AFFINITY BIOSEPARATION AND BIOSENSING USING MAGNETIC PARTICLES FOR FOOD SAFETY

PhD Thesis

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List of Abbreviations and Symbols

§	Section
ʎ	Wavelenght
µA	Microamper
mV	Milivolt
E	Potential
i	Intensity current
A	Adenine
AD	Alkaline phosphatase
AntiDig-HRP	Anti-digoxigenin antibody HRP labelled
AntiFlu-HRP	Anti-fluorescein antibody HRP labelled
AU	Absorbance unit
Av	Avidin
BHI	Brain heart infusion broth
Bio	Biotin
bp	Base pair
BSA	Bovine serum albumin
BTB	Bovine tuberculosis
C	Cytosine
cDNA	Complementary DNA
CFU	Colony-forming unit
Da	Dalton (g mol^{-1})
Dig	Digoxigenin
DPV	Differential Pulse Voltammetry
dsDNA	Double-stranded DNA
EIA	Enzyme immunoassay
EL	Enzymatic labelling
ELIME	Enzyme-linked immunomagnetic electrochemical method
ELISA	Enzyme-linked immunosorbent assays
F	Forward primer
Fab	Antigen binding fragment of an antibody
Flu	Fluorescein
G	Guanine
GEB	Graphite-epoxy biocomposite
GEC	Graphite-epoxy composite
HACCP	Hazard analysis and critical control points
HQ	Hydroquinone
HRP	Horseradish peroxidase enzyme
IA	Immunoassay
i.d.	Internal diameter
IMS	Immunomagnetic separation
ISFET	Ion-sensitive field-effect transistor
LB	Luria-Bertani broth
LOD	Limit of detection
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-associated laser desorption/ionisation time of flight mass spectrometry
m-	magneto
MOI	Multiplicity of infection
MNB	Magnetic nanobead
MP	Magnetic particle

mPCR	Multiplex polymerase chain reaction
n	Number of replicates
OD	Optical density
o.d.	Outer diameter
pa	Anodic peak
pc	Cathodic peak
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
PMS	Phagomagnetic separation
PPD	Purified protein derivative
QCM	Quartz crystal microbalance
QD	Quantum dots
R	Reverse primer
rpm	Revolutions per minute
RSD	Relative standard deviation
RT	Room temperature
scFv	Single-chain variable fragment of an antibody
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SPR	Surface Plasmon resonance
ssDNA	Single-stranded DNA
Strep-HRP	Streptavidin HRP labelled
T	Timina
T*aq*	*Thermus aquaticus* DNA polymerase
TMB	3,3',5,5'-tetramethylbiphenyl-4,4'-diamine
TSP	Tailspike protein
TST	Tuberculin skin test
v/v	Volume-to-volume ratio
w/v	Weight-to-volume ratio

SUMMARY

SUMMARY

Infectious diseases including food-borne illnesses caused by microorganisms, among them bacteria, are a large, widespread and growing public health problem. Moreover, conventional microbiological detection methods are laborious and time-consuming. To overcome these drawbacks rapid and sensitive methods for the detection, identification and quantification of pathogens have been developed. The recent advances in biosensor technology enable the rapid detection of pathogens. However, the majority of them are based on fluorescence spectroscopy, surface plasmon resonance and other methods that require specialised bulky and expensive instruments, which will increase the overall production cost. Therefore, the need of reducing time and complexity from the existing methods is still an issue.

This dissertation reports the design and evaluation of novel strategies, based on both optical and electrochemical detection, for the rapid detection of pathogenic bacteria in food safety applications. Different electrochemical platforms, based on the coupling of magnetic particles with magneto graphite-epoxy composite as well as graphite-epoxy biocomposite, were explored. Magnetic particles which allow covalent or electrostatic immobilisation were used, *e.g*. silica, streptavidin, antibody and phage-modified magnetic particles. Thus, several bioaffinity interactions such as biotin-avidin, antibody-antigen and bacteriophage-bacterial surface receptors were evaluated.

Firstly, a rapid method for screening-out of bovine tuberculosis in milk and dairy products based on electrochemical genosensing of DNA specific of *M. bovis* is presented. In this study, two different platforms for electrochemical genosensing were evaluated: i) an avidin-biocomposite (Av-GEB), and ii) streptavidin-modified magnetic particles coupled with a magneto-electrode based on graphite-epoxy composite (m-GEC). The comparison with the tuberculin skin test and an inter-laboratory PCR assay was performed with raw milk samples collected from local dairy farm tanks.

Moreover, different biosensing strategies for pathogenic bacteria were evaluated by using *Salmonella* as a model. The first approach relies on a double biorecognition of the bacteria, *i.e.* immunological and genetic biorecognition. The bacteria were captured and pre-concentrated from food samples with magnetic particles by the immunological reaction with the specific antibody against *Salmonella*. After the immunomagnetic separation (IMS), the captured bacteria were lysed, and further amplification of the genetic material by Polymerase Chain

Reaction (PCR) with a double-tagging set of primers was performed to confirm the identity of the bacteria. The double-tagged amplicon was then detected by electrochemical magneto-genosensing.

The second strategy is based, for the first time, on the use of bacteriophages, as biorecognition element for the magnetic separation of pathogenic bacteria. Phages offer several analytical advantages as biorecognition element, since they are inexpensive, highly specific and strong binders, resistant to high temperatures and environmental stresses. The phage capabilities were explored by using the phage P22 towards *Salmonella* as a model which was immobilised in an oriented way on magnetic particles. The bacteria were then captured and pre-concentrated by the phage-modified magnetic particles throughout the phage-host interaction. To confirm the identity of the bacteria, further double-tagging PCR amplification of the captured bacteria DNA and electrochemical magneto-genosensing of the amplicon were performed.

The third strategy for *Salmonella* detection relies on the detection of the whole bacteria by a double immunological recognition. The bacteria were captured from food samples and pre-concentrated by immunomagnetic separation. After the IMS, the enzymatic labelling of the bacteria was also performed using a specific antibody against *Salmonella*, performing thus the electrochemical magneto-immunosensing. Although higher LODs were obtained, the assay time and complexity of the procedure were reduced considerably. The analytical features of the magneto-immunosensing of *Salmonella* were evaluated not only for electrochemical but also for optical detection, developing thus an optical magneto-immunoassay as the last strategy presented for *Salmonella* detection in this dissertation.

Finally, the last strategy developed is based on the electrochemical magneto-genosensing of the three most common pathogenic bacteria in food safety (*Salmonella, Listeria* and *E. coli*). This approach was performed by the release of the bacteria genome followed by PCR in order to obtain the tagged amplicons by using three different coding tags. The tagged amplicons were then immobilised on silica magnetic particles. To confirm the identity of the three bacteria, the tagged amplicons were detected by electrochemical magneto-genosensing using three different electrochemical reporters.

It is important to highlight that biosensing devices based on these strategies are ideal tools for being used as an alarm to rapidly detect the risk of contamination by pathogens in an inexpensive and sensitive manner and in a wide variety of matrixes.

1

INTRODUCTION

The development of analytical biosystems involves deepening knowledge of analytical and biological chemistry, enzyme and materials technology, genetics, microbiology and immunology. All these converging technologies have opened new horizons in electrochemical biosensors, which are the main aim of study of this dissertation. This general introduction of the field includes an overview of food safety, food-borne pathogens and related infectious diseases, conventional and other rapid methodologies used in bacterial pathogen detection as well as biosensors and new trends in biosensing. In addition, a compilation of the state-of-the-art is presented as well.

1.1. FOOD SAFETY, FOOD-BORNE PATHOGENS AND RELATED INFECTIOUS DISEASES

Food-borne and infectious diseases are a global problem, causing considerable morbidity and mortality annually. For the past decade, the increase in food-borne infections has become an important public health concern worldwide being bacteria the most important agents of food-borne illness. Many factors have contributed to food-safety problems over recent decades such as intensive farming, globalisation of the food industry, and changes in consumer demands.

There are three main types of agents in food with the potential to cause an adverse health effect, as shown in Table 1.1:

Regarding the biological hazards, they include pathogenic strains of bacteria, viruses, helminthes, protozoa and algae, and certain toxic products they may produce. Food-borne infections are caused when microorganisms are ingested, and these can multiply in the human body. Intoxications result when microbial or naturally occurring toxins are consumed in contaminated foods. Microorganisms or toxins may be introduced directly from infected food animals or from workers, other foods, or the environment during the preparation or processing of food.

Chemical hazards may result from pollution arising from industrial and other human activities, from agricultural practice or from food processing and packaging. These contaminants may present a potential hazard for human health if exposure exceeds tolerable levels. They can be categorised according to their chemical nature in inorganic or organic chemical hazards.

Physical hazards are related with foreign matter, including dirt and glass, which accidentally get into food. They can be the result of environmental contamination during production, processing, storage, packaging, and transport, or from fraudulent practices. The potential for ionising radiation to have long-term health effects, not only on the people living nearby a nuclear reactor but also the health of the ecosystem, is considerable.

Table 1.1. Main biological, chemical and physical hazards [1].

Biological Hazards	Chemical Hazards	Physical Hazards
BACTERIA (spore-forming) *C. botulinum* *C.perfringens* *B. cereus* BACTERIA (nonspore-forming) *Campylobacter* spp. Pathogenic *E. coli* *L. monocytogenes* *Salmonella* spp. *S. dysentetiae* *S. aureus* *S. pyogenes* *V. cholera* *V. parahaemolyticus* VIRUSES Hepatitis A and E Norwalk virus group Rotavirus PROTOZOA AND PARASITES *C. parvum* *Diphyllabothrium* *E. histolytica* *G. lamblia* *A. lumbricoides*	INDUSTRIAL / ENVIRONMENTAL POLLUTION Polychlorinated biphenyls (PCBs) Agricultural chemicals (pesticides, fertilizers, antibiotics, growth hormones) Toxic elements and compounds INORGANIC CHEMICALS Lead, Tin Mercury, Zinc Cadmium, Arsenic Cyanide ADDITIVES Feed additives Veterinary drugs Food additives Vitamins and minerals CONTAMINANTS Lubricants Cleaners Pest control chemicals Coatings Paints CHEMICAL MIGRATING FROM PACKAGING Plasticizers, Vinyl chloride Printing/coding inks Adhesives, Lead, Tin	GLASS Bottle, jars, light fixtures, gauge covers WOOD Field sources, pallets, boxes, building materials STONES Fields, buildings METAL Machinery, fields, wire INSULATION Building materials BONE Improper processing PLASTIC Packaging, pallets, equipment PERSONAL EFFECTS Employees

The European Food Safety Authority (EFSA) is the keystone of European Union (EU) risk assessment regarding food and feed safety. The EFSA was set up in 2002, following a series of food crises in the late 1990s, as an independent source of scientific advice and communication on risks associated with the food chain. Similarly, the Food and Drug Administration (FDA) organisation ensures the food safety in the United States (US).

Despite the effort of all organisations involved, the lasting solution to the problems which have shaken the food industry in recent years can only be overcome if farmers, food processors, wholesalers, transporters, retailers, caterers, scientist, regulators, and government work together towards common goals. The investigation and control of food-borne disease outbreaks are multi-disciplinary tasks requiring skills in the areas of clinical medicine,

epidemiology, laboratory medicine, food microbiology, chemistry, food safety, food control, risk communication and management [2, 3]. Along with emerging biological, chemical and physical issues in food safety, new technologies have been developed which have contributed to containing the rise in food-borne illnesses. However, solutions to completely eliminate such hazards from food are complex and not readily available. Changes in the food production environment, food processing with new product formulations, failures in proper food handling practices, and people's interests in eating raw or undercooked foods will continue to promulgate the occurrence of food-borne outbreaks in the new millennium. Innovative technologies for hazard control, from reducing contamination to treating foods to inactivate food-borne pathogens and retain freshness and flavours, are being developed. Such new techniques are crucial to enhancing the safety of the food supply of the future [4].

The ultimate objective of the food industry and government regulators is to ensure that food reaching the consumer is safe and wholesome. The Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) have complementary food-safety mandates to protect the health of consumers, to prevent the spread of disease and to ensure that the procedures followed in food trade are fair. In this regards, the FAO and WHO created the Codex Alimentarius Commission in 1963 to develop food standards, guidelines and texts such as codes of practice under the Joint FAO/WHO Food Standards Program. Approaches such as Hazard Analysis Critical Control Point (HACCP) can play an important role in reducing food-borne illnesses.

The HACCP system is a program of preventative food safety assurance originally developed in the USA by the Pillsbury Company working with the National Aeronautics and Space Administration (NASA) and the US army laboratories at Natick in order to ensure the safety of astronauts' food. In the last three decades, HACCP has been internationally recognised and accepted as a proven method for food safety assurance. Its original purpose was to ensure microbiological safety of foodstuffs, and then it was further broadened to include physical and chemical hazards for a wide variety of food and associated industries. The system consists of seven principles, detailed by the Codex Alimentarius Commission which outlines how to establish, implement, and maintain a HACCP plan. As enforcers, regulatory authorities such as EFSA, WHO, FDA and FAO, assess the appropriate implementation of the HACCP plans and confirm that they are properly designed and implemented [3, 5-7].

The implementation of an HACCP system is closely connected with microbiological quality control. Rapid microbiological methods are being extensively developed, as explained in § 1.2. These methods can be used when the HACCP system is developed, implemented and maintained. Successful combination of the HACCP program and rapid microbiological methods may help food industry to find new ways of obtaining reliable results more efficiently [8].

1.1.1. A brief description of infection diseases and food-borne pathogens

The targets chosen as a model for the development of the strategies presented in this dissertation were *Mycobacterium bovis,* the causative agent of bovine tuberculosis (BTB), an important animal pathogen with public health implications as it is a zoonosis; *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* the most harmful food-borne pathogens. In this section a brief description of the pathogens and the diseases that cause is presented.

1.1.1.1. Mycobacterium bovis and bovine tuberculosis

Mycobacterium bovis is a member of the *Mycobacterium* tuberculosis complex, a group that also includes *M. tuberculosis, M. africanum, M. canetti*, and *M. microti*, and the recently described *M. caprae* and *M. pinnipedii* [9]. These bacteria are acid-fast, aerobic, non-spore forming and non-motile. They form slightly curved or straight rods which may branch (0.2 to 0.6 µm by 1.0 to 10 µm). They are slow growers, *i.e.* they require more than 7 days to form colonies [10]. *M. bovis*, the etiopathogenic agent of tuberculosis in cattle, is also a zoonotic disease for humans and a large number of other animals (wild, farmed, feral, and domestic animals). Bovine tuberculosis (BTB) is a disease characterised by progressive development of specific granulomatous lesions or tubercles in lung tissue, lymph nodes or other organs. Humans are also susceptible to the disease, the highest risk groups are individuals with concomitant HIV/AIDS infection [11, 12].

Main ways of transmission are both nosocomial or airborne. Infected animals can spread the infection to laboratory workers through aerosols, vomits or bites. Bovine tuberculosis can occur from exposure to infected cattle (airborne, ingestion of raw milk or dairy products). The bacteria need 4 – 6 weeks of incubation period and are highly transmissible, infected humans can transmit the disease to cattle and vice versa [13]. Thus, zoonotic infection is a driver for disease control in animal hosts.

1.1.1.2. *Salmonella, Listeria monocytogenes, Escherichia coli and diarrhoeal diseases*

Salmonella, *Listeria monocytogenes* and *Escherichia coli* are the most harmful food-borne pathogens that cause diarrhoeal diseases. A brief description of each pathogen is presented next:

***Salmonella* spp.**

The genus *Salmonella* is a member of the family Enterobacteriaceae. The genus is composed of Gram-negative bacilli that are mesophilic, facultative anaerobic, non-spore forming and motile (flagellated). The growing occurs between 5 – 47 °C, although the optimum is at 37 °C. The illness usually lasts 4 – 7 days and includes fever, abdominal pain, diarrhoea, nausea and vomiting. Most people recover without treatment, however, in some people, the diarrhoea may be so severe that the patient needs to be hospitalised. In these patients, the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics. The elderly, infants, and those with impaired immune systems are more likely to have a severe illness. A small number of people with *Salmonella* infection develop pain in their joints, irritation of the eyes, and painful urination, a syndrome called reactive arthritis which can also lead to the difficult to treat chronic arthritis.

The symptoms of *Salmonella* infection usually appear 12 – 72 hours after infection. The main route of transmission is by ingestion of the organisms in food (milk, meat, poultry, and eggs). Food can also be contaminated by infected food handlers, pets and pests, or by cross-involved in contamination owing to poor hygiene. Contamination of food and water may also occur from the faeces of an infected animal or person. Problems caused by initial contamination may be exacerbated by prolonged storage at temperatures at which the organism may grow. Direct person-to-person transmission may also occur during the course of the infection. Consumers, particularly vulnerable groups, should avoid raw and undercooked meat and poultry, as well as raw milk and eggs and foods containing raw eggs.

Listeria monocytogenes

Listeria monocytogenes is a bacterium in the division Firmicutes. It is a Gram-positive, non-spore-forming, facultative anaerobic and rod-shaping bacterium. The bacterium is capable of

growing in the presence of 10 % salt, and in a temperature range of 3 – 42 °C, but optimally at about 30 – 35 °C. Listeriosis primarily affects older adults, pregnant women, newborns, and adults with weakened immune systems. A person with listeriosis usually has fever and muscle aches, sometimes preceded by diarrhoea or other gastrointestinal symptoms. Almost everyone who is diagnosed with listeriosis has invasive infection, in which the bacteria spread beyond the gastrointestinal tract. The symptoms can have duration of days – weeks and vary with the infected person.

The incubation period needed ranges from few days to several weeks. *Listeria monocytogenes* is commonly found in soil and water. Animals can carry the bacterium without appearing ill and can contaminate foods of animal origin, such as meats and dairy products. Most human infections follow consumption of contaminated food. Rare cases of nosocomial transmission have been reported. *L. monocytogenes* is killed by pasteurisation and cooking; however, in some ready-to-eat foods, such as hot dogs and deli meats, contamination may occur after factory cooking but before packaging. Unlike most bacteria, *Listeria* can grow and multiply in some foods in the refrigerator.

Escherichia coli

E. coli consists of a diverse group of bacteria. Pathogenic *E. coli* strains are categorised into pathotypes. Six pathotypes are associated with diarrhoea and collectively are referred to as diarrhoeagenic *E. coli:*

- Shiga toxin-producing *E. coli* (STEC)—STEC may also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC).
- Enterotoxigenic *E. coli* (ETEC)
- Enteropathogenic *E. coli* (EPEC)
- Enteroaggregative *E. coli* (EAEC)
- Enteroinvasive *E. coli* (EIEC)
- Diffusely adherent *E. coli* (DAEC)

In general, the bacteria are Gram-negative, non-spore-forming, facultative anaerobic rods, which belong of the agent to the family Enterobacteriaceae. Typically mesophile, the bacteria grow from about 7 – 10 °C up to 50 °C, with the optimum at 37 °C. Most *E. coli* are harmless inhabitants of the gut of humans and other warm-blooded animals, however the strains

mentioned above may cause diseases. Some kinds of *E. coli* can cause diarrhoea, while others cause urinary tract infections, respiratory illness and pneumonia, and other illnesses. Still other kinds of *E. coli* are used as markers for water contamination. Moreover, the infection of *E. coli* has been linked to haemolytic uremic syndrome and hemorrhagic colitis in humans, and these illnesses cause diarrhoea, kidney failure, seizure, and death.

The types of *E. coli* that can cause diarrhoea can be transmitted through contaminated water or food or through contact with animals or people in a short incubation period of 1 to 4 days depending on the pathotype. The infection of *E. coli* is mostly associated with a contaminated beef, sprouts, iceberg lettuce, salami, milk, juices, and water [3, 14].

1.1.2. Importance and worldwide prevalence

According to a report of the World Health Organisation, hundreds of millions of people worldwide suffer from infectious and food-borne diseases. Since diarrhoea is a common clinical symptom in food-borne diseases, many of these diseases are referred to as "diarrhoeal diseases". As shown in Table 1.2, diarrhoeal diseases and tuberculosis, which are diseases caused by the pathogenic bacteria studied in this dissertation, are among the main causes of death worldwide.

Table 1.2. Main causes of death according to world health statistics 2013 (WHO).

Cause of death	Deaths (millions)	Deaths (%)
Ischaemic heart disease	7.25	12.8
Stroke and other cerebrovascular disease	6.15	10.8
Lower respiratory infections	3.46	6.1
Chronic obstructive pulmonary disease	3.28	5.8
Diarrhoeal diseases	2.46	4.3
HIV/AIDS	1.78	3.1
Trachea, bronchus, lung cancers	1.39	2.4
Tuberculosis	1.34	2.4
Diabetes mellitus	1.26	2.2
Road traffic accidents	1.21	2.1

Tuberculosis (TB) is one of the world's deadliest diseases, one third of the world's population are infected with TB. In 2011, nearly 9 million people worldwide became sick with TB disease, most of whom (82 %) live in one of the 22 high burden countries for TB. TB is a leading killer of people living with HIV. While significant progress has been made toward the

elimination of TB in some countries, this disease remains an urgent public health problem in many other parts of the world.

The annual global number of new cases of TB has been slowly declining since 2007, and between 2010 and 2011 the number of such cases fell by 2.2 %. Of the estimated 8.7 million new cases in 2011, about 13 % involved people living with HIV. In all six WHO regions (Africa, America, South-East Asia, Europe, Eastern Mediterranean and Western Pacific), the incidence of TB is falling (Figure 1.1). Mortality due to TB has also fallen by 41 % since 1990 and the trend indicates that it will reach a 50 % reduction by 2015. However, TB remains one of the most devastating infectious diseases and its eradication is still unattainable [15]. *M. tuberculosis* is the causal agent of tuberculosis in humans; however, the zoonotic transmission of *M. bovis* from animals to humans, mainly immunocompromised individuals, is of great impact in public health. In industrialised countries, approximately 0.5 – 1.5 % of all the human isolates from TB patients are *M. bovis*. Although most countries are officially free of *M. bovis*, residual infections are still present as for instance occurred with the outbreak in Birmingham, England in 2004 [16]. Moreover, in 2011, 132 cases of *M. bovis* infection were reported in European Union Member States, representing a decrease compared with 2010 (165 cases), but still being an important issue (Figure 1.2, A) [17].

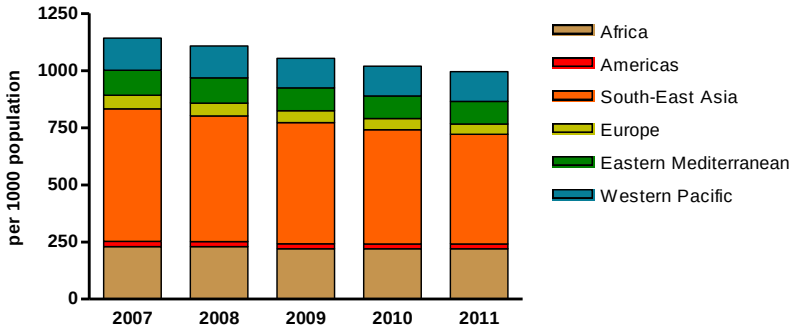

Figure 1.1. Estimated number of deaths due to tuberculosis, excluding HIV.

In this dissertation, besides *Mycobacterium bovis* that causes bovine tuberculosis, *Salmonella, Listeria* and *E. coli* were selected as a representation of biological agents that cause diarrhoeal diseases for being among the five most studied pathogens that account for over 90 % of estimated food-related deaths: *Salmonella* (31 %), *Listeria* (28 %), *Toxoplasma* (21 %), Norwalk-like viruses (7 %), *Campylobacter* (5 %), and *E. coli* O157:H7 (3 %) [18].

According to the European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks [17], a total of 5,648 food-borne outbreaks were reported in the European Union, resulting in 69,553 human cases, 7,125 hospitalisations and 93 deaths only in 2011. Most of the reported outbreaks were caused by *Salmonella*, *Listeria* and *E. coli* infections, and among other bacterial agents *M. bovis* was also present (reported cases in 2007 – 2011 showed in Figure 1.2).

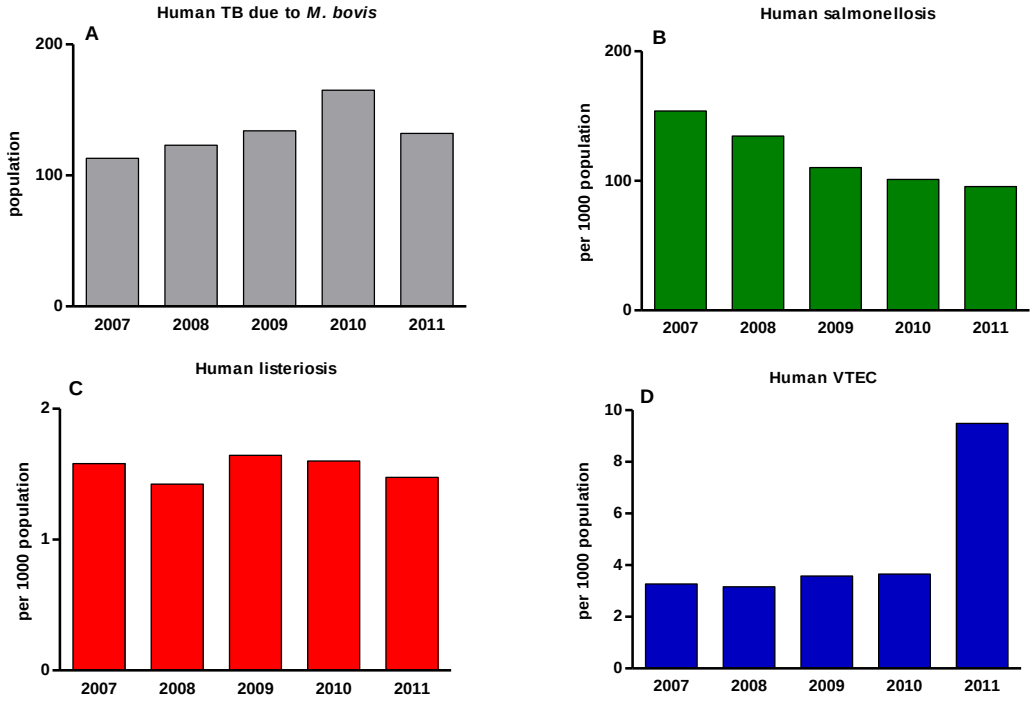

Figure 1.2. Reported cases of tuberculosis due to *M. bovis* (A), salmonellosis (B), listeriosis (C) and Verocytoxigenic *Escherichia coli* (VTEC) infections (D) in 2007 – 2011 [17].

It is worth enhancing the recent *E. coli* outbreak in Germany in 2011 (Figure 1.2, D), as an example of the significant threat to human public health that food-borne pathogens symbolise, leading to a substantial loss of investments from both public and private sector annually worldwide. It appears that the *E. coli* O104:H4 strain in Germany was spread mostly by contaminated sprouts, and in just a few cases, from close contact with a sick person. This outbreak underscores the critical importance of all aspects of public health, including the following: continuous public health surveillance to detect disease outbreaks; rapid epidemiological investigation of outbreaks; public health reference laboratories that can examine and identify uncommon organisms that sometimes cause disease; and food safety authorities that take appropriate measures to control the source of the infection and to prevent similar events from happening in the future.

1.2. CONVENTIONAL METHODS FOR PATHOGENIC BACTERIA DETECTION

The purpose of the next section is to review the traditional methods employed for pathogenic bacteria detection over the past decades to the recent year, by highlighting their strengths and weakness. An overview of these methods is presented, particularly, conventional culture (§ 1.2.1), and rapid methods based on molecular (§ 1.2.2) and immunological techniques (§ 1.2.3).

Over the last fifty years, microbiologists have developed reliable culture-based techniques to detect pathogens. Although these are considered to be the "gold-standard," they remain cumbersome and time-consuming. More recently, standard culture-based pathogen detection methods have been refined and even improved, with an eye towards reducing time to detection. This is generally done by replacing the selective and differential plating step with more rapid immunological or molecular-based assays. Among these, the enzyme-linked immunosorbent assay (ELISA), DNA hybridisation, and polymerase chain reaction (PCR) methods are the most notable. These "rapid" assays must include a number of essential features. Firstly, they must exhibit a high degree of sensitivity, defined as the ability to detect the pathogen when it is actually present in the sample. This is required to prevent false negative results and hence assure that a contaminated unit is identified accurately. Routinely, detection limits of a single viable cell of contaminating pathogen per sample unit are considered essential. A high level of test specificity, or the ability to classify a sample as negative if the pathogen is absent, is no less important as it reduces the likelihood of having to spend additional time and resources confirming results on products which do not represent a risk to public health. Although rapid methods can be highly accurate (some are over 98 % in agreement with a reference culture method), normally they are not considered definitive because they usually do not produce an isolate. Rapid methods that exhibit both high specificity and high sensitivity can be used as a screening tool when they are performed in tandem with the culture method.

Table 1.3 compares the main advantages and drawbacks of each method, and Figure 1.3 displays, as an example, the comparison of time needed with different methods for *Salmonella* spp. detection. In detail, classical cultural techniques are highly labour-intensive and require skilled microbiologist to carry out the analysis. Negative results are obtained after approximately 4 – 5 days and confirmation of presumptive-positives requires a further 2 days. In the case of ELISAs, negative results are obtained as early as 24 – 30 h and confirmation of

presumptive-positive requires a further 2 days. Although the reagents and material costs in cultural techniques are generally lower than ELISAs, the labour costs are likely to be higher. Among the different methods, immunological techniques are promising because of their sensitivity and rapidity. However, even rapid detection tests normally require enrichment of the target bacteria to the level of the assay's detection limit. A major disadvantage of the immunological techniques is that every step in the assay requires a rigorous washing procedure, which is labour-intensive and difficult to automate.

Concerning molecular methods based on nucleic acid probes and PCR, the total time frame of the analysis is still several hours. However, these are generally an order of magnitude more sensitive and exhibit better specificity than the immunological techniques, and the results obtained are usually definitive with no requirement for confirmation by classical cultural techniques. A major drawback is that molecular techniques require high levels of technical skill, special laboratory facilities to avoid PCR contamination problems, generally high capital equipment costs and are prone to PCR inhibition depending on the matrix analysed.

Table 1.3. Comparative features of conventional methods for pathogenic bacteria detection.

Detection method	Advantage	Disadvantage
Culture methods	• relatively inexpensive • simple • specific • widely accepted method (gold-standard)	• time-consuming • no detection of non-cultivable microbes • low sample throughput • laborious
Molecular methods	• high sample throughput • sensitive • specific • quantitative	• no live/dead cell differentiation • sensitive to matrix interference (high extraction effort) • susceptible to polymerase inhibition
Immunological methods	• differentiation of serotypes or subspecies • high sample throughput (96-microwell plates) • quantitative and qualitative	• lower sensitivity • lower specificity, higher cross-reactivity • slow and expensive assay development

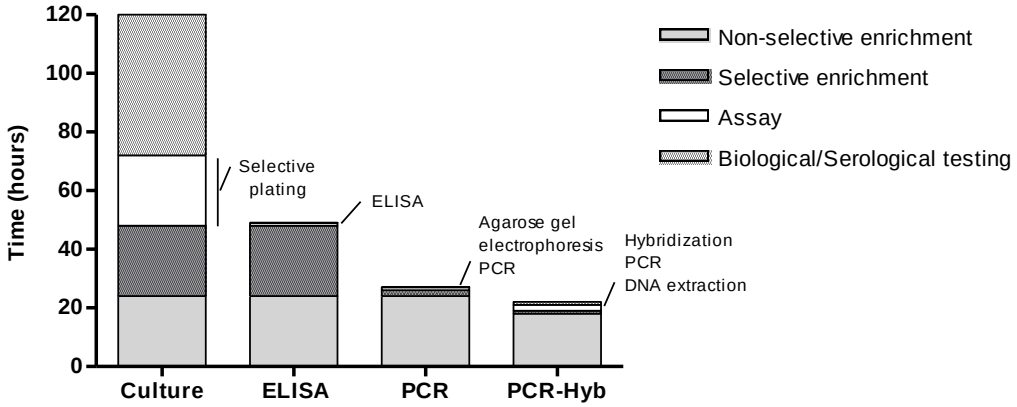

Figure 1.3. Comparison of maximum assay times for the detection of *Salmonella* spp. [19].

All the current generation of tests, both molecular and immunological, usually requires at least an overnight pre-enrichment step before analysis. Despite the advent of these rapid detection methods, it is clear that reduction and/or elimination of cultural enrichment will be essential in the quest for truly real-time detection methods.

The development of new methods that provide confirmed results in 1 day are still necessary for the consumer protection, and the quality of these results should be at least as reliable as those of the reference method. Rapid methods still require 1 to 3 days and often lack specificity or sensitivity. In recent years, some developments became accepted to reduce the time for gaining a result while enhancing sensitivity and specificity. However, there is still a need of finding a way to improve them, in terms of reducing their time assay and complexity.

In conclusion, methods such as ELISA, DNA hybridisation and PCR, although rapid and reliable, must be thoroughly compared to standard methods before use for routine analysis. Currently only a limited number of rapid methods have been validated by collaborative studies and approved by the Association of Analytical Communities (AOAC International). These rapid methods can only be used for screening. Negative results are regarded as definitive, but positive results are considered presumptive and must be confirmed by standard methods [20-23].

1.2.1. Culture methods

Over the last 50 years, microbiologists have developed reliable techniques to detect pathogens from different kind of samples including contaminated foods and environmental samples. These culture-based methods are considered to be the “gold-standard” and are

known for their cost effectiveness, sensitivity, ability to confirm cell viability and ease of standardisation. Culture-based assays are designed to be able to detect a single target cell in the sample. Sample sizes typically vary from a low of 10 g to 375 g or more. These methods are definitive and result in a bacterial isolate. Most of the culture methods consist of the following five steps:

- *Pre-enrichment*. This preliminary step is performed with a non-selective medium to increase the number of the target organism, without suppressing the growth of other organisms. Moreover, the resuscitation and proliferation of stressed or injured cells to detectable levels is also achieved, since cells are injured due to the exposure to adverse conditions during food processing, such as chilling, freezing or drying. If a resuscitation step is not included, the stressed cells may be missed.

- *Selective enrichment*. This step is used to increase the population of the specific organisms to be detected while suppressing the growth of competitors.

- *Selective/differential plating*. Selective enrichment broths are streaked on to selective solid media, both to suppress the growth of competitors and to allow for the isolation of discrete presumptive colonies. The result of selective and differential plating is the isolation of one or more colonies that fulfil the presumptive positive criteria. In the absence of typical colonies, the analysis is completed and the results are reported as negative.

- *Biochemical testing.* Suspect colonies from the selective agar plates are transferred to tubes of differential agar for the preliminary biochemical characterisation of the isolates.

- *Serological testing.* The results obtained after the biochemical testing provide a relatively reliable indication of the bacteria presence. However, final confirmation is performed serologically by determining their antigenic composition.

The combined tandem “enrichment and plating” takes 24 – 48 hours to be performed, which means that presumptive detection of a pathogen can take about 4 days. The confirmation of a positive sample can take up to a week or more. Many alternative methods have consequently been introduced in recent years to reduce analytical time and also save staff time and media requirements. The most outstanding methods are based on nucleic-acid and immunological techniques, both presented in §§ 1.2.2 and 1.2.3 [20, 22, 24, 25].

1.2.2. Molecular methods

Recently molecular methods have been developed which offer considerable potential for obtaining a rapid result. These methods include the use of DNA hybridisation (§ 1.2.2.1) and DNA amplification by the Polymerase Chain Reaction technique (§ 1.2.2.2) [26].

1.2.2.1. DNA hybridisation

Hybridisation is a specific method and has been used to detect several pathogens based on the specific DNA sequence of the bacteria genome. The DNA-DNA hybridisation method requires the selection of a specific target gene of the bacteria and a complementary specific probe. The DNA probes require to be labelled with different tags, such as radioisotopes, enzymes, or other small moieties or functional groups. Radioactive labels are extremely sensitive but they have the disadvantages of short shelf life, possible exposure of personnel to radiation, cost, and storage and disposal problems. On the other hand, non-radioactive probes, such as enzymatic or luminescence labels, are less sensitive and flexible in terms of design and application [27]. Probes can be directed to either DNA or RNA targets and may be oligonucleotide probes (often 20 – 50 bp) or DNA probes (50 to thousands of base pairs). Oligonucleotides can be synthesised chemically and easily purified while long DNA probes can be obtained by molecular cloning techniques.

Under the proper conditions, a single-stranded DNA (ssDNA) molecule will hybridise selectively with a complementary sequence of DNA, forming double-stranded DNA (dsDNA). The use of a tagged ssDNA molecule (*i.e.*, the DNA probe) allows the detection of specific, complementary, nucleic acid sequences. DNA probes can be designed with different levels of specificity to detect to species level, or beyond species level to particular pathogenic strains, depending on the selected DNA sequence to be interrogated.

For hybridisation to occur between a DNA probe and the target sequence, the target dsDNA must be denatured into two separate strands by increasing the temperature. When the temperature is lowered, the strands will reform a double helix if strands contain similar sequences. The temperature and salt concentration used for hybridisation are critical. If the temperature is too low, hybrids can be formed by strands that are not exactly complementary (low stringency). If the temperature is too high, strands that are exactly complementary may

not hybridise, resulting in a negative reaction. The salt concentration is also adjusted to increase or decrease the stringency of the hybridisation. Together, temperature and salt concentrations are used to optimise the hybridisation conditions so that only the probe and the complementary nucleic acid will bind to each other [28].

Concerning formats, hybridisation can be homogeneous, performed in liquid solution with no separation step needed prior to measurement (label free, colorimetric or chemiluminiscence) or heterogeneous, performed in a solid support where a separation step is required (by using radioisotope, fluorescence or enzyme labels). This separation can be achieved by biochemical means (adsorption chromatography, differential precipitation and electrophoresis) or by immunological means (affinity chromatography and immuno-capture). The ssDNA immobilised on the solid support can be the target analyte, which would hybridise with the tagged probe, or the probe, which would hybridise with the tagged target analyte. As shown in Figure 1.4, hybridisation can be direct (Figure 1.4, i), when the label on the probe DNA is able to give analytical signal (enzyme, fluorescent and chemiluminiscent substrates), or indirect (Figure 1.4, ii), when the label on the probe DNA needs a second recognition to give analytical signal (system biotin/streptavidin or immunological recognition). In the sandwich assay format (Figure 1.4, iii), the probe DNA is immobilised on the solid support to specifically hybridise with the target DNA from the medium under assay, then a second hybridisation is performed with the tagged probe, that also is complementary to the target DNA achieving thus a second recognition of the analyte. The last format is the competitive hybridisation (Figure 1.4, iv) which has different possible designs. For instance, when the probe DNA is immobilise on the solid support, the tagged probe and the target DNA are in solution in such a way that the target analyte is competing with the tagged probe for the hybridisation with the immobilised probe [29-31].

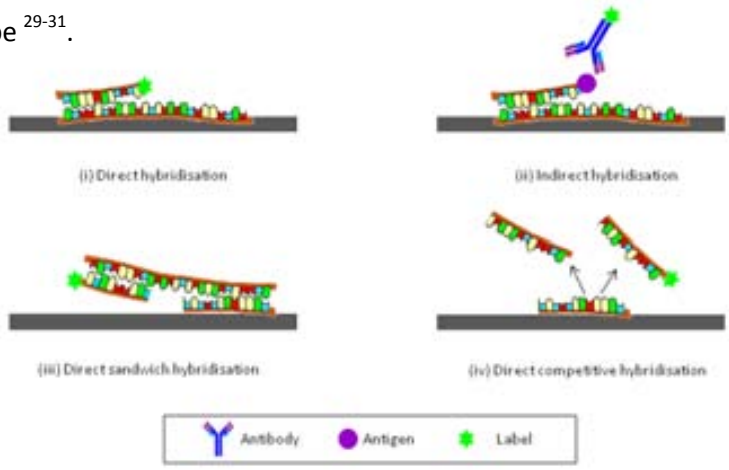

Figure 1.4. Schematic representation of the different hybridisation formats: (i) direct, (ii) indirect, (iii) direct sandwich and (iv) direct competitive.

1.2.2.2. Polymerase Chain Reaction

In April 1983, Kary Mullis of Cetus Corporation conceived the basic idea for the Polymerase Chain Reaction (PCR) which is a technique for amplifying a specific target DNA sequence *in vitro*. This technique of impressive sensitivity can amplified a single copy of a particular sequence of DNA for further detection. As outlined in Figure 1.5, each round of PCR cycle involves three steps: denaturation, annealing and extension. The PCR cycle is repeated, usually 30 times, to amplify the genetic material. The repetition of the cycle is the key to achieve exponential amplification. Because the primer extension products synthesised in a given cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle. Thus, in only 20 cycles, PCR can produce about a million (2^{20}) copies of the target. In detail, the PCR requires two oligonucleotide primers, complementary to sequences on opposite DNA strands. The two strands of the target DNA are separated by an increase in temperature (denaturation at 95 ºC), and the primers are allowed to anneal to the complementary sequences in the denatured target DNA at a reduced temperature (annealing at 50 – 70 ºC). A thermally stable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (*Taq* polymerase) is used to synthesise the complementary strand (at 72 ºC), although other polymerases can be used. Two double-stranded DNA molecules have now been created from a single double-stranded molecule. The process is repeated, with each amplification step doubling the number of strands of target DNA [28, 32].

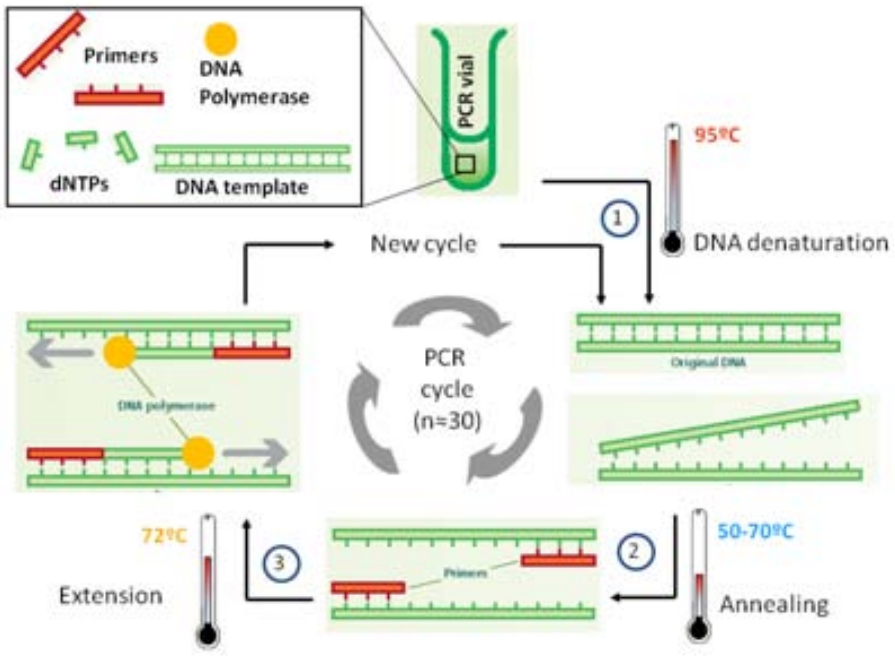

Figure 1.5. The PCR procedure. Each cycle involves the following three steps: (1) DNA denaturation, (2) primers annealing and (3) DNA extension. When the cycle is repeated several times, the net result is a rapid increase in the total number of copies of the target DNA.

Agarose gel electrophoresis and a DNA-binding dye such as ethidium bromide is commonly used for the detection of the amplified PCR products. However, hybridisation has also been investigated for amplicon confirmation. The use of PCR technique was extended to the amplification of RNA as well by using a reverse transcriptase to convert the RNA into cDNA, and then a thermostable DNA polymerase is used to amplify the cDNA to detectable levels. This technique made it possible to use PCR to detect and analyse mRNA transcripts and other RNAs present in low abundance.

This type of PCR assays is named “end-point”, because they are usually done after 30 to 40 cycles. End-point analysis is not very suitable for quantitative PCR because it is done in the *plateau* phase of PCR where the reaction no longer follows exponential kinetics. In this phase, the reaction can no longer be described by a mathematical formula. Thus, it is not possible to directly correlate the end-point signal with the initial template amount or target copy number. Real-time PCR (also named quantitative PCR, qPCR) offers an alternative method for both qualitative and quantitative analysis. This type of analysis allows the amplification and fluorescent detection to be performed by single instruments in a single tube with data recorded online. In these automated technique, the identity of PCR products is determined by the incorporation in the assay of a fluorescently labelled moiety which binds to the target while PCR is occurring; such binding results in a fluorescent signal that is read automatically by the PCR thermocycler. Hence, this method results in the simultaneous enrichment and detection/confirmation of target-specific DNA.

In early qPCR assay formats, the amplicon was detected using a DNA intercalating dye such as SYBR® Green. SYBR® Green binds within the minor grooves of dsDNA but not to ssDNA and fluoresces several times brighter in the bound state than as a free dye. Hence, the SYBR® Green signal increases with the increase in double stranded product generated during PCR. In real-time PCR, products can be detected using not only fluorescent dyes that bind to dsDNA such as SYBR® Green, but also fluorescently labelled sequence-specific probes such as molecular beacons, TaqMan or FRET probes [20, 33].

In all these formats, PCR technique is highly specific to target cells, because it relies on a primer DNA, which is complementary to a part of the sequence in the bacterial genome. In addition, since PCR can amplify several sets of DNAs simultaneously within a few hours, it can be a useful technique for multiple target detection [34].

1.2.3. Immunological methods

Immunological methods, or immunoassays, are analytical techniques based on the specific and high affinity binding characteristics of antibodies with particular target molecules called antigens. The basis of every immunoassay is the detection and measurement of the primary antigen-antibody reaction. The classification of immunoassay methods is based on (a) whether they are homogeneous, with no separation step needed prior to measurement, or heterogeneous, where a separation step is required; (b) which species, antibody or antigen, is labelled; and (c) the type of label employed (examples shown in Table 1.4).

Table 1.4. Common antibody labels for immunochemical techniques [35].

Label	Examples	Main uses(s)
Radioisotope	^{125}I	Competitive and non-competitive RIA
Enzymes	Alkaline phosphatase (AP)	Immunohistochemistry, EIA, immunoblotting
	β-Galactosidase	As above
	Horseradish peroxidase (HRP)	As above, immunoelectron microscopy
	Glucose oxidase	Immunohistochemistry
	Urease	EIA
Fluorochromes	Fluorescein	Immunohisto/cytochemistry, flow cytofluorimetry, fluorimetric assays
	Rhodamine	Immunohisto/cytochemistry, flow cytofluorimetry
	Phycoerythrin	Flow cytofluorimetry
	Texas Red	Flow cytofluorimetry
	7-Amino-4-methylcoumarin-3-acetate (AMCA)	Flow cytofluorimetry
	BODIPY derivatives	Flow cytofluorimetry
	Cascade Blue	Flow cytofluorimetry
Electron dense	Gold	Immunoelectron microscopy
	Ferritin	As above

Immunological assays based on polyclonal or monoclonal antibodies can be divided in Radioimmunoassays (RIAs); Enzyme Immunoassays (EIAs), in particular Enzyme-Linked Immunosorbent Assays (ELISAs); Immunofluorescent Assays (IFA), Chemiluminescence Immunoassay (CLIA) or Latex Agglutination tests (LA) [8, 36-42], as it is explained in detail next.

1.2.3.1. Radioimmunoassays

In 1960, radioimmunoassays (RIAs) were the earliest of the immunoassays developed for the estimation of small quantities of biological substances. This technique is based on the antigen or antibody labelling with a radioisotopic tracer. The label has virtually no effect on antibody–antigen binding. However, radioisotopes are costly, hazardous, and require inconvenient monitoring and disposal procedures. In addition, isotopic decay necessitates the regular replacement of the labelled component. In general, the isotope ^{125}I is used as a label for large-protein antigen and the isotope ^{3}H is commonly used for hapten labelling. Some specialised assays employ isotopes of Co, Fe, and Se. The RIA detection limits for antigens are approximately 10^{-12} mol L^{-1}, and are equalled only by the enzymatic labels, since they are capable of catalytic signal amplification.

1.2.3.2. Enzyme Immunoassays

Enzymes are currently the most widely used and investigated labels for immunoassays, because a single enzyme label can amplify the signal due to the catalytic activity and its turnover rate. This catalytic amplification results in immunoassay detection limits that rival those of radioimmunoassay without the storage and disposal problems associated with radioisotopes.

In homogeneous immunoassays, where there is no need to separate the two phases, the enzyme coupled to an antigen or antibody is modified in its activity after the reaction. In such a case separation of immune complex from the reaction mixture is not required. Such formats are used mainly in drug assays and are called Enzyme Multiplied Immunoassay Technique (EMIT). On the contrary, in heterogeneous immunoassays, separation of immune complex from the reactants is an essential feature. These assays have been named Enzyme-Linked Immunosorbent Assays (ELISAs). The immunoassay is called competitive when the analyte is in excess and other reagents are constant and limited, and non-competitive when the reagents employed are in excess and there is no competition involved. In Figure 1.6, some formats for an ELISA immunoassay are outlined. In detail: (i) direct competitive assay, when the antibody is coated on the solid phase and the antigen is captured, the assay uses competition of two types of antigen, one labelled and the other unlabelled (analyte); (ii) direct competitive assay, when the antigen is coated on the solid phase and the labelled antibody either binds the free antigen (analyte) or the antigen bound to the solid phase; (iii) indirect competitive (double antibody)

assay, when the antigen is coated on the solid phase, a primary non-labelled antibody that either binds the free antigen (analyte) or the antigen bound to the solid phase is added and a secondary antibody, an anti-antibody, is used as a labelled reagent; (iv) non-competitive sandwich assay (two-sites assay) when the antibody is coated on the solid support, the antigen is captured and a second antibody conjugated with a marker is then added, and (v) indirect non-competitive sandwich assay (double antibody) when the antibody is coated on the solid support, after the antigen is captured a primary non-labelled antibody was added and a second antibody, an anti-antibody, is used as a labelled reagent.

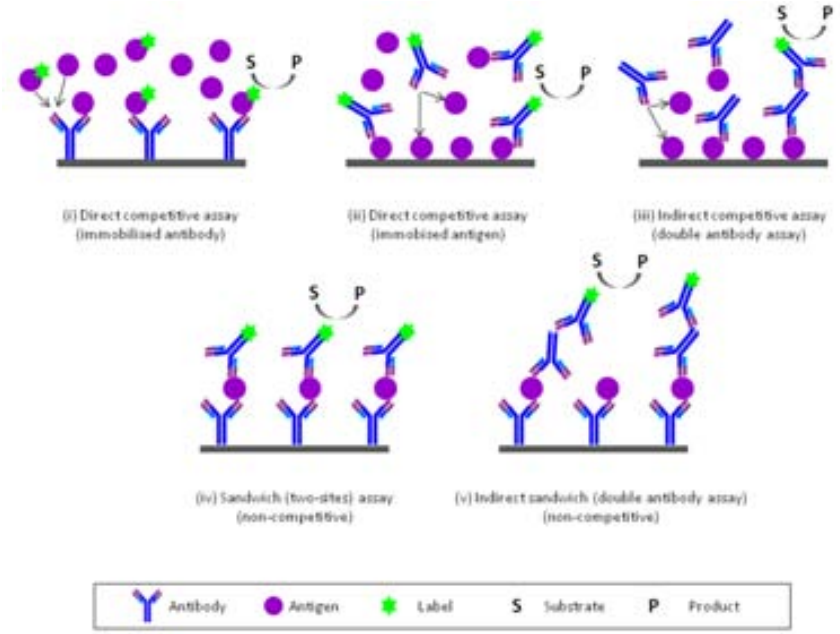

Figure 1.6. Schematic representation of the different ELISAs formats: (i) Direct competitive assay; (ii) Indirect competitive assay; (iii) Indirect competitive double antibody assay; (iv) Sandwich assay (non-competitive), and (v) Sandwich double antibody assay.

In a competitive ELISA, enzyme-labelled antigen competes with free antigen (the analyte) for a fixed, insufficient quantity of immobilised antibody binding sites, as illustrated in Figure 1.6 (i). After incubation, the support is rinsed to remove unbound species, and the enzyme substrate is added in saturating concentration. The conversion of substrate to product may be measured continuously, in a kinetic assay, in which the rate of conversion decreases with increasing free antigen concentration. More often, a fixed-time approach is used; after a given incubation time, the reaction is stopped by the addition of strong acid or base that denatures the enzyme. Product quantification then yields a calibration curve in which product concentration decreases with increasing free antigen concentration. Non-competitive ELISA methods are based on sandwich assays, as shown in Figure 1.6 (iv and v). An immobilised

primary antibody is present in excess, and quantitatively binds antigen. Directly or indirectly, an enzyme-labelled second antibody is then allowed to react with the bound antigen or with the primary non-labelled antibody respectively, forming a sandwich that is detected by measuring enzyme activity bound to the surface of the support. Non-competitive ELISAs yield calibration curves in which enzyme activity increases with increasing free antigen concentration.

In both types of EIA, homogeneous and heterogeneous, an ideal labelling enzyme should have a high turnover number (kcat), a product that is easily measured, and a substrate that does not interfere with measurement. In addition, it should be inexpensive, stable, and resistant to interferences that may be present in biological samples. The enzyme, and its substrate and product should not normally be present in samples for assay. Five enzymes are commonly employed as labelling enzymes in immunoassays: horseradish peroxidase, alkaline phosphatase, β-galactosidase, glucose-6-phosphate dehydrogenase and urease. The most widely used is horseradish peroxidase, because of its high specific activity (4500 U mg^{-1} at 37 º C) and because this 40 kDa enzyme is relatively non-specific for its secondary substrate. This means that a variety of reduced dyes may be used as substrates of this enzyme being converted to their highly absorbing oxidised forms able to be measured.

1.2.3.3. Immunofluorescent assays

Antibodies can also be coupled to fluorescent dyes, allowing detection of bound antibodies by fluorescence. Commonly used fluorescent labels include fluorescein, rhodamine, and umbelliferone derivatives. Recently, a group of proteins isolated from algae have been used as immunoassay labels due to their high molar absorptivity (at least 10 times that of fluorescein), these are the phycobiliproteins, called phycoerythrin, phycocyanin, and allophycocyanin. Fluorescent labels are safe, and require no licensing for their use. Antigen detection limits of about 10^{-10} mol L^{-1} are normal with fluorescent labels, two orders of magnitude higher than those of radioactive labels, as a result of scattering, quenching, and background fluorescence from biological samples, especially those containing significant quantities of protein. Fluorescent labels offer many options for signal generation in heterogeneous immunoassays, and may be classified as indirect, competitive, or sandwich methods (similar to ELISA assays, Figure 1.6).

1.2.3.4. Chemiluminescence Immunoassay

Chemiluminescence is the light emission produced in certain chemical oxidation reactions. The light emission arises from the decay of chemo-excited intermediates or product molecules to the electronic ground state. The most extensively studied chemiluminescent reaction is the oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). Both heterogeneous immunoassays (competitive and sandwich assays) that require a separation of bound and free labelled fractions, and homogeneous (non-separation) immunoassays, which do not require a separation step, have been described. Proteins in biological samples quench chemiluminescence, and so the separation and washing steps in sandwich-type immunoassays provide a means of eliminating these interferences. A continuing trend is the development of chemiluminescent end-points assays for enzyme. The new end-points assays are more sensitive than the colorimetric and fluorometric alternatives, and have been adopted for the detection of alkaline phosphatase, peroxidase, and glucose oxidase labels.

1.2.3.5. Latex agglutination

In this technique, latex particles coated with antibodies are mixed with sample solutions or extracts. If the antigen is present, it will agglutinate the latex particles within minutes, giving a macroscopically visible result. Agglutination occurs when an antibody interacts with a multivalent, particulate antigen (*i.e.,* an insoluble particle), resulting in cross-linking of the antigen particles by the antibody. This eventually leads to agglutination, and will not occur with haptens. Whether or not agglutination occurs depends on the relative concentrations of antigens and antibodies, and critically depends on ionic force. Although the primary use of LA reagents is in the confirmation of colonies, identification and serotyping at the end of a cultural procedure, it is possible to apply them at earlier stages to enrichment cultures as a tool to screen for the likely presence or absence of bacteria. In this way, the time, labour and materials associated with further processing of large numbers of negative cultures are reduced. LA assays are cost effective, easy to perform and provide rapid presumptive results for the identification of pathogenic bacteria. As a confirmation technique, the use of latex particles greatly facilitates the visual observation of the agglutination reaction when compared to traditional slide agglutination using glass slides. As a screening technology, LA is simple and cheap. Although a potential and significant limitation on the use of LA for screening of enrichment broth cultures is the lack of sensitivity (10^7 – 10^8 CFU mL^{-1}).

1.3. BIOSENSORS FOR PATHOGENIC BACTERIA DETECTION

Traditional pathogen detection methods, although sensitive enough, are often too slow to be of any use. Therefore, new methods are needed that exceed their performance. Over the recent years, a lot of effort has gone into the study and development of biosensors of the most diverse nature as an alternative to classical and rapid methods, but their performance still needs improvement. In this section, the definition, general classification (§ 1.3.1) and worldwide overview (§ 1.3.2) of biosensors as analytical devices for pathogen detection are presented.

1.3.1. Definition and classification of biosensors

Biosensors are a relatively new area in the automated food microbiology. They are analytical devices, which convert a biological response into an electrical signal. As shown in Figure 1.7, biosensors consist of two main components: a bioreceptor or biorecognition element, which recognises the target analyte and a transducer, for converting the recognition event into a measurable electrical signal. A bioreceptor can be a tissue, microorganism, organelle, cell, enzyme, antibody, nucleic acid, biomimetic or phage, and the transduction may be optical, electrochemical, thermometric, piezoelectric, magnetic, micromechanical or combinations of one or more of the above techniques. The bioreceptor recognises the target analyte and the corresponding biological responses are then converted into equivalent electrical signals by the transducer. The amplifier in the biosensor responds to the small input signal from the transducer and delivers a large output signal that contains the essential waveform features of an input signal. The amplified signal is then processed by the signal processor where it can later be stored, displayed and analysed. Biosensors have been widely applied to a variety of analytical problems in medicine, the environment, food, process industries, security and defence.

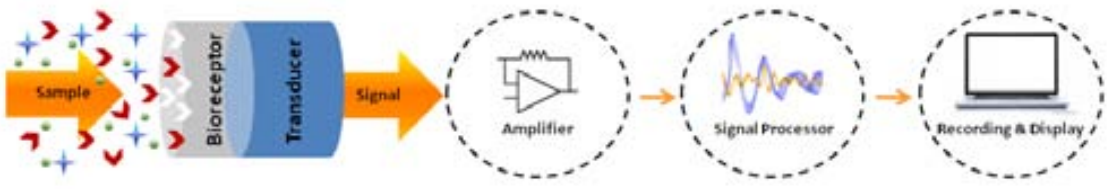

Figure 1.7. Schematic diagram of a biosensor.

Biosensors can be classified according to the type of recognition element used as well as their transducer type, as outlined in Figure 1.8.

