz. The biofunctionalization process causes a small decrease in the permittivity. Total number of samples: 7. Shaded area surrounding the measurement curves: standard error (σ).

The permittivity observed for the nitrocellulose membranes can be described by simple modelling of the membrane actual permittivity. Since the measurements were performed under dry conditions, the pores are filled with air, and both the fibers and the pore content are insulating materials. Modeling the permittivity of the bulk material is challenging due to its membranous structure, making it difficult to extract the permittivity of the nitrocellulose porous matrix as a standalone parameter. The nitrocellulose matrix and the voids within the pores form an interconnected network. Based on mixing rules for dielectric constants, we can approximate the

bulk permittivity of dried nitrocellulose sheets using a linear volumetric average of both values:

$$\epsilon_{dry,NC} = v\epsilon_{air} * (1 - v)\epsilon_{NC} \quad (6.13)$$

with v representing the void fraction of the membrane. Based on the bulk permittivity value of nitrocellulose ($\epsilon_{NC} = 6.4$, as reported by Dixon⁴ and an approximate porosity of 80% for the membranes (as provided by Sartorius, the membrane supplier), we can predict a permittivity value of $\epsilon_{dry,NC} = 2$.

The measured permittivity using the parallel plate test-fixture is approximately 1.52, which is slightly lower than the predicted value. Several hypotheses emerge to explain this difference, including variations in dielectric properties between the nitrate cellulose used for prediction and the actual permittivity values of nitrocellulose, a real void volume different from the nominal value provided by the supplier, and so on.

The difference between the two dielectric constant for raw and biofunctionalized nitrocellulose membranes is small (between 1 and 3%). Second, the bio-functionalization protocol can substantially modify the permittivity of the NC sheets by altering its pore surface properties. Indeed, under conditions of low humidity reached after the desiccator step in the protocol, nitrocellulose membranes accumulate a significant static charge, affecting the dielectric measurements. In addition, the difference in permittivity could be explained by the electrostatic charge inherent to every protein. A study by Low *et al.* shown that the charge of a NC surface with immobilised proteins changed to be almost the same charge as that of the protein. Their finding strongly suggested that the protein (BSA) was adsorbed onto the membrane pore walls and dominated the surface charge of the NC membrane, which is therefore controlled by the protein properties [175].

However, such small difference between the two membrane could simply result from a slight difference in handling due to the pressure applied by the parallel-plate setup used with the Novocontrol measurement device on the sample. Despite the presence of a micrometer screw to ensure a consistently identical gap during measurements, slightly different conditions could lead to variations in nitrocellulose thickness. Indeed, since the mem-

⁴<https://www.dixonvalve.com/sites/default/files/product/files/brochures-literature/dielectric-constant-values.pdf>

branes undergo a biofunctionalization process, the functionalized membrane may undergo modifications such as slight swelling, although nitrocellulose membranes are relatively robust to these microstructural variations as they are not composed of cellulose fibers per se (unlike cellulose sheets). The swelling, consequence of the drying of the nitrocellulose membrane during the process steps, could lead to thickness modifications, or even the reorganization of fibers following drying (however, as mentioned above, nitrocellulose is relatively robust and less susceptible to microstructural property variations than cellulose sheets). At the light of all these factors, it is difficult to assert that the permittivity of nitrocellulose membranes is significantly modified after the biofunctionalization process.

Finally, the shaded area around the measurement curves in Figure 6.5 express the standard errors bars, indicate a certain variability in the measurements. It could be explained since membranes present a foam-like structure (Figure 3.8) resulting in structural anisotropy and surface inhomogeneity (the fibers within the matrix are not uniformly arranged), which renders absolute measures of the permittivity difficult as the solver algorithm assumes that the material under test is homogeneous [241].

6.5.2 Calibration of the Parallel-Plate Sensor to Electrolyte Conductivity

In order to understand the response of the parallel-plate sensing system and allow to discriminate bacteria electrical contribution, it is of utmost importance to characterize the effect of the background ionic noise resulting from remaining dissolved salt in the biological suspensions. Not monitoring or controlling the ionic concentration of the electrolyte where bacteria are suspended could lead to misinterpretation of the electrical results, as the supposed detection of elements could be caused by changes in the background ionic strength. To this end, we first characterized and calibrated the response of the parallel plate setup to saline solutions.

The concentrations of the saline solutions were chosen to represent background ionic noise for different water sources of interest (Table 6.1). Impedance measurements were first carried out to investigate the impedance modulus and phase dependence upon the salt concentration between 1 kHz and 1 MHz. Figures 6.6A and B show that the sensor discriminates the different saline concentrations through shifts of both impedance magnitude and phase. A global decrease of the impedance magnitude is observed with increasing NaCl concentration, since increased salt concentration increases the conductivity σ_{sol} . This magnitude decrease is higher between 10 kHz and 1 MHz, where the impedance phase is mostly resistive: this is where R_{NC} has the most effect on the total complex impedance. Even if these

peaks tend towards resistive impedance angles, they stay lower than -45° given that no direct conduction path exists between the two parallel electrodes due to the isolating backing (Figure 6.4A). At both sides of these peaks, the phase decrease indicates a transition from a mixed to an exclusively capacitive behavior, led by the volume capacity C_{NC} at higher frequencies. We observe a frequency shift of the peak, shifting towards higher frequencies when the salt concentration increases. This can be explained by equations 6.8 and 6.10: f_1 and f_3 correspond to the middle of the upwards and downwards flank of the peak. These cut-off frequencies increase when the salt concentration increases, given the variation of R_{NC} and C_{NC} in Table 6.2.

The working frequencies of the sensor towards detection of saline solutions corresponding to ionic noise of interest (Table 6.2) lies in the range 10-200 kHz. The limit of detection of the system (LOD) lies between 10^{-5} M and 10^{-4} M since the sensing device was not able to differentiate significantly ($< 3\sigma$) impedance modulus and phases. This LOD corresponds to very low salinity electrolytes, less conductive than most of the buffers considered in biological detection schemes, which is beyond the scope of interest for the sensor-applications.

In order to quantify the impact of changes in salt concentration on the global system impedance and quantitatively calibrate the sensor response to the saline solutions, we extracted the values of R_{NC} and C_{NC} for the different salt concentrations based on the simple electrical equivalent model of the parallel-plate setup (Figure 6.4A). The values of the components, extracted from the fitting of the raw impedance data depicted on Figure 6.6, are reported in Appendices Table E.1. Figure 6.7 and Table 6.2 summarize the observed changes in the parallel-plate equivalent circuit elements for different NaCl concentrations. Between 1kHz and 1MHz, we observe that the impedance measurements are particularly sensitive to the electrical conductivity (σ_{sol}) of the electrolyte, as it directly influences R_{NC} .

The system is sensitive to both resistive and capacitive effects, even though, comparatively, the increasing ionic strength of the solution is more sensed through R_{NC} than C_{NC} (Table 6.2). In addition, the quantitative evaluation of R_{NC} and C_{NC} also supports the LOD of 10^{-4} M, as the difference in impedance, resistance and capacitance with the 10^{-5} M solution is less than 3 times the standard deviation (σ).

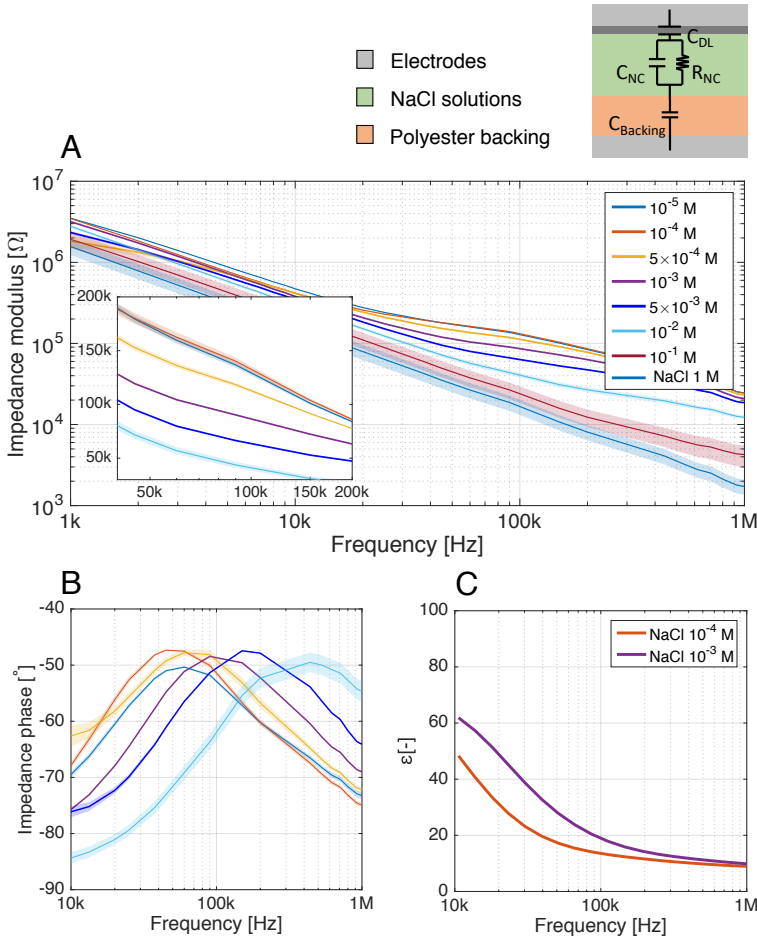


Fig. 6.6 (A) Impedance measurements of the nitrocellulose (NC) membrane with isolating backing as seen by the parallel-plate setup. The NC membrane is saturated with saline solutions of different molar concentrations, modelling the dielectric properties of the relevant water samples described in Table 6.1. A significant decrease in the impedance modulus results from an increase of the ionic strength, expressed as a drop in the membrane resistance R_{NC} . (B) The impedance phase evolution over 10 kHz – 1 MHz highlights the contribution of both R_{NC} and C_{NC} to the impedance of the system. As the resistive peak in the phase shifts with the ionic strength, the system is therefore sensitive to saline electrolyte through both R_{NC} and C_{NC} changes. (C) Dielectric relative permittivity of the system as seen by the parallel-plate setup, with different diluted salt solutions in the nitrocellulose membrane. Number of samples: 12 from two independent experiments. Shaded area surrounding the measurements curves: standard deviation (σ). σ smaller than the measurement line thickness if not visible on the graph.

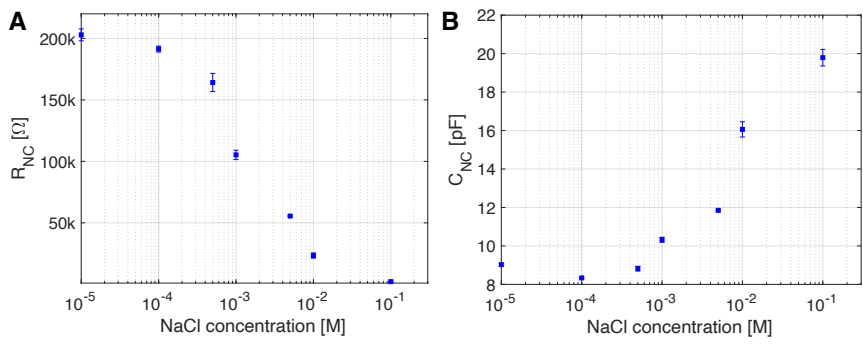


Fig. 6.7 Evolution of the nitrocellulose membrane **A.** resistance R_{NC} and **B.** capacitance C_{NC} subjected to NaCl solutions, whose concentration where selected to model different types of water samples through their conductivities. The circuit element values are obtained by fitting the simple electrical models to impedance data on Figure 6.6.

c_{NaCl} [M]	10^{-4}	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}	10^{-1}
ΔR_{NC}	$1.9 \times 10^4 \Omega$	-15%	-35%	-47%	-57%	-90%
ΔC_{NC}	8.34 pF	+5%	+17%	+15%	+35%	+22%

Table 6.2 Relative representation of the nitrocellulose membrane resistance R_{NC} and capacitance C_{NC} evolution depicted on Figure 6.7, showing consecutive relative decreases and increments with the concentration of the NaCl solutions used to model different types of water samples through their conductivities. The parallel-plate setup is more sensitive to R_{NC} , but is still responsive to capacitance changes of the membrane.

Dielectric measurements were carried out to corroborate the impedance measurements. Relative permittivity of NC membranes soaked with 10^{-4} M and 10^{-3} M NaCl solutions, modelling respectively highly diluted PBS (PBS:1000) and slightly saline solutions, was extracted over the frequency range of interest (Figure 6.6C). The permittivity of the saturated membrane with backing shows an increase of the system permittivity with the salt concentration. This is reflected in the increasing value of C_{NC} extracted from the impedance measurements (Table 6.2), even if the proportions are different since this system permittivity considers also the double layer and backing permittivity while C_{NC} only incorporates the volume of the nitrocellulose.

6.5.3 Detection of *B. thuringiensis* Cells with the Parallel-Plate Setup

After the characterization of the sensor response to ionic background noise in aqueous solutions, impedance measurements of *B. thuringiensis* resuspended in the low-salt buffer PBS:1000 was investigated to assess the electrical model proposed in Section 6.4.3 to the detection of label-free, whole bacterial cells. As the impedance measurements are extremely sensitive to the electrical conductivity σ_{sol} of the electrolyte, it is almost impossible without labels to directly predict the bacterial concentration from a single measure since two samples with identical bacterial loads but different conductivities would result in different signals. Effective discrimination of the electrical footprint of the bacterial cells from the ionic background noise thus requires comparing the sensor signal to an appropriate control value.

We therefore performed differential measurements, comparing the signal obtained with samples of 10^8 , 10^7 and 10^6 CFU mL⁻¹ of *B. thuringiensis* to blank PBS:1000 measurements (Figure 6.8A). Significant differences with 10^8 CFU mL⁻¹ and without bacterial cells were observed between 10 kHz and 1 MHz for both impedance modulus and phase, where the membranes soaked with bacterial solutions show a lower value of impedance modulus than the blank reference PBS:1000 buffer. The measurements for bacterial cells and PBS:1000 follow the same typical decrease of impedance modulus than observed for low salinity solutions (Figure 6.6A). The phase peak of the membrane filled with 10^8 CFU mL⁻¹ bacterial suspension (Figure 6.8B) is also subjected to a shift towards higher frequencies. The bacteria and PBS:1000 impedance modulus and phase curves show good fitting with the impedance modulus and phase of the NaCl solutions. In particular, PBS:1000 impedance curves lie in between the impedance curves of 2×10^{-4} M (not shown) and 5×10^{-4} M NaCl solutions, while the impedance of the solution containing the 10^8 CFU mL⁻¹ bacterial suspension lies between the 5×10^{-4} M and 10^{-3} M curves. Regarding the PBS:1000 buffer, the approximative correspondence of its dielectric properties to the range of 2.5×10^{-4} M NaCl solution is confirmed as the salt concentration used to model this buffer is precisely 1.6×10^{-4} (Table 6.1). To deepen the comparison between the bacterial and saline solutions, we have extracted the membrane resistance R_{NC} and capacitance C_{NC} of the electrical model under the PBS:1000 and 10^8 CFU mL⁻¹ bacteria conditions (see Appendices Table E.2 for the data). R_{NC} and C_{NC} extracted for the PBS:1000 condition diverges of around 5% from the 5×10^{-4} M NaCl condition, against about 10% between the solution containing 10^8 CFU mL⁻¹ bacteria and the 10^{-3} M NaCl model solution. For their part, the 10^7 and 10^6 CFU mL⁻¹ bacterial suspensions show a significant drop in impedance modulus (Figure 6.8A, inset) relative to the PBS:1000 buffer. However, unlike the 10^8 CFU

mL^{-1} suspensions, the shift in the phase peak is not significant for the 10^7 and 10^6 CFU mL^{-1} bacteria solutions (Figure 6.8B) and can therefore not be used to assess the bacteria presence in the membrane. In addition, the impedance modulus and phases of 10^7 and 10^6 CFU mL^{-1} suspensions are overlapping over the whole spectrum. **This suggests an intrinsic limit of detection of the parallel plate towards bacteria detection about 10^7 CFU mL^{-1} .**

Dielectric measurements were carried out to substantiate the bacterial detection results through impedance measurements (Figure 6.8C). Here again, the relative shift between PBS:1000 with and without bacteria follows a similar tendency than the model saline solutions. This tends to confirm the similarities in dielectric property variations between solutions with and without bacteria, and the differences in salt concentration. **It is thus consistent with the hypothesis that bacteria are sensed through increase in ionic concentration.**

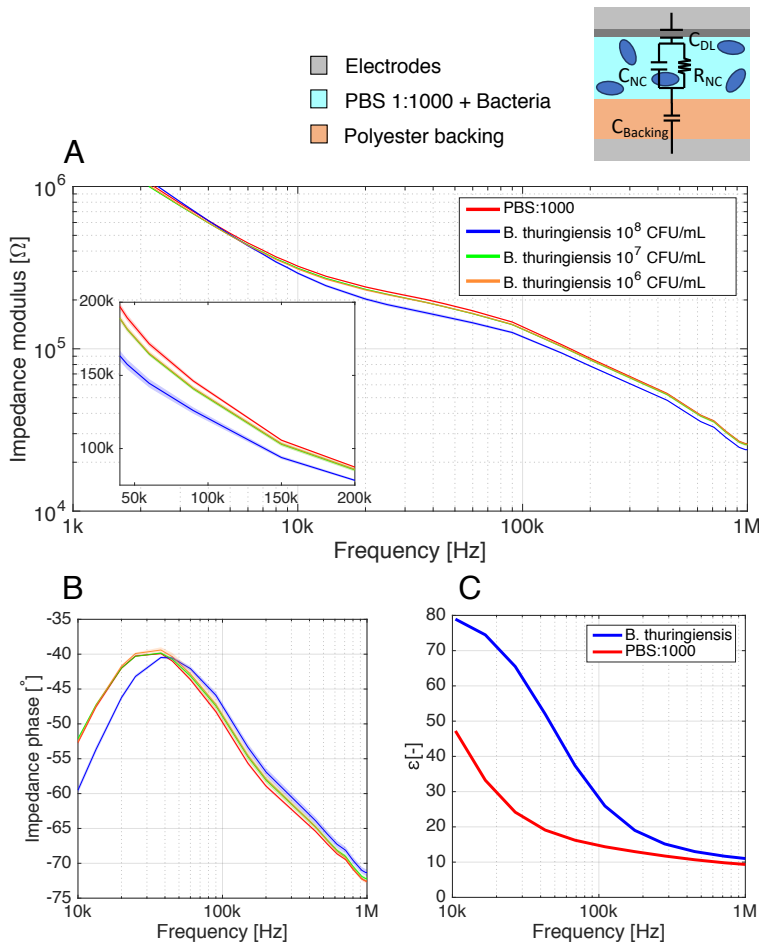


Fig. 6.8 (A) Impedance measurements performed on a nitrocellulose (NC) membrane as seen by the parallel-plate setup. The NC membrane is saturated with PBS:1000 (reference buffer) or with PBS:1000 containing 10^8 , 10^7 and 10^6 CFU mL $^{-1}$ of *B. thuringiensis* cells. The global impedance is decreasing, and the phase is subjected to a shift in presence of bacterial cells in the buffer, showing high similarities with the response of slightly saline solutions. (B) The impedance phase evolution over 10 kHz – 1 MHz, presenting a phase peak, indicates an interplay of R_{NC} and C_{NC} in the impedance of the system when subjected to bacteria. (C) Dielectric permittivity of the system as seen by the parallel-plate setup, when subjected to low-salt buffer with and without bacterial cells. Number of samples: 12 from two independent experiments. Shaded area surrounding the measurements curves: standard deviation (σ).

6.5.4 Comparison with Another System: *B. thuringiensis* Detection with the IDE Setup

In order to assess the detection results obtained with the plug-and-play parallel-plate system where the electrodes are deposited, we considered to deposit metallic IDE directly on top of the nitrocellulose membrane to reach an integrated sensing device.

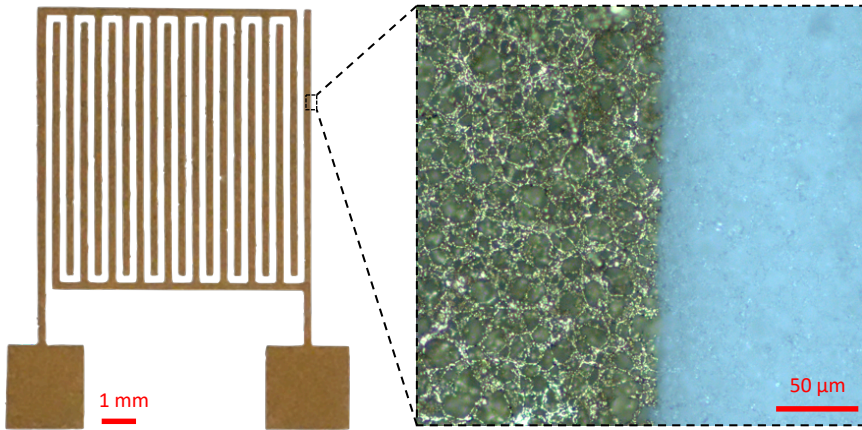


Fig. 6.9 Optical microscopy images of Au-IDE (200 μm of interdigit gap) deposited on a nitrocellulose membrane. The inset is a zoom in image (magnification $\times 20$) of the electrode showing the Au deposition on the membrane surface as well as in the first microns of the membrane thickness due to NC porosity.

Gold IDE Deposited on Nitrocellulose Membranes

Au-IDE were successfully deposited on top of the NC membranes, showing good adherence with the support (Figure 6.9). The Au-deposited thin-film follows the porous microstructure of the membrane, and shows good conductivity.

*Detection of *B. thuringiensis* with the IDE setup*

Interdigital electrodes, which are among the most commonly used periodic electrode structures for fringing field detection [237], were used for impedance measurements in order to substantiate the parallel-plate measurements results. An important advantage of this electrode design is that only a single-side access to the test material is required.

Figure 6.10A shows a lower impedance modulus due to bacterial presence, with a resistive angle. This express that bacterial solutions are sensed through a decrease in the solution resistance resulting from an increase in conductivity. Given the single-side access to the material, the conductive phenomena of the nitrocellulose soaked with PBS:1000 are not shielded by the isolating backing layer, resulting in highly resistive impedance phase over the 1kHz – 1 MHz range. The sensitivity towards 10^8 CFU mL⁻¹ *B. thuringiensis* cells was evaluated over the whole spectrum, and presents a plateau of > 18% at 10 kHz – 0.3 MHz, resulting essentially from the almost stable difference of the impedance modulus in this frequency range. Due to the very resistive nature of the phase over this range, the modulus remains quasi-independent of the frequency.

Regarding the possible influence of electrode roughness on the impedance measurements, we stated that their particular microstructure following the nitrocellulose membrane porosity does not have a critical influence on our detection system. Indeed, since the non-Faradaic impedance measurements are performed above 1kHzn they are much less sensitive to surface phenomena occurring at the electrolyte-metal interface (e.g., measuring changes in properties near the electrode surface due to variations in double-layer capacitance C_{DL} or charge transfer resistance R_{ct}) than to bulk electrolyte properties within the volume of the nitrocellulose membrane.

Sensitivities towards B. thuringiensis cells

The sensitivities towards 10^8 CFU mL⁻¹ *B. thuringiensis* cells in low salinity buffers were evaluated over the whole spectrum for both parallel-plate and IDE setups. The spectral sensitivities of the systems were computed from the complex impedance of the blank PBS:1000 buffer and the bacterial suspension (Figure 6.11).

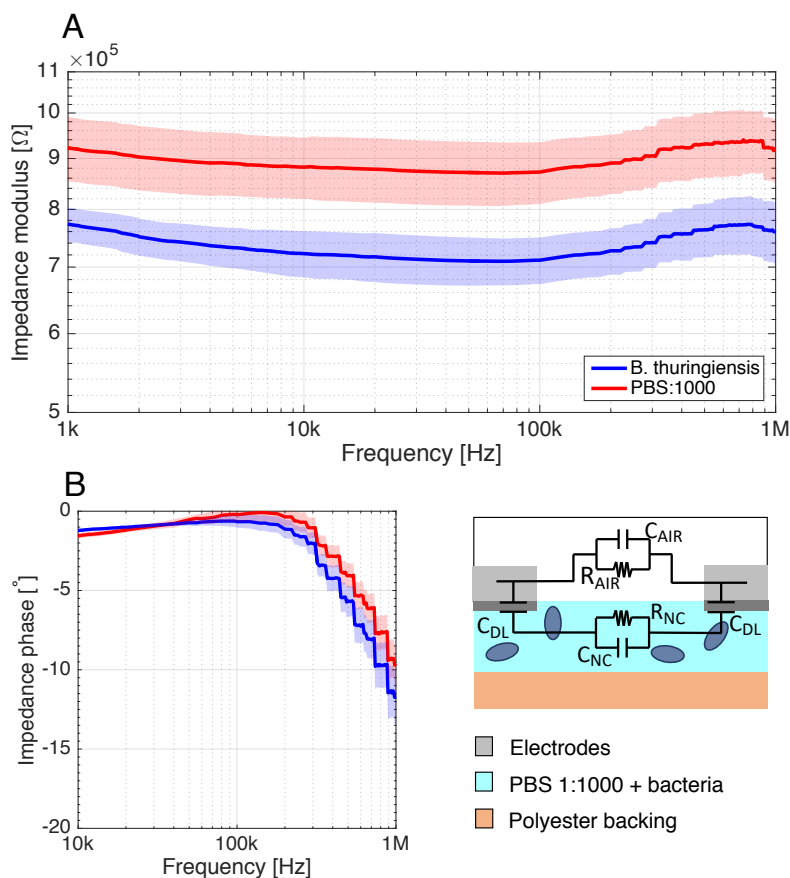


Fig. 6.10 (A) Impedance measurements performed on nitrocellulose (NC) membrane as seen by the IDE, with and without bacterial cells in PBS:1000 buffers. The impedance modulus presents a constant impedance shift representing a decrease in the solution resistance. (B) Since a direct conduction path exists between their electrodes, the impedance seen by the IDE has a character that is rather resistive as expressed by the highly resistive phase over a large spectrum. Number of samples: 12 from two independent experiments. Shaded area surrounding the measurements curves: standard deviation (σ).

Regarding the parallel-plate (Figure 6.11A), a maximal sensitivity of about 21% is observed around 30 kHz for the parallel-plate setup. The sensitivity computed from the complex impedance is superior to the modulus sensitivity (around 17% at 40 kHz) as it includes both contribution from the impedance modulus change and phase shift around this frequency

(depending on the interplay between R_{NC} and C_{NC}). In this system, the sensitivity does not remain constant over the spectrum as C_{NC} has a non-negligible contribution to the detection.

Contrary to the parallel-plate, the sensitivity of the IDE setup presents a plateau of $> 18\%$ at 10 kHz – 0.3 MHz (Figure 6.11B), resulting essentially from the almost stable difference of the impedance modulus in this frequency range. Due to the very resistive nature of the phase over this range, the modulus remains quasi-independent of the frequency.