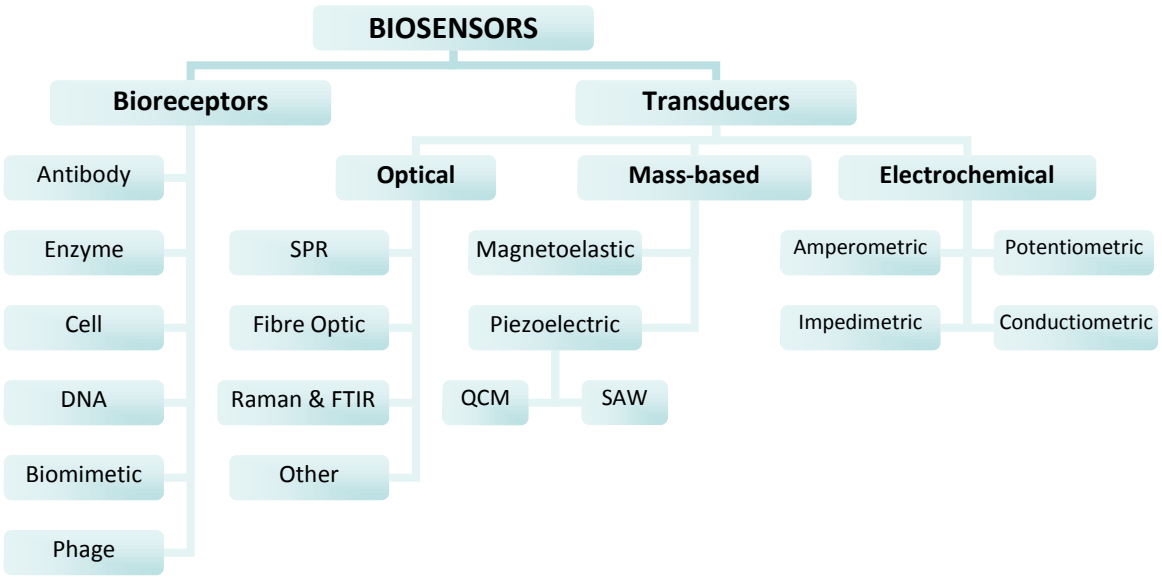

Figure 1.8. Classification of biosensors, adapted from Velusamy, V. *et al.* [43].

Bioreceptors can generally be classified into five different major categories. These categories include antibody/antigen, enzymes, nucleic acids/DNA, cellular structures/cells, biomimetic and bacteriophage (phage). Among them, antibodies and phages are also named affinity-based recognition elements. They specifically bind to individual targets or groups of structurally related targets. Affinity-based sensors are very sensitive, selective and versatile since affinity-based recognition elements can be generated of a wide range of targets. Antibodies have long been the most popular affinity-based recognition elements.

Enzymes, antibodies and nucleic acids are the main classes of bioreceptors which are widely used in biosensor applications. Enzymes were the first recognition elements included in biosensors. Enzymatic biosensors measure the selective inhibition or the catalysis of enzymes by a specific target. They have been extensively used for the detection of contaminants in food and environmental samples. In the field of pathogen detection, using enzymes as bioreceptors not only provides biosensors with a high degree of specificity, but their catalytic activity can amplify the pathogenic bacteria being detected and measured, allowing for sensitive analyses. In most of the cases enzymes are used also to function as labels than the actual bioreceptor. Owing to the improvements in enzyme-labelling methods during the past decades, enzyme-labelled antigens and antibodies have been increasingly used. The use of enzymes as labels has

gained more popularity in immunoassay detection than other labelling methods such as radioisotope and fluorescent tags. Enzymes offer the advantages of high sensitivity, possibility of direct visualisation and are stable for years. But there are some disadvantages found when using enzymes as labels, which include multiple assay steps and the possibility of interference from endogenous enzymes.

Antibodies are common bioreceptors used in biosensors as well. They may be polyclonal, monoclonal or recombinant, depending on their selective properties and the way they are synthesised. In any case, antibodies are generally immobilised on a substrate, which can be the detector surface, its vicinity, or a carrier. The way in which an antigen and an antigen-specific antibody interact is similar to a lock and key fit. An antigen-specific antibody fits its unique antigen in a highly specific manner, so that the three-dimensional structures of antigen and antibody molecules are matching. This unique property of antibodies is the key that makes the immunosensors a powerful analytical tool and their ability to recognise molecular structures allows the development of antibodies that bind specifically to chemicals, biomolecules, or microorganisms. Antibodies can be covalently modified in many ways to suit the purpose of a particular assay. Many immunological methods involve the use of labelled antibodies. Enzymes, biotin, fluorophores and radioactive isotopes are commonly used labels to provide a detection signal in biological assays (Table 1.4, § 1.2.3). Covalently attaching such a label to an antibody combines the unique specificity of the antibody with a sensitive means for detection, thus creating an ideal probe molecule.

In the case of nucleic acid bioreceptors, the identification of a target analyte's nucleic acid is achieved by matching the complementary base pairs that are the genetic components of an organism. Since each organism has unique DNA sequences, any self-replicating microorganism can be easily identified. They are mainly based on the natural affinity of ssDNA to its complementary strand. The classical nucleic acid biosensors measure the hybridisation of ssDNA present in the sample to a complementary strand immobilised onto the sensor chip surface. Biosensors based on nucleic acid as biorecognition element are simple, rapid, and inexpensive and hence it is widely used in pathogen detection. In contrast to enzyme or antibodies bioreceptors, nucleic acid recognition layers can be readily synthesised and regenerated. DNA damage is one of the most important factors to be considered when nucleic acid bioreceptor are used. Hundreds of compounds bind and interact with DNA. Detection of chemicals may cause irreversible damage to DNA by changing the structure of DNA and the base sequence, which in turn disturbs the DNA replication. DNA hybridisation microarrays

have been suggested as a platform for the parallel detection of multiple pathogenic microorganisms in food in a relatively short time. Recent advances in nucleic acid recognition, like the introduction of peptide nucleic acid (PNA) and aptamer technology, have opened up exciting opportunities for DNA biosensors. Due to their high binding affinity, simple synthesis, easy storage, and wide applicability, nucleic acid sensor recognition elements have gained popularity and can substitute the commonly used antibody biosensor recognition elements.

Another frequently used biorecognition element, especially for the monitoring of environmental pollutants, are whole cells such as bacteria, fungi, yeast, animal or plant cells. The ability of cells to recognise and respond to stimuli has made them attractive components for incorporation into biosensors. These whole cell biosensors detect responses of cells after exposure to a sample, which are related to its toxicity. These (toxic) responses can be non-specific, such as DNA damage, heat shock and oxidative stress or specific to a class of environmental pollutants, such as metals, organic compounds and compounds with biological importance (such as nitrate, ammonia and antibiotics). In cell-based biosensors (CBB), a whole cell serves as the molecular recognition element and requires two transduction phases. The cells serve as the primary transducer, converting the detected analyte into a cellular response. A second transducer is required to convert the cellular signal into an electronic signal that can be processed and analysed. The second transduction is dependent on the type of cellular signal to be monitored. There are many reasons why living cells are well-suited for recognition. First, cells provide sensitivity to a wide range of biochemical stimuli since they contain many highly evolved biochemical pathways. Secondly, cells provide a functional assay for biochemical agents. Because CBB make use of direct measurements of physiologic function (and changes induced by toxins), they provide detection capability for previously unknown agents as well. The third major advantage associated with cells as bioreceptors for incorporation into biosensors, is that the detection limits can be very low, because of signal amplification. The above properties distinguish CBB from molecular biosensors that rely on the detection of molecular events such as antibody binding, DNA hybridisation, or enzymatic reactions. Though the CBB were reported by many researchers for the identification and quantification of the pathogenicity induced by various bacteria, they have some major difficulties as well. Because, the cells need a specific environment to function normally and also, as they are subject to biological variability, the cells duplicate which leads to the reproducibility of sensor responses. Moreover, their self-life is very short and preservation is not easy. In addition to the above disadvantages, it is difficult to classify the type of agent

based on functional activity of the cells, because multiple biochemical pathways can lead to the same cellular response. However, due to the development of genetically-engineered cell-based biosensor (GECBB) this has been overcome.

In addition, a receptor that is fabricated and designed to mimic a bioreceptor (antibody, enzyme, cell or nucleic acids) is often termed a biomimetic receptor. Though there are several methods, such as genetically engineered molecules and artificial membrane fabrication, the molecular imprinting technique has emerged as an attractive and highly accepted tool for the development of artificial recognition agents. Molecular imprinting is one of the techniques of producing artificial recognition sites by forming a polymer around a molecule which can be used as a template. Molecular imprinted polymers (MIPs) are synthetic cross-linked materials with artificially generated recognition sites able to specifically bind a target molecule whereas the affinity for closely related compounds should be minor. In that manner, they mimic the biological activity of natural receptors such as antibodies. MIPs can, in principle, be synthesised for any analyte molecule and are capable of binding target molecules with affinities and specificities on a par with biological recognition elements. However, MIPs possess many disadvantages as well. For example, it is hard to completely remove the template from MIPs, the imprinted polymer is insoluble, and although the polymer contains many imprinted cavities, only some are really good and match the template molecule.

From the past decades to date, enzymes, antibodies, nucleic acids and biomimetic materials are used as biomolecular recognition elements and they have both merits and demerits when compared to one another. Recently, bacteriophages are employed as biorecognition elements for the identification of various pathogenic microorganisms. These powerful bacteriophages (phages) are viruses that bind to specific receptors on the bacterial surface in order to inject their genetic material inside the bacteria. These entities are typically of 20–200 nm in size. Phages recognise the bacterial receptors through its tail spike proteins. Since the recognition is highly specific it can be used for the typing of bacteria and hence opened the path for the development of specific pathogen detection technologies (§ 1.4.3).

Biosensors can also be classified depending on the method of signal transduction. Although there are new types of transducers constantly being developed for use in biosensors, the transduction methods such as optical, electrochemical and mass-based are the most popular and common methods. Each of these three main classes contains many different subclasses and they can be further divided into label and label-free (non-labelled) methods. Where, the

labelled methods depend on the detection of a specific label and the label-free detection is based on the direct measurement of a phenomenon occurring during the biochemical reactions on a transducer surface. The sensitivity of a particular biosensor system varies depending on the transducer's properties and the biological recognising elements. An ideal biosensing device for the rapid detection of food contaminants should be fully automated, inexpensive, and able to be used routinely in the field as well as in the laboratory. Optical transducers are particularly attractive since they allow direct label-free and real-time detection. Surface plasmon resonance (SPR) has shown good biosensing potential, with many commercial SPR systems now available. Among these, the Pharmacia BIAcore (a commercial SPR system), which is based on optical transducing, is by far the most frequently reported method for biosensing food residues in food. However, as analytical systems, electrochemically based transduction devices are more robust, easy to use, portable, and inexpensive [44].

Among electrochemical transduction types, amperometric transduction is the most common electrochemical detection method which has been used for pathogen detection and it has superior sensitivity than potentiometic method. In amperometric based detection the sensor potential is set at a value where the analyte produces current. Thus, the applied potential serves as the driving force for the electron transfer reaction, and the current produced is a direct measure of the rate of electron transfer (as explained in detail in § 3.4.1).

Table 1.5 compares the main features of traditional analytical techniques and biosensors. Major advantages of biosensors, over traditional analytical techniques for the detection of food and environmental contaminants, are their cost-effective, fast and portable detection, which makes *in situ* and real time monitoring possible, without extensive sample preparation. In general, biosensors are small-integrated, specific, sensitive, simple and fast instruments. However, most biosensors still have a few drawbacks. Mostly they allow the detection of just one analyte, although recently some multi-analyte sensors have also been developed. So far, biosensors that are used to measure food and environmental contaminants are not all commercially available, in contrast to biosensors for medical applications such as glucose sensors. However, much of the instrumentation developed for medical diagnostics could be adapted for food and environmental applications. Biosensing devices, especially those based on electrochemical transduction can be considered as ideal tools to be used as an "alarm" to rapidly detect the risk of contamination by pathogens in a rapid, inexpensive and sensitive manner [20, 43-46].

Table 1.5. Comparison of the characteristics between traditional analytical techniques and biosensors.

Traditional Analytical Techniques	Biosensors
- Time consuming	+ Rapid, real-time detection
- Expensive	+ Cost-effective
- Laboratory monitoring	+ Portable (in situ monitoring)
- Trained laboratory personnel	+ Simple use
- High-tech equipment	+ Simple apparatus
- Extensive sample preparation	+ Limited sample preparation
- More organic solvent consumption	+ Less organic solvent consumption
+ Multi-analyte detection possible	- Limited multi-analyte detection
+ Commercial availability	- Limited commercial availability
+ Standardised	- Non standardised
+ Sensitive	+ Sensitive
+ Specific	+ Specific
+ Reusable	+ Reusable

1.3.2. Worldwide overview of biosensors

The basic concept of the biosensor was first elucidated by Leyland C. Clark in 1962, in his seminal description of an ‘‘enzyme electrode’’. Building on his earlier invention of the Clark oxygen electrode, he reasoned that electrochemical detection of oxygen or hydrogen peroxide could be used as the basis for broad range of bioanalytical instruments, by the incorporation of appropriate immobilised enzymes. The classic example was immobilised glucose oxidase (GOx), which converted a simple platinum electrode into a powerful analytical instrument for the detection of glucose in human samples from people with diabetes [47, 48]. Two decades later, optical transducers were harnessed in conjunction with antibodies to create real time bioaffinity monitors. These immunosensors laid the foundation for the second major evolutionary line of biosensing instrumentation. Both the enzyme electrode and the bioaffinity sensors originally found utility in laboratory instruments, but advances in manufacturing coupled with mediated electrochemistry, launched the enzyme-based systems along a new and highly successful trajectory of home use, which was to lead to a turnover currently in excess of 13 billion US$ and engaged the full attention of the world’s major diagnostics companies [49]. Hence, electrochemistry has come to dominate distributed diagnostics, while optical techniques have found their niche principally in R&D. To complete the picture concerning transduction strategies, advances in acoustic resonance devices are certainly

worthy of note, but both thermometric and magnetic transduction have failed to have any serious practical impact to date [50].

The biosensors industry is now worth billions of United States dollars, the topic attracts the attention of national initiatives across the world and thousands of papers have been published in the area. A search of the Web of Knowledge database for the topic "biosensor" with publication dates between 2000 and 2012, returns 44,389 hits. Refining this search with the topic "food" returns 2,196 hits. This indicates that only approximately 5 % of the biosensor literature between 2000 and 2012 was food-related. As displayed in Figure 1.9, the growth in the field of biosensors as well as in food-related biosensors has been phenomenal during last years. Moreover, approximately 8,720 results are found in the worldwide patents database (Espacenet) for "biosensors" being 104 of them food-related, that fact shows the implication of industry in this field.

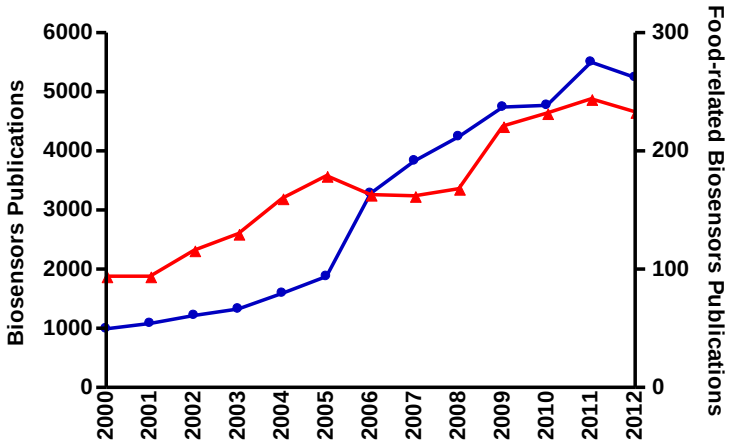

Figure 1.9. Graph of a search on the term "biosensor" (red line, left axis) and after refining with the term "food" (blue line, right axis) during the period 2000 to 2011, using the Web of Knowledge database.

The demand for rapid, real-time, simple, selective and low cost techniques for the detection of food hazards has led to rapid advancements in biosensors. Pathogen detection is of the utmost importance primarily for health and safety reasons. As shown in Figure 1.10, most of the reported biosensors on detection of pathogenic bacteria deal with *Salmonella, Listeria* and *E. coli* [51].

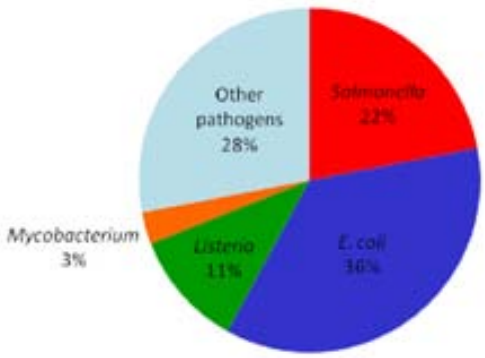

Figure 1.10. Distribution by microorganism of the relative number of works appeared in the literature on biosensors for pathogenic bacteria detection, using the Web of Knowledge database.

Despite the vast numbers of papers published, the field of biosensors may be viewed as comprising essentially two broad categories of instrumentation: (a) sophisticated, high-throughput laboratory machines capable of rapid, accurate and convenient measurement of complex biological interactions and components; and (b) easy-to-use, portable devices for use by non-specialists for decentralised, *in situ* or home analysis. The former are expensive and the latter are mass produced and inexpensive. Biosensors have achieved considerable success both in the commercial and academic arenas and that the need for new, easy-to-use, home and decentralised diagnostics is greater than ever. The enormous success of the glucose sensor serves as a model for future possibilities and should not overshadow the multifarious other applications that this versatile technology can address.

1.4. NEW TRENDS FOR PATHOGENIC BACTERIA DETECTION

The integration of micro- and nanostructured materials within biosensing devices is providing excellent analytical performances in the detection of pathogens. New trends are addressed towards not only the integration of new materials in biosensing but also the design of portable platforms incorporating all the necessary preparation and fluidic processes, rapid diagnostic tests, low-cost instrumentation and point-of-care devices. A brief overview of the aforementioned new trends is presented in next sections.

1.4.1. Integration of nanomaterials in biosensing

Nanoscience is a new conceptual and methodological platform in which chemistry, physics, and biology merge into a single form of knowledge. In particular, bionanotechnology or nanobiotechnology are terms that are often alternated and refer to the intersection of nanotechnology and biology [52]. In recent years, particular attention has been paid to the use of nanomaterials, which can be used in various aspects of the detection system including capture probes, reporting molecules, electrode fabrication, and electrode coatings. These materials offer improved biocompatibility, additional binding sites and higher signal intensities (via enhanced electrical properties) compared with traditional materials in electrochemical sensors.

The need of more flexible, reliable and sensitive targeting of pathogens has promoted research on the potential of nanomaterials and their incorporation into biosensor systems. Thus, the primary objective of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. In this perspective, the use of nanotechnology based bioreceptors could translate into superior sensitivity and may also aid in reducing time of diagnosis. The typically nanostructures used as in-vitro nanodiagnostic tools are highlighted next [53-56]:

- *Liposomes*: are small vesicles consisting of one or more concentric lipid bilayers surrounding aqueous compartments. Particle size and physicochemical characteristics of liposomes can be manipulated for specific applications.

- *Carbon nanotubes (CNTs):* are considered as a sheet of graphite rolled into a tube with bonds at the end of the sheet that close the tube. A single walled nanotube (SWNT) can

have a diameter of 2 nm and a length of 100 µm, making it effectively a one dimensional structure called as nanowire. The multiwalled nanotubes (MWNTs) can be considered as SWNTs kept one inside of another. These nanotubes lend themselves for biofunctionalisation with multiple copies of biomolecules (*e.g.* carbohydrates and antibodies) for an enhanced detection of the antigen in the analyte. In particular, the unique chemical, structural, mechanical and electronic properties of CNTs have been exploited for the development of several microbial biosensors. However, toxic effects of CNTs on biological systems may compromise their use on a larger scale in disposable systems.

- *Dendrimers*: are hyperbranched, tree-like rigid structures and have compartmentalised chemical polymers with unique structural and topological features. They contain far more surface groups capable of being functionalised compared with proteins of similar size. In contrast to other linear, cross-linked, and branched polymers, the three-dimensional structure of dendrimers gives them a variety of unique properties such as low polydispersity and high functionality. Thus a wide range of potential applications using dendrimers as nanoscopic objects have been explored.

- *Gold nanoparticles (AuNPs):* are nano-sized particles that can be prepared with different geometries, a range of shapes are available such as nanospheres, nanoshells, nanorods or nanocages. The unique physical and chemical properties and high surface to volumen ratio enable AuNPs to be an ideal material for adsorption of biomolecules without compromising their biological activities. AuNPs-functionalised antigens or antibodies can serve as optical labels, electrochemical markers, surface plasmonic amplifiers or signal transfer mediator for the quantitative analysis of ligands. AuNPs in combination with other signal generators or other nanoparticles could doubly amplify the signal being a versatile metal nanoparticle for diagnosis.

- *Conducting polymeric nanoparticles:* are an attractive alternative to silicon nanowires and CNTs since the discovery that conjugated polymers can be made to conduct electricity through doping. The conductivity of polymeric NPs is controlled chemically and have shown to possess unique electrical, electronic, magnetic and optical properties, apart from its features such as flexibility, chemical diversity, more rapid electrochemical switching speed and ease of processing makes them promising sensing material for ultra sensitive, trace-level biological and chemical nanosensors. A thin film of conducting

polymer having both high conductivity, oriented microstructure and the high surface area is a suitable component for the fabrication of enzyme electrodes. Moreover, the relative stability is increased due to the efficient bonding of the enzyme on the transducer surface which gives it better reproducibility.

- *Polystyrene nanoparticles:* are used as a carrier for europium (Eu) III ions that serve as fluorescent reporter. Eu (III) has unique photophysical characteristics (such as sharp line-like emission peaks, longer lifetime, large stokes shift, etc.) that are useful for sensitive detection of biological targets.

- *Superparamagnetic nanoparticles/ferrofluids*: are coloidal solutions (25 – 100 nm in radius) consist of an inorganic core of iron oxide (magnetite, maghemite or other insoluble ferrites) coated with polymer to confer stability (such as dextran, polyacrylic acid or silica), with added functional groups (such as amino and carboxylic acids) to make subsequent conjugations easy. As superparamagnetic particles are widely used in the strategies developed in this dissertation, further detailed explanation of them is presented in § 1.4.2.

- *Quantum dots (QDs):* are nanocrystals composed of a core of a semiconductor material generally of atoms from groups II and VI (*i.e.* CdSe, CdS, and CdTe etc.) or III and V (*i.e.* such as InP) of the periodic table, enclosed within a shell of another semiconductor that has a larger spectral band gap (such as ZnS and CdS). The shell prevents the surface quenching of excitons in the emissive core and hence, increase the photostability and quantum yield for emission. They are neither atomic nor bulk semiconductors, since their properties originate from their physical size, which ranges from 10 to 100 Å in radius. They have high sensitivity, broad excitation spectra, stable-bright fluorescence with simple excitation and no need for lasers. These characteristics make them suitable for various biomedical applications such as sensing and detection of biomarkers including antigens and pathogens, immunolabelling of cells and tissues. QDs have become one of the most promising and interesting materials for diagnostic applications of bioimaging, labelling, and sensing, due to their exceptional optical properties.

- *Bacteriophage particles:* are viruses that infect and replicate within bacteria. Though many methods are available, the low cost and ready production of large numbers of bacteriophage, along with their specificity for the target bacterial species make them ideal

for detecting bacteria. Extended explanation of advantages and disadvantages of using bacteriophages in biosensing are presented in § 1.4.3.

Nanostructured materials represent an exciting area in analytical chemistry due to their improved characteristics compared to their non-nanostructured counterpart. Among the different nanomaterials for biosensing aforementioned, magnetic particles (§ 1.4.2) and bacteriophages (§ 1.4.3) are of special interest in this work, due to they were integrated in different procedures and biological reactions.

1.4.2. Integration of magnetic particles in biosensing

Magnetic particles (MPs) have been commercially available for many years (*e.g.* BioMag® [57], Dynabeads® [58], Adembeads® [59] and SiMAG® [60]) and are widely used in laboratories to extract desired biological components, such as cells, organelles or DNA, from a fluid. Magnetic particles can have any size from a few nanometres to a few micrometres and can contain magnetic materials such as iron, nickel, cobalt, neodymium-iron-boron, samarium-cobalt or magnetite. Often the particles are coated with polymers to prevent the formation of aggregates and to facilitate biological functionalisation. Nano-sized particles (5 – 50 nm) are usually composed of a single magnetic core with a polymer shell around it. Larger particles (30 nm – 10 µm) can be composed of multiple magnetic cores inside a polymer matrix. In the last decade extensive research has been done on the use of magnetic particles for a novel generation of biosensors. Magnetic particles can be used for efficient transport, faster assay kinetics, improved binding specificity and as labels for detection [61].

In particular, superparamagnetic particles are highly attractive for use in biosensors due to their capability to magnetise under an applied magnetic field. This capacity has led to the use of biorecognition agent functionalised magnetic particles for the separation and pre-concentration of whole organisms from complex media [62]. As shown in Figure 1.11, they consist of an inorganic core of iron oxide (magnetite Fe_3O_4, maghemite ɣ-Fe_2O_3 or other insoluble ferrites) coated with polymer to confer stability (such as polystyrene, dextran, polyacrylic acid or silica), with added functional groups (such as amino and carboxylic acids) to make subsequent conjugations easy. Hence, iron oxide particles can carry diverse ligands, such as peptides, small molecules, proteins, antibodies and nucleic acids.

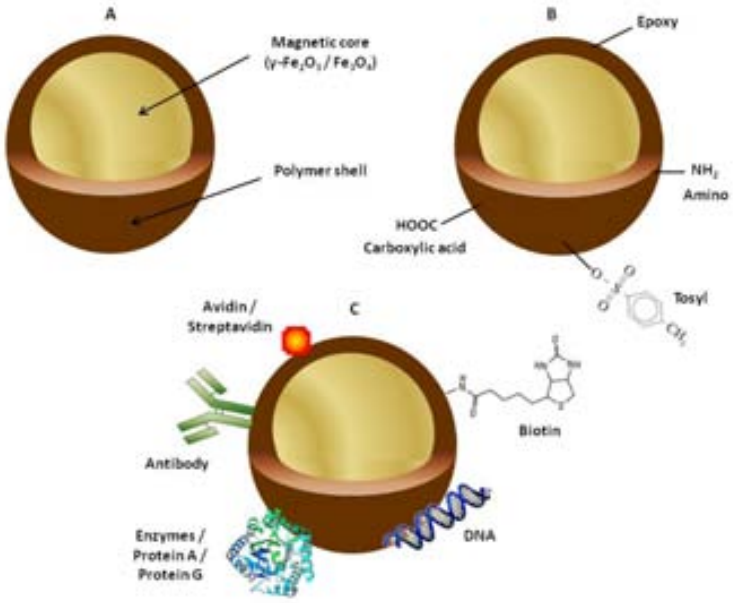

Figure 1.11. Schematic representation of magnetic particles (A), activated with functional groups (B) and conjugated to biological molecules (C) [63].

These particles are superparamagnetic, meaning that they have neither magnetic permanence nor hysteresis. In other words, they respond to a magnetic field, but demagnetise completely when the field is removed. Thus, the particles can be separated easily from the liquid phase with a small magnet, but can be redispersed immediately after the magnet is removed [64]. They confer a number of benefits, including ease of separation and suitability for automation. When coated with recognition molecules, magnetic spheres are ideal for the efficient capture and separation of target. Unwanted sample constituents may be washed away following a simple magnetic separation step. In particular, antibody-coated paramagnetic particles are used for the immunomagnetic separation (IMS) of nucleic acids, proteins, viruses, bacteria and cells and it forms the basis of several tests. Immunomagnetic separation has proved to be very efficient method for separating target organisms from food materials and background flora. A number of procedures may be used for subsequent final detection, such as conventional culturing, microscopy, impedance technology, ELISA, latex agglutination or DNA hybridisation, partly involving amplification techniques. In addition to the short separation and concentration time, IMS technology also overcomes the problem associated with unwanted inhibition due to selective media components. Since IMS can be used in conjunction with many final detection technologies it is expected that several automated analytical procedures will make use of this potent technique in the near future [28, 65, 66].

In recent years, the magnetic properties of MPs have also been used as labels in biosensing. This approach for magnetic detection is inspired by the binding effect of the substrates on the superparamagnetic particles that can align under an applied magnetic field. Therefore, uniformly aligned particles are detected by magnetic detectors while others that are randomly oriented are ignored. As a result, this technique does not require a washing step before imaging, because other non-specific moieties inside the same such as buffer or sample will not bind to these particles and thus will not affect the imaging. Bound to a suitable antibody, they are used to label specific molecules, cell populations and structures or microorganisms.

Besides all these applications, MPs are particularly suitable for integration in microfluidic devices. Due to their composition (iron oxide content of 70 %) they ensure an ionic and hydrophilic environment favourable for preserving the biological activity of biological molecules. The transport of magnetic particles in microfluidic systems or biosensors is investigated in several ways, such as using mechanically moving permanent magnets, sets of electromagnets with specific actuation schemes, or micro-patterned and integrated current wires. To optimally use magnetic particles to improve assay kinetics, it should be noted that the size of the particles is important. Small particles have a high diffusivity (rotational, translational), which increases the rate of finding reactive sites. However, large particles are generally more magnetic and thus easier to manipulate or detect. For different types of biosensors, particle diameters between 50 nm and 3 µm have been used. A technique to improve binding specificity using magnetic particles was recently developed showing that in a surface-immunoassay involving magnetic particles, a magnetic force can be used to remove magnetic particles that are settled onto the surface by gravity or by weak non-specific interactions. Particles that are specifically bound to the surface via a target molecule and antibodies remain at the surface, because the specific binding force is higher than the applied magnetic force [61, 67].

1.4.3. The use of bacteriophages in biosensing

Bacteriophages (or phages) were first described in 1915 by the British microbiologist Frederick Twort and independently two year later by the Canadian microbiologist Felix d'Herelle. Phages are natural host-specific, self-reproducing, and self-assembling nanostructured particles, with both structure and function encrypted in the genomic DNA. They bind to specific receptors on the bacterial surface in order to inject the genetic material

inside the bacteria, using the host's own replication machinery for multiplication. The replicated virions are eventually released, killing the bacteria and allowing the infection of other host cells. The use of phages to treat bacterial infections, and research using phage therapy to overcome the problem of increasing levels of antibiotic resistance has become widely published. Besides the promising features of phage therapy, much work has been carried out to develop phage-based methods for rapid detection of pathogens in foods.

Bacteriophages have the ability to display peptides or proteins on their surface, this technology is called phage display. With phage display it is possible to screen for peptides or proteins with affinity for all kinds of targets, ranging from small molecules to proteins and even cells. Therefore, phage libraries consisting of a high number of different phages (10^8 – 10^{10}) that each displays a different peptide or protein on their surface are used. Different types of phage libraries exist, which display different types of peptides or proteins: peptide, cellular proteins (from cDNA libraries) or antibody fragments, like single chains variable fragments (scFv) and antigen binding fragment (Fab). Among the huge number of phages in these phage libraries, the ones with high affinity and specificity for a target can be isolated in an affinity selection procedure and can be used as a target specific biorecognition element of a biosensor. Besides the target specific phages, the peptides or proteins that are identified by phage display as good binders can also be directly used as a biorecognition element. The peptides or proteins are then chemically synthesised or produced by recombinant expression in bacterial cells.

When phages are used to detect bacteria, it is not always necessary to use phages that display specific binding peptides or proteins because the phage itself can specifically recognise its particular bacterial host strain. The phages identify their host by specific receptor molecules on the outside of the bacterial cell. Once the phages recognise their specific receptors, they bind to the bacterial cells and infect them. The binding between phages and bacterial cells can be so specific, that only certain strains of a single species can be infected. Thus, bacteriophage-based diagnostic is attracting much interest due to the high specificity of phages, which makes them ideal agents not only for the detection of bacteria, but also for the detection of almost all kinds of targets, ranging from small molecules to proteins and even cells, by using the phage display technique.

The phage amplification technique is another candidate method for food-borne pathogen detection. This is based on the principle that lytic phages produce a large number of progeny upon rupture of the host cell, being these progeny phages an indirect signal of host cell

infection, and hence the presence of a target bacterium. Amplified bacteriophage can be detected using a variety of techniques such as live/dead exogenous fluorochromic cell staining, matrix-associated laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF) or competitive enzyme-linked immunosorbent assay (cELISA). The advantage of detection with bacteriophage amplification technique is that they usually detect living bacteria thereby avoiding the false positives that often arise from the use of approaches such as PCR.

In general, there are three different advantages of the use of phages as biorecognition elements in biosensors compared with antibodies. The first important advantage of phages is their sensitivity and specificity. Phages can very specifically recognise a target bacterial cell and they can display target specific peptides or proteins on their surface. Phages can be selected out of libraries with a very high diversity, even higher than the diversity of antibody repertoires screened with the hybridoma technique. By using strong selection conditions and counter selections with related targets, a specific selection can be performed, leading to sensitive and specific binding phages. Moreover, the sensitivity and specificity of the selected phages can be increased after the selection procedure by genetic modification. A second important advantage is that phages are fast, cheap and animal-friendly producible. They are cost-efficiently mass produced by infecting bacteria, which takes only a few hours. The animal-free production also allows selection of phages against toxic or poorly immunogenic targets as the process does not rely on the induction of an animal immune response, contrary to antibody generation. The third advantage which makes phages suitable as biorecognition elements for biosensors is their stability. Phages are stable in a variety of harsh conditions including pH and temperature. Phages can even be used in the presence of nucleases or proteolytic enzymes, without degradation. The high stability of phages in a variety of environmental conditions makes them suitable for *in situ* monitoring of food and environmental contaminants. These naturally occurring nanoparticles have other interesting properties in comparison with synthetic nanoparticles: all bacteriophages are nearly identical, being monodisperse in shape and size, a fact difficult to achieve by laboratory synthesis. Moreover, these nanoparticles are self-synthesised in their specific host, by producing a large amount of viral coat proteins with a large surface for further chemical modification. Thus, bacteriophages can be used not only as a biorecognition element but also as a label for biosensing applications.

Although phage-based technologies offer promise for pathogen concentration and detection, further investigation is needed before the more widespread use of these technologies can ensue. Critical factors for consideration include the identification of suitable

phage, determination of phage orientation, and the choice of appropriate solid support. Another important consideration is minimising undesired consequences of bacteriophage interactions with target cells, such as target cell lysis and degradation of DNA [20, 46, 68-69].

1.4.4. Towards rapid diagnostic tests (RDT)

In recent years, the evolution of pathogen detection and diagnosis techniques has originated a new series of requirements that leads directly to the search of the simplification of several standard medical processes and treatments. In this sense, the idea of integrating current diagnosis techniques into a simple and portable device has been reinforced, promoting the development of the lab-on-a-chip (LOC) concept. Efforts have been focused on the detection and quantification of specific infectious biological specimens in food samples, a task that requires reliability and short processing periods in order to obtain high efficiency [70].

Research and development into biosensors is focused on designs compatible with technologies, such as screen-printing techniques, which allow for the industrial production of low-cost devices. The same technology that has given us microelectronic devices can be used for the microfabrication of microsensor devices and analytical microinstruments, such as labs-on-a-chip, paving the way for a miniaturisation of the analytical process. This means that biosensors could become low-cost instruments of mass use, for personal use or, sometimes, simply disposable instruments [52].

Owing to recent advances in microfabrication techniques, it is also now possible to prepare microelectrodes of various sizes and geometries as well as to construct parallel arrays of microsensors on the same chip. Such systems are powerful tools able to fulfil most of the environmental monitoring requirements such as rapidity of response, sensitivity and parallel analysis of a large number of parameters and samples. Moreover, the small size is useful for the design of portable biosensors intended for in-field applications. Microelectrodes, fabricated by lithographic or screen-printing technologies, offer many advantages compared with macroelectrodes of conventional size, including lower consumption of biological material as well as higher sensitivity and decreased analysis time owing to the reduction of macrokinetic transport effects. Compared with thin-film technologies, the screen-printing (thick-film) technique is simpler, less expensive, and suitable for mass production. Several types of screen-printed electrodes (SPEs), functionalised or not, are now commercially

available [71] and many laboratories have their own facilities for in-house production. The low cost of SPEs makes them disposable. Issues arising from electrode fouling, contamination among samples and recalibration can thus be avoided. Moreover, advances in nanotechnology, microelectroelectronics and microfluidics have permitted the miniaturisation of electrochemical biosensors and the fabrication of high-density arrays, particularly interesting for the real-time parallel monitoring of multiple chemicals or biological parameters, or the monitoring of a single parameter in several samples. The miniaturisation of sensing platforms can provide a number of benefits, including a reduction of the amounts of both bioreceptor and sample, increased sensitivity and high throughput analysis. Combination of several types of detection on the same platform is also possible [55]. Ultimately, the end goal of biosensor development is to construct a total analysis system for rapid biosensing, which incorporates sample pre-treatment, sample delivery, and detection.

The current trend is moving towards low-cost and easy to use point-of-care diagnostics [72]. A point-of-care biosensor is a small, preferably hand-held, device that can detect the presence of a certain molecule in a body fluid (*e.g.,* blood glucose sensor and pregnancy test urine). To make a successful point-of-care biosensor, the device has to detect the analyte fast, in seconds to minutes, and in a small amount of body fluid, such as a drop of blood from a finger prick. It has to be sensitive and specific, as many other molecules will also be present in the body fluid, and it should give a reliable diagnosis with hardly any false negatives or false positives. It should also be easy to operate and be robust against external circumstances like temperature [61]. In this regards, it is worth enhancing the non-profit enterprise Diagnostics for All (DFA) [73] and the organisation Program for Appropriate Technology in Health (PATH) in collaboration with the centre for Point-of-Care Diagnostics for Global Health (GHDx Center) [74] that work to improve the availability, accessibility, and affordability of essential point-of-care diagnostic tests for use in low-resource settings around the world. Their goal is to develop simple and inexpensive devices requiring minimal training to be used, practically no sample preparation, and no electricity or additional equipment to process a sample. These devices, based on lateral-flow technique, are made primarily of paper which is significantly less expensive than other materials typically used in diagnostic devices and is compatible with a variety of existing diagnostic tests. The main goal is the simplicity of these devices, they are easy-to-use and easy-to-read, minimal training is required to use them and produce minimal invasion, no syringes are involved and neither clean water nor sample preparation is needed. Results are displayed in an easily understood manner, as through a colorimetric scale.

In general, the strengths of rapid diagnostics tests (RDTs) mostly based on lateral flow tests, agglutination assays, flow-through, solid-phase (dipstick assays), microfluidic chips, immunosensors and labs-on-a-chip include their ease of use, minimum training requirements, rapid results, and limited need for instrumentation and infrastructure. On the other hand, the general weaknesses of RDTs include their subjective interpretation of readout, low throughput, often limited sensitivity relative to laboratory or reference tests, and need for quality control mechanisms [75]. The common goal of all the tests aforementioned is the development of assured diagnostics, being ASSURED an acronym that describes the most valuable diagnostic test characteristics [76]:

- Affordable (by those at risk of infection)
- Sensitive (few false negatives)
- Specific (few false positive)
- User-friendly (simple, can be performed by people with minimal training)
- Rapid (quickly generates results) and Robust (does not require refrigerated storage)
- Equipment-free (easily collected non-invasive specimens, *e.g.* urine, saliva)
- Delivered (delivered to end-users, portable, acceptable to population)

1.5. STATE OF THE ART

This PhD dissertation is focused on the use of biosensors to detect pathogenic bacteria. In recent years significant improvements in the methodology for microbiological analysis have been made. However, the development of new methods with the advantages of rapid response, sensitivity and ease of multiplexing is still a challenge for food hygiene inspection [65].

Most of the currently developed biosensors for pathogenic bacteria detection are based on the specific antigen-antibody binding reactions, where the antibody is immobilised on the sensor platform to capture the bacteria that are of interest. Then the detection is measured through electrochemical, optical, or piezoelectric signals. Moreover, the genetic biorecognition is also widely used in biosensing as well as the combination of both, immunological and genetic biorecognition, mostly based on immunomagnetic separation (IMS). Recently, approaches based on IMS coupled with culture, PCR or immunoassay has been developed [77]. In next sections, a general overview of the rapid approaches for bacteria detection focusing on *Mycobacterium bovis* (§ 1.5.1)*, Salmonella* spp. (§ 1.5.2) and multiplex bacterial detection (§ 1.5.3) is presented.

1.5.1. Rapid approaches for *Mycobacterium bovis* detection

Currently, the diagnosis of bovine tuberculosis (BTB) caused by *M. bovis* is based on the caudal fold test of the Tuberculin Skin Test (TST). This test is based on the inoculation of *M. bovis* antigens called purified protein derivative (PPD). Although the tuberculin skin test is highly sensitive and specific, it requires 48 – 72 h to process, and veterinarians must be specially trained to perform the assay [78]. The culture of milk samples is another approach to the detection of *M. bovis*, but while it provides acceptable sensitivity and specificity it is labour-intensive, with up to 6 weeks required to detect positive specimens. Moreover, the low sensitivity of cultured milk has been reported, which can be attributed to the drastic pre-culture milk decontamination procedures and to the presence of mammary macrophages able to kill *M. bovis* bacilli. More recent approaches for the rapid detection of *M. bovis* include chromatographic and molecular methods, such as PCR, which have advantages of speed, sensitivity, and specificity; however, they require adequately trained personnel and have high associated costs (reagents and equipment) [79]. Biosensors, by contrast, offer an exciting

alternative, allowing the rapid and multiple analyses essential for the detection of bacteria in food.

Table 1.6 shows a brief compilation of the main recent rapid approaches for *M. bovis* detection. All of them are PCR-based methods and electrophoresis gel dependent with limits of detection ranging from 3 to 50 CFU mL^{-1}. Other works combined PCR amplification with immunomagnetic separation (IMS) obtaining similar LODs in reduced time. So far, biosensors were developed for Mycobacterium tuberculosis complex detection [80] and in particular for *Mycobacterium tuberculosis* [81], but further work has to be done for *M. bovis* biosensing.

Table 1.6. Main features of rapid approaches for the detection of *Mycobacterium bovis.*

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
IMS and PCR	Agarose gel electrophoresis	PBS and bovine lymph node matrices (inoculated)	Not-performed	2 h	3 CFU mL^{-1}	82
IMS and PCR	Agarose gel electrophoresis	PBS and organs (inoculated)	Not-performed	24 h	10 CFU mL^{-1}	83
PCR	Agarose gel electrophoresis	Raw milk samples (inoculated)	Not-performed	17 h	9 CFU 40 mL^{-1}	84
PCR	Agarose gel electrophoresis	Nasal swabs and milk samples (real and inoculated)	Not-performed	18 h	9 – 900 CFU	9
IMS and PCR	Agarose gel electrophoresis	Milk samples (inoculated)	Not-performed	2 h 30 min	20 – 50 CFU mL^{-1}	85

1.5.2. Rapid approaches for *Salmonella* spp. detection

Several rapid methods have been developed for testing foods for the presence of *Salmonella* spp. In this section, approaches based on immunological, genetic and bacteriophage-based biorecognition as well as commercial kits available are discussed. Tables 1.7 – 1.12 show an extended compilation of the main bibliography reported since 2009 onward and some outstanding previous works. Earlier work was reported in detail by Lermo, A. [86] and in several reviews [20, 87, 88].

Table 1.7 summarises the rapid approaches based on immunological biorecognition. It must be highlighted the improvement that involves the use of immunomagnetic separation (IMS). This step, as explained in detail in § 1.4.2, consist in the use of magnetic particles to capture target bacteria -through an immunological reaction- from contaminating microflora and interfering food components, and to concentrate them into smaller volumes for further testing. Approaches based on IMS coupled with quantum dots (QDs) or gold nanoparticles (AuNPs) labelling have been developed obtaining excellent limits of detection (10^2 CFU mL^{-1}) in less than 2 hours of assay. Similar LODs were obtained for electrochemical approaches using screen-printing technique. The most used optical detection techniques were fluorescence or absorbance, and regarding electrochemical techniques amperometry or impedance. Concerning colorimetric assays, based on ELISA format, the pathogen detection sensitivity was improved with the incorporation of nanomaterials such as single-walled carbon nanotubes (SWCNTs) or QDs.

The combination of ELISA with IMS step with aiming to pre-concentrate cells from mixed cultures has been previously used in some works, nevertheless, the detection sensitivity was considered close to that of a conventional ELISA (10^5 – 10^6 CFU mL^{-1}). In Table 1.8 a compilation of the main enzyme-linked immunomagnetic assays (IMS-ELISA) is shown. The integration of nanomaterials such as QDs or AuNPs as well as the coupling of the magneto-immunoassay with electrochemical detection in what is named ELIME (Enzyme-Linked Immunomagnetic Electrochemical method) improved the typical sensitivity up to 1 – 10^2 CFU mL^{-1} in a considerably reduced time.

Table 1.7. Main features of rapid approaches based on immunological biorecognition for the detection of *Salmonella* spp.

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
ELISA using modified polyacrylo-nitrile fibers (PAN)	Absorbance	PBS, milk and juice matrix (inoculated)	Not-performed	2 h 30 min	10 CFU mL^{-1}	89
IMS with AuNP labelling	Differential Pulse Voltammetry (DPV)	PBS-Tween (inoculated)	Not-performed	1 h 30 min	143 CFU mL^{-1}	90
Solid-phase sandwich ELISA	Absorbance	PBS (inoculated)	Not-performed	21 h	2×10^3 CFU mL^{-1}	91
Sandwich immunoassay	Ion-Sensitive Field-Effect Transistor (ISFET)	NaCl solution (inoculated)	Not-performed	30 min	2 – 3 CFU mL^{-1}	92
Screen-printing technique immunosensing	Electrochemical Impedance Spectroscopy (EIS)	PBS (inoculated)	Not-performed	6 min	5×10^2 CFU mL^{-1}	93
IMS with Magnetic Nanobeads (MNBs) and QDs labelling	Fluorescence	PBS, ground beef, chicken carcasses, fresh-cut broccoli and lettuce (inoculated)	Not-performed	2 h	20 – 50 CFU mL^{-1}	94
Direct and sandwich ELISA with SWCNTs labelling platform	Absorbance	PBS, UHT milk (inoculated)	Not-performed	4 h	$10^3 – 10^4$ CFU mL^{-1}	95
IMS screen-printing technique and enzymatic detection	EIS and amperometry	Peptone water (inoculated)	Not-performed	1 h	$10^2 – 10^5$ CFU mL^{-1}	96

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
Automated IMS and Enzyme Immunoassay (EIA)	Absorbance	Poultry environmental samples	18-24 h	48 h	$10^4 – 10^6$ CFU mL^{-1}	97
Immunosensing by using macroporous silicon trapping array	EIS	PBS (inoculated)	Not-performed	30 min	10^3 CFU mL^{-1}	98
IMS and label free detection	IR fingerprinting	2 % milk and spinach extract (inoculated)	Not-performed	30 min	10^5 CFU mL^{-1}	99
Fibre-optic immunosensor	Evanescent wave, Time-Resolved Fluorescence (TRF)	Egg and chicken breast (inoculated)	2-6 h	<8 h	10^4 CFU mL^{-1}	100
Immunosensing on screen-printed gold electrodes	Amperometry	PBS and chicken breast (inoculated)	18-24 h	27 h	21 CFU mL^{-1}	23
Multichannel electrochemical immunosensor (MEI) using screen-printed sensor array	Intermittent Pulse Amperometry (IPA)	NaCl solution (inoculated)	Not-performed	3 h	2×10^6 CFU mL^{-1}	101
Array-Based Immunosensor	Fluorescence	PBS, poultry, chicken excretal samples (inoculated)	Not-performed	1 h	$10^3 – 10^6$ CFU mL^{-1}	102
Electrochemical ELISA	Electrochemical Flow Injection Analysis (FIA) and Intermittent Pulse Amperometry (IPA)	PBS, pork, chicken and beef (inoculated)	5 h	8 h	1 – 10 CFU $25g^{-1}$	103

Table 1.8. Main features of rapid approaches based on enzyme-linked immunomagnetic assay for the detection of *Salmonella* spp.

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
IMS with immuno-AuNP network	Absorbance	PBS, fat milk, ground beef, pineapple juice (inoculated)	2 h	4 h 30 min	3 CFU mL^{-1}	104
IMS with immuno-QDs	Fluorescence	PBS (inoculated)	Not-performed	30 min	500 CFU mL^{-1}	105
IMS-ELIME	IPA	Pork, chicken, beef, and turkey (real and inoculated)	6 h	8 h	1 – 10 CFU 2 g^{-1}	106
IMS-ELISA	Absorbance	Skimmed milk powder in buffered peptone water (BPW) (inoculated)	18 – 24 h	24 h	10^5 – 10^6 CFU mL^{-1}	107
IMS-ELIMC / IMS-ELIME	Absorbance / Osteryoung square wave voltammetry (OSWV)	PBS (inoculated)	Not-performed	80 min	2 x 10^4 CFU mL^{-1} (ELIMC) / 8 x 10^3 CFU mL^{-1} (ELIME)	108
IMS-ELISA	Absorbance	Eggs	18 – 24 h	24 h	10^5 – 10^6 CFU mL^{-1}	109
IMS-ELISA	Absorbance	Eggs and chicken meat	24 h	26 h	10^5 CFU mL^{-1}	110

Recent rapid approaches based on genetic biorecognition are outlined in Table 1.9. The majority of the methods developed were based on nucleic acid amplification techniques such as PCR coupled with hybridisation techniques. Except some works based on Surface Plasmon Resonance detection technique, the vast majority of methods were based on electrochemical detection and in particular on Differential Pulse Voltammetry (DPV). Although most methods were tested only with synthetic oligonucleotides, the limits of detection determined for inoculated bacteria were ranged from 10 to 10^4 CFU mL^{-1}. Moreover, some rapid approaches were based on both immunological and genetic biorecognition. As shown in Table 1.10, IMS in conjunction with PCR was evaluated for detection of *Salmonella* spp. The limit of detection demanded by legislation (1 – 10 CFU 25 g^{-1}) was reached in all cases by using this combination coupled with agarose gel electrophoresis detection technique.

Table 1.9. Main features of rapid approaches based on genetic biorecognition for the detection of *Salmonella*

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
Gene-based electrochemical DNA biosensor based on thin-film gold electrodes	DPV	PBS (Synthetic oligonucleotides)	Not-performed	1 h	0.2 µmol L^{-1}	[111]
PCR and gene-based electrochemical DNA biosensor	DPV	Luria-Bertani broth (inoculated)	Not-performed	3.5 h	0.5 pmol L^{-1}/ 10 CFU mL^{-1}	[112]
AuNP-DNA biosensor using Screen-Printed Carbon Electrodes (SPCEs)	DPV	Luria-Bertani broth (inoculated), 2 % milk, 100 % Orange juice	Not-performed	6 h	100 ng mL^{-1} / 10^4 CFU mL^{-1}	[113]

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
PCR and DNA biosensor label-free	SPR	Luria-Bertani broth (inoculated)	Not-performed	4.5 h	0.5 nmol L^{-1}/ 10^2 CFU mL^{-1}	114
DNA biosensor based on polystyrene-modified glassy carbon electrodes	Osteryoung Square Wave Voltammetry (OSWV)	PBS (Synthetic oligonucleotides)	Not-performed	12 h	0.55µmol L^{-1}	115
DNA biosensor based on modified single walled carbon nanotube electrode (SWNTs)	EIS	Phosphate buffer solution (Synthetic oligonucleotides)	Not-performed	20 min	1 nmol L^{-1}	116
PCR and optical thin-film DNA biosensor	Human eye	Luria-Bertani broth and pork (inoculated)	18-24 h	21.5 /28.5 h	8.5 x 10^{1} CFU mL^{-1} (LB) / 0.4 CFU g^{-1} (pork)	117
PCR and hybridisation in screen-printed gold electrodes	DPV	PBS (Synthetic oligonucleotides)	Not-performed	3 h	5 nmol L^{-1}	118
PCR and hybridisation in screen-printed electrodes	DPV	PBS (Synthetic oligonucleotides)	Not-performed	3 h	0.3 nmol L^{-1}	119

Table 1.10. Main features of rapid approaches based on both immunological and genetic biorecognition for the detection of *Salmonella*

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
IMS and PCR	Agarose gel electrophoresis	Brain Heart Infusion broth, minced beef, pork and chicken meats (inoculated)	16-18 h and 6h post-enrichment after IMS	26 h	1 – 10 CFU / 25 g	[120]
IMS and PCR	Agarose gel electrophoresis	Chicken meats (inoculated)	12 h	16 h	1 – 10 CFU / 25 g	[121]

On the other hand, rapid approaches based on bacteriophage biorecognition are summarised in Table 1.11. The reported methods for bacteria detection using bacteriophages include (i) expression of bacteriophage-encoded bioluminescent genes which produce visible products within the specific target cells (lux-bacteriophage strategy), (ii) fluorescence-labelled phage, which can be combined with immunomagnetic separation (labelled phage strategy), (iii) detection of bacteria by the intracellular replication of specific bacteriophages (named "phage amplification" strategy), and the (iv) detection of the phage-mediated bacterial lysis and release of host enzymes (*e.g.,* adenylate kinase) or ATP (termed "lysin-release ATP bioluminescence strategy") [122].

Bacteriophages, as explained in detail in § 1.4.3, recognise the bacterial receptors through their tail spike proteins. This biorecognition is highly specific and has been employed for the typing of bacteria. This level of specificity and selectivity opens avenues for the development of specific pathogen detection technologies and for the creation of biosensing platforms. Biosensing approaches based on quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) as transduction platform were reported [123]. These early reports relied on physical adsorption of the bacteriophage on the sensor surface. Other works reported the electrostatically-facilitated physisorption on silica particles [124]. Single-point, oriented, covalent attachment of the bacteriophages on different surfaces and transducers was also reported in order to yield better coverage and to improve the performance of these devices. Streptavidin-mediated attachment of bacteriophages that were genetically modified to directly express

biotin on their capsid was reported [125]. Covalent immobilisation of bacteriophages on gold surface [126], screen-printed carbon electrode [127], and glass substrates [128] for biosensor application was also reported. Other approaches are addressed towards the chemical modification of the viral capsid, such as biotinylation for further immobilisation on biosensor surfaces [129]. In particular for *Salmonella* detection the LODs obtained with rapid approaches based on bacteriophage biorecognition were ranged from 10^2 to 10^5 CFU mL^{-1}, and the time assay was less than 16 hours (Table 1.11).

Table 1.11. Main features of rapid approaches based on bacteriophage biorecognition for the detection of *Salmonella* spp.

Phage	Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
E2	Phage-coated sensor	Magnetoelastic (ME) biosensor	Eggshells (inoculated)	Not-performed	30 min	160 CFU cm^{-2}	[130]
E2	Phage-coated sensor	ME biosensor	Fresh tomato (inoculated)	Not-performed	30 min	5×10^2 CFU mL^{-1}	[131]
P22	Phage-based biosorbent (genetically engineered tailspike proteins (TSPs) on gold surface)	SPR	Luria-Bertani broth (inoculated)	Not-performed	30 min	10^3 CFU mL^{-1}	[132]
E2	Phage-coated sensor	ME biosensor	Water (inoculated)	Not-performed	1 h	5×10^3 CFU mL^{-1}	[133]
P22	Phage-based biosorbent (monolayer) and ELISA	Absorbance	Luria-Bertani broth (inoculated)	Not-performed	2.5 h	-	[128]

Phage	Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
P22	Recombinant P22::luxAB phage	Bioluminiscence	Luria-Bertani broth and poultry and feed samples (inoculated)	12 – 14 h	16 h	1.65 x 10^3 CFU mL^{-1}	[134]
E2	Phage-based biosorbent (physical adsorption)	ME biosensor	Skimmed milk and water (inoculated)	Not-performed	20 min	5 x 10^3 CFU mL^{-1}	[135]
E2	Piezoelectric platform (physical adsorption)	Quartz Cristal Microbalance (QCM)	PBS (inoculated)	Not-performed	-	10^2 CFU mL^{-1}	[136]
SJ2	Phage-mediated cell lysis	Bioluminiscence	Trypticase Soy Broth (inoculated)	Not-performed	2 h	10^3 CFU mL^{-1}	[137]
SJ2	IMS and phage amplification assay	Fluorescence or optical density	Luria-Bertani broth (inoculated)	Not-performed	4-5 h	10^4 CFU mL^{-1}	[138]
SJ2	Phage-based biosorbent (streptavidin magnetic beads and biotinylated phage)	Bioluminiscence	Luria-Bertani broth (inoculated)	Not-performed	40 min	4 x 10^3 CFU mL^{-1}	[139]
Sapp-hire	Phage-based biosorbent (passive immobilisation on polystirene strips)	PCR / Agarose gel electrophoresis	Luria-Bertani broth (inoculated)	Not-performed	2 h	10^5 CFU mL^{-1}	[140]

Concerning commercial available kits, PCR and ELISA systems are the most frequently commercialised. Immunodiffusion, hybridisation and dip-stick technology are also outstanding (Table 1.12). The majority of the methods are based on optical detection (fluorescence or absorbance). To the best of our knowledge the only electrochemical commercial kit is the QFast™ *Salmonella* from iMICROQ, Tarragona, Spain. Methods based on nucleic acid hybridisation are on the market for several organisms including *Salmonella* spp. However, the detection level of nucleic acid hybridisation methods is about 10^5 – 10^6 CFU mL^{-1}, and enrichment steps are therefore needed for food samples. For food testing in general there are other available kits based on PCR. These include Bax2 from Qualicon, Wilmington, DE, USA; TaqMan2 from Perkin Elmer Applied Biosystems, Foster City, CA, USA, and Probelia2 from Sano® Diagnostics Pasteur, Marnes La Coquette, France [19].

Currently, there are numerous ELISA-based assay systems for the detection of *Salmonella* spp. Some of these tests have the advantage of being able to process numerous samples at once in 96 well microtitre plates, and some such as the Tecra™ *Salmonella* Visual Immunoassay (3M), provide a visual indication of detection without the use of colorimetric equipment. In addition, ELISA systems have been automated to facilitate routine laboratory testing such as the EIAFoss (Foss Electronics) and the VitekImmuno Diagnostic Assay System (VIDAS) (BioMerieux). Nevertheless, ELISA methods are not without disadvantages, some of which include high limits of sensitivity of $>10^5$ CFU mL^{-1}, variable cell surface antigen production, cross reactivity, and changes to antigens due to acetylation and changing recognition by assay antibodies. Newer ELISA-based techniques utilise fluorogenic, electrochemiluminescent, and real-time PCR reporters to create quantifiable signals.

The most successful approach for the separation and concentration of target organisms has been the use of immunomagnetic separation. IMS is used in conjunction with other rapid detection methods, including ELISA, conductance microbiology, electrochemiluminescence, and PCR to further increase its analytical sensitivity. The most commonly used commercial IMS bead for the recovery of *Salmonella* from food is Captivate *Salmonella* (Lab M), Tecra *Salmonella* Unique (3M), as well as for specific serovars such as *S. enteritidis*, via Rapidchek Confirm *S. enteritidis* IMS kit (SDIX). IMS can also be automated using automated IMS separators such as the BeadRetriever (Invitrogen), Kingfisher IMS separator (Thermofisher) or Mag Max (Life Technologies) capable of processing up to 100 samples with the capability of re-suspending the IMS target complex in microtitre plates for further testing by PCR, or ELISA. Another IMS variation was also developed by Pathatrix (Matrix MicroScience Ltd) combining

IMS and a recirculation step (Flow Through Immunocapture or FTI), to further increase the sensitivity of detection since larger enrichment volumes can be reacted with IMS beads [141].

Table 1.12. Main features of commercial kits available for the detection of *Salmonella* spp.

Commercial kit	Assay format	Applicable to	Pre-enrichment	Total assay time	LOD	Company
VIDAS® Easy SLM	Sandwich immunoassay (fluorescence)	Environmental sampling	16 – 22 h (x2)	45 h	1 – 5 CFU / 25 g	BioMérieux
VIDAS® UP *Salmonella*	Sandwich immunoassay using phage recombinant protein (fluorescence)	Food, feed, environmental sampling	18 – 24 h	25 h	1 – 5 CFU / 25 g	BioMérieux
TRANSIA® PLATE *Salmonella* Gold	ELISA Sandwich immunoassay (LPS detection)	Food, feed, environmental sampling	18 – 20 h	24 h	1 – 5 CFU / 25 g	BioControl
RIDASCREEN® *Salmonella* ELISA	ELISA Sandwich immunoassay	Food, feed, environmental sampling	16 – 20 h	< 23 h	1 – 5 CFU / 25 g	R-Biopharm
LOCATE® *Salmonella* ELISA	ELISA monoclonal antibody (O somatic antigen detection)	Food commodities	46 h	< 48 h	1 – 5 CFU / 25 g	R-Biopharm
TECRA® ULTIMA™ *Salmonella*	ELISA	Raw meats and carcass swabs	16 – 20 h (x2)	42 h	1 – 5 CFU / 25 g	3M
3M™ Tecra™ *Salmonella* Visual Immunoassay	ELISA	Raw materials, finished products and environmental surfaces	16 – 20 h (x2)	42 h	1 – 5 CFU / 25 g	3M

Commercial kit	Assay format	Applicable to	Pre-enrichment	Total assay time	LOD	Company
LightCycler ® foodproof *Salmonella* Detection	Real-time PCR	>100 tested food matrices and environmental samples	16 – 20 h	18 – 22 h	1 – 5 CFU / 25 g	Roche Diagnostics GmbH Roche Applied Science
Foodproof® *Salmonella* PCR	Real-time PCR	Food, environmental samples and beverages	16 – 20 h	< 24 h	1 – 5 CFU / 25 g	Merck-Millipore
MicroSEQ® *Salmonella* spp. Detection Kit	Real-time PCR	Food, beverage and animal feed	16 – 24 h	18 – 27 h	1 – 5 CFU / 25 g	Applied Biosystems, Life Technologies
HybriScan™D *Salmonella*	RNA sandwich hybridisation and enzyme-linked optical detection	Food samples	18 h – 24 h	44.5 h	1 – 5 CFU / 25 g	Sigma-Aldrich
RapidChek® SELECT™ *Salmonella* enteritidis	Test strip, sandwich immunoassay using colloidal gold	Chicken house drag swabs, egg pool samples and chicken rinse samples	16 – 22 h (x2)	32 – 48 h	1 – 5 CFU / 25 g	SDIX
QFast™ *Salmonella*	IMS and electrochemical detection	Skin and chicken meat, raw materials (cereals, nuts, extracts)	20 – 24 h	< 24 h	1 – 5 CFU / 25 g	iMICROQ

1.5.3. Rapid approaches for simultaneous pathogenic bacteria detection

The most developed methodology for simultaneous bacterial detection is the multiplex Polymerase Chain Reaction (mPCR). In spite of its high sensitivity, PCR-based methodologies still have some drawbacks such as price, sensitivity to matrix interference and no live/dead cell differentiation. Recently, some papers review last developments in bioanalytical multiplex technologies [142, 143]. Microarrays and multi-channel platforms offer high multiplexing capabilities for the biological binding assays. Other methods based on electrochemical sensors incorporate other platforms such as screen-printed electrodes. These devices usually involve antibody-antigen and DNA hybridisation specific interactions. Table 1.13 shows a brief summary of some rapid approaches for simultaneous detection of pathogenic bacteria. The most prominent detection methods are the optical, mostly fluorescence and chemiluminescence. Better LODs were obtained when immunoassays were combined with IMS and multiplexed PCR for lateral flow or optical detection ($10 – 10^3$ CFU mL^{-1}). At present, the commercially available test kits are designed for a single pathogen, thus to test a product for multiple bacteria, multiple assay kits must be used. Therefore, a long path for improvement in multiplexed pathogenic microorganisms' detection methods still needs to be done.

Table 1.13. Main features of rapid approaches for simultaneous detection of pathogenic bacteria.

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
Ten bacteria and toxins; Immunoassay using fluorescent coded microspheres	Microflow cytometer/ Fluorescence	PBS, serum and nasal wash (inoculated)	Not-performed	< 2 h	$10^4 – 10^6$ CFU mL^{-1}	144
Listeria, E. coli and *Salmonella;* Immunoassay with fluorescent antibodies reporters	Evanescent-based fibre optic sensor/ Fluorescence	BHI, beef, chicken and turkey meats (inoculated)	18 h	22 h	10^3 CFU mL^{-1}	145

Assay format	Detection technique	Test matrix	Pre-enrichment	Total assay time	LOD	Ref.
Salmonella and *Cronobacter;* DNA array, PCR, hybridisation	DVD driver	Powder skimmed milk (inoculated)	Not-performed	2 h	$10^0 - 10^2$ CFU mL^{-1}	146
E. coli, *Bacillus subtilis* and *Salmonella;* IMS and bioactive paper strips	Lateral flow colorimetric	Milk, orange juice, lettuce (inoculated)	7 h	8 h	1 CFU 100 mL^{-1}	147
E. coli and *Salmonella;* IMS-mPCR	Agarose gel electro-phoresis	Ground beef and whole milk (inoculated)	16-20 h	< 24 h	$10^3 - 10^4$ CFU mL^{-1}	148
Listeria, E. coli and *Salmonella;* Sandwich immunoassay. IMS and QDs labelling	Fluorescence	BHI, chicken carcasses, ground beef, fresh cut broccoli, and fresh-cut lettuce (inoculated)	Not-performed	1 h 30 min	20 – 50 CFU mL^{-1}	94
Nineplexed; sandwich immunoassay using fluorescent coded microspheres	Microflow cytometer/ Fluorescence	PBS (inoculated)	Not-performed	1 h 15 min	10^5 CFU mL^{-1}	149
Sixplexed; sandwich immunoassay. Microspheres and signal amplification	Microflow cytometer/ Fluorescence	PBS (inoculated)	Not-performed	2 h 30 min	10^4 CFU mL^{-1}	150
E. coli, Yersinia, Salmonella and *Listeria*; sandwich EIA	Chemilumi-nescence	Human fecal and bovine meat samples (inoculated)	9 h	10 h	$10^4 - 10^5$ CFU mL^{-1}	151
E. coli and *Salmonella;* Sandwich immunoassay with IMS	Electrochemi-luminescence	Milk, juices, serum, ground beef, chicken, fish and freshwater	Not-performed	< 1 h	$10^2 - 10^3$ CFU mL^{-1}	152

1.5.4. General overview

For the past several decades, significant advancements in the microbial analysis of foods and environmental samples have been made. All advancements are aimed at achieving sensitive and specific detection of pathogens, but despite these efforts, methods are not yet quantitative nor have they resulted in the elimination of lengthy cultural enrichment steps. In fact, the biggest hurdle in the development of more rapid detection methods is the dependency on culture. It appears that the only way around this dilemma is to begin to apply pre-analytical sample processing methods to separate and concentrate microbial targets from the sample in preparation for direct downstream detection. Ideally, such methods must be able to selectively recover all target microbial cells from the matrix. Further, the methods should be simple and broadly applicable to multiple sample matrices having different physico-chemical properties. In the near term, techniques such as IMS and alternative bioaffinity ligands such as bacteriophage are promising approaches to explore for improved target capture and sample preparation. These sorts of methods open avenues for rapid microbial detection from farm-to-table using simple, integrated platforms contained in automated, miniaturised and portable devices. Although these novel technologies such as the application of biosensors, microarrays, and nanotechnology are currently in the research stage, these are likely to become available for routine testing of food and food ingredients within the next decade.

The application of rapid methods for the detection, identification, and characterisation of pathogenic bacteria provides a useful tool for assessment of the safety of food products when used in conjunction with food safety programs such as the Hazard Analysis Critical Control Point for the assessment of raw materials and food ingredients used in food processing and production. Further improvements to rapid methods for isolation and detection of microbial pathogens will continue to focus on sample enrichment and preparation procedures to reduce test turnaround times and increase the sensitivity of detection, and also on the application of novel technologies such as biosensors, microarrays and nanotechnology for pathogen detection in foods [141].

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2

AIMS OF RESEARCH

2. AIMS OF RESEARCH

The overall aim of this dissertation was the design and development of biosensing strategies for the detection of pathogenic bacteria with improved analytical features, mainly in terms of cost, rapidity and sensitivity. The research presented in the dissertation is focus on the exploitation of novel biorecognition and transduction strategies through the integration of converging technologies such as nanotechnology, biotechnology and analytical chemistry.

For this purpose, optical and electrochemical-based strategies for pathogenic bacteria detection were investigated. The specific objectives of this work were:

1) The evaluation and comparison in terms of their electrochemical performance of different electrochemical transducers based on i) biocomposites, and ii) magneto-composite coupled with magnetic particles.

2) The integration of magnetic particles in the bioanalytical procedure, not only for bioseparation and pre-concentration, but also as a platform for immobilisation of the bioreceptor.

3) The exploration of different affinity biorecognition elements coupled to magnetic particles such as streptavidin –for biotinylated bioreceptors– as well as commercial antibodies for the specific detection of bacteria.

4) The immobilisation in an oriented way of bacteriophages on magnetic particles as biorecognition element in biosensing approaches.

5) The development of different bioanalytical approaches, immunosensing and genosensing, for the ultrasensitive detection of bacteria.

6) The characterisation of the analytical performance of the aforementioned strategies and comparison with the gold standard reference methodology.

7) The comparison of the analytical features in terms of cost, rapidity, sensitivity and specificity of the developed strategies with the state-of-the art of the biosensing for pathogenic bacteria mainly in food safety applications.

3

MATERIALS AND METHODS

This dissertation is focused on the design and evaluation of novel strategies for the rapid detection of pathogenic bacteria. The targets chosen as a model for the development of these strategies were *Mycobacterium bovis, Salmonella*, *Escherichia coli* and *Listeria monocytogenes*. *Mycobacterium bovis* was analyzed with different electrochemical platforms such as affinity biocomposite as well as magnetic particles integrated on magneto electrodes. After the evaluation of the analytical performance, further analysis were perform on magnetic particles. Several approaches based on immunosensing and genosensing, with optical and electrochemical detection were explored. Figure 3.1 shows a general scheme of all the strategies developed. Further experimental details of all the strategies developed are explained in the following sections.

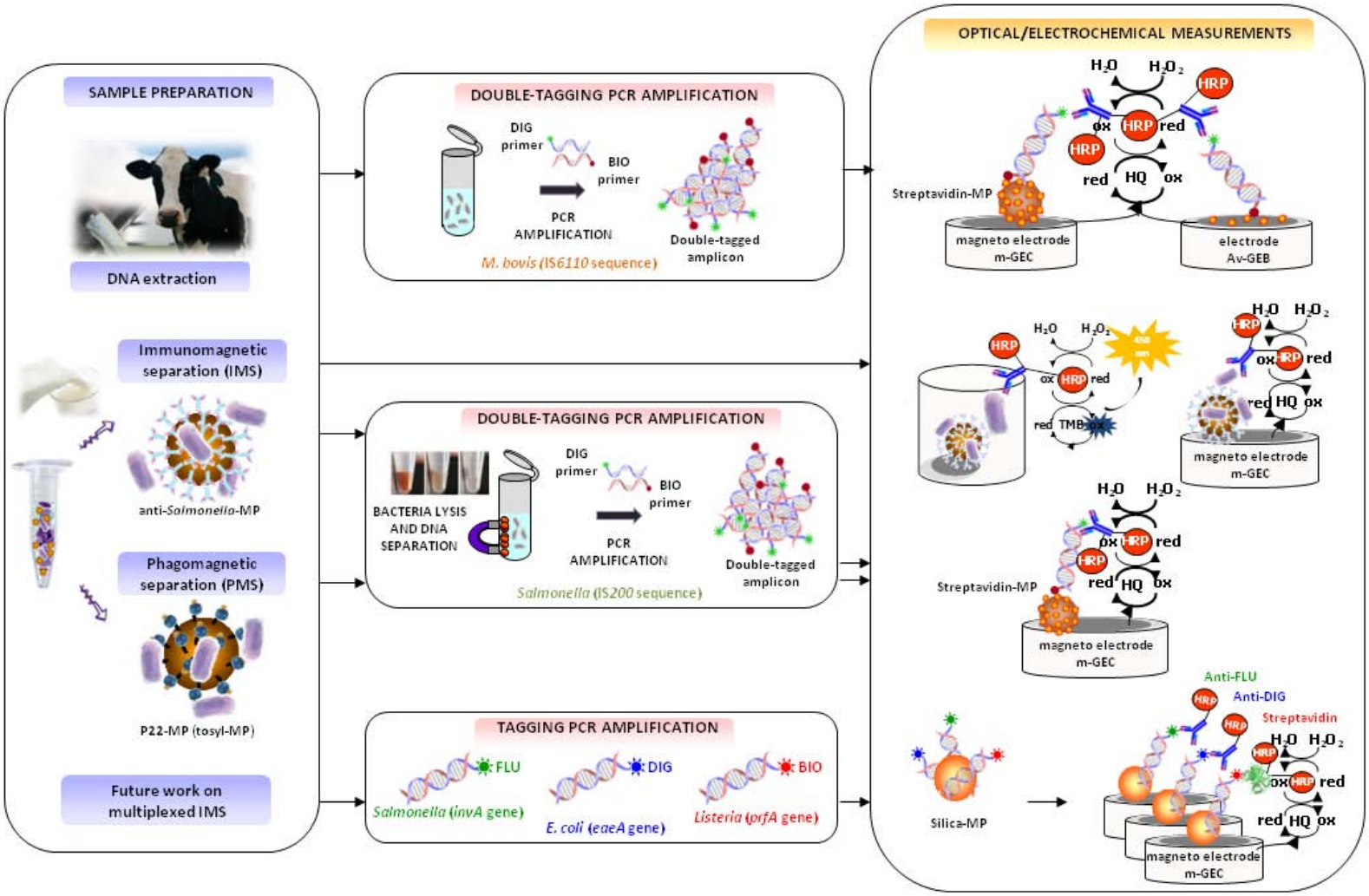

Figure 3.1. Schematic representation of the immunosensing and genosensing strategies for food safety applications.

3.1. INSTRUMENTATION

The amperometric measurements were performed in all cases with a LC-4C amperometric controller (BAS Bioanalytical Systems, USA). A three-electrode setup was used, comprising a platinum auxiliary electrode (Crison 52-67 1, Spain), a double junction Ag/AgCl reference electrode (Orion 900200) with 0.1 mol L^{-1} KCl as the external reference solution, and a working electrode (m-GEC or Av-GEB electrode, detailed preparation described in § 3.3). The amperometric signals were registered using a DUO-18 data recording system (WPI, UK) and the cyclic voltammetry for the characterisation of the working electrodes using Autolab PGSTAT (Eco Chemie, The Netherlands). The SEM images were taken with the scanning electron microscope Hitachi LTD S-570 (Hitachi LTD, Tokyo, Japan). The gold sputtering of the samples was performed with the E5000 Sputter Coater Polaron Equipment Limited (Watford, UK). The K850 Critical Point Drier Emitech (Ashford, UK) was also used for the preparation of the samples for SEM.

Polypropylene and polystyrene microtitre plates were purchased from Corning (Catalogue Nº 153364) and Nunc (Catalogue Nº 269787, Roskilde, DK), respectively. The incubations and washing steps with the microtitre plates were performed under shaking conditions using a Minishaker MS1 (IKA, Germany). Optical measurements were performed on a TECAN Sunrise microplate reader using Magellan v4.0 software. Temperature-controlled incubations were performed in an Eppendorf Thermomixer compact (Product Nº 5350 000.013). The magnetic separation of the particles during the washing steps was performed using a magnetic separator for Eppendorf tubes Dynal MPC-S (Product Nº 120.20D, Dynal Biotech ASA, Norway) or a 96-well plate magnet (Product Nº 21358, Thermo Fisher Scientific, Waltham, USA). When needed, DNA quantification after the lysis of the bacteria and the DNA purification was performed by a micro-volume spectrophotometer (Nanodrop2000spectophotometer; Thermo Scientific). The PCR reactions were carried out in a thermal cycler (Product Nº 2720, Applied Biosystems, Life Technologies Corporation). The P22 bacteriophages lysates were concentrated using 25 x 89 mm ultracentrifuge tubes (Ultra-ClearTM Tubes, Beckman, California, USA) in an ultracentrifuge (Optima^{TM} L-80, Beckman, California, USA). The ultracentrifugation was performed using the SW28 Ti rotor (Beckman, California, USA). P22 bacteriophages were inactivated when needed with a germicidal lamp (Philips, TUV15W/GU15-T8). The covalent immobilisation of P22 bacteriophage on tosylactivated magnetic particles was performed using a rotor for test tubes (Micro Bio Tec-TTR 79).

3.2. CHEMICALS, BIOCHEMICALS AND MATERIALS

3.2.1. Commercial kits

The commercial kits used along the work developed in this dissertation were:

- Expand High Fidelity PCR System kit from Roche Diagnostics S.L., Sant Cugat del Vallès, Spain (Product Nº 11732641001)
- Coomassie (Bradford) Protein Assay kit from Pierce, USA (Product Nº 23200)
- DNeasy Tissue and Blood kit from Qiagen, Hospitalet de Llobregat, Spain (Product Nº 69504)
- Dynabeads SILANE genomic DNA kit from Life technologies, Invitrogen Dynal AS (Oslo, Norway) (Product Nº 370.12D)

3.2.2. Magnetic particles

The magnetic particles used in this work were purchased from Life Technologies, Invitrogen Dynal AS (Oslo, Norway). Their main characteristics are shown in Table 3.1 and a brief description of each type of particle is listed below:

- Anti-*Salmonella* magnetic particles (anti-*Salmonell*a-MPs) (Dynabeads anti-*Salmonella,* Product Nº 710.02) are superparamagnetic, polystyrene beads with affinity-purified antibodies against *Salmonella* covalently bound to the surface.
- Streptavidin magnetic particles (streptavidin-MPs) (Dynabeads M-280 Streptavidin, Product Nº 112.05) are superparamagnetic beads with a streptavidin monolayer covalently coupled to the surface.
- Tosylactivated magnetic particles (tosyl-MPs) (Dynabeads M-280, Product Nº 142.03) are superparamagnetic, polystyrene beads coated with a polyurethane layer. The hydroxyl groups are activated by reaction with p-toluensulphonyl chloride resulting in a sulphonyl ester able to react covalently with proteins or other ligands containing amino or sulfhydryl groups.
- Silica magnetic particles (silica-MPs) (Dynabeads MyOne Silane, Product Nº 370.02D) are superparamagnetic, polystyrene beads coated with optimised silica-like chemistry (silanol groups).

Table 3.1. Particle stock characteristics, from Life Technologies.

	anti-*Salmonell*a-MPs	streptavidin-MPs	tosyl-MPs	silica-MPs
Product Nº	710.02	112.05	142.03	370.02D
Diameter (μm)	2.8	2.8	2.8	1
mg mL^{-1}	5	10	30	40
MP mL^{-1}	3 x 10^{8}	6.5 x 10^{8}	2 x 10^{9}	4 x 10^{10}

3.2.3. Electrochemical and optical reporters

In order to perform both the electrochemical and the optical detection, an enzymatic conjugate labelled with horseradish peroxidase enzyme (HRP) as electrochemical and optical reporter were used in all the cases. With this purpose, the following conjugates based on antibodies and affinity proteins were used:

- Anti-Digoxigenin-POD Fab fragments (AntiDig-HRP) (Product Nº 11207733910), purchased from Roche Diagnostics GmbH (Mannheim, Germany).
- Anti-Fluorescein-POD Fab fragments (AntiFlu-HRP) (Product Nº 11426346910) purchased from Roche Diagnostics GmbH (Mannheim, Germany).
- Streptavidin-POD conjugate (Strep-HRP) (Product Nº 1089153001) purchased from Roche Diagnostics GmbH (Mannheim, Germany).
- Anti-*Salmonella*-HRP, rabbit polyclonal antibody (Product Nº ab20771, stock solution of 1 mg mL^{-1}) purchased from Abcam (Cambridge, UK).

3.2.4. Oligonucleotide sequences

The primers for the tagging PCR amplification in the genosensing strategies were obtained from TIB-Molbiol GmbH (Berlin, Germany). These primers were selected for the specific amplification of the IS*6110* insertion sequence [1, 2] related to *Mycobacterium bovis,* the IS*200* insertion sequence [3, 4] and *invA* gene related to *Salmonella* spp., the *prfA* gene related to *Listeria monocytogenes* and the *eaeA* gene related to *E. coli* 0157:H7 [5]. Biotin (BIO), digoxigenin (DIG) and fluorescein (FLU) were used as labels, all them inserted in 5' end of the primers. The primer sequences as well as the tags used for the PCR amplification are shown in Table 3.2.

Table 3.2. Primer pairs and labels selected for the tagging PCR amplifications.

Target	Sequence/ Gene	Sequence (5' – 3')	Type	Labelling	Amplicon size (bp)
M. bovis	IS*6110*	GCG TAG GCG TCG GTG ACA AA	forward	BIO	245
		CGT GAG GGC ATC GAG GTG GC	reverse	DIG	
***Salmonella* spp.**	IS*200*	ATG GGG GAC GAA AAG AGC TTA GC	forward	BIO	201
		CTC CAG AAG CAT GTG AAT ATG	reverse	DIG	
S. enterica	*invA*	AATTATCGCCACGTTCGGGCAA	forward	FLU	278
		TCGCACCGTCAAAGGAACC	reverse	-	
L. monocytogenes	*prfA*	TCATCGACGGCAACCTCGG	forward	-	217
		TGAGCAACGTATCCTCCAGAGT	reverse	BIO	
***E. coli* 0157:H7**	*eaeA*	GGCGGATAAGACTTCGGCTA	forward	DIG	151
		CGTTTTGGCACTATTTGCCC	reverse	-	

3.2.5. Materials for the construction of the m-GEC and Av-GEB electrodes

The magneto-electrodes based on graphite-epoxy composite (m-GEC) and the electrodes based on graphite-epoxy biocomposite (Av-GEB) were prepared with the materials and reagent listed below:

- Graphite thin powder (Product Nº 1.04206.2500, Merck, Darmstadt, Germany)
- Epoxy resin and hardener (Product Nº Epo-Tek H77, Epoxy Technology, USA)
- Female electric connector, with a metal end of 2 mm diameter (Product Nº 224CN, Onda radio, Spain)
- Cylindrical PVC tubes of 6 mm i.d., 8 mm o.d. and 22 mm long (local retail store)
- Copper disk with a diameter of 6 mm (local retail store)
- Tin solder wire (local retail store)
- Abrasive paper of different thickness (local retail store)
- Neodymium magnets of 3 mm diameter and 1.5 mm long, only for m-GEC preparation (Product Nº N35D315, Halde Gac, Spain)
- Avidin, only for GEB preparation (Product Nº A9275, Sigma, Steinheim, Germany)

3.2.6. Other reagents, materials and solutions

Other reagents and materials needed for the development of this work were:

- Cellulose membranes (Product Nº D-9277, Dialysis Tubing, Sigma, Steinheim, Germany) used for dialysis when needed.
- Nucleopore Track-Etched Membranes, Whatman, (25 mm Ø, 0.2 µm pore size) (Product Nº 110606, ALCO, Spain) used as a support for SEM as well as in the bacteriophage filtration.
- Hydroquinone (Product Nº H9003, Sigma-Aldrich), as a mediator in the electrochemical measurements.
- Hydrogen peroxide 30 % (Product Nº 1.07209.0250, Merck, Germany), as a substrate of the enzyme HRP in the electrochemical measurements.

All buffer solutions were prepared with milli-Q water and all reagents were of the highest available grade (supplied from Sigma and Merck). The composition of these solutions was:

- tris buffer (TB): 0.1 mol L^{-1} Tris-hydrochloride, 0.15 mol L^{-1} NaCl, pH 7.5
- blocking tris buffer (BTB): 2 % w/v BSA, 0.1 % w/v Tween 20, 5 mmol L^{-1} EDTA, in Tris Buffer
- tris washing buffer (TWB): 10 mmol L^{-1} Tris-hydrochloride, pH 4.5
- phosphate buffer for the electrochemical cell (PBE): 0.1 mol L^{-1} sodium phosphate, 0.1 mol L^{-1} KCl, pH 7.0
- blocking phosphate buffer (BPB): 10 mmol L^{-1} sodium phosphate buffer, 0.8 % w/v NaCl, 0.05 % v/v of Tween 20, pH 7.4
- skimmed milk blocking phosphate buffer (1 %-BPB): 1 % w/v skimmed milk powder in BPB solution, pH 7.4
- citrate buffer (CB): 0.75 mol L^{-1} NaCl, 75 mmol L^{-1} trisodium citrate, pH 7.0
- borate buffer (BB): 0.1 mol L^{-1} boric acid, pH 8.5
- immobilisation phosphate buffer (IPB): 10 mmol L^{-1} sodium phosphate, 0.1 % w/v BSA, pH 7.4
- immobilisation tris buffer (ITB): 0.2 mol L^{-1} Tris-hydrochloride, 0.1 % w/v BSA, pH 8.5
- storage phosphate buffer (SPB): 10 mmol L^{-1} sodium phosphate, 0.1 % w/v BSA, 0.02 % w/v sodium azide, pH 7.4
- SEM fixation buffer: 3 % v/v glutaraldehyde, 0.1 mol L^{-1} sodium phosphate, pH 7.4

- SEM post-fixation buffer: 1 % w/v OsO_4, 0.1 mol L^{-1} sodium phosphate, pH 7.4
- binding buffer (BB): 0.1 mol L^{-1} Tris-hydrochloride, 5 mol L^{-1} GuSCN, pH 6.4
- Triton-binding buffer (TBB): 0.1 mol L^{-1} Tris-hydrochloride, 5 mol L^{-1} GuSCN, 0.1 mol L^{-1} Triton X-100, 40 mmol L^{-1} EDTA, pH 6.4
- TAE buffer: 0.04 mol L^{-1} Tris-hydrochloride, 0.1 % v/v acetic acid, 1 mmol L^{-1} EDTA, pH 8.0
- NTE buffer: 10 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris-hydrochloride, 1m mol L^{-1} EDTA, pH 7.4
- substrate TMB/H_2O_2 solution: 0.004 % v/v H_2O_2, 0.01 % w/v TMB (dissolved in DMSO), citrate buffer 0.04 mol L^{-1}, pH 5.5

3.2.7. Bacterial strains and culturing

All bacterial strains used in this work were routinely grown in Luria Bertani (LB) pH 7.5 broth (yeast extract 5.0 g L^{-1}; peptone from casein (tryptone) 10.0 g L^{-1}; sodium chloride 10.0 g L^{-1}) or agar plates for 18 h at 37 ºC. Bacterial viable counts were determined by plating on LB plates followed by incubation at 37 ºC for 24 h. When needed, the lysis of the bacteria and the DNA extraction and purification was performed according to the kit manufacturer (DNeasy Tissue and Blood Kit, Qiagen). The extraction and purification efficacy was evaluated by spectrophotometric analysis as UV absorption at 260 nm. The three bacterial strains used for the development of the strategies presented are listed below:

- *Salmonella enterica* Typhimurium LT2
- *Listeria monocytogenes* DSM20600 (DSMZ)
- *Escherichia coli* K12

3.2.8. Bacteriophage culturing and purification

The P22 bacteriophage (ATCC® 19585-B1™), a temperate virus that infects *Salmonella* groups A, B and D_1 was used as a model in the development of a genosensing strategy for *Salmonella*. To obtain the lysates of this bacteriophage, exponential cultures of *Salmonella* Typhimurium LT2 (10^8 CFU mL^{-1}) grown in LB medium at 37 ºC were infected with P22 bacteriophage at a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU) per colony-forming unit (CFU) and incubated at 37 ºC for 5 h. Afterwards, infected cultures were centrifuged at 8,000 × g for 10 min and the supernatants were filtered through 0.22-µm Nucleopore membrane to remove any remaining bacteria in the solution.

The bacteriophage titre was determined by plating adequate dilutions using double agar layered method (Figure 3.12, B), as follows: 100 µL of each dilution were blended with 100 µL of 10^8 CFU mL^{-1} *Salmonella* Typhimurium LT2 dilution and 2.5 mL of soft agar. The mixture was spilt on LB agar plates and after incubation for 18 – 24 h at 37 ºC bacteriophage plaques were counted. Normally, between 5 x 10^{10} and 1 x 10^{11} pfu mL^{-1} were obtained and P22 bacteriophages needed to be concentrated by ultracentrifugation at 27,000 × g for 2 h and resuspended in 10 mmol L^{-1} $MgSO_4$ milli-Q water to obtain bacteriophage titres of 10^{12} PFU mL^{-1}. Then, the bacteriophage lysate was purified with a cesium chloride gradient (adapted from Sambrook *et al.)* [6]. Briefly, 15 mL of concentrated bacteriophage suspension was overlaid onto a three-step CsCl gradient containing 7.6 mL of each 1.6 g mL^{-1} CsCl, 1.5 g mL^{-1} CsCl, and 1.45 g mL^{-1} CsCl, respectively, in the ultracentrifuge tubes. Afterwards, P22 bacteriophages were centrifuged for 2 h at 87,000 × g at 4 ºC in a rotor. Phage-containing bands (translucent white/grey) were extracted through the wall of the centrifuge tube by puncturing with a needle, and the CsCl was subsequently removed by dialysis using a cellulose membrane for 16 hours with three changes of $MgSO_4$ 10 mmol L^{-1} at 4 ºC. The bacteriophage titre was determined as above described. The phage stock solutions were maintained in $MgSO_4$ 10 mmol L^{-1} in milli-Q water solution at 4 ºC retaining a constant titre for several months.

When specified, the P22 bacteriophages were inactivated in order to avoid the lytic cycle. To inactivate the P22 bacteriophages, 1 ml of a lysate at 10^{12} PFU mL^{-1} in 10 mmol L^{-1} $MgSO_4$ milli-Q water was exposed to UV-C (254 nm) of a germicidal lamp at a light intensity of 10 $J/m^2/s$.

3.2.9. Safety considerations

All the procedures involving the manipulation of potentially infectious materials or cultures were performed following the guidelines for safe handling and containment of infectious microorganism [7]. Strict compliance with BSL-2 practices was followed in all experiments involving P22 bacteriophage, *Salmonella enterica* Typhimurium LT2, *Listeria monocytogenes* DSM20600 (DSMZ) and *Escherichia coli* K12, and proper containment equipment and facilities were used. The ultimate disposal was performed according to local regulations.

3.3. CONSTRUCTION AND CHARACTERISATION OF ELECTRODES BASED ON GRAPHITE-EPOXY COMPOSITES AND GRAPHITE-EPOXY BIOCOMPOSITES

The construction and characterisation of the electrodes based on graphite-epoxy composite and biocomposite are described in this section. The electrodes used in this work were based on magneto graphite-epoxy composite (m-GEC) and avidin graphite-epoxy biocomposite (Av-GEB), both electrodes were designed in the Sensors and Biosensors Group and their detailed preparation has been extensively described previously [8-14]. The steps of the construction are outlined in Figure 3.2 and briefly described in this section.

3.3.1. Construction of the electrode body

The body of the electrodes in all the instances consisted of a female electric connector with a metal end of 2 mm diameter where a copper disk of 5.9 mm of diameter was welded at the end by using solder wire, as it is shown in Figure 3.2 (i – iii). Before welding the copper disk, it was cleaned by dipping in milli-Q water: HNO_3 (1:1) for a few seconds, in order to remove the copper oxide formed that can increase the electric current resistance reducing thus the sensitivity of the transducer. This connector was set inside a cylindrical PVC tube of 6 mm i.d., 8 mm o.d. and 22 mm long (Figure 3.2, iv) using a hammer. A gap with a depth of 3 mm was thus obtained in the end of the electrode basis. Both composite and biocomposite, were prepared and added to this gap as explained in §§ 3.3.2 and 3.3.3.

3.3.2. Construction of the magneto-electrodes based on graphite-epoxy composite

The graphite-epoxy composite (GEC) paste was prepared by hand mixing the epoxy resin and the hardener at a 20:3 (w/w) ratio, according to the manufacturer. When the resin and hardener were well mixed, the graphite powder was added in a 1:4 (w/w) ratio. The resulting paste was softly mixed thoroughly again until it becomes homogenous (approximately for 30 min).

Once the paste was homogeneous, a thin layer of the resulting soft paste was placed in the gap of the PVC cylindrical basis, which has the electrical contact to a depth of 3 mm, to isolate the copper disc. A 3 mm diameter neodymium magnet was placed in the centre and further filling and tight packing of the gap with the soft GEC paste was done (Figure 3.2, va). After the

construction, the magneto electrodes based on the graphite epoxy composite (m-GEC) were cured at 80 ºC for 1 week until the paste become completely rigid.

Before each use, the electrode surface of m-GEC electrodes was renewed by a simple polishing procedure. The electrode surface was wetted with milli-Q water and then thoroughly polished with abrasive paper of different thickness to give a smooth mirror finish with a fresh renewable surface. When not in use the electrodes were stored in a dried place at room temperature.

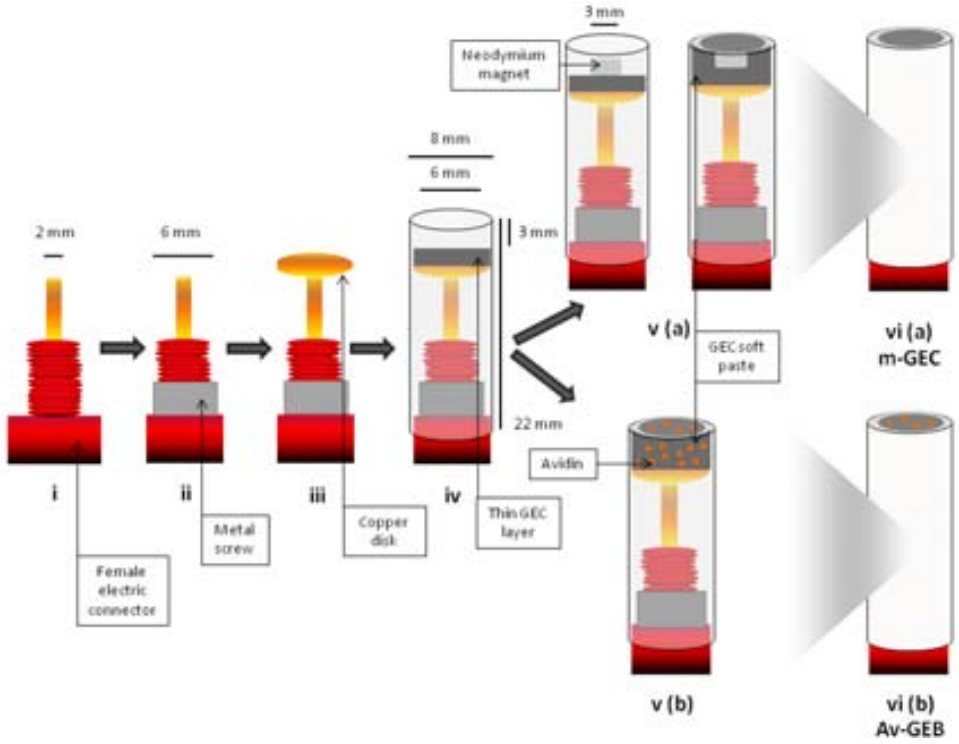

Figure 3.2. Schematic representation for the construction of m-GEC (a) and Av-GEB (b) electrodes.

3.3.3. Construction of electrodes based on avidin graphite-epoxy biocomposite

The avidin graphite-epoxy biocomposite (Av-GEB) paste was prepared as detailed for m-GEC in § 3.3.2 by hand mixing the epoxy resin, the hardener and the graphite powder. In this case, for every gram of graphite/epoxy mixture, an additional 10 mg of avidin was added—resulting in a 1 % (w/w) avidin-graphite-epoxy biocomposite (Av-GEB). This mixture was thoroughly hand mixed to ensure the uniform dispersion of the avidin and graphite throughout the polymer.

The resulting paste was placed in the gap of the PVC cylindrical basis, but in this case without including a magnet inside (Figure 3.2, vb). After filling the electrode body gap completely with the soft paste, the electrode was tightly packed and cured at 40 ºC for 1 week. As the avidin molecule is highly resistant to a wide range of chemicals, pH range variations, and high temperature [15, 16], the avidin within the Av-GEB can resist the curing temperature without any loss in its binding capacity for biotinylated molecules. When not in use, the biocomposite electrodes were stored at 4 ºC.

Prior to each use, the electrode surface of Av-GEB electrodes were renewed as previously mentioned for m-GEC electrodes. The reproducibility of the construction of both m-GEC and av-GEB electrodes, as well as the polishing procedure have been previously reported [13, 17].

3.3.4. Electrochemical characterisation of the electrodes by cyclic voltammetry

Both type of electrodes used in this dissertation, based on magneto graphite-epoxy composite (m-GEC) and avidin graphite-epoxy biocomposite (Av-GEB), were characterised by cyclic voltammetry. This technique is widely used for acquiring qualitative information about redox reactions and transducer materials. In particular, it offers a rapid location of redox potentials of the electroactive species, and convenient evaluation of the effect of media upon the redox process.

Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode (in a non-stirred solution) using a triangular potential waveform (Figure 3.3, A). Depending on the information sought, single or multiple cycles can be used. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The plot of current versus potential is termed cyclic voltammogram, where the potential (E) and current (i) of the anodic (E_{pa}, i_{pa}) and cathodic (E_{pc}, i_{pc}) peaks are shown, corresponding to the oxidation and reduction reactions respectively (Figure 3.3, B) [18].

Due to the fact that the amperometric measurements were performed using hydroquinone as a mediator (§ 3.4.1), the electrochemical behaviour of the electrodes (m-GEC and Av-GEB) was evaluated through the redox couple benzoquinone/hydroquinone (Figure 3.3, C). For the characterisation of both electrodes, the three-electrode setup described in § 3.1 was immersed into the electrochemical cell containing 20 mL of phosphate buffer with 1.81 mmol L^{-1} hydroquinone. The scan rate was 100 mV s^{-1}. This characterisation allows the determination

of the reduction potential needed during the amperometric measurements. The reproducibility of the construction and renewal of the surface, as well as the optimisation of the curing temperature for the construction of the electrodes were also evaluated.

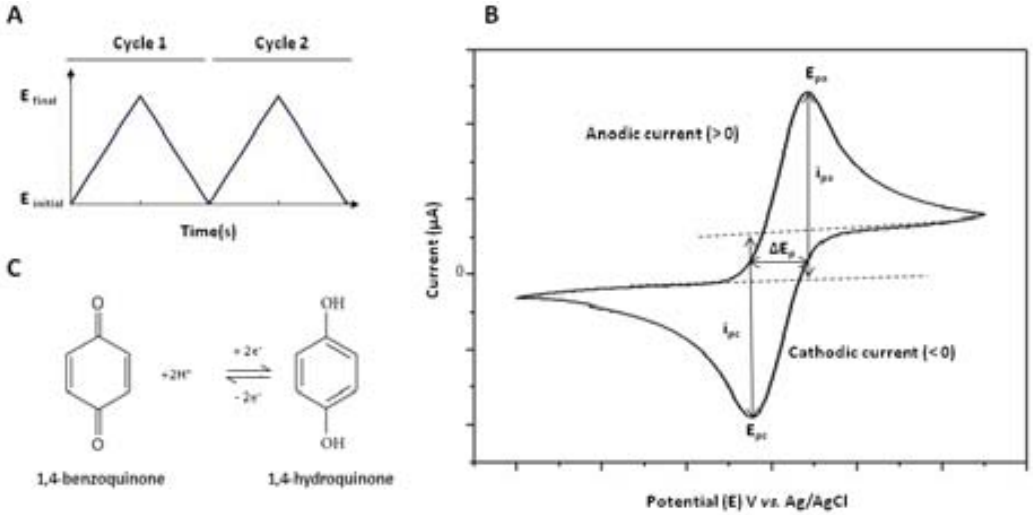

Figure 3.3. Cyclic voltammetry potential waveform (A), a typical cyclic voltammogram showing the oxidation or anodic (*pa*) and the reduction or cathodic (*pc*) peaks (B), and the benzoquinone/hydroquinone redox reaction (C).

3.4. ELECTROCHEMICAL AND OPTICAL MEASUREMENTS

In the strategies presented in this dissertation, the final measurements were performed by electrochemical or optical methods. For the electrochemical measurements, amperometry method was chosen in which a constant potential is applied to the working electrode, and current is measured as a function of time. For the optical measurements, the absorption of visible radiation was used for determining the analyte's concentration. In following sections both techniques are explained in detail.

3.4.1. Amperometric measurements

In the electrochemical genosensing and immunosensing strategies, the amperometric detection based on the horseradish peroxidase enzyme (HRP) activity by adding hydrogen peroxide (H_2O_2) as a substrate and using hydroquinone (HQ) as a mediator was performed. The mediator was regenerated by applying a reduction potential on the surface of the electrode being the current measured directly proportional to the concentration of HRP, when saturated substrate conditions (H_2O_2) were used, as showed in the following equation:

$$\mathbf{H_2O_{2(ox)} + peroxidase_{(red)} \rightarrow H_2O_{(red)} + peroxidase_{(ox)}}$$

$$\mathbf{peroxidase_{(ox)} + mediator_{(red)} \rightarrow peroxidase_{(red)} + mediator_{(ox)}}$$

$$\mathbf{mediator_{(ox)} + ne^- \rightarrow mediator_{(red)}}$$

where $mediator_{(red)}$ corresponds to the reduced form 1,4-hydroquinone, and $mediator_{(ox)}$ to the oxidised form 1,4-benzoquinone. However, this mechanism is only valid when the mediator provides rapid reaction with the enzyme, rapid electron transfer and low regeneration potential and stability in both structures oxidised and reduced. All these conditions are ensured, among others, by the system benzoquinone/hydroquinone.

Figure 3.4 shows the three-electrode setup used for the amperometric measurements comprised by a platinum auxiliary electrode, a double junction Ag/AgCl reference electrode with 0.1 mol L^{-1} KCl as external reference solution, and a working electrode (m-GEC or Av-GEB electrode, detailed preparation described in § 3.3). When Av-GEB platform was used, different steps of the procedure were performed directly on the surface of the electrode by dipping the Av-GEB electrode in an Eppendorf tube containing the different solutions needed. While when

m-GEC platform was used, all the steps were performed in solution on the surface of the magnetic particles and at the end, the modified magnetic particles were captured by dipping the magneto-electrode (m-GEC) inside the reaction tube (as shown in Figure 3.9, § 3.5).

The modified m-GEC or Av-GEB electrode were immersed into the electrochemical cell containing 20 mL of phosphate buffer with 1.81 mmol L^{-1} hydroquinone, and under continuous magnetic stirring, a potential of -0.100 or -0.150 V (*vs*. Ag/AgCl) was applied, respectively. When a stable baseline was reached, 500 µL of hydrogen peroxide was added into the electrochemical cell to a final concentration of 4.90 mmol L^{-1}, which corresponds to the H_2O_2 concentration capable of saturating the whole enzyme amount employed in the labelling procedure. The current was measured when the steady state current was reached (normally by 1 min of H_2O_2 addition). This steady-state current was used in all the electrochemical data shown in § 4.

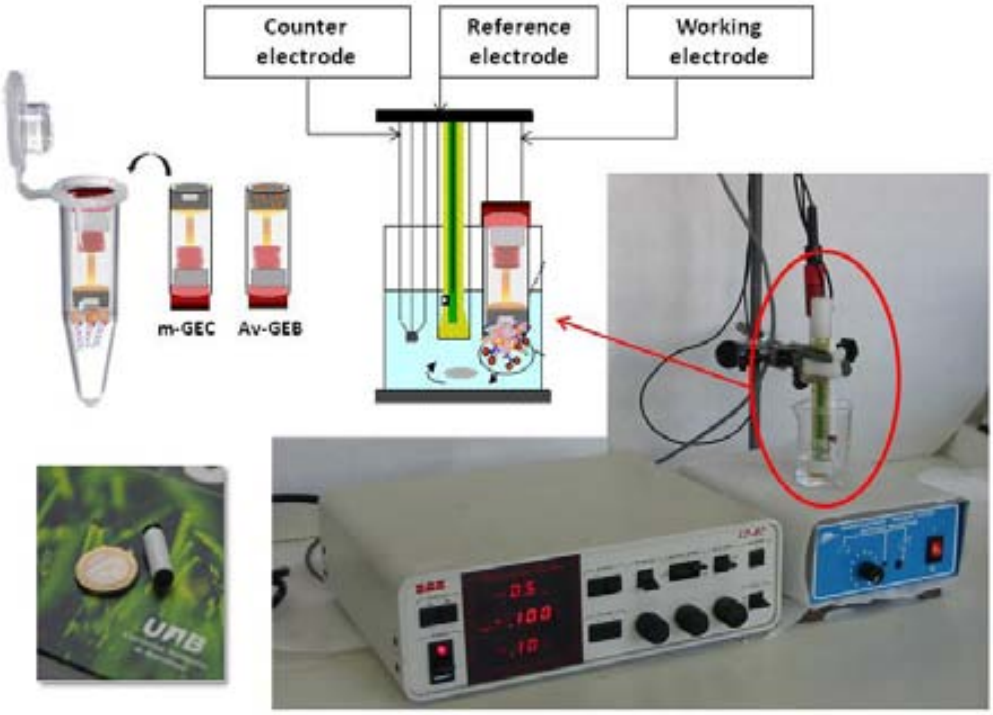

Figure 3.4. Schematic representation and photograph of the electrochemical setup, the m-GEC and Av-GEB electrodes.

3.4.2. Absorbance measurements

The optical detection was used for the Coomassie-based protein assay (Bradford assay) [19] as well as for the magneto-immunoassay. Both methods are based on the intensity of transmitted light by the generation of absorbing species in proportion to the quantity of protein present in the sample. Figure 3.7 shows the microplate reader used for the optical measurements.

Coomassie-based protein assay or Bradford assay is a rapid method for the estimation of protein concentration. As shown in Figure 3.5, Bradford method is based on the non-covalent binding of the anionic form of the dye Coomassie Blue G-250 with a protein. The dye reacts chiefly with arginine residues, which have a positively charged side chain, and slight interactions have also been observed with basic residues (histidine and lysine) and aromatic residues (tyrosine, tryptophan, and phenylalanine). In the absence of protein, the dye reagent is a pale red (absorbance maximum at 465 nm), and upon binding to protein, a blue colour is generated (absorbance maximum at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, so that is the optimal wavelength to measure the blue colour from the Coomassie dye-protein complex [20]. The Bradford assay was performed according to the kit manufacturer and 620 nm was the wavelength used, corresponding to available filters [21].

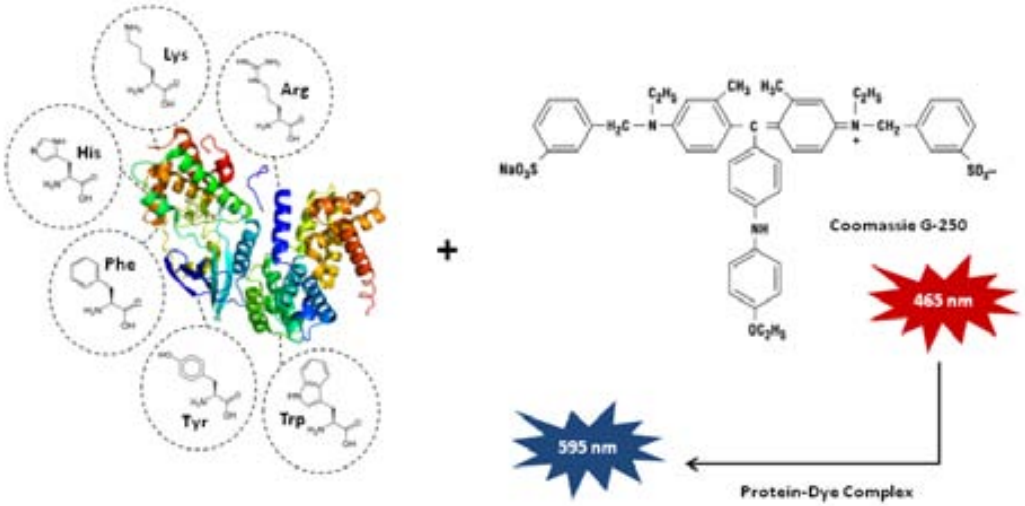

Figure 3.5. Schematic reaction of the Coomassie-based protein assay (Bradford assay).

The optical detection for the magneto-immunoassay was based on the horseradish peroxidase enzyme (HRP) activity by adding hydrogen peroxide (H_2O_2) as a substrate and using TMB (3,3',5,5'-tetramethylbiphenyl-4,4'-diamine) as a chromogen. As can be seen in Figure 3.6, TMB yields a blue colour when oxidised, typically as a result of oxygen radicals produced by the hydrolysis of hydrogen peroxide by HRP. For kinetic or non-stopped ELISA assays, the TMB

chromogen has maximal absorbance at 370 nm and 652 nm. The colour then changes to yellow with the addition of sulphuric or phosphoric acid with maximum absorbance at 450 nm. A green reaction product may result from partial conversion to the yellow product from the blue intermediate. TMB is very sensitive ELISA substrate and is more quickly oxidised than other HRP substrates, resulting in faster colour development [20, 22-24].

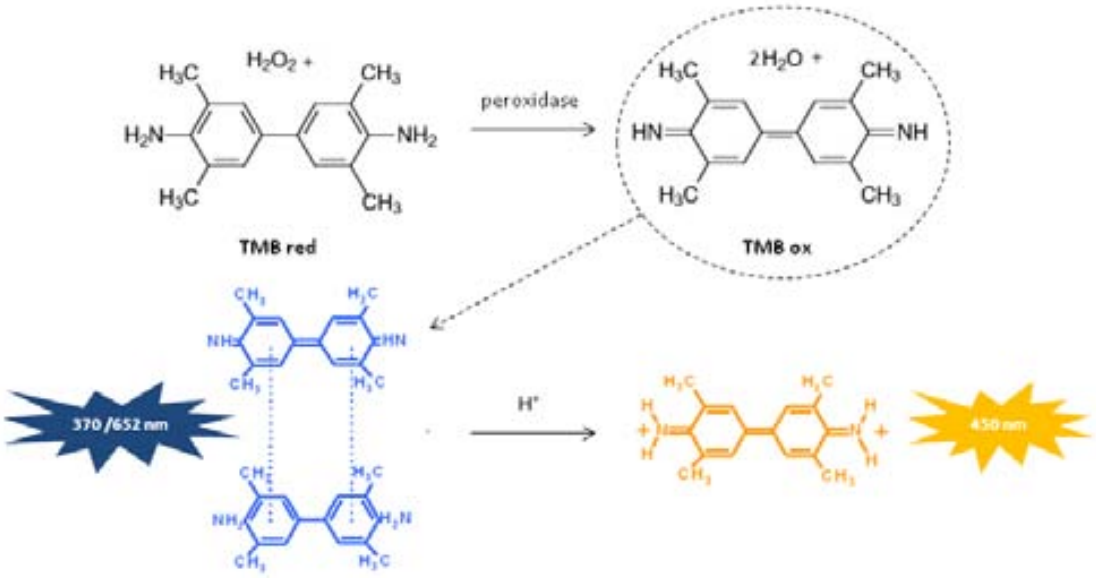

Figure 3.6. Schematic mechanism of the optical detection when hydrogen peroxide (H_2O_2) was used as a substrate for the HRP enzyme and TMB (3,3',5,5'-tetramethylbiphenyl-4,4'-diamine) as a chromogen.

The optical detection for the magneto-immunoassay was performed adding 100 µL of substrate TMB/H_2O_2 solution to the well and incubating the microplate for 30 min at room temperature under dark conditions. The enzymatic reaction was stopped by adding 50 µL of H_2SO_4 (2 mol L^{-1}) and the absorbance measurement of the supernatants was performed at 450 nm. This absorbance value was used in the optical data shown in § 4.

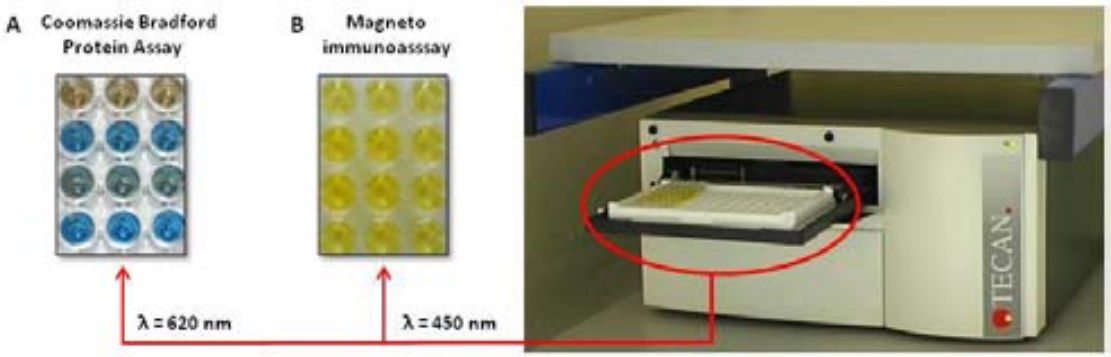

Figure 3.7. Photograph of the 96-well microplate reader (right) and, the appearance of the microplates after performing the Bradford assay (A) and the magneto-immunoassay (B) (left).

3.5. ELECTROCHEMICAL GENOSENSING OF *MYCOBACTERIUM BOVIS*

The aim of this study was to develop a rapid method for screening-out of tuberculosis (TB) in milk and dairy products based on electrochemical genosensing of DNA specific of *Micobacterium bovis*. Two different electrochemical platforms for genosensing were evaluated: i) an avidin biocomposite (Av-GEB), and ii) a magneto-composite (m-GEC) coupled with streptavidin magnetic particles. The assay was performed, as shown in Figure 3.8, by the specific amplification and double-tagging of the IS*6110* insertion sequence highly related to *Mycobacterium bovis,* followed by the electrochemical detection of the amplified product. The amplification was performed by PCR using a labelled set of primers, obtaining a double-tagged amplicon with biotin and digoxigenin in each extreme. In both cases, when Av-GEB and m-GEC where used, the immobilisation of the double-tagged amplicon was achieved through the biotinylated end of the amplicon while the electrochemical detection was performed through the digoxigenin end by using an AntiDig-HRP conjugate. The results obtained were discussed and compared with tuberculin skin test, the current gold standard for identifying cattle exposed to *M. bovis*, and inter-laboratory PCR assays performed in collaboration with the Universidad Nacional del Litoral, Santa Fe, Argentina and the Centro Nacional de Investigaciones Agropecuarias (CNIA-INTA), Castelar, Buenos Aires, Argentina.

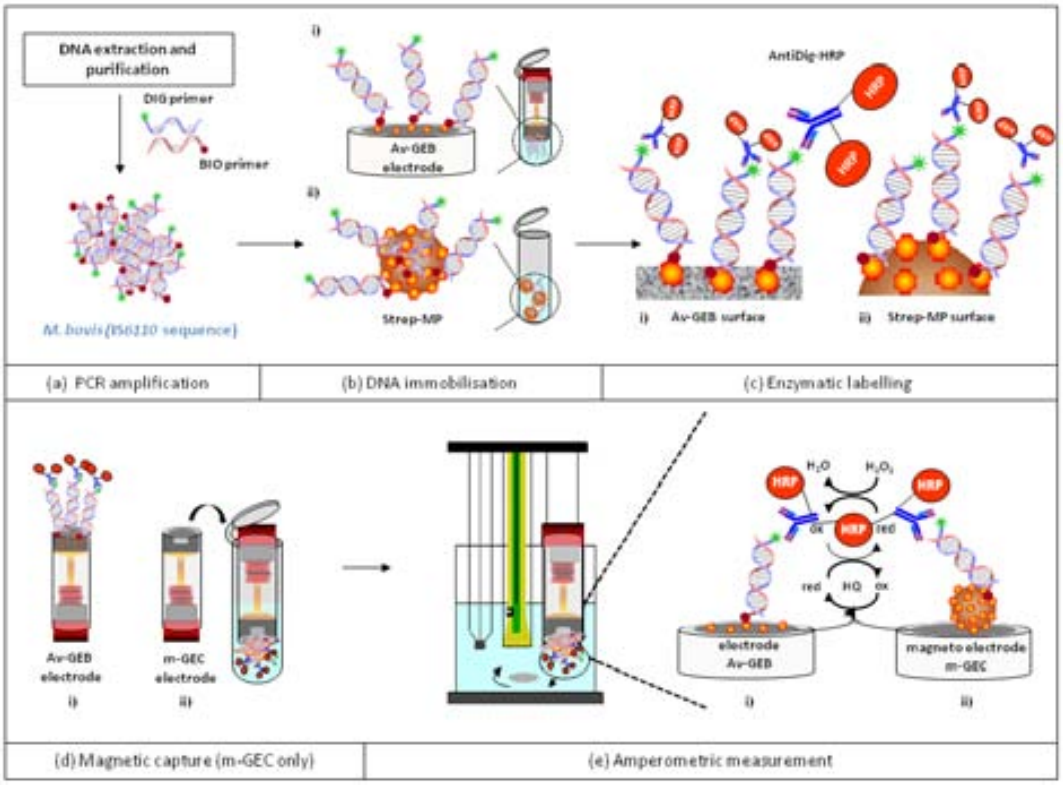

Figure 3.8. Schematic representation for the electrochemical genosensing of *Mycobacterium bovis*.

3.5.1. Sample preparation and extraction of bacterial genomic DNA

Raw milk samples used in this study were collected from local dairy farm tanks originally from the state of Santa Fe in Argentina. The samples were transported refrigerated to the laboratory and deactivated at 70 ºC for 70 min and stored at -20 ºC until they were used. A volume of 125 mL of the sample was centrifuged at 3,000 rpm for 15 min, collecting both the fat layer and somatic and/or bacterial cells. In order to remove the fat layer, the cell layer obtained was washed and resuspended in 1 mL of phosphate buffer. The cellular suspension was diluted 1:2 in NTE buffer with 10 % SDS and incubated at 37 ºC for 1 h. Then, the mixture was incubated with proteinase K 1 % at 37 ºC overnight. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with chloroform:isoamyl alcohol (24:1, v/v). The DNA collected in the aqueous phase was precipitated with NaCl 5 mol L^{-1} and isopropanol. Then it was kept at -20 ºC overnight. The precipitated DNA was washed with 1 mL of ethanol 70 % and resuspended in 40 µL of RNAse-free water [1, 25].