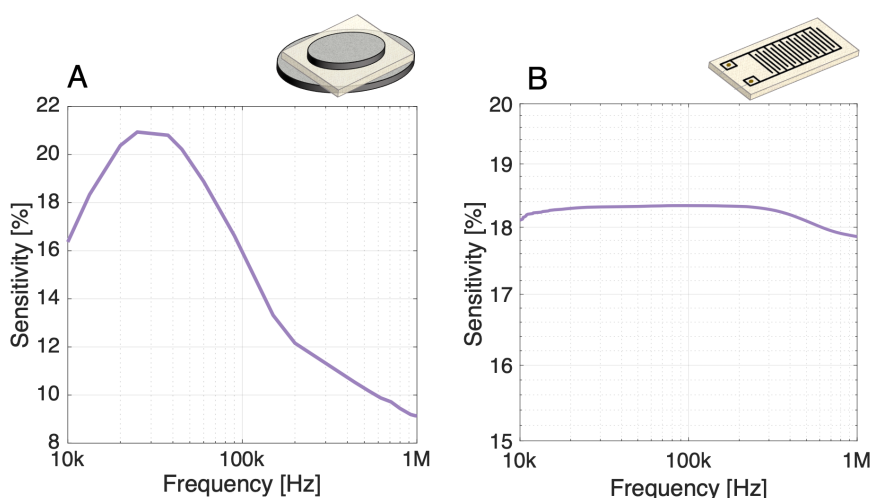


Fig. 6.11 Spectral sensitivities of the (A) parallel-plate and (B) IDE setup towards 10^8 CFU mL⁻¹ *B. thuringiensis* cells in low salinity buffers. The sensitivity includes both contribution from the modulus and the phase shift. The IDE sensitivity expresses their high response towards resistance changes through a plateau between 10 kHz and 0.3 MHz, while the parallel-plate sensitivity presents a peak because of the interplay between R_{NC} and C_{NC} in the sensing mechanism.

6.6 Discussion

The main objective of this work is to accommodate electrical sensing on paper sub-strates towards simple, rapid, quantitative bacteria detection in aqueous solutions.

First, we took advantage of the NC support to develop an innovative **volume-based electrical detection setup** by applying the membrane

between the electrodes of a parallel-plate prober for dielectric measurements. This setup forms a simple plug-and-play sensing device which is responsive to the electrical properties of the NC membrane. A nitro-cellulose membrane functionalized with a specific biointerface is indeed an adequate substrate to support the volume-based electrical detection of bacterial suspensions. Such a detection scheme is attractive as the whole sample volume contribute to the sensing, increasing the contribution from targeted bacteria with respect to surface-based methods. The parallel-plate detection scheme based on impedance spectroscopy proposed in this work is much more straightforward both in fabrication and handling than most of the paper-based electrical biosensors encountered in the literature. They often rely on direct current monitoring requiring to apply more complex electrode systems to the paper (usually three electrodes, for reference, working and counter electrode) and involving electrochemical reactions which are relatively complex to interpret in ionic medium [242, 243]. These paper-based sensors usually rely either on the application of conducting pastes showing high electrode and contact resistances (generally requiring custom, hand-made electrical connections) [243, 244], or more complex and expansive fabrication processes from the microelectronics industry.

Then, we quantified the presence of 10^8 and 10^7 CFU mL^{-1} *B. thuringiensis* in diluted physiological buffer (PBS diluted 1000 x). An important feature is the rapidity of the sensing mechanism: the whole detection protocol lasts less than 5 min. Using a simple electrical model including the electrical properties of the NC membrane and the capacitive contribution of the polyester backing, the interplay of both capacitive and resistive properties of the electrolyte are observed. The detection mechanism, based on ion concentration, was determined by the parallel-plate characterization of NC membrane filled with different saline solutions, and helped us understanding the sensor differential response for various electrolyte solutions. Similarities can be drawn in the complex impedance difference between the saline solutions and for biological buffer with and without bacterial cells. In particular, close correspondence is observed between electrical properties of PBS:1000 and $\sim 2 \times 10^{-4}$ M NaCl solution, while signals from the 10^8 CFU mL^{-1} bacterial suspensions are representative to those of the $\sim 10^{-4}$ M condition. These results, supported by dielectric measurements, suggest that the electrical model proposed for the sensing of NC membranes soaked with ionic solutions can be extended to bacterial solutions. Further, it also endorses the concept of bacteria detection through its surroundings ions, which has already been discussed in [45]: the differences in sensor response are attributed to the slight difference in ionic content, i.e., electrical conductivity, between sterile PBS:1000 and bacterial

resuspension in PBS:1000. Indeed, centrifugation steps lead bacterial cells to release ions due to osmotic pressure and damaged cell walls [245].

Both for saline solutions and bacterial suspensions, an interesting phase shift occurs in the phase peaks between 10 kHz and 1 MHz, driven by changes in f_3 due to both C_{NC} and R_{NC} variations. Although the resistive character of the sensor is predominant (changes in R_{NC} affect the total module more than C_{NC}), the possibility to monitor NC membrane property changes through variation of C_{NC} renders this setup versatile in use and potentially more robust again ionic noise, strongly affecting R_{NC} . To assess the relative advantage of the parallel-plate setup over traditional paper-based sensors, we also applied gold microelectrodes (IDE) to the NC membrane using a standard microfabrication process. The IDE sensor is only reactive towards R_{NC} , and shows a high sensitivity to bacteria over a larger frequency range than the parallel-plate. Indeed, the electrode disposition does not prevent a direct conduction path between the two electrodes.

Finally, the biointerface obtained by functionalizing the NC membrane with endolysin cell-wall binding domain (CBD) aimed at specifically capturing bacterial cells through the whole membrane pore surface. Although specific immobilization capacity towards this bacteria within NC membranes was already demonstrated in chapter 5, the specificity of electrical biosensors towards whole-cell pathogens is usually assessed with the absence of electrical signal in absence of bioreceptors or in presence of only non-target pathogens. Therefore, **the detection specificity of our system remains to be studied**, and will be the topic of future works. When considering specificity of the detection, it will be of utmost importance to use a complete lateral flow assay with controlled flow as support to perform the electrical detection and a related robust detection protocol integrating the necessary washing and subsequent incubation steps. A **relevant protocol for the specific electrical detection of whole-cell bacteria with the parallel-plate setup** could consist in (i) bacterial suspensions deposition and capture on **two different NC pads** : one functionalized with bioreceptors and one non-functionalized, (ii) **washing steps** with de-ionized water to remove non-specific unbound pathogens and ions non-related to bacterial presence (e.g. ions from the buffers), (iii) a **short incubation step** would allow for ion release from the captured bacteria due to osmotic pressure, and (iv) a **differential electrical measurement** between the functionalized and the non-functionalized area.

6.7 Limitations and Perspectives

Despite its dependence on bacterial concentration, **two reasons make the detection of bacteria through higher ionic content unsuitable for point-of-care specific and highly sensitive bacterial sensing in paper-based sensors.** First, the resulting signal is strongly affected by experimental procedures such as manipulation, affecting the baseline sample conductivity. Secondly, the sensing principle in the form presented here is inadequate for real-world applications involving the detection of pathogens in complex saline solutions. The high electrical conductivity of these solutions is hardly affected by bacterial ion release. In addition, the proposed detection method lacks robustness in environments with various living organisms that are potential ion sources. As a result, the signal-to-noise ratio (SNR) is low. **The specificity and sensitivity of the electrical detection can be improved by nanoparticles (NP) to specifically label whole bacterial cells.** In [246, 46, 247], *E. coli* O157:H7 were detected through amplified conductance and permittivity changes by means of to the conjugation of specific graphene or gold NPs to the bacteria. In other works, the highly intensive response of Si-NPs and Au-NPs conjugated to bioanalytes at radio frequencies (RF) was used to amplify the dielectric contrast of bioanalytes in solutions [248, 249] or to act as microantenna in NC membranes [250].

The ability for the parallel-plate setup to monitor changes of both conductive and dielectric properties in the NC membrane makes it possible to select the type of NP (conductive or dielectric) that increases the SNR the most. **Conjugating diverse types of NPs with bacterial cells offers promising perspectives for highly specific electrical bacterial detection on lateral flow assays,** and will be the focus of the next chapter.

Beyond the handling of electrical measurements on paper sensors, which was one of the objectives of this chapter, perspectives can emerge from the limitations we have identified from our system. **Possible applications for our system are worth to be explored in the field of cell counting.** Indeed, cell counting is currently performed by **impedance flow cytometry (ICF)**, which involves the detection of dielectric footprint of bacterial cells flown in μm -scale microfluidics channels, in between two parallel, planar electrodes in a volume-based detection setup. Flow cytometry is currently performed with empty microfluidic channels, enabling an accurate counting and statistical size analysis of a large number of cells ($> 10,000$ in less than 2 min) [251]. We can consider a paper-based alternative to flow cytometry by optimizing the volume-based electrical detection system described here. A limitation of flow cytometry is its inability to specifically detect

bacterial cells. Our paper-based approach can extend the volume-based detection of flow cytometry to specific detection through capture in the nitrocellulose membrane. However, the washing steps must be critical and controlled, and a system set up for differential measurements compared to a controlled blank.

Finally, since 1950s it is known that **the number of the red blood cells (RBC) are correlated to the blood conductivity** [252]. Recently, simple RBC quantification procedures based on impedance measurements have been proposed. Since our system is sensitive to the conductivity of the solution in the piece of nitrocellulose, this system could be used to quantify the number of RBC present in a blood sample, through a simplified handling of sample directly deposited on the piece of cellulose material. Of course, a calibration curve is required, relating the amount of ions (and thus the conductivity of the liquid) to a number of cells. Electrical sensing of blood conductivity was also proposed to quantify the blood damage (hemolysis), i.e. the rupture of red blood cells that can occur during clinical procedures. As red blood cells rupture, they release their hemoglobin and raise the conductivity of the entire fluid. Change in blood conductivity can be related directly to the level of hemolysis [253]. With a paper microfluidics support instead of usual capillaries, the spontaneous separation of the blood cells from the plasma could be included thanks to the paper capillarity and its blood filtration properties, which were already demonstrated [254].

6.8 Conclusion

In this chapter, an innovative method for the electrical characterization of cellulose-based membranes is proposed towards the development of a quantitative paper-based biosensors. It consists in the use of a simple parallel-plate setup as sensor to perform impedance measurements on the membrane inserted within the electrodes. The sensing principle was studied and validated by detecting saline solutions of different molar concentrations spread within nitrocellulose membranes. We then demonstrated the proof-of-concept of the detection of bacteria: impedance-based detection of 10^8 CFU mL⁻¹ of *B. thuringiensis* cells was presented without labelling nor signal enhancement strategies. The bacteria are detected through an overall increase in ions in the membrane caused by their presence. Impedance measurements were also performed with interdigital electrodes integrated on the membrane and confirmed the parallel-plates results. Finally, newly discovered endolysin CBD are introduced as specific bioreceptors and de-

posited inside the nitrocellulose membrane, enabling successful bacterial cell capture over the whole membrane volume.

In conclusion, by combining the benefits of cellulose-based membrane, novel protein bioreceptors and precise impedance measurements with a reusable plug-and-play setup, we obtained promising results towards the development of an simple and sensitive electrical biosensor for whole-cell pathogen detection with a speed of response under 5 min. To overcome the limitations presented by our system (specificity of the detection) and reach the very low limit of detection required for example for drinking water assessment, it is necessary to integrate signal enhancement strategies. The next chapter will explore the use of nanoparticles as labels to increase the analytical performances of the device. The development of such versatile tools creates novel opportunities in situations that require rapid and frequent pathogen detection, such as the detection of *E. coli* in drinking water. Sensing applications could be extended to the detection of various pathogens and viruses as well, through appropriate and direct modification of the biointerface.

7

Towards impedance lateral flow assays



Chapter objectives

In chapter 6, we introduced impedance measurements to accommodate the electrical detection of pathogens on paper-substrates with a simple setup, although presenting many functional limitations for its intended use as specific and sensitive pathogen biosensor. The LFA advantageous properties of rapidity, integration of functional nanoparticles and passive microfluidics, enabling washings and thus detection specificity, are essential to address these limitations. In this chapter, we apply the detection methodology used in chapter 6 to the LFAs developed in chapter 4, with the aim of discovering the challenges, opportunities and key best practices for designing high-performance electrical LFA sensors.

The work in this chapter was partly carried out during a research stay at Prof. Arben Merkoçi's Nano-Bioelectronics & Biosensors group in Barcelona (Spain) during summer 2021, a collaboration that is still ongoing. The results were presented at the World Congress on Biosensors 2023.

7.1 Introduction & motivation

By means of a plug-and-play yet stable parallel plate setup, **in chapter 6 we rapidly detected volumic impedance property change of nitrocellulose membranes** due to the presence of 10^8 CFU mL⁻¹ bacterial cells in low conductive buffers (representative water samples). The non-Faradaic impedance spectroscopy measurements (1kHz to 1Mhz) showed high sensitivity to the ionic content of the samples. These label-free detection results, although preliminary and based on a very simplified system, raise the fair comparison with a classical IDE electrode system fabricated on NC membranes. Therefore, it suggests that this external (i.e. non-integrated) and reusable system could be a sensitive and very practical alternative to the conventional electrode systems embedded in the paper substrate.

However, **the clear challenges offered by the system**, i.e. (i) the non-specific detection of bacteria through their ion release and (ii) the static detection setup in NC that implies no control on the microfluidics sample buffer properties (e.g. conductivity), offering only a slight control on the bacterial capture or the washing steps, make this detection method inadequate (complicated to implement + experimental bias), thus **motivating the integration of electrical measures with a complete LFA setup**. Indeed, LFAs involve label nanoparticles that specifically binds to the target bacteria, assumes a proper and specific immobilization of the targets, allow the control of some of the microfluidic properties such as sample buffer conductivity, and enable washing steps. In addition, due to the LFA geometry, it facilitates the integration of a differential measurement on the same device, as advised in section 6.6.

The objective of this chapter is to take advantage of the LFA dynamic properties and capabilities in order to imagine new electrical detection systems on LFAs, and propose concrete guidelines to overcome the challenges mentioned above. It is organized as follows. In section 7.2, we review the state-of-the-art of the electrical transducers applied to LFAs, in particular for the detection of gold nanoparticles (AuNPs). Based on the state-of-the-art conclusions, section 7.3 propose a step-by-step strategy to tackle the challenges offered by integration of electrical measurements on AuNP-based LFA. We apply this strategy in section 7.4 in order to explore electronic systems (sensing modalities, materials and devices) that would be the most relevant to develop. Finally, the implementation outcomes and the lessons learned are put into perspective with other relevant work in the section 7.5, and future research directions are proposed.

7.2 State-of-the-art

In this section, we present the main types of nanoparticles used in LFA as transducer labels and the corresponding detection modalities that can be operated. It is then followed by an overview of the state of the art of LFA integrated with electrical, electrochemical or electromagnetic readers, in order to identify the major trends in the design of non-optical LFAs. Finally, since our work focus on the use of gold nanoparticles, we review the sensing modalities that have already been exploited to sense AuNPs, especially under LFA formats.

7.2.1 Co-integration of electronic readers and nanoparticle-based LFAs

Colorimetric LFAs mainly take advantage of the color change of nanoparticles, due to the surface plasmonic resonance. However, nanomaterials offer a large number of other unique properties due to the confinement of electrons within them. The use of different types of nanoparticles makes it possible to take advantage of their excellent physico-chemical properties (surface plasmon, very high conductivity, appearance of magnetism, redox behavior, etc) in order to detect them through different detection modalities. Many excellent reviews offer detailed viewpoints of the highly-performing transducers that can be integrated in LFAs with regard to the NP properties [115, 149, 151, 152, 183, 195, 255, 256, 257, 258, 259] : The literature provides information on four families of nanoparticle-based transduction modes that can be applied to lateral flow assays (Figure 7.1):

1. **Optical** LFAs, mainly integrating gold nanoparticles (colorimetric detection), or carbon nanoparticles such as graphene expressing fluorescence under certain excitation conditions;
2. **Thermal contrast** LFA;
3. **Magnetic** LFAs, mainly based on the detection of magnetic iron oxide nanoparticles (MNPs) accumulation to the test line by means of sensitive probes (Hall effect sensor, giant magnetoresistance (GMR) sensor);
4. **Electrical and electrochemical** LFAs, based on the use of metallic nanoparticles (Au, Ag), oxides or materials with catalytic properties (Pt).

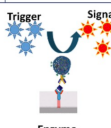
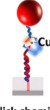
	Optical	Thermal	Magnetic	Electrochemical
Sensing Modality	Colorimetric Fluorescence Surface-enhanced Raman scattering Chemiluminescence	Thermal imaging Photoacoustic Speckle imaging	Giant magnetoresistance Tunneling magnetoresistance Magnetic particle quantification	Amperometry Cyclic voltammetry Impedimetry Electro-chemiluminescence
Tracer	 AuNP  AgNP  MNP  IrO ₂ NP  CNT  CNB  CuONP  Polymer  GO	 AuNP  AgNP  CNT  BP	 MNP	 AuNP  AuNP@Pt  MNP  GO
Recognition/ Amplification Strategy	 Antibody  Enzyme  Aptamer  Click chemistry  Antibody  Enzyme  Aptamer  DNA  Target  Alkyne-azide cycloaddition			

Fig. 7.1 Major existing detection methods on LFA taking advantage of the original properties of nanoparticles (NP). Detection methods are categorized on the basis of the type of transduction offered by NPs, which can serve both as a direct marker for detection or as an element for triggering / amplifying another signal. Reproduced from [257] (CNT: carbon nanotubes, GO : graphene oxide, MNP : magnetic nanoparticles, CNB : cellulose nanobeads, BP : black phosphor)

According to Figure 7.1, LFAs with non-optical readout are thus also possible. In fact, lower detection limits and more quantitative results can be achieved in comparison with colorimetric-based LFAs, but they require specific readers.

In order to understand the dynamics of the developments implemented to improve the performance of LFAs, it is useful to analyze the distribution of the different detection methods and types of nanoparticles mainly used in rapid tests at the present time to realize the omnipresence of the use of gold nanoparticles within LFAs. Colorimetric detection methods currently represent more than 80% of developments, fluorescence around 15%, while electrochemical and magnetic methods share the remaining 5%. This is also reflected in the distribution of the markers used in the LFA tests (Figure 7.2). **Electric and magnetic detection modes are not yet strongly represented among LFAs.** Indeed, the apparent barriers of these methods are that the research and their use can be initially more expensive and more complex than their optical counterparts, which has constrained their development [260].

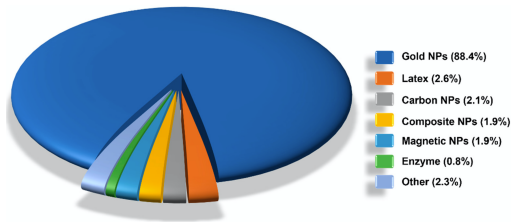


Fig. 7.2 Overview of the labels used within the LFAs over the period 2010-2019. This distribution is quite representative of the distribution of the different detection methods used on the LFAs. Reproduced from [116].

However, they are the subject of intense research and development at present due to their high potential for performance (quantitative, sensitive, and digital measurements), miniaturization (through integration), portability and cost reduction compared to optical readers. The interest of electrical and magnetic detection techniques compared to the use of (optical) colorimetry can be justified by the diagram in Figure 7.3: where only the nanoparticles present in the first 10 μm of the thickness of the test line participate in the generation of the signal (surface detection), all of the nanoparticles present at the test line interact with the electric and magnetic fields respectively. Therefore, in this volume-based detection, a greater number of markers participate in the disturbance of the electric or magnetic field probing the zone, making these latter modes of detection more sensitive than their optical counterparts.

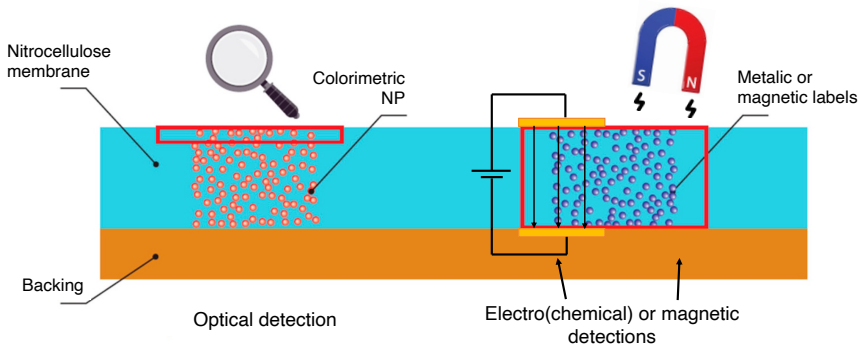


Fig. 7.3 Comparison of **optical, electrical and magnetic detection methods for nanoparticle-based LFA**. The electric and magnetic transducers are sensitive to all the markers (NP) accumulated in the detection zone, unlike the optical detection modes sensitive only to the nanoparticles present in the first 10 μm . These methods are therefore more sensitive, enabling lower detection limits. Adapted from [261]

Comparison A comparative overview of relevant electro(chemical), thermal and magnetic LFAs in the literature is provided in Table 7.1. A performance comparison is proposed with their optical counterparts. Because each sensor is designed specifically for its application, such as the sensor principle, electrode dispositions or nanoparticles, it is difficult to provide an objective comparison of these works. However, some key conclusions can be drawn :

- It is shown that the use of alternative sensing modality to colorimetry by means of additional readers leads to lower LoD ($\sim 10\text{-}100 \times$) for each type of target (proteins or whole-cell pathogens) as compared to the colorimetric LFA reference.
- As a striking example , we can cite the work of Sinawang *et al.* [262] aimed at obtaining quantitative detection and lowering the detection limits of optical LFAs [263] for the detection of a virus protein (dengue virus NS1 protein) by means of adding an electrochemical reader unit to the test line on the LFA where the AuNP are accumulated.
- Interestingly, in another example combining electrochemical and colorimetric detection on the same AuNP-based LFA [264], the LoD for the biomarker was the in the same range for both the colorimetric and the electrochemical response of the test line. In that case, the authors justified the interest of their dual detection methods as it can provide double confidence on the same assay, and that the integration could potentially avoid false results coming from one method, since they previously shown that the colorimetric detection of 8-OHdG was regularly leading to false results.
- In the case of electrical detection modalities, the choice of electrodes is often focused on screen-printed electrodes, either carbon- or metal-based (Au, Ag) inks, that are directly integrated onto the LFA substrate or under the substrate on the plastic backing. This can probably be explained by the accessibility and the diffusion of this manufacturing technique, low-cost and simple to implement, even on inhomogeneous substrates such as paper.
- Finally, the use of AuNPs as a signal enhancement strategy in the different sensing modalities is still widely spread, either of course for their colorimetric properties, but also as an electrochemical reaction catalyst increasing charge transfert [265, 262], for their ease of functionalization with the electroactive reagent, which is therefore carried by the AuNP and specifically delivered to the target, or for their thermal properties [266].

Table 7.1 Overview of relevant electro(chemical), thermal and magnetic LFAs in the literature, and performance comparison with their optical counterparts. (*Electr.:* reader system or electrodes, *Integr.:* integration, *Steps:* additional steps, *QD:* Quantum Dot, *SPE:* screen-printed carbon electrodes, *SPGE:* screen printed gold electrodes, *Enhanc.:* enhancement, *MNP:* magnetic nanoparticles, *MPQ:* MNP reader (coil), *Prep.:* sample preparation steps, *PA:* conductive polyaniline, *PA-MNP:* polyaniline coated MNP, $\otimes$: not available.)

Target	Medium	Principle	NP type	Electr.	Integr.	Time	LoD	Steps	Ref.
Human IgG	aqueous buffer aqueous buffer aqueous buffer	Colorimetry Colorimetry Electrochemical	AuNP AuNP QD (Cd)	- - SPCE	- - yes	15 min 15 min 20 min	10 ng/mL 28 ng/mL 10 pg/mL	- - yes	[267] Us [268]
8-OHdG protein	aqueous buffer aqueous buffer	Colorimetric Electrochemical	AuNP AuNP	- SPCE	- yes	10 min 10 min	2.07 ng/mL 3.11 ng/mL	- -	[264] [264]
PSA	aqueous buffer aqueous buffer aqueous buffer aqueous buffer human serum human serum	Colorimetric Colorimetric Magnetic Magnetic Electrochemical	AuNP AuNP MNP MNP QD (Cd)	- - - PCB MPQ SPCE	- - no no yes	20 min 20 min 10 min 30 min 20 min	0.5 ng/mL 0.1 ng/mL 0.25 ng/mL 25 pg/mL 20 pg/mL	- - Enhanc. - - yes	[269] [269] [270] [271] [268]
Dengue NS1 prot.	aqueous buffer aqueous buffer	Colorimetric Electrochemical	AuNP AuNP	- SPGE	- yes	15 min 20 min	5 ng/mL 0.5 ng/mL	- -	[263] [262]
<i>E. coli</i>	aqueous buffer aqueous buffer aqueous buffer aqueous buffer aqueous buffer	Colorimetric Thermal Electrochemical Electrochemical	AuNP AuNP PA-MNP AuNP	- Camera Ag SPCE	- no yes yes	10 min 10 min 60 min $\otimes$	10 ⁴ CFU/mL 2·10 ⁴ CFU/mL 61 CFU/mL 6 CFU/mL	Filtration Prep. Prep. -	[47] [266] [272] [265]
<i>B. cereus</i>	aqueous buffer aqueous buffer	Colorimetric Electrochemical	AuNP PA	- Ag	- yes	20 min 6 min	10 ⁴ CFU/mL 10 ¹⁻² CFU/mL	yes -	[118] [222]

In view of the previous sections, **it seems interesting to exploit the properties of gold nanoparticles to improve LFA detection systems**, for several reasons: (i) they still equip most LFAs at present and are therefore very well established within the various LFA manufacturers and development processes, (ii) their manufacturing process is largely mature, and they can be produced in large quantities in high quality and stability, at lower costs compared to other NPs, and (iii) they offer numerous electrical detection schemes (impedimetric and electrochemical) [155].

7.2.2 Electrochemical detection techniques of AuNPs

In this section, we describe in more detail the physical principles of the electro(chemical) detection modalities available to detect gold nanoparticles in biosensor technologies [233, 273, 274, 275, 276, 277]. Methods for the electrical detection of AuNPs within biosensors in the literature are mainly divided into impedimetric detection modes, analytical electrochemistry methods and detection at higher frequencies.

Impedimetry

The complex impedance behaviour was introduced in section 6.3. Impedimetric detection modes are divided into electrical impedance spectroscopy (no electron transfer occurs at the solid-electrolyte interface, non-Faradaic), and (ii) electrochemical impedance spectroscopy (in presence of electroactive species, Faradaic).

In non-Faradaic **electrical impedance spectroscopy**, the detection information is mainly contained within the dielectric properties of the system. In this sense, it is also called *electrochemical capacitance spectroscopy*. Applied to the detection of gold nanoparticles within an LFA, two electrodes can be applied on either side of the test area and measure the difference in capacitance induced by the presence of the metallic labels (AuNPs) within nitrocellulose composing the LFA (Figure 7.4).

The value of the volume capacity of the test area can be approximated to $C = \frac{\epsilon S}{d}$, with S the surface of the electrodes, d the distance between the two electrodes, and ϵ is the dielectric constant of the rapid test volume at the test area, which is the physical parameter mainly modified by the presence of AuNPs. The working frequency ranges must be well defined, typically between 1 kHz and 1 MHz. The correlation between the variation of the capacitance (and therefore of the overall impedance) and the concentration of nanoparticles makes it possible to obtain quantitative values of

the presence of contaminants in the test.

Other examples of non-Faradaic impedance sensors based on AuNPs are depicted in [274, 278, 279]. Although not already applied to an LFA, Pal *et al.* interestingly used microelectrodes to detect AuNP-labelled pathogen through changes in R_s at 0.1 MHz [222].