3.5.2. Double-tagging PCR amplification and gel electrophoresis detection

As shown in Figure 3.8 (a), a pair of labelled primers with biotin and digoxigenin respectively (see Table 3.2, § 3.2.4) was used for the amplification and the double-tagging of the bacterial DNA extracted. The PCR was performed in 100 µL of reaction mixture containing 8 µL of purified DNA coming from *M. bovis*. Each reaction contained 100 µmol L^{-1} of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 0.1 µmol L^{-1} of the double-tagged set of primers (biotinylated IS*6110* forward and digoxigenated IS*6110* reverse) and 5.6 U of polymerase. The reaction was carried out in Expand High Fidelity Kit 1x buffer containing 1.5 mmol L^{-1} of $MgCl_2$ and 5 % v/v DMSO. The amplification mixtures were treated, as outlined in Table 3.3, with an initial step at 95 ºC for 2 min followed by 30 cycles at 95 ºC for 30 s, 64 ºC for 30 s, and 72 ºC for 30 s, and a last step of 7 min at 72 ºC. The resulting samples were stored at 4 ºC.

Table 3.3. Double-tagging PCR protocol for the amplification of *M. bovis* DNA. *Performed 30 cycles.

	Initial step	DNA denaturation*	Annealing*	Extension*	Last step
Temperature (ºC)	95	95	64	72	72
Time (sec)	120	30	30	30	420

In all the instances, a negative control was included, which contained all reagents except *M. bovis* template in the PCR mixture. The amplification products were analysed by electrophoresis on 2 % agarose gel in TAE buffer containing 0.5 μg ml^{-1} ethidium bromide. ΦX174-Hinf I genome consisting of DNA fragments ranged from 24 to 726 bp was used as a molecular weight marker. The DNA bands were visualised by UV transillumination. As the primers were labelled with biotin and digoxigenin, the amplified DNA (amplicon) was expected to be double-tagged as well with both biotin and digoxigenin in each extreme, respectively.

3.5.3. Electrochemical genosensing of the double-tagged amplicon based on Av-GEB electrodes

The electrochemical genosensing based on Av-GEB electrodes consisted briefly of the following steps, as schematically depicted in Figure 3.8: 1) Immobilisation of the double-tagged amplicon on the Av-GEB electrode (Figure 3.8, bi), where the biotin end of the dsDNA amplicon was attached to the surface of the electrode because of the avidin-biotin interaction; 2) Enzymatic labelling using AntiDig–HRP conjugate able to be attached in the digoxigenin end of the dsDNA amplicon (Figure 3.8, ci), and 3) Amperometric measurements (Figure 3.8, ei).

The protocol previously optimised [13, 26] is schematised in Table 3.4. After the double-tagging PCR amplification, the PCR amplicon was diluted in milli-Q water (1/15, 1/8, 1/4 and 1/2) and 10 μL were incubated in citrate buffer (CB) for 15 min at 42 ºC. Then, the immobilisation of the double-tagged amplicon was achieved by dipping the Av-GEB electrode in an Eppendorf tube containing the diluted amplicon. The immobilisation was performed in CB at a final volume of 140 μL for 30 min at 42 ºC, followed by two washing steps for 10 min at 42 ºC in 140 μL CB. After that, the enzymatic labelling was performed by incubating the Av-GEB surface with AntiDig–HRP (60 μg) in blocking tris buffer at a final volume of 140 μL for 30 min at 42 ºC, followed by two washing steps for 10 min at 42 ºC in 140 μL of tris buffer. Different steps of the procedure were performed directly on the surface of the electrode, dipping the Av-GEB electrode in an Eppendorf tube containing the different solutions needed, as shown in Figure 3.9 (i). The electrochemical measurements were performed as described in § 3.4.1, using the modified Av-GEB electrode as working electrode.

Table 3.4. Protocol for the electrochemical genosensing based on Av-GEB electrodes.

Step	Description	Temperature	Time (min)	Shaking (rpm)
Conditioning temperature	10 µL diluted amplicon + 130 µL CB	42 ºC	15	700
Immobilisation	Dipping the Av-GEB electrode in the Epp tube (140 µL)	42 ºC	30	700
Washing	2 x 140 µL CB	42 ºC	10	700
Enzymatic labelling	140 µL AntiDig-HRP (60 µg)	42 ºC	30	700
Washing	2 x 140 µL TB	42 ºC	10	700

3.5.4. Electrochemical genosensing of the double-tagged amplicon based on m-GEC electrodes

The electrochemical genosensing based on m-GEC electrodes consisted briefly of the following steps, as schematically depicted in Figure 3.8: 1) Immobilisation of the double-tagged amplicon on streptavidin magnetic particles, in which the biotin end of the dsDNA amplicon was immobilised on the streptavidin magnetic particles (Figure 3.8, bii); 2) Enzymatic labelling using AntiDig–HRP conjugate able to be attached in the digoxigenin end of the dsDNA amplicon (Figure 3.8, cii); 3) Magnetic capture of the modified magnetic particles by the m-GEC electrode (Figure 3.8, dii), and 4) Amperometric measurements (Figure 3.8, eii).

The protocol previously optimised [26, 27] is schematised in Table 3.5. As in the case of Av-GEB procedure, after the double-tagging PCR amplification, the PCR amplicon was diluted in milli-Q water (1/960, 1/480, 1/240, 1/120, 1/60, 1/30, 1/15, 1/8 and 1/4) and 10 µL were incubated in CB for 15 min at 42 ºC. Then, the immobilisation of the double-tagged amplicon was achieved by adding 6.5 x 10^6 streptavidin-MPs in an Eppendorf tube with the diluted amplicon in CB solution at a final volume of 140 µL for 30 min at 42 ºC. Two washing steps were then performed with 140 µL CB for 10 min at 42 ºC. After that, the enzymatic labelling was performed by incubating the streptavidin-MPs with AntiDig–HRP (60 µg) in blocking tris buffer at a final volume of 140 µL for 30 min at 42 ºC. Two washing steps were then performed for 10 min at 42 ºC in 140 µL of tris buffer. After each incubation or washing step, the magnetic particles were separated from the supernatant on the sidewall by placing the Eppendorf tubes in a magnet separator until the particles were migrated to the tube sides and the liquid was

clear. After the final washing step, the modified magnetic particles were captured by dipping the magneto-electrode (m-GEC) inside the reaction tube as shown in Figure 3.8 (dii) and 3.9 (ii). The electrochemical measurements were performed as described in § 3.4.1, using the modified m-GEC electrode as working electrode.

Table 3.5. Protocol for the electrochemical genosensing based on m-GEC electrodes.

Step	Description	Temperature	Time (min)	Shaking (rpm)
Conditioning temperature	10 µL diluted amplicon + 110 µL CB	42 ºC	15	700
Immobilisation	+ 20 µL streptavidin-MPs (6.5 x 10^6 MPs, 140 µL in total)	42 ºC	30	700
Washing	2 x 140 µL CB	42 ºC	10	700
Enzymatic labelling	140 µL AntiDig-HRP (60 µg)	42 ºC	30	700
Washing	2 x 140 µL TB	42 ºC	10	700

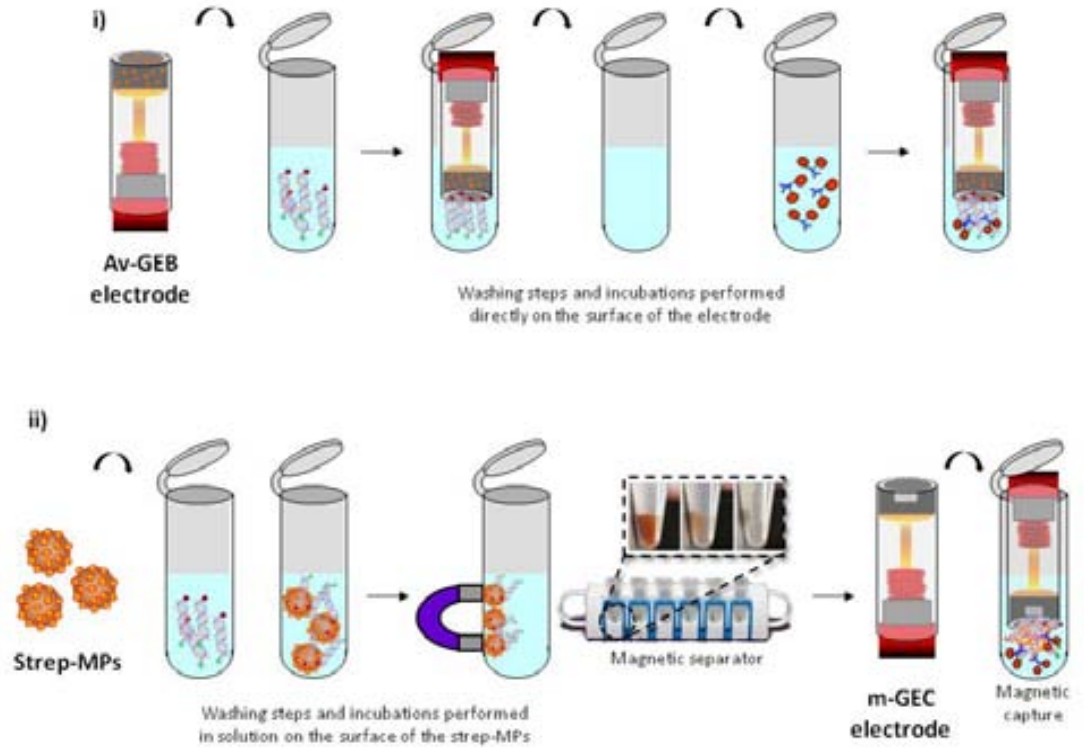

Figure 3.9. Schematic representation of both Av-GEB (i) and m-GEC (ii) electrodes handling.

3.6. ELECTROCHEMICAL GENOSENSING AND IMMUNOSENSING OF *SALMONELLA*

Next section describes three different electrochemical strategies for the detection of the bacteria *Salmonella* based on magnetic particles. As outlined in Figure 3.10, magnetic particles were used to achieve the magnetic separation from complex matrix of the bacteria using different biorecognition reactions (immunomagnetic (IMS) and phagomagnetic (PMS) separations). Moreover, the magnetic particles were also used to achieve the electrochemical genosensing and immunosensing of the bacteria with improved analytical features. The following strategies based on magnetic particles and using *Salmonella* as a model were evaluated: 1) "IMS/double-tagging PCR/m-GEC electrochemical genosensing"; 2) "PMS/double-tagging PCR/m-GEC electrochemical genosensing", and 3) "IMS/m-GEC electrochemical immunosensing". In detail, these strategies comprise the following steps:

1) "IMS/double-tagging PCR/m-GEC electrochemical genosensing" (Figure 3.10, a – f): (i) Immunomagnetic separation (IMS); (ii) Double-tagging PCR amplification; (iii) Immobilisation of the double-tagged amplicon on streptavidin magnetic particles; (iv) Enzymatic labelling with the electrochemical reporter AntiDig-HRP; (v) Magnetic capture of the modified magnetic particles by the m-GEC electrodes, and (vi) Amperometric measurement.

2) "PMS/double-tagging PCR/m-GEC electrochemical genosensing" (Figure 3.10, a – f): (i) Phagomagnetic separation (PMS); (ii) Double-tagging PCR amplification; (iii) Immobilisation of the double-tagged amplicon on streptavidin magnetic particles; (iv) Enzymatic labelling with the electrochemical reporter AntiDig-HRP; (v) Magnetic capture of the modified magnetic particles by the m-GEC electrodes, and (vi) Amperometric measurement.

3) "IMS/m-GEC electrochemical immunosensing" (Figure 3.10, a, d – f): (i) Immunomagnetic separation (IMS); (ii) Enzymatic labelling with the electrochemical reporter Anti-*Salmonella*-HRP; (iii) Magnetic capture of the modified magnetic particles by the m-GEC electrodes, and (iv) Amperometric measurement.

In all cases, magneto graphite-epoxy composite (m-GEC) electrodes were used as a working electrode for the electrochemical measurements. The experimental details of all the steps involved in these strategies, such as magnetic separation (IMS and PMS) and electrochemical magneto genosensing and immunosensing are presented next.

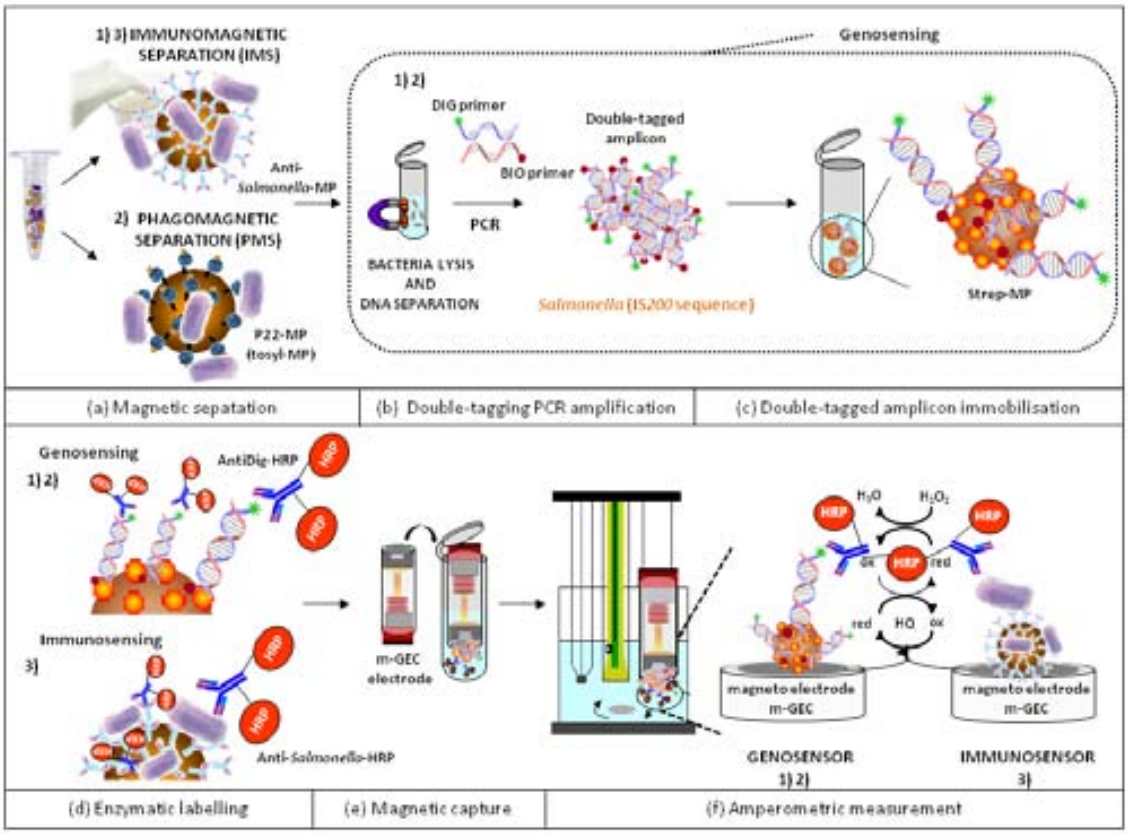

Figure 3.10. Schematic representation of the electrochemical strategies for *Salmonella* spp. detection: 1) "IMS/double-tagging PCR/m-GEC electrochemical genosensing", 2) "PMS/double-tagging PCR/m-GEC electrochemical genosensing", and 3) "IMS/m-GEC electrochemical immunosensing".

3.6.1. Immunomagnetic separation

In both strategies, 1) "IMS/double-tagging PCR/m-GEC electrochemical genosensing", and 3) "IMS/m-GEC electrochemical immunosensing", the bacteria were captured by using «immunomagnetic separation (IMS)» (Figure 3.10 (a) 1 and 3), based on immunological reaction with magnetic particles modified with specific antibody against *Salmonella* (anti-*Salmonell*a-MPs, § 3.2.2). The immunomagnetic separation of *Salmonella* ranged from 10^0 to 10^7 CFU mL^{-1} was performed in both LB broth and skimmed milk (diluted 1/10 in LB broth). A negative control was also included for both samples, which contained all reagents except *Salmonella*. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar.

The protocol is schematised in Table 3.6 and described next. A volume of 10 µL of anti-*Salmonell*a-MPs was added to a volume of 500 µL of the pure culture or skimmed milk diluted 1/10 in LB broth. An incubation step was performed for 10 min with slight agitation at room temperature. After that, the magnetic particles with the attached-bacteria were separated

with a magnet, and then washed with 500 µL of blocking phosphate buffer twice for 10 min at room temperature. Finally, the collected modified MPs were resuspended in 150 µL of milli-Q water. Magnetic particles manipulation is detailed in § 3.5. The evaluation of the IMS was performed by Scanning Electron Microscopy (SEM) and by microbiological culture, as explained in following section.

Table 3.6. Protocol for the immunomagnetic separation step (IMS).

Step	Description	Temperature	Time (min)	Shaking (rpm)
IMS	500 µL sample + 10 µL anti-*Salmonell*a-MPs (0.1 mg mL^{-1})	RT	10	700
Washing	2 x 500 µL BPB	RT	10	700
Storage until further use	Resuspension in 150 µL of milli-Q water	-	-	-

3.6.1.1. Evaluation of the IMS by Scanning Electron Microscopy and microbiological culture

For the microscopic evaluation of the attached bacteria on the magnetic particles by SEM, the IMS was performed as mentioned in § 3.6.1 with a concentration of bacteria of 10^4 CFU mL^{-1} in LB broth, a negative control was included, which contained all reagents except *Salmonella*. After that, 5 mL of milli-Q water containing the modified magnetic particles were filtered through a Nucleopore membrane as a support for the SEM microscopy. The filters were then treated following a protocol provided by the *Servei de Microscopia* at UAB. The filters were fixed by immersing them in 3 mL of SEM fixation buffer for 2 hours at 4 ºC. Four washing steps in phosphate buffer for 10 min were performed. Then, the filters were postfixed with SEM postfixation buffer for 2 hours at 4 ºC. Four washing steps in phosphate buffer for 10 min each were then performed. Afterwards, dehydration with ethanol was performed as follows: 15 minutes in 30 % ethanol; 30 minutes in 50 % ethanol; 12 hours at 4 ºC in 2 % acetate uranyl in 70 % ethanol; 30 minutes in 90 % ethanol, and twice 30 minutes in 100 % ethanol. Any remained ethanol was removed by critical point drying with CO_2. The filters were finally submitted to metallisation with gold in order to improve the electric conductivity and the electron emission.

In order to study the efficiency of the immunomagnetic separation step (§ 3.6.1) by microbiological culture, the modified magnetic particles were plated. For each concentration of bacteria ranged from 10^0 to 10^7 CFU mL^{-1} in both LB broth and skimmed milk (diluted 1/10 in LB broth), a volume of 50 µL of the MPs with the attached bacteria was plated in LB agar. In all cases, in order to achieve statistically reliable counting, between 15 and 300 colonies per plate, the appropriate dilutions were plated and after the incubation for 18 – 24 h at 37 °C the grown colonies were counted.

3.6.2. Phagomagnetic separation

In the second strategy, "PMS/double-tagging PCR/m-GEC electrochemical genosensing", the bacteria were captured by using «phagomagnetic separation (PMS)» (Figure 3.10 (a) 2) instead of «immunomagnetic separation (IMS)» (Figure 3.10 (a) 1 and 3). The PMS is based on the use of bacteriophage as specific biorecognition element for the capture and pre-concentration of pathogenic bacteria. The icosahedral-shaped bacteriophage P22 specific to the pathogenic bacteria *Salmonella* (serotypes A, B, and D_1) was studied as a model [28]. Bacterial solutions ranged from 3.2 x 10^0 to 3.2 x 10^6 CFU mL^{-1} in LB broth and a negative control containing all reagents except *Salmonella* were processed. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar.

The protocol followed is outlined in Table 3.7 and described next. A volume of 50 µL of P22 phage-modified MPs was added to a volume of 500 µL of the tenfold dilution of *Salmonella*. An incubation step was performed for 30 min at 37°C without agitation. After that, the magnetic particles with the attached-bacteria were separated with a magnet, and then washed with 500 µL of blocking phosphate buffer for 5 min at room temperature three times. Finally, the modified magnetic particles were resuspended in 80 µL of milli-Q water. Magnetic particles manipulation is detailed in § 3.5.

Before the PMS, the purification of the bacteriophages was evaluated in order to obtain bacteriophages without any debris of the host bacteria that can interfere the analysis. Then, the immobilisation of the native –non-modified– P22 phage on tosylated magnetic particles (tosyl-MPs) was achieved. The coupling efficiency was evaluated by the Coomassie-based protein assay (Bradford assay) and microbiological cultures. Further appraisal of the correct orientation of the immobilised P22 bacteriophages was performed by microscopic techniques

(SEM) as well as by microbiological cultures. After the correct immobilisation of the P22 bacteriophage on the magnetic particles, the phagomagnetic separation was performed as above described and evaluated as well by SEM and microbiological cultures. The following sections describe in detail all the aforementioned procedures.

Table 3.7. Protocol for the phagomagnetic separation step (PMS).

Step	Description	Temperature	Time (min)	Shaking (rpm)
PMS	500 µL sample + 50 µL P22-MPs (0.1 mg mL^{-1})	37 ºC	30	700
Washing	3 x 500 µL BPB	RT	5	700
Storage until further use	Resuspension in 80 µL of milli-Q water	-	-	-

3.6.2.1. Evaluation of the bacteriophage purification by electrochemical magneto-immunosensing

As previously mentioned (§ 3.2.8), the lysates of P22 bacteriophage were obtained by infecting exponential cultures of *Salmonella* Typhimurium. After the infection, the bacteria crude phage culture contains, apart from the phages themselves, the bacterial debris, proteins, nucleic acids and endotoxins or lipopolysaccharides (LPSs). Further purification steps are mandatory, as described in § 3.2.8.

In this dissertation, a novel method for the evaluation of bacterial debris, specifically LPSs, in bacteriophage cultures based on electrochemical magneto-immunosensing is presented. To obtain phage lysate with different amount of LPSs, the following purifications were performed: dialysis-purified bacteriophages and full-purified bacteriophages following the procedure described in § 3.2.8. Non-purified bacteriophages and a negative control containing the phage media ($MgSO_4$ 10 mmol L^{-1} in milli-Q water) were also processed as outlined in Table 3.8 and Figure 3.11.

Firstly, the IMS was performed as described in § 3.6.1, a volume of 10 µL of anti-*Salmonell*a-MPs was added to a volume of 140 µL of the sample. An incubation step was performed for 10 min with slight agitation at room temperature. After that, the magnetic particles with the attached-bacteria debris were separated with a magnet and, after removing the supernatant, were incubated with 140 µL of anti-*Salmonella*-HRP antibody (diluted 1/1000 in LB broth) for

30 minutes at room temperature with slight agitation and without any previous washing step. Then the MPs were washed with 140 µL of blocking phosphate buffer twice for 10 min at room temperature. After the final washing step, the modified magnetic particles were captured by dipping the magneto-electrode (m-GEC) inside the reaction tube (as shown in Figure 3.9 ii), and the amount of bacteria debris present was determined by amperometric detection as described in § 3.4.1, using the modified m-GEC electrode as working electrode.

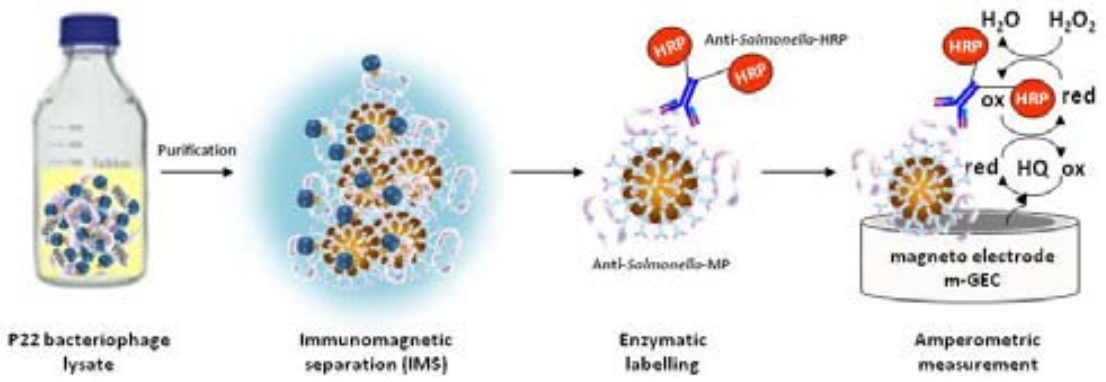

Figure 3.11. Schematic representation of the electrochemical strategy for the evaluation of bacteriophage purification.

Table 3.8. Protocol for the evaluation of the bacteriophage purification.

Step	Description	Temperature	Time (min)	Shaking (rpm)
IMS	140 µL bacteriophage + 10 µL anti-*Salmonell*a-MPs (0.1 mg mL^{-1})	RT	10	700
Enzymatic labelling	140 µL anti-*Salmonella*-HRP (1/1000 in LB broth)	RT	30	700
Washing	2 x 140 µL BPB	RT	10	700

3.6.2.2. Covalent immobilisation of P22 bacteriophage on magnetic particles and evaluation by Bradford assay, Scanning Electron Microscopy and microbiological culture

The native and purified P22 phage nanoparticles were covalently coupled for the first time to tosyl-activated magnetic particles by an amine linkage, as schematically outlined in Figure 3.12. In order to obtain the greatest ratio tosyl-MP/P22 phage during the immobilisation, the optimisation of different conditions such as tosyl-MP and P22 phage concentrations was

performed. P22 phage-modified magnetic particle conjugate (P22-MP) was obtained as explained in detail below.

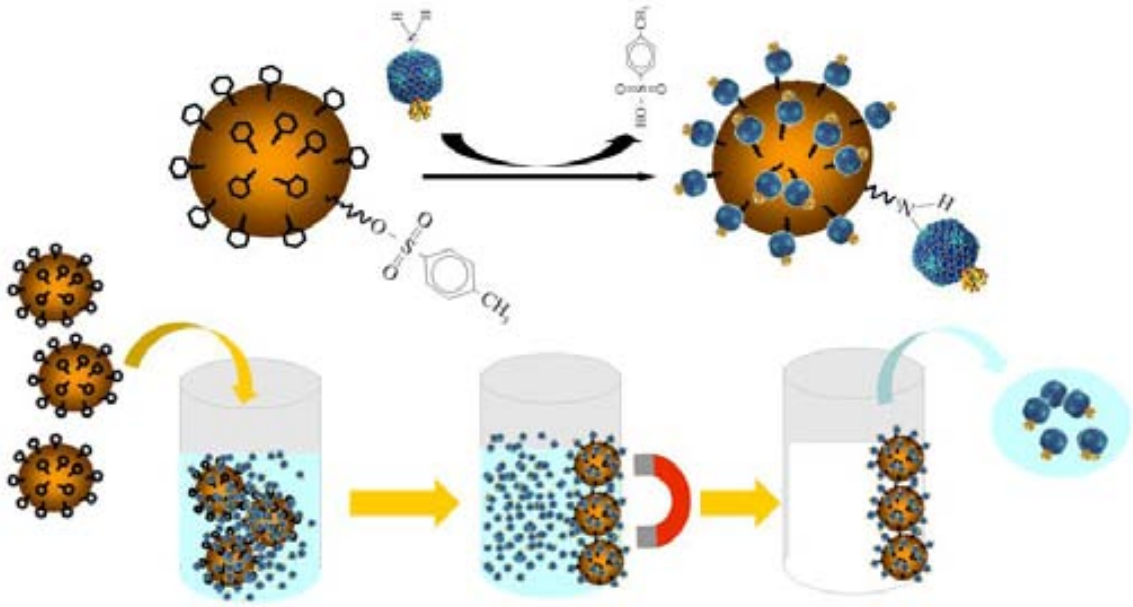

Figure 3.12. Schematic representation of the strategy for immobilising native P22 bacteriophage on tosyl-MPs.

In order to achieve the binding, a volume of 35 or 350 µL of tosylactivated magnetic particles (tosyl-MPs, § 3.2.2) corresponding to 7 x 10^7 and 7 x 10^8 MPs respectively, was washed twice with 1 mL of borate buffer, avoiding foaming. Afterwards, the magnetic particles were resuspended in borate buffer at a final volume of 800 µL or 500 µL. Then, 200 µL or 500 µL of the purified P22 bacteriophage solution (2 x 10^{12} PFU mL^{-1}) was added, reaching a concentration in the immobilisation solution of 4 x 10^{11} PFU mL^{-1} or 1 x 10^{12} PFU mL^{-1} respectively in a total volume of 1 mL. The bacteriophage titre was previously determined by serial dilutions plating onto LB plates using the double agar layered method. The same protocol was performed with active P22 phage as well as with UV-inactivated P22 phage, exposed to UV-C (254 nm) germicidal lamp at a light intensity of 10 $J/m^2/s$, in order to avoid the lytic process of the bacteria when needed. The magnetic particles were incubated during 24 h, firstly at 37 ºC for 8 h and at room temperature for further 16 h, with slow tilt rotation, using a rotor for test tubes. After incubation, the supernatant was removed and placed in another tube to perform the quantification of the remaining protein by Bradford test or to count the active phages by double agar layered method.

The P22 phage-modified magnetic particles (P22-MPs) were then washed twice in 1 mL of immobilisation phosphate buffer for 5 minutes at 4 ºC, and then resuspended in 1 mL of

immobilisation tris buffer, and incubated overnight at room temperature, for inactivating the remaining tosyl groups. The P22-MPs were then submitted to a further washing step for 5 minutes at 4 ºC in immobilisation phosphate buffer, and finally resuspended in storage phosphate buffer to reach a 1.0 mg mL^{-1} stock concentration, which was stored at 4 ºC. Before each assay, a volume of P22-MPs corresponding to the desired amount was washed twice with blocking phosphate buffer and resuspended in the appropriate volume. Table 3.9 shows the protocol followed in further sections.

Table 3.9. Optimised protocol for the covalent immobilisation of P22 bacteriophage on tosyl-MPs.

Step	Description	Temp.	Time	Shaking (rpm)
Immobilisation	7 x 10^7 tosyl-MPs in 800 µL BB + 200 µL P22 phage (2 x 10^{12} PFU mL^{-1}) (1 mL in total)	37 ºC / RT	8 h / 16 h	Thermomixer (700) / Rotor (30)
Washing	2 x 1 mL IPB	4 ºC	5 min	Thermomixer (700)
Blocking	1 mL ITB	RT	24 h	Rotor (30)
Washing	1 mL IPB	4 ºC	5 min	Thermomixer (700)
Storage until further use	Resuspension 1mL SPB (1.0 mg mL^{-1})	-	-	-

In order to analyse the efficiency of the P22 bacteriophage coupling to the magnetic particles, Coomassie-based protein assay (Bradford assay) [19] to determine the amount of viral protein and microbiological culture to quantify the bacteriophages present after the covalent attachment were performed.

The protein concentration of the P22 phage capsid in the supernatant before and after the immobilisation step was determined by the Coomassie-based protein assay (Bradford assay) (§ 3.4.2), as schematically shown in Figure 3.12 (down). With this purpose, a bacteriophage calibration curve was prepared with concentrations ranged from 2 x 10^{10} to 5 x 10^{11} PFU mL^{-1}, in 40 mmol L^{-1} borate buffer and 2 mmol L^{-1} $MgSO_4$ by considering the phage titre as previously determined by serial dilutions plating onto LB plates using the double agar layered method (§ 3.2.8). The Bradford assay was performed according to the kit manufacturer. After the reaction, the plate was shaken for 30 seconds and then incubated for 10 minutes at room temperature. Finally, the absorbance was measured at 620 nm after a short shaking and the standard curve was obtained plotting Abs *vs*. logarithm of phage concentration (PFU mL^{-1}).

A similar approach was performed by the double agar layered method for counting active phages. In this approach, tenfold dilutions of the supernatant after the covalent attachment were plated through double agar layered method as outlined in § 3.2.8 and Figure 3.13 (A and B). In order to obtain better images plates were dyed with tetrazolium 0.01 % and counted after this overnight incubation.

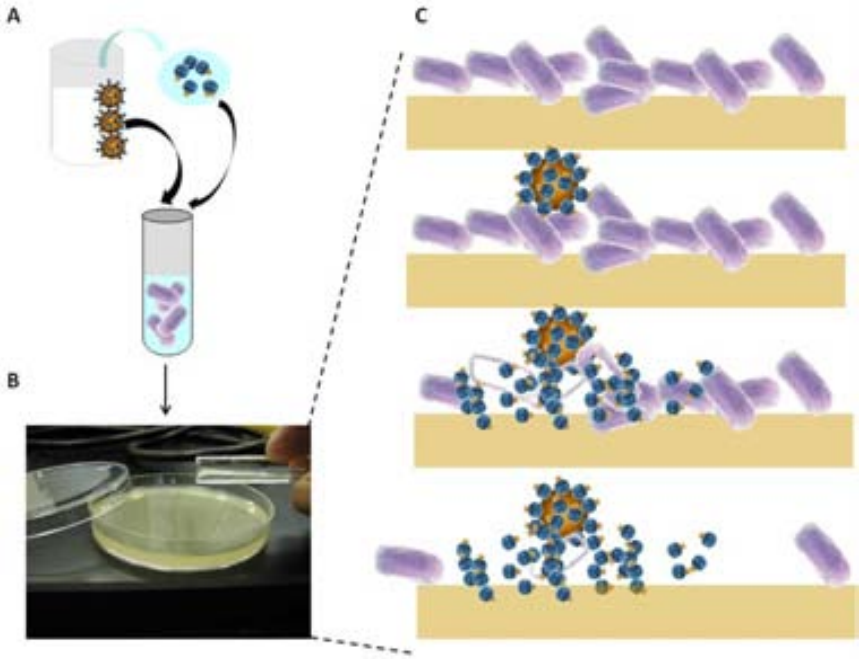

Figure 3.13. Schematic representation of the double agar layered method for counting active phages (A, B), and the formation of plaques in the double agar by active P22 phages immobilised on magnetic particles (C).

After the evaluation of the coupling efficiency, further study of the immobilised P22 bacteriophages was performed by microscopic techniques (SEM) as well as by microbiological cultures, as detailed next.

For the microscopic evaluation by Scanning Electron Microscopy (SEM), a volume of 10 µL of the P22 phage-modified magnetic particles (P22-MPs) in 5 mL of milli-Q water was filtered through a Nucleopore membrane as a support for the SEM microscopy. Before the observation the filters were treated following the *Servei de Microscopia* (UAB) protocol as described in § 3.6.1.1.

As previously addressed, the orientation of the bacteriophages on the solid support is an important issue to consider. This orientation was studied by the double agar layered method and enumeration of plaques by culturing the P22-MPs, since oriented phages immobilised on magnetic particles will produce bacteria attachment and further infection of viable bacteria,

producing the plaques, as outlined in Figure 3.13 (C). To achieved this task, tenfold dilutions of the P22 phage-modified magnetic particles (P22-MPs) were plated through double agar layered method as described in § 3.2.8 and Figure 3.13.

3.6.2.3. Evaluation of the PMS by Scanning Electron Microscopy and microbiological culture

Once the immobilisation of the bacteriophages was performed and the correct orientation of them was evaluated, the bacteria were captured by using «phagomagnetic separation (PMS)» as a first step for the second strategy, "PMS/double-tagging PCR/m-GEC electrochemical genosensing". The evaluation of the PMS was performed by Scanning Electron Microscopy and microbiological cultures. Inactivated bacteriophages by UV radiation were used for the PMS to avoid the lytic cycle in order to keep the attached bacteria as a whole cell while being captured, pre-concentrated and cultured since both SEM and culturing require non-infected bacteria.

For the microscopic evaluation of the attached bacteria on the magnetic particles by SEM, the PMS was performed as previously mentioned in § 3.6.2, Table 3.7 with a concentration of bacteria of 2.9 x 10^7 CFU mL^{-1} in LB broth, a negative control was included as well which contained all reagents except *Salmonella*. After that, 5 mL of milli-Q water containing 10 µL of the P22-MPs with the attached-bacteria were filtered through a Nucleopore membrane as a support for the SEM microscopy. Before the observation the filters were treated following the *Servei de Microscopia* (UAB) protocol as described in § 3.6.1.1.

In order to study the efficiency of the phagomagnetic separation step by microbiological culture, the P22-MPs with the bacteria attached were plated. For each concentration of bacteria ranged from 3.2 x 10^0 to 3.2 x 10^6 CFU mL^{-1} in LB broth including a negative control, a volume of 10 µL of P22-MPs with the attached-bacteria was plated in LB agar. In all cases, in order to achieve statistically reliable counting, between 15 and 300 colonies per plate, the appropriate dilutions were plated and after the incubation for 18 – 24 h at 37 °C the grown colonies were counted.

3.6.3. Electrochemical magneto-genosensing

Both immunomagnetic (IMS) and phagomagnetic (PMS) separations were followed by double-tagging PCR amplication and electrochemical magneto-genosensing (Figure 3.10, strategies 1 and 2) thus achieving the approaches "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and "PMS/double-tagging PCR/m-GEC electrochemical genosensing".

In the case of PMS, UV-inactivated P22 phage nanoparticles were used to avoid the lytic cycle and the release of the genomic DNA of the bacteria while being captured and pre-concentrated, since DNA is required for the double-tagging PCR amplification. For every concentration of bacteria in LB or milk samples captured following §§ 3.6.1 and 3.6.2, the lysis of the attached bacteria on the MPs was performed at 99 °C for 20 min in order to break the cells and to achieve the releasing of the genomic DNA to the solution for the PCR amplification and further electrochemical magneto-genosensing (Figure 3.10, b – f), as described in following sections.

3.6.3.1. Double-tagging PCR amplification and gel electrophoresis detection

As shown in Figure 3.10 (b), a pair of tagged primers with biotin and digoxigenin (Table 3.2, § 3.2.4) was used for the amplification and the double-tagging of the bacterial DNA coming from the bacteria captured by the magnetic particles. The PCR was performed in 50 µL of reaction mixture containing the DNA coming from *Salmonella* captured. Each reaction contained 200 µmol L^{-1} of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 0.5 µmol L^{-1} of the double-tagged set of primers (biotinylated IS*200* forward and digoxigenated IS*200* reverse) and 5 U of polymerase. The reaction was carried out in Expand High Fidelity Kit 1x buffer containing 1.5 mmol L^{-1} of $MgCl_2$. The amplification mixture was exposed, as outlined in Table 3.10, to an initial step at 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and a last step of 7 min at 72 °C. The resulting samples were stored at 4 ºC.

All instances included a blank as a control, which contained LB broth instead of *Salmonella* dilution, a PCR negative control, which contained all reagents except *Salmonella* template in the PCR mixture, as well as a PCR positive control. The amplification products were analysed by electrophoresis on 2 % agarose gel in TAE buffer containing 0.5 µg ml^{-1} ethidium bromide.

ΦX174-Hinf I genome consisting of DNA fragments ranged from 24 to 726 bp was used as a molecular weight marker. The DNA bands were visualised by UV transillumination. As the primers were tagged with biotin and digoxigenin, the amplified DNA (amplicon) was expected to be double-tagged as well with both biotin and digoxigenin in each extreme, respectively.

Table 3.10. Double-tagging PCR protocol for the amplification of *Salmonella* DNA. *Performed 30 cycles.

	Initial step	**DNA denaturation***	**Annealing***	**Extension***	**Last step**
Temperature (ºC)	95	95	53	72	72
Time (sec)	120	30	30	30	420

3.6.3.2. Electrochemical magneto-genosensing of the double-tagged amplicon

This strategy comprises the following steps: 1) Immobilisation of the double-tagged amplicon on streptavidin magnetic particles, where the biotin end of the dsDNA amplicon was immobilised on the streptavidin magnetic particles (Figure 3.10, c); 2) Enzymatic labelling using AntiDig–HRP conjugate able to be attached in the digoxigenin end of the dsDNA amplicon (Figure 3.10, d); 3) Magnetic capture of the modified magnetic particles by the m-GEC electrode (Figure 3.10, e); 4) Amperometric measurements (Figure 3.10, f).

The protocol is described in detail in § 3.5.4. In order to obtain the optimal conditions for the electrochemical genosensing of *Salmonellam,* 1/10 and 1/15 dilutions of the PCR amplicon in milli-Q water, as well as non-diluted amplicon were evaluated.

3.6.3.3. Detection limit, matrix effect and specificity studies

Further studies were also performed, such as limit of detection (LOD), matrix effect and specificity. The LOD was evaluated for both approaches, "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and "PMS/double-tagging PCR/m-GEC electrochemical genosensing" by following the above procedures with solutions ranged from 10^0 to 10^4 CFU mL^{-1} of *Salmonella* in LB broth, a negative control was included which contained all reagents except *Salmonella*. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar. The matrix effect was also

evaluated by processing, as above explained, solutions ranged from 10^0 to 10^4 CFU mL^{-1} of *Salmonella* in both LB broth and skimmed milk (diluted 1/10 in LB broth).

In order to verify the specificity of both approaches, "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and "PMS/double-tagging PCR/m-GEC electrochemical genosensing", the above procedures were also performed with 10^5 CFU mL^{-1} of *Escherichia coli*, 10^5 CFU mL^{-1} of *Salmonella*, a sample containing both bacterial species (10^5 CFU mL^{-1} of each bacterial specie) artificially inoculated in LB and, finally a negative control containing LB broth.

3.6.3.4. Pre-enrichment of milk samples

A pre-enrichment step must be included to fulfil the legislation requirements for milk (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each [29, 30]). In conventional cultural techniques, a pre-enrichment step (18 – 24 h) is usually included to achieve the proliferation of stressed *Salmonella* cells. During food processing, the cells can be injured when exposed to adverse conditions, such as chilling, freezing or drying, thus if a resuscitation step is not included these cells may be missed [31, 32]. The goal of this study was the evaluation of the pre-enrichment time needed not only to achieve the detection limits required by legislation but also to resuscitate injured cells.

The time of pre-enrichment needed to accomplish the legislation was evaluated for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" strategy following the next procedure. A volume of 250 mL of skimmed milk was spiked with 10 CFU of *Salmonella*, corresponding statistically to 1 CFU per 25 mL, five 5-mL portions were diluted 1/10 in LB broth, used as a non-selective broth medium. All samples were incubated at 37 ºC and assayed after 0, 6, 12, and 24 h of pre-enrichment following the procedure described in §§ 3.6.1 and 3.6.3. A positive control (10 CFU per 25 mL) as well as two negative controls (0 CFU mL^{-1}) were also included.

3.6.4. Electrochemical magneto-immunosensing

In the third strategy developed, "IMS/m-GEC electrochemical immunosensing", the detection of the whole bacteria was performed by a double immunological recognition. After the IMS, the enzymatic labelling of the bacteria was also performed using a specific antibody against *Salmonella* labelled with HRP, performing thus the electrochemical magneto-immunosensing as shown in Figure 3.10 a, d – f (3). For the optimisation of the "IMS/m-GEC electrochemical immunosensing" approach, four different immunological procedures were evaluated, which vary in the order of the immunological and the washing steps. The concentration of the anti-*Salmonella*-HRP antibody was also optimised. All these optimisations are described in following section. Other parameters of the assay such as the limit of detection (LOD), matrix effect, specificity and pre-enrichment time needed were also evaluated.

3.6.4.1. Optimisation of the electrochemical magneto-immunosensing procedure

Samples containing 10^6 CFU mL^{-1} of *Salmonella* were processed for each procedure described briefly next (Figure 3.14), the optimal conditions are outlined in Table 3.11. In the procedure Nº 1, the IMS and the enzymatic labelling were performed in one-step. A volume of 10 µL of anti-*Salmonell*a-MPs was added to 500 µL of a sample as well as 140 µL of anti-*Salmonella*-HRP antibody (diluted 1/150; 1/500; 1/1000; 1/3000; 1/5000; 1/6000 in milli-Q water and 1/1000 in LB), and were incubated for 30 min in slight agitation at room temperature.

In the procedure Nº 2, the IMS was performed after a pre-incubation step of the bacteria with the anti-*Salmonella*-HRP antibody. A volume of 500 µL of a sample was preincubated with 140 µL of anti-*Salmonella*-HRP antibody (diluted 1/150; 1/500; 1/1000; 1/3000; 1/5000; 1/6000 in milli-Q water and 1/1000 in LB) for 30 min in slight agitation at room temperature. Without any washing or separation step, 10 µL of anti-*Salmonell*a-MPs were added and incubated for further 10 minutes.

In the procedure Nº 3, the enzymatic labelling was performed after the IMS. A volume of 500 µL of a sample was pre-incubated with the 10 µL of anti-*Salmonell*a-MPs for 10 min in slight agitation at room temperature. After discarding the supernatant, the collected MPs were further incubated with 140 µL of anti-*Salmonella*-HRP antibody (diluted 1/1000 in LB or milli-Q water) were added and incubated for further 30 minutes.

Finally, the procedure Nº 4 is a variation of procedure Nº 3 including washing steps. First, a volume of 500 µL of a sample was incubated with the 10 µL of anti-*Salmonell*a-MPs for 10 min in slight agitation at room temperature. Two washing steps were performed for 10 min with 500 µL of BPB in slight agitation at room temperature. After discarding the supernatant, the collected MPs were further incubated with 140 µL of anti-*Salmonella*-HRP antibody (diluted 1/150; 1/500; 1/1000; 1/3000; 1/5000; 1/6000 in milli-Q water and 1/1000) for 30 minutes.

In all cases (procedures Nº 1 to 4), the collected modified MPs were washed twice for 10 min with 650 µL of BPB in slight agitation at room temperature and finally resuspended in 140 µL of BPB. The modified MPs were captured by dipping the magneto-electrode (m-GEC) inside the reaction tube, as shown in Figure 3.10 (e) and finally, the electrochemical measurements were performed as described in § 3.4.1 using the modified m-GEC electrode as working electrode (Figure 3.10 (f)).

Table 3.11. Optimised protocol for the "IMS/m-GEC electrochemical immunosensing" approach (Procedure Nº 3).

Step	Description	Temperature	Time	Shaking (rpm)
IMS	500 µL sample + 10 µL anti-*Salmonell*a-MPs (0.1 mg mL^{-1})	RT	10	700
Enzymatic labelling	140 µL of anti-*Salmonella*-HRP antibody (1/1000 in LB)	RT	30	700
Washing	2 x 650 µL BPB	RT	10	700

3.6.4.2. Detection limit, matrix effect and specificity studies

Other parameters of the assay such as limit of detection (LOD), matrix effect and specificity were also evaluated. The LOD and the matrix effect were evaluated for the "IMS/m-GEC electrochemical immunosensing" approach by following the procedure Nº 3 (Table 3.11) with solutions ranged from 10^0 to 10^6 CFU mL^{-1} of *Salmonella* in both LB broth and skimmed milk (diluted 1/10 in LB broth), a negative control was included for both media which contained all reagents except *Salmonella*. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar.

In order to verify the specificity of the "IMS/m-GEC electrochemical immunosensing" approach, the procedure Nº 4 was performed with 10^6 CFU mL^{-1} of *Escherichia coli*, 10^6 CFU

mL^{-1} of *Salmonella*, a sample containing both bacterial species (10^6 CFU mL^{-1} of each bacterial specie) artificially inoculated in LB and, finally, a negative control containing LB broth.

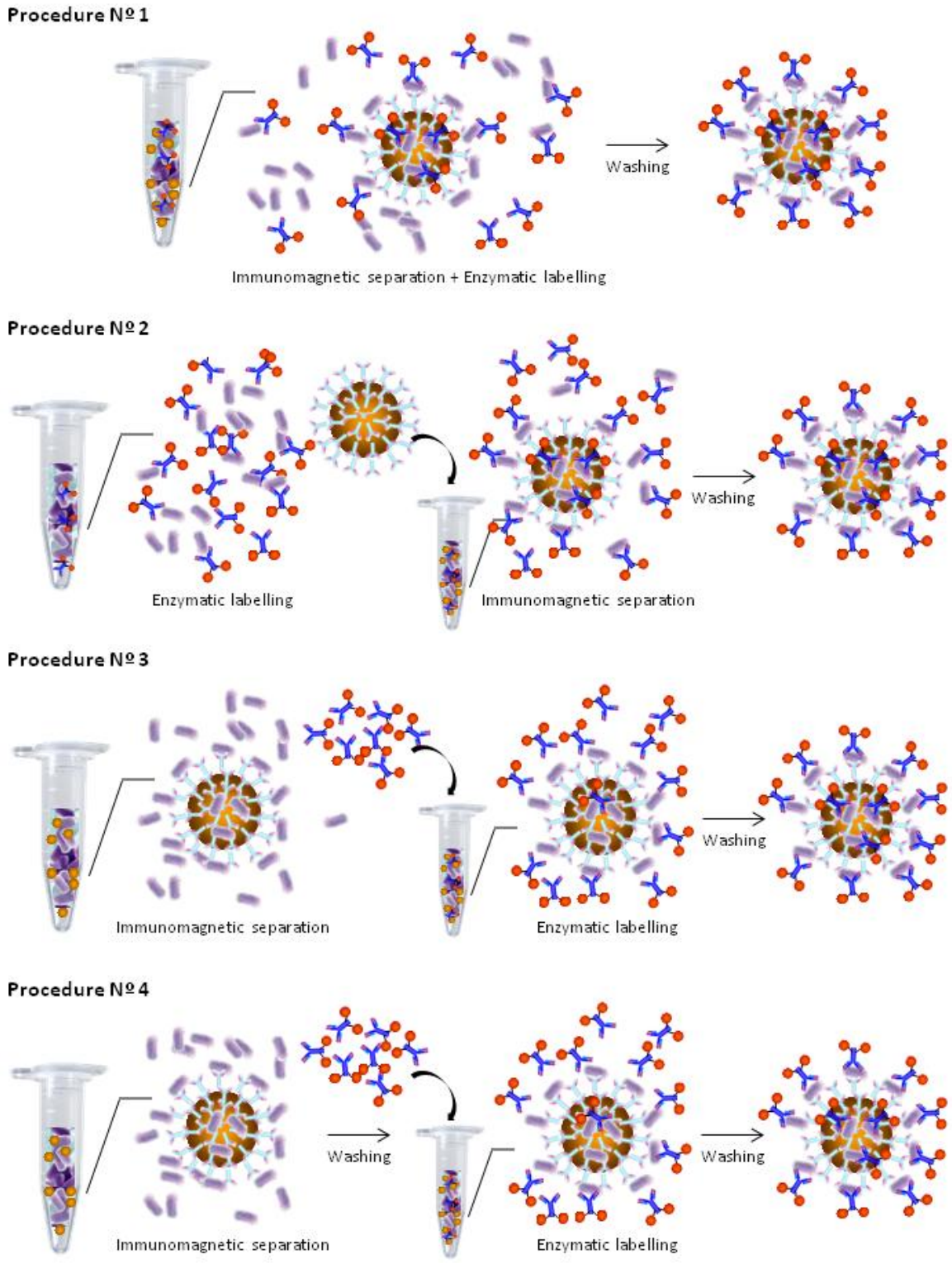

Figure 3.14. Schematic representation of the different procedures evaluated for the "IMS/m-GEC electrochemical immunosensing" approach.

3.6.4.3. Pre-enrichment of milk samples

As explained in § 3.6.3.4, a pre-enrichment step must be included to fulfil the legislation requirements for milk (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each [29, 30]). The pre-enrichment time needed to accomplish the legislation was evaluated for the "IMS/m-GEC electrochemical immunosensing" strategy following the next procedure. A volume of 250 mL of skimmed milk was spiked with 10 CFU of *Salmonella*, corresponding statistically to 1 CFU per 25 mL, five 5-mL portions were diluted 1/10 in LB broth, used as a non-selective broth medium. All samples were incubated at 37 ºC and assayed after 0, 4, 6, 8, 12, and 24 h of pre-enrichment following the procedure Nº 3 described in Table 3.11. A positive control (10 CFU per 25 mL) and a negative control (0 CFU mL^{-1}) were also evaluated.

3.7. OPTICAL IMMUNOASSAY OF *SALMONELLA*

In this section, an optical immunoassay for the detection of *Salmonella* based on magnetic particles is described. The detection of the whole bacteria was performed by a double immunological recognition, as previously explained for the electrochemical magneto-immunosensing in § 3.6.4. Following previous optimisations, the bacteria were captured from samples and pre-concentrated by immunomagnetic separation. After the IMS, the enzymatic labelling of the bacteria was also performed using a specific antibody against *Salmonella* labelled with HRP, performing thus the optical magneto-immunoassay.

The optical magneto-immunoassay consisted briefly of the following steps, as schematically depicted in Figure 3.15: a) Immunomagnetic Separation (IMS); b) Enzymatic labelling using anti-*Salmonella*-HRP antibody, and c) Absorbance measurements.

3.7.1. Magneto-immunoassay optimisation

As schematised in Figure 3.16, in order to select optimal concentrations of both anti-*Salmonell*a-MPs and anti-*Salmonella*-HRP antibody, two-dimensional (2D) serial dilution experiments were performed using the sandwich magneto-immunoassay format with optical detection outlined in Figure 3.15. Optimal concentrations were chosen to produce a signal about 1 absorbance unit. With this aim, decreasing concentrations of anti-*Salmonell*a-MPs were assayed with different concentrations of anti-*Salmonella*-HRP antibody while the amount of bacteria was fixed in 10^7 CFU mL^{-1} (Figure 3.16, A). Afterwards, the anti-*Salmonell*a-MPs concentration was fixed in 0.125 mg mL^{-1}, in agreement with the manufacturer recommendations, and tenfold dilutions of *Salmonella* ranged from 10^0 to 10^8 CFU mL^{-1} were processed with different concentrations of anti-*Salmonella*-HRP antibody (Figure 3.16, B). The 2D experiments were made covering a large range of magnetic particles concentration (ranged from 0.02 to 0.25 mg mL^{-1}) and anti-*Salmonella*-HRP antibody dilutions (ranged from 1/1000 to 1/20000) to get a global overview of the behaviour and make the first adjustments. Other experimental parameters (like surfactant concentration, ionic strength and pH) were used as optimised in previous works [33].

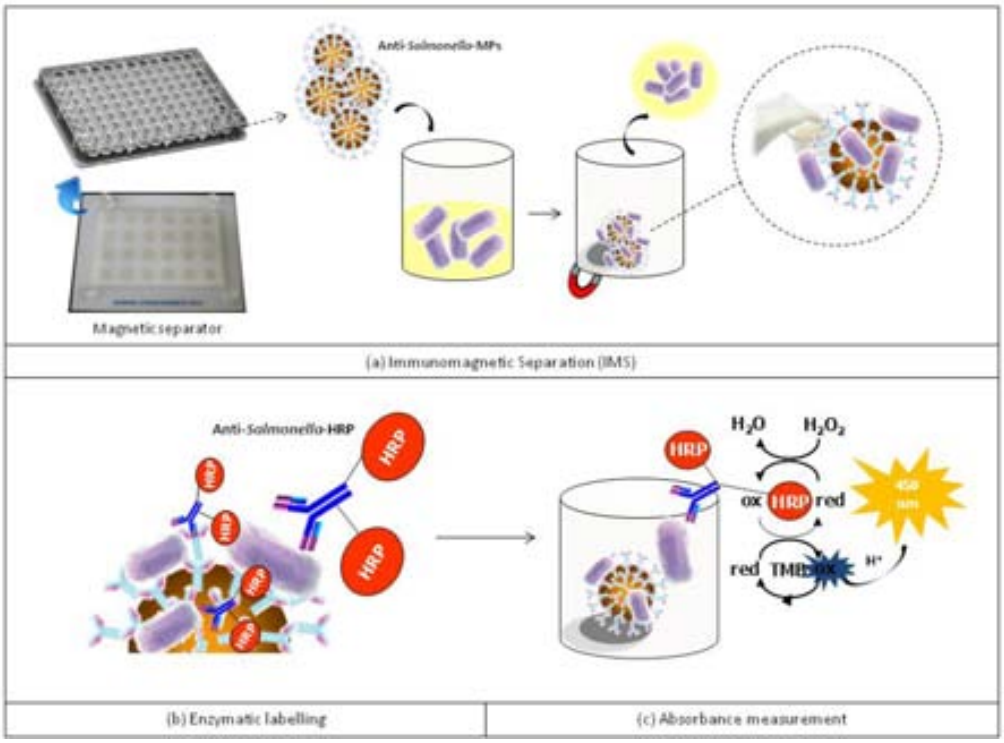

Figure 3.15. Schematic representation of the optical magneto-immunoassay for *Salmonella* spp. detection.

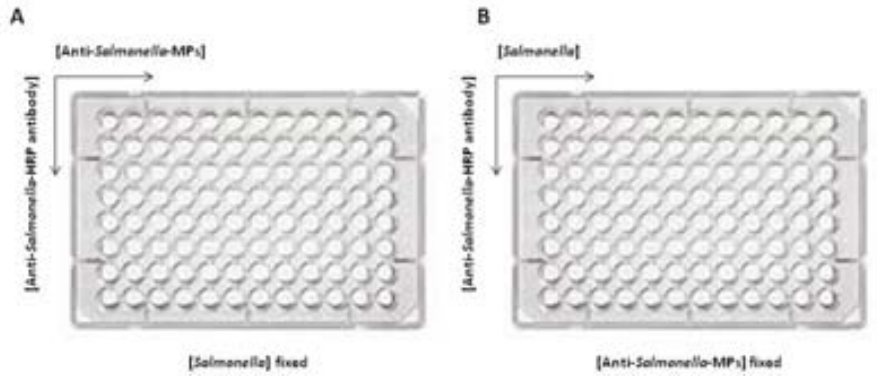

Figure 3.16. Schematic representation of the two–dimensional (2D) serial dilution experiments for the optimisation of the magneto-immunoassay.

The magneto-immunoassay procedure is briefly described next and outlined in Table 3.12 (all the referred quantities are "the amounts added per well"). Firstly, the anti-*Salmonell*a-MPs were added to 100 µL of sample (bacterium dilution or LB broth) and was incubated for 10 min at room temperature while shaking (Figure 3.15, a). After the IMS, two washing steps were performed for 5 min with 100 µL of 1 %-BPB in slight agitation at room temperature. After discarding the supernatant, the enzymatic labelling was performed by incubating with 100 µL of the anti-*Salmonella*-HRP antibody diluted in 1 %-BPB for 30 min at room temperature while shaking (Figure 3.15, b). Three washing steps for 5 min with 100 µL of BPB in slight agitation at room temperature were performed. After each incubation or washing step, a 96-well magnet

plate separator was positioned under the microtitre plate until pellet formation on the bottom corner, followed by supernatant separation. Finally, the optical detection was performed, as explained in § 3.4.2, by adding 100 µL of substrate TMB/H_2O_2 solution to the well and incubating the microplate for 30 min at room temperature under dark conditions. The enzymatic reaction was stopped by adding 50 µL of H_2SO_4 (2 mol L^{-1}) and the absorbance measurement of the supernatants was performed at 450 nm after transferring a volume of 140 µL to polystyrene microtitre plates (Figure 3.15, c).