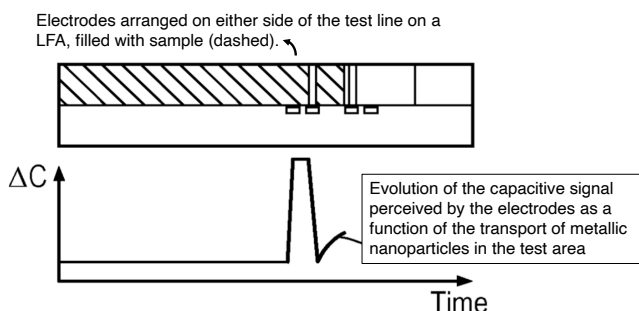


Fig. 7.4 Illustrative diagram of the concept of a capacitive LFA for the continuous monitoring of the LFA. Modified from patent Nguyen, V., *et al.* (2013) An electronic lateral flow test arrangement and method (WO2013083686A1).

For their part, sensors based on **electrochemical impedance spectroscopy** (Faradaic) take advantage of the electrochemical reactions between the species present in the sample (the contaminants directly, or the nanoparticles) and the electroactive species added to the medium (often $[\text{Fe}(\text{CN})_6]^{3-/4-}$). In this case, the transfer of electrons between the electrodes is quantified by means of the charge transfer resistance R_{ct} at the surface of the electrodes, modified in the presence of the AuNPs.

In the study by Jolly *et al.* [280] aimed at detecting DNA strands, they partly used impedance spectroscopy in Faradaic mode to detect changes in R_{ct} due to the introduction of AuNPs near the surface. Figure 7.5 illustrates the recorded impedance plots: a decrease of about 30% in impedance was observed due to the reduction of R_{ct} after incubation of AuNPs on the biosensor. This decrease in resistance is the result of the attachment of AuNPs (positively charged) to the surface of the electrode, which results in a decrease in the resistance to the negative redox couple in the solution. The need for surface effect diminishes the advantages of volume sensing mentioned above. Wan *et al.* present a similar detection principle [205].

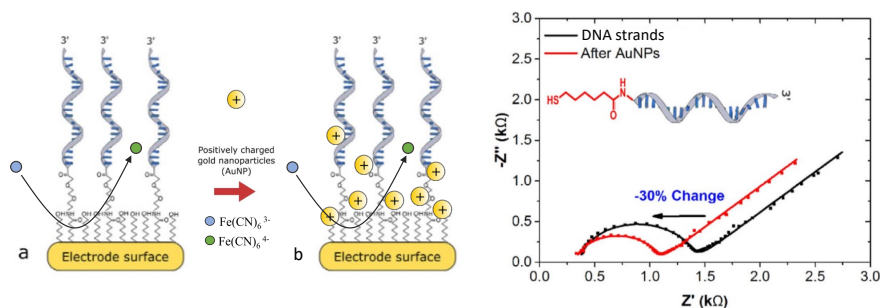


Fig. 7.5 Illustration of the principle of detection of gold nanoparticles by means of Faradaic impedance spectroscopy. The AuNPs must be in close proximity to the surface in order to influence (decrease) the resistance to the transfer of R_{ct} charge of the solution containing the sample (here DNA strands attached to the surface of the electrode) and the electroactive species (redox). Modified from [280].

Analytical electrochemical methods

Other analytical methods have been used for AuNP detection purposes. AuNPs can also be chemically oxidized in Br_2 or HCl to form an electrochemically active species, AuCl_4^- , which can then be detected using analytical electrochemistry modes such as differential pulse voltammetry (DPV). This is the scheme put in place by Ng *et al.* [281], Figure 7.6. The higher the number of nanoparticles (proportional to the number of pathogens in the sample), the higher will be its oxidation peak observed by DPV. The author obtained a quantitative detection with extremely low detection limits for the detection of bacillus *Mycobacterium tuberculosis* (1 CFU mL^{-1}), a pathogen responsible for tuberculosis and of interest to be detected in order to prevent and stem the spread of the disease. Similar examples are described in the excellent works of de la Escosura *et al.* [276, 282, 247].

Detection of AuNPs at high frequencies

Finally, it is worth mentioning that beyond detection techniques by impedimetry or analytical electrochemistry, studies have attempted to detect gold nanoparticles at higher frequencies on a nitrocellulose membrane. In this study, the gold nanoparticle, subjected to a silver enhancement process, were behaving like antennae on nitrocellulose supports using a reader without RFID wire (915 MHz) to detect very low concentrations of IgG proteins (Figure 7.7 [250]).

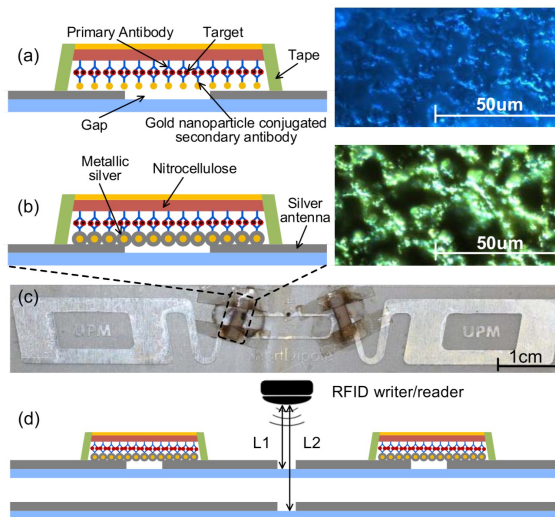
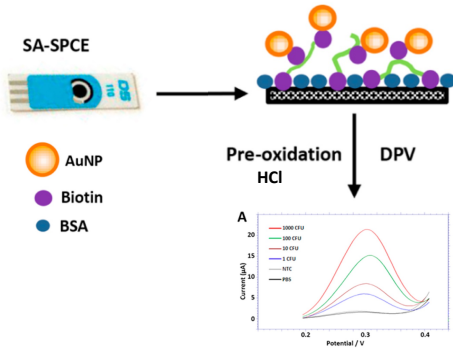


Fig. 7.7 Working principle of dipole antenna biosensor based on gold nanoparticle capture on a nitrocellulose membrane [249]: (a) cross-sectional view of the antenna gap with gold nanoparticle conjugated anti-rabbit IgG forming a sandwich structure (top-view of NC membrane microphotograph); (b) cross-sectional view after the gap is bridged using silver enhancement (top-view of NC membrane microphotograph); (c) 915 MHz dipole antenna based RFID biosensor; (d) illustration of the differential RFID based detection system, where L1 and L2 correspond to the farthest distance the RFID tag can be interrogated by an RFID reader.

7.3 State-of-the-art conclusion and chapter contributions

To overcome the limitations of colorimetric LFAs, electrical and magnetic transduction make it possible to take advantage of the signal generated by all the markers present in the reaction zone (test line). In particular, several electrical detection principles, essentially based on impedimetry, take advantage of the electrical properties of gold nanoparticles to generate an electrical signal sufficiently defined to obtain high sensitivities and quantitative detections of analytes. There is great interest in combining these detection methods with existing conventional LFAs in order to improve their performance. However, very few of these detection principles are already integrated on LFAs, which leaves many challenges and opens the door to the study of their integration. The study of the state of the art shows that:

1. Gold nanoparticles could be a good candidate to detect electrically, given their ubiquity.
2. Faradaic electrochemical impedimetry for the detection of gold nanoparticles can achieve high sensitivities. However, the incorporation of a redox reagent complicates the manipulations (influencing the reliability of the test, crucial to ensure the competitiveness of rapid tests), and makes necessary a three-electrode system more complex to integrate into the LFAs due to the LFA geometry (the electrodes are often directly printed and does not support reusing).
3. Non-Faradaic impedance detections, where the dielectric fingerprint of the AuNPs is used directly to identify and quantify the presence of contaminants, was already demonstrated and would be simpler to implement on LFA. However, to the best of our knowledge there is no such sensor yet for the detection of AuNPs within LFAs.
4. Detection methods must be studied and evaluated on the basis of several criteria: response time, detection limit, quantification range, signal-to-noise ratio, and ease of interfacing the sensitive probes with LFA microfluidics. The costs and reliability of the tests must be taken into account as well. Since electrical sensing modalities are sensitive to many species in the samples, validity of the electrical tests results must be a priority, for example by an optical control.

Chapters 4 and 5 concluded that the intrinsic LoD of AuNP-based LFAs are driven by the interactions between the targets, the labels and their accumulation to the test line. Chapter 6 showed that non-faradaic impedance spectroscopy is a powerful tool for highly sensitive biosensors (easy implementation of simple electrodes, well documented), but demands delicate

interpretation given its extreme sensitivity to electrical properties of the buffers involved in LFA.

Taken all together, **the study of impedance detection on AuNP-based LFA seems like a reasonable choice to initiate the implementation of electrical LFAs and gain experience on the key features of their interfacing.**

Chapter contributions Here, we propose a **step-by-step strategy to tackle the challenges offered by the integration of electrical measurements on AuNP-based LFAs**. In particular, this chapter introduces the necessary characterizations to qualify and model the LFAs and the interactions they involve, enabling the design of nanoparticle detection strategies.

1. First, a **LFA design suitable with non-faradaic impedance measurements is proposed**, taking advantage of the plug-and-play parallel plate electrode disposition and the flexibility offered by the LFA fabrication process. This innovative design allows for both impedimetric and optical detection in the same device, and integrate a differential detection scheme for control measurement on the same device. Basic characterizations are performed to optimize the impedance measurement parameters, such as the frequency range. An equivalent electrical model of the LFA test zones is established. The impact of the porous membrane on the model parameters is considered by means of a simple analytical model.
2. Second, we **calibrate the impedance response of conventional LFAs**. The calibration provides insights into the intrinsic electrical noise and electro-fluidic phenomena occurring within the detection area, which result from the specific LFA microfluidics (i.e. capillarity) and the high complexity of the buffers required for proper LFA functionalities. The calibration is performed with respect to the buffer conductivity and to the operating time of the LFA.
3. Then, the **combination of colorimetric and electrical characterizations of an engineered LFA design** is proposed as a mean to study and **control the electro-fluidic properties of the LFAs**. The electrical phenomena observed can thus accurately be linked to the propagation of fluids successively added in the LFA, enabling the design of effective washing steps.
4. These results are leveraged to **set up a strategy to reveal the dielectric footprint of the AuNPs and their accumulation at the test line**. As a prospective study, we evaluate a simplified system made of conjugated AuNPs that accumulate on a test line made human IgG protein. To date, the sensitive electrical detection of AuNPs within a LFA still remains to be demonstrated. Further works are currently conducted to this end.

Finally, we compare relevant works on electrical transduction of LFAs in the light of our methodology. The lessons learned from our implementation are used to propose a research path.

7.4 Results

7.4.1 Implementation

It is desirable to perform affinity impedance measurements on gold nanoparticle-based LFAs since this kind of LFA is the most widely developed, used and accepted. To design the prototype impedance-based LFA, we leveraged the experience from chapter 4 to adapt the AuNP-based LFA for human IgG (H IgG) colorimetric detection with the outcomes of chapter 6 to design a plug-and-play setup relying on the parallel plate approach (Figure 7.8 A). In this design, the LFA is inserted in a cassette designed specifically for our application, and integrating electrodes made of sputtered gold (500 nm) on a plastic support (PET). The choice of gold was made in order to avoid oxidation of the electrodes, a problem which had been revealed with the IDEs in the chapter 6. A 500 nm-thickness was supposed to be enough in order to withstand handling of the PET, cutting and attachment in the cassette.

This setup is design in such a way that two pairs of parallel and planar electrodes are disposed on the LFA. A first pair of electrodes is the *detection pair*, and disposed at the conventional colorimetric test line, such that it becomes the "impedance test line" as well. A second pair of electrodes is disposed at the end of the detection membrane, closer to the absorbent pad, and serve as the impedance control (blank) since no particles are supposed to bind at that place. The two electrode pairs are forming a differential measurement setup. In order to secure a visual control on the proper flow of the LFA, just like any colorimetric LFA, the colorimetric test line is still visible thanks to a aperture in the cassette. In addition, the design of the cassette allow to observe visually the accumulation of the AuNP at the TL from the sides of the electrode notches.

Since our goal is to demonstrate the impedimetric detection of AuNP concentrated at the TL, we simplify the sandwich immunoassay to an *accumulation assay*. In this configuration, which is a "competitive-like" assay, the human IgG target proteins are disposed at the TL, and AuNP conjugates with anti-human IgG antibodies can thus reliably accumulate at the TL when properly flown into the LFA (Figure 7.8 B).

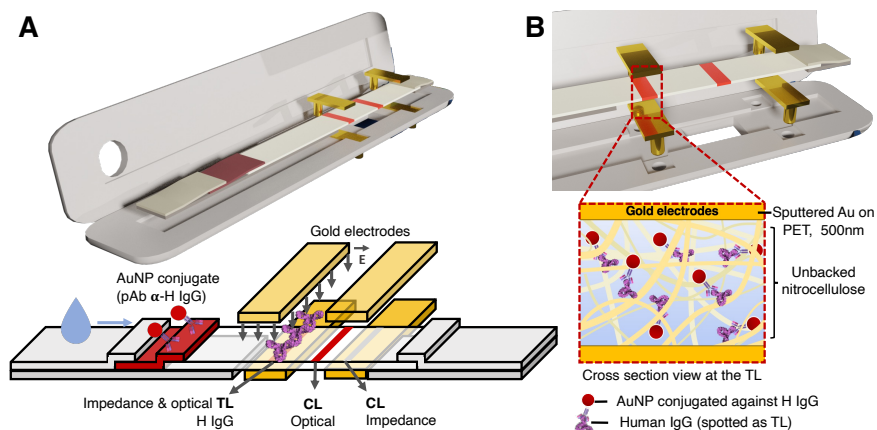


Fig. 7.8 Implementation of non-Faradaic impedance measurements on AuNP-based LFA. **(A)** The experience with the parallel-plate in chapter 6 is leveraged to design a reusable and plug-and-play differential scheme with two electrodes pairs, integrated in an ad-hoc cassette, that can provide impedance measurements at the test line and farther on the LFA in a location without labels, thus serving as blank measurement. The colorimetric test line was kept accessible to allow visual assessment of the LFA flow, and help the interpretation of electrical measurements. **(B)** The fabrication protocol of AuNP-LFA from chapter 4 where modified to ensure the accumulation of a significant number of gold labels at the test line, by direct functionalization of the TL with the target to be detected, i.e. human IgG.

Figure 7.9 shows the reusable, 3D printed cassette with the gold planar electrodes disposed in the differential scheme. To perform the impedance measurements, each electrode pairs was connected to an independent impedance meter (Metrohm μ STAT Multi-4 potentiostat, a 4-channel potentiostat with frequency response analyzer modules). Since the quality of the measurement is highly dependent of the close contact between the electrodes and the nitrocellulose membrane, clamping screws are added to control the pressure and ensure reproducible clamping.

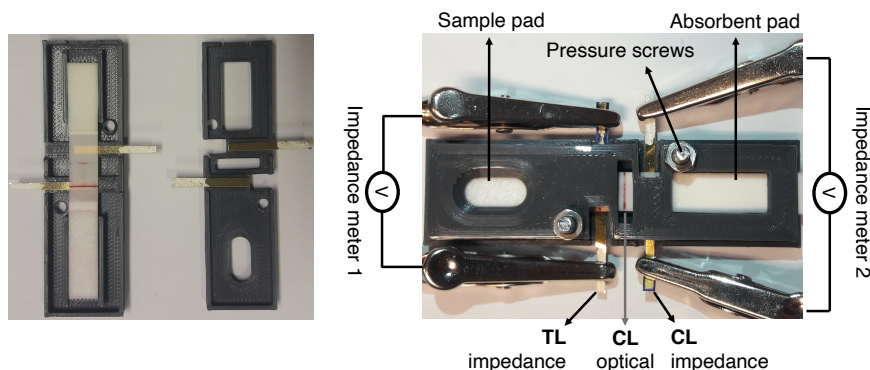


Fig. 7.9 3D-printed setup for non-Faradaic impedance measurements on LFAs, enabling real-time optical and electrical monitoring.

Sensor modelling to support LFA design choices

Conventional AuNP-based LFAs use nitrocellulose membranes casted on a polyester support, or *backing*, which eases the handling. All the pads are then laminated on a second plastic support card. In chapter 6, we performed the impedance measurements directly on the nitrocellulose membranes with a backing, providing a sensitivity to ionic concentration through the dielectric properties of the membrane soaked with electrolyte (both resistance and capacitance changes). Here, the electrodes are produced in a much more homemade, uncontrolled, and unshielded way compared to the probe parallel plate. Therefore, the interfacing of simple gold planar electrodes with the backed NC membrane was assessed and compared to the use of unbacked NC membrane, that are produced and supplied without the polyester backing. In order to make the unbacked nitrocellulose accessible to the electrodes on either side of the membrane, an opening was inserted in the supporting card, and the membrane was stuck on both sides of the support card to form a bridge between the sample pad and the absorbent pads sides, ensuring a proper overlap over the entire strip.

As depicted on Figure F.1 (Appendix F), the resolution of impedance measurements in the presence of two model fluids with opposite resistive dielectric properties (DI water is highly resistive whereas PBS, highly saline, tolerates the ionic polarisation of its medium very well) suggests the need to use NC membrane without backing for our system in order to remove the capacitive effect of the backing, which drastically increases the global impedance and prevent to be sensitive to any other element of the proposed sensor equivalent circuits.

The comparison of the two setups, with backed or unbacked nitrocellulose membranes, was further studied based on the electrical equivalent models depicted on Figure F.1. Their corresponding total impedance expression are given in Equation 7.1 and 7.2, respectively, highlighting the significant contribution of the backing capacitance to the impedance of the system.

$$\begin{aligned} Z_{\text{backed}} &= \frac{1}{j\omega C_{\text{Backing}}} + \frac{1}{j\omega C_{dl}} + \left(\frac{1}{R_{LFA}} + j\omega C_{LFA} \right)^{-1} \\ &= \frac{j\omega R_{LFA} C_{LFA} (C_{dl} + C_{\text{Backing}}) + j\omega R_{LFA} C_{\text{Backing}} C_{dl} + C_{\text{Backing}} + C_{dl}}{j\omega C_{dl} C_{\text{Backing}} (j\omega R_{LFA} C_{LFA} + 1)} \end{aligned} \quad (7.1)$$

$$\begin{aligned} Z_{\text{unbacked}} &= \frac{1}{j\omega C_{dl}} + \left(\frac{1}{R_{LFA}} + j\omega C_{LFA} \right)^{-1} \\ &= \frac{1 + j\omega R_{LFA} (C_{LFA} + C_{dl})}{j\omega C_{dl} (1 + j\omega C_{LFA} R_{LFA})} \end{aligned} \quad (7.2)$$

In chapter 6, at saline conditions (typically PBS buffers $\sigma_{\text{PBS}} \simeq 1.6$ [S/m]) we saw that due to the high electrolyte permittivity and ionic concentrations, C_{dl} is extremely large ($\simeq 1\text{-}100 \mu\text{F}/\text{cm}^2$). Since $C_{\text{Backing}} \simeq 100$ pF, the backing capacitance dominates in series with C_{dl} . The double layer is screened by the backing insulator layer [13], the latter implying a high total impedance of the system, even at high ionic concentrations. However, when the backing is removed, the global impedance of the system decreases as the displacement current, generated by the polarization arising from the alternative electric field, can flow through the solid-electrolyte interfaces much more easily. Therefore, the measured impedance is lower and driven by R_{LFA} , i.e. the conductivity of the buffers, over a wider range and at lower frequencies.

Figure 7.10 depicts the impedance spectra of the electrical equivalent models, including or not an insulating layer characterised by a $C_{\text{backing}} = 100$ pF (measured). The volume capacitance of the NC membrane, soaked with aqueous electrolytes, is approximated to $C_{LFA} = \frac{\epsilon_0 \epsilon_r A}{d} \sim 100$ pF with $\epsilon_0 = 8.85 \times 10^{-12}$ F.m⁻¹ the permittivity of free-space, $\epsilon_r \sim 80$ F.m⁻¹ the permittivity of water, $A = 10^{-5}$ m² the surface area of the electrodes at the test and control lines, and $d = 1.4 \times 10^{-4}$ m the thickness of the unbacked nitrocellulose membrane. The resistance value of the NC membrane soaked in PBS and deionized water was estimated as $R_{LFA} = \frac{\rho d}{A}$

with ρ the resistivity of the solution, d the thickness of the NC membrane, A the surface area of the electrodes. The dielectric properties of the PBS are $\sigma_{PBS} \sim 15 \text{ mS/cm}$, and $\rho_{PBS} \sim 0.66 \Omega\cdot\text{m}$. Therefore, in unbacked disposition, R_{PBS} can be approximated with 10Ω . For DI water, the R_{DI} estimation based on the theoretical resistivity value reflects less the observations. DI water is supposed to be highly resistive, i.e. $\rho_{DI} \sim 10 [\text{M}\Omega\cdot\text{cm}]$. However, in practice we see that the resistance measured with DI water is often in the same range as the one of PBS diluted $1000 \times$. Therefore, the experimental manipulations result in a measured resistance DI water solutions being maximum $10^4 \Omega$, which is used in the model. For the backed NC membranes, the R_{PBS} and R_{DI} used in the model are 10^3 and $10^5 \Omega$, respectively, reflecting our previous experimental observations.

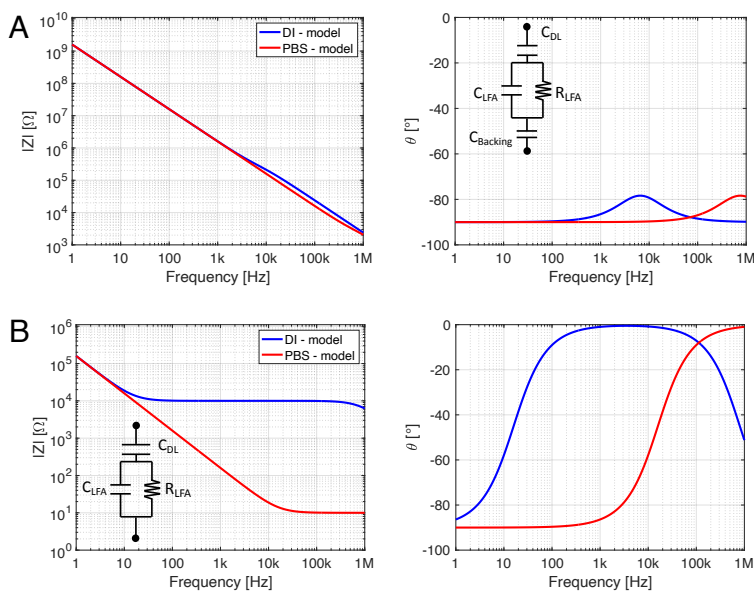


Fig. 7.10 Modelling of **A.** backed and **B.** unbacked nitrocellulose membranes, with representative parameters (see text). These models confirm the experimental observations made on Figure F.1. The observation of a significant shift between the DI and PBS samples over the frequency range of interest suggests to consider the use of an unbacked NC membrane in the impedance LFA prototype. DI and PBS buffers can be considered as the lower and higher bound in term of electrical conductivity among all the electrolytes that will be applied to the LFA.

As highlighted in the literature (section 7.2.2), one possible way to detect pathogens or specific labels in solution with non-Faradaic impedance

measurements is to be sensitive to changes in the system resistance R_{LFA} due to their perturbing effect in the local electrical field. This strategy is not widely spread, or demonstrated with microelectrodes benefiting from significant electrical field alterations when bioanalytes manage to bind in their close vicinity. In this work, we chose to leverage the sensitivity of the prototype setup to the fluid dielectric properties to try extending the use of non-Faradaic impedance to AuNP-based LFAs. Therefore, the final LFA design involved the use of unbacked nitrocellulose membranes.

Selection of impedance spectroscopy parameters

The first set of experiments was performed by taking impedance measurement of buffers alone in the LFA strip using a frequency sweep from 0.1 Hz to 1 MHz (Figure 7.11 in order to calibrate impedance response under conditions relevant to LFA operation. PBS buffer and DI water were used as lower limit and upper limits of fluid resistivities, respectively, as any buffer used in LFAs are dilutions of these buffers and have intermediate resistivities that are proportional to the PBS or DI. They are depicted on Figure 7.11, mainly as dilutions of the 10 mM PBS stock solution.

Figure 7.11, described by equation 7.2 explains the properties of impedance measurement of the LFA soaked with electrolytes, which depends on frequency. In the low frequency zone ($f < 10$ kHz), the double layer capacitance is the main contributor to the high impedance modulus $|Z|$ and is the main driver of total impedance, so that the resistance of the medium can be ignored (Figure 7.11, C_{dl} region). At high frequencies ($f > 10$ kHz), the resistance of the medium is the most important contribution to total impedance, which is independent from the frequency. This region is defined as the resistive region, in which free ion conduction in the medium dominates the total impedance response of the system (Figure 7.11, R_s region). Therefore, changes of medium resistance due to accumulation of analytes or labels can be measured at the highest signal to noise ratio without the capacitance buildup at the electrodes by operating at a single frequency, e.g. 100 kHz.

Further analysis of the phase angle θ was carried out to distinguish between impedance change due to conductivity and impedance change due to capacitance changes. If there is a change in impedance but no change in the phase angle, which remains highly resistive (i.e. close to 0°), then the change is due to the resistivity of the medium. However, if the change in modulus is also accompanied by a change in θ , so the system also displays a capacitive change. This was clearly observed with the setup used in chapter 6. Here, at 100 kHz, the phase angle is significantly close to 0 for every conditions, such that the impedance changes are mainly sensed

through R_{LFA} .

This also confirmed that the resistance value observed for the DI water sample lies in the same range as $1000\times$ diluted PBS, although resistivity of DI water was supposed to be significantly higher, suggesting contamination of the sample and influence of other parameters (such as the NC membrane) once the measurements are performed on the LFA.

Then, we extracted the parameters corresponding to the equivalent circuit (Figure 7.12 A) from recorded impedance spectra from Figure 7.11. Fitting the spectra, conducted with Zview software, enabled the extraction of R_{LFA} and comparison with the recorded impedance modulus $|Z|$ to demonstrate the accuracy of the hypothesis of sensing mainly through R_{LFA} at 100 kHz (Figure 7.12 B): R_{LFA} extracted very similar to the impedance modulus at 100 kHz because impedance is dominated by the medium resistance at high frequencies (electrolyte + nitrocellulose membrane).

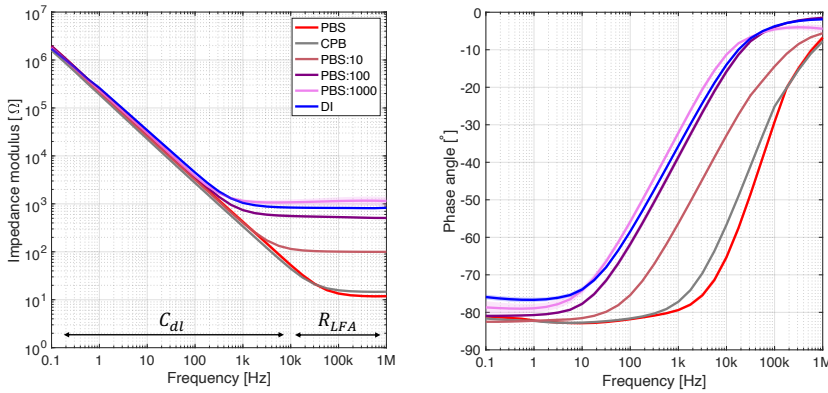


Fig. 7.11 Selection of impedance spectroscopy parameters. Impedance spectrum measurements on LFA test line (0.1Hz - 1MHz, 10 mV) subjected to various electrolyte, with PBS and DI water as the lower and upper resistivity limits as any buffer used in LFAs have intermediate dielectric properties. *PBS*: buffers are dilutions of the initial PBS 10 mM stock solution. *CPB* is the conjugate pad buffer, i.e. the main buffer influencing the physico-chemical properties during the LFA operation.

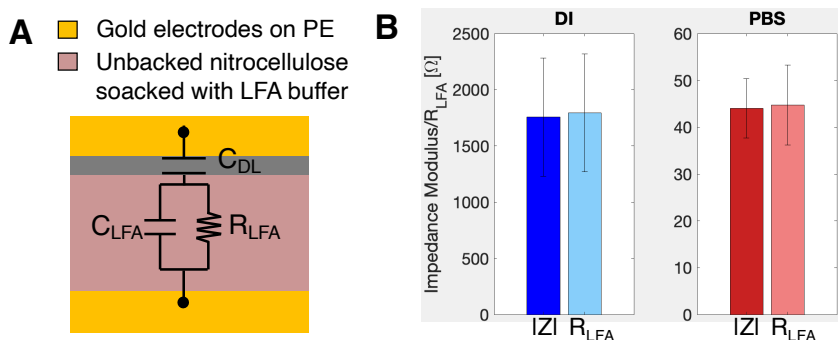


Fig. 7.12 **A.** Equivalent circuit of the impedance-based LFA Impedance. **B.** Values of the equivalent circuit R_{LFA} parameter as extracted by fitting the model to the PBS and DI curves displayed on Figure 7.11. The highly close value between the value of the resistance extracted and the impedance modulus at 0.1 MHz confirms the mainly resistive behaviour of the unbacked nitrocellulose LFA at this frequency.

Data analysis

To account for fabrication differences among the electrode pairs used (geometries), among the fabricated LFAs and from the variability of LFA interfacing in the cassette (pressure differences), the results are typically based on a subsets of the performed measurements :

- Non-working channels (poor contact/pression, bad connections) were excluded based on noise and impedance amplitude evaluation.
- Evaluation of the channel quality based on the background noise (rough or smooth curve).
- Variability within and between working electrodes pairs was studied by statistical measures (means and standards deviation of impedance amplitude for different frequencies) and depicted on Figures F.2 and F.3.

The **variability analysis** (Appendix 6.2) suggests strong influence of the LFA fabrication protocol and the electrode pair used on the recorded impedance. This calls to avoid absolute comparison of the recorded impedances between the electrodes pairs (for example comparison of the test and control line electrodes), or between different LFAs. Therefore, the development of the LFA testing protocol must take this consideration into account in order to ensure the accuracy of the measurements interpretation.

7.4.2 Calibration

In view of the results of chapter 6 and the sensitivity analysis carried out in section 7.4.1, it is therefore anticipated that the impedance measurements

will be very sensitive to the specificities of full LFA tests. It includes the inhomogeneity related to its manufacture, its materials, or the complex design with interconnection of several substrates (SP-CP-NC-AP) that influence the microfluidics of the system). One can also expect a variable sensitivity to the buffers involved to enable the LFA functionalities (the CPB introduced in section 7.4.1 for the release and proper fluidics of the NPs within the LFA, residual BSA related to blocking, etc).

All of these factors contribute to the complexity of interpreting the results of impedance measurements from LFAs, and contribute to significant variability between measurements. It is therefore critical to **characterise the system behaviour in experimental conditions representative to LFA operation**.

To this end, we further monitored the response of our impedance LFA prototype to model saline solutions (dilutions of the 10 mM PBS stock buffer). Since the calibration to the electrolyte conductivity conducted in section 7.4.1 was performed in a *static* disposition, i.e. 15 min after the drop-casting on the sample on the LFA, no more flow was expected within the LFA. However, here, **we performed continuous measurements of the impedance at 100 kHz over the complete LFA operating time in order to understand the effect of the LFA fluidics on the impedance measurements**. This calibration is used to determine the impedance "background noise" generated by the LFA in various electrolytes.

This **study of the electro-fluidic dynamics of the LFA**, which aims to understand the physical phenomena within the LFA and the influence on the interpretation of the measurements, includes analysis of the evolution of the flow within the first moments after release, the dynamics of mass and charge transport (ionic displacement) within it and the influence of evaporation on the results. These studies on LFA are usually carried out empirically by means of optimisation via colorimetric tests or liquid mass content analysis. The characterization of such parameters with non-faradaic impedance measurements has not been reported before, to the best of our knowledge.

Temporal response highlights different fluidic regimes

Figure 7.13 shows the evolution of the impedance modulus measured at 1kHz over the 1000 first seconds of the LFA operation time. It can be observed that when the fluid arrives at the electrode area, an initial drop of impedance is measured during a period of 100 seconds, followed by a progressive stabilization of the impedance. This impedance curve shows high similarities with the flow rate evolution over the LFA time duration reported by Gasperino *et al.* (Figure 7.14). They identified 4 main fluidic regimes during an LFA operation : (1) fast flow during the NC membrane wetting, (2) the absorbent pad impregnation from the membrane, (3) absorbent pad filling, and (4) progressive drying of the LFA from the LFA sides.

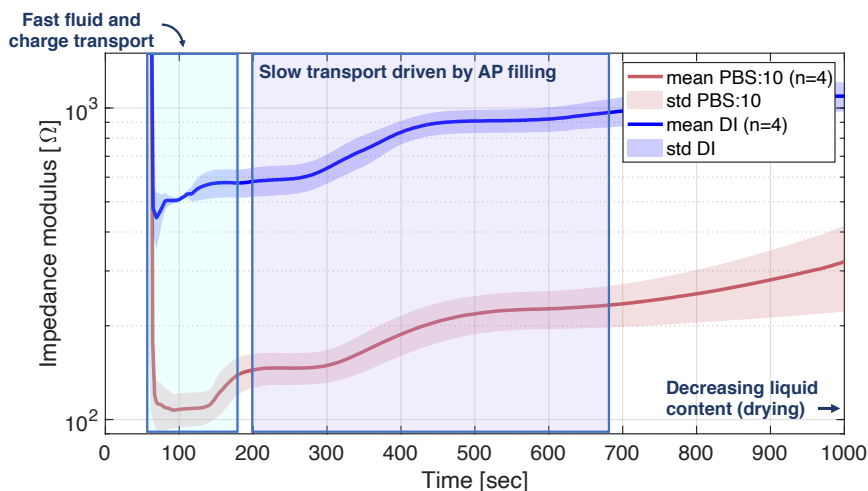


Fig. 7.13 Different fluidic regimes highlighted by monitoring impedance evolution over the LFA operation time. After an initial drop of about 100 sec, the impedance progressively increases to stabilize after about 7 min, once the absorbent pad drives the capillarity action. The initial decrease in impedance can be attributed to the higher number of ions transported by the fluid during the first fast fluidic regime driven by the membrane wetting.

In order to understand the origin of the impedance drop, we need to understand the charge transport dynamic happening in the LFA at that moment. **Charge transport in microfluidics** is described by the Péclet

number, Pe , which is defined as

$$Pe = \frac{\text{convection rate}}{\text{diffusion rate}} = \frac{V \cdot r_C}{D} \quad (7.3)$$

with V which is the velocity of the moving fluids through the LFA membrane, r_C is the characteristic size of the pores inside the membrane, and D is the diffusivity of the species transported in the fluids.

To obtain an estimation of the velocity of the moving fluid, we recorded the advance of the fluid front over time during the first seconds of the LFA operation (see Appendices Figure F.4). From these measurements, we computed a fluid flow velocity $V \simeq 8 \times 10^{-4}$ m/s. Since the diffusion coefficients of the NaCl solution D_{NaCl} is 1.61×10^{-9} m²/s [283], and the mean pore size $r_C \simeq 11.5$ μ m computed in section 3.6.2 for CN95 membranes, $Pe \simeq 5.96$, and the transport of ions in the LFA is dominated by the convection, i.e. convection rate due to capillarity is higher than the diffusion rate. This initial fast fluid transport, resulting in fast ionic charge transport between the electrodes, could explain the subsequent lower monitored impedance during this very precise moment of the LFA operation.

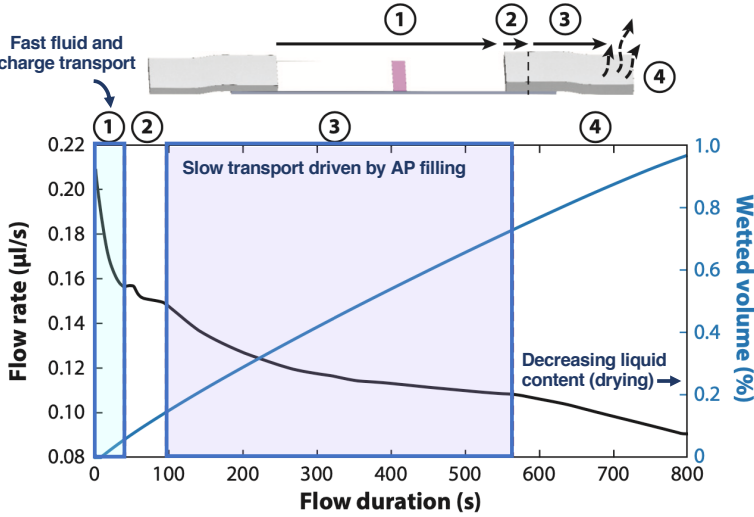


Fig. 7.14 Gasperino *et al.* also reported 4 main fluidic regimes, including the initial fast fluidic transport over the first 50 seconds. The duration of the initial fast fluid transport depends on the LFA geometry. Reprinted from [179]

Finally, we also measured the impedance evolution over a much longer

period of time ($\sim 3\text{h}$), which revealed the drying process of the LFA (Figure 7.15). This study allows us to select the appropriate timeframe during LFA operation to perform the measurements at a moment where the LFA fluidic is not influenced by transient effects such that the initial fast flow or the drying of the LFA. In particular, the timeframe between 15 min and $\sim 2\text{h}$ looks promising to keep a constant, steady flow of ions and remove background noise of the LFA operation.

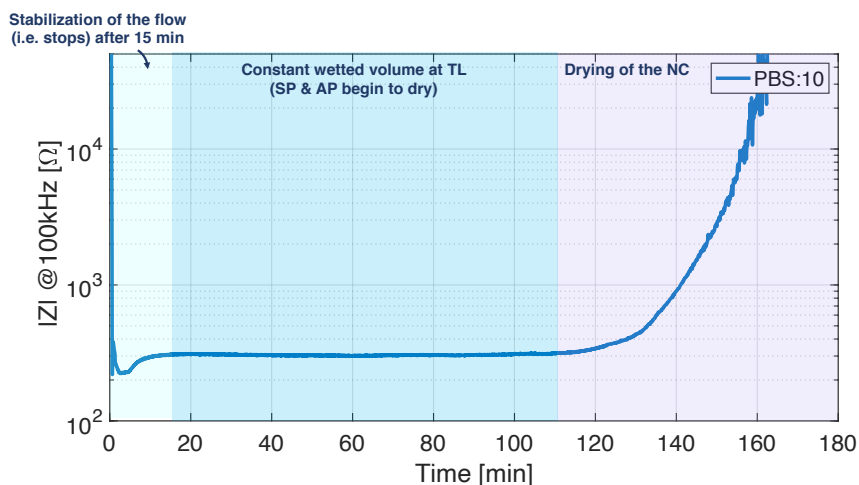


Fig. 7.15 LFA drying study by impedance measurements. The timeframe between 15 min and $\sim 2\text{h}$ indicates a constant, steady flow of ions through the LFA, i.e. in between the electrodes, which thus remove some of the background noise of the LFA operation

7.4.3 Control of LFA electro-fluidic properties

In order to control the dielectric properties of the LFA, in this section we assessed the possibility of **washing the LFA ionic content during the LFA operation time**. In conventional microfluidics (i.e. plastic microchannel actuated with a pump), washing of the test area is achieved by progressively adding new buffers in the microfluidic circuit to force the buffer present at the test area to move further downstream in the circuit, thus replacing the properties of this fluid at the test area by those of the new buffer. In our case, we try to apply this strategy to the LFA case, by continuously monitoring the impedance response of the test zone. We apply a procedure consisting in a **successive addition of small buffer volumes of different resistivity**, alternating between a highly conductive PBS with high ionic

content and low resistivity, and PBS diluted 1000× in non-conductive DI water.

Coupling optical and electrical measures highlights fluid mixing in LFAs

First, we studied the dynamic impedance response to fluid resistivity in conventional LFA formats (60 mm × 5 mm, cellulose sample and absorbent pads). The results, depicted on Figure 7.16 A for the impedance modulus show a progressive decoupling of the impedance response with the added buffer resistivity. In addition, an unexpected decrease of the impedance was observed at the end of the last washing step, inconsistent with the expectations (i.e. increasing impedance). Monitoring of the phase angle over the whole operation is depicted on Figure F.5.

In order to understand the origin of the progressive decoupling of the impedance response with the added buffer and the inconsistent increasing impedance after 2h, we simulated the progressive propagation of PBS and PBS:1000 buffers by means of coloured fluids in order to observe their flows and to highlight their possible interactions (Figure 7.16 B). We observed that the addition of the red fluid, simulating the PBS, was leading to an increasing mixing of the buffers at the test line (dashed rectangle on Figure 7.16 B), seen at 45 and 105 min, explaining the global decrease of impedance over time.

These observations indicate that the microfluidic principle of conventional LFA format, i.e. capillarity with a limited bed volume, cannot be approximated with the operation of conventional microfluidic circuits (plastic-based) for a precise control of the fluid properties at a certain place of the channel. Indeed, due to the limited wetting capacity of the absorbent pad, the flow will eventually stops (more or less quickly depending on the AP size), leading to a mixing of the fluids within the LFA. These results suggest that **conventional LFAs are not appropriate for controlling the electrofluidic properties of the flow.**

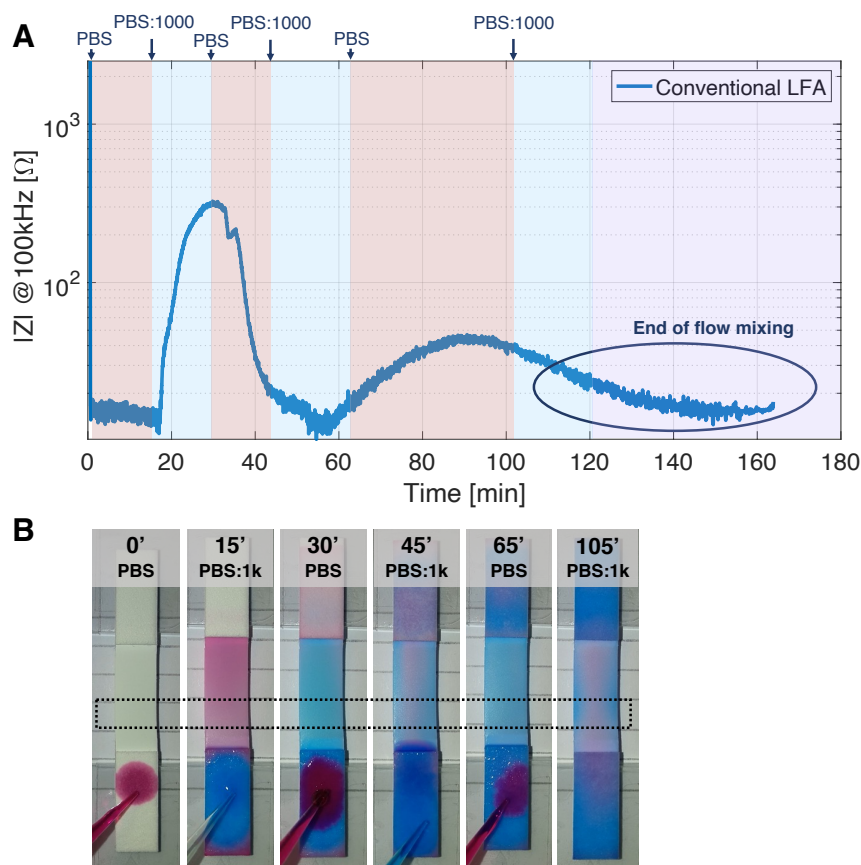


Fig. 7.16 Impedance sensing of successive buffers in conventional LFA. **A.** Each coloured area starts when the corresponding buffer is added to the LFA (red: PBS, blue: PBS:1000). Here, we drop-casted 75 μL of PBS to start the LFA operation. Then, we successively alternated the addition 50 μL buffers (PBS or diluted PBS:1000) on the LFA at specific time intervals : PBS:1000 (15 min), PBS (15 min), PBS:1000 (15 min), PBS (20 min). The last addition consisted in a PBS:1000 washing (40 min). The impedance-LFA showed a response consistent with the resistivity of the buffers added during the first 3 injections. Then, an increasing response time to the last sample buffer was observed until the resistivity of the added fluids and the resistance of the measured nitrocellulose membrane completely decoupled. Strikingly, whereas we expected to completely wash the LFA of the majority of its ions with highly resistive PBS:1000 at the end of the LFA test time, a drop in resistance was observed, uncorrelated with the added PBS:1000. **B.** Simulation of the successive buffers highlights fluid mixing (at 45 and 105 min), consistent with the impedance measurements. Therefore, a conventional LFA is not suitable for precise control of the ionic content at the test line area.

Engineering LFA design to control LFA electro-fluidic properties

To overcome the limitations presented by the conventional LFA format, in this section we introduce an **innovative LFA design enabling the precise control of the ionic content in the LFA** on a timescale adequate for the proper functioning of the impedance-LFA.

LFA design engineering Since we observed that the bed volume was the limiting factor for controlling dynamic washing steps, that involve successive addition of buffers, here we propose to engineer the absorbent pad of conventional LFA to obtain an LFA design suitable for true flow control, similar to what is obtained for conventional microfluidic channels. The idea of flow control was already developed for other paper-based application, mainly the development of paper-based batteries [184, 284]. In that case, the requirement of a continuous supply of reactive species, in order to keep a constant ionic concentration at the reaction zone, involved the design of a paper microfluidic channel that can maintain the same flow velocity over a long period of time. This was obtained by designing the paper microfluidics channel as a fan, with a large surface area, such that the increase in viscous forces due to the fluid progression in the paper channel is compensated for by the increase in capillary force thanks to the larger pore contact area available to the fluid, see section 3.8. Very recently, a first study of an electrochemical paper-based sensor leveraged this approach to allow the continuous-flow electrochemical detection of biomolecules in a three-electrodes configuration [285].

Here, we take advantage of this strategy by integrating a fan-like absorbent pad, aimed at increasing the available bed volume and enabling precise control on the LFA flow over the period of time necessary to perform the impedance measurements.

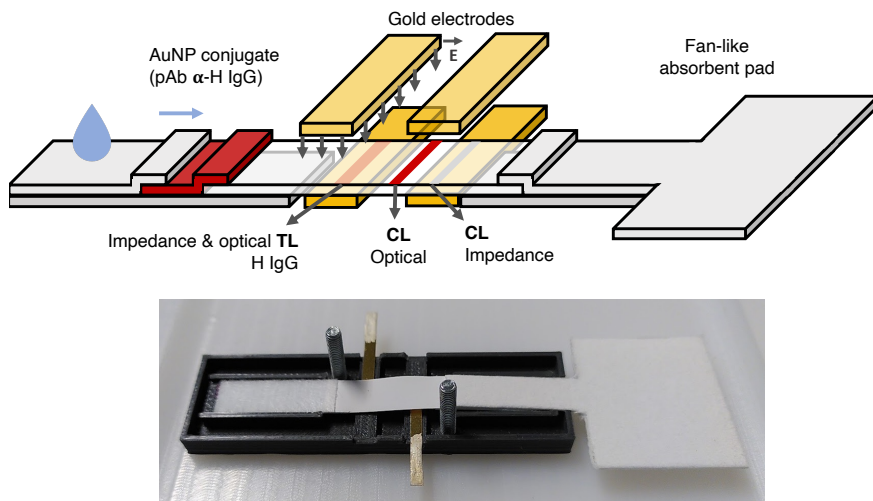


Fig. 7.17 LFA design engineering for efficient control on ionic content. The fan absorbent pad increases the total bed volume of the LFA, i.e. capacity, and thus permits a continuous flow for a much larger sample volume.

Device characterization with model fluids Figure 7.18 depicts that the flow control in the fan-LFA results in the possibility to control the dielectric properties of the flow through the implementation of consistent washing steps. Dynamics of washings in fan LFA 1 - 15 min period (except first). The phase angle evolution is shown on Figure F.6. + limitations

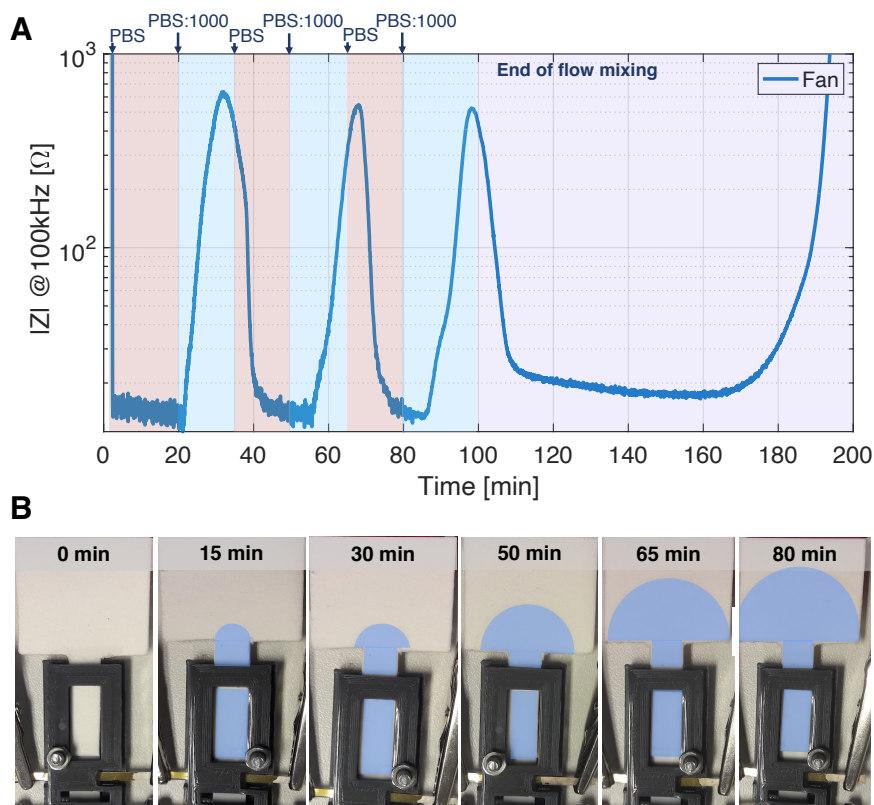


Fig. 7.18 **A.** Characterization of the engineered LFA design for efficient control on ionic content. This is reflected in the impedance response, which is driven by the addition of the buffer in a much more precise way than for conventional LFA (depicted on Figure 7.16). Therefore, adequate washing steps are possible to implement with this setup. **B.** The fan absorbent pad increases the total bed volume of the LFA, i.e. capacity, and thus permits a continuous flow for a much larger sample volume than conventional LFA.

Optimisation of time and sample volume In order to fully characterize the concept of controlling the dielectric properties of LFA flow by washing ionic species in LFA with successive buffers, we perform a second dynamic impedance measurement on the engineered LFA, with a time period of 20 min between two injection of buffers (Figure 7.19 A). The colorimetric experiment carried out in parallel (Figure 7.19 B) allowed to visualise the reason for the time lag between the injection of the buffer and its visualisation thanks to the impedimetric signal of the test line: the time required for the new liquid front to clear the previous buffer in the nitrocellulose mem-

brane increases with the cycles (dashed area). Therefore, the dynamics of the washing steps must be taken into account when integrating washings in the detection protocol. The phase angle evolution with time is reported on Figure F.7.

Another interesting feature of the engineered fan-LFA is the possibility to perform staircase calibration of the sensor response to electrolyte of different conductivities (Figure 7.20).

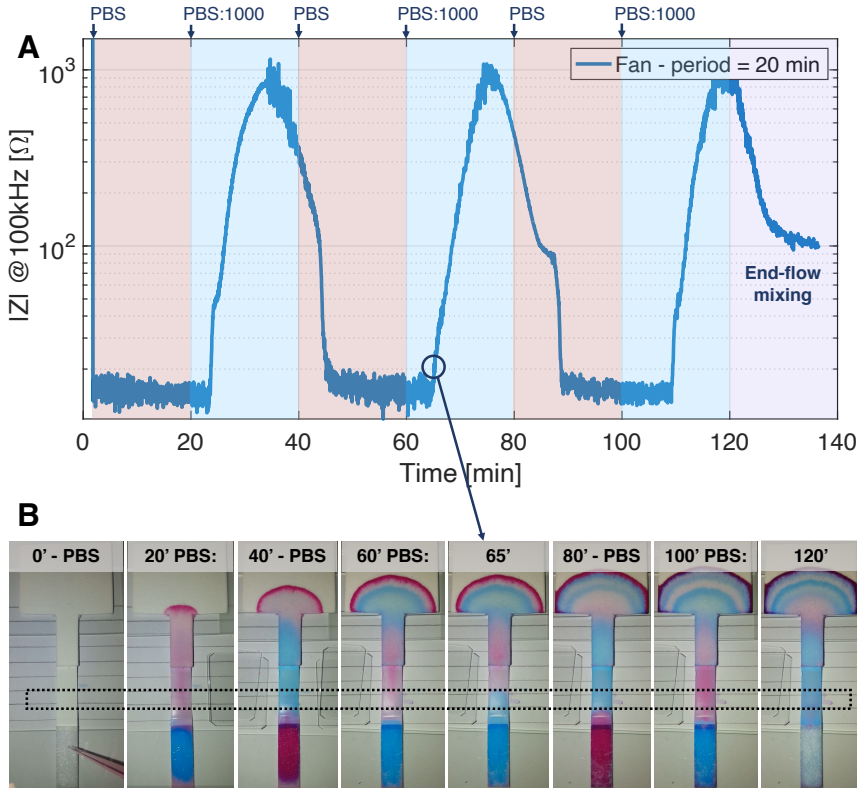


Fig. 7.19 Characterization of the engineered LFA design for efficient control on ionic content. This is reflected in the impedance response, which is driven by the addition of the buffer in a much more precise way than for conventional LFA (depicted on Figure 7.16). Therefore, adequate washing steps are possible to implement with this setup.

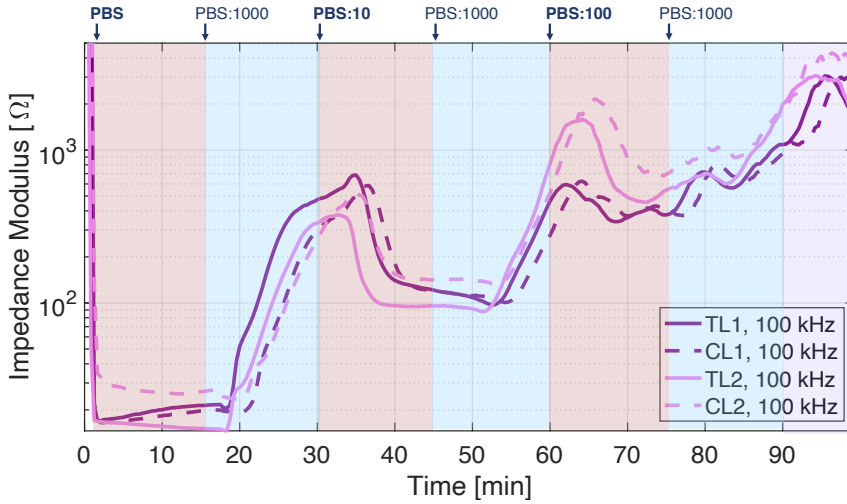


Fig. 7.20 Example of staircase calibration enabled by the engineered fan-LFA.