Table 3.12. Optimised protocol for the optical magneto-immunoassay of *Salmonella.*

Step	Description	Temperature	Time	Shaking (rpm)
IMS	100 µL sample + 2.5 µL anti-*Salmonell*a-MPs (0.125 mg mL^{-1})	RT	10	700
Washing	2 x 100 µL 1 %-BPB	RT	5	700
Enzymatic labelling	100 µL of anti-*Salmonella*-HRP antibody (1/4000 or 1/8000 diluted in 1 %-BPB)	RT	30	700
Washing	3 x 100 µL BPB	RT	5	700
Substrate addition	100 µL of substrate TMB/H_2O_2 solution (darkness)	RT	30	700
Stopping	50 µL of H_2SO_4 (2 mol L^{-1})	-	-	-

3.7.2. Detection limit, matrix effect and specificity studies

After the optimisation of the immunoreagents, other features of the assay such as limit of detection (LOD), matrix effect and specificity were also evaluated. The LOD and the matrix effect were evaluated for the optical magneto-immunoassay of *Salmonella* by following the above procedure (Table 3.12) with optimal antibody dilutions (1/4000 and 1/8000) and solutions ranged from 10^0 to 10^6 CFU mL^{-1} of *Salmonella* in both LB broth and skimmed milk (diluted 1/10 in LB broth). A negative control was also included for both media, which contained all reagents except *Salmonella*. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar.

In order to verify the specificity of this approach, the procedure outlined in Table 3.12 was performed with 1/8000 antibody dilution for 10^5 CFU mL^{-1} of *Escherichia coli*, 10^5 CFU mL^{-1} of *Salmonella*, a sample containing both bacterial species (10^5 CFU mL^{-1} of each bacterial specie) artificially inoculated in LB and, finally, a negative control containing LB broth.

3.8. ELECTROCHEMICAL GENOSENSING OF *SALMONELLA, LISTERIA* AND *E. COLI*

The last strategy presented in this dissertation is based on the electrochemical magneto-genosensing of the three most common pathogenic bacteria in food safety (*Salmonella, Listeria* and *E. coli*). As outlined in Figure 3.17, after the lysis of the bacteria, further amplification of the genetic material by tagging PCR with a labelled set of specific primers for each pathogen was performed. Labelled primers were selected for the specific amplification of the *invA* (278 bp), *prfA* (217 bp) and *eaeA* (151 bp) genes related to *S. enterica, L. monocytogenes* and *E. coli* 0157:H7 respectively (Table 3.2, § 3.2.4). The tagged amplicons were then immobilised on silica magnetic particles based on the nucleic acid-binding properties of silica particles in the presence of the chaotropic agent guanidinium thiocyanate. To confirm the identity of the bacteria, three different electrochemical reporters were used: AntiFluorescein-HRP, Streptavidin-HRP and AntiDigoxigenin-HRP for *Salmonella, Listeria* and *E. coli* amplicons, respectively. The optimisation of the silica magnetic particles as platform for electrochemical genosensing is described in following sections.

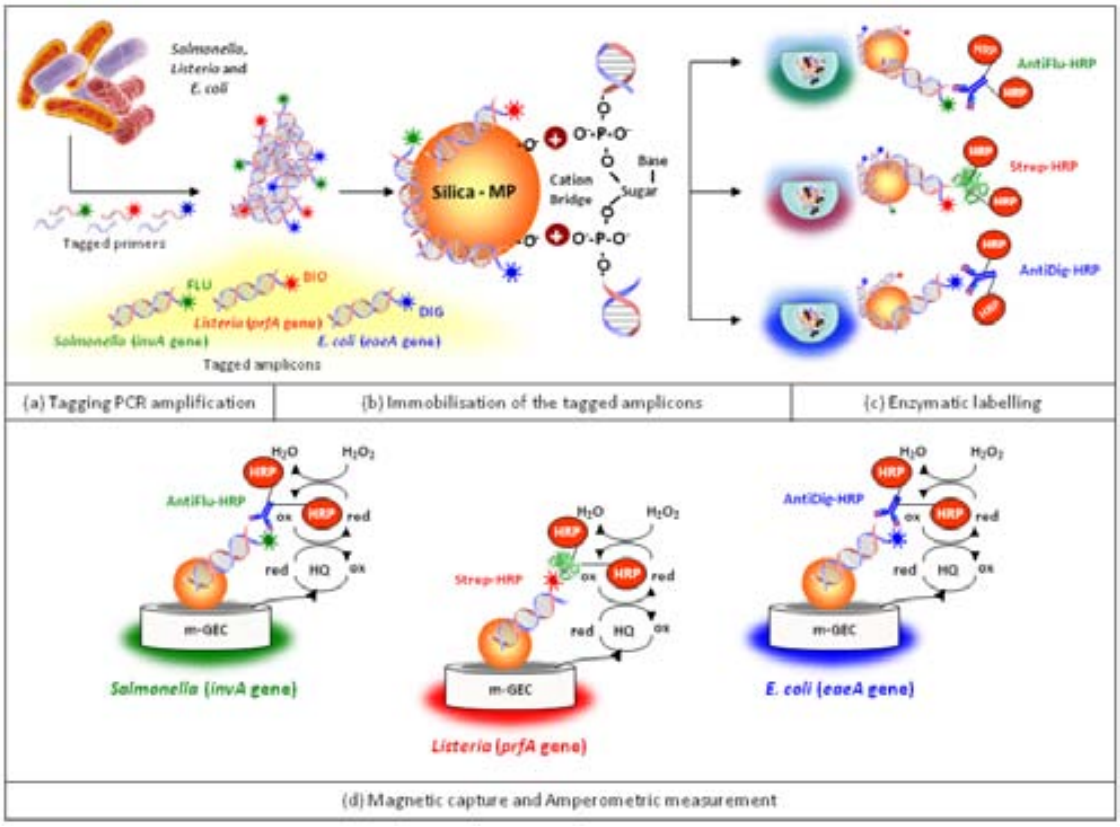

Figure 3.17. Schematic representation of the electrochemical magneto-genosensing for *Salmonella, Listeria* and *E. coli*.

3.8.1. Tagging PCR amplification and gel electrophoresis detection

The gene targets chosen for the amplification of different specific sequence related to *Salmonella, Listeria* and *E. coli* were the invA (invasion protein A) gene for *S. enterica*, the prfA (transcriptional activator of the virulence factor) gene for *L. monocytogenes* and the eaeA (attaching and effacing A) gene for *E. coli* 0157:H7, since these are described in the recent literature [5] as being among the most specific and reliable genetic targets for the considered microorganisms. As shown in Figure 3.17 (a), tagged primers with fluorescein, biotin and digoxigenin (see Table 3.2, § 3.2.4) were used for the amplification and tagging of the bacterial DNA. All PCR reactions were performed similar as reported previously [5]. PCR final volume was 100 µL using 100 ng of purified total DNA of each microorganism as template (extracted and purified as explained in § 3.2.7). Each reaction contained 0.4 mmol L^{-1} of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP); 0.5 µmol L^{-1} of the invA-F(FLU) and invA-R primers; 0.3 µmol L^{-1} of the prfA-F, prfA (BIO)-R, eae (DIG)-F and eae-R primers, and 0.1 U μL^{-1} of polymerase. The reaction was carried out in Expand High Fidelity Kit 1x buffer containing 4 mmol L^{-1} of $MgCl_2$. The amplification mixtures were submitted, as outlined in Table 3.13, to an initial step at 95 ºC for 7 min followed by 40 cycles consisting of dsDNA denaturation at 95 ºC for 50 s, primer annealing at 54 ºC for 40 s, primer extension at 72 ºC for 50 s and final elongation at 72 ºC for 5 min. The resulting samples were stored at 4 ºC.

The tagged amplicons were analysed by conventional gel electrophoresis on 4 % agarose gel containing 0.5 X TAE and ethidium bromide staining. ΦX174-Hinf I genome consisting of DNA fragments ranged from 24 to 726 bp was used as a molecular weight marker. The DNA bands were visualised by UV transillumination. As the primers were tagged with fluorescein, biotin and digoxigenin, the amplified DNA (amplicon) was expected to be tagged as well, and thus able to be analysed also by electrochemical magneto-genosensing by using the m-GEC electrodes (§ 3.8.3).

DNA extracts from the three pathogens were compared to the same test performed on the pathogens individually. In all cases, 100 ng of each pathogen and only corresponding primers were used. All these amplifications included several negative controls, which contained all the PCR reagents adding milli-Q water instead of *Salmonella, Listeria* and *E. coli* DNA templates.

Table 3.13. Tagging PCR protocol for the amplification of *Salmonella, Listeria* and *E. coli* DNA. *Performed 40 cycles

	Initial step	DNA denaturation*	Annealing*	Extension*	Last step
Temperature (ºC)	95	95	54	72	72
Time (sec)	420	50	40	50	300

3.8.2. Optimisation of the electrochemical magneto-genosensing procedure

The electrochemical magneto-genosensing strategy of the tagged amplicons comprises the following steps, as outlined in Figure 3.17 (b – d): (i) Immobilisation of the tagged amplicons on silica magnetic particles by electrostatic forces and hydrogen bond formation; (ii) Enzymatic labelling with the electrochemical reporters AntiFlu-HRP, Strep-HRP and AntiDig–HRP, in three different reaction chambers, labelled as green, red and blue; (iii) Magnetic capture of the modified magnetic particles by the three m-GEC electrodes, labelled as green, red and blue for the detection of *Salmonella, Listeria and E. coli,* respectively, and amperometric measurements.

The immobilisation of tagged amplicons on silica magnetic particles was evaluated by comparing four different procedures (named A to D). Further optimisation of other experimental parameters such as concentration of magnetic particles, agitation, temperature incubation and concentration of enzymatic labels was performed as well, as described in detail in following sections.

3.8.2.1. Optimisation of the tagged amplicon immobilisation on silica magnetic particles

The immobilisation of the tagged amplicons on silica magnetic particles (Figure 3.17, b) was optimised taking the DIG-tagged amplicon of the *eaeA* gene for *E. coli* detection as a model of the other coding tags. Four different procedures (named A to D) with some slight modifications of the Gonzalez *et al.* [34] and Boom *et al.* [35] previous works as well as a commercial approach (Dynabeads SILANE genomic DNA kit), were evaluated in terms of immobilisation performance. In all cases, the negative as well as the positive amplification products labelled with digoxigenin were processed. All procedures are described next:

- *Procedure A.* According to the kit manufacturer (Dynabeads SILANE genomic DNA kit), a volume of 5 µL of silica-MPs (stock, 40 mg mL^{-1}) was washed in binding buffer and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 90 µL of lysing/binding buffer with isopropanol and incubated for 10 min at room temperature without shaking. Two washing steps were then performed in 120 µL of washing buffer 1 followed by two more washing steps performed in 120 µL of washing buffer 2. All buffers provided with the kit.

- *Procedure B.* According to Gonzalez *et al.* a volume of 5 µL of silica-MPs (stock, 40 mg mL^{-1}) was washed in binding buffer (BB) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at room temperature without shaking. Two washing steps were then performed in 80 µL of tris washing buffer (TWB).

- *Procedure C.* According to Gonzalez *et al.* a volume of 5 µL of silica-MPs (stock, 40 mg mL^{-1}) was washed in BB and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of Triton-binding buffer (TBB) for 10 min at room temperature without shaking. A washing step was then performed in 80 µL of BB followed by another washing step in 80 µL of TWB.

- *Procedure D.* According to Boom *et al.* a volume of 5 µL of silica-MPs (stock, 40 mg mL^{-1}) was washed in BB and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of TBB for 10 min at room temperature without shaking. Silica-MPs were then washed as follows: twice with BB, twice with ethanol 70 % (v/v), and once with acetone. After all the washing steps, the silica particles inside tubes were dried for 10 min at 56 ºC.

In all cases (procedures A to D), after the immobilisation of the amplicon on silica-MPs the enzymatic labelling (Figure 3.17, c) was performed by incubating with AntiDig–HRP (60 µg) in BTB at a final volume of 140 µL for 30 min at 42 ºC. Two washing steps were then performed for 10 min at 42 ºC in 140 µL of TB. The modified MPs were captured by dipping the magneto-electrode (m-GEC) inside the reaction tube, and finally, the electrochemical measurements were performed as described in § 3.4.1 using the modified m-GEC electrode as working electrode (Figure 3.17, d).

Once different procedures were evaluated, further optimisation of the procedure B, based on Gonzalez *et al.* including the incubation temperature (room temperature –procedures B1

and B2–, or 55 ºC –procedures B3 and B4–) and shaking conditions (with 700 rpm agitation –procedures B1 and B3–, or without 700 rpm agitation –procedures B2 and B4–) during the tagged-amplicon immobilisation on silica-MPs was performed, as detailed next:

- *Procedure B1:* 5 µL of silica-MPs washed in BB (stock, 40 mg mL^{-1}) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at room temperature with shaking at 700 rpm. Two washing steps were then performed in 80 µL of TWB.

- *Procedure B2:* 5 µL of silica-MPs washed in BB (stock, 40 mg mL^{-1}) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at room temperature without shaking. Two washing steps were then performed in 80 µL of TWB.

- *Procedure B3:* 5 µL of silica-MPs washed in BB (stock, 40 mg mL^{-1}) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at 55 ºC with shaking at 700 rpm. Two washing steps were then performed in 80 µL of TWB.

- *Procedure B4:* 5 µL of silica-MPs washed in BB (stock, 40 mg mL^{-1}) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at 55 ºC without shaking. Two washing steps were then performed in 80 µL of TWB.

In all cases (procedures B1 to B4), after the immobilisation of the amplicon on silica magnetic particles the enzymatic labelling and the amperometric measurements were performed as above described.

3.8.2.2. Optimisation of the amount of silica magnetic particles

Besides the binding procedure, another important parameter for the electrochemical magneto-genosensing strategy is the concentration of silica magnetic particles. The optimal amount of particles was evaluated by Scanning Electron Microscopy as well as by electrochemical magneto-genosensing.

The SEM technique was used to evaluate the distribution of silica magnetic particles in different concentrations on the surface of m-GEC with the purpose of confirming the optimal amount of silica magnetic particles for the electrochemical signal transduction. A volume of 5 µL of silica-MPs from different stock solutions (40 mg mL^{-1}, 20 mg mL^{-1} and 10 mg mL^{-1}, washed

and diluted in BB) were resuspended at a final volume of 140 µL of milli-Q water. The non-modified silica-MPs were captured by dipping the magneto-electrode (m-GEC) inside the tube. In all cases, samples were observed at the same acceleration voltage (30 kV) with different resolutions (2, 5 and 15 µm).

The electrochemical magneto-genosensing was performed by processing both the negative and the positive amplification products of the DNA extract from the mixture of the three pathogens following procedure B4 as follows: 5 µL of silica-MPs from different stock solutions (40 mg mL^{-1}, 20 mg mL^{-1} and 10 mg mL^{-1}, washed and diluted in BB) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at 55 ºC without shaking. Two washing steps were then performed in 80 µL of TWB. Further enzymatic labelling was performed by using AntiFlu-HRP (60 µg), Strep-HRP (60 µg) or AntiDig–HRP (60 µg), in BTB at a final volume of 140 µL for 30 min at 42 ºC. Two washing steps were then performed for 10 min at 42 ºC in 140 µL of TB. After the final washing step, the modified MPs were captured and the electrochemical measurements were performed as described in § 3.4.1.

3.8.2.3. Optimisation of the enzymatic labelling step

The enzymatic labelling with the electrochemical reporters AntiFlu-HRP, Strep-HRP and AntiDig–HRP able to detect the fluorescein, biotin and digoxigenin coding tags of the dsDNA amplicons was also optimised. Both negative and positive amplification products of the DNA extract from the mixture of the three pathogens were processed following procedure B4 as follows: 5 µL of silica-MPs (10 mg mL^{-1}, washed and diluted in BB) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at 55 ºC without shaking. Two washing steps were then performed in 80 µL of TWB. Further enzymatic labelling was performed by adding variable quantities of AntiFlu-HRP, Strep-HRP or AntiDig–HRP (60 µg, 30 µg and 10 µg), in BTB at a final volume of 140 µL for 30 min. Two washing steps were then performed for 10 min in 140 µL of TB. Both incubation and washing steps were performed at 42 ºC as well as 25 ºC for each amount of reporter. After the final washing step, the modified MPs were captured and the electrochemical measurements were performed as described in § 3.4.1.

3.8.3. Electrochemical magneto-genosensing of *Salmonella, Listeria* and *E. coli* tagged amplicons

After optimising the main parameters for the electrochemical magneto-genosensing of the tagged amplicons on silica magnetic particles using the DIG-tagged amplicon of the *eaeA* gene for *E. coli* detection as a model of the other coding tags, the evaluation of the DNA extracts from the three pathogens simultaneously (*Salmonella, Listeria and E. coli*) as well as the pathogens individually were processed. For the immobilisation of the tagged amplicons on silica magnetic particles (Figure 3.17, b) 5 µL of silica-MPs (10 mg mL^{-1}, washed and diluted in BB) and 30 µL of the diluted amplicon (1/10) were added in an Eppendorf tube with 45 µL of BB for 10 min at 55 ºC without shaking. Two washing steps were then performed in 80 µL of TWB. The enzymatic labelling (Figure 3.17, c) was performed in three different reaction chambers, labelled as green, red and blue by using AntiFlu-HRP (10 µg), Strep-HRP (10 µg) or AntiDig–HRP (10 µg), respectively, in BTB at a final volume of 140 µL for 30 min at 25 ºC. Two washing steps were then performed for 10 min at 25 ºC in 140 µL of TB. After the final washing step, the modified magnetic particles were captured by the three m-GEC electrodes, labelled as green, red and blue for *Salmonella, Listeria and E. coli* respectively, and the amperometric measurements (Figure 3.17, d) were performed as described in § 3.4.1.

Table 3.14. Optimised protocol for the electrochemical magneto-genosensing of *Salmonella, Listeria* and *E. coli.*

Step	Description	Temperature	Time	Shaking (rpm)
Immobilisation	5 µL of silica-MPs (0.05 mg) + 30 µL amplicon (diluted 1/10) + 45 µL of BB	55 ºC	10	-
Washing	2 x 80 µL TWB	RT	1	-
Enzymatic labelling	AntiFlu-HRP (10 µg), Strep-HRP (10 µg) or AntiDig–HRP (10 µg) in BTB (final volume 140 µL)	RT	30	700
Washing	2 x 140 µL TB	RT	10	700

3.9. REFERENCES

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4

RESULTS AND DISCUSSION

The results obtained for all the strategies formerly described are presented and discussed in the following sections. In addition to the detailed description of the results given in the published articles (§ 6), an extended discussion of all the strategies developed is presented. Briefly, the electrochemical characterisation of different electrodes (Av-GEB and m-GEC), the evaluation of different electrochemical platforms for genosensing applications, electrochemical genosensing based on several magnetic particles for single and simultaneous bacterial detection and both electrochemical immunosensing and optical magneto-immunoassay, including their application in food samples are presented in the following sections.

4.1. CONSTRUCTION AND CHARACTERISATION OF ELECTRODES BASED ON GRAPHITE-EXPOXY COMPOSITES AND GRAPHITE-EXPOXY BIOCOMPOSITES

4.1.1. Electrochemical characterisation of the electrodes by cyclic voltammetry

The electrodes used in this dissertation, based on magneto graphite-epoxy composite (m-GEC) and avidine graphite-epoxy biocomposite (Av-GEB), have been thoroughly studied in the group. Variations in the electrochemical behaviour after modification of the composite with a magnet or biological compounds such as avidin were formerly studied [1]. In this work, the reproducibility of the construction and renewal of the surface for both electrodes (m-GEC and Av-GEB), as well as the optimisation of the curing temperature for the construction of the m-GEC electrodes were evaluated.

As previously mentioned in § 3.3.4, both types of electrodes were characterised by cyclic voltammetry using the redox couple benzoquinone/hydroquinone. This characterisation allows not only the determination of the reduction potential needed during the amperometric measurements (§ 3.4.1), but also the evaluation of the electrochemical performance.

Cyclic voltammograms displayed in Figures 4.1 – 4.3 are the result of plotting current measured versus potential applied. Two measured parameters of interest on these cyclic voltammograms are the ratio of peak currents i_{pa}/i_{pc} and the separation of peak potentials ($\Delta E_p = E_{pa} - E_{pc}$). When the charge–transfer reaction is reversible, there is no surface interaction between the electrode and the reagents, and the redox products are stable (at least in the time frame of the experiment), resulting in anodic and cathodic current peaks of similar values ($i_{pa}/i_{pc} = 1.0$). In addition, for such a system it can be shown that the separation between peaks is following diffusion-controlled Nernst behaviour giving values of 59/n mV (for a n electron transfer reaction) at all scan rates. Due to the two electrons transferred in the redox couple benzoquinone/hydroquinone (Figure 3.3, C), a value of 30 mV of separation between peaks is expected. However, the measured value for a reversible process is generally higher due to uncompensated solution resistance and non-linear diffusion [2].

Figure 4.1 shows the comparison of the electrochemical behaviour for m-GEC electrodes cured at different temperatures. The performance of one m-GEC electrode cured at 42 ºC for 1 week (blue), the same m-GEC electrode cured for an additional 24 h at 80 ºC (red) and another m-GEC electrode from a different batch cured at 80 ºC for 1 week (green) are compared. Table 4.1 outlines the parameters calculated from these voltammograms. In all cases, similar ratio

about 1.0 of anodic to cathodic peak current (i_{pa}/i_{pc}) was obtained. The importance of the curing temperature should be emphasised, the more temperature is applied the more the anodic and cathodic current are raised and the higher reduction in the difference between peak potentials is obtained (from 634 to 144 mV), showing thus better charge-transfer of the material. From these values, it is clear that better electrochemical performance was achieved when the composite is more cured or mature, and this effect can be achieved by means of the time or, shorter, by increasing the temperature of curing.

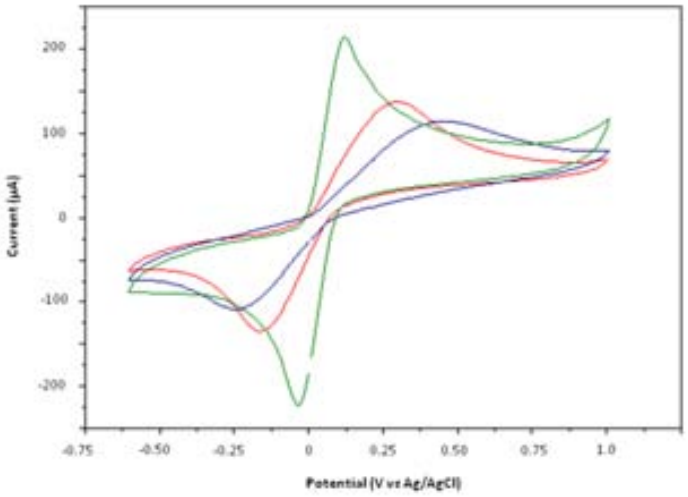

Figure 4.1. Cyclic voltammograms using as working electrode the m-GEC cured at 42 ºC for 1 week (blue), the same m-GEC cured for an additional 24 h at 80 ºC (red) and a different m-GEC cured at 80 ºC for 1 week (green). In all cases, a volume of 20 mL of phosphate buffer with 1.8 mmol L^{-1} of hydroquinone was used. The scan rate was 100 mV s^{-1}.

Table 4.1. Potential and current values of the anodic and cathodic peaks, separation between peaks (ΔE_p) and ratio of anodic to cathodic peak current (i_{pa}/i_{pc}) obtained from cyclic voltammetries of m-GEC electrodes at different curing temperatures.

Time	Temp. (ºC)	E_{pa} (mV)	E_{pc} (mV)	ΔE_p (mV)	i_{pa} (µA)	i_{pc} (µA)	i_{pa}/i_{pc} (µA)
1 week	42	393	-241	634	74.3	-75.5	0.98
1 week / 24 h	42 / 80	279	-153	432	120.7	-117.4	1.03
1 week	80	117	-27	144	206.3	-216.4	0.95

In Figure 4.2 (A) the plot obtained for Av-GEB electrode (red) is compared with that obtained for m-GEC electrode (blue) both cured at 42 ºC for a week, whereas Figure 4.2 (B) compares both electrodes cured at 42 ºC (Av-GEB) and 80 ºC (m-GEC). Table 4.2 outlines the parameters calculated from these voltammograms. When the same curing temperature was applied, the difference in anodic and cathodic current (about 30 µA) and in the separation

between peak potentials (237 mV) may be attributed to the presence of avidin, which may affect the conductivity, surface properties and therefore the location of the formal potential [3]. It is important to enhance that although higher curing temperature improves the reversibility of the system, as shown in Figure 4.1 and 4.2 (B), in the case of Av-GEB electrodes this condition might not be applied in order to preserve the stability of the protein.

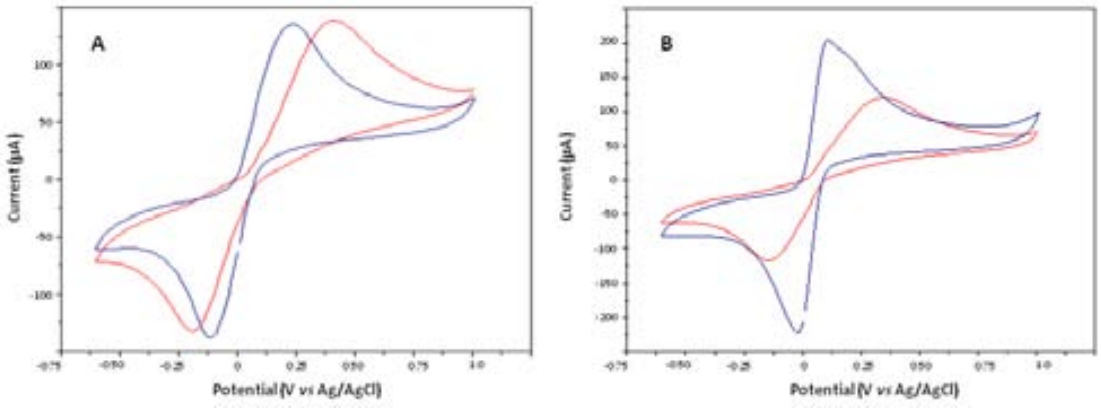

Figure 4.2. Cyclic voltammograms using as working electrode (A) Av-GEB (red) and m-GEC (blue) both cured at 42 ºC for a week, and (B) Av-GEB (red) and m-GEC (blue) at optimal curing conditions 42 ºC and 80 ºC for a week, respectively. All other conditions as in Figure 4.1.

Table 4.2. Potential and current values of the anodic and cathodic peaks, separation between peaks (ΔE_p) and ratio of anodic to cathodic peak current (i_{pa}/i_{pc}) obtained from cyclic voltammetries of Av-GEB and m-GEC electrodes at different curing temperatures.

	Electrode	Time	Temp. (ºC)	E_{pa} (mV)	E_{pc} (mV)	ΔE_p (mV)	i_{pa} (µA)	i_{pc} (µA)	i_{pa}/i_{pc} (µA)
A	Av-GEB	1 week	42	375	-187	562	98.21	103.6	0.95
	m-GEC	1 week	42	200	-125	325	135.7	132.1	1.03
B	Av-GEB	1 week	42	375	-187	562	98.21	103.6	0.95
	m-GEC	1 week	80	108	-18	126	201.3	-216.5	0.93

In addition, the reproducibility study for both electrodes was also performed. Figure 4.3 comparatively shows the electrochemical signal of four different Av-GEB electrodes cured at 42 ºC for 1 week (A) and four different m-GEC electrodes cured at 80 ºC for 1 week (B). Table 4.3 summarises the potential and currents values of the cathodic and anodic peaks extracted from the voltammograms. For the Av-GEB electrodes, values of 8 % and 26 % of relative standard deviation (RSD) were obtained for the anodic and cathodic peak respectively. Whereas in the case of the m-GEC electrodes, a RSD of 2 % was obtained for both peaks, showing greater reproducibility. Thus, although the homogeneity of avidin-composites could

be a drawback, both electrodes show an excellent reproducibility of construction and polishing, in such a case that different electrodes from the same batch could be used to perform replicates. According to these figures, a working potential of -0.150 V (*vs*. Ag/AgCl) for Av-GEB and -0.100 V (*vs.* Ag/AgCl) for m-GEC were selected for the amperometric measurements.

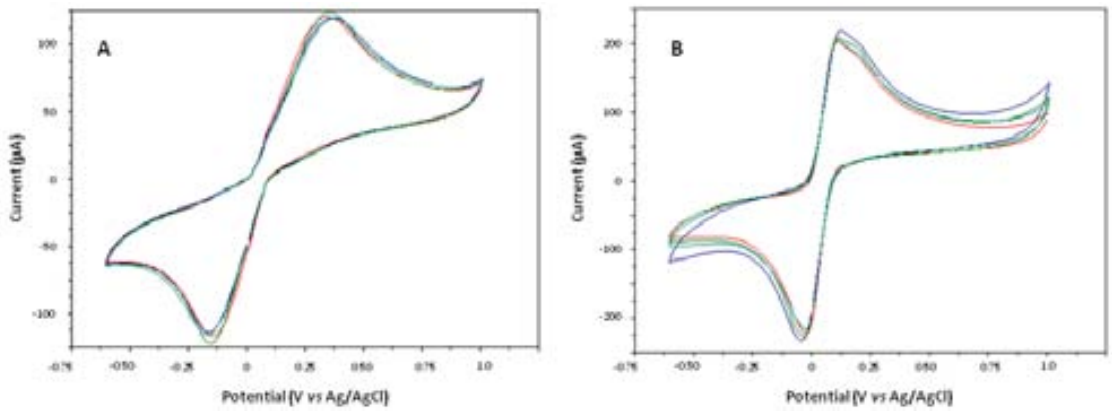

Figure 4.3. Cyclic voltammograms using as working electrode: (A) Av-GEB cured at 42 ºC for 1 week (n=4), and (B) m-GEC cured at 80 ºC for 1 week (n=4). All other conditions as in Figure 4.1.

Table 4.3. Potential and current values of the anodic and cathodic peaks, separation between peaks (ΔE_p) and ratio of anodic to cathodic peak current (i_{pa}/i_{pc}) obtained from cyclic voltammetries of Av-GEB and m-GEC electrodes.

	Electrode	E_{pa} (mV)	E_{pc} (mV)	ΔE_p (mV)	i_{pa} (µA)	i_{pc} (µA)	i_{pa}/i_{pc} (µA)
Av-GEB	1	342	-144	486	64.80	-60.17	1.08
	2	360	-162	522	65.46	-60.17	1.09
	3	351	-144	495	77.27	-98.97	0.78
	4	369	-153	522	70.14	-91.99	0.76
m-GEC	1	108	-18	126	201.3	-216.5	0.93
	2	117	-36	153	203.7	-212.2	0.96
	3	108	-27	135	200.8	-216.3	0.93
	4	108	-18	126	194.9	-205.4	0.95

4.2. ELECTROCHEMICAL GENOSENSING OF *MYCOBACTERIUM BOVIS*

In this section, the results obtained for the electrochemical genosensing of *Micobacterium bovis* based on the specific amplification and double-tagging of the IS*6110* insertion sequence highly related to this bacteria are presented. The comparison between both electrochemical platforms, based on m-GEC and Av-GEB electrodes, as well as the evaluation of milk samples through both strategies is shown. The results are discussed in next sections by comparing them with tuberculin skin test, the current gold-standard for identifying cattle exposed to *M. bovis*, and inter-laboratory PCR assays performed in collaboration with the Universidad Nacional del Litoral, Santa Fe, Argentina and the Centro Nacional de Investigaciones Agropecuarias (CNIA-INTA), Castelar, Buenos Aires, Argentina.

4.2.1. Double-tagging PCR amplification and gel electrophoresis detection

The extracted bacterial DNA was amplified by double-tagging PCR. As shown in Figure 4.4, after the annealing of both 5' labelled primers with the template, a new DNA strand was enzymatically assembled by the Taq polymerase, by the addition of nucleotides to the 3' end of both primers. The primers, and thus their tags, were included in the amplicon in such a way that the DNA was not only amplified but also labelled.

The efficacy of the IS*6110* insertion sequence amplification is evaluated by electrophoresis agarose gel. This technique determines the presence or absence of PCR products and quantifies the size (length of the DNA molecule) of the product by comparison with the fragments of a DNA size marker. The amplification (absence of PCR inhibitors, primers effectiveness and experimental conditions) and the exclusivity of the obtained band are confirmed by this technique.

As shown in Figure 4.5, under the PCR conditions used here, the double-tagged set of primers exclusively amplified the IS*6110* insertion sequence. A unique positive electrophoresis band was observed in the third lane, the expected 245 bp fragment band, corresponding to the amplification of the IS*6110* insertion sequence specific for *M. bovis.* Figure 4.5 also shows no bands in the negative PCR control sample (lane 1), which contained all reagents except *M. bovis* template.

Thus, the protocol described in § 3.5.2 is effective and the primers selected show good specificity, no primer dimmers or other non-specific amplifications were observed. Moreover, as the primers were labelled with biotin and digoxigenin, the amplified DNA (amplicon) was expected to be double-tagged as well with both biotin and digoxigenin in each extreme, respectively.

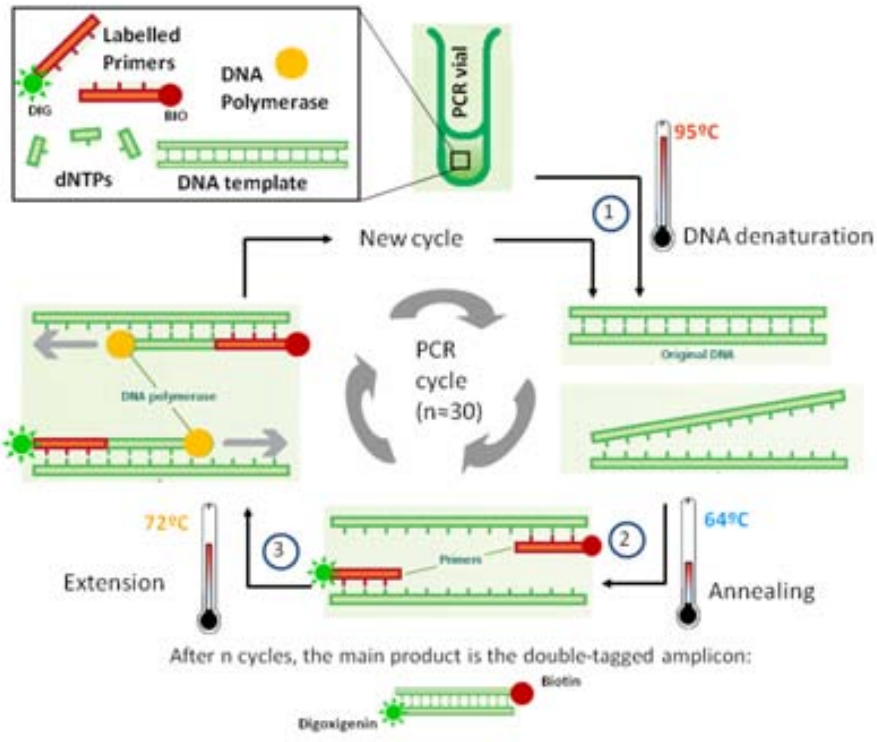

Figure 4.4. Schematic representation of the double-tagging PCR amplification, in order to obtain a double-tagged amplicon labelled with both biotin and digoxigenin tags, from a *M. bovis* genome template.

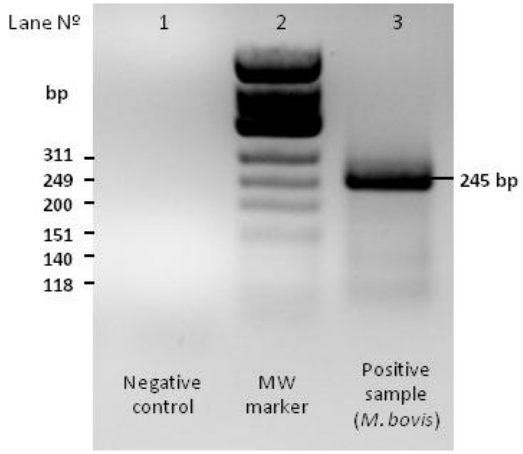

Figure 4.5. Agarose gel electrophoresis of double-tagged PCR amplicon of IS*6110* insertion sequence (lane 3) compared with a negative control (lane 1) and the molecular weight marker ΦX174-Hinf I genome (lane 2).

4.2.2. Electrochemical genosensing of the double-tagged amplicon based on Av-GEB and m-GEC electrodes

The double-tagged IS*6110* PCR amplicon was evaluated by two electrochemical genosensing strategies (§§ 3.5.3 and 3.5.4) based on Av-GEB and m-GEC electrodes. Figure 4.6 (A) shows the responses obtained with the different dilutions of double-tagged amplicon using the Av-GEB (1/15, 1/8, 1/4, and 1/2) and m-GEC (1/960, 1/480, 1/240, 1/120, 1/60, 1/30, 1/15, 1/8, and 1/4) electrodes plotted against the PCR amplicon concentration determined spectrophotometrically at 260 nm. The electrochemical signals were obtained under conditions at which the enzyme was saturated with the substrate, as explained in § 3.4.1. For each measurement, a steady-state current was obtained after the addition of hydroquinone and hydrogen peroxide (normally after 1 min of addition of the latter), as displayed in Figure 4.6 (B). This steady-state current was also used for the electrochemical signal plotted in following electrochemical data presented along the dissertation.

As shown in Figure 4.6 (A), the analytical response of both electrodes increased quantitatively with the amount of double-tagged amplicon, but the sensitivity of the assay based on the m-GEC electrodes (black line) was higher than that obtained with the Av-GEB electrode (dotted line). The inset in the figure shows in detail the responses obtained with the two electrodes at the lowest concentration range. The lowest amount of analyte producing a meaningful analytical signal was 620 fmol for the Av-GEB electrode and 10 fmol for the m-GEC electrode.

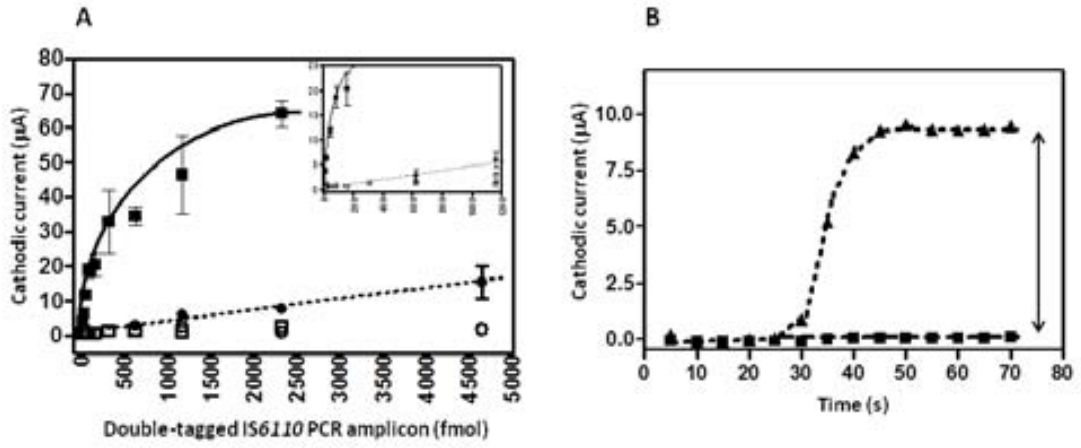

Figure 4.6. (A) Electrochemical signals of the *M. bovis* amplicon detection based on Av-GEB and m-GEC electrodes. Closed circles, positive *M. bovis* sample Av-GEB; open circles, negative control Av-GEB; closed squares, positive *M. bovis* sample m-GEC; open squares, negative control m-GEC. (B) The typical amperometric curve, showing the enzyme saturation signal. Closed triangles, positive *M. bovis* sample; closed squares, negative control. 6.5 x 10^6 streptavidin-MPs were used for the m-GEC electrodes. In all cases, 60 µg of AntiDig-HRP were used (n = 3). Medium: phosphate buffer. Mediator: hydroquinone 1.8 mmol L^{-1}. Substrate: H_2O_2 4.9 mmol L^{-1}. Applied potential= -0.100 V (*vs*. Ag/AgCl).

Figure 4.7 presents the electrochemical response provided by five different milk samples from dairy farms using double-tagging PCR combined with either the Av-GEB or the m- GEC strategy, as an indicator of infected cattle. In all cases, 1/4 dilution factor of the amplicon was processed. In order to screen-out negative samples, a cut-off value was established by using both electrochemical genosensing strategies to analyse a negative milk sample (negative confirmed by two inter-laboratory PCR assays and by the tuberculin skin test). Accordingly, four replicates of the negative control were processed, obtaining a mean value of 1.4 µA with a standard deviation of 0.8 µA for the Av-GEB assay and a mean value of 3.3 µA with a standard deviation of 0.3 µA for the m-GEC assay. The cut-off value was then extracted using a one-tailed t test at a 95 % confidence level, giving a value of 3.9 µA and 4.2 µA for the Av-GEB and m-GEC strategies, respectively (shown in Figure 4.7 (A) as a dotted line). The assay based on the m-GEC electrode showed the presence of *M. bovis* in samples 3, 4, and 5, whereas only sample 5 was positive in the Av-GEB based assay (3 and 4 gave negative results). Figure 4.7 (B) shows in detail the responses obtained for samples 3 and 4. Therefore, the higher sensitivity of the electrochemical genosensing strategy based on m-GEC electrodes decreases the probability of obtaining false-negatives in comparison with the electrochemical genosensing strategy based on Av-GEB electrodes.

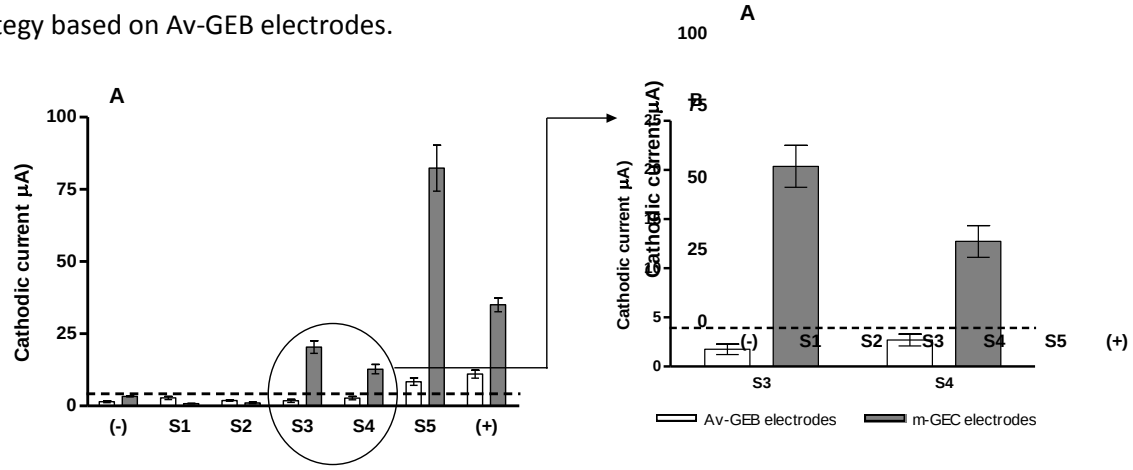

Figure 4.7. Electrochemical signals of the *M. bovis* amplicon detection in milk samples based on the Av-GEB (white bars) and m-GEC (grey bars) electrodes. The dotted line shows the cut-off value, n = 4. (S1, sample 1; S2, sample 2; S3, sample 3; S4, sample 4; S5, sample 5). All other conditions as in Figure 4.6.

4.2.3. Tuberculin skin test and inter-laboratory PCR assays

The results obtained for the five milk samples processed in § 4.2.2 were compared in Table 4.4 with those obtained by inter-laboratory PCR assays and administering the tuberculin skin test to the animals.

Tuberculin skin tests have been used for the diagnosis of tuberculosis in cattle for more than 100 years and their estimation of the sensitivity ranges from 68 – 95 % while specificity is estimated to be 96 – 99 % [4]. Due to the high specificity, samples 3 and 4 (Figure 4.7) with positive tuberculin skin test results (Table 4.4), in all likelihood came from infected animals. However, inter-laboratory PCR assays as well as electrochemical genosensing of the double-tagged amplicon based on the Av-GEB platform gave false-negative results for these samples. As a false-negative can be the source of misdiagnoses, with severe consequences, the poor analytical performance in screening-out negative samples is noteworthy. By contrast, positive results, consistent with the tuberculin skin tests were obtained for samples 3 and 4 by electrochemical genosensing of the double-tagged amplicon using the m-GEC platform combined with streptavidin magnetic particles. The discrepancy in the electrochemical genosensing results was likely due to the fact that the m-GEC approach has a better LOD than the Av-GEB approach (10 *vs.* 620 fmol of double-tagged amplicon, respectively), allowing the identification of samples 3 and 4 as positive with higher sensitivity. The negative result obtained for sample 5 with the tuberculin skin test but not by electrochemical genosensing with either the m-GEC or the Av-GEB electrodes (as displayed in Figure 4.7 and Table 4.4) could be ascribed to the lower sensitivity of the traditional test. Accordingly, sample 5 should be further investigated. However, as the primary use of electrochemical genosensing of the double-tagged amplicon based on m-GEC is to screen-out negative samples, the most important parameter is the LOD, and thus to consider any negative results as definitive. By contrast, positive test results always should be considered presumptive and must be confirmed by an approved culture method.

Table 4.4. Results of inter-laboratory PCR assays and tuberculin skin tests of milk samples screened for *Mycobacterium bovis.*

	Inter-laboratory PCR assays			**Electrochemical assays**	
Sample	**Lab 1**	**Lab 2**	**Tuberculin skin test**	**m-GEC**	**Av-GEB**
1	positive	positive	negative PPD	negative	negative
2	positive	positive	negative PPD	negative	negative
3	negative	positive	positive PPD	positive	negative
4	negative	negative	positive PPD	positive	negative
5	positive	negative	negative PPD	positive	positive

4.2.4. General discussion

The aim of this study was to develop a rapid method for screening-out of tuberculosis (TB) in milk and dairy products based on electrochemical genosensing of DNA specific of *Mycobacterium bovis*. Two different electrochemical platforms for genosensing were evaluated: i) an avidin-biocomposite (Av-GEB), and ii) a magneto-composite (m-GEC) coupled with streptavidin magnetic particles. The features of both platforms have been previously studied [5], however, this is the first time that DNA extracted from raw milk samples was evaluated.

One of the accomplished goals was the successful incorporation of both the biotin and the digoxigenin moieties into the PCR product using a set of 5' labelled primers in such a way that the DNA was not only amplified but also labelled. In the electrochemical genosensing procedure based on the Av-GEB electrodes, the double-tagged amplicon was immobilised on the surface of the avidin biocomposite platform, while in the electrochemical genosensing procedure based on the m-GEC electrodes, the double-tagged amplicon was immobilised on streptavidin magnetic particles and then captured on the surface of the magneto-electrode (m-GEC). In both cases, the electrochemical response of the double-tagged product was due to the electrochemical reporter AntiDig-HRP. As shown previously, the electrochemical genosensing strategy based on m-GEC had a higher sensitivity, obtained by using streptavidin magnetic particles, which immobilised the biotinylated amplified DNA on the m-GEC surface and permitted rapid magnetic separation of the unbound components. However, non-specific adsorption for both electrodes, as determined with the negative PCR control, was low and almost the same throughout the evaluated concentration range. The results showed that both electrochemical genosensing developed, using Av-GEB and m-GEC electrodes, were suitable for the detection of amplified PCR amplicon, although a better limit of detection was achieved with streptavidin magnetic particles coupled with m-GEC electrodes.

The high specificity of the tuberculin skin test (96 – 99 %, *i.e.,* the proportion of negatives that are correctly identified) ensures the correct identification of a negative sample. Nevertheless, the test is unable to ensure the total absence of *M. bovis* in milk samples, as its sensitivity ranges from 68 – 95 % [4], obtaining thus false-negatives. This fact can be resolved by the implementation of any of the methodologies described in this section, as it was proved with Sample 5 (Table 4.4), which gave negative results by tuberculin skin test but positive with both genosensing strategies. Since screening assays are used on large sample populations,

often with the aim of determining those samples requiring further investigation, false-positives are not as problematic as false-negatives, since the former will be further examined. Between both strategies, electrochemical genosensing based on m-GEC electrodes shows greater analytical features suggesting this approach as a promising strategy to screen-out negative dairy samples and thereby to isolate negative cattle from presumptive infected animals. The combination of genome amplification by double-tagging PCR, capture of the double-tagged amplicon, and electrochemical genosensing detection using the sensitive m-GEC electrode provides a rapid, cheap, and sensitive assay for the screening-out of samples contaminated with *M. bovis.*

4.3. ELECTROCHEMICAL GENOSENSING AND IMMUNOSENSING OF *SALMONELLA*

In this section, the main features of the electrochemical genosensing and immunosensing for *Salmonella* are presented and discussed.

The first approach, "IMS/double-tagging PCR/m-GEC electrochemical genosensing", was based on a double biorecognition of the bacteria, in this case immunological followed by genetic biorecognition. The bacteria were captured and pre-concentrated from food samples with magnetic particles through the immunological reaction with the specific antibody against *Salmonella*. After the immunomagnetic separation, the bacteria were lysed and further amplification of the genetic material by Polymerase Chain Reaction (PCR) with a double-tagged set of primers was performed to confirm the identity of the bacteria. The double-tagged amplicon was then detected by electrochemical magneto-genosensing.

The second strategy, "PMS/double-tagging PCR/m-GEC electrochemical genosensing", was based on the use of bacteriophages, which offer several analytical advantages as biorecognition element for the magnetic separation of pathogenic bacteria. The phage capabilities as biorrecognition element were explored by using the model phage nanoparticle P22 towards *Salmonella*. Both active and inactive (UV irradiated) P22 bacteriophage were immobilised on tosyl-activated magnetic particles in an oriented way. The bacteria were then captured and pre-concentrated by the phage-modified magnetic particles throughout the phage-host interaction. To confirm the identity of the bacteria, further double-tagging PCR amplification of the captured bacteria DNA and electrochemical magneto-genosensing of the amplicon were performed.

In the third strategy, "IMS/m-GEC electrochemical immunosensing", the detection of the bacteria was performed by a double immunological recognition. The bacteria were captured from food samples and pre-concentrated by immunomagnetic separation. After the IMS, the enzymatic labelling of the bacteria was also performed using a specific antibody against *Salmonella* labelled with HRP, performing thus the electrochemical magneto-immunosensing.

The results obtained for all the steps involved in these strategies, such as the magnetic separation based on different affinity biorecognition (IMS and PMS), as well as the electrochemical magneto-biosensing (genosensing and immunosensing) are presented and discussed in the next sections.

4.3.1. Immunomagnetic separation

The first step in both strategies, the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" as well as the "IMS/m-GEC electrochemical immunosensing" was the immunomagnetic separation (IMS) of the bacteria *Salmonella*. This step allowed the pre-concentration of the bacteria in a rapid and selective way directly from the sample. The results obtained for the evaluation of the IMS, performed by Scanning Electron Microscopy (SEM) and by microbiological culture, are presented next.

4.3.1.1. Evaluation of the IMS by Scanning Electron Microscopy and microbiological culture

The microscopic characterisation by SEM was performed for the evaluation of the immunological attachment of the bacteria to the magnetic particles. The Figure 4.8 shows that the binding was achieved with more than one specific binding site of the bacteria to the magnetic particle. In some cases, the whole surface of the bacterium was completely attached to the particle (Figure 4.8 A and F). Moreover, a unique magnetic particle was able to attach more than one bacterium (Figure 4.8 B, C, and F), although no more than three cells per magnetic particle were observed at a bacterial concentration of 10^4 CFU mL^{-1}. Some particle aggregates were also observed due to the binding of two different magnetic particles by a unique bacterium cell (Figure 4.8 E), in accordance with the multivalency of both magnetic particle and bacteria.

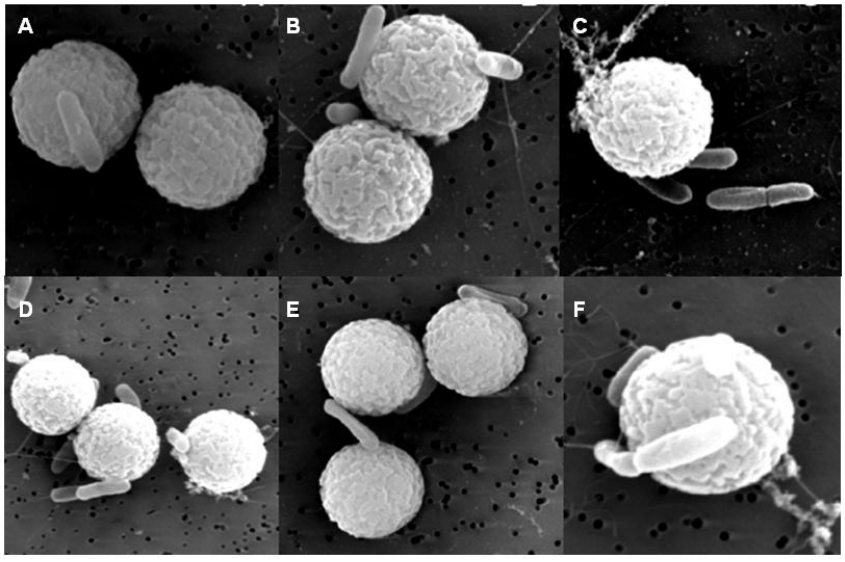

Figure 4.8. Evaluation of the IMS by SEM at a *Salmonella* concentration of 10^4 CFU mL^{-1}. The images show the *Salmonella* cells attached to the magnetic particles. In all cases, identical acceleration voltage (15 KV) was used.

The efficiency of the IMS procedure was also evaluated by microbiological culture growing the bacteria attached on magnetic particles for 18 – 24 h at 37 ºC. Colony counting was clearly decreasing from 2.9 x 10^6 to 2.9 x 10^0 CFUs, as shown in Table 4.5. As a result of the high aggregation of the magnetic particles, the IMS efficiency should be better estimated by the supernatant plating. This evaluation was further performed obtaining recovery values about 90 – 100 % (results not shown).

Table 4.5. Colony counting after IMS and culture in LB for 18 – 24 h at 37 °C

	Counted CFUs				
CFUs expected	**10^0 original solution**	**10^{-2} dilution**	**10^{-4} dilution**	**CFUs found**	**IMS Recovery (%)**
2.9 x 10^6	uncountable	uncountable	225	2.3 x 10^6	79.3
2.9 x 10^5	uncountable	uncountable	21	2.1 x 10^5	72.4
2.9 x 10^4	uncountable	179	3	1.8 x 10^4	62.1
2.9 x 10^3	uncountable	19	0	1.9 x 10^3	65.5
2.9 x 10^2	159	1	-	1.6 x 10^2	55.2
2.9 x 10^1	16	0	-	1.3 x 10^1	55.2
2.9 x 10^0	4	0	-	4.0 x 10^0	137.9

The corresponding plates from 2.9 x 10^0 CFUs (plate F) to 2.9 x 10^4 CFUs (plate B) are also shown in Figure 4.9, displaying the characteristic colony features of *Salmonella* in LB media. A negative control is also shown (plate A, Figure 4.9). An underestimation of the expected amount of bacteria was observed in all the concentration range, except for the more diluted concentration corresponding to 2.9 x 10^0 CFU in 500 µL of sample (5 CFU mL^{-1}). The efficiency of the counting was found to be between 60 and 80 % of the expected amount that may be related with the formation of the aggregates observed by SEM, formed by several bacterium cells but growing at a unique colony point in the agar plate. As low as 5 CFU mL^{-1} were effectively captured and detected by culturing the bacteria attached to the magnetic particles after the IMS. Similar results were obtained when *Salmonella* was artificially inoculated into skimmed milk samples and other accompanied flora was not observed in the plate. Thus, no matrix effect for the IMS step was found.

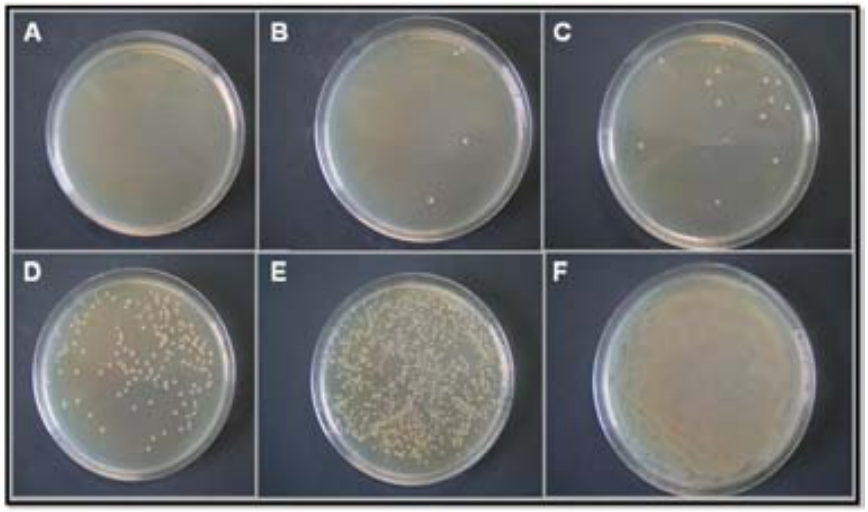

Figure 4.9. Evaluation of the IMS by microbiological culture. Culture plates of *Salmonella* cells attached to magnetic particles for concentrations ranged from 0 (plate A) to 2.9×10^4 (plate F) showing the typical colony features of *Salmonella*.

4.3.2. Phagomagnetic separation

In the second strategy, the "PMS/double-tagging PCR/m-GEC electrochemical genosensing", the first step was the phagomagnetic separation (PMS) of *Salmonella*. In this case, instead of using antibody-modified magnetic particles, the concentration of the bacteria was achieved by using bacteriophage-modified magnetic particles. The results for the evaluation of the covalent immobilisation of P22 bacteriophage on magnetic particles and their correct orientation obtained by Coomassie-based protein assay (Bradford assay), microbiological culture and Scanning Electron Microscopy (SEM), as well as the evaluation of the PMS performed by SEM and microbiological culture, are discussed in following sections.

4.3.2.1. Evaluation of the bacteriophage purification by electrochemical magneto-immunosensing

Firstly, the purification of the bacteriophages was evaluated by an electrochemical magneto-immunosensing approach in order to obtain antigen-free bacteriophages. As bacteriophages are obtained from bacterial infection in cultures containing 10^8 CFU mL^{-1}, the phage lysate always include a mixture in which bacterial antigens are highly concentrated. Among bacterial debris, the removal of endotoxins or lipopolysaccharides (LPSs) has always been a challenge. LPSs are located in the outer cell membrane of Gram-negative bacteria such as *Salmonella* being somatic antigen O part of the moiety [6, 7], in such a case that further

bioanalysis can be worsen by their presence, providing higher levels of background signal. Therefore, further purification is essential to remove these antigens from bacteriophages not only for bioanalysis, but also for animal or human applications. The most typical phage purification method used for small, laboratory-scale preparations is based on procedures used for molecular work with lambda phage [8], being other methodologies extensively described in literature [9]. The goal of this section is to demonstrate the capability of the strategy based on electrochemical magneto-immunosensing as an evaluation test for the bacteriophage purification by comparing different bacteriophage purification approaches which come of different amount of remaining LPSs.

Figure 4.10 shows the data obtained by the detection method of LPSs. Different amounts of LPSs obtained by purifying the phage lysates in two different ways (dialysis and full-purified bacteriophages following the procedure described in § 3.2.8) are compared with the non-purified bacteriophages and a negative control. As shown, a clear decreasing in the amount of bacterial antigen was achieved with the complete purification procedure. A correlation between the amount of LPSs present in the samples and the signal obtained is observed, confirming that the purification by cesium chloride gradient adapted from Sambrook *et al.* [8] with a signal similar to the negative control contains the lower amount of LPSs, and purification only through dialysis was not sufficient to obtain low background signal.