Differential measurements estimates the ionic fluid velocity

A delay of 200 seconds between the TL and the CL is reported from the differential impedance measurements when the highly conductive PBS buffer is injected in the LFA. However, is the delay due to the flow convection across the LFA due to the capillarity, or rather due to diffusion in the flow due to the presence of two fluids with different concentrations ?

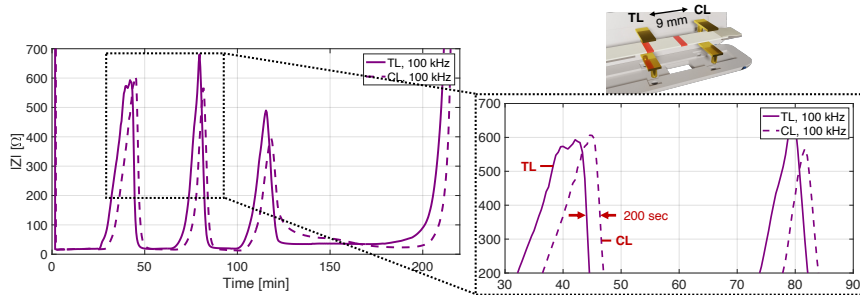


Fig. 7.21 Characterization of the engineered LFA design for efficient control on ionic content. This is reflected in the impedance response, which is driven by the addition of the buffer in a much more precise way than for conventional LFA (depicted on Figure 7.16). Therefore, adequate washing steps are possible to implement with this setup.

Origin of charge transport in LFA

Finally, to complete the characterization of the prototype impedance-LFA setup, we need to determine the origin of the ionic velocity previously computed. Indeed, even though Figures 7.18 and 7.19 clearly reported a control over the ionic content at the test line location, the kinetics of the charge transport within the LFA is not determined, and could still potentially mix convection and diffusion of the ions in the LFA.

We used again the Péclet number from Equation 7.3 to obtain insights into the dominant charge transport mechanism within the LFA. We determined the velocity of the flow by comparing the velocity of the moving fluids from the colorimetric measurements (Figure 7.19 B) and from the impedance response delay between the TL and the CL observed in the impedance differential measurements (Figure 7.21), which were 0.5×10^{-4} and 0.45×10^{-4} m/s, respectively. Therefore, according to the average fluid flow velocity $V \simeq 0.475 \times 10^{-4}$ m/s, the diffusion coefficients of the NaCl solution D_{NaCl} is 1.61×10^{-9} m²/s and the mean pore size $r_C \simeq 11.5$ μ m for CN95 membranes, $Pe \simeq 0.57$, and the **transport of ions in the LFA during the successive depositions is dominated by the diffusion**, i.e. the diffusion rate is higher than the convection rate due to capillarity.

7.4.4 Towards the specific impedimetric detection of AuNPs in LFA

Concept

The various challenges raised in Chapter 6 and in this chapter, which are in order of appearance:

- The need to be able to make differential measurements between a test line and an impedimetric control line to ensure that the observed impedance variation is due to the presence of targets at the test line.
- The high variability of the observed results due to the variability of the electrodes, the manufacturing process of the LFAs, and the intrinsic inhomogeneity of the LFAs prevents the results obtained with one pair of electrodes from being compared absolutely with the measured impedance.
- The particular nature of LFA microfluidics based on capillarity and its mode of operation of the LFA, which implies that it is almost impossible to exert perfect control over the electrical properties of the LFA, or that it is at best possible to approach control, but only over a certain period of time (before end-flow mixing).

This section proposes a strategy for impedimetric detection of AuNPs incorporating measurements to address the above challenges. Performing an impedimetric detection measurement on the LFA requires taking many precautions, including the steps to prepare the ideal detection medium within the LFA (i.e. controlled dielectric properties), depicted on Figure 7.22 :

- Always perform differential measurements as proposed in section 7.4.3. Care should be taken to allow sufficient time for equilibration so that the fluid in which the measurement is to be made is evenly distributed under the test line and the control line.
- Relative impedance measurements must be made for each electrode, as it is the difference in impedance between a controlled situation and the situation of accumulation of AuNPs that must be made visible. In this way, taking advantage of the relative impedance measurements of the presence of AuNPs at the test line, the two relative signals from the TL and CL can be compared.
- Finally, it is critical to select the timeframe over which the impedance measurement can be made within the LFA, to ensure that undesirable effects such as end-of-flow mixing do not occur and screen the detection signal.

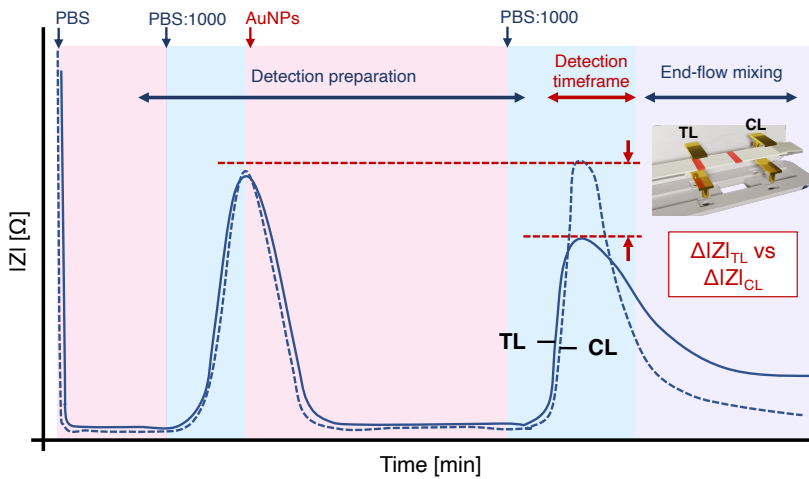


Fig. 7.22 Proposed methodology for specific, differential impedance detection of AuNPs in an engineered LFA.

First detection results

Typical impedance detection results of accumulation of AuNPs at the TL are depicted on Figure 7.23 and F.8. Figure 7.23 shows the overlay results for test line and control line impedance measurements at 100kHz performed on two independent cassettes (cassette 1 and cassette 2) recorded at the same time since our multi-channel impedance meter possesses 4 channels. The proper binding of the AuNP to the TL were ensure by visually check that the CL has well reacted (Figure 7.23 photo) First, we can notice that the impedance modulus measured by the different electrode pairs are different, as observed in the variability analysis. It supports the relative impedance change analysis. Second, we observe the impedance delay of the fluid between the TL and the CL, for each point of the measurement. Third, we observed quite a lot of variability in the response of the end-flow mixing area, suggesting not to use the impedance plateau observed in this timescale as a proper detection "detection zone".

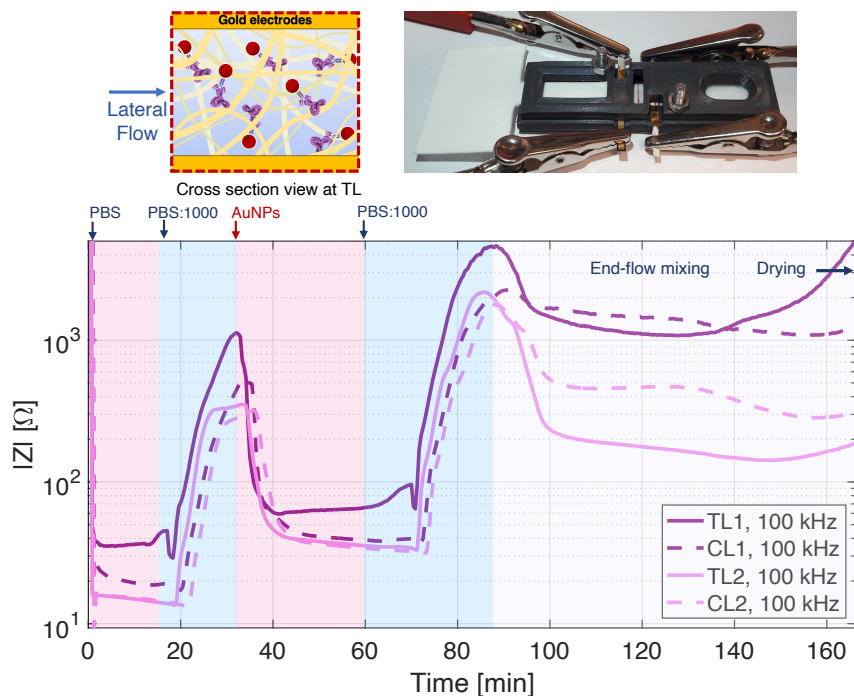


Fig. 7.23 First results of AuNP impedance detection (impedance modulus). The results for two impedance-LFA cassettes are reported, which were tested simultaneously.

Controls

In order to quickly compare the results obtained for the accumulation of AuNPs with a reference measurement, we performed negative control experiments by removing the conjugated AuNP from the solution and conducting the impedance measurements at 100kHz with LFA wicking only with the running buffer. Typical results are depicted on Figure 7.24 and F.9, showing again the delay between the TL and the CL and the same characteristic curve than with the accumulation of AuNPs at the TL, with highly variable end-flow mixing area.

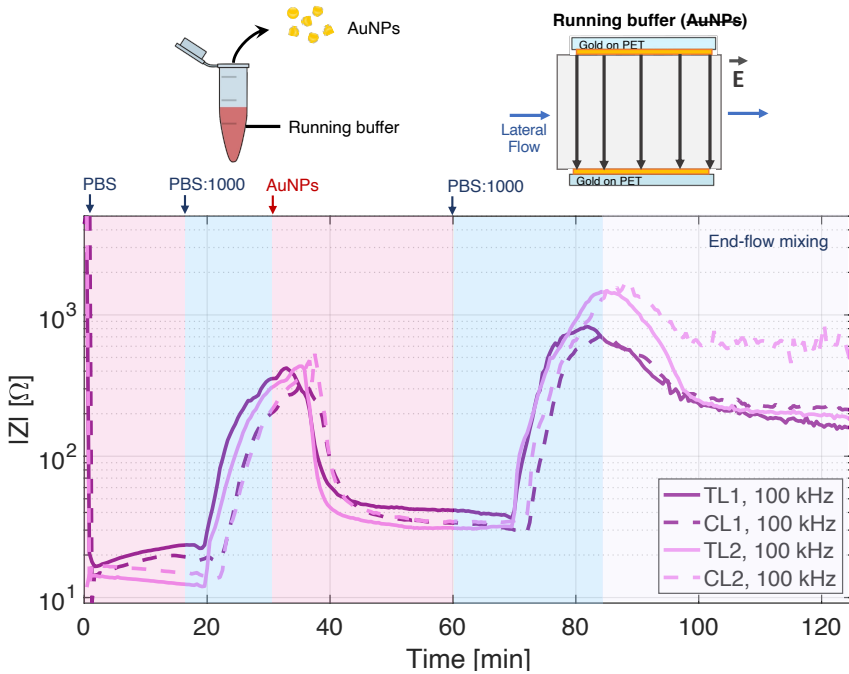


Fig. 7.24 As negative control for the impedance detection of the AuNPs, we removed the conjugated AuNP from their running buffer, which was then used as a buffer for the LFA.

7.5 Discussion and Perspectives

The characterization methodology presented in this chapter allowed for a detailed understanding of certain charge transport phenomena occurring within the LFA, which significantly influence the non-Faradaic impedance measurements proposed as detection mode. Taking into account this electrical background noise of the LFA, a detection scheme was proposed to highlight the accumulation of gold nanoparticles within an LFA using simple resistive impedance measurements via parallel plate electrodes placed on either side of the TL.

However, no conclusions can be drawn from the initial measurements presented at the end of the chapter regarding the dielectric detection of the AuNPs accumulated in the LFA. As this chapter's topic is still under research, a more detailed analysis on a much larger number of measurements must still be carried out. Nevertheless, the small size of the gold nanoparticle (20-40 nm) could make them difficult to be detected by volumic non-Faradaic impedance spectroscopy because they represent only a tiny proportion of the total liquid volume at the test line, and so have little influence on the volume dielectric properties of the LFA at the test line and on the impedance measurements. Different strategies can be applied to enhance the signals from the LFAs nanoparticle, including chemical enhancement methods (silver enhancement, double gold conjugation and induced gold aggregation) or the use of the gold nanoparticle catalytic properties to enhance an electrochemical reaction (substrate + H_2O_2), generating a larger current.

Using the silver enhancement reaction on the gold nanoparticles, the AuNP size can increase up to 10 microns [207]) through silver growth amplification: Ag is nucleated on captured GNPs in the test region by flowing Ag-reducing reagents through an LFA after a normal assay. The resulting Ag layer on the AuNP label surface increase the dimensions of the nanoparticles up to a few microns. Figure 7.25 describes in detail the principle of the silver enhancement for the LFA test developed by Fujifilm. In colorimetric LFA, it has been shown that the size increase due to the Ag reduction amplifies the color intensity of the test region. With this method, a colorimetric sensitivity gain of approximately 10-fold relative to that without Ag enhancement has been achieved.

In our case, we can expect that the larger size of the Ag-enhanced AuNP could induce a more significant effect on the change in dielectric proper-

ties of the volume located in between the electrodes than individual 20-nm AuNPs, and therefore expect an increase of the sensitivity of the non-Faradaic impedance measurements to the AuNPs at the test line.

To showcase the concept of size enhancement, a first Ag size enhancement test was performed on gold nanoparticles bound to the test line of the LFA. This first test is intended to evaluate the relevance of the signal increase and/or cumbersomeness of the additional steps in view of the final application of the impedance LFA. After having accumulated gold nanoparticles at the TL with an “accumulation assay” as presented in section 7.4.1 (Figure 7.26 A), the LFAs presenting the red test lines were dip 10 min in a mix of silver nitrate (0.3 % w/v in water) and other of hydroquinone (3 % w/v in 0.5 M citrate buffer pH 4.0). The resulting TL, after chemical enhancement, shows a significantly stronger colorimetric signal at the TL (Figure 7.26 B), especially for low quantities of AuNPs in the LFA (4-6 g/mL), suggesting size increase of the AuNPs through silver reduction.

In order to validate the increase of the size of the nanoparticles bound at the TL, direct visual analysis of the nanoparticles size at the TL must be performed by SEM images. The next step is then to apply the electrodes at the test line of the LFA with larger-sized Ag-enhanced AuNPs in order to investigate the change of dielectric properties brought by the size increase. This is the subject of future work on this system.

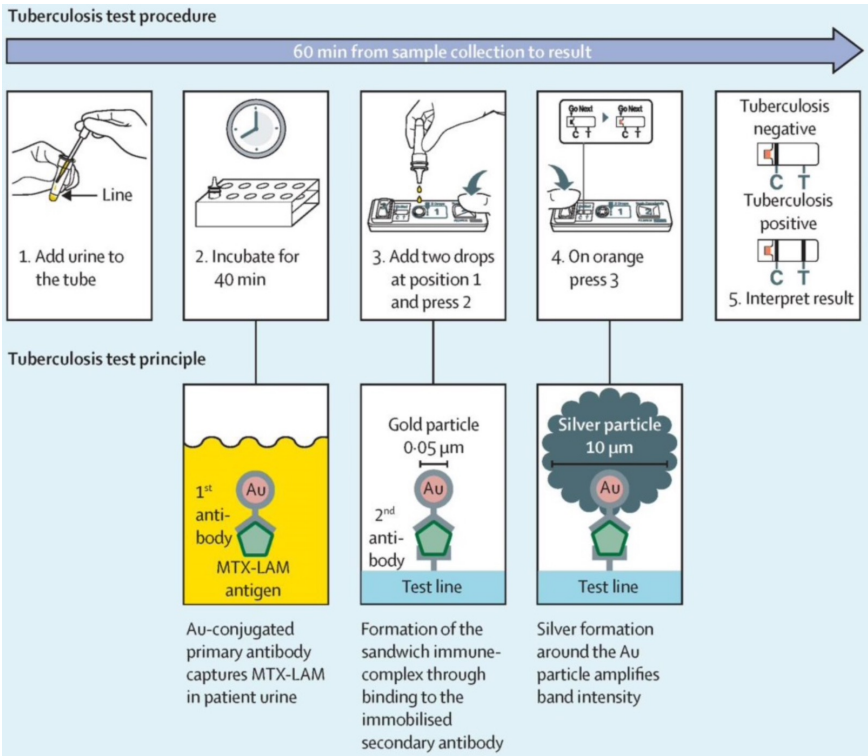


Fig. 7.25 The principle, procedure, and test device of Fujifilm SILVAMP TB LAM [286]. Different from the conventional test strip, this modified device contains two additional buttons located at both ends, which are used to store the silver ion reagent and reducing agent. Consequently, silver growth amplification can be easily achieved by completely pushing the two buttons to release the corresponding growth solution. Compared with the AlereLAM assay for the diagnosis of tuberculosis in people with HIV as recommended by the WHO, FujiLAM displays superior diagnostic sensitivity thanks to the increasing size of the nanoparticle without compromising specificity and simplicity, although an additional signal amplification step is required.

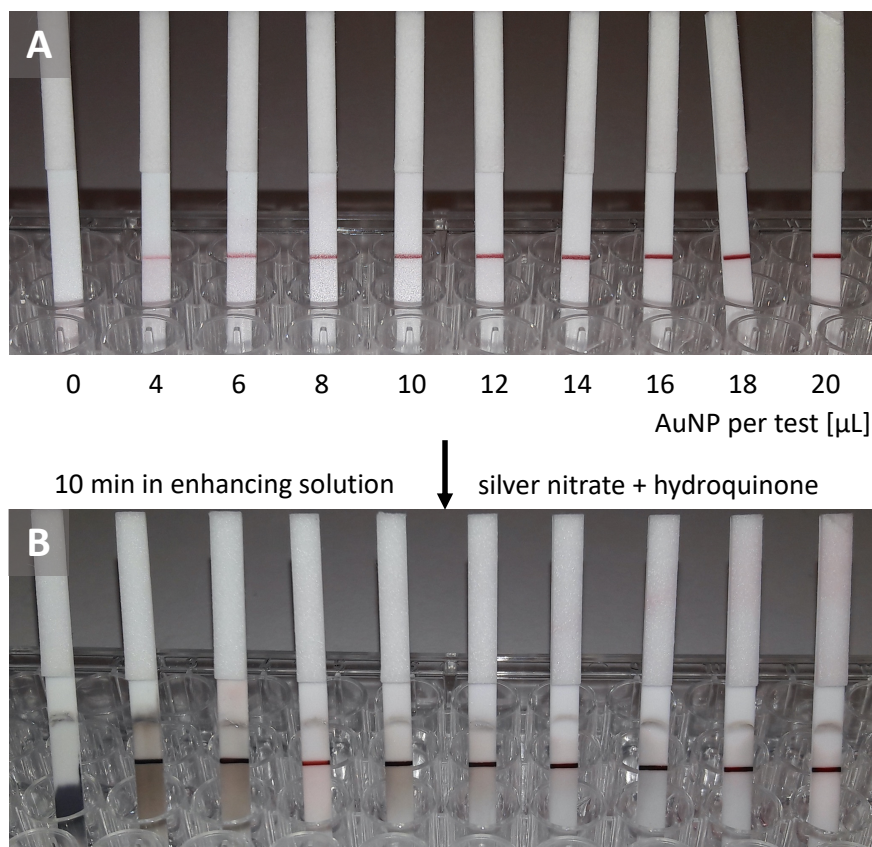


Fig. 7.26 The chemical silver enhancement method increases the sensitivity of a simple colorimetric assay of 20 nm gold nanoparticles accumulated at the test line (A.), thanks to the larger size of the silver-coated gold nanoparticles expressing a stronger colour (especially seen for 4-6 $\mu\text{g/mL}$ of AuNPs in the LFA) (B.). The failure of silver enhancement for 8 $\mu\text{g/mL}$ is due to experimental errors. Next experimental validations of the size-enhancement include direct visual assessment of the nanoparticle morphologies by SEM observation. Then, the goal is to apply the electrodes at the test line of the LFA with larger-sized Ag-enhanced AuNPs to investigate the change of dielectric properties brought by the size increase.

8

Conclusion and Perspectives

In this thesis, we investigated the design of point-of-care (PoC) biosensors for pathogen detection, mainly intended for critical situations. The main aim of this work was to investigate the development of an *appropriate* technology to fulfil this need. The appropriateness of the technology was studied by mean of a *systemic research methodology*, as proposed by Ashby *et al.* [5]. In this thesis, *systemic* can be summarized as identifying the links and barriers between all the dimensions that define our research question, which are depicted on Figure 8.2. This methodology imply successive stages in order to cover the essential dimensions of a systemic analysis. Therefore, the organisation and the key aspects investigated in this thesis are closely linked to this methodology. Each investigated aspect has resulted in innovations that address existing challenges in the field of point-of-care (PoC) biosensing and paper-based biosensor technologies. The main contributions are outlined hereafter.

In the first part of this thesis, a critical overview of the state-of-the-art of biosensors for the detection of pathogens was proposed, complemented by an analysis of environmental impacts in order to help guide the design choices (material and manufacturing process) minimising environmental burdens. From these chapters, we selected the paper-based sensors to initiate the development of the PoC pathogen biosensor.

In chapter 1, the **state of the art** observed that each class of PoC diagnostic devices has individual advantages on certain figure of merits, and that the most promising PoC devices are those that combine technologies

to combine their advantages. In particular, colorimetric lateral flow assays (LFAs) are well suited to be disseminated thanks to their user-friendliness, while electrical biosensors have shown superior performances.

The challenge lies in integrating the advantages of different sensor technologies, making appropriate integration choices, and anticipating the impacts, including environmental aspects. The current state-of-the-art in PoC sensors primarily focuses on traditional performance criteria, neglecting other important factors, which hinders a holistic decision-making process. To enable informed choices, it is essential to incorporate additional aspects, specifically environmental considerations, into design practices and establish a methodology for their implementation.

Chapter 2 tackles this challenge by proposing a innovative framework to **integrate eco-design questions right from the start of the research process**, yet over-viewing all the development stages of a technology (TRLs 0 to 9). This framework proposes to (i) include more systemic figure of merit, such as environmental metrics, in the initial design choices, and (ii) to integrate simplified Life Cycle Analysis during the biosensor research process, in order to guide the design choices (materials and manufacturing techniques) towards to most appropriate technology, both in term of technical and environmental performances. In the chapter and the proposed framework, special attention has been drawn to gather knowledge, data and references that could be further used by other researchers to apply this methodology in a simplified way on other use-cases. The main findings of this chapter demonstrate that the use of paper or related substrates such as nitrocellulose can reduce the environmental costs of PoC sensors by relying on materials and fabrication techniques that are less energy-intensive than sensors fully integrated on plastic or silicon substrates.

However, the conclusions drawn from such a methodology combining technical and environmental assessments must be approached with caution and critical thinking, as it is often challenging to compare two devices of different technologies with the exact same functionalities. One notable example is the difficulty in achieving technical performance of paper-based sensors that is on par with silicon-based sensors.

Nevertheless, paper-based technology appears promising for developing relevant sensors to meet our needs while potentially offering environmental advantages.

In the second part of this thesis, we introduced in detail the most conventional paper-based sensor technology, i.e. the colorimetric lateral flow assay (LFA). A detailed study has provided insight into the physical origins of the high detection limits observed in colorimetric LFAs. Furthermore,

based on the understanding gained from these chapters, new bioreceptors are proposed to facilitate the development of LFA prototypes for the detection of *Bacillus cereus* cells.

In chapter 3, we perform a **complete overview of the multidisciplinary colorimetric LFA technology**, with a special attention to attentively describe all the technical challenges involved in the LFA development process. This chapter provides the necessary foundation to undertake the design of colorimetric LFAs for any biochemical target.

We leveraged this knowledge in chapter 4 to design, develop and operate a **colorimetric LFA for the quantitative detection of a model protein, human IgG**. In addition to having taken over the complete development of an LFA, the significant contribution of this work was to understand the intrinsic detection limits of gold nanoparticle (AuNP)-based LFAs, in the case of protein detection. The main aim is to extend this defined model to other analytes, such as pathogens in Chapter 5. The importance of understanding the intrinsic limitations of the sensors is justified so as to be able to imagine new design scenarios to overcome these limitations which is the topic of Chapters 6 and 7.

In chapter 5, we accommodate the previously described **AuNP-based LFAs to the detection of whole-cell pathogens**. Taking advantage of innovative bioreceptors derive from bacteriophages, we perform a complete characterization of the necessary biointerfaces for LFA, from the molecular to the device level. The results, showing rapid (15min) detection of *B.cereus* are promising in the quest for user-friendly while specific LFAs for pathogen detection in food safety of environmental diagnostics.

Then, after having explored the details of colorimetric LFAs for pathogen detection, in the third part of the thesis we introduced new transduction mechanisms with the long-term goal of improving the previously found performances of colorimetric LFAs.

In chapter 6, we **accommodate electrical detection of pathogen onto simple paper-like substrates**. The use of a particularly appropriate electrical transducer (external electrodes accommodated to nitrocellulose membranes in a plug-and-play system) was validated with conventional electrical sensor to detect label-free *B. cereus* cells in aqueous samples. The major limitation of this label-free detection technique is the lost of the specific detection obtained on LFAs in chapter 5.

Therefore, chapter 7 aims at tackling this challenge by **integration this plug-and-play electrical setup onto LFAs**, with the objective of reaching specific detection of the gold nanoparticles used as label in most of the LFAs. Although the specific detection of AuNP is still to be demonstrated, the in-depth electrical characterizations of LFAs have allowed us to understand the main constraints associated with integrating impedance measurement systems into conventional LFAs. The combination of the biochemical constraints inherent to LFAs with the sensitivity of impedance measurements to the components of LFAs necessitates a re-engineering of LFA platforms to incorporate electrical measurement systems. This includes enlarging the absorbent pad to allow for longer measurement times beyond the conventional 15 minutes of colorimetric LFAs and achieving stabilization of the electrical responses of LFAs. Promising research avenues have been initiated to reduce the interference between biochemical and electrical constraints, primarily focusing on increasing the sensitivity of measurements to the presence of nanoparticles.

Taken all together, **the works performed in this thesis have essentially contributed to :**

- Having build successful and highly relevant links and collaborations between the different disciplines involved in the design and manufacture of the LFAs studied in this thesis. In particular, the success in sharing a common language between disciplines such as biology, engineering and nanoscience, which allowed researchers from different backgrounds to express all their skills, is one of the greatest strengths of the research carried out during this thesis. In the hope that it will be useful to others.
- Then, thanks to the many collaborations that have taken place within this thesis, the implementation of LFA R&D in our laboratory at UCLouvain could be leveraged towards the development of paper-based sensors for other applications.
- In particular, the development of a LFA for pathogen detection based on innovative, highly performing bioreceptors shows potential for further development. One could imagine this device to be used in food-safety diagnostics in the coming years.
- Although being deeply different sciences, the integration of electrical sciences and biotechnologies offers a lot of opportunities. At the research level and beyond.
- Finally, the investigation of an eco-design framework at the early-research stage has been a highly prospective process regarding the few exemplary studies. This process, although already interesting as an example, would benefit enormously from being applied to other case studies,

being pursued in wider impact research projects (such as the microelectronics sector), or even from being pursued in the development of higher TRLs.

8.1 Technical perspectives

In light of the hybrid biotechnology-engineering work method employed in this thesis, two main technical perspectives have been identified.

Firstly, the possibility of expanding the use of phage proteins to wider applications. Their strength, which lies in their ability to be engineered and produced at low cost, could be used to develop multiplexed LFAs, enabling the simultaneous detection of multiple pathogens on a single LFA (Figure 8.1 A). The expected benefits of this technology include a potential reduction in global diagnostic costs and time, and the opening up of new applications that are currently inaccessible [105, 259].

Second, we have seen that conventional biosensor systems still require cumbersome components (pumps, sample processors, optical/electrical detectors) and complex setups for operation [34]. This, in addition to bulkiness and overall cost of the readout devices, hinders for portable and cost-effective biodetection. To address the challenge, researchers have attempted to integrate smartphone with (bio)sensors and bioelectronics to reduce the overall size and cost of the system [38]. The recent concept of using mobile phones for improving healthcare, particularly in developing countries, has been proposed due to their portability and their ubiquitous availability [287]. By end 2014, 55% of all mobile-broadband subscriptions were estimated to be in the developing world¹. Mobile phones are also equipped with most of the bulky functional blocs of a sensor system, such as the necessary circuits for processing, storing and communicating the results as well as the power requirements, i.e. processor, visual display and battery, depicted on Figure 8.1 B. Utilizing its built-in function modules, smartphone could be employed as controller, analyzer, and displayer for rapid, real-time, and point-of-care monitoring, which can significantly simplify electronic design, minimize volume size and reduce cost of the detecting systems [38]. Therefore, utilizing mobile phones for diagnostics applications (e.g. water quality assessment or food control) offers a unique approach to increase the availability and accessibility of diagnostic test-

¹ICTFacts and Figures. The world in 2014. [Online] <https://www.itu.int/en/ITU-D/Statistics/Documents/facts/ICTFactsFigures2014-e.pdf>

ing, especially in developing countries that lack adequate resources and laboratory facilities [288]. Smartphone-based electrochemical sensors were reported in the literature to detect nitrate, heavy metals and various bacterial antigens in water for in-field environmental monitoring [288, 289, 290].

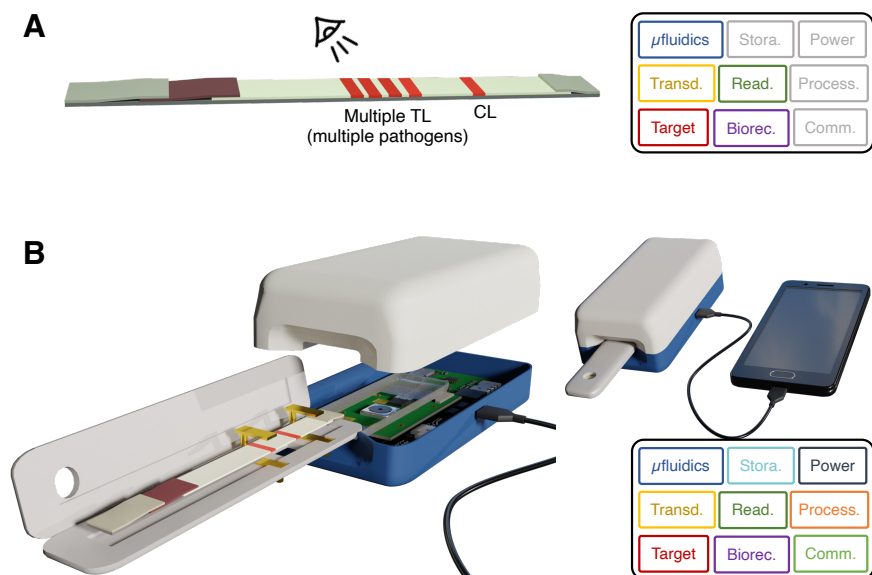


Fig. 8.1 Perspectives of the works conducted in this thesis. **A.** "Biotechnology" perspectives include the opportunity to combine multiple phage proteins, specific to different bacterial strains, to enable the *multiplexed* detection of different bacterial strains in the same sample, which is expected to significantly help screening pathogen contamination in high-risk environments, such as the agri-food sector. **B.** The "engineering" perspectives mainly include the integration of highly *sensitive transducers* to the conventional LFA, mainly to increase their sensitivity, which is expected to decrease their LoD. Many different transducers are worth being explored, such as magnetic transducers, high performance cameras, or judiciously developed electrochemical modes. Critical care is required to keep the sensor user-friendliness, even with such transducers. In addition, as it can be seen on the picture, interfacing such handheld devices with the ubiquitous yet powerful *smartphones* would allow to meet all the functionalities necessary for a high performance sensor which are not present in the current colorimetric sensors (tab in A).

8.2 Towards systemic perspectives for PoC paper-based sensors

The systemic discussion which should be conducted to **conclude the 5-step methodology for systemic assessment of a technology** consist in finding and discussing facts on different critical aspects of the PoC paper-based sensors diagnostics considered in this thesis. The steps 1 and 2 were briefly presented in the introduction of this manuscript. On Figure 8.2, we see that the fact-finding (step 3) related to the **Design, Materials & Manufacture** and **Environment** were conducted and discussed within the different chapters of this thesis (chapters 1, 3, 4, 5, 6, 7 for Design, and chapter 2 for Materials & Manufacture and Environment). Therefore, the systemic discussion would include facts from the three not yet covered dimensions from Figure 8.2 : **Society, Economics** and **Regulations**. Some of these key-words have already started to be explored with the help of other scientists and would merit inclusion in a broader discussion on anticipating the impacts of the technological innovations presented in this thesis.

Reflections about the environmental impacts have already been initiated in the eco-design methodology in chapter 2 section 2.4.2. This methodology can serve as an excellent basis for initiating systemic perspectives. To shift from an eco-design primarily focused on anticipating environmental impacts to an eco-design that evaluates multi-dimensional impacts, i.e. societal-level impacts, the last steps of eco-design methodology must be taken over by **interdisciplinary and transdisciplinary teams of scientists** that conduct systemic analyses and who are accustomed to studying in a cross-cutting way, through articulated design practices, the various externalities related to the manufacture and use of the PoC sensors. At these steps, we are thus at pivotal stages in the development of the sensors where it is necessary to go beyond the framework of environmental impacts to study technological choices in the light of issues of societal responsibility. Examples of critical reflections may include:

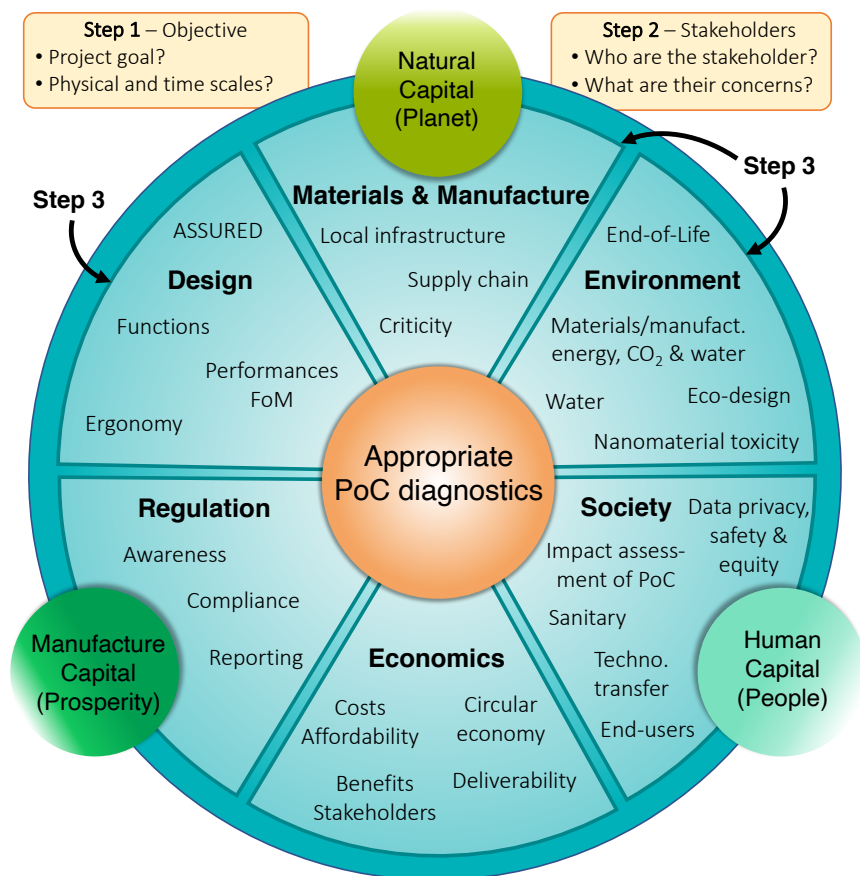


Fig. 8.2 Necessary components of an appropriate technology, from a systemic perspective. The role of this thesis was to explore the design and environmental facts associated with the quest for the appropriate technology for PoC pathogen diagnostics. The other dimensions should be explored with inter- and trans-disciplinary teams of scientists to result in a complete multidimensional study assessing the impacts of the proposed innovations in this thesis.

- The **end-of-life perspectives**, which are extremely difficult to realistically take into account within an LCA. Several barriers explain the difficulty of integrating end-of-life stages (recycling, collection, recovery, reconditioning, etc): they are subject to enormous heterogeneity between different countries due to varying legislations and opaque modes of operation. Generally, considerations for safe and sustainable disposal have typically been excluded from design requirements so far. Accuracy, reliability, usability, and affordability are the main drivers in the indus-

trial diagnostic device sector [57]. However, change is on the horizon. Regulators around the world are now requiring more sustainability for single-use products, for example in the US [291] or the EU [292].

- The **perceptions and the social acceptability** of innovations must be studied. Priority is given to questions related to the use and dissemination of nanomaterials in biosensors, which are very little taken into consideration and for which very little information is disclosed about their impacts, both on humans and on the ecosystems [64].
- The questions of **implementation of the developed innovations**. In particular, are we dependent on a particular production chain that would make the innovation non-resilient in the event of a supply chain disruption? Are there any particular constraints on production, such as the need to rely to specialized techniques?
- The **regulatory considerations** can present bottlenecks that delay the uptake of PoC devices on a global scale, especially in crisis situation. For instance, lack of regulatory harmonization between regions mean tests approved in one jurisdiction could not granted wider authorization [105].
- The question of **equity in accessibility or distribution** of the developed technologies for the users and beneficiaries identified during step 1 of the methodology [105]. Typically, for such PoC diagnostic applications whose objective is to benefit a large number of people, especially in low-income countries, the question of the investments required for the manufacture, supply and dissemination of the sensors arises seriously (related to the *Deliverable* criteria in Table 1.4). Similarly, the possibilities of technology transfers must be considered to allow users to appropriate these technologies. As a reminder, in the context of PoC diagnostic tests, the user and the beneficiary are often the same person (see Figure 1). According to the relatively low initial investment required to acquire a sufficient R&D chain for laboratory-scale productions of paper-based biosensors, it is anticipated that the development of these relatively affordable technologies could be easily transferred to countries with fewer resources, and benefit a larger population [213].

PART

Appendices



Complementary Informations for Environmental Assessments

1.1 The Product Design Process (PDP)

Traditionally, the design of a product starts with the identification of a market need, or the customer requirements; i.e., identifying the need for a function. Concepts (general working principles) are identified by the designer to fill the need during the conceptual design stage. The most promising concepts are elaborated in the configuration of a technical system with technical requirements, layout, materials and scale (embodiment design). One or more of these are selected for detailed development, analyzing performance, safety and cost (detailed design). The detailed design enables the construction of a prototype that, after testing and development, goes to manufacture, market launch and, eventually, to the product customer verification. [59]

1.2 Eco-design tools

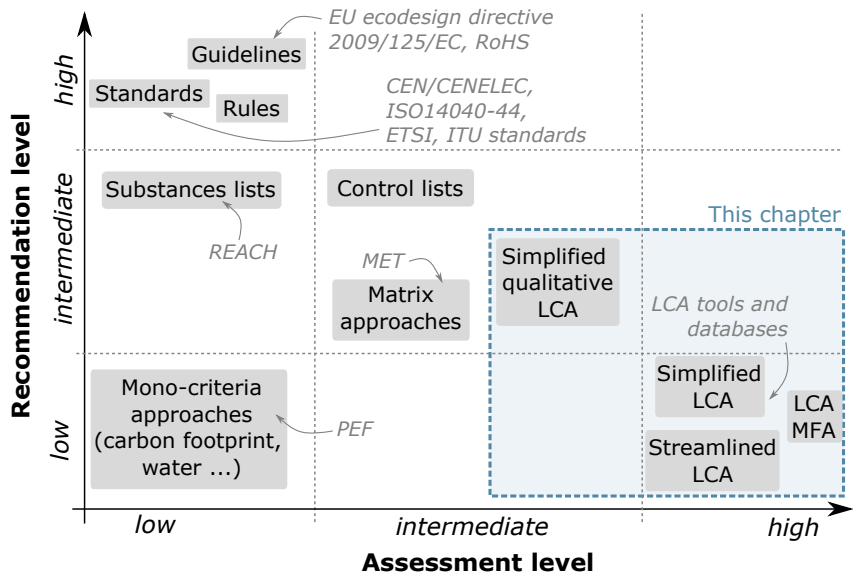


Fig. A.1 Overview of generic ecodesign tools with their associated recommendation level and assessment level. Figure adapted from [293].

1.3 LCA methodology

According to ISO14040 [294], LCA is a standard quantification method that facilitates the estimation of multiple impact categories related to a product. Specifically, a LCA study aims at modeling several life cycles phases (i.e., modeling of extractions and acquisition of raw materials, production, use and waste treatments), including all the energy and additional materials, products and emissions involved in these phases. The transfer of potential impacts between life cycle phases, impact categories, or particular processes can therefore be identified and limited (or avoided).

The iterative steps for conducting a LCA study are presented in Figure A.2 (a) and the fundamental concepts of LCA are introduced in the rest of this section. The goal must define clearly the intended application of the study because it will frame the further steps of the methodology. The

goal may be showing the environmental impact of a product based on an individual or comparison analysis, evaluating it based on a norm or a standard, evaluating its redesign alternatives, or facilitating the construction of ecological policies around it. On the other hand, the scope definition or the system boundary includes the limits of the product system (the life cycle model of a product in terms of unit processes; and elementary, product and intermediate flows) and the level of details of the environmental assessment, which depends on the functional unit. The functional unit defines the quantification of selected function in a LCA study and guides the definition of a product system. The definition of functional units is generally given as a sentence and needs to be detailed enough to assure appropriate impact estimation. For instance, “monitoring the domestic water consumption over a year”. Functional units quantify the performance characteristics of a product to provide a reference to which the inputs and outputs are related. The definition of the functional unit is critical as it allows the identification of the reference flow, which is the quantity of product systems, material, energy, or even additional products needed to fulfil the functional unit as it is expressed.

The inventory analysis step involves the description and the data collection processes needed to model a product system, according to the functional unit evaluated by a LCA study. It includes the calculation procedures to quantify relevant inputs and outputs of unit processes of a product system and its outcome catalogues the flows crossing the system boundary. The inventory analysis includes key calculations of material and primary energy inputs for a product system and key calculations of its output emissions to air (e.g., CO_2 emissions), earth and water (e.g., toxic nanomaterials).

The impact assessment step consists of applying a Life Cycle Impact Assessment (LCIA) method to estimate the environmental impacts of a product system by associating the outcomes of the inventory analysis with specific impact categories and impact characterization factors. LCA databases include the majority of global LCIA methods (e.g., ReCiPe), local LCIA methods (e.g., CML-IA for Europe), and Single-impact-based LCIA methods (e.g., IPCC).

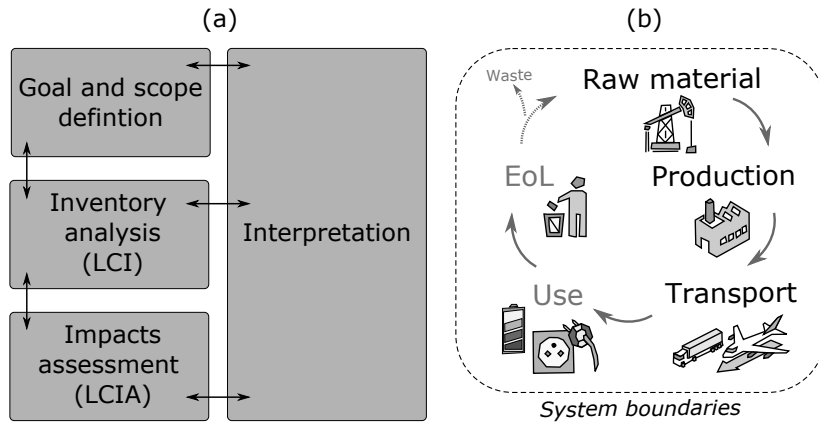


Fig. A.2 (a) Life Cycle Assessment framework (adapted from ISO14040 [58]). (b) Generic representation of the different life-cycle phases and the system boundary of a cradle-to-grave LCA study. It considers unit processes from the raw material extraction to the production, transport, use phase and end-of-life.

1.4 Additional details of impact factors used in this thesis

This section presents some of the details about the embodied energy, carbon footprint and water consumption of material production and manufacturing techniques used alongside with Figures 2.5, 2.6 in the eco-design methodology.

1.4.1 Energy requirements of manufacturing techniques

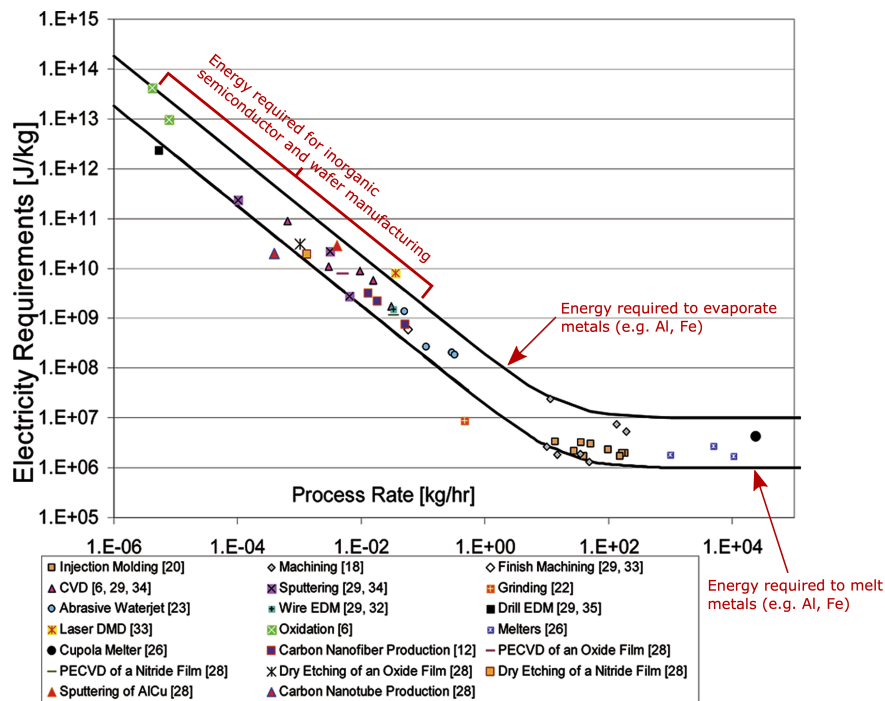


Fig. A.3 Work in the form of electricity used per unit of material processed expended in twenty typical manufacturing techniques, as a function of the rate of material processed (numbers in the brackets represent the reference cited therein). Modified from [295].

1.4.2 Modelling nitrocellulose fabrication process

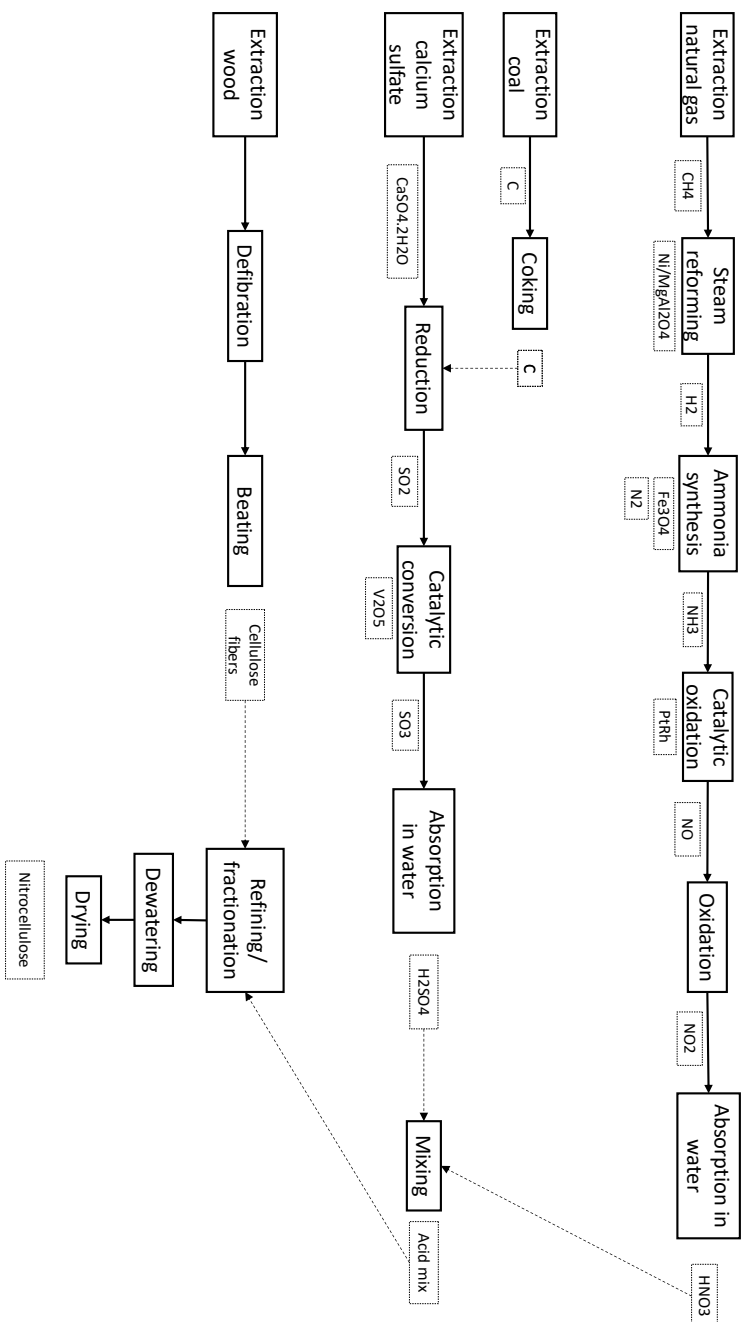


Fig. A.4 System boundary and life cycle inventory considered for the modelling of the nitrocellulose fabrication process. Material (including chemicals) and energy flows were computed to derive the whole energy consumed and emissions related to the nitrocellulose fabrication process.

1.5 Eco-design of pathogen PoC biosensors

1.5.1 Step 2 - Exploration of conceptual designs

CNT- μ PEDs		Al- μ PEDs	
Electricity	2.25 kWh	Electricity	390 kWh
Paper	16 kg	Paper	16 kg
CNTs	0.04 kg	Al	0.15 kg
Water	22 kg	Ag	0.2 kg
Surfactant	0.9 kg	Ar	6.03 kg
		N_2	3.465 kg
		H_2	0.225 kg
		He	0.405 kg
		HNO_3	0.2 kg
		Ethanol	0.2 kg
		Formaldehyde	0.02 kg

Table A.1 Inputs for the manufacturing of 1000 CNT- μ PEDs and 1000 Al- μ PEDs.

Material/Energy	Consumption	Material/Energy	Consumption
Electricity	226 kWh	TMAH	0.02 kg
Wafer	0.08 kg	Ethyl Lactate	0.186 kg
Al	0.003 kg	HMDS	0.154 kg
Al_2O_3	0.001 kg	C_4F_8	0.003 kg
PDMS	1.5 kg	CHF_3	0.001 kg
H_2O_2	4.6 kg	CH_4	2.5 kg
H_2SO_4	16 kg	NF_3	0.06 kg
Ar	0.27 kg	NH_3	0.11 kg
N_2	88.75 kg	HF	4.6 kg
He	0.018 kg	IPA	12 kg
H_2	0.01 kg	H_3PO_4	12 kg

Table A.2 Main inputs for the manufacturing of 1000 Si-PDMS sensors.

1.5.2 Inventory for specific embodied energies and carbon footprints

The specific embodied energies and carbon footprints for the raw materials and energy supply used in this work are available in the literature (see [92]) and extracted from CES EduPack Sustainability Edition database.

Material	EE [MJ/kg]	CFP [$\frac{\text{kg CO}_2}{\text{kg}}$]	Material	EE [MJ/kg]	CFP [$\frac{\text{kg CO}_2}{\text{kg}}$]
Electricity	10.5	0.447	Paper	51.5	1.17
	[MJ/kWh]	[$\frac{\text{kg CO}_2}{\text{kWh}}$]			
Wafer	7657	372	Ethyl Lact.	3.1	0.26
Al	225	17	HMDS	3.1	0.26
Ag	1450	100	HNO_3	15	1.3
CNTs	11925	600	Surfact.	17	1.4
Al_2O_3	53	2.7	C_4F_8	1	86
PDMS	140	7.3	CHF_3	59	5.1
H_2O_2	12	1	CH_4	38	3.3
H_2SO_4	0.04	0	NF_3	40	3.4
Ar	3.6	0.31	NH_3	31	2.7
N_2	0.66	0.06	H_3PO_4	3.1	0.26
He	0.83	0.07	HF	18	1.5
H_2	8.5	67	IPA	3.1	0.26
Ethanol	3.1	0.26	Formald.	3.1	0.26

Table A.3 Inventory for specific embodied energies (EE) and carbon footprints (CFP).



Complementary informations for Fundamentals on LFAs

2.1 Evolution of LFA research dynamics

About half a century after its development, the LFA technique is increasingly used thanks to its overall evolution and its characteristics that reduce costs and analytical workload, while improving efficiency. workflow and turnaround times. The LFIA has become one of the reference tests for obtaining results quickly without the need for sophisticated and expensive laboratory equipment, whether at the level of clinical tests, food safety, veterinary and environmental monitoring. Although they can be criticized for too low reliabilities or sensitivities, the interest of the scientific community for LFAs remains high, as shown by the growing number of research articles aimed at improving one or more aspects of the technique. The improvement of sensitivity and the possibility of reporting quantitative results have been the most studied aspects in the last decades.

Interestingly, over the past 10 years, Food Safety has seen the highest growth with a CAGR of 21%, while Clinical Application has seen a CAGR of 16%, followed by veterinary application (13%) and environmental application (9%). Researchers' efforts to push LFA technologies forward are evidenced by the 18% average annual growth rate of articles published on technical improvement issues (sensitivity, quantification, reliability) [116].

It thus appears that the original research carried out within the framework of this project is relevant in view of the growing needs for more efficient diagnostic technologies for the food safety sector, but not only as shown in Figure B.1.