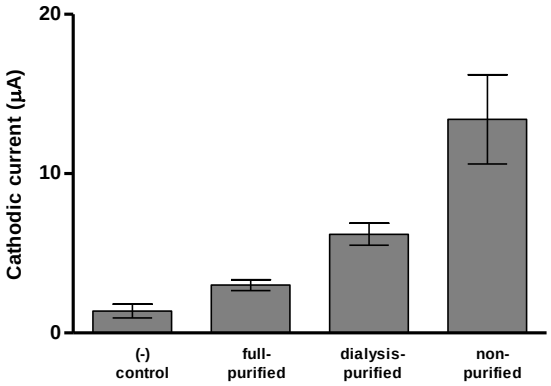

Figure 4.10. Evaluation of the bacteriophage purification by electrochemical magneto-immunosensing. Grey bars show the electrochemical signal corresponding to non-purified (n=2), dialysis-purified (n=2) and full-purified following procedure in § 3.2.8 (n=4) bacteriophages. A negative control is also shown (n=4). In all cases, 0.1 mg mL^{-1} of anti-*Salmonella*-MPs and 1 µg $mL^{-1}$of anti-*Salmonella*-HRP were used. Medium: phosphate buffer. Mediator: hydroquinone 1.8 mmol L^{-1}. Substrate: H_2O_2 4.9 mmol L^{-1}. Applied potential= -0.100 V (*vs*. Ag/AgCl).

This methodology based on immunological biorecognition and electrochemical detection results in a fast, robust and cheap technique to evaluate the phage purification. Comparing with the current methodology for endotoxin detection, Limulus Amebocyte Lysate (LAL) test [10]

and other commercial approaches such as EndoLISA® or EndoZyme® from Hyglos GmbH company, the three tests require much more expensive equipment to perform the detection due to the fact that are based on chromogenic or fluorescent detection techniques. Another advantage of the methodology presented come from the use of magnetic particles, the available tests for endotoxin are based on ELISA technology while by using magnetic particles, among other advantages, sensitivity and precision problems resulting from desorption of antibodies (LAL test) or bacteriophages (EndoLISA® or EndoZyme®) during the assay are avoided. Thus, the evaluation of the bacteriophages purification by the electrochemical magneto-immunosensing was chosen to verify the bacteriophage purity before being used.

4.3.2.2. Covalent immobilisation of P22 bacteriophage on magnetic particles and evaluation by Bradford assay, Scanning Electron Microscopy and microbiological culture

The native and antigen-free P22 phage nanoparticles were covalently coupled for the first time to tosyl-activated magnetic particles by the reaction of aminated aminoacidic lysine moieties of the main capsid monomeric protein (gp5) [11] by an amine linkage, as outlined in Figure 3.12 (§ 3.6.2.2). In order to obtain the best ratio tosyl-MP/P22 phage during the immobilisation, the optimisation of different conditions such as tosyl-MP and P22 phage concentrations was performed. The bioconjugation of the P22 phage to magnetic particle (P22-MP) was evaluated by several techniques such as Coomassie-based protein assay (Bradford assay), microbiological culture and Scanning Electron Microscopy (SEM). The data obtained from all the evaluations is discussed next.

The coupling efficiency of the P22 bacteriophage to the magnetic particles was evaluated by Bradford assay and microbiological culture. In both cases, the amount of bacteriophage, as phage protein or phage unit (PFU) respectively, immobilised on the magnetic particles was determined as the difference between the initial amount of bacteriophage and the quantity present in the supernatant after the immobilisation (Figure 3.12, § 3.6.2.2). The coupling efficiency, summarised in Table 4.6, was calculated following the equation:

$$\% \text{ immobilisation} = \frac{\text{(total amount of protein or PFU in supernatant before immobilisation} - \text{total amount of protein or PFU in supernatant after immobilisation)}}{\text{total amount of protein or PFU in supernatant before immobilisation}} \times 100$$

Table 4.6. Comparative results obtained with Bradford assay and microbiological culture for the coupling efficiency of the P22 phages covalent immobilisation on tosyl-MPs.

Assay	P22 phage (PFU)	Tosyl-MPs	Coupling efficiency (%)	Ratio PFU/MP
	4×10^{11}	7×10^{8}	100	626
Bradford	4×10^{11}	7×10^{7}	24	1924
	1×10^{12}	7×10^{7}	26	2163
Culture	1.44×10^{11}	7×10^{7}	37	757

The amount of viral protein present in the supernatant before and after the immobilisation step was determined by the Bradford assay to quantify the coupling efficiency of the capsid protein to the magnetic particle. Bradford assay is very popular because it is rapid and simple, involves a single addition of the dye reagent to the sample, as described in detail in § 3.4.2. As shown in Figure 4.11, a calibration curve (r = 0.968) was obtained with Bradford assay for the P22 phage nanoparticles showing good reproducibility at each concentration level (n=3) and a linear range from 2×10^{10} to 5×10^{11} PFU mL^{-1}. By comparing the phage concentration before and after immobilisation, the coupling efficiency of non-modified P22 bacteriophages (4×10^{11} PFU mL^{-1}) on tosyl-activated magnetic particles on both 7×10^{8} and 7×10^{7} magnetic particle units was found to be 100 % and 24 %, respectively, with ratios of 626 and 1924 P22 phage nanoparticles (PFU) immobilised per magnetic particle, respectively. Moreover, the immobilisation of an increased amount of P22 phage nanoparticles (1×10^{12} PFU) on the same amount a magnetic particles (7×10^{7}), showed a similar coupling efficiency (25.6 %), with a ratio of 2163 P22 phage nanoparticles (PFU) per each magnetic particle, indicating a plateau in the immobilisation efficiency in approximately 2000 PFU/MP.

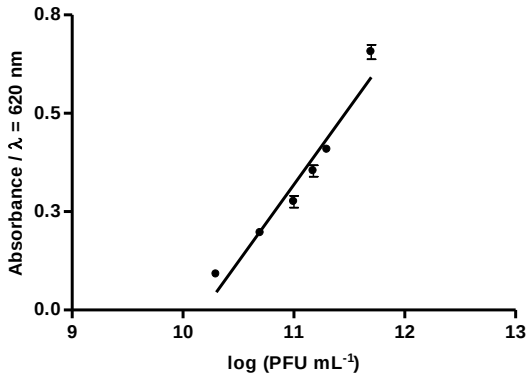

Figure 4.11. Calibration curve of P22 phage nanoparticles in 40 mmol L^{-1} borate buffer and 2 mmol L^{-1} $MgSO_4$ for Bradford assay. The error bars show the standard deviation for n=3.

A similar approach for coupling efficiency study was performed by quantifying the plaque forming units (PFU) in the supernatant before and after the immobilisation step by the double agar layered method for counting active phages (Figure 4.12). After comparing the bacteriophage counting (PFU) before and after the immobilisation, the coupling efficiency for 1.44 x 10^{11} PFU on 7 x 10^{7} magnetic particle units was found to be 37 %, with a ratio of 757 phage nanoparticles (PFU) immobilised in each magnetic particle. The results are comparable to those obtained by the Bradford assay, considering that, in this last case, the starting amount of phage for immobilisation (1.44 x 10^{11} PFU) was around 35 % of the amount used for Bradford assay (4 x 10^{11} PFU, the saturating phage concentration), being thus also the immobilised phages on 7 x 10^{7} MPs approximately 35 % (757 PFU per MP) of the saturated value (2000 PFU phage nanoparticles per magnetic particle) obtained by Bradford test. The Bradford assay showed thus good performance as a rapid alternative for the time consuming microbiological methods in order to estimate the coupling efficiency of phage nanoparticles, not only on magnetic particles, but also in other supports. Finally, the optimal ratio to achieve the higher covering of P22 bacteriophages on 7 x 10^{7} magnetic particles was found to be 4 x 10^{11} PFU mL^{-1}, reaching a coupling efficiency of around 25 % with approximately 2000 PFU per MP, and ful coverage of the magnetic particles.

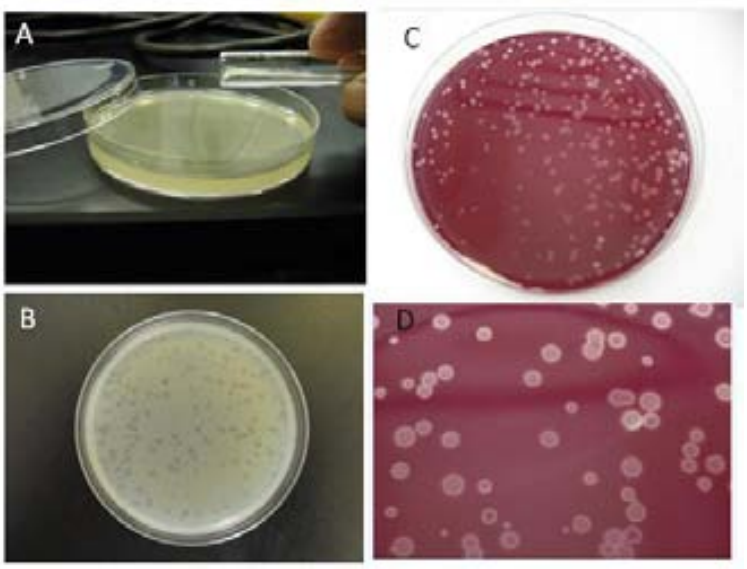

Figure 4.12. Schematic representation of the double agar layered method for counting active phages (A). Aspect of the plaques without (B), and with (C and D) tetrazolium dye.

The covalent immobilisation of P22 bacteriophage on the magnetic particles was also evaluated by analysing the magnetic particles instead of the supernatants with both Scanning Electron Microscopy and microbiological culture.

Figure 4.13 shows the microscopic characterisation by SEM of non-modified (A, B and E) and modified (C, D, F to H) magnetic particles with P22 phages nanoparticles. The Figure 4.13 shows the spherical structures of P22 bacteriophages (~ 600 – 700 Å in diameter [12]) (F, G and H) uniformly distributed on the surface of the magnetic particles (C and D).

Although the success in the immobilisation of P22 phage nanoparticles on magnetic particles was demonstrated by different methodologies (Bradford assay, phage counting on the supernatant by the double agar layered method, and SEM), none of these methods can ensure the orientation of the tail spikes away the solid support. This orientation was studied by the double agar layered method and enumeration of plaques by culturing the P22 phage-modified magnetic particles (P22-MPs), since oriented phages immobilised on magnetic particles will produce bacteria attachment and further infection of viable bacteria, producing the plaques, as shown in Figure 4.12. The quantification of the number of bacteriophages per magnetic particle is not possible by plating the P22-MPs conjugates, due to the fact that all the bacteriophages attached on the same magnetic particle will produce a unique plaque, as outlined in Figure 3.13 (C). However, the phage counting for the immobilisation of 1.44×10^{11} PFU on 7×10^{7} magnetic particle units was found to be 5.3×10^{7}, which demonstrated lytic activity in at least 75 % of the magnetic particles, and, as such, the confirmation of the oriented immobilisation of the phages on the magnetic particles. The P22 phage-modified magnetic particles were stable at least during a year, without any loss in their analytical performance, showing the same lytic activity.

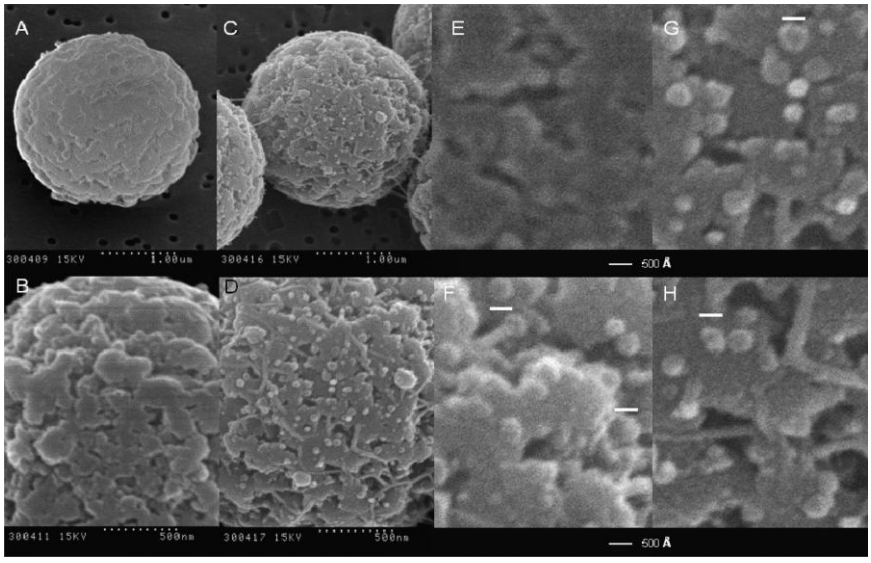

Figure 4.13. Evaluation of the immobilised P22 bacteriophage on magnetic particles by SEM (1924 PFU/MP). Images C, D, and F – H show, at different resolution levels, the P22 bacteriophages attached to the magnetic particles. Panels A, B, and E show the magnetic particle without modification as a negative control. In all cases, identical acceleration voltage (15 kV) was used.

4.3.2.3. Evaluation of the PMS by Scanning Electron Microscopy and microbiological culture

Once the immobilisation of the bacteriophages was performed and the correct orientation of them was evaluated, the bacteria were captured by using «phagomagnetic separation (PMS)» as a first step for the third strategy, "PMS/double-tagging PCR/m-GEC electrochemical genosensing". The evaluation of the PMS was performed by Scanning Electron Microscopy and microbiological culture. Inactivated bacteriophages by UV radiation were used for the PMS to avoid the lytic cycle in order to keep the attached bacteria as a whole cell while being captured, pre-concentrated and cultured since both SEM and culture require non-infected bacteria.

The microscopic characterisation by SEM was performed for the evaluation of the phagomagnetic separation. Figure 4.14 shows that the binding was achieved with more than one specific binding site of the bacteria to the magnetic particle (Figure 4.14 B). Single-point attachment of the bacteria to the magnetic particle was mostly observed. Moreover, a unique magnetic particle was able to attach more than one bacterium (Figure 4.14 E). Finally, some aggregates were observed due to the binding of two or more different magnetic particles by a unique bacterium cell (Figure 4.14 A, C and D), in accordance with multivalency of both bacteria and magnetic particle.

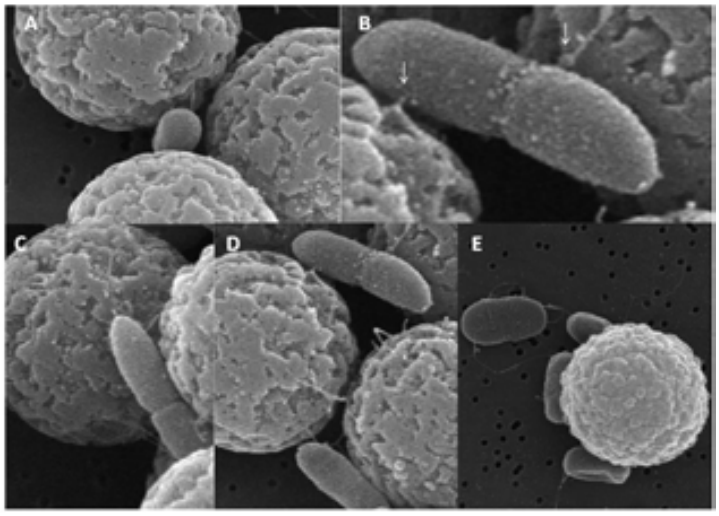

Figure 4.14. Evaluation of the PMS by SEM at a *Salmonella* concentration of 2.9 x 10^7 CFU mL^{-1}. The images show the *Salmonella* cells attached to the magnetic particles through the tail spikes. In all cases, identical acceleration voltage (15 KV) was used.

The PMS was also evaluated by microbiological culture. As shown in Table 4.7, colony counting corresponding to the bacteria attached on magnetic particle was clearly decreasing from 1.9 x 10^4 to 7 CFUs.

Table 4.7. Colony counting after PMS and culture in LB for 18 – 24 h at 37 °C.

	Counted CFUs				
CFUs expected	**10^0 original solution**	**10^{-1} dilution**	**10^{-2} dilution**	**CFUs found**	**IMS Recovery (%)**
2×10^5	uncountable	uncountable	188	1.9×10^4	9.5
2×10^4	uncountable	93	19	9.3×10^2	4.7
2×10^3	66	15	-	6.6×10^1	3.3
2×10^2	7	1	-	7.0×10^0	3.5
2×10^1	0	-	-	0	0
2×10^0	0	-	-	0	0

The corresponding plates are also shown in Figure 4.15, displaying the characteristic colony features of *Salmonella* in LB media. However, an underestimation of the expected amount of bacteria was observed in all the concentration range. The counted colony number was found to be in all cases under 10 % of the expected amount, which may be related with the aggregates observed by SEM, formed by several bacterium cells but growing at a unique colony point in the agar plate, or, for instance, due to infection of remaining active bacteriophages and, thus, under growing of the attached bacteria. As a result of the high aggregation of the magnetic particles, the PMS efficiency should be better estimated by the supernatant plating. This evaluation was further performed obtaining recovery values about 95 % (results not shown).

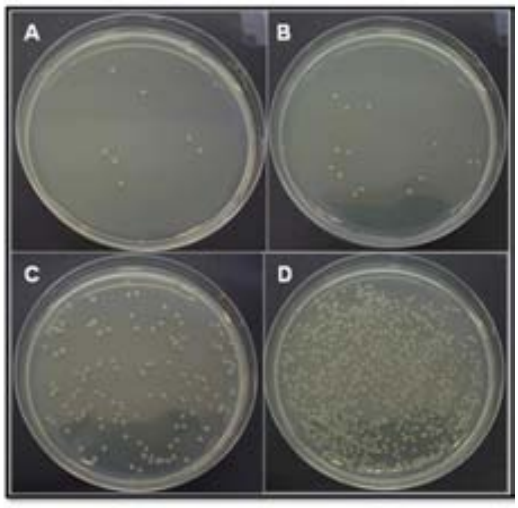

Figure 4.15. Evaluation of the PMS by microbiological culture. Culture plates of *Salmonella* cells attached to magnetic particles for concentrations ranged from 2×10^2 (plate A) to 2×10^5 (plate D) showing the typical colony features of *Salmonella*.

4.3.3. Electrochemical magneto-genosensing

In both strategies, namely the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing", after the magnetic separation based on different affinity biorrecognitions (*i.e.* immunomagnetic (IMS) or phagomagnetic (PMS)), the pre-concentrated bacteria were lysed in order to release the genomic DNA for further double-tagging PCR amplification. Once the bacterial DNA was amplified and labelled, the amplicon was detected by gel electrophoresis and electrochemical magneto-genosensing. The results obtained for both genosensing approaches are discussed and compared in following sections, as well as other features of the strategies such as the limit of detection (LOD), matrix effect, specificity and pre-enrichment time needed.

4.3.3.1. Double-tagging PCR amplification and gel electrophoresis detection

The second step in both strategies presented in this section, the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing", is the double-tagging PCR for the amplification of the *Salmonella* spp. genome which is finally detected by electrochemical magneto-genosensing. During the PCR, as explained previously for the *M. bovis* amplification (§ 4.2.1), not only the amplification of the pathogenic bacteria genome was achieved, but also the double tagging of the amplicon ends with (i) the biotinylated capture primer to achieve the immobilisation on streptavidin-modified magnetic particles and (ii) the digoxigenin signalling primer to achieve the enzymatic detection through AntiDig-HRP reporter.

After the amplification, the double-tagged amplicon was evaluated by the conventional agarose gel electrophoresis. Figure 4.16 compares the gel electrophoresis obtained for both approaches, "IMS/double-tagging PCR/electrophoresis" and "PMS/double-tagging PCR/electrophoresis". In both cases, the chosen set of primers amplified exclusively the IS*200* insertion sequence, producing only the expected 201 bp fragment and no bands were observed for the negative controls (0 CFU mL^{-1}) performed with LB broth (Figure 4.16, (A) lanes 9 and 10; (B) lanes 10 to 13).

As shown in Figure 4.16 (A) for the concentrations ranged from 10^7 to 10^2 CFU mL^{-1} of *Salmonella* artificially inoculated in LB broth, a gradient in the band intensity which can be correlated with the bacterial concentration, and thus with the amount of template for double-

tagging PCR, is observed. The LOD for the "IMS/double-tagging PCR/electrophoresis" approach was found to be 10^2 CFU mL^{-1} (lane 6 in the gel electrophoresis). For the "PMS/double-tagging PCR/electrophoresis" approach (Figure 4.16, B), the gradient in the band intensity is also observed for the concentrations ranged from 10^6 to 10^3 CFU mL^{-1} of *Salmonella* artificially inoculated in LB broth and the LOD in this case was found to be 10^3 CFU mL^{-1} (lane 5 in the gel electrophoresis). The higher LOD found in the "PMS/double-tagging PCR/electrophoresis" approach might be related to the lower magnetic capture efficiency observed with the phagomagnetic separation (under 10 %) compared with the immunomagnetic separation (between 60 and 80 %). However, these capture efficiencies are interestingly an estimation of the agglomeration observed by Scanning Electron Microscopy (§§ 4.3.1.1 and 4.3.2.3) and should not be considered as a real efficiency due to the fact that an unique colony can be formed by several agglomerated magnetic particles providing a miscounting of the total captured cells.

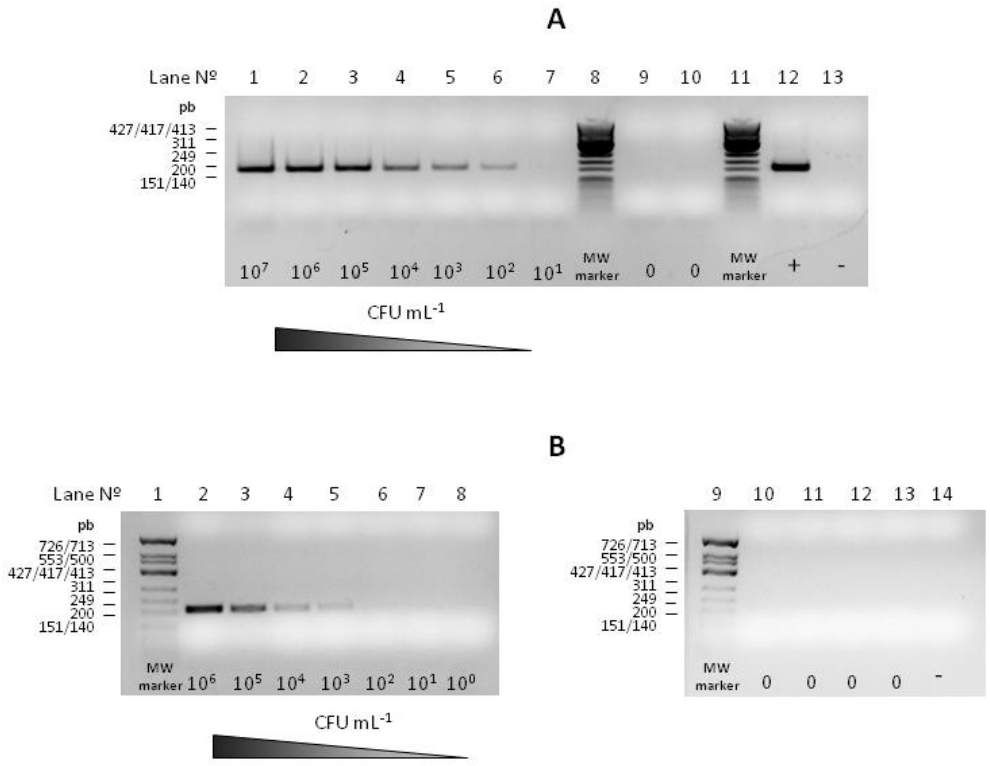

Figure 4.16. (A) Agarose gel electrophoresis of the double-tagged PCR amplicon obtained with the "IMS/double-tagging PCR/electrophoresis" approach. Lanes 1 to 7 are the corresponding tenfold dilutions ranged from 10^7 to 10^1 CFU mL^{-1}. Lanes 9 and 10 are negative control assays (0 CFU mL^{-1}). A positive (lane 12) and a negative (lane 13) PCR controls are also shown. Lanes 8 and 11 are the molecular weight marker (ΦX174- Hinf I genome). (B) Agarose gel electrophoresis of the double-tagged PCR amplicon obtained with the "PMS/double-tagging PCR/electrophoresis" approach. Lanes 2 to 8 are tenfold dilutions ranged from 10^6 to 10^0 CFU mL^{-1}. Lanes 10 to 13 are negative controls (0 CFU mL^{-1}), while lane 14 is the PCR negative control. Lanes 1 and 9 are the molecular weight marker (ΦX174-HinfI genome).

4.3.3.2. Electrochemical magneto-genosensing of the double-tagged amplicon

The double-tagging PCR amplification was also evaluated by electrochemical magneto-genosensing. In order to obtain improved LODs, both strategies proposed in this section, the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing", were implemented for the replacement of the conventional gel electrophoresis detection by the electrochemical magneto-genosensing of the double-tagged amplicon. In these approaches, streptavidin magnetic particles modified with the double-tagged amplicon were easily captured by a magneto-electrode (m-GEC) which was also used as the transducer for the electrochemical detection.

Figure 4.17 shows the electrochemical response obtained for different dilutions of *Salmonella* amplicon, 1/10 and 1/15 in milli-Q water, as well as non-diluted amplicon. The optimisation of the amplicon dilution used is essential in order to be able of discriminating among different concentrations of DNA. A constant signal-to-background ratio was obtained when non-diluted amplicon was processed for *Salmonella* concentrations ranged from 10^1 to 10^7 CFU mL^{-1} (Figure 4.17, black bars). This fact can be ascribed to an excess of amplicon present in the sample, which overloads the magnetic particles and results in no difference among different concentrations. However, diluted amplicons allowed better discrimination among the range of concentration tested. As pointed out in Figure 4.17, amplicons diluted 1/10 (dark grey bars) gave better differentiation between concentrations nearby the limit of detection, thus 1/10 dilution was selected for further assays.

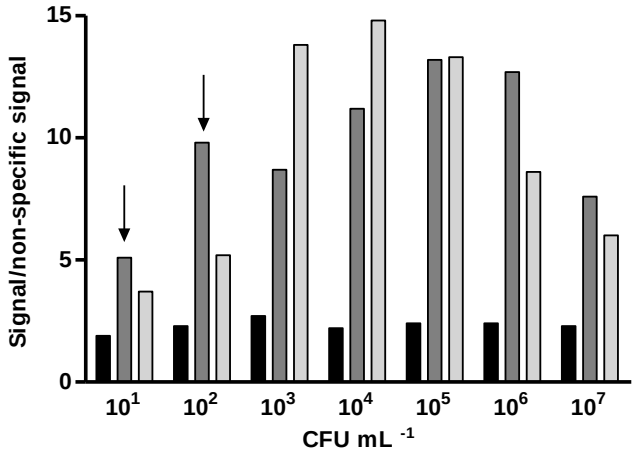

Figure 4.17. Electrochemical signals for the optimisation of the double-tagged amplicon immobilisation on streptavidin magnetic particles. Bars show the signal-to-background ratio for the non-diluted (black), 1/10 diluted (dark grey) and 1/15 diluted (light grey) amplicon. In all cases, 6.5 x 10^6 streptavidin-MPs and 60 µg of AntiDig-HRP were used. Medium: phosphate buffer. Mediator: hydroquinone 1.8 mmol L^{-1}. Substrate: H_2O_2 4.9 mmol L^{-1}. Applied potential= -0.100 V (*vs*. Ag/AgCl).

Moreover, it is important to enhance the high-dose hook effect [13] observed for high bacterial concentration (Figure 4.17, $10^6 – 10^7$ CFU mL^{-1}). This effect consists in a decreasing of signal when high analyte concentration is processed and can be due to the excess of bacterial DNA that not only exceed the finite amount of AntiDig-HRP reporter, but also produce electrostatic repulsion because of the negative charge density of the sugar-phosphate backbone which prevents the binding of the double-tagged amplicon with streptavidin magnetic particles.

4.3.3.3. Detection limit, matrix effect and specificity studies

The electrochemical magneto-genosensing of the double-tagged amplicon was performed for both the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" strategies. Figure 4.18 compares the amperometric responses obtained for both approaches. The amperometric signal corresponding to the LOD was estimated by processing the negative control samples (0 CFU mL^{-1}) using six magneto-electrode devices from different batches and performing different single inter-day assays. A mean value of 2.2 µA with a standard deviation of 0.7 µA was obtained for the approach based on immunomagnetic separation, while a mean value of 0.8 µA with a standard deviation of 0.2 µA was obtained for approach based on phagomagnetic separation. The amperometric signals corresponding to the LOD values were then extracted with a one-tailed t test at a 99 % confidence level, giving values of 3.8 µA and 1.3 µA respectively (shown in Figure 4.18 as the solid and dotted horizontal lines).

Despite the fact that the bacteriophage-based approach gave lower amperometric signals in the whole range, it gave also significant lower background values for the negative control compared with the antibody-based approach (0.8 *vs.* 2.2 µA, respectively), better standard deviation values (0.2, n=8 *vs.* 0.7, n=35), and thus lower amperometric signal corresponding to the LOD value (1.3 *vs.* 3.8 µA) allowing better discrimination at low concentration levels. As shown in Figure 4.18 for the samples artificially inoculated with *Salmonella*, both strategies were able to give a clear positive signal for 10^1 CFU mL^{-1}, whereas the electrophoresis at the same concentration shows no band for any of them (Figure 4.16). On the other hand, in both strategies as low as 1 CFU mL^{-1} was detected as a positive sample in a total assay time of 3 h without the use of any pre-enrichment or selective enrichment steps, with higher sensitivity than PCR followed by electrophoresis or magnetic separation followed by microbiological

culture. According to the anti-*Salmonella*-MPs manufacturer, the accuracy of the IMS coupled with microbiological culture is not measurable since IMS is a qualitative, not a quantitative method. Agglomerated particles and several target bacteria bound to the same particle give rise to only one colony forming unit (CFU) on the plating media. Therefore, by coupling IMS or PMS with double-tagged PCR amplification and electrochemical magneto-genosensing quantitative methods were achieved, due to the fact that a single cell is detected and these methods are not affected by the formation of aggregates. Compared with other biosensing methodologies for detecting pathogenic bacteria (Tables 1.7 – 1.12, § 1.5.2) excellent detection limits were achieved with both procedures. It should be also pointed out that remarkable improved LOD was also achieved with the bacteriophage-based approach compared with LODs reported for other biosensing approaches using bacteriophages (Table 1.1, § 1.5.2). This fact can be ascribed to the combined used of the magnetic separation and the sensitivity of the amplicon detection with the m-GEC electrochemical genosensing strategy. Although both methodologies are able to detect as low as 1 CFU mL^{-1}, it is important to highlight that the strategy based on phagomagnetic separation not only achieves better discrimination between concentrations nearby the limit of detection but also provides the advantages of using bacteriophages instead of antibodies such as a cost-efficient and animal-free production, as well as their outstanding stability, overcoming thus the main challenges of the biorecognition elements in biosensing devices.

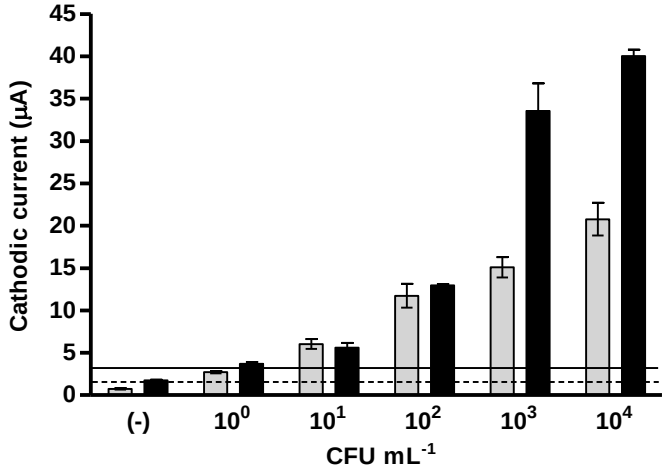

Figure 4.18. Electrochemical signals for the magneto-genosensing of the double-tagged amplicons of the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach (black bars) and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach (grey bars) by increasing the amount of *Salmonella* ranged from 10^0 to 10^4 CFU mL^{-1} artificially inoculated in LB broth. In all cases, n = 4 except for the 0 CFU mL^{-1} negative control (n = 35, for the IMS-based approach, and n = 8 for the PMS-based approach). LODs are shown as solid line (IMS) and dotted line (PMS). All other conditions as in Figure 4.17.

Another important feature to evaluate is the matrix effect, which is related with the sum of all interference effects that influence the final results. Due to the composition of milk, involving high contents of fat, protein and minerals [14], it is expected that this complex matrix content will affect the biorecognition of the bacteria. For this reason, the matrix effect was evaluated in skimmed milk obtained from local retail stores for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" strategy.

Figure 4.19 compares the electrochemical signals obtained for the assay performed in both LB broth and skimmed milk (diluted 1/10 in LB). As previously detailed, the amperometric signal corresponding to the negative control samples (0 CFU mL^{-1}) for the assay performed in LB broth gave a mean value of 2.2 µA (Figure 4.19 A dotted line) with a standard deviation of 0.7 µA. Whereas, for the assay performed in skimmed milk diluted 1/10 in LB, a mean value of 2.2 µA (Figure 4.19 B dotted line) with a standard deviation of 0.35 µA was obtained. The amperometric signal corresponding to the LOD value was then extracted with a one-tailed t test at a 99 % confidence level, giving a value of 3.8 and 3.1 µA, respectively (shown in Figure 4.19 A and B as the solid horizontal lines).

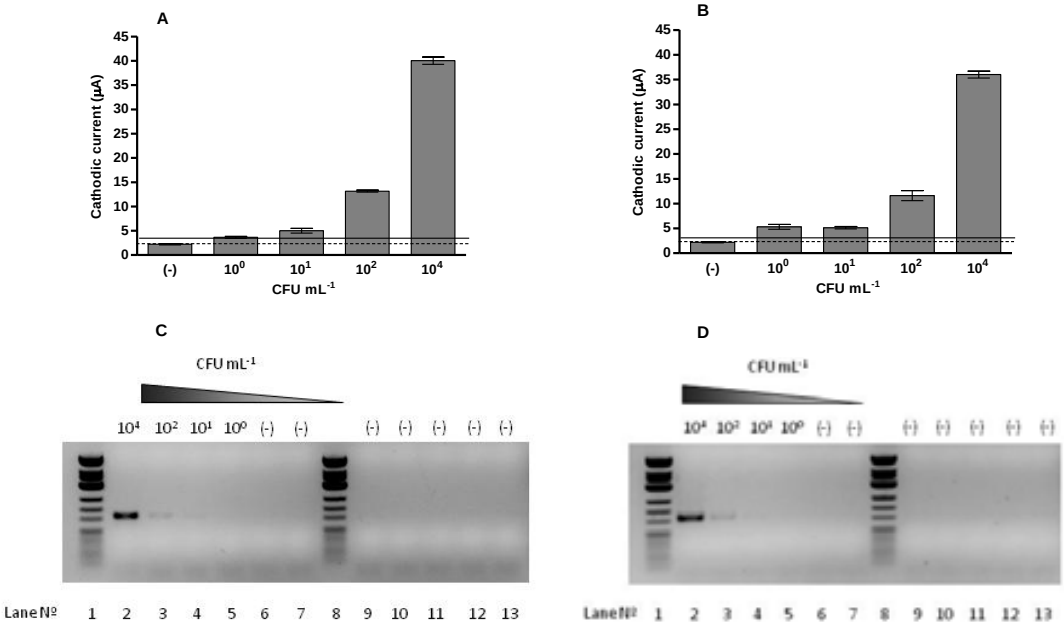

Figure 4.19. Up: Electrochemical signals for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach. Grey bars show the signal by increasing the amount of *Salmonella* ranged from 10^0 to 10^4 CFU mL^{-1} artificially inoculated in LB broth (A) and in skimmed milk diluted 1/10 in LB broth (B). A negative control is also shown. In all cases, n = 4, except for the negative control (n = 35). All other conditions as in Figure 4.17. Down: Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "IMS/double-tagging PCR/electrophoresis" approach, performed in LB broth (C) and in skimmed milk diluted 1/10 in LB broth (D). Lanes 2 – 5 correspond to 10-fold dilutions from 10^4 to 10^0 CFU mL^{-1}. Lanes 6, 7, and 9 to 13 are negative controls. Lanes 1 and 8 are the molecular weight marker (ΦX174-Hinf I genome).

No significant differences in the electrochemical signal were observed for the samples artificially inoculated with *Salmonella*, regardless the sample (LB or milk diluted 1/10 in LB). In both matrixes, as low as 1 CFU mL^{-1} was detected while no band was observed for the same concentration in the corresponding gel electrophoresis (Figure 4.19 C and D, lanes 5). No matrix effect was thus observed for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach performed in milk.

On the other hand, it is important to enhance that both the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approaches, showed high specificity. Figure 4.20 shows the electrochemical results of both approaches for LB artificially inoculated, in all cases, with 10^5 CFU mL^{-1} of *E. coli, Salmonella* and, a mix containing both bacteria *E. coli* and *Salmonella*, as well as a negative control (IMS-based approach, A; PMS-based approach, B). The corresponding electrophoresis images of the double-tagged amplicon are also shown (IMS-based approach, C; PMS-based approach, D).

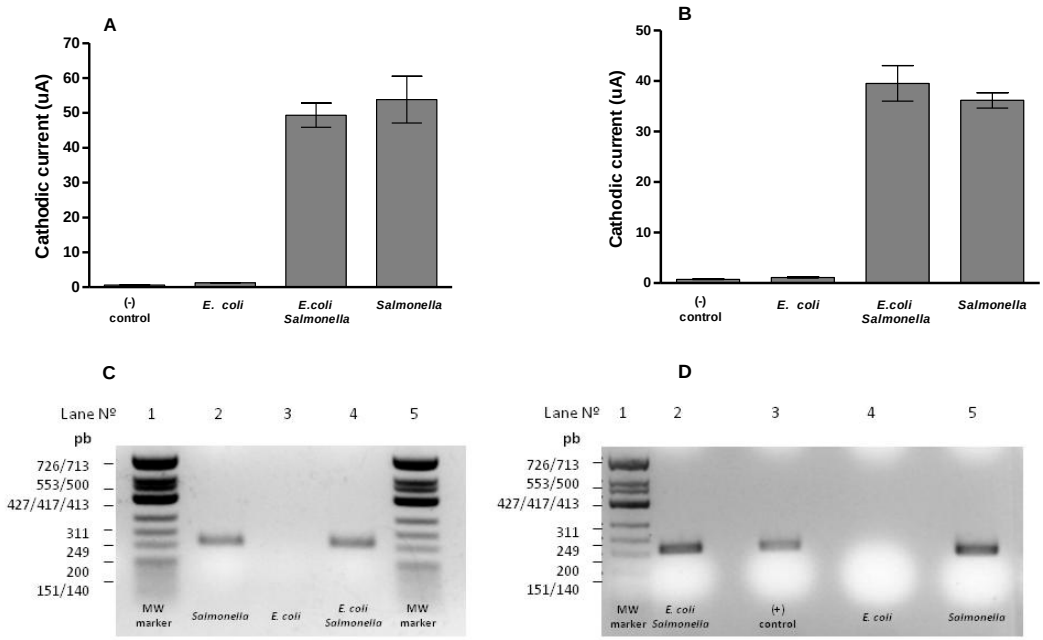

Figure 4.20. Up: Specificity study for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" (A) and "PMS/double-tagging PCR/m-GEC electrochemical genosensing" (B) approaches. Grey bars show the electrochemical signal for LB artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control), 10^5 CFU mL^{-1} of *E. coli*, *E. coli* and *Salmonella*, and *Salmonella*. All other conditions as in Figure 4.17. Down: Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "IMS/double-tagging PCR/electrophoresis" (C) and "PMS/double-tagging PCR/electrophoresis" (D) approaches. ΦX174-Hinf I genome was used as a molecular weight marker.

In both cases, as expected, the electrochemical signal obtained for *E. coli* is similar that for the negative assay, whereas the mix of both pathogens gave a similar signal that the sample spiked just with *Salmonella*. Similarly, no electrophoresis band was observed for *E. coli* (Figure 4.20, lane 3 -C- and lane 4 -D-), whereas the mix of both pathogens (Figure 4.20, lane 4 -C- and lane 2 -D-) and the *Salmonella* sample (Figure 4.20, lane 2 -C- and lane 5 -D-) gave a unique positive electrophoresis band producing only the expected 201 bp fragment, corresponding to the amplification of the IS*200* insertion sequence specific for *Salmonella*. The same results were obtained by microbiological culture by plating the bacteria attached to the magnetic particles. No growing was observed for *E. coli*, whereas typical colony features of *Salmonella* were observed for the mix of both pathogens as well as for just *Salmonella*.

These results confirm that the specificity of the approaches presented is coming mainly from the magnetic separation step, due to the specific antibody toward *Salmonella* which coated the magnetic particles for the IMS, or bacteriophages for the PMS. According to the anti-*Salmonella*-MPs suppliers, the magnetic particles reacts with all current *Salmonella* serovars of importance as the cause of human and animal disease occurring in food, feed and environmental samples, comprising the somatic groups from B to Z with variable reactivity depending on the serotype. For the bacteriophage-based strategy, the tailspike proteins present in the P22 bacteriophage specific to *Salmonella* which coated the magnetic particles specifically recognise the repetitive O-antigen part present in the lipopolysaccharides (LPSs) of *Salmonella* serotypes A, B and D1 outer membrane [15]. Another source of specificity for both electrochemical magneto-genosensing approaches to detect *Salmonella* spp. is coming from the double-tagging PCR. In this case, the chosen set of primers amplified exclusively the IS*200* insertion sequence, a transposable element present in more than 90 % of the pathogenic or food-poisoning isolates of *Salmonella* spp. [16, 17]. The selection of the IS*200* specific set of primers provides an additional source of specificity for the assay, particularly for those bacteria antigenically related to salmonellae genus. This fact could be especially useful in other applications when antibodies or bacteriophages with low specificity are involved in the magnetic separation step.

4.3.3.4. Pre-enrichment of milk samples

As cells are injured when exposed to adverse conditions during food processing, a pre-enrichment step is usually included in conventional methods to achieve the proliferation of

stressed *Salmonella* spp. cells. If this step is not included in the method, then stressed cells that have not been fully repaired may be missed [18]. Besides, a pre-enrichment step must be included to fulfil the legislation requirements for milk (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each [19, 20]). The "IMS/double-tagging PCR/m-GEC electrochemical genosensing" strategy was performed as an example of the evaluation of the limit of detection with a pre-enrichment step. The milk sample (25 mL) was spiked with just 1 CFU, divided in five 5-mL portions and processed as explained in § 3.6.3.4. The samples were pre-enriched in LB broth and assayed at 0, 6, 12, and 24 h of pre-enrichment, the positive control (7 CFU mL^{-1}) and two negative controls (0 CFU mL^{-1}) were also evaluated. As expected, just one of the five portions (Sampling 1), the one containing statistically 1 CFU of bacteria, gave positive results after 6 h (as shown in Figures 4.21 and 4.22). Figure 4.21 (A) shows the amperometric signal for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach after 6 h of pre-enrichment, for the five-5 mL samples, as well as for the positive and negative controls. Clearly, just the sample number 1 gave a positive signal due to the presence of as low as 1 CFU in 25 mL (0.04 CFU mL^{-1}). Figure 4.21 (B) shows the amperometric signal of the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach for the positive sample (S1) at 0, 6, 12, and 24 h of pre-enrichment. As a conclusion, after the pre-enrichment in LB, the procedure is able to detect as low as 0.04 CFU mL^{-1}, according to the legislation (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each in different points), with an amperometric signal of above 40 µA and with a signal-to-background ratio of 20.

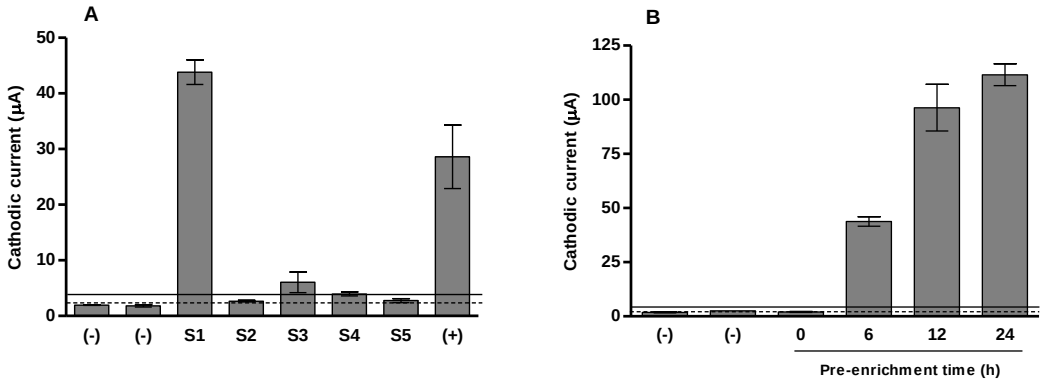

Figure 4.21. (A) Electrochemical signals for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach with a pre-enrichment step of 6 h for artificially inoculated skimmed milk. Five 5-mL portions (S1 to S5) of skimmed milk, two negative controls (0 CFU mL^{-1}), and a positive control (7 CFU mL^{-1}) are shown. (B) Electrochemical signals for the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach with a pre-enrichment step for the positive sample (S1) of 0, 6, 12, and 24 h. Two negative controls (0 CFU mL^{-1}) are also shown. In all cases, n = 4. All other conditions as in Figure 4.17.

The gel electrophoresis shown in Figure 4.22 is in agreement with the electrochemical results above explained. No bands were observed for any sampling from 2 to 5, or negative controls. Only sampling 1 and the positive controls produce the expected 201 bp fragment corresponding to the IS*200* insertion sequence. In this manner, 6 hours was determined as the pre-enrichment time needed not only to resuscitate injured cells but also to achieve the detection limits required by legislation.

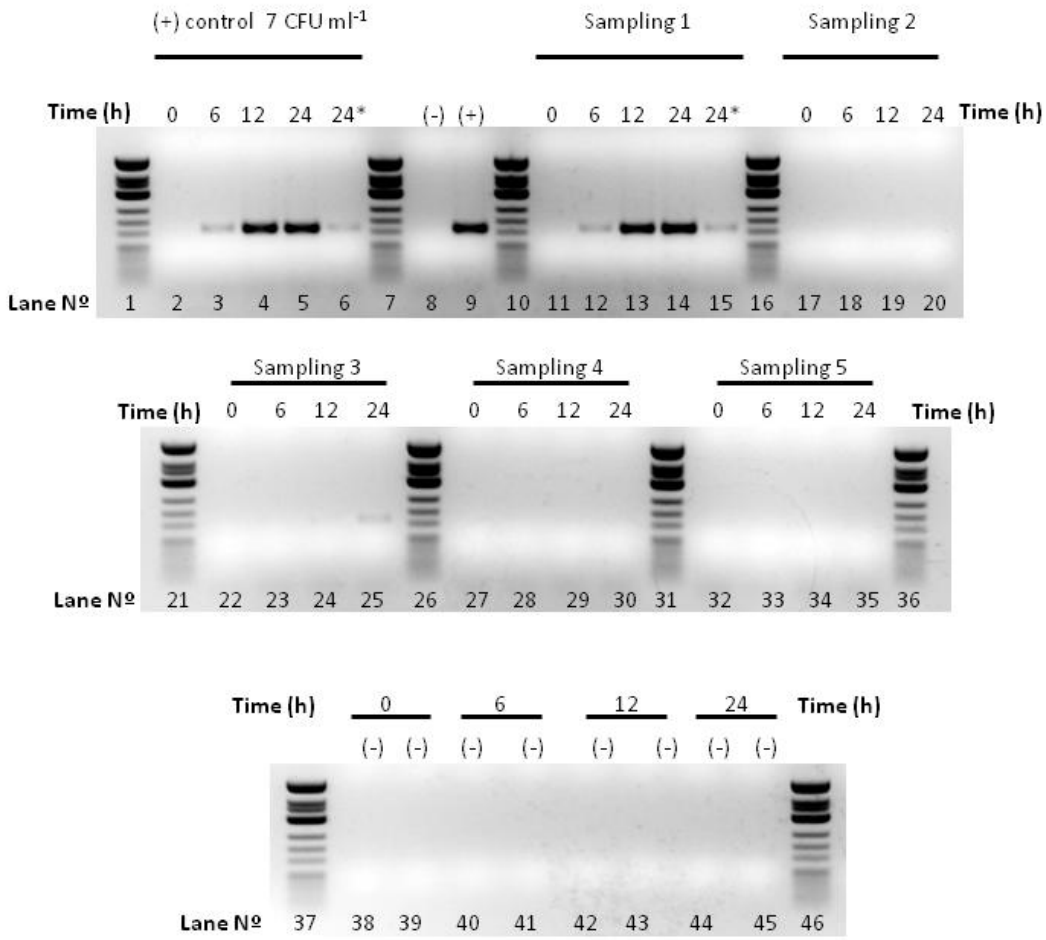

Figure 4.22. Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "IMS/double-tagging PCR/electrophoresis" approach with pre-enrichment step of 0, 6, 12 and 24 h. Five 5-mL portions (Sampling 1 to 5), two negative controls (0 CFU mL^{-1}, lanes 38 to 45) and a positive control (7 CFU mL^{-1}, lanes 2 to 6) are shown at all the pre-enrichment times (0, 6, 12 and 24 h). A 10-fold dilution of the 24 h pre-enriched samples is also shown (lanes 6 and 15, signalled as 24*). A positive (lane 9) and a negative (lane 8) PCR controls are also shown, as well as the molecular weight marker (ΦX174-Hinf I genome, lanes 1, 7, 10, 16, 21, 26, 31, 36, 37 and 46). Results were obtained for artificially inoculated skimmed milk.

4.3.4. Electrochemical magneto-immunosensing

As the specificity of the IMS showed good results, a simplification of the analytical procedure was performed. In this strategy, "IMS/m-GEC electrochemical immunosensing", after the immunomagnetic separation step instead of lysing the concentrated bacteria to detect the bacterial genome, a second immunological biorecognition of the whole cell was performed. In order to optimise the electrochemical magneto-immunosensing strategy four different immunological procedures were evaluated. The results obtained for all these optimisations are discussed and compared in following sections, as well as other parameters of the assay such as the limit of detection (LOD), matrix effect, specificity and pre-enrichment time needed.

4.3.4.1. Optimisation of the electrochemical magneto-immunosensing procedure

The optimisation of the immunological reactions for the "IMS/m-GEC electrochemical immunosensing" approach, was performed by evaluating different procedures for the detection of 7.2×10^6 CFU mL^{-1}, by varying the order of the immunological and the washing steps, being procedures Nº 1 to 3 one or two-step assays without washing steps in between while, in the procedure Nº 4, two succeeding immunological reactions were sequentially performed with washing steps in between (Figure 3.14, § 3.6.4.1). The incubation time used for the IMS step and for the enzymatic labelling was recommended by the suppliers as well as the amount of magnetic particles used, while the concentration of the anti-*Salmonella*-HRP antibody was optimised, for the procedures 1, 2 and 4 since procedure 3 is similar to procedure 1 and 4 in the steps order. Figure 4.23 shows the results obtained for procedures Nº 1, 2 and 4 for different dilutions of the anti-*Salmonella*-HRP antibody in milli-Q water. Although the specific signal was higher for smaller antibody dilutions (grey bars), the non-specific adsorption was also higher (white bars). As a consequence, the signal-to-background ratio was found to be better for anti-*Salmonella*-HRP antibody diluted 1/1000 in milli-Q water in all cases.

After the anti-*Salmonella*-HRP antibody concentration was optimised in 1 µg mL^{-1} (1/1000 dilution in milli-Q water) further optimisation of all the procedures was performed in order to evaluate the importance of the order of both steps, immunomagnetic separation and enzymatic labelling, as well as the effect of the media during the enzymatic labelling.

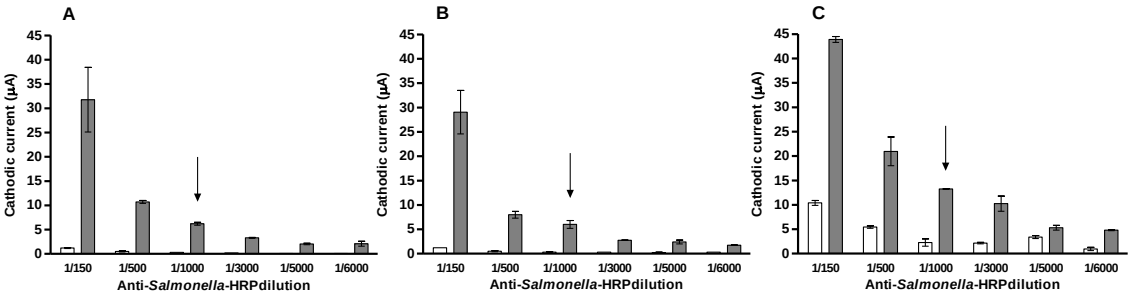

Figure 4.23. Evaluation of the enzymatic labelling for the "IMS/m-GEC electrochemical immunosensing" approach following procedures Nº 1 (A), 2 (B) and 4 (C). Grey bars show the electrochemical signal for 7.2 x 10^6 CFU mL^{-1} of *Salmonella* in LB broth. Negative controls are also shown as white bars. In all cases, 10 µL of commercial anti-*Salmonella*-MPs were used. Medium: phosphate buffer. Mediator: hydroquinone 1.8 mmol L^{-1}. Substrate: H_2O_2 4.9 mmol L^{-1}. Applied potential= -0.100 V (*vs*. Ag/AgCl). The error bars show the standard deviation for n=3.

As shown in Figure 4.24, the four proposed procedures were useful for the detection of *Salmonella* spp., although better results were achieved with procedure Nº 3 (IMS followed by enzymatic labelling without washing steps in between) and Nº 4 (IMS followed by enzymatic labelling with washing steps in between). Moreover, lower level of non-specific adsorption was achieved when the enzymatic conjugate anti-*Salmonella*-HRP antibody was diluted in LB broth, which seems to act not only as a growing media for the bacteria, but also as a blocking agent for the enzymatic labelling step. This blocking effect of LB broth is especially important for procedure Nº 4 during the immunological reaction with the anti-*Salmonella*-HRP antibody. In this case, the LB broth was able to reduce the non-specific electrochemical signal of the negative control from 5.5 (Figure 4.24 (A) Nº 4) to 1.0 µA (Figure 4.24 (B) Nº 4).

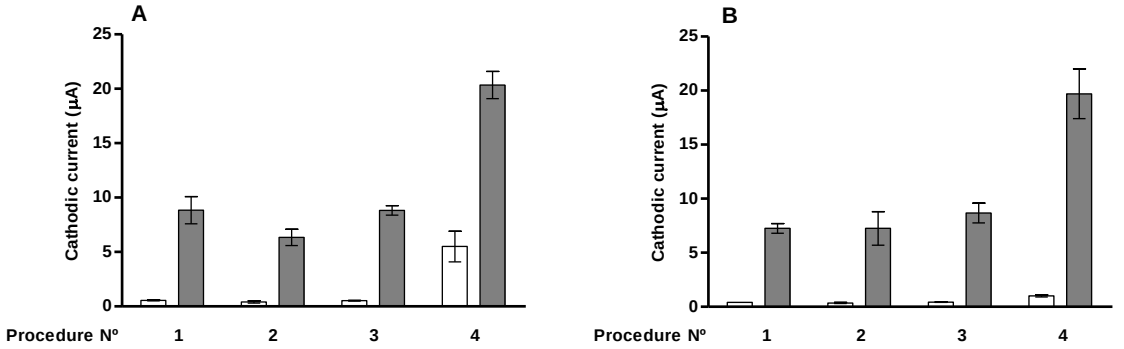

Figure 4.24. Electrochemical signals for the "IMS/m-GEC electrochemical immunosensing" approach by performing four different procedures, as detailed explained in Figure 3.14 (§ 3.6.4.1). Grey bars show the electrochemical signal for 7.2 x 10^6 CFU mL^{-1} of *Salmonella* in LB broth. Negative controls are also shown as white bars. In all cases, 10 µL of anti-*Salmonella*-MPs were used as well as anti-*Salmonella*-HRP antibody, diluted 1/1000 in milli-Q water (A) or in LB broth (B). The error bars show the standard deviation for n=3. All other experimental conditions as in Figure 4.23.

A comparison of the results obtained with all the procedures is shown in Table 4.8. The best result was achieved with procedure Nº 3 (two-steps, no washing). Although the specific signal was higher (19.7 *vs*. 9.8 µA) for the procedure Nº 4, the non-specific adsorption was also higher (1.0 *vs*. 0.5 µA), as a consequence, the signal-to-background ratio was slightly better for the procedure Nº 3 (21.7 *vs*. 19.7). Comparing the procedure Nº 1 (Figure 4.24 (B) Nº 1) and the procedure Nº 3 (Figure 4.24 (B) Nº 3), the pre-incubation step for 10 min of the magnetic particles with the bacteria improved slightly the electrochemical signal while keeping constant the non-specific signal. Also, the procedure Nº 2 gave signal-to-background slightly lower than procedure Nº 3 (Table 4.8) and worse results in terms of reproducibility (Figure 4.24 (B) Nº 2 and 3). Considering that the enzymatic labelling in procedure Nº 3 is performed after the IMS without prior washing steps, taking a total time of 60 min, this procedure demonstrated the best performance in terms of rapidity and simplicity, when the presence of interfering flora is not expected to be an issue. On the contrary, the two-steps procedure Nº 4, including washing steps, should be performed when high level of accompanying microflora is expected, in order to favour the specificity of the assay, taking a total time of 80 min. Thus, the procedure Nº 3 was selected for further quantification studies, while the specificity study was performed with the procedure Nº 4.

Table 4.8. Comparative results obtained for the "IMS/m-GEC electrochemical immunosensing" approach by performing four different procedures. (IMS: immunomagnetic separation; EL: enzymatic labelling).

Procedure Nº	**1**	**2**	**3**	**4**
Description	One-step (no washing): IMS + EL	One-step (no washing): EL+ IMS	Two-steps (no washing): IMS + EL	Two-steps (in between washing): IMS + EL
Time (min)	50	60	60	80
Signal-to-background ratio (milli-Q water)	16	16	17	4
Signal-to-background ratio (LB broth)	18	21	22	20

4.3.4.2. Detection limit, matrix effect and specificity studies

The amperometric response of the "IMS/m-GEC electrochemical immunosensing" approach for artificially inoculated *Salmonella* (ranged from 10^0 to 10^6 CFU mL^{-1}) in LB broth is shown in Figure 4.25. The amperometric response was adjusted to a sigmoidal curve with a $R^2 = 0.9849$.