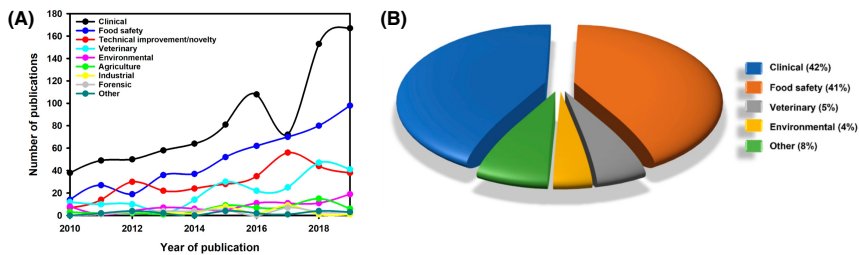


Fig. B.1 Overview of (A) LFAs and (B) multiplex LFAs application fields over the period 2010-2019. The food security sector occupies an important place. From [116]



Supplementary Materials for LFA H-IgG detection

3.1 Additional recommendations for UV-vis experiments

Differences in the AuNP solution number densities observed in Table 4.3 could be caused by the UV-vis measurement itself, as it is not recommended to trust recorded OD above 1 since the linearity of the relationship between sample concentration and recorded OD is not linear anymore. Therefore, it is necessary to extrapolate OD above 1 with measurements of sample dilutions, which could be source of dilution inaccuracies.

Typically, such an experimental measurement error was observed in Table 4.3 were the measured A_{450} for the original batch of Unisensor AuNPs was 6.62, instead of the ~ 4 expected. In this case, the inaccurate measured value can be attributed to the inaccurate measurements with the spectrophotometer.

3.2 Additional coated NC membrane characterizations

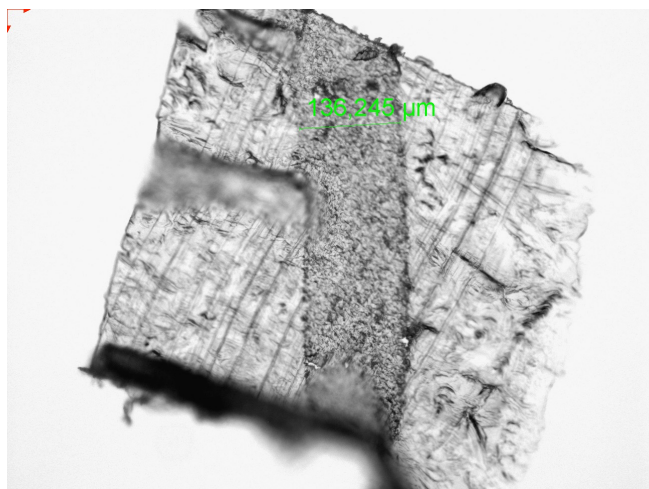


Fig. C.1 Side-view of CN140 unbacked nitrocellulose membrane. Microtomy sections were prepared and observed under optical microscope.

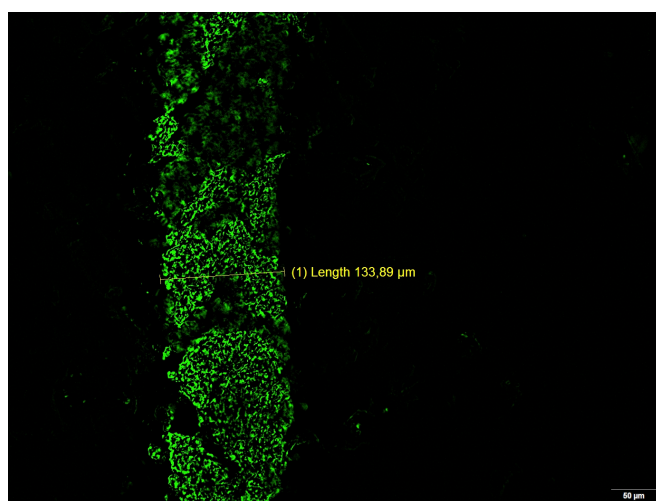


Fig. C.2 Side-view of CN140 unbacked nitrocellulose membrane functionalized with GFP-tagged Abs and observed under epi-fluorescent microscopy.

3.3 Hill model and calibration curve fitting

The Hill model is used to fit signal intensities from LFA quantitative assessment. The binding properties of a bioreceptor-ligand system can be quantitatively described by binding affinity models such as introduced in section 3.4. They study the propensity of a protein to bind to its target, in function of the target concentration. From Figure 4.6 we can study the degree of response of the capture antibody to the concentration of the human IgG in the sample, i.e. the binding affinity between the receptor and the target.

When a protein possesses more than one binding site, just as it is the case for antibodies (see Figure 3.4), the binding of one site to the target often facilitates the binding of the others (i.e. increases the affinities of any remaining unfilled binding sites), in a process called **cooperative ligand binding**. Such cooperative binding has some important physiological functions as it permits a much more sensitive response to the target concentration, and it can be observed directly on the calibration curve by the shape of the curve. Cooperative binding is characterized by a sigmoidal binding curve. **Quantitative model of the binding affinities**, such as the **Hill model**, have been derived and can be fitted to the quantitative data in order to extract the binding affinity of the system.

For a protein (e.g. antibody) with n binding sites, the equilibrium reaction with its ligand introduced in equation 3.1 becomes

$$[P][L]^n = K_D[PL_n] \quad (C.1)$$

Studying the evolution of a protein binding affinity to its target in function of the target concentration implies to determine the fraction θ of ligand-binding sites on the protein that are occupied by the ligand :

$$\theta = \frac{\text{binding sites occupied}}{\text{total binding sites}} = \frac{[PL_n]}{[PL_n] + [P]} \quad (C.2)$$

Expressing equation C.2 in terms of the target concentration, it comes that

$$\theta = \frac{[L]^n}{[L]^n + K_D} \quad (C.3)$$

Therefore, the binding affinity of the bioreceptor antibody depends on the ligand (i.e. target) concentration, modulated by n , the Hill coefficient, which is actually more a measure of the degree of cooperativity than the

exact number of binding sites on the protein : if n equals 1, ligand binding is not cooperative. However, a n greater than 1 indicates positive cooperativity in ligand binding, i.e. that the binding of one molecule of ligand facilitates the binding of the others (via conformational changes and protein-protein interactions).

From equation C.3, it is observed that the fraction of ligand-binding sites occupied approaches saturation asymptotically as $[L]$ increases, generating a sigmoid binding curve. The $[L]$ at which half of the available ligand-binding sites are occupied (at $\theta = 0.5$) corresponds to K_D .

Figure 4.6 actually expresses this fraction of target-binding sites of the test line antibodies occupied with respect to the target concentration, and we can use a **four-parameter logistic curve** (a sigmoidal curve) representative of the Hill equation :

$$y = start + (end - start) \times \frac{x^n}{x^n + k^n} \quad (C.4)$$

with y the signal intensity from the TL (i.e. fraction of target-binding sites of the test line antibodies occupied), x the ligand/target concentration (i.e. human IgG concentration in the sample), k corresponding to K_D , and with the *start* and *end* values corresponding to the non-responsive and saturated signals, respectively. The possibility to fit the data with a four parameter logistic curve thus requires studying a range of target concentrations sufficiently wide to generates both non-responsive and saturated signals.

Generally, sigmoidal approximation is accepted for competitive immunoassays (since they more closely represent an Hill model than an immune sandwich assay). Sometimes, linear approximation of the initial part of concentration dependence could be preferred for immune sandwich assays [104]. If the fit is not more appropriate using higher and lower concentration, it is still possible to approximate the fit to a linear curve on a range of target concentrations focused on the dynamic range of the fitted sigmoidal curve.

3.3.1 Molar expression of K_D

To determine the molar $[M]$ expression of K_D from the fitted value $[ng/mL]$, we used a molecular mass of IgG typically quoted at 150,000 daltons (150 kDa), which is equivalent to 150 000 g/mol. Thus, 1 ng/mL IgG = 0.006 nM.

3.3.2 Sensor response calibration in terms of AuNP number

From section 3.5.2, we know that the 20-nm AuNP density at DO 1 is $\sim 1 \times 10^{12}$ [AuNP/mL]. During the conjugation process, 1.5 mL of the AuNP solution is used, i.e. 1.5×10^{12} AuNPs. The conjugated AuNPs are concentrated 3 $\times$, leading to 0.5 mL of coated 20-nm AuNPs at $\sim 3 \times 10^{12}$ [AuNP/mL] (DO 3). 0.5 mL of conjugated AuNPs is used to fill 8 cm of the CP glass fiber, which corresponds to 24 LFAs (3-mm width). **Each LFA strip therefore contains $\sim 21 \mu\text{L}$ of AuNPs, or 62.5×10^9 AuNPs.**

From the human IgG concentrations [ng/mL] used and the volume of sample solution used (70 μL), **the corresponding number of H IgG molecules** can be determined via the H IgG molar mass (their size of 150 kDa means a molar mass of about 150 000 [g/mol]¹) and Avogadro number $N_{Av} = 6.023 \times 10^{23}$ [# /mol]. Table C.1 express the conversion :

H IgG [ng/mL]	H IgG [mol/mL]	H IgG [mol/sample]	H IgG [# /sample]
1000 (satur.)	6.67×10^{-12}	4.67×10^{-13}	281×10^9
400 (high DR)	2.67×10^{-12}	1.87×10^{-13}	112×10^9
34 (low DR)	2.27×10^{-13}	1.58×10^{-14}	9.56×10^9
28 (LoD)	1.86×10^{-13}	1.3×10^{-14}	7.9×10^9
1 (non-respons.)	6.67×10^{-15}	4.67×10^{-16}	2.81×10^8

Table C.1 Conversion of the H IgG concentrations in molecule number. (satur. : saturation, non-respons.: non-responsive, # : protein macromolecule)

A similar discussion about the number of hCG molecules as the cutoff of pregnancy LFAs test is available at this url : https://dcndx.com/blog/antibody-labeling-analyte-detection/#_ftn1.

¹This number comes from IgG's tetrametric structure. IgG contains two heavy chains (50 kDa each) and two light chains (23.5 kDa). $50 + 50 + 23.5 + 23.5 = 150$ kDa. See <https://www.aatbio.com/resources/faq-frequently-asked-questions/What-is-the-molecular-weight-mw-of-Immunoglobulin-G-IgG>



Supplementary Materials for phage protein-based LFAs

4.1 Materials and Methods

4.1.1 Phage protein production, purification and affinity testing

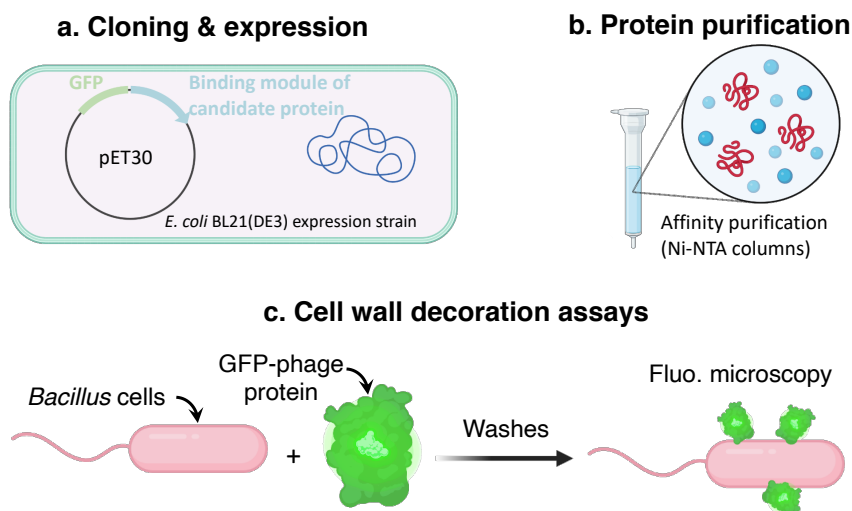


Fig. D.1 Schematic summary of the production process and binding affinity analysis of the two different proteins used in this work (PlyB221 and evoDit-Gp28). **a.** the genome coding for the part of the phage protein of interest (e.g. the binding module) is inserted into a plasmid. If necessary, a sequence coding for a green fluorescent protein (GFP) is included. The plasmid is then inserted into an *E. coli* expression strain and the phage protein fused with the GFP is recombinantly produced (by bacteria replication). **b.** Then, the protein are separated, or purified, from the bacteria metabolites and proteins on affinity purification columns. **c.** Finally, the phage protein fused with a GFP is incubated with fresh bacterial culture and observed at the fluorescence microscopy in a Cell Wall Decoration Assay (CWDA) to evaluate the degree of the phage protein binding onto the bacterial cell surface, i.e. adsorption on the outer membranes, which reveals their binding activity for the bacterial strain in the culture. Illustrations from Manon Nuytten.

4.2 Phage protein binding spectra

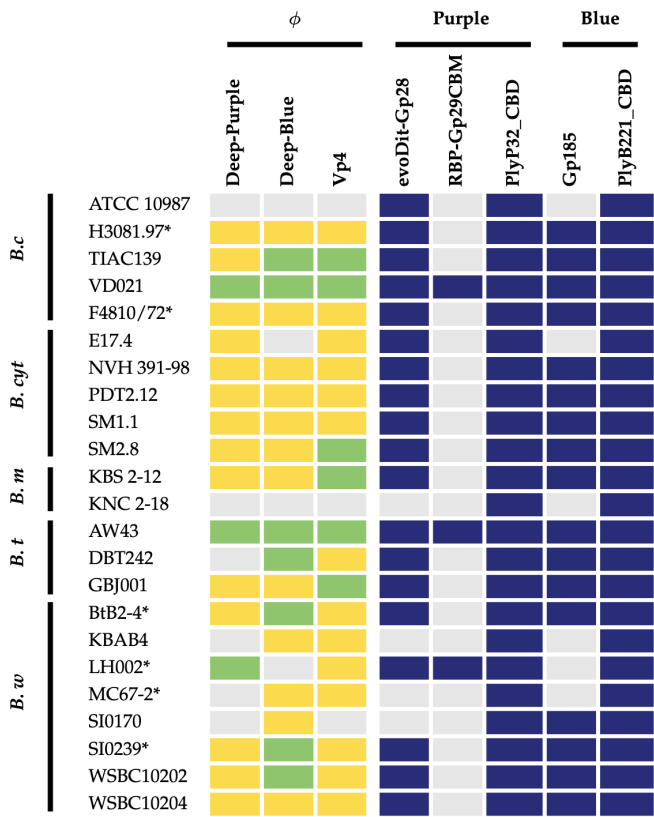


Fig. D.2 Summary of the binding spectra exhibited by the two different proteins used in this work (PlyB221_CBD from Deep-Blue and evoDit-Gp28 from Deep-Purple bacteriophages) with the host spectra of their related phages. The host sensitivity is indicated as follows: green: sensitive; yellow: affected by lysis at high phage concentrations; gray: insensitive. Regarding the proteins, the results of the cell wall decoration assays are represented with blue if binding of the protein was observed and gray if there was no binding. Stars indicate emetic strains. The binding spectra identified for the two proteins are broader than their parental phage host spectra. The protein affinities are therefore advantageous to envision specific detection of many strain members from the *Bacillus cereus* family. Reprinted from Audrey Leprince's PhD thesis [209].

4.3 Supplementary materials for the *in silico* analyses

4.3.1 *In silico* analysis of PlyB221

PlyB221 Enzymatically Active Domaine (EAD, aa 1-135) consists of five α -helices and one β -sheet containing three antiparallel β -strands. Two helices ($\alpha 3$ and $\alpha 4$) are supposedly forming a flap over the subdomain comprising the β -sheet and three α -helices ($\alpha 1$, $\alpha 2$ and $\alpha 5$) (Figure D.3 A-C).

PlyB221 CBD consists of two tandem SH3 domains displaying eight β -strands which are composed into two anti-parallel β -sheets (Figure 5.2 A-C). The two tandem SH3 domains of PlyB221 CBD exhibit 76% amino acid sequence identity and can be superposed with a Root Mean Square Deviation (RMSD) of 0.190Å for 60 C α positions. Overall, Dali server highlights that the predicted structure of the PlyB221 SH3 domains is very similar (RMSD: 1,156Å between 39 pruned atom pairs) to that of the SH3 domains of LysPBC5, the endolysin encoded by the *B. cereus* PBC5 phage (Figure 5.2 D), for which crystal structure indicated that the CBD binds to glycan strands of the peptidoglycan (PG) in a bidentate manner via distal β -sheet motif of the tandem SH3 domains conferring the host specificity to the endolysin [198].

The whole structure of PlyB221 was predicted by AlphaFold2 with high confidence (predicted local distance difference test - pLDDT, above 80 which is comparable to experimental results) with the only exception of the linker regions (Figure D.3 D). The relative orientation of the different domains has also to be interpreted with caution as shown by the Predicted Aligned Error (PAE) metric (Figure D.3 E).

4.3.2 *In silico* analysis of EvoDit-Gp28

Further bioinformatic analyses of EvoDit-Gp28 confirmed the presence of a Carbohydrate Binding domain (CBM) fold comprising 16 β -strands and several long stretches and loops (Figure 5.3 A-C). The core of the structure is a β -sandwich of 2 β -sheets, each gathering 6 β -strands. The N- and C-termini form two antiparallel β -strands ($\beta 1$ and $\beta 16$) that join the CBM fold to the rest of the protein. Dali server indicated that the predicted structure of the EvoDit-Gp28 CBM fold is very similar (RMSD: 1,354Å between 108 pruned atom pairs) to the CBM2 fold of *Lactobacillus casei* phage J-1 (Figure 5.3 D) [200]. Similarly to the J-1 CBM2 fold, Dali server highlighted a significant EvoDit-Gp28 CBM hit with an acidic lectin (PDB ID: 1FAY, RMSD:

2.8A) that has been co-crystallized with saccharides (i.e. methyl- α -D-galactose), suggesting that the region involved in binding to bacterial surface saccharides may be a cavity opposite to the N- and C-termini. The whole structure of EvoDit-Gp28 was predicted by AlphaFold2 with high confidence (pLDDT <90, Figure D.4 A) and even the relative orientation of the different domains is predicted with high confidence (Figure D.4 B). The N-terminus of the EvoDit-Gp28 (i.e. belt domain) is involved in the formation of the hub structure of the Dit upon hexameric assembly (Figure D.4 C-D), the CBM supposedly pointing out of the structure for proper interaction with the bacterial surface.

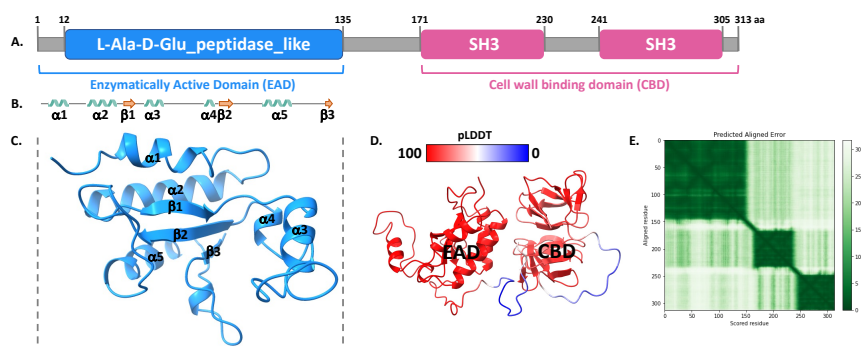


Fig. D.3 *In silico* analysis of PlyB221. **A.** PlyB221 two domain organization defined by BLASTp search. **B.** Predicted PlyB221 EAD secondary structure (α helices in green, β strands as orange arrows and disordered regions as grey lines) **C.** Ribbon representations of EAD predicted structure. The domain boundaries predicted by AlphaFold2 is indicated with dotted lines. **D.** AlphaFold2 PlyB221 model coloured in red-blue scale representing the pLDDT score for residue, with blue representing low pLDDT and red high pLDDT **E.** Predicted aligned error for PlyB221. Figures were generated with ChimeraX (Pettersen et al, 2021).

D | Supplementary Materials for phage protein-based LFAs

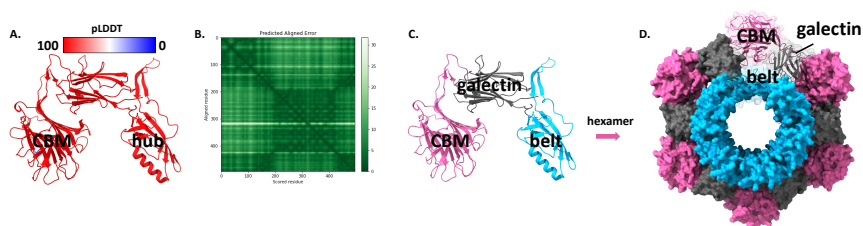


Fig. D.4 *In silico* analysis of EvoDit-Gp28. **A.** AlphaFold2 EvoDit-Gp28 model coloured in red-blue scale representing the pLDDT score for residue, with blue representing low pLDDT and red high pLDDT. **B.** Predicted aligned error (PAE) for EvoDit-Gp28. **C.** Ribbon representation of EvoDit-Gp28 with domains colouring: belt in blue (aa 1-121), galectin in dark grey (122-182 / 436-495) and CBM in pink (aa 182-435). **D.** Surface representation of EvoDit-Gp28 hexameric assembly predicted by Symmdock (top view) and ribbon representation of one EvoDit-Gp28 monomer. Figures were generated with ChimeraX [?](Pettersen et al, 2021).

4.4 Gel of purified phage proteins

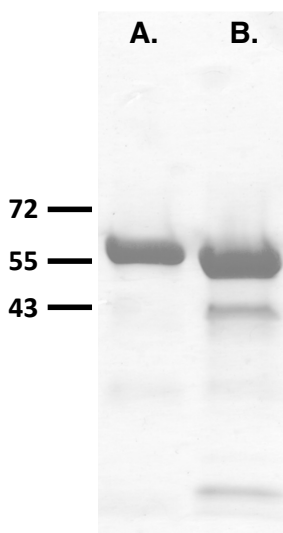


Fig. D.5 SDS-page gel of purified 6His::EvoDit-Gp28 (62,6 kDa - **A.**) 6His::GFP::PlyB221_CBD (50,5 kDa - **B.**). Proteins were expressed in *E. coli* BL21(DE3) and purified using affinity techniques (Ni-NTA columns) thanks to their hexa-histidine tag. Relevant protein sizes (kDa) are shown on the left.

4.5 PlyB221 capture biointerface on NC membrane

3D CLSM images of the membrane were acquired without (Figure D.6 A) and with bacterial cell incubation (Figure D.6 B) to serve as control. 3D images of the membrane structure makes it possible to observe in detail the preferential choices of location for bacterial capture as a result of the size of the pores in the membrane and its morphology.

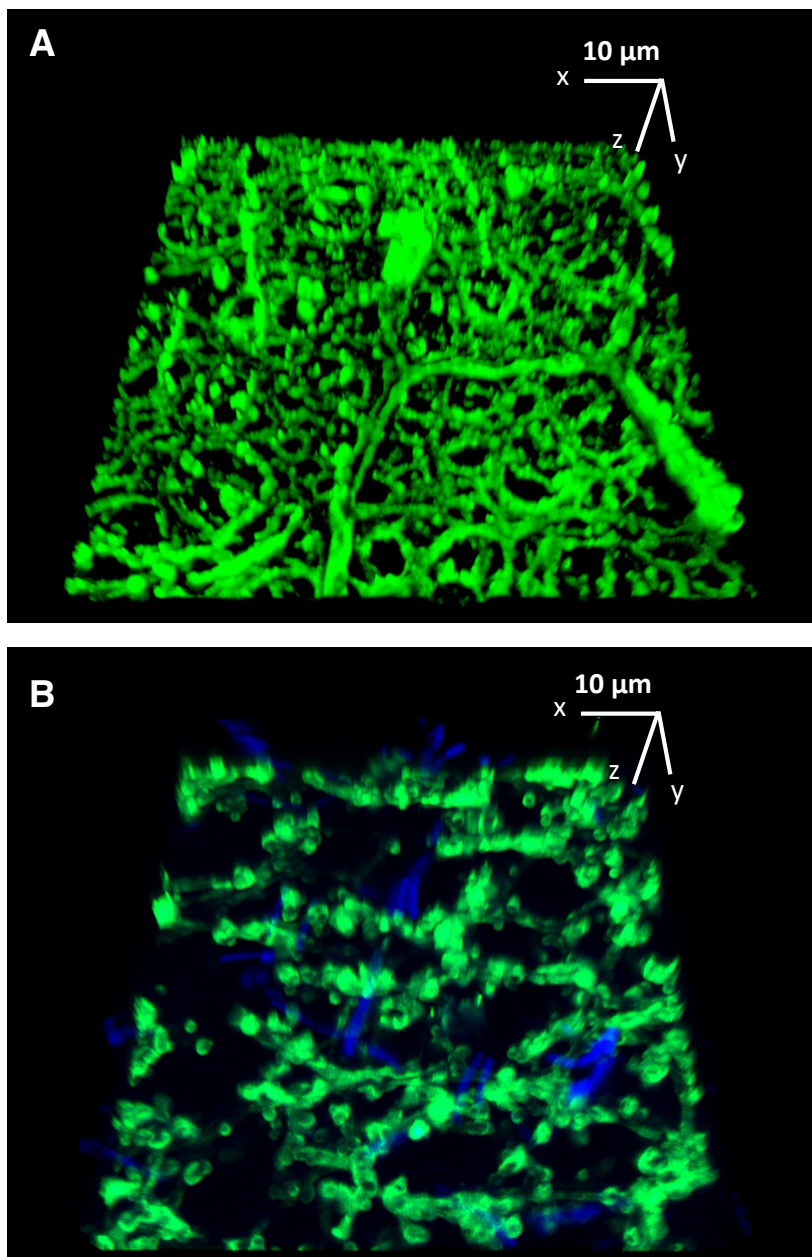


Fig. D.6 3D image of the functionalized NC membrane surface **A.** without and **B.** with incubated, washed and captured bacterial cells, built from Z-stack CLSM images on 15 μm height.

4.6 AuNP-EvoDit-Gp28 as specific detection biointerface

4.6.1 Results of the gold aggregation test

Figure D.7 depicts the results of a gold aggregation test for 40-nm diameter AuNPs and purified EvoDit-Gp28 phage binding proteins. **(a-c)** Photos of the resulting gold aggregation test. The color gradient after addition of salt to the different EvoDit-Gp28 concentrations incubated with AuNPs indicates the aggregation for AuNP coated with a too low protein concentrations. **(d-f)** UV-vis spectra obtained for the different tested conditions. It is important to note that the peak position for stable conjugates will be slightly shifted to higher wavelengths (in this case, 528 nm) due to different optoelectronic properties of the conjugates (AuNPs + EvoDit-Gp28) compared to that of bare AuNPs (in this case, 520 nm), see Figure 5.8 E. The higher the calculated value the narrower the peak, indicating more monodispersed AuNPs. On the contrary, lower and broader peaks indicates aggregation of the AuNPs. **(g-i)** Bar charts, relative to the photos in **a-c** and the spectra in **d-f**, obtained by subtracting the absorbance value at 520 nm from the one at 580 nm for each concentration (i.e. on each recorded curve). This analysis allows for estimation of the minimum EvoDit-Gp28 concentration to give stable conjugates. In this case, the pH showing the best optical properties of the conjugate (i.e. low aggregation) is 8, and the lowest EvoDit-Gp28 concentration showing retained optical properties of the conjugated AuNPs is 80 $\mu\text{g/mL}$. (Abs, absorbance; a.u., arbitrary units.)