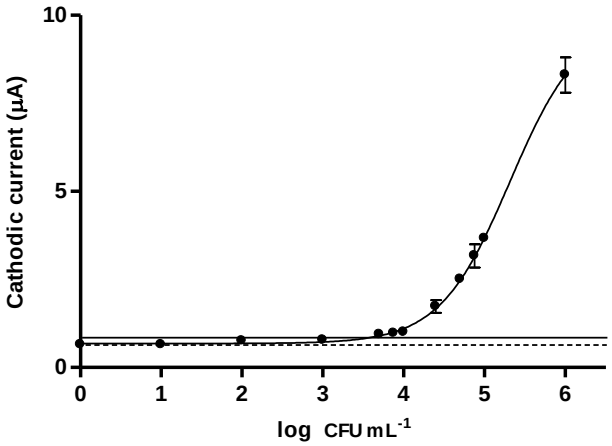

Figure 4.25. Eletrochemical signals for the "IMS/m-GEC electrochemical immunosensing" approach by increasing the amount of *Salmonella* ranged from 10^0 to 10^6 CFU mL^{-1} artificially inoculated in LB broth performing the procedure Nº 3. In all cases, 10 µL of anti-*Salmonell*a-MPs and anti-*Salmonella*-HRP antibody diluted 1/1000 in LB broth were used. Medium: phosphate buffer. Mediator: hydroquinone 1.8 mmol L^{-1}. Substrate: H_2O_2 4.9 mmol L^{-1}. Applied potential= -0.100 V (*vs*. Ag/AgCl). The error bars show the standard deviation for n=3, except for the negative control (n=10). The mean value for the negative control (dotted line) and the LOD value (solid line) are also displayed.

Moreover, in Figure 4.26, the detailed amperometric response at lower concentration, near the LOD, for artificially inoculated *Salmonella* in both LB broth and in skimmed milk dilute 1/10 in LB is compared. A linear response was obtained in both cases with a R^2 = 0.9225 and 0.7919, respectively. In the case of the assay performed in LB broth, the amperometric signal corresponding to the LOD was estimated by processing 10 negative control samples (0 CFU mL^{-1}) in two different single inter-day assays, and using six batches of magneto-electrode devices, obtaining a mean value of 0.7 µA (Figure 4.25 and 4.26 (A) dotted line) with a standard deviation of 0.06 µA. In the case of the skimmed milk, 15 negative control samples were processed, obtaining a mean value of 0.3 µA (Figure 4.26 (B) dotted line) with a standard deviation of 0.09 µA. The amperometric signals corresponding to the LOD values were then extracted with a one-tailed t-test at a 99 % confidence level, giving a value of 0.8 and 0.5 µA, respectively (shown in Figure 4.26 A and B as the solid horizontal lines). The "IMS/m-GEC electrochemical immunosensing" approach is able to detect 5.0 × 10^3 CFU mL^{-1} (in LB broth) and 7.5 × 10^3 CFU mL^{-1} (in skimmed milk diluted 1/10 in LB) artificially inoculated with *Salmonella*, in 60min.

Comparing with the microbiological culture (Table 4.5, § 4.3.1.1) as low as 5 CFU mL^{-1} were effectively captured and detected after the immunomagnetic separation followed by plating the magnetic particles with the attached bacteria in LB agar, however, an overnight incubation was needed for this method while the "IMS/m-GEC electrochemical immunosensing" approach is performed in only 60 min. As shown in Figure 4.26, a slight decrease in the slope of the

amperometric response in all the concentration range was observed due to the matrix effect of the milk sample. If this methodology were used for the quantification of *Salmonella* spp. in a sample different from milk, a quantification curve with a negative sample would be performed for each type of food matrix, prior to quantification. However, as the primary use of the "IMS/m-GEC electrochemical immunosensing" is to screen-out negative samples, the most important parameter is the LOD, in order to consider definitive any negative results. Positive test results should be always considered presumptive, and must be confirmed by an approved culture method.

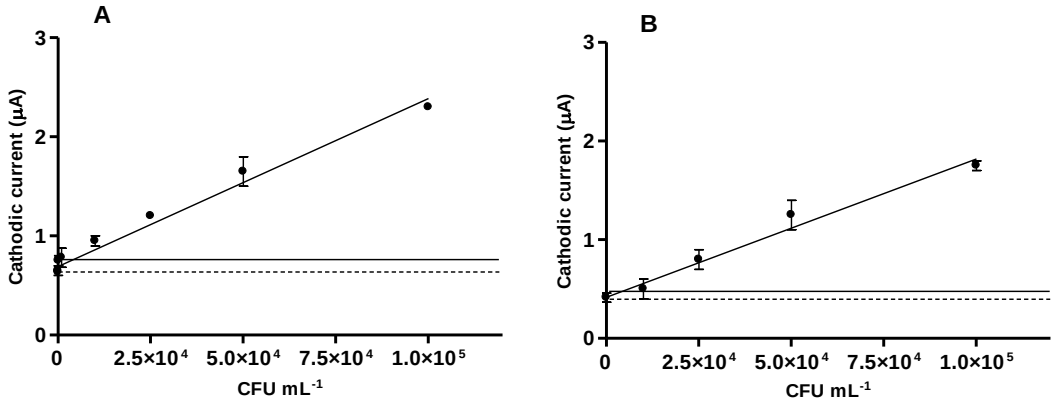

Figure 4.26. Electrochemical signals for the "IMS/m-GEC electrochemical immunosensing" approach for the detection of *Salmonella* cells from 10^0 to 10^5 CFU mL^{-1} artificially inoculated in (A) LB broth and (B) skimmed milk diluted 1/10 in LB, performing the procedure Nº 3. All other experimental conditions as in Figure 4.25. The error bars show the standard deviation for n = 3, except for the negative control (n = 10 for LB broth and n = 15 for skimmed milk diluted 1/10 in LB). The mean value for the negative control (dotted line) and the LOD value (solid line) are also displayed in both cases.

As previously mentioned, the two-steps procedure Nº 4 should be performed when high level of accompanying microflora is expected, in order to favour the specificity of the assay, by including the in-between washing steps, taking a total time of 80 min. As a consequence, the specificity study was performed by using the two-steps procedure Nº 4, the results are shown in Figure 4.27. As expected, the electrochemical signal obtained for *E. coli* (2.8×10^6 CFU mL^{-1}) was similar to the signal for the negative control (0 CFU mL^{-1}), while the mix of both pathogens (1.4×10^6 CFU mL^{-1} *E. coli* and 4.65×10^6 CFU mL^{-1} *Salmonella*) gave signals similar to the sample spiked just with *Salmonella* (5.2×10^6 CFU mL^{-1}). Same results were obtained by plating the magnetic particles with the attached bacteria in LB agar, no growing was observed for the *E. coli* sample, while typical colony features of *Salmonella* were observed for both the mix of both pathogens as well as just for *Salmonella*. These results confirm that the specificity of the "IMS/m-GEC electrochemical immunosensing" approach is coming mainly from the IMS step, due to the specific antibody towards *Salmonella* which coated the magnetic particles.

However, the selection of a specific enzymatic conjugate anti-*Salmonella*-HRP antibody could provide not only the electrochemical reporter, but also an additional source of specificity for the assay.

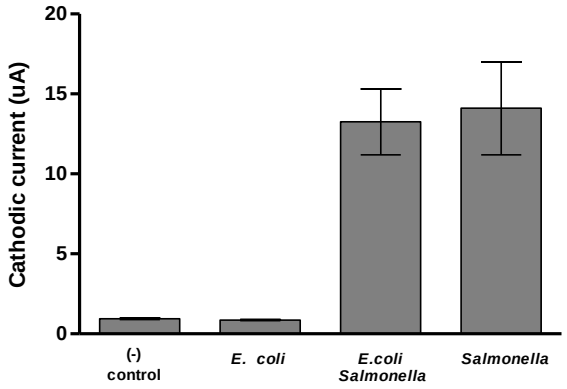

Figure 4.27. Specificity study for the "IMS/ m-GEC electrochemical immunosensing" approach. Grey bars show the electrochemical signal for LB artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control), 10^6 CFU mL^{-1} of *E. coli*, *E. coli* and *Salmonella*, and *Salmonella*. In all cases, 10 µL of anti-*Salmonella*-MPs were used as well as anti-*Salmonella*-HRP antibody, diluted 1/1000 in LB broth. All other conditions, as in Figure 4.25. The error bars show the standard deviation for n=3.

4.3.4.3. Pre-enrichment of milk samples

As previously mentioned in § 4.3.3.4, a pre-enrichment step is usually included in conventional methods to achieve the proliferation of stressed *Salmonella* spp. cells. If this step is not included, small amount of stressed cells that have been not fully repaired but still able to produce pathogenesis may be missed [18]. Beside this, and accordingly to the results for the LODs obtained in milk (7.5×10^3 CFU mL^{-1}), a pre-enrichment step should be included to fulfil the legislation requirements for milk (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each in different points [19, 20]). The pre-enrichment step was studied with a non-selective broth medium, in this case LB broth. The milk sample (25 mL) was spiked with just 2.7 CFU, divided in five 5-mL portions and processed as explained in § 3.6.4.3. The samples were pre-enriched in LB broth and assayed at 0, 4, 6, 8, 12, and 24 h of pre-enrichment, the positive control (1.4 CFU mL^{-1}) and the negative control (0 CFU mL^{-1}) were also evaluated. Figure 4.28 (A) and (B), shows that two of the five portions –as expected, those statistically containing the 1 CFU of *Salmonella* – gave positive results after 8 h, due to the presence of as low as 2.7 CFU in 25 mL (0.108 CFU mL^{-1}). Figure 4.28 (C) and (D) shows the amperometric signal of the "IMS/m-GEC electrochemical immunosensing" approach for the positive samples S2 and S4 respectively at 0, 4, 6, 8, 12, and 24 h of pre-enrichment. Although the method is able to detect 0.108 CFU mL^{-1} according to the legislation (absence of *Salmonella* in 25 g, sampled in

five portions of 5 g each in different points) with 8 h of pre-enrichment, remarkable improvement of the signal is achieved between 8 and 12 h of pre-enrichment. As a conclusion, after the pre-enrichment in LB, the procedure is able to detect as low as 0.108 CFU mL^{-1}, if the milk sample is pre-enriched for at least 8 h. In this manner, 8 hours was determined as the pre-enrichment time needed not only to resuscitate injured cells but also to achieve the detection limits required by legislation.

Comparing with commercial PCR assays for the screening of *Salmonella* without IMS (Table 1.12, § 1.5.2) the main advantage of this procedure is that free DNA released from death cells during food processing are not detected with this strategy, because of the IMS, which separates and pre-concentrates whole bacteria cells – but not DNA –, from food samples. Taking into account that a "positive screen test result" leads to a confirmatory culture assay, this fact is particularly important to avoid unnecessary confirmation testing. Comparing with other biosensing methodologies for detecting pathogenic bacteria in food (Table 1.7 – 1.12, § 1.5.2) excellent detection limits were achieved with this procedure in 60 min. Moreover, this method is more rapid and simple than other rapid antibody-based and nucleic acid-based polymerase chain reaction (PCR) methods that have been previously reported (Table 1.10, § 1.5.2).

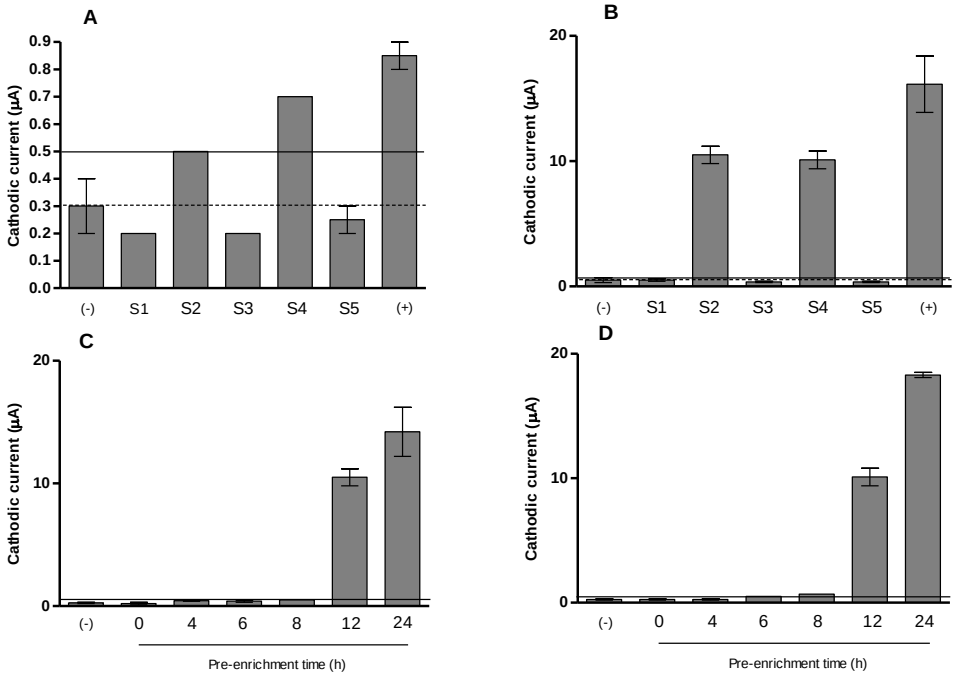

Figure 4.28. Electrochemical signals for the "IMS/m-GEC electrochemical immunosensing" approach after a pre-enrichment step of 8 (A) and 12 hours (B) for artificially inoculated with *Salmonella* skimmed milk. Five 5-mL portions (S1 to S5) of skimmed milk, a negative control (0 CFU mL^{-1}), and a positive control (1.4 CFU mL^{-1}) are shown. The electrochemical signals for the positive samples are also shown, S2 (C) and S4 (D) at a 0, 4, 6, 8, 12, and 24 h of pre-enrichment. In all cases, n = 3. All other conditions as in Figure 4.25.

4.3.5. General discussion

All the electrochemical strategies for *Salmonella* detection presented rely on the use of magnetic particles due to the fact that magnetic particles allow better analytical performance, as it was discussed in § 4.2 for the electrochemical detection of *M. bovis.* In addition, the advantages of using magnetic particles such as shortening of assay time, increasing of sensitivity as well as improving the reproducibility are well described in literature (§ 1.4.2). In the strategies discussed, the magnetic particles were used not only for the magnetic separation, IMS (anti-*Salmonella*-MPs) or PMS (P22 phage-modified MPs), but also for the electrochemical genosensing (streptavidin-MPs) and immunosensing (anti-*Salmonella*-MPs). Among the advantages of using magnetic particles, one of the most important is the capability of being separated easily from the liquid phase with a magnetic field, while being dispersed immediately after removed it. Higher reproducibility and improved LODs are thus achieved by the use of MPs that can easily bind the target while being dispersed in solution avoiding sensitivity and precision problems resulting from more desorption of antibodies during the assay or less diffusion of the analyte to the surface of the solid support, such as microplates.

In this section, magnetic separation based on different affinity biorecognition principles were evaluated, *i.e.* immunomagnetic and phagomagnetic separation. Although similar analytical performance were obtained, the use of bacteriophages as a biorecognition element offers additional advantages, such as low-cost, rapidity and animal-friendly production of the bacteriophages, among others (§ 1.4.3). It must be highlighted that for the first time non-modified bacteriophages are covalently coupled to magnetic particles. Moreover, in both strategies, namely the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" the magnetic separation steps are followed by the electrochemical magneto-genosensing. Improved LODs (1 CFU mL^{-1}) were obtained in both cases if compared with the IMS and PMS followed by conventional gel electrophoresis (10^2 and 10^3 CFU mL^{-1}, respectively) as well as a significant reduction of the assay time if compared with IMS and PMS followed by microbiological culture method (3 h *vs*. 18 – 24 h *vs*.), in which an underestimation of the bacteria counting was observed in the whole concentration range. The accuracy of the magnetic separation step coupled with microbiological culture is not measurable since magnetic separation is a qualitative, not a quantitative method. Agglomerated particles and several target bacteria bound to the same particle give rise to only one colony forming unit (CFU) on the plating media. Therefore, by coupling IMS or PMS with double-tagged PCR amplification and electrochemical magneto-

genosensing quantitative methods were achieved, due to the fact that a single cell is detected and these methods were not affected by the formation of aggregates. The double-tagging PCR also allows the amplification of the analytical signal by the amplification of the bacterial genome in a rapid way, instead of the multiplication of the bacteria number by growing in traditional culturing methods. The magnetic separation and the double-tagging PCR provide specificity, as well as versatility of the assay, by selecting different capture antibodies, bacteriophages or tagged primers. Therefore, the models described in this section can be widening to other bacterial targets.

The last strategy, namely the "IMS/m-GEC electrochemical immunosensing", represents a simplification in the analytical methodology in which the detection of the bacteria was performed by a double immunological recognition. After the IMS, the bacteria was detected by a second immunological biorecognition, reducing considerably the assay time from 3 h to 60 min as well as the complexity of the procedure compared with the electrochemical magneto-genosensing strategy that is PCR-dependent. On the other hand, worse LOD as well as slight matrix effect was obtained with this strategy (5 x 10^3 CFU mL^{-1} in LB broth and 7.5 x 10^3 CFU mL^{-1} in skimmed milk diluted 1/10 in LB broth). On the contrary, this method present better features for being implemented in microfluidic systems, due to its simplicity. In terms of specificity, both genosensing and immunosensing approaches, result in good performance due to the magnetic separation, however, it must be emphasised that although the fact of being PCR-dependent increases the complexity of the assay the selection of specific primers in the genosensing approach gives greater selectivity to the strategy.

Despite the differences, a real shortening of the analytical time is obtained for both genosensing and immunosensing approaches by the IMS or PMS followed by the double-tagging PCR with electrochemical magneto-genosensing, or by the serological confirmation with electrochemical magneto-immunosensing for the confirmation of the bacteria, as an alternative for the gold-standard microbiological culture method, in which the whole procedure (selective enrichment, differential plating culture, biochemical and serological confirmation testing) are time consuming. The strategies designed in this section fulfil the LOD required by the legislation (absence of *Salmonella* in 25 g of sample). Comparing with traditional methodologies, a significant improvement in total assay time has been achieved from 3 – 5 days to 9 hours in both cases, when the pre-enrichment step is included. In spite of the higher LOD obtained for the "IMS/m-GEC electrochemical immunosensing" approach compared with the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" (10^3 *vs*. 1

CFU mL^{-1}), after the pre-enrichment step, the same LOD demanded by legislation (1 CFU in 25 mL) was achieved in approximately 9 hours of total assay time for both strategies, having in this manner no advantages among them in terms of assay time.

All the approaches presented in this section, are more rapid and show better LODs than other rapid antibody-based and nucleic acid-based PCR methods previously reported (§ 1.5.2). As an example, enzyme-linked electrochemical detection coupled with IMS generally gave detection limits of 10^3 CFU mL^{-1}, whereas PCR methods could achieve LODs ranging from 10^1 to 10^4 CFU mL^{-1} depending on the efficiency of the DNA extraction, with or without enrichment step, and the nature of the food samples. Comparing with other commercial PCR assays for the detection of *Salmonella* without magnetic separation the main advantage of the "IMS/double-tagging PCR/m-GEC electrochemical genosensing" and "PMS/double-tagging PCR/m-GEC electrochemical genosensing" procedures is that free DNA coming from death or injured cells during food processing are not detected with this strategy, because of the IMS or PMS, which separate and pre-concentrate whole bacteria cells but not DNA from food samples. Moreover, as the bacteria are pre-concentrate and separate from the original matrix, the PCR inhibitors are also avoided, overcoming thus one of the most important issues of PCR-based assays. The amplicon detection with the electrochemical magneto-genosensing strategies demonstrated improved sensitivity than other approaches for detecting DNA. Regarding other rapid approaches based on genetic recognition, most of them are demonstrated with synthetic oligonucleotides, and only few procedures are based on inoculated bacteria detection obtaining LODs ranged from 10 to 10^4 CFU mL^{-1} (Table 1.9, § 1.5.2). Other rapid approaches based on immunological recognition coupled with electrochemical impedance spectroscopy or fluorescence detection are able to detect the bacteria faster (ranging from 6 min to 2.5 h), but with significantly higher LODs (from 10^2 to 10^5 CFU mL^{-1}) (Table 1.7, § 1.5.2). To the best of our knowledge, only detection techniques based on fluorescence are able to obtain similar features in terms of sensitivity to the approaches presented in this section.

These novel procedures are suitable for the rapid and sensitive on-site screening-out of *Salmonella* in HACCP. Since screening assays are used on large sample populations, often with the aim of determining which samples require further investigation and confirmation of the results, these approaches are promising strategies to screen-out negative samples and thereby to isolate negative from presumptive contaminated samples. Positive test results should be always considered presumptive and must be confirmed by an approved microbiological method, which is still considered the gold-standard for bacteria detection.

4.4. OPTICAL IMMUNOSASSAY OF *SALMONELLA*

In this section, a strategy based on the optical magneto-immunoassay of *Salmonella* based on magnetic particles is presented as an alternative for the electrochemical detection. The whole bacteria were detected by a double immunological recognition as in the "IMS/m-GEC electrochemical immunosensing" approach. After the IMS, the enzymatic labelling of the bacteria was also performed using a specific antibody against *Salmonella* labelled with HRP, in this case as optical reporter. The optimisation of the experimental factors that can affect the magneto-immunoassay was performed as well as other features of the assay such as the limit of detection, matrix effect and specificity studies. The results obtained for all these optimisations are discussed in following sections.

4.4.1. Magneto-immunoassay optimisation

The optical magneto-immunoassay was performed as previously optimised for the electrochemical magneto-immunosensing of *Salmonella* including the washing steps needed for the optical measurements. The sandwich magneto-immunoassay with optical detection (Figure 3.15, § 3.7), was performed in 96-well microtitre plates for the optimisation of the main factors affecting the immunological reaction, including the use of magnetic particles as solid support and the second antibody labelled with the enzyme HRP. Figure 4.29 displays the two-dimensional (2D) results obtained -for variable amount of anti-*Salmonella*-MPs and anti-*Salmonella*-HRP antibody while fixed concentration of *Salmonella* (10^7 CFU mL^{-1}) was added (Figure 4.29, A) and, variable concentration of the bacteria and anti-*Salmonella*-HRP antibody when fixed amount of anti-*Salmonella*-MPs (0.125 mg mL^{-1}) were used (Figure 4.29, B). In order to determine the optimal conditions, two factors were taken into account, the limitations of the Beer's Law [21] which suggest optimal signals about 1 absorbance unit, and the high-dose hook effect [13, 22, 23], that occurs when an immunoassay system is overwhelmed resulting in lower than expected absorbance readings. Accordingly to that, an amount of 0.125 mg mL^{-1} of anti-*Salmonella*-MPs, in agreement with the manufacturer recommendations, was chosen for resulting the value corresponding to the saturated signal to the majority of the antibody dilutions tested (Figure 4.29, A). Moreover, Figure 4.29 (B) shows the curves obtained for different anti-*Salmonella*-HRP antibody concentration and tenfold dilutions of the bacteria while the amount of anti-*Salmonella*-MPs used was the optimised 0.125 mg mL^{-1}. The response was adjusted to a sigmoidal curve for all the antibody dilutions tested with R^2 values of 0.9195 (1/1000), 0.9803 (1/2000), 0.9972 (1/4000), 0.9969 (1/6000), 0.9989 (1/8000), 0.9991

(1/10000) and 0.9960 (1/20000). It can be observed that lower dilutions (1/1000 and 1/2000) gave higher non-specific adsorption (0 CFU mL^{-1}), however, when the antibody is over diluted (1/10000 and 1/20000) the slope of the curve close to the LOD region (10^4 – 10^5 CFU mL^{-1}) is lower, resulting in a decrease of sensitivity of the assay. Hence, 1/4000 and 1/8000 antibody dilutions were chosen for further experimental assays.

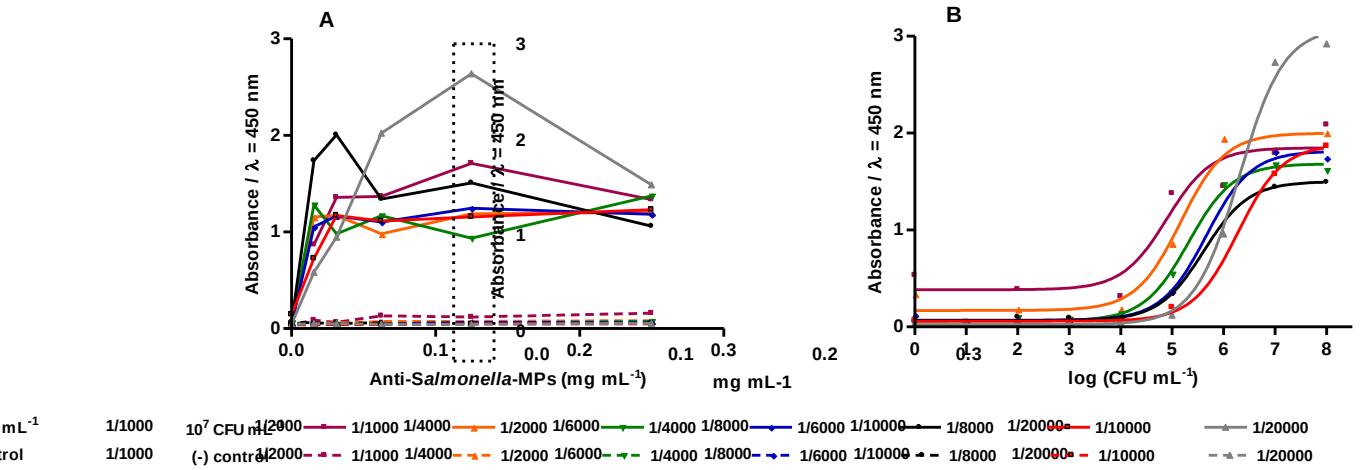

Figure 4.29. Optimisation of the immunoreagent concentrations by two-dimensional serial dilution experiments for the optical magneto-immunoassay. (A) Anti-*Salmonella*-MPs ranged from 0.02 to 0.25 mg mL^{-1} and *Salmonella* concentration fixed at 10^7 CFU mL^{-1} were used. (B) Tenfold dilutions of *Salmonella* ranged from 10^0 to 10^8 CFU mL^{-1} and anti-*Salmonella*-MPs fixed in 0.125 mg mL^{-1} were used. A negative control was included (0 CFU mL^{-1}). In all cases, anti-*Salmonella*-HRP antibody dilutions in 1 %-BPB ranged from 1/1000 to 1/20000 were used.

4.4.2. Detection limit, matrix effect and specificity studies

After all these preliminary optimisations, other features of the assay such as limit of detection (LOD), matrix effect and specificity were also studied.

In Figure 4.30 the results obtained for the optical magneto-immunoassay performed with optimal antibody dilutions (1/4000 and 1/8000) for *Salmonella* concentration ranging from 10^0 to 10^6 CFU mL^{-1} in both LB broth and skimmed milk (diluted 1/10 in LB broth) are presented. In the case of the assay performed with 1/4000 antibody dilution, the absorbance signal corresponding to the LOD was estimated by processing 12 negative control samples (0 CFU mL^{-1}) obtaining a mean value of 0.049 AU with a standard deviation of 0.007 AU for LB broth, and a mean value of 0.047 AU with a standard deviation of 0.005 AU for skimmed milk (diluted 1/10 in LB broth) (Figure 4.30, A). Moreover, for the assay performed with 1/8000 antibody dilution, the absorbance signal corresponding to the LOD was estimated by processing 9 negative control samples (0 CFU mL^{-1}) obtaining a mean value of 0.043 AU with a standard deviation of 0.003 AU for LB broth, and a mean value of 0.044 AU with a standard deviation of

0.004 AU for skimmed milk (diluted 1/10 in LB broth) (Figure 4.30, B). The signals corresponding to the LOD values were then extracted with a one-tailed t-test at a 99 % confidence level, giving values of 0.068 AU (in LB broth) and 0.060 AU (in skimmed milk diluted in LB broth) for 1/4000 antibody dilution and 0.051 AU (in LB broth) and 0.056 AU (in skimmed milk diluted in LB broth) for 1/8000 antibody dilution (shown in Figure 4.30 A and B respectively as the solid horizontal lines). Similar optical signals values as well as reproducibility were obtained independently of the antibody dilution tested, however, less matrix effect was observed for 1/8000 antibody dilution. Thus, it can be concluded that when 0.125 mg mL^{-1} of anti-*Salmonella*-MPs and 0.125 µg mL^{-1} of anti-*Salmonella*-HRP antibody are used the optical magneto-immunoassay is able to detect 10^4 CFU mL^{-1} of *Salmonella* artificially inoculated in both, LB broth and skimmed milk (diluted 1/10 in LB broth) in 90 min assay time.

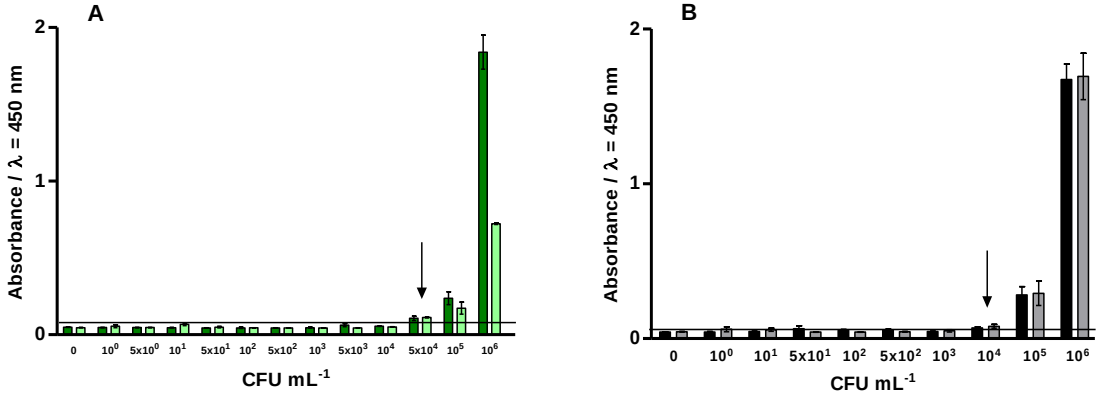

Figure 4.30. Comparative results obtained for the optical magneto-immunoassay for the detection of *Salmonella* cells ranged from 10^0 to 10^6 CFU mL^{-1} artificially inoculated in LB broth (dark green and black bars) and skimmed milk diluted 1/10 in LB broth (light green and grey bars). In all cases, 0.125 mg mL^{-1} of anti-*Salmonella*-MPs were used while anti-*Salmonella*-HRP antibody dilution was fixed in 1/4000 (A) and 1/8000 (B). The error bars show the standard deviation for n = 3, except for the negative controls, n = 12 (A) and n = 9 (B). The LOD values (solid lines) are also displayed in both cases.

Finally, the specificity of the optical magneto-immunoassay approach was verify by performing the assay with the best conditions above described for *Salmonella*, *E. coli* and a sample containing both bacterial species. Figure 4.31 compares the absorbance measurements obtained, as expected, signal obtained for *E. coli* (10^5 CFU mL^{-1}) was similar to the signal for the negative control (0 CFU mL^{-1}), while the mix of both pathogens (10^5 CFU mL^{-1} *E. coli* and *Salmonella*) gave signals similar to the sample spiked just with *Salmonella* (10^5 CFU mL^{-1}). These results are in agreement with the previous results for the electrochemical magneto-immunosensing and genosensing, due to the fact that the specificity is coming mainly from the IMS step, common to all the strategies, due to the specific antibody towards *Salmonella* which coated the magnetic particles.

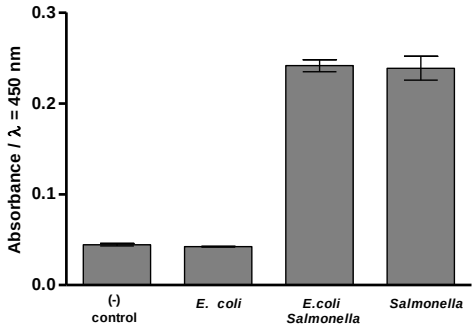

Figure 4.31. Specificity study for the optical magneto-immunoassay. Grey bars show the signal for LB broth artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control), 10^5 CFU mL^{-1} of *E. coli*, *E. coli* and *Salmonella*, and *Salmonella*. In all cases, 0.125 mg mL^{-1} of anti-*Salmonella*-MPs were used as well as anti-*Salmonella*-HRP antibody diluted 1/8000 in 1 %-BPB. The error bars show the standard deviation for n=3.

4.4.3. General discussion

In this study a novel optical magneto-immunoassay for the detection of *Salmonella* in food such as skimmed milk was developed, as an alternative for the electrochemical detection. This strategy was also useful for to the rapid optimisation of the main parameters as well as to compare with other existing ELISA-type assays.

The Enzyme Linked Immunosorbent Assays (ELISAs) are popular and widely implemented technique to detect pathogenic bacteria in routine analysis but a typical ELISA yields a sensitivity of 10^6 – 10^7 CFU mL^{-1}. The combination of the ELISA with the immunomagnetic separation step with aiming to pre-concentrate cells from mixed cultures has been previously reported, nevertheless, the detection sensitivity was considered close to that of a conventional ELISA (10^5 – 10^6 CFU mL^{-1}). Considerably improvement of the LOD was achieved with the optical magneto-immunoassay developed in this section (10^4 CFU mL^{-1}) taking into account that only recent works based on the use of carbon nanotubes as signal amplifiers (10^4 CFU mL^{-1}) or polyacrylonitrile fibres as alternative matrix for antibody immobilisation (10 CFU mL^{-1}), and those based on fluorescence or electrochemical detection (1 – 10^2 CFU mL^{-1}) are able to improve the detection limits of the popular ELISA technique (Table 1.7 and 1.8, § 1.5.2). Comparing with conventional ELISA assay, the main advantage of this strategy is the use of antibodies covalently attached to magnetic particles, avoiding desorption of antibodies during the assay as in the case of other common platforms, such as polystyrene microplates, in which the random physical adsorption of the antibodies is performed [24]. The magnetic particles serve as solid support in the ELISA system, and the uniform dispersion of particles throughout the reaction mixture allows for rapid reaction kinetics resulting in the improvement of the analytical features of the strategy [25].

4.5. ELECTROCHEMICAL GENOSENSING *OF SALMONELLA, LISTERIA* AND *E. COLI*

Finally, the last strategy presented in this dissertation is based on the electrochemical magneto-genosensing of the three most common pathogenic bacteria in food safety (*Salmonella, Listeria* and *E. coli*). This approach was performed by the release of the bacteria genome of *Salmonella, Listeria* and *E. coli* followed by PCR in order to obtain the tagged amplicons by using fluorescein, biotin and digoxigenin as coding tags for one of the primers of each set. The amplicons, tagged with fluorescein, biotin and digoxigenin for *Salmonella, Listeria* and *E. coli* respectively, were then immobilised on silica magnetic particles. To confirm the identity of the three bacteria, the tagged amplicons were detected by electrochemical magneto-genosensing using three different electrochemical reporters, Anti-Fluorescein-HRP, Streptavidin-HRP and Anti-Digoxigenin-HRP conjugates respectively. The optimisation of the silica magnetic particles as platform for electrochemical genosensing is described, including the main parameters to selectively attach longer dsDNA fragments instead of shorter ssDNA primers based on their negative charge density of the sugar-phosphate backbone. After optimising the main parameters for the electrochemical magneto-genosensing of the tagged amplicons with fluorescein, biotin and digoxigenin on silica magnetic particles, the evaluation of the DNA extracts from the three pathogens simultaneously (*Salmonella, Listeria and E. coli*) as well as the pathogens individually were processed. Further discussion of all the results obtained is presented in next sections.

4.5.1. Tagging PCR amplification and gel electrophoresis detection

The amplification of the *invA, prfA* and *eaeA* genes related to *S. enterica*, *L. monocytogenes* and *E. coli* respectively, was performed in order to obtain the tagged amplicons with fluorescein, biotin and digoxigenin by polymerase chain reaction (PCR) using the tagged primers. As explained previously for *M. bovis* electrochemical genosensing (§ 4.2.1), during the PCR, not only the amplification of pathogenic bacteria genome was achieved, but also the tagging of the amplicon end with (i) fluorescein to achieve the enzymatic detection through antiFlu-HRP reporter (*invA* gene, forward primer) (ii) biotin to achieve the enzymatic detection through Strep-HRP reporter (*prfA* gene, reverse primer) and (iii) digoxigenin to achieve the enzymatic detection through AntiDig-HRP reporter (*eaeA* gene, forward primer). The extracted bacterial DNA was amplified by tagging PCR. In this case, only one primer of the set was labelled, as schematised in Figure 4.32. After the annealing of 5' labelled primers with the

template, a new DNA strand was enzymatically assembled by the Taq polymerase, by the addition of nucleotides to the 3' end of both primers. The primers, and thus their tags, were included in the amplicon in such a way that the DNA was not only amplified but also labelled.

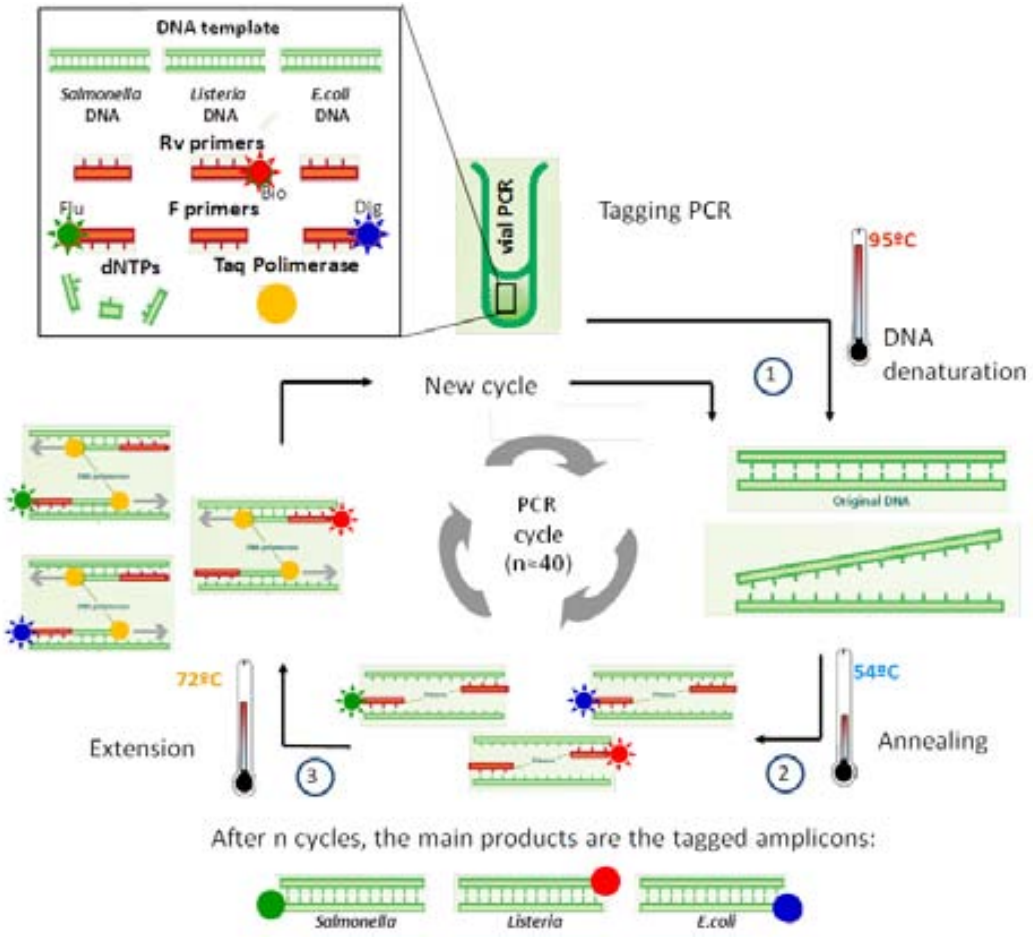

Figure 4.32. Schematic representation of the tagging PCR amplification, in order to obtain the amplicons tagged with fluorescein, biotin and digoxigenin coding tags, from a *Salmonella, Listeria* and *E. coli* genome templates, respectively.

As the set of primers were selected for the amplification of *invA* (278 bp), *prfA* (217 bp) and *eaeA* (151 bp) genes, in order to produce tagged amplicons of different molecular weight, the gel electrophoresis is a complementary tool for the evaluation of the performance of the selected primers towards *S. enterica*, *L. monocytogenes* and *E. coli* 0157:H7, respectively. This technique determines the presence or absence of PCR products and quantifies the size (length of the DNA molecule) of the product by comparison with the fragments of a DNA size marker. The amplification (absence of PCR inhibitors, primers effectiveness and experimental conditions) and the exclusivity of the obtained band are evaluated by this technique.

According to the agarose gel electrophoresis shown in Figure 4.33, the chosen set of primers amplified the *invA, pfrA* and *eaeA* genes, producing the expected 278 bp, 271 bp and 151 bp fragments, related to *S. enterica, L. monocytogenes* and *E. coli* 0157:H7 respectively (Figure 4.33, lanes Nº 2, 4, 5 and 6). No bands were observed for the negative controls performed with milli-Q water instead of *S. enterica*, *L. Monocytogenes* and *E. coli* 0157:H7 DNA templates (Figure 4.33, lanes Nº 8 to 10). A critical feature of this system is that specific amplification during PCR is mandatory, since the amplicon will be detected without any further hybridisation to interrogate the sequence. Although when the template of the three pathogens were amplified simultaneously, the PCR showed only the specific bands (as shown in Figure 4.33, lane Nº 2), the binary combination of *E. coli* and *Salmonella,* and the individual amplification of *E. coli* and *Salmonella* showed non-specific bands (results not showed) suggesting the non-specific annealing of one of the set of primers when its specific target (*Listeria*) was absent in the PCR mix. As a consequence, a deeper study for the selections of primers for the multiplex PCR should be performed if the triple-tagging multiplex PCR would be used for the simultaneous detection of the three pathogens. However, in this dissertation a full optimisation of the parameters for the attachment of the tagged-amplicon on silica-MPs was performed by using the amplicons that showed only the expected bands by gel electrophoresis and thus, specific amplification, *i.e.* the PCR coming from the mix of the three pathogens (Figure 4.33, lane Nº 2) as well as the interrogation of the pathogens individually amplified by using only corresponding primers (Figure 4.33, lanes Nº 4 to 6), while those showing non-specific bands (mainly the binary combination of *E. coli* and *Salmonella,* and the individual amplification of *E. coli* and *Salmonella*) were discarded.

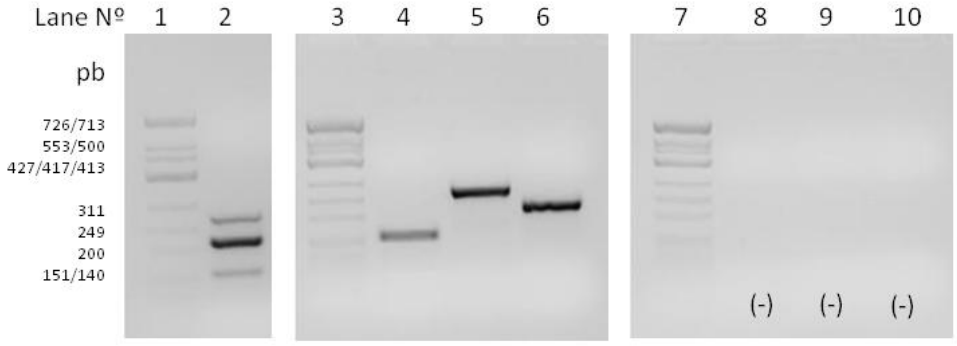

Figure 4.33. Agarose gel electrophoresis of the tagged amplicons obtained by PCR amplification. Lane 2 corresponds to the amplification of *invA* (278 bp)*, pfrA* (271 bp) and *eaeA* (151 bp) genes simultaneously. Lanes 4 to 6 correspond to the individual amplification of genes, in detail, lane 4 is the *eaeA* amplification, lane 5 is the *invA* amplification, and lane 6 is the *pfrA* amplification. Lanes 8 to 10 are the PCR negative control. Lanes 1, 3 and 7 are the molecular weight marker (ΦX174-Hinf I genome). The combinations showing non-specifics bands are not shown.

4.5.2. Optimisation of the electrochemical magneto-genosensing procedure

The immobilisation of tagged amplicons on silica magnetic particles was evaluated by comparing four different procedures (named A to D). Further optimisation of other experimental parameters such as concentration of magnetic particles, agitation, temperature incubation and concentration of enzymatic labels was performed as well, as described in detail in following sections.

4.5.2.1. Optimisation of the tagged amplicon immobilisation on silica magnetic particles

As the DNA is attracted to silica by weak, non-specific adsorptive forces, and this interaction is attributed to negative charge density of the sugar-phosphate backbone, the dsDNA amplicon as well as the ssDNA primers could be both theoretically attracted by silica magnetic particles. Thereby, the conditions for the attachment of longer dsDNA amplicons instead of shorter ssDNA primers based on their negative charge density was carefully studied and optimised for the first time. This optimisation was firstly performed by processing the DIG-tagged amplicon of the *eaeA* gene for *E. coli* detection as a model in four different procedures (named A to D, § 3.8.2.1).

Figure 4.34 shows comparatively the electrochemical signal obtained by the adsorption of the dsDNA DIG-tagged amplicon (white bar) as well as the negative control containing the ssDNA DIG-tagged primer (grey bar). Although the amount of digoxigenin tag was the same in the positive and the negative control, the signal coming from the DIG-tagged amplicon was significantly higher in all the procedures tested. These results confirm that when the shorter ssDNA tagged primers are incorporated by PCR in longer dsDNA fragments, the adsorption on silica increases due to the higher charge density of the sugar-phosphate backbone. Moreover, and as shown in Figure 4.34 (left), among the different procedures, procedure B provided a better amperometric signal of above 7 µA and with a signal-to-background ratio of 3. The binding buffer used in procedure B contains a high concentration of the chaotropic agent guanidinium thiocyanate, used for its capability to disrupt the association of nucleic acids with water and favouring silica adsorption. According to Mao *et al.*, the binding takes place despite the fact that the silanol and phosphate moieties involved are both acidic and thus negatively charged in an aqueous environment [26]. Counter ions, either in the buffer or as metal impurities

on the silica surface, may be involved in lessening the energy barrier that must be overcome before hydrogen bonding takes place. Besides the chaotropes as agents favouring the adsorption of DNA on silica, some detergents such as Triton X-100 can be also used (as in the case of procedure A and C). However, poorer analytical performance, in terms of net amperometric signal and signal-to-background ratio, was obtained in both cases compared with procedure B. Regarding procedure D, ethanol and acetone were added for the removal of the chaotropic salts producing also worse amperometric signal and signal-to-background ratio.

Once the procedures were evaluated, further optimisation of the selected procedure B was performed, by the study of the effect of the temperature and agitation during the amplicon immobilisation on silica-MPs. These parameters were also evaluated by processing both the negative and the positive amplification products of the *eaeA* gene labelled with digoxigenin with several variations of procedure B. Figure 4.34 (right) shows that the higher rate of binding of tagged-dsDNA amplicon was achieved at 55 ºC (procedures B3 and B4) compared with room temperature (procedures B1 and B2), while keeping approximately the same adsorption value for the ssDNA tagged-primers. Comparing the results of the optimisation for the shaking conditions (with 700 rpm agitation –procedure B3–, or without agitation –procedure B4–) similar results were obtained in both cases. The procedure B4 was chosen for being more reproducible and easy to perform, besides, incubations performed without shaking are a clear advantage in terms of automation and miniaturisation.

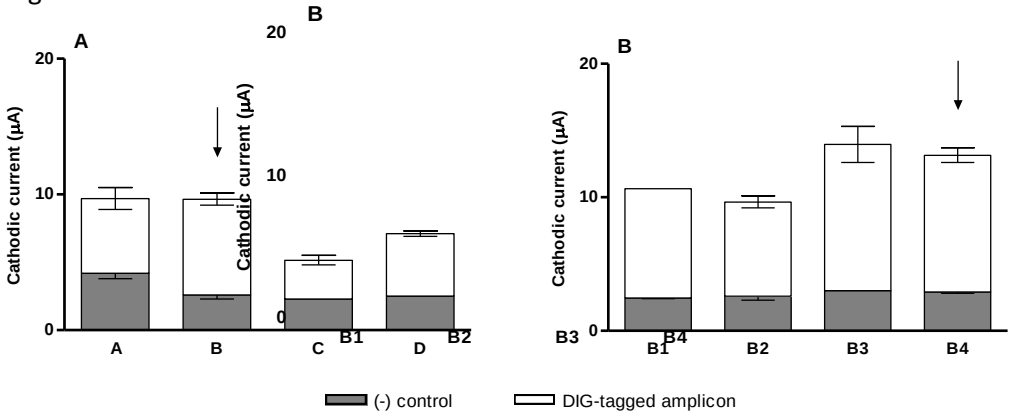

Figure 4.34. Electrochemical signals for the optimisation of the tagged amplicon immobilisation on silica-MPs. (A) Comparison of different procedures (A) based on Dynabeads SILANE genomic DNA kit, (B) and (C) based on Gonzalez *et al.* and (D) based on Boom *et al.* (B) Electrochemical signals corresponding to procedure B with different experimental conditions, including the incubation temperature (room temperature –procedures B1 and B2–, and 55 ºC –procedures B3 and B4–) and shaking conditions (with 700 rpm agitation –procedures B1 and B3–, or without 700 rpm agitation –procedures B2 and B4–). In all cases, grey bars correspond to the non-specific adsoption of the DIG-tagged ssDNA primer while the white stacked bars show the signal corresponding to the DIG-tagged dsDNA amplicon of the *eaeA* gene (n=2). In all cases, 0.2 mg of silica-MPs and 60 µg AntiDig-HRP were used. Medium: phosphate buffer. Mediator: hydroquinone 1.81 mmol L^{-1}. Substrate: H_2O_2 4.90 mmol L^{-1}. Applied potential= -0.100 V (*vs*. Ag/AgCl).

4.5.2.2. Optimisation of the amount of silica magnetic particles

Besides the binding procedure, an important parameter for the electrochemical magneto-genosensing strategy is the concentration of silica magnetic particles which was also optimised to improve the strategy. The optimal amount of particles was evaluated by electrochemical magneto-genosensing, processing both the negative and the positive amplification products of the DNA extract from the mixture of the three pathogens, by challenging them with the different electrochemical tracers, namely AntiFlu-HRP, Strep-HRP and AntiDig-HRP, to detect, respectively, *Salmonella*, *Listeria*, and *E. coli*. As shown in Figure 4.35 (right), better analytical performance in terms of signal-to-background response was obtained in all cases, when using the stock solution of 10 mg mL^{-1} of silica-MPs (0.05 mg per reaction).

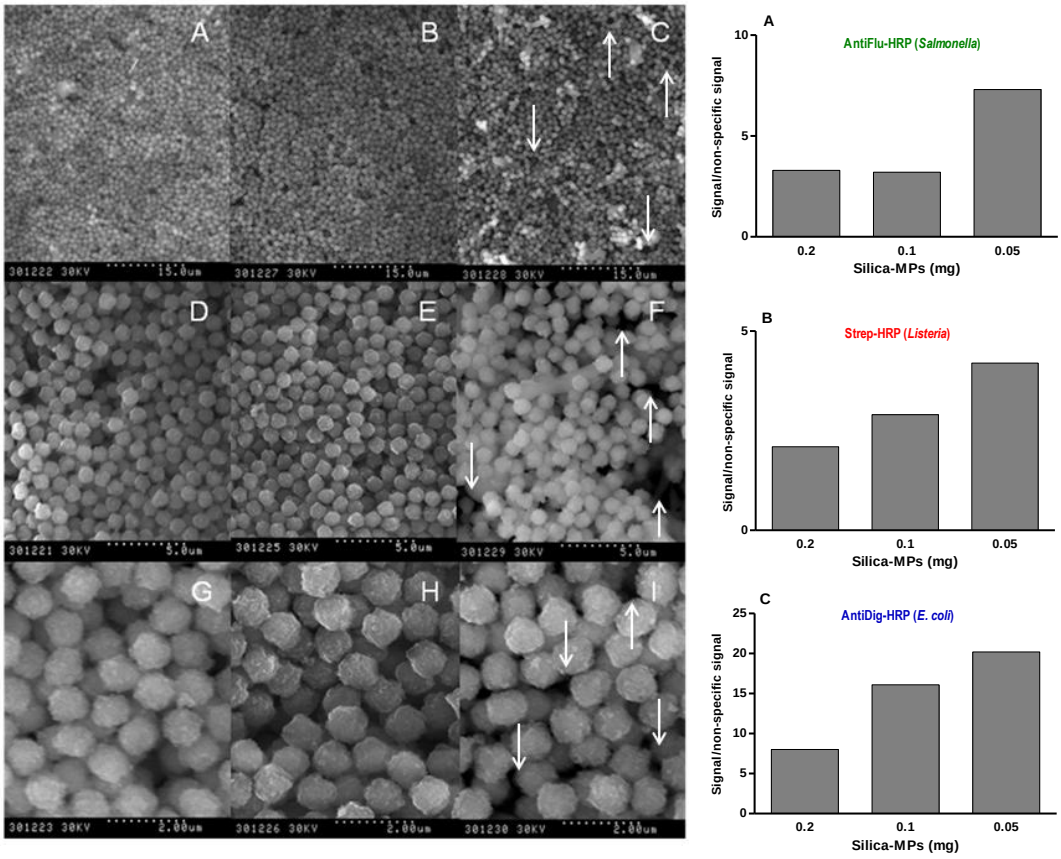

Figure 4.35. Left. Scanning electron microphotographs showing the captured silica-MPs on the surface of m-GEC magneto-electrode. Resolution 15 µm (A, B and C), 5 µm (D, E and F) and 2 µm (G, H and I). In all cases, identical acceleration voltage was used (30 kV). Number of magnetic particles are 2 x 10^8 (0.2 mg) for A, D and G images; 1 x 10^8 (0.1 mg) for B, E and H images; 5 x 10^7 (0.05 mg) for C, F and I images. Right. Electrochemical signals for the optimisation of the tagged amplicon immobilisation on silica-MPs. Grey bars show the signal-to-background ratio for 0.2, 0.1 and 0.05 mg of silica-MPs when AntiFlu-HRP (A), Strep-HRP (B) or AntiDig-HRP (C) were used. In all cases, amplicon coming from the mixture of the three pathogens and 60 µg of reporters were used (n=2). All other conditions as in Figure 4.34.

These results are in accordance with the qualitative information about the distribution of different amount of silica magnetic particles on m-GEC surface provided by Scanning Electron Microscopy. Figure 4.35 (left) shows comparatively the SEM images of m-GEC with different amount (0.2, 0.1 and 0.05 mg) of silica magnetic particles. As can be seen in the microphotographs, at lower amount of magnetic particles, bare transducer areas of the magneto-electrode can be observed, which can be easily reached by the electrochemical mediator hydroquinone (HQ) without hindrance, and thus, improving the electrochemical transduction in terms of signal-to-background response which allows better discrimination between concentrations nearby the limit of detection. Taking these results into account, 0.05 mg that corresponds to 5 x 10^7 silica-MPs were used for further experimental assays.

4.5.2.3. Optimisation of the enzymatic labelling step

The enzymatic labelling was evaluated for the electrochemical reporters AntiFlu-HRP, Strep-HRP and AntiDig-HRP able to detect the fluorescein, biotin and digoxigenin coding tags at the end of the dsDNA amplicons. Figure 4.36 shows the signal-to-background ratio obtained with different quantities of enzymatic label as well as different temperature incubation tested.

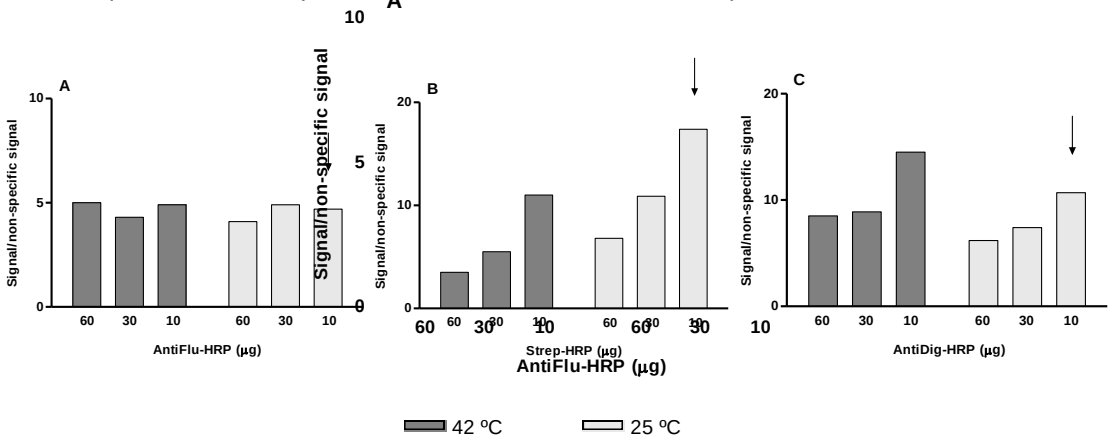

Figure 4.36. Electrochemical signals for the optimisation of the enzymatic labelling. Bars show the signal-to-background ratio for incubations performed at 42 ºC (dark grey) and 25 ºC (light grey). Variable quantities (60 µg, 30 µg and 10 µg) of AntiFlu-HRP (A), Strep-HRP (B) or AntiDig–HRP (C) were used (n=2). In all cases, 0.05 mg of silica-MPs were used. Medium: phosphate buffer. Mediator: hydroquinone 1.81 mmol L^{-1}. Substrate: H_2O_2 4.90 mmol L^{-1}. Applied potential= -0.100 V (*vs.* Ag/AgCl).

In general, better signal-to-background ratio was obtained when using 10 µg of enzymatic labels, except when AntiFlu-HRP was used (Figure 4.36, A) that similar values were obtained for all the quantities tested. Regarding the incubation temperature, the signal-to-background ratio obtained when AntiFlu-HRP was used was about 5 in all conditions. However, the results

when Strep-HRP was used were significantly better for incubations at room temperature than at 42 ºC (Figure 4.36, B), 17 *vs.* 11 (10 µg of Strep-HRP). By contrast, when AntiDig-HRP was used, the data obtained at room temperature was slightly lower than at 42 ºC (Figure 4.36, C), 11 *vs*. 14 (10 µg of AntiDig-HRP). Thus, room temperature was chosen as the optimal parameter for being as well an advantage for future implementation in microfluidic devices.

4.5.3. Electrochemical magneto-genosensing of *Salmonella, Listeria* and *E. coli* tagged amplicons

After optimising the main parameters for the electrochemical magneto-genosensing of the tagged amplicons on silica magnetic particles using the DIG-tagged amplicon of the *eaeA* gene for *E. coli* detection as a model of the other coding tags, the evaluation of the DNA extracts from the PCR mixtures showing only specific amplification, *i.e.* the three pathogens together (*Salmonella, Listeria and E. coli*) as well as the individual pathogens, were analysed by electrochemical magneto-genosensing with the m-GEC electrodes.

Figure 4.37 shows the amperometric response when the electrochemical reporters (AntiFlu-HRP, Strep-HRP and AntiDig-HRP) were used for the simultaneous detection of the three pathogens. The amperometric signal corresponding to the negative controls, which contains the tagged ssDNA primers, was estimated by processing 15 samples, performing seven different single inter-day assays, and using 12 magneto-electrode devices, obtaining a mean value of 1.0 µA with a standard deviation of 0.3 µA (for *Salmonella* detection), 1.2 µA with a standard deviation of 0.4 µA (for *Listeria* detection) and 0.9 µA with a standard deviation of 0.3 µA (for *E. coli* detection). The cut-off values were then determined with a one-tailed t test at a 95 % confidence level, giving values of 1.6, 1.8 and 1.4 µA respectively (shown in Figure 4.37, as the dotted lines). A clear positive signal was obtained for all the pathogens with an amperometric signal of 9.8, 12.9 and 6.6 µA and with a signal-to-background ratio of 10, 11 and 7 for *Salmonella*, *Listeria* and *E. coli*, respectively, confirming a selective attachment of longer dsDNA amplicons instead of shorter ssDNA primers based on their negative charge density.