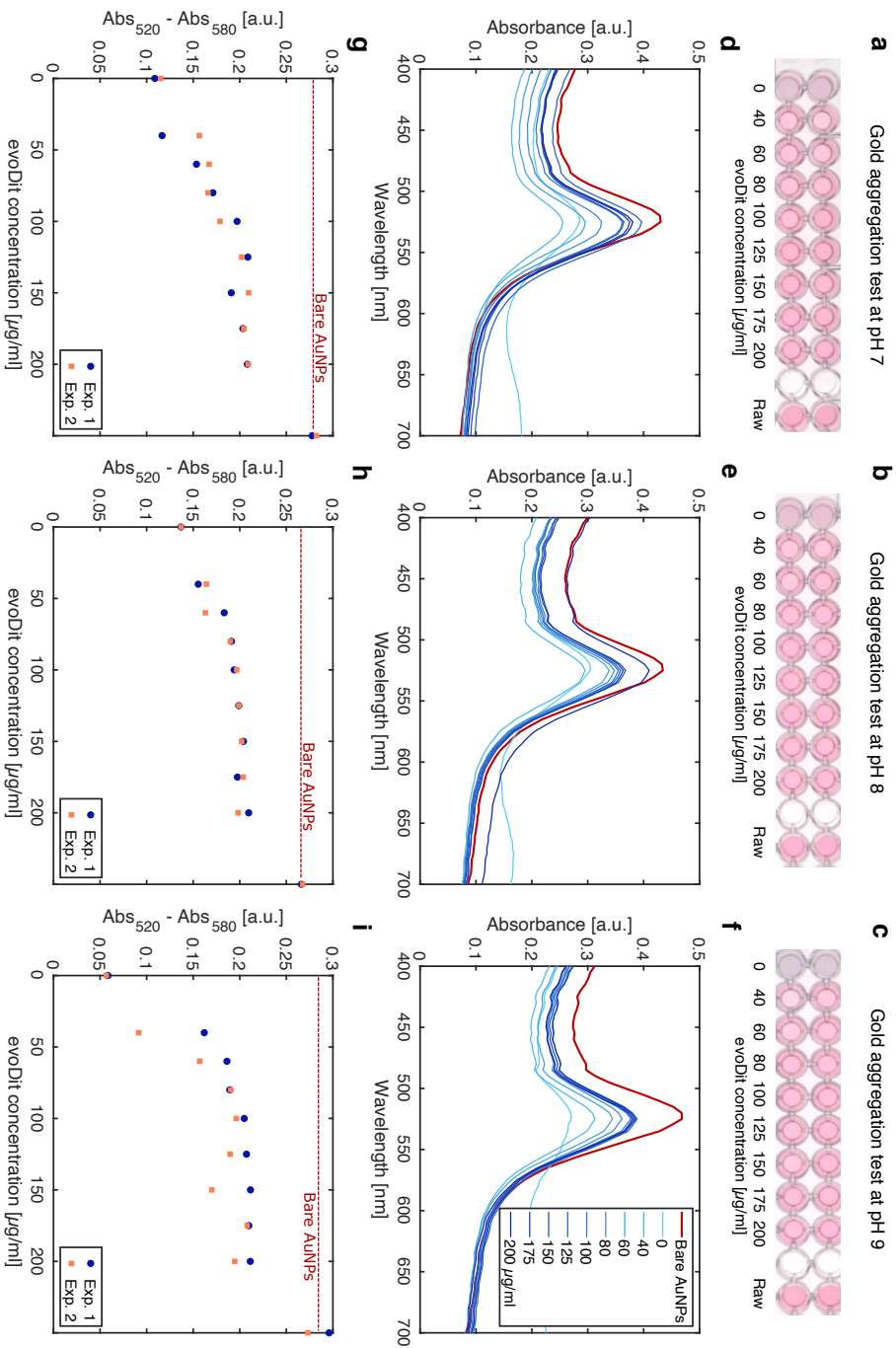


Fig. D.7 Gold aggregation test for 40-nm AuNPs and EvoDit-Gp28 phage binding proteins. Description in Appendices D section 4.6.1.

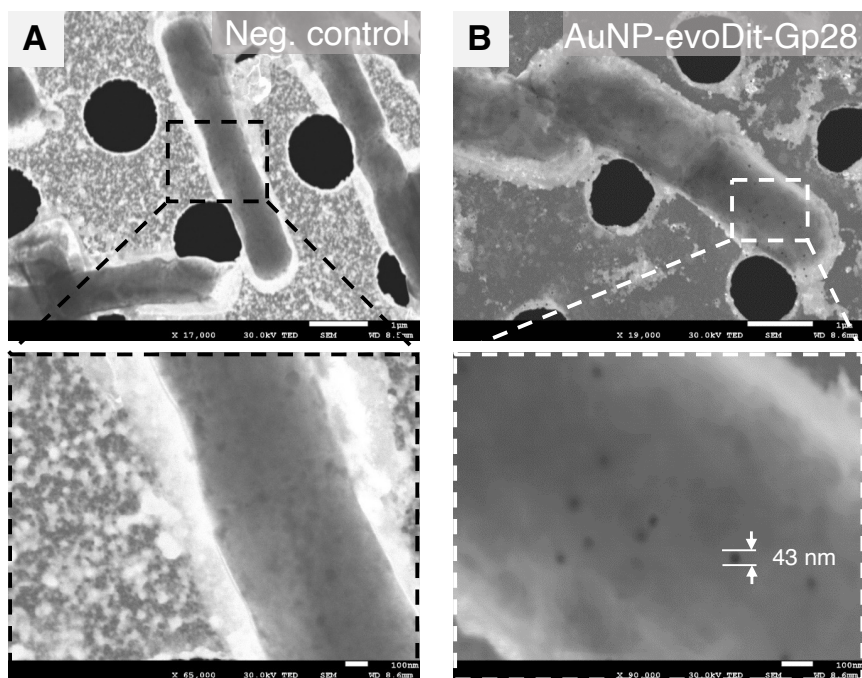
4.6.2 AuNP labelling of *B. cereus* cells

Fig. D.8 STEM images of bacterial cells incubated with AuNP conjugates, without glutaraldehyde fixation. The bacterial residues on the pictures prevent the proper AuNP discrimination on the bacterial surface and provide a highly inhomogeneous background, complicating the image analysis process. This problem was solved by bacterial cells fixation with glutaraldehyde.

AuNP labelling quantification

Here, we detail the computations of the number of AuNP attached to the bacterial cells from the STEM images in section 5.4.3, showing incubated bacterial cells with different AuNP concentrations. For simplifications, and because results from Table 4.3 obtained by our UV-vis measurements may be more approximate to the actual number densities of AuNPs than the ones performed in industrial facilities with calibrated protocols (see Appendices 3.3.2), the nanoparticle number density from the BBI tables are used (Table 4.3). From their optical densities, the number density of particles of 40 nm AuNP of OD 1, 4 and 9 is approximately equivalent to $\sim 9 \times 10^{10}$, $\sim 4 \times 10^{11}$ and $\sim 9 \times 10^{11}$ AuNP/mL.

For the STEM imaging experiments, samples were prepared by incubation of 150 μL of overnight bacterial suspensions and 35 μL of AuNP conjugates at the OD of interest. The number of bacteria and AuNPs in the samples are reported on Table 5.1. Since the mean surface area covered by the AuNPs on the bacterial surface was quantified with image analysis software (ImageJ) from the STEM images, the number of AuNP per bacteria can be computed by dividing this mean surface area by the visible surface area of a single 40-nm AuNP on the pictures, which is $4 * \pi * 20^2 = 1256 \text{ nm}^2$. These results are reported on Table 5.1 for each AuNP OD condition.

Since 150 μL of overnight bacterial suspensions at 10^7 CFU/mL were incubated with the AuNP conjugates, it means that the total number of AuNP that have bound to bacteria during incubation is approximately 1.5×10^6 bacteria multiplied by the mean number of AuNP bound to the bacterial surface. The proportion of the initial AuNP solution in the sample that have bound during the incubation is directly derived from the initial number of AuNP in the sample and the the number of bound AuNP, and reported in Table 5.1.



Supplementary Materials for the parallel-plate response calibration to saline solutions

E | Supplementary Materials for the parallel-plate response calibration to saline solutions

Saline solution	R_{NC} [Ω]	C_{NC} [pF]	$C_{backing}$ [pF]
c_{NaCl} [mol/L]	Mean (μ) $\pm$ s.d. (σ)	Mean (μ) $\pm$ s.d. (σ)	
10^{-5}	202 923 $\pm$ 4855	9.028 $\pm$ 0.071	100
10^{-4}	191 393 $\pm$ 2426	8.336 $\pm$ 0.024	100
5×10^{-4}	164 160 $\pm$ 7400	8.819 $\pm$ 0.123	100
10^{-3}	105 279 $\pm$ 3813	10.32 $\pm$ 0.13	100
5×10^{-3}	55 512 $\pm$ 322	11.85 $\pm$ 0.051	100
10^{-2}	23 422 $\pm$ 2096	16.08 $\pm$ 0.397	100
10^{-1}	2165 $\pm$ 76	19.75 $\pm$ 0.431	100

Table E.1 Results from the fitting of impedance data (Figure 6.6) to the simple electrical equivalent model used for the parallel-plate setup. All fit were accurate ($R^2 < 0.2$). Mean (μ) $\pm$ standard deviation (s. d.) on twelve measurements from two independent experiments.

Biological solution	R_{NC} [Ω]	C_{NC} [pF]	$C_{backing}$ [pF]
	Mean (μ) $\pm$ s.d. (σ)	Mean (μ) $\pm$ s.d. (σ)	
PBS:1000	156 313 $\pm$ 1580	8.223 $\pm$ 0.025	100
<i>B. thuringiensis</i> 10^8 CFU mL $^{-1}$	118 317 $\pm$ 2426	9.312 $\pm$ 0.018	100

Table E.2 Results from the fitting of impedance data (Figure ??) to the simple electrical equivalent model used for the parallel-plate setup. All fit were accurate ($R^2 < 0.2$). Mean (μ) $\pm$ standard deviation (s. d.) on twelve measurements from two independent experiments.

F

Supplementary Materials for the implementation of the prototype impedance LFA

6.1 Implementation

6.1.1 Sensor response with and without NC membrane backing

The accommodation of non-Faradaic impedance measurements on a functional LFA suggested to favor the use of a plug-and-play, reusable electrode setup similar to the parallel-plate explored in chapter 6. The resulting setup is depicted on Figure 7.8. The integration of the LFA to this setup forces us to question the applicability of the backing currently applied under the membrane for its structural support to allow sensitive detection via direct access of the probes to the test areas of the membrane.

In section 7.4, **the response of the prototype impedance LFA with and without backing of the NC membranes towards changes in the fluid resistivity was studied and modelled and reported on Figure F.1.** In order to provide sensitivity to the labels and analytes within the LFA, which are supposed to modify R_{LFA} , a setup with unbacked nitrocellulose membrane was selected.

F | Supplementary Materials for the implementation of the prototype impedance LFA

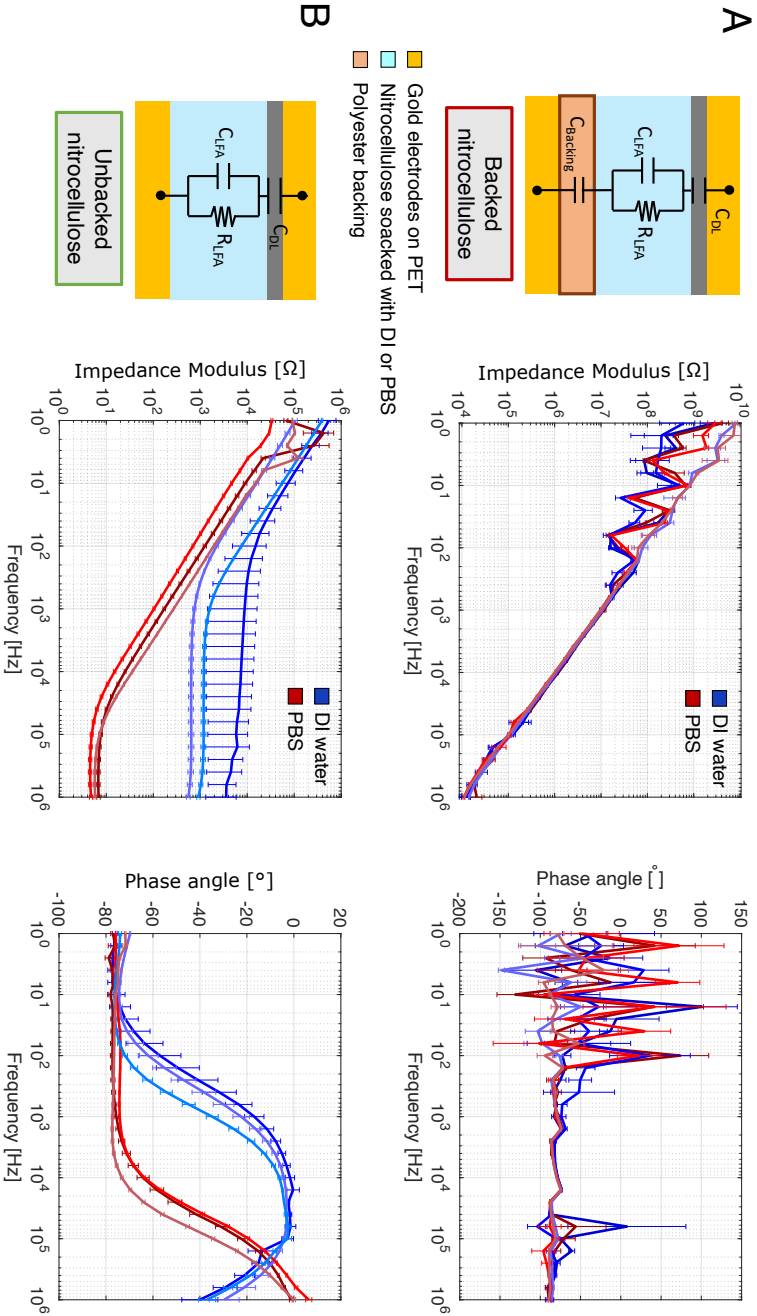


Fig. F.1 Comparison of impedance measurements on backed and unbacked NC membranes. Each red- or blue-ish curve represent an independent tested device, either with deionized water of PBS solution. Each device was tested minimum 3 times ($n = 3$). The displayed curves represent the mean of these n measurements, and the error bars represent the standard deviations.

6.2 Variability analysis of electrode pairs and LFAs

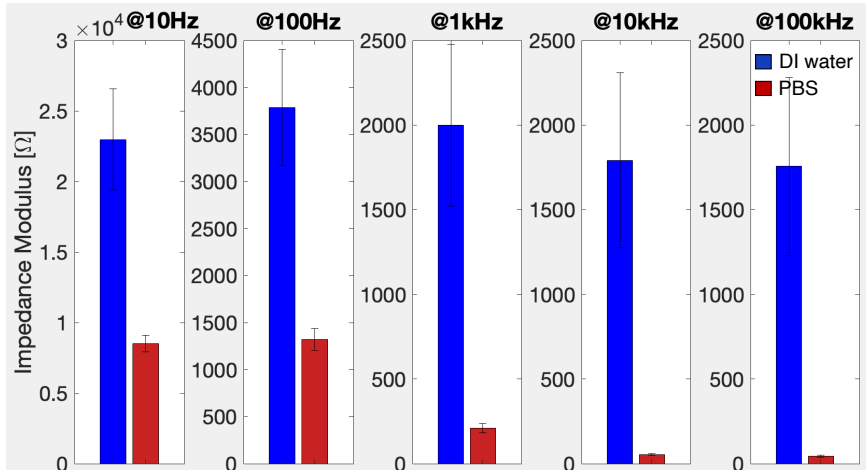


Fig. F.2 Intrinsic variability (intra-variability) for impedance measurements on LFA soaked with PBS buffer and DI water with the same electrode pair. 5 measurements ($n = 5$) were performed on independent LFAs with the same electrode pair. The difference between the LFA was more seen with high resistivity electrolyte (DI Water). Each measurement was performed after 15 min of an operating LFA filled with the buffer.

F | Supplementary Materials for the implementation of the prototype impedance LFA

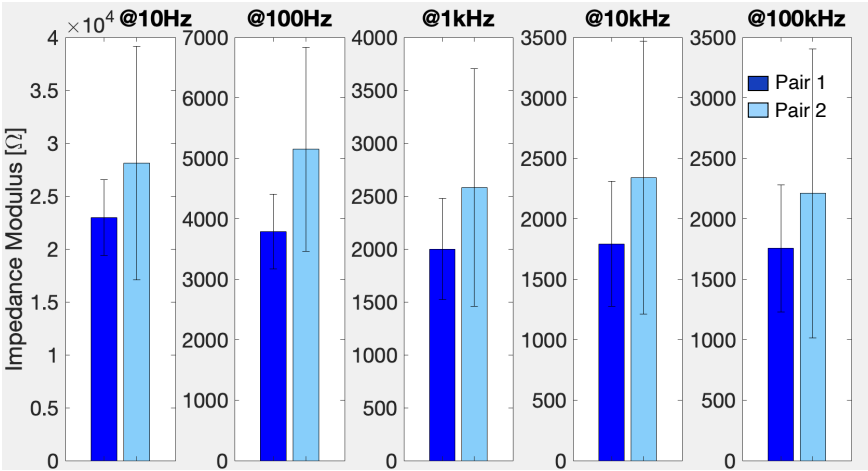


Fig. F.3 Variability between different electrode pairs (inter-variability) was assessed by impedance measurements of independent LFAs ($n = 5$) soaked with DI water, after 15 min of LFA operation. These measurements showed difference in the absolute measured impedance from the two electrode pairs, and in their respective intrinsic variability, highlighting the complexity of direct comparison between measurements resulting from two electrode pairs.

6.3 Calibration

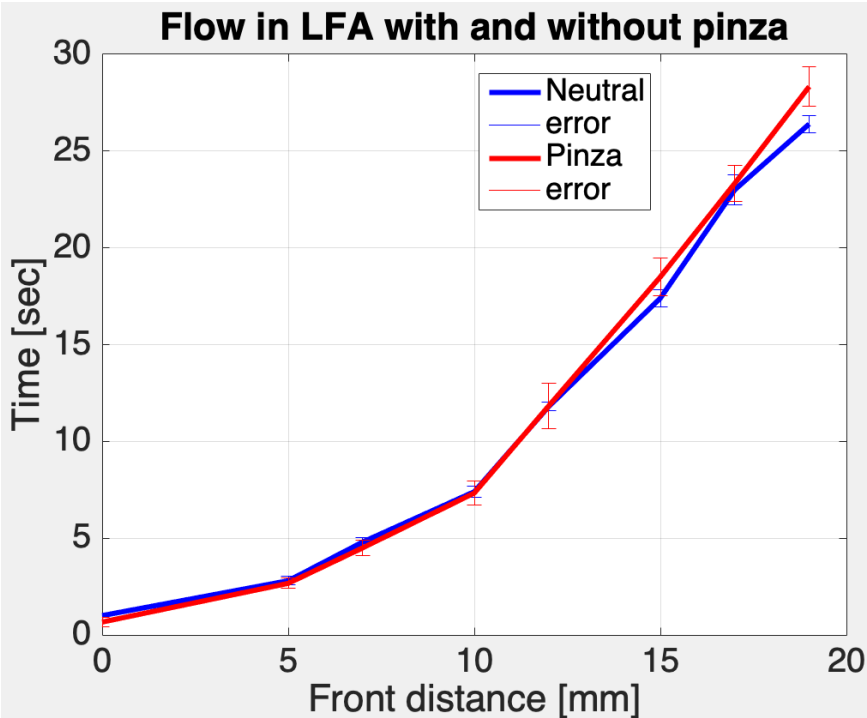


Fig. F4 Flow velocity in LFA first seconds, with and without the cassette containing the gold electrodes.

6.4 Control of LFA electro-fluidic properties

6.4.1 Coupling optics and impedance highlights fluid mixing in LFAs

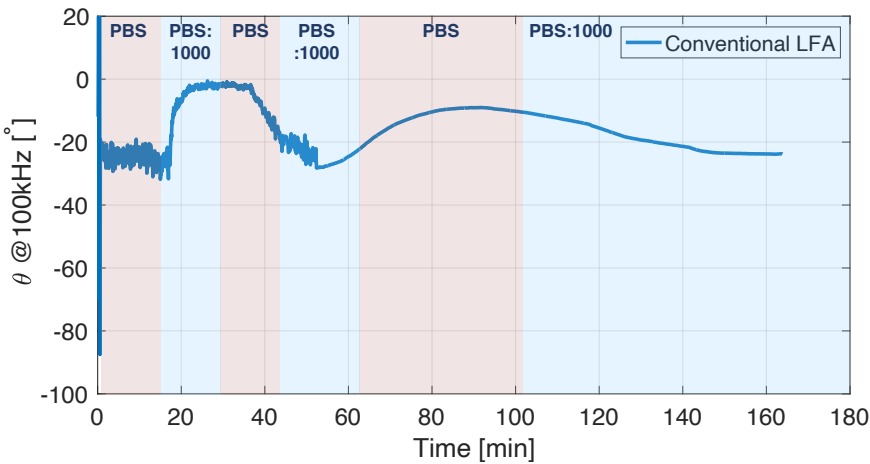


Fig. F.5 Impedance sensing of successive buffers in conventional LFA : evolution of the phase angle.

6.4.2 Engineered LFA device characterization with model fluids

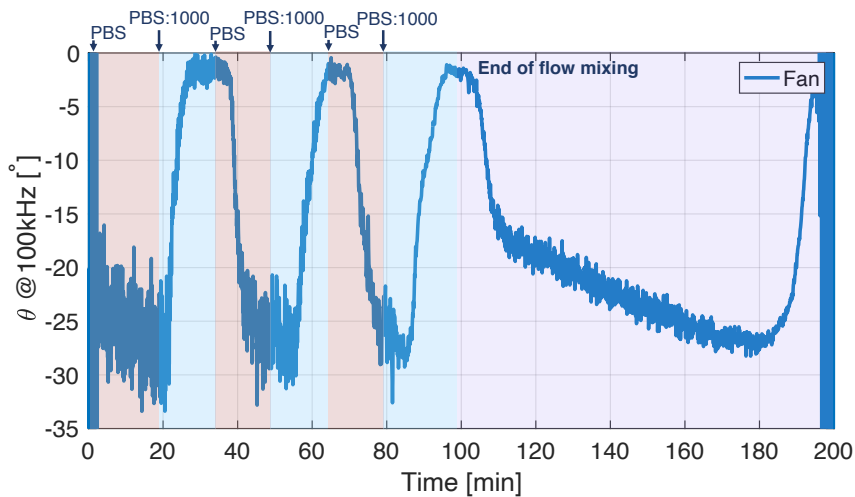


Fig. F.6 Characteriation of the engineered LFA design for efficient control on ionic content. This is reflected in the impedance response, which is driven by the addition of the buffer in a much more precise way than for conventional LFA (depitced on Figure 7.16). Therefore, adequate washing steps are possible to implement with this setup.

F | Supplementary Materials for the implementation of the prototype impedance LFA

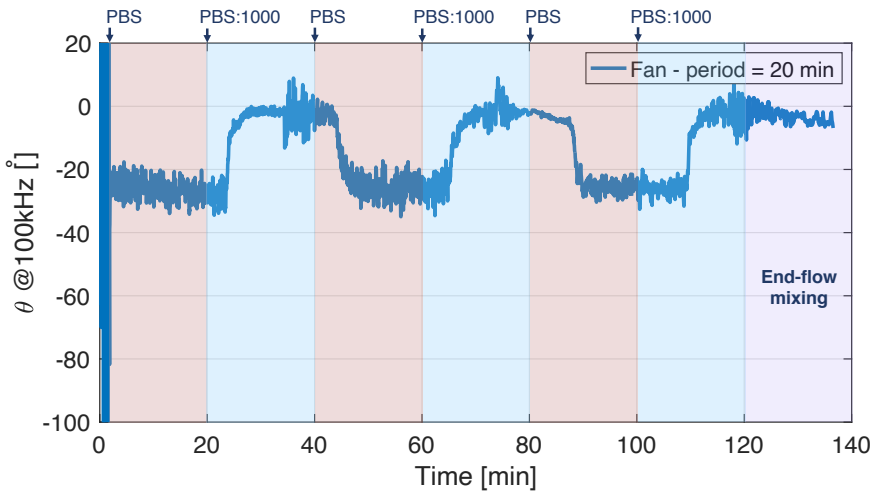


Fig. F.7 Characterization of the engineered LFA design for efficient control on ionic content. This is reflected in the impedance response, which is driven by the addition of the buffer in a much more precise way than for conventional LFA (depicted on Figure 7.16). Therefore, adequate washing steps are possible to implement with this setup.

6.5 Towards the specific impedimetric detection of AuNPs in LFA

First detection results

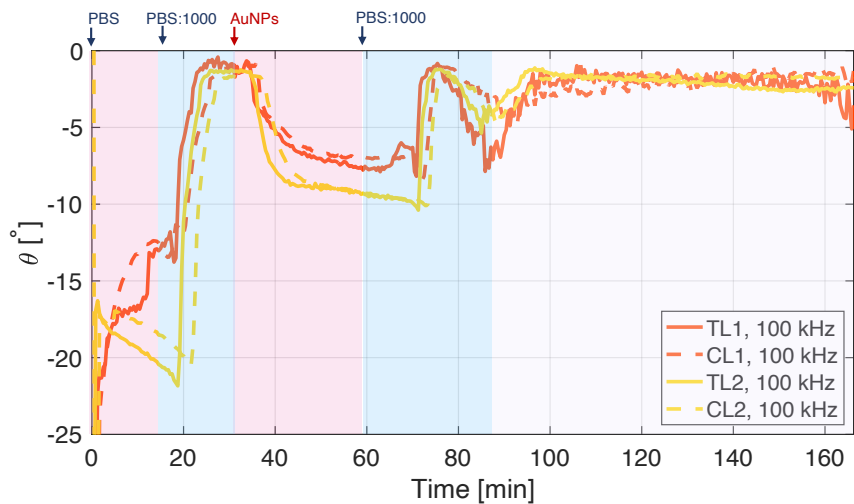


Fig. E8 First results of AuNP impedance detection (impedance modulus).

F | Supplementary Materials for the implementation of the prototype impedance LFA

Controls

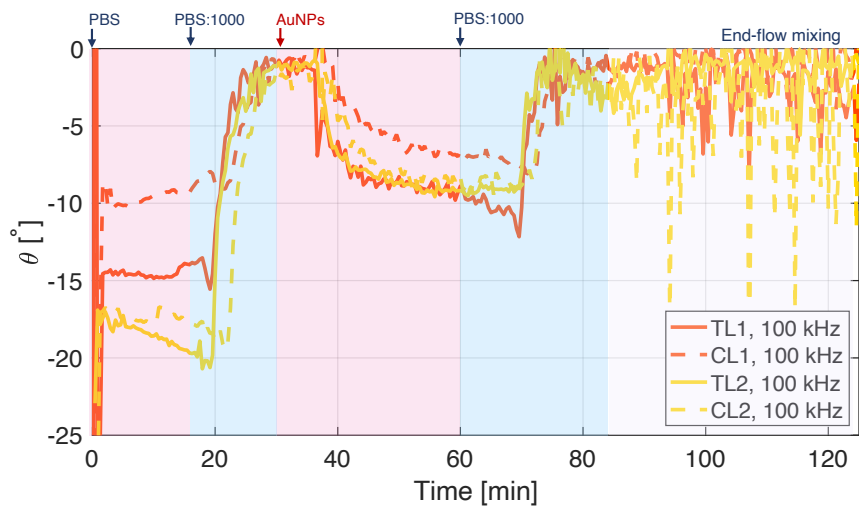


Fig. F.9 As negative control for the impedance detection of the AuNPs, we removed the conjugated AuNP from their running buffer, which was then used as a buffer for the LFA. Impedance phase angle.

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