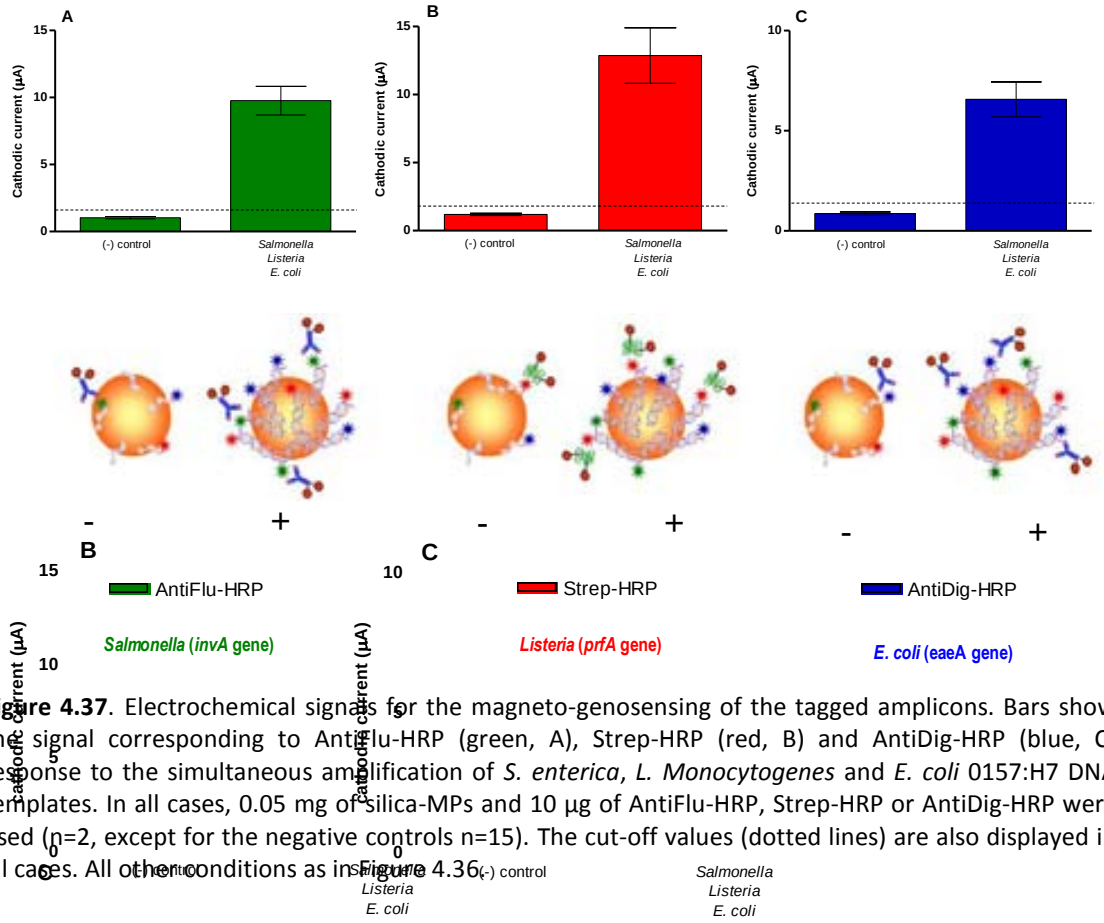

Figure 4.37. Electrochemical signals for the magneto-genosensing of the tagged amplicons. Bars show the signal corresponding to AntiFlu-HRP (green, A), Strep-HRP (red, B) and AntiDig-HRP (blue, C) response to the simultaneous amplification of *S. enterica*, *L. Monocytogenes* and *E. coli* 0157:H7 DNA templates. In all cases, 0.05 mg of silica-MPs and 10 μg of AntiFlu-HRP, Strep-HRP or AntiDig-HRP were used (n=2, except for the negative controls n=15). The cut-off values (dotted lines) are also displayed in all cases. All other conditions as in Figure 4.36.

Figure 4.38 shows the results obtained for amplifications of the bacteria individually. The electrochemical magneto-genosensing approach was able to give a clear positive signal only for the corresponding electrochemical reporters. A clear positive signal was obtained for all the pathogens with an amperometric signal of 6.1, 4.0 and 8.1 μA for *Salmonella*, *Listeria* and *E. coli*, respectively.

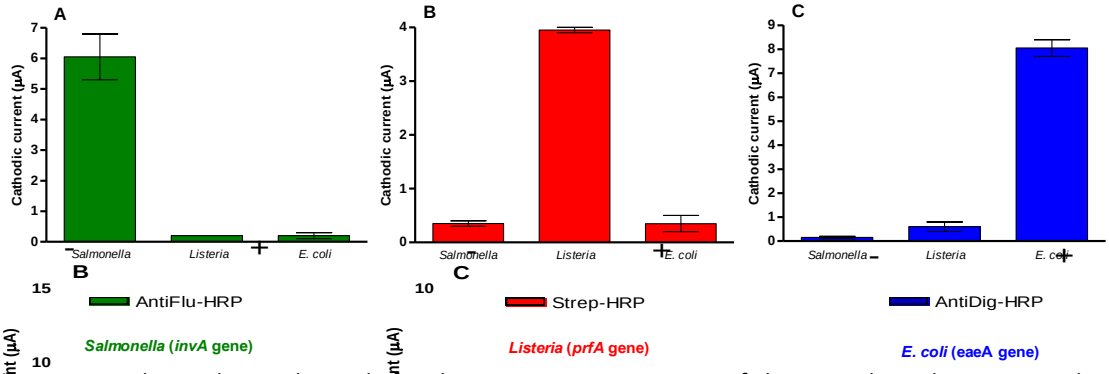

Figure 4.38. Electrochemical signals for the magneto-genosensing of the tagged amplicons. Bars show the signal corresponding to AntiFlu-HRP (green, A), Strep-HRP (red, B) and AntiDig-HRP (blue, C) response to the amplification of single bacteria (*Salmonella*, *Listeria* and *E. coli*). In all cases, 0.05 mg of silica-MPs and 10 μg of AntiFlu-HRP, Strep-HRP or AntiDig-HRP were used (n=2). All other conditions as in Figure 4.36.

4.5.4. General discussion

In this section a novel strategy for the detection of *S. enterica*, *L. Monocytogenes* and *E. coli* 0157:H7 is presented. The method is based on PCR amplification by using fluorescein, biotin and digoxigenin tagged-primers for coding *Salmonella, Listeria* or *E. coli,* respectively. For the first time, silica-MPs were used as a platform for DNA immobilisation followed by electrochemical genosensing. The non-specific attachment of DNA on silica is based on the weak adsorptive forces due to the negative charge density of the sugar-phosphate backbone. The tagged amplicons were immobilised on silica-MPs based on the nucleic acid-binding properties of silica particles in the presence of the chaotropic agent guanidinium thiocyanate. It is important to enhance that in all assays, although the amount of tagged primers were the same, longer dsDNA tagged-amplicons showed in all cases higher affinity to silica surface than shorter ssDNA tagged-primers present mainly in the negative controls, fact that can be ascribed to the higher charge density of the sugar-phosphate backbone present in longer dsDNA fragments. This feature makes silica-MPs a robust platform for the electrochemical detection of amplicons without the interference of remaining primers. This methodology is able to overcome disadvantages coming from the use of the gel electrophoresis for the detection of PCR products such as difficulties in automation of the assay or rapid screening of samples, limitation of number of samples that can be analysed at one time, and the hazard for routine use in laboratories that ethidium bromide used to stain the agarose gels supposes.

The electrochemical magneto-genosensing on silica-MPs is able to detect at least 1 ng μL^{-1} of *Salmonella, Listeria* and *E. coli* in a considerably reduced time of 3 h. Once modified with the tagged DNA, the silica-MPs will be divided in three reaction chambers for the reaction with the specific electrochemical reporters coding for each bacteria, and finally placed them in three different magneto-electrodes for the electrochemical detection using the same mediator and substrate in a unique electrochemical cell, with a multichannel potentiostat. This system showed also very promising features for a multiplex triple-tagged PCR combined with electrochemical genosensing in silica magnetic particles. As the selected primers showed non-specific annealing of one of the set of primers when its specific target was absent in the PCR mix, further optimisation of the PCR should be performed, since the specific amplification during PCR is mandatory for this assay. A similar approach can be also easily implemented for optical detection using three different fluorescent coding tags. All of them are promising features for being implemented as a microfluidic system mainly for food industry application.

4.6. GLOBAL DISCUSSION OF RESULTS

This dissertation reports for the first time the design and evaluation of novel strategies, based on both optical and electrochemical detection, for the rapid detection of pathogenic bacteria in food safety applications. Different electrochemical platforms, based on the coupling of magnetic particles with magneto graphite-epoxy composite as well as graphite-epoxy biocomposite, were explored. Different magnetic particles which allow covalent or electrostatic immobilisation were evaluated such as non-modified silica magnetic particles and modified magnetic particles with streptavidin, tosyl group or specific antibody against *Salmonella*. Regarding the bioaffinity interactions, biotin-streptavidin, antibody-antigen and bacteriophage-bacterial surface receptors were also discussed.

Tables 4.9 and 4.10 display the overall description and the analytical features of all the strategies, genosensing and immunosensing, developed in this dissertation. A real shortening of the assay time was obtained for all the strategies based on the use of magnetic particles comparing with conventional techniques that last form 3 to 5 days. As compared in Table 4.9, the detection of *M. bovis* was the most time-consuming assay; however, results were obtained in less than two days. In this case, the DNA extraction was the complex step, fact that was improved by the use of magnetic separation in the rest of the strategies developed whose excellent limits of detection were obtained in less than 9 h.

It is important to highlight that although the different electrochemical platforms evaluated for *M. bovis* detection were studied previously in the group by using synthetic oligonucleotides or DNA extracted from bacterial culture, this is the first time that DNA extracted from raw milk samples was evaluated and compared with an inter-laboratory PCR assay. As shown in Table 4.10 (A and B), greater analytical features were obtained with the strategy based on the coupling of magnetic particles with magneto graphite-epoxy composite providing a rapid, cheap, and sensitive assay for the screening-out of samples contaminated with *M. bovis.*

Moreover, the double-tagging PCR followed by magneto-genosensing of the amplicon was related to the bacteria captured by IMS or PMS for the first time in the group. Therefore, the amplicon detected can be directly related to the cells captured and pre-concentrated by the magnetic particles. Excellent analytical features were achieved for *Salmonella* detection by combining the evaluation of the double-tagging PCR performed previously in the group with both recent immunomagnetic and phagomagnetic separations (Table 4.10, C and D).

An additional novel aspect of this dissertation is the covalent immobilisation of bacteriophages on magnetic particles. Bacteriophage capabilities as a biorecognition element were explored being, up to our knowledge, the first time that whole non-modified bacteriophages are used in phagomagnetic separation coupled with electrochemical magneto-genosensing. Table 4.10 (C and D) shows the improvement achieved in the analytical features of this strategy compared with the electrochemical magneto-genosensing based on IMS, although both strategies were able to detect as low as 1 CFU mL^{-1}.

It should be also pointed out that the electrochemical magneto-immunosensing developed results in a more reproducible, rapid and easier procedure than the magneto-genosensing approaches (Table 4.9 and 4.10, C, D and E). In spite of the worse detection limits obtained (10^3 *vs*. 1 CFU mL^{-1}), this method presents better features for being implemented in microfluidic systems, due to its simplicity, and coupled with a pre-enrichment step, similar detection limits were achieved in the same total assay time (Table 4.10, C and E).

In both cases, electrochemical magneto-genosensing and immunosensing of *Salmonella*, the accomplishment of the requirements demanded by legislation were studied for the first time in the group. Exceptional results were obtained for both strategies that were able to fulfil the legislation requirements for milk by detecting 1 CFU in 25 mL of milk in less than 9 h (Table 4.10, C and E).

Another concern that must be highlighted is the development of the optical magneto-immunoassay of *Salmonella* (Table 4.9 and 4.10, F). Furthermore, greater LOD was obtained compared with conventional ELISA techniques when the magnetic particles are integrated to the immunoassay.

The last remarkable issue of this work is related to the first steps done towards multi-coding genosensing of pathogenic bacteria (Table 4.9 and 4.10, G). For the first time, the high affinity of DNA to silica was used not only for the DNA binding but also as a platform for further electrochemical magneto-genosensing. The well-known double-tagging PCR amplification was evolved in triple-tagged PCR amplification with the aim of multiplexing pathogens in a near future. So far, promising features were achieved for the electrochemical magneto-genosensing based on silica magnetic particles.

Table 4.9. Overall description of the immunosensing and genosensing strategies developed for food safety applications

Strategy	Description	Target	Detection Technique	Platform	Magnetic Particles	Assay time	Pre-treatment time	Total time
A	**DNA extraction + Double-tagging PCR + Genosensing**	***M. bovis***	Amperometry	Av-GEB	-	2 h 30 min	38 h (DNA extraction)	40 h 30 min
B	**DNA extraction + Double-tagging PCR + magneto-genosensing**	***M. bovis***	Amperometry	m-GEC	Streptavidin-MPs	2 h 30 min	38 h (DNA extraction)	40 h 30 min
C	**IMS + Double-tagging PCR + magneto-genosensing**	***Salmonella***	Amperometry	m-GEC	Anti-*Salmonella*-MP and streptavidin-MPs	3 h	6 h (Pre-enrichment)	9 h
D	**PMS + Double-tagging PCR + magneto-genosensing**	***Salmonella***	Amperometry	m-GEC	P22-MP and streptavidin-MPs	3 h 15 min	-	3 h 15 min
E	**IMS + magneto-immunosensing**	***Salmonella***	Amperometry	m-GEC	Anti-*Salmonella*-MPs	1 h	8 h (Pre-enrichment)	9 h
F	**IMS + magneto-immunoassay**	***Salmonella***	Absorbance	96-well microplate	Anti-*Salmonella*-MPs	1 h 30 min	-	1 h 30 min
G	**DNA extraction + Tagging PCR + magneto-genosensing**	***Salmonella, Listeria* and *E. coli***	Amperometry	m-GEC	Silica-MPs	2 h 45 min	DNA extraction	2 h 45 min

Table 4.10. Comparative analytical features of the immunosensing and genosensing strategies for food safety applications

Strategy	LB broth						Skimmed milk (diluted 1/10 in LB broth)					
	(-) control signal	SD	n	RSD (%)	Cut-off values	LOD	(-) control signal	SD	n	RSD (%)	Cut-off values	LOD
A	1.4 µA	0.8 µA	4	57	3.9 µA	620 fmol	-	-	-	-	-	-
B	3.3 µA	0.3 µA	4	9	4.2 µA	10 fmol	-	-	-	-	-	-
C	2.2 µA	0.7 µA	35	32	3.8 µA	1 CFU mL^{-1}	2.2 µA	0.35 µA	35	16	3.1 µA	1 CFU mL^{-1} (3 h) 1 CFU 25 mL^{-1} (9 h)
D	0.8 µA	0.2 µA	8	25	1.3 µA	1 CFU mL^{-1}	-	-	-	-	-	-
E	0.7 µA	0.1 µA	9	14	0.8 µA	5 x 10^3 CFU mL^{-1}	0.3 µA	0.1 µA	15	30	0.5 µA	7.5 x 10^3 CFU mL^{-1} (1 h) 1 CFU 25 mL^{-1} (9 h)
F	0.043 AU	0.003 AU	9	7	0.051 AU	10^4 CFU mL^{-1}	0.044 AU	0.004 AU	9	9	0.056 AU	10^4 CFU mL^{-1}
G	1.0 µA (S) 1.2 µA (L) 0.9 µA (E)	0.3 µA (S) 0.4 µA (L) 0.3 µA (E)	15	30 (S) 33 (L) 33 (E)	1.6 µA (S) 1.8 µA (L) 1.4 µA (E)	-	-	-	-	-	-	-

4.7. REFERENCES

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5

CONCLUDING REMARKS

5.1. OVERALL CONCLUSIONS

Conventional methods for pathogen detection usually rely on microbiological and biochemical analyses that enable accurate identification of the pathogens. These methods, however, are time-consuming, laborious, need trained personnel and are non amenable to developing point-of-care diagnostic tools. Novel pathogen detection methods have therefore been sought to overcome these limitations. Biosensors are a promising alternative to conventional and recent rapid methodologies. Therefore, the present dissertation was focused on the development of novel biorecognition and transduction strategies for pathogenic bacteria biosensing.

Different electrochemical platforms were evaluated, based on affinity biocomposite, and magneto-composite coupled with magnetic particles. Very promising results were obtained by the integration of magnetic particles in biosensor analytical systems based on magneto-electrodes (m-GEC) designed in our group. The magnetic particles were integrated in the magnetic bioseparation and pre-concentration of the analyte from a complex sample, as well as in the biosensing as a platform for immobilisation of the bioreceptor. Different affinity biorecognition elements coupled to magnetic particles were explored, *i.e.* streptavidin, antibodies and bacteriophages. Modified magnetic particles were used not only for the magneto separation step by comparing both immunomagnetic separation (IMS) and phagomagnetic separation (PMS), but also for the biotinylated-nucleic acid immobilisation with streptavidin-modified magnetic particles.

Magnetic selective separation is a procedure that can facilitate or accelerate separation and purification processes and can be efficiently combined with the majority of other procedures used in biosciences. Comparing with other assays the main advantage of this procedure is that free DNA released from death cells during food processing are not detected with this strategy, because of the IMS or PMS, which separates and pre-concentrates whole bacteria cells, but not DNA, from food samples. The high sensitivity of the approaches given by the electrochemical magneto-genosensing or immunosensing coupled with magnetic separation, results in extremely specific, rapid, robust and sensitive procedures, all of them promising features for being implemented as microfluidic system mainly for food industry applications. These methodologies were able to avoid the underestimation of the bacteria counting observed when magnetic separation was coupled with microbiological culture, due to the formation of agglomerates that give rise to only one colony forming unit (CFU) on the plating media. Different antibodies or a phage cocktail can be employed by using the same strategies to

increase the host range of these assays or for multiplexing the bacteria detection towards other pathogens.

Moreover, a novel electrochemical platform for magneto-genosensing based on the high affinity of nucleic acids to silica surfaces was evaluated as bedrock for future multi-coding genosensing of pathogenic bacteria. Promising features were achieved for the electrochemical magneto-genosensing based on nucleic acid immobilisation on silica magnetic particles.

Besides the electrochemical approaches, an optical magneto-immunoassay was also developed and compared with the analytical features of the previous electrochemical strategies. Despite the fact that the electrochemical strategies showed better analytical performance, great improvement of sensitivity was achieved by comparing with the conventional immunoassay methodologies.

In conclusion, the novel procedures presented here are suitable for the rapid and sensitive on-site screening-out of bacteria in HACCP. Since screening assays are used on large sample populations, often with the aim of determining which samples require further investigation, these approaches are promising strategies to screen-out negative samples and thereby to isolate negative from presumptive infected samples. Positive test results should be always considered presumptive and must be confirmed by an approved microbiological method. Taking into account that a “positive screen test result” leads to a confirmatory culture assay, this fact is particularly important to avoid unnecessary confirmation testing.

5.2. FUTURE PERSPECTIVES

After outlining the main conclusions of the present work, some future considerations that give continuity to this line of research are detailed. During this dissertation many techniques and concepts were encountered which hold great promise for further research.

One of the main issues is the use of bacteriophages as a biorecognition element in biosensors that is still in its beginnings. Future efforts should aim at better understanding their capabilities not only as capture element but also as a tag. Other approaches based on phagomagnetic separation followed by electrochemical magneto-immunosensing, as well as the use of phage as tags to increase the sensitivity of the detection, are currently being studied. Further development of this topic, such as bioconjugation of the bacteriophage to fluorescent coding tags will result in new molecular probes for pathogenic bacteria detection.

The large experience obtained in single bacteria detection opens up the perspective of performing multiplexed detection of bacteria. Further work will be focused on the optimisation of a multiplexed triple-tagging PCR, incorporating also immunomagnetic separation of the bacteria in order to increase the limit of detection of the previous approaches. On the other hand, electrochemical multiplexed magneto-immunosensing of pathogenic bacteria is currently being studied as well. A similar approach can be also easily implemented for optical detection using three different fluorescent coding tags.

In addition, the modification of the methodologies presented to include disposable, low-cost screen-printed electrodes is of great interest due to the fact that allows the development of disposable electrodes for in-field, low-cost and user-friendly detection of multiple food pathogens affecting food safety. Moreover, future challenges are also focus on further analytical validation of these assays by processing a higher number of samples artificially inoculated as well as in naturally contaminated meats, poultry, dairy products, and environmental samples.

The most important outstanding issue that deserves special mention is the miniaturisation of all the strategies presented. Much effort has been done regarding optimisation of the genosensing and immunosensing strategies, obtaining excellent analytical features for all the strategies presented. Therefore, further steps towards the implementation of these procedures in microfluidic systems for food industry applications will be done.

6

PUBLICATIONS

All the work presented resulted in four peer-reviewed journal publications. An additional publication is in preparation. Another paper including the work performed during the three-month internship at Philips Research, The Netherlands was also published. A total of 34 presentations in conferences, both national and international, are also the result of this work (10 oral communications, 17 posters and 7 poster-short communications). As a continuity of the research lines described in this dissertation a conference proceeding was also published and another peer-reviewed publication is in preparation.

PUBLICATIONS

I. Magneto immunoseparation of pathogenic bacteria and electrochemical magneto genosensing of the double-tagged amplicon. Susana Liébana, Anabel Lermo, Susana Campoy, Jordi Barbé, Salvador Alegret, and María Isabel Pividori.

Analytical Chemistry **2009** 81 (14), 5812 – 5820.

II. Rapid detection of *Salmonella* in milk by electrochemical magneto-immunosensing. Susana Liébana, Anabel Lermo, Susana Campoy, María Pilar Cortés, Salvador Alegret, and María Isabel Pividori.

Biosensors and Bioelectronics **2009** (25), 510 – 513.

III. A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* in dairy farms by double-tagging PCR and electrochemical genosensing. Anabel Lermo, Susana Liébana, Susana Campoy, Silvia Fabiano, M. Inés García, Adriana Soutullo, Martín J. Zumárraga, Salvador Alegret and María Isabel Pividori.

International Microbiology **2010** 13 (2), 91 – 97.

IV. Phagomagnetic separation and electrochemical magneto-genosensing of pathogenic bacteria. Susana Liébana, Denis A. Spricigo, María Pilar Cortés, Jordi Barbé, Montserrat Llagostera, Salvador Alegret and María Isabel Pividori.

Analytical Chemistry **2013** 85 (6), 3079 – 3086.

V. Magneto-capillary valve for integrated purification and enrichment of nucleic acids and proteins. Remco C. den Dulk, Kristiane A. Schmidt, Gwénola Sabatté, Susana Liébana and Menno W. J. Prins.

Lab on a chip **2013** (13), 106 – 118.

Other works related to the research developed in this dissertation:

i. Electrochemical magneto-immunosensing of *Salmonella* based on nano and micro-sized magnetic particles. Delfina Brandão, Susana Liébana, Susana Campoy, Pilar Cortés, Salvador Alegret and María Isabel Pividori.

Journal of Physics: Conference Series **2013** (421), 012020.

ii. Electrochemical genosensing of *Salmonella, Listeria* and *E. coli* on silica magnetic particles. Susana Liébana, Delfina Brandão, Pilar Cortés, Susana Campoy, Salvador Alegret and María Isabel Pividori. *In preparation.*

iii. Rapid detection of *Salmonella* in milk by electrochemical magneto-immunosensing using nano and micro-sized magnetic particles. Delfina Brandão, Susana Liébana, Susana Campoy, Pilar Cortés, Salvador Alegret and María Isabel Pividori. *In preparation.*

CONGRESSES

- 5th European Conference on Chemistry for Life Sciences. 10 – 12th June 2013, Barcelona, Spain.
- 3rd International Bio-Sensing Technology Conference. 12 – 15th May 2013, Sitges, Spain.
- 8th Ibero-American Congress on Sensors, Ibersensor 2012. 16 – 19th October 2012, Carolina, Puerto Rico.
- *XVII Trobada Transfronterera Sobre Sensors i Biosensors.* 20 – 21th September 2012, Tarragona, Spain.
- Southern Catalonia Nobel Campus. 1 – 4th July 2012, Tarragona, Spain.
- First Workshop on Nanomedicine UAB CEI. 5th June 2012, Bellaterra, Spain.
- *13as Jornadas de Análisis Instrumental.* 14 – 16th November 2011, Barcelona, Spain.
- *XVI Trobada Transfronterera Sobre Sensors i Biosensors.* 29 – 30th September 2011, Toulousse, France.
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- *Primeres Jornades Doctorals del Departament de Química de la Universitat Autònoma de Barcelona.*1 – 2nd June 2011.
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- *IV Workshop Nanociencia y Nanotecnología Analíticas.* 7 – 9th September 2010. Zaragoza, Spain.
- II International Workshop on Analytical miniaturization (lab-on-a-chip). 7 – 8th June 2010. Oviedo, Spain.

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- *XVI Jornades de Biologia Molecular.* 19 – 20th June 2008. Barcelona, Spain.

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- NanoSpain 2008. 14 – 18th April 2008. Braga, Portugal.

APPENDICES

Magneto immunoseparation of pathogenic bacteria and electrochemical magneto genosensing of the double-tagged amplicon

Susana Liébana, Anabel Lermo, Susana Campoy, Jordi Barbé, Salvador Alegret, and María Isabel Pividori. *Analytical Chemistry* **2009** 81 (14), 5812 – 5820.

Anal. Chem. **2009,** *81,* 5812–5820

Magneto Immunoseparation of Pathogenic Bacteria and Electrochemical Magneto Genosensing of the Double-Tagged Amplicon

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A rapid and sensitive method for the detection of food pathogenic bacteria is reported. In this approach, the bacteria are captured and preconcentrated from food samples with magnetic beads by immunological reaction with the specific antibody against *Salmonella*. After the lysis of the captured bacteria, further amplification of the genetic material by PCR with a double-tagging set of primers is performed to confirm the identity of the bacteria. Both steps are rapid alternatives to the time-consuming classical selective enrichment and biochemical/serological tests. The double-tagged amplicon is then detected by electrochemical magneto genosensing. The "IMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is used for the first time for the sensitive detection of *Salmonella* artificially inoculated into skim milk samples. A limit of detection of 1 CFU mL^{-1} was obtained in 3.5 h without any pretreatment, in LB broth and in milk diluted 1/10 in LB. If the skim milk is pre-enriched for 6 h, the method is able to feasibly detect as low as 0.04 CFU mL^{-1} (1 CFU in 25 g of milk) with a signal-to-background ratio of 20. Moreover, the method is able to clearly distinguish between pathogenic bacteria such as *Salmonella* and *Escherichia coli*. The features of this approach are discussed and compared with classical culture methods and PCR-based assay.

INTRODUCTION

The increasing incidence of food poisoning is a significant public health concern for customers worldwide.[1] Among food pathogens such as *Listeria monocytogenes*, *Vibrio vulnificus*, and *Escherichia coli* O157:H7, *Salmonella enteritidis* has been the source of many outbreaks, while *Salmonella typhimurium* and other antibiotic-resistant salmonellae have also recently become a concern.[2]

Many factors have contributed to recent food emergencies, such as the increasingly complexity of the food production chain because of mass production. Food regulatory agencies have thus established control programs in order to avoid food pathogens from entering the food supply. One of the most effective ways for the food sector to protect public health is to found their food management programs on hazard analysis and critical control point (HACCP), which require rapid method for enabling manufactures to take corrective actions immediately during the course of the manufacturing process. In recent years significant improvements in the methodology for food microbiological analysis have been made. However, the development of new methods with the advantages of rapid response, sensitivity and ease of multiplexing is still a challenge for food hygiene inspection.[3] One improvement involves the use of "immunomagnetic separation" (IMS), that is, the use of magnetic beads to capture target bacteria -through an immunological reaction- from contaminating microflora and interfering food components, and to concentrate them into smaller volumes for further testing.[4] Biosensing devices, especially those based on electrochemical transduction, can be also considered as ideal tools to be implemented in HACCP programs, for being used as an "alarm" to rapidly detect the risk of contamination by food pathogens in a rapid, inexpensive and sensitive manner and in a wide variety of food matrixes.[5]

In this work, a rapid and sensitive strategy for the detection of pathogenic bacteria in food is presented. In this approach, the bacteria are captured from food samples and preconcentrated by immunomagnetic separation. After that, the bacteria attached to the magnetic beads are lysed by a heating treatment and the genomic DNA is thus released. The amplification of the genetic material with a double-tagging set of primers is then performed to confirm the identity of the bacteria by an electrochemical magneto genosensing strategy. A real shortening of the analytical time is obtained by replacing the time-consuming *tandem* "selective enrichment/differential plating culture steps" by the "immu-

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nomagnetic separation", while the classical "biochemical/serological testing assays" are replaced by the double-tagging PCR with electrochemical magneto genosensing, for the confirmation of the bacteria. The PCR also allows the amplification of the analytical signal in a rapid way instead of the classical culture amplification of the bacteria. This methodology (IMS/double-tagging PCR/m-GEC electrochemical genosensing) is used for the first time in the sensitive detection of *Salmonella* in skim milk samples. The results obtained with this new approach are compared with classical cultured methods as well as with PCR strategies.

EXPERIMENTAL SECTION

Instrumentation. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems Inc., U.S.). A three-electrode setup was used comprising a platinum auxiliary electrode (Crison 52-67 1), a double junction Ag/AgCl reference electrode (Orion 900200) with 0.1 mol L^{-1} KCl as the external reference solution and a working electrode (the magneto electrode, mGEC). The detailed preparation of the m-GEC electrodes has been extensively described by Pividori et al.[6] Temperature-controlled incubations in Eppendorf tubes were performed in an Eppendorf Thermomixer compact. The magnetic separation during the washing steps was performed using a magnetic separator Dynal MPC-S (Prod. N° 120.20D, Dynal Biotech ASA, Norway). The PCR reaction was carried out in an Eppendorf Mastercycler Personal thermocycler. The SEM images were taken with the scanning electron microscope Hitachi LTD S-570 (Hitachi LTD, Tokyo, Japan). The gold sputtering of the samples was performed with the E5000 Sputter Coater Polaron Equipment Limited (Watford, UK). The K850 Critical Point Drier Emitech (Ashford, UK) was also used for the preparation of the samples for SEM.

Chemicals and Biochemicals. The graphite-epoxy composite was prepared with graphite powder of 50 μm particle size (BDH, UK) and Epo-Tek H77 (epoxy resin and hardener both from Epoxy Technology, U.S.). Dynabeads anti-Salmonella magnetic beads (Prod. N° 710.02) and streptavidin magnetic beads (Prod. N° 112.06) were purchased from Invitrogen Dynal AS (Oslo, Norway). Anti-digoxigenin-POD (AntiDig-HRP) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). *Salmonella enterica serovar Typhimurium LT 2* and *Escherichia coli* K-12 strains were grown at 37 °C in Luria-Bertoni (LB) pH 7.5 broth or agar.

The filters used as a support for SEM were Nucleopore Track-Etched Membranes, Whatman, (25 mm Ø, 0.2 μm pore size) (Product N° 110606, ALCO, Spain). The primers for the PCR amplification in the genosensing strategies were obtained from TIB-MOLBIOL (Berlin, Germany). These primers were selected for the specific amplification of the IS200 insertion fragment related to *Salmonella* spp. The primer sequences were Biotinylated IS200 up: 5′ bio- ATG GGG GAC GAA AAG AGC TTA GC 3′, DIG-IS200 down: 5′ DIG- CTC CAG AAG CAT GTG AAT ATG 3′. The Expand High Fidelity PCR System kit (Roche Molecular Biochemicals) was used for performing the PCR. All buffer solutions were prepared with bidistilled water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions were 5 × SSC (0.75 mol L^{-1} NaCl, 75 mmol L^{-1} trisodium citrate, pH 7.0); Tris Buffer (0.1 mol L^{-1} Tris, 0.15 mol L^{-1} NaCl, pH 7.5); blocking Tris buffer (2% w/v BSA, 0.1% w/v Tween 20, 5 mmol L^{-1} EDTA, in Tris Buffer); PBST (10 mmol L^{-1} phosphate buffer, 0.8% w/v NaCl, pH 7.5, 0.05% v/v of Tween 20); PBSE (0.1 mol L^{-1} phosphate buffer, 0.1 mol L^{-1} KCl, pH 7.0). Skim milk samples were purchased in a local retail store.

Immunomagnetic Separation of *Salmonella* spp. The schematic procedure is outlined in Figure 1. For the immunocapturing of *Salmonella enterica serovar Typhimurium* LT 2, solutions from a concentration of 10^7 to 10^0 CFU mL^{-1} in both LB broth and milk (diluted 1/10 in LB), were performed. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar. The pure culture or skim milk diluted 1/10 in LB samples (500 μL) were added to 10 μL of anti-Salmonella magnetic bead (Figure 1A). An incubation step was performed for 10 min slight agitation. After that, the magnetic beads with the attached-bacteria were separated with a magnet, and then washed with PBST twice for 10 min. Finally, the collected modified MBs were resuspended in 150 μL of milli-Q water.

Evaluation of the IMS by SEM and Classical Culture Methods. The evaluation of the immunomagnetic separation (Figure 1A) was performed by SEM and by classical culture methods, as schematically outlined in the Figure 2A. For the microscopic evaluation of the attached bacteria on the magnetic bead by SEM, the IMS was performed with a concentration of bacteria of 10^4 CFU mL^{-1}. After that, 5 mL of the solution containing the modified magnetic bead was filtered through a Nucleopore membrane (used as a support for the SEM microscopy). The filters were then fixed with glutaraldehyde and postfixed with osmium tetroxide.[7]

In order to study the efficiency of the immunomagnetic separation step, 500 μL of different concentration of bacteria (ranged from 2.9×10^6 to 2.9×10^{-3} CFUs), in both LB broth and milk (diluted 1/10 in LB) were captured with 10 μL of anti-Salmonella magnetic beads, for 10 min in slight agitation and the MB were resuspended in 50 μL milli-Q water. Finally, the initial solutions, as well as 10^{-2} and 10^{-4} dilutions of the MBs with the attached bacteria were plated in LB agar and grown for 18−24 h at 37 °C (Figure 2A).

Confirmation by Double-Tagging PCR Amplification and Electrochemical Magneto Genosensing of the Amplicon. For every concentration of bacteria in LB or milk samples, the lysis of the attached bacteria on the MBs was performed at 99 °C for 20 min in order to break the cells and to achieve the releasing of the genomic DNA and the cellular debris to the solution for the PCR amplification (Figure 1B). The electrochemical genosensing strategy for the confirmation of *Salmonella* spp. was preceded by the amplification of a specific sequence for the IS200 insertion fragment related to *Salmonella* spp.[8] (Figure 1C). The amplification was performed by a double-tagging polymerase chain reaction

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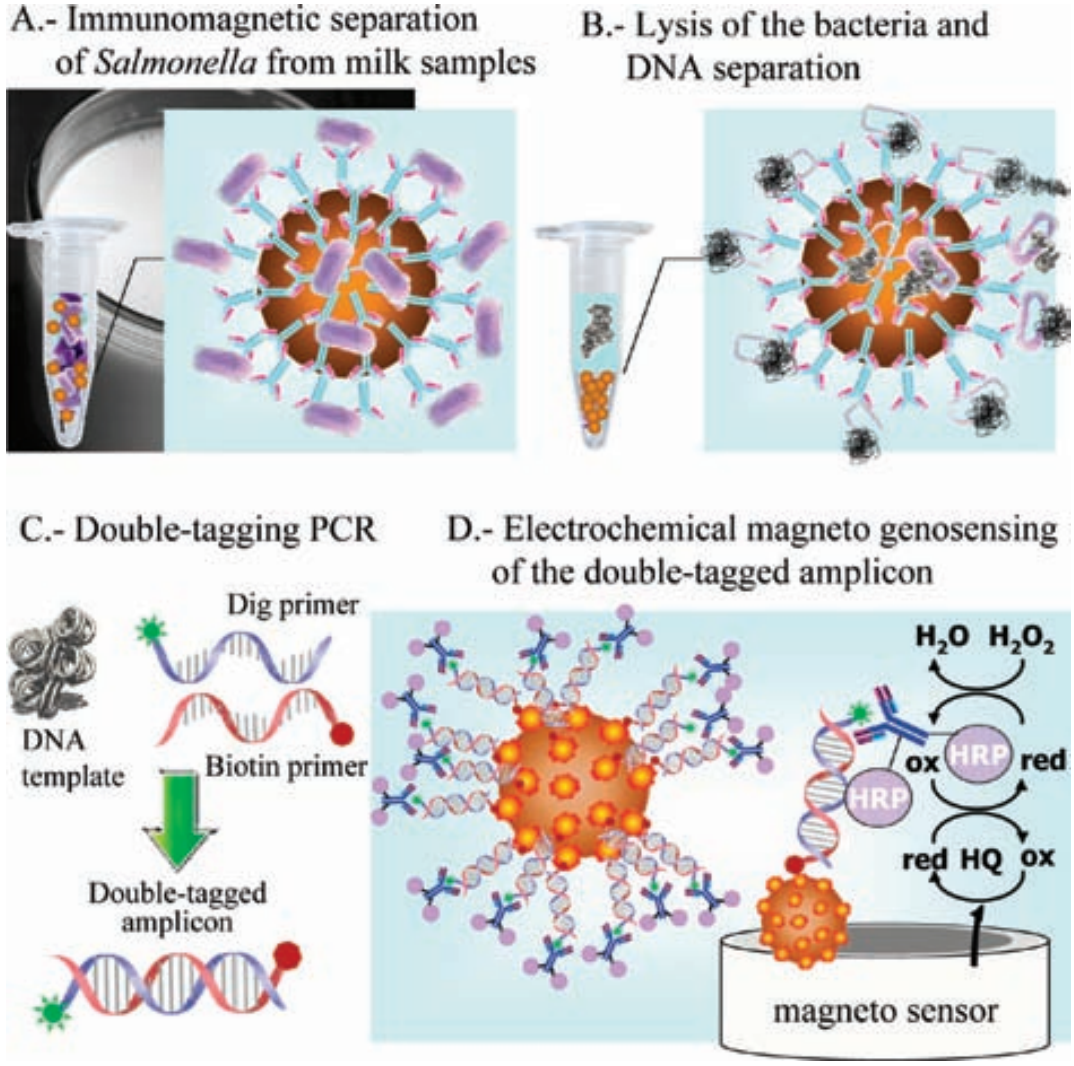

Figure 1. Schematic representation of the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach. Details of parts C and D are provided in the expanded version of the Figure in the Supporting Information.

(PCR) using two labeled-primers with biotin and digoxigenin in each extreme, respectively[9] (detailed outlined in the expanded version of Figure 1C in the Supporting Information). During the PCR, not only the amplification of pathogenic bacteria genome was achieved, but also the double tagging of the amplicon ends with (i) the biotinylated capture primer to achieve the immobilization on streptavidin-modified magnetic beads and (ii) the digoxigenin signaling primer to achieve the enzymatic detection through AntiDig-HRP reporter.

The PCR was performed in a 50 μL of reaction mixture containing the DNA coming from *Salmonella enterica serovar Typhimurium*, previously attached onto the MBs. Each reaction contained 200 μmol L^{-1} of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 0.5 μmol L^{-1} of the double-tagging set of primers and 5 U o fpolymerase. The reaction was carried out in buffer containing 1.5 mmol L^{-1} $MgCl_2$. The amplification mixtures were exposed to an initial step at 95 °C for 120 s followed by 30 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and a last step of 420 s at 72 °C.

All of these amplifications included a blank as a control, which contained the samples (LB broth or skim milk) without *Salmonella* spp. template, as well as a positive control. The double-tagged amplicon was analyzed by electrochemical genosensing with the m-GEC electrodes as well as with the classical gel electrophoresis.

The electrochemical genosensing strategy of the double-tagged amplicon (Figure 1D) comprises the follow steps, as detailed outlined in Figure 1D in the Supporting Information: (a) Immobilization of the double-tagged amplicon in which the 5′ biotin end was immobilized on the streptavidin magnetic beads; (b) Enzymatic labeling with the antibody AntiDig-HRP able to bond the 3′ digoxigenin end of the ds-DNA amplicon; (c) Magnetic capture of the modified magnetic beads by the m-GEC electrode; (d) Amperometric determination.

The immobilization of the double-tagged amplicon was achieved by adding 6.2×10^6 streptavidin magnetic beads in an Eppendorf tube with the diluted amplicon in 5 × SSC for 30 min at 42 °C and at a final volume of 140 μL. Two washing steps were then performed with 140 μL of 5 × SSC for 2 min at 42 °C. After that, the enzymatic labeling was performed by using antiDig-HRP (60 μg) in blocking Tris buffer at a final volume of 140 μL for 30 min at 42 °C. Two washing steps were then performed

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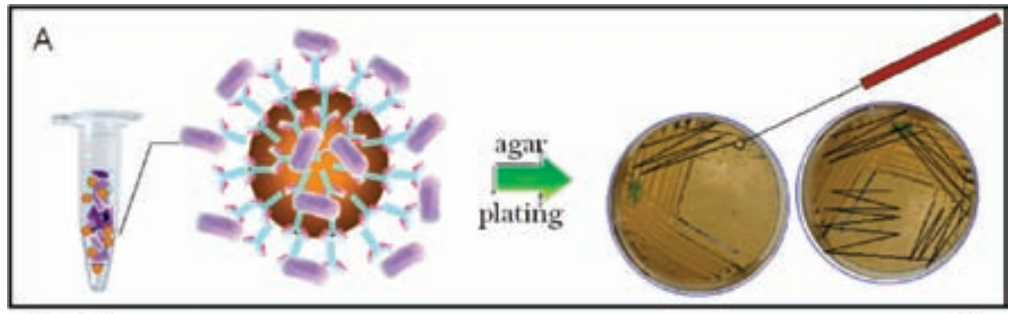

Table A

CFUs (expected amount)	Counted CFUs			CFUs (found)	IMS Recovery (%)
	10^0 (original solution)	10^{-2} dilution	10^{-4} dilution		
$2.9\ 10^4$	uncounted	uncounted	225	$2.3\ 10^6$	79.3
$2.9\ 10^3$	uncounted	uncounted	21	$2.1\ 10^5$	72.4
$2.9\ 10^2$	unc (plate N° 1)	179	3	$1.8\ 10^4$	62.1
$2.9\ 10^1$	unc (plate N° 2)	19	0	$1.9\ 10^3$	65.5
$2.9\ 10^0$	159 (plate N° 3)	1	n/d	$1.6\ 10^3$	55.2
$2.9\ 10^{-1}$	16 (plate N° 4)	0	n/d	$1.3\ 10^3$	55.2
$2.9\ 10^{-2}$	4 (plate N° 5)	0	n/d	$4.0\ 10^3$	137.9
$2.9\ 10^{-3}$	0 (plate N° 6)	n/d	n/d	0	0
$2.9\ 10^{-4}$	0	n/d	n/d	0	0
$2.9\ 10^{-5}$	0	n/d	n/d	0	0

B

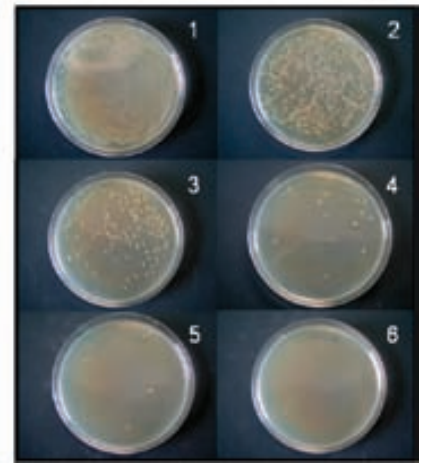

Figure 2. A: Evaluation of the IMS by classical culture method. Table A: Counted colony number after IMS and plating in LB for 18−24 h at 37 °C. B: Culture plates of *Salmonella* cells attached to magnetic beads (concentrations ranged from 2.9×10^{-1} (plate N° 6) to 2.9×10^4 (plate N° 1) showing the typical colony features of *Salmonella*.

for 5 min at 42 °C in 140 μL of Tris buffer. After each incubation or washing step, the magnetic beads were separated from the supernatant on the side wall by placing the Eppendorf tubes in a magnet separator until the beads were migrated to the tube sides and the liquid was clear. After the final washing step, the modified magnetic beads were captured by dipping the magneto electrode (m-GEC) inside the reaction tube (as shown in Figure 1D in the Supporting Information section). The modified m-GEC electrode was immersed into the electrochemical cell containing 20 mL of PBSE buffer with 1.81 mmol L^{-1} hydroquinone, and under continuous magnetic stirring, a potential of −0.100 V vs Ag/AgCl was applied. When a stable baseline was reached, 1 mL of H_2O_2 was added into the electrochemical cell to a final concentration of 4.90 mmol L^{-1} (which corresponds to the H_2O_2 concentration capable to saturate the whole enzyme amount employed in the labeling procedure), and the current was measured until the steady state current was reached (normally after 1 min of H_2O_2 addition). This steady-state current was used for Figures 4, 5, and 6.

Safety Considerations. All the procedures involving the manipulation of potentially infectious materials or cultures were performed following the safe handling and containment of infectious microorganism's guidelines.[10] According to these guidelines, the experiments involving *Salmonella typhimurium* and *E. coli* were performed in a Biosafety Level 2 Laboratory. Strict compliance with BSL-2 practices was followed and proper containment equipment and facilities were used. Contaminated disposable pipet tips were carefully placed in conveniently located puncture-resistant containers used for sharps disposal. All cultures, stocks, laboratory waste, laboratory glassware and other potentially infectious materials were decontaminated before final disposal by autoclaving. The ultimate disposal was performed according to local regulations.

RESULTS AND DISCUSSION

Immunomagnetic Separation of *Salmonella* spp. Evaluation by SEM and Classical Culture Methods. The first step in the IMS/double-tagging PCR/m-GEC electrochemical genosensing strategy is the immunomagnetic separation of *Salmonella*. The microscopic characterization by SEM was performed for the evaluation of the immunological attachment of the bacteria to the magnetic beads. Figure 3 shows that the binding was achieved with more than one specific binding site of the bacteria to the magnetic bead. In some cases, the whole surface of the bacterium was completely attached to the magnetic bead (Figure 3A and F). Moreover, a unique magnetic bead was able to attach more than one bacterium (Figure 3B, C, and F). However, no more than three cells per magnetic beads were observed at a bacterial concentration of 10^4 CFU mL^{-1}. Finally, some aggregates were observed due to the binding of two different magnetic beads by a unique bacterium cell (Figure 3E).

In order to evaluate the efficiency of the IMS procedure, classical culture method was also performed by growing the bacteria attached on magnetic beads for 18−24 h at 37 °C, as

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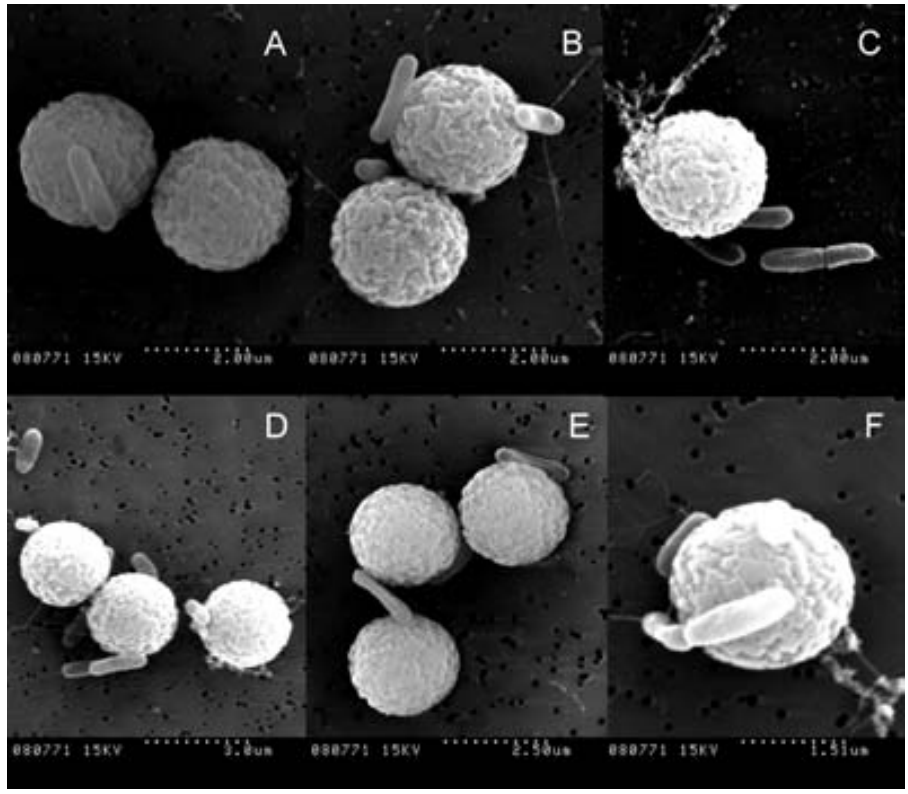

Figure 3. Evaluation of the IMS by SEM at a *Salmonella* concentration of 10^4 CFU mL^{-1}. The images show the *Salmonella* cells attached to the magnetic beads. In all cases, identical acceleration voltage (15 KV) were used.

schematically outlined in Figure 2A. Colony counting was clearly decreasing from 2.9×10^6 to 2.9×10^0 CFUs, as shown in Figure 2, Table B. The corresponding plates from 2.9×10^4 CFUs (plate 1) to 2.9×10^{-1} CFUs (plate 6) are also shown in Figure 4B, displaying the characteristic colony features of *Salmonella* in LB media. An underestimation of the expected amount of bacteria was observed in all the concentration range, except for the more diluted concentration corresponding to 2.9×10^0 CFU in 500 μL of sample (5 CFU mL^{-1}). The counted colony number was found to be between 60 and 80% of the expected amount, perhaps due to the formation of the aggregates observed by SEM, formed by several bacterium cells but growing at a unique colony point in the agar plate. As low as 5 CFU mL^{-1} were effectively captured and detected with this strategy. Similar results were obtained when *Salmonella* was artificially inoculated into skim milk samples. In that case, not other accompanying flora was observed in the plate, as expected for UHT milk as well as for the IMS procedure.

Confirmation by Double-Tagging PCR Amplification and Electrochemical Magneto Genosensing of the Amplicon. The second step in the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach is the double-tagging PCR for the amplification of the *Salmonella* spp. genome for the genosensing detection.[9] Regarding the double-tagging PCR, the chosen set of primers amplified exclusively the IS200 insertion sequence, producing only the expected 201 bp fragment, according to the agarose gel electrophoresis shown in the expanded version of Figure 4 in the Supporting Information, for the concentration range from 10^7 to 10^2 CFU mL^{-1} in skim milk diluted 1/10 in LB broth and artificially inoculated with *Salmonella*. As shown in the expanded version of Figure 4 in Supporting Information, the LOD for the IMS/double-tagging PCR/electrophoresis was found to be 10^2 CFU mL^{-1} (lane N° 6 in the gel electrophoresis). No bands were observed for the negative controls (0 CFU mL^{-1}) performed with skim milk diluted 1/10 in LB broth (expanded version of Figure 4, Supporting Information, lanes N° 9 and 10). In order to increase the sensitivity of the assay, instead of the IMS/double-tagging PCR/electrophoresis approach, the proposed methodology is based on the IMS/double-tagging PCR/m-GEC electrochemical genosensing, by replacing the electrophoresis detection for the electrochemical magneto genosensing of the double-tagged amplicon[9] (expanded version of Figure 1D, Supporting Information). The amperometric response of the doubly labeled product was evaluated for artificially inoculated bacteria in LB as well as in milk diluted 1/10 in LB (Figure 4A and B). For the assay performed in LB broth the amperometric signal corresponding to the LOD was estimated by processing 35 negative control samples and performing seven different single interday assays, and using six batches of magneto electrode devices, obtaining a mean value of 2.2 μA (Figure 4A dotted line) with a standard deviation of 0.65 μA. For the assay performed in skim milk dilute 1/10 in LB, a mean value of 2.2 μA (Figure 4B dotted line) with a standard deviation of 0.35 μA was obtained. The amperometric signal corresponding to the LOD value was then extracted with a one-tailed *t* test at a 99% confidence level, giving a value of 3.78 and 3.10 μA, respectively (shown in Figure 4A and B as the solid horizontal lines). As shown in Figure 4A and B for the samples artificially inoculated with *Salmonella*, the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach is able to give a

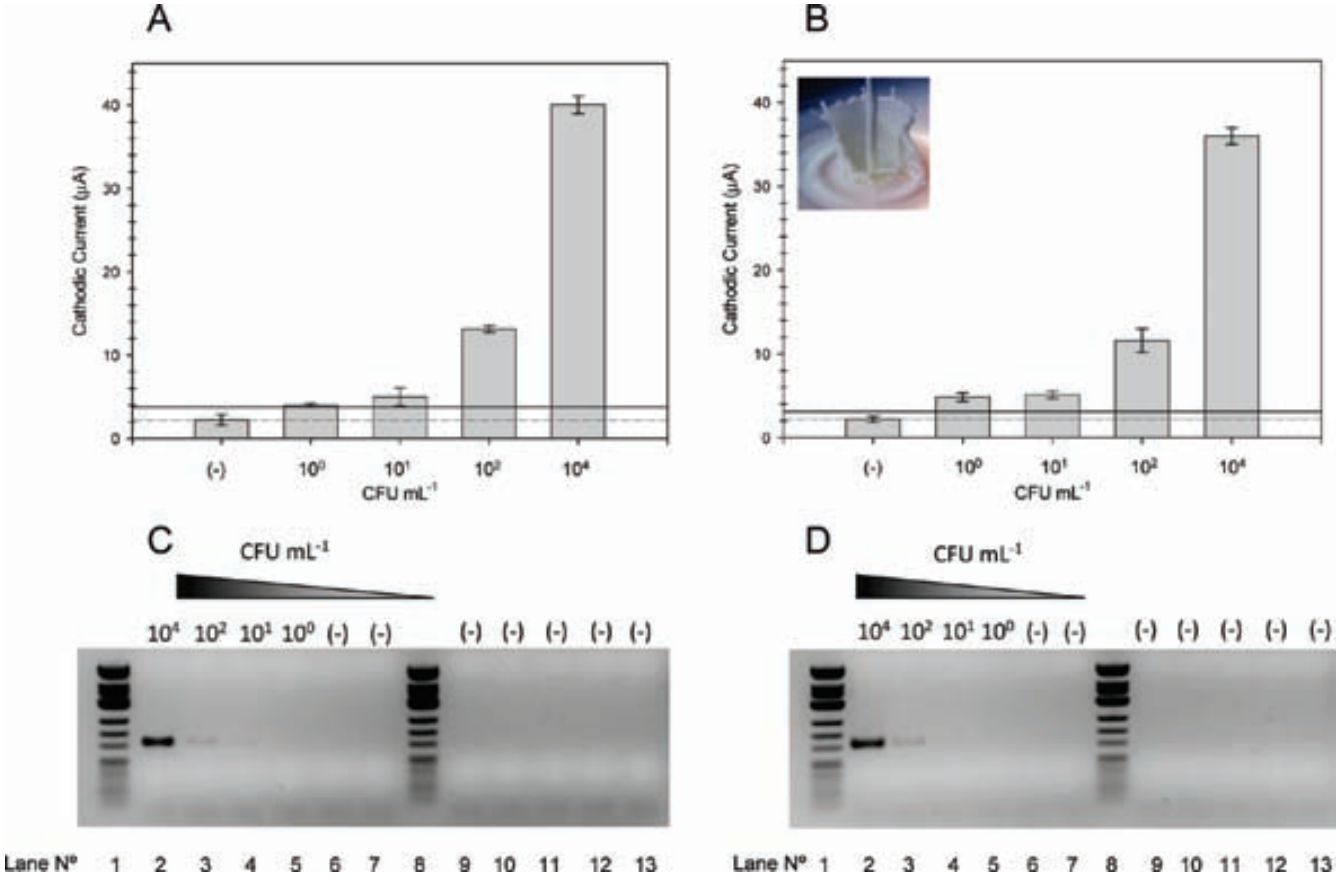

Figure 4. Up. Electrochemical signals for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach. Gray bars show the signal by increasing the amount of *Salmonella* ranged from 10^0 to 10^4 CFU mL^{-1} artificially inoculated in LB broth (A) and in skim milk diluted 1/10 in LB broth (B). A negative control is also shown. In all cases, 60 μg AntiDig-HRP and 6.2×10^6 magnetic beads were used. Medium: phosphate buffer 0.1 M, KCl 0.1 M, pH 7.0. Mediator: hydroquinone 1.81 mM. Substrate: H_2O_2 4.90 mM. Applied potential = −0.100 V (vs Ag/AgCl). In all cases, $n = 4$, except for the negative control ($n = 35$). Down. Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the IMS/double-tagging PCR/electrophoresis approach, performed in LB broth (C) and in milk (D) artificially inoculated samples. Lanes 2−5 correspond to 10-fold dilutions from 10^4 to 10^0 CFU mL^{-1}. Lanes 6, 7, and 9−13 are negative controls. Lanes 1 and 8 are the molecular weight marker (ΦX174-Hinf I genome).

clear positive signal for 10^2 CFU mL^{-1}, whereas the electrophoresis at the same concentration shows a doubtful positive band (Figure 4C and D). On the other hand, as low as 1 CFU mL^{-1} was detected as a positive sample with a total assay time of 3.5 h for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach. No significant differences in the electrochemical signal were observed for the samples artificially inoculated with *Salmonella*, regardless the sample (LB or milk diluted 1/10 in LB). No matrix effect was thus observed for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach performed in milk.

Compared with other biosensing methodologies for detecting pathogenic bacteria in food,[11] excellent detection limits were achieved with this procedure. Moreover, this method is more rapid and sensitive than other rapid antibody-based and nucleic acid-based polymerase chain reaction (PCR) methods that have been previously reported (Table B, Supporting Information). As an example, enzyme-linked electrochemical detection coupled with IMS generally gave detection limits at 10^3 CFU mL^{-1}, whereas PCR methods could achieve detection limits ranging from 10^1 to 10^4 CFU mL^{-1} depending on the efficiency of DNA extraction, with or without enrichment step, and the nature of the food samples. Moreover, the procedure was able to detect 1 CFU mL^{-1} in 3.5 h without the use of any pre-enrichment or selective enrichment steps, with higher sensitivity than PCR followed by electrophoresis, and classical culture method. Comparing with other commercial PCR assays for the detection of *Salmonella* without IMS,[12] the main advantage of this procedure is that free DNA coming from death or injured cells during food processing are not detected with this strategy, because of the IMS, which separates and preconcentrates whole bacteria cells—but not DNA—from food samples. It should be also pointed out that better LODs were also achieved with the IMS/double-tagging PCR/m-GEC electrochemical

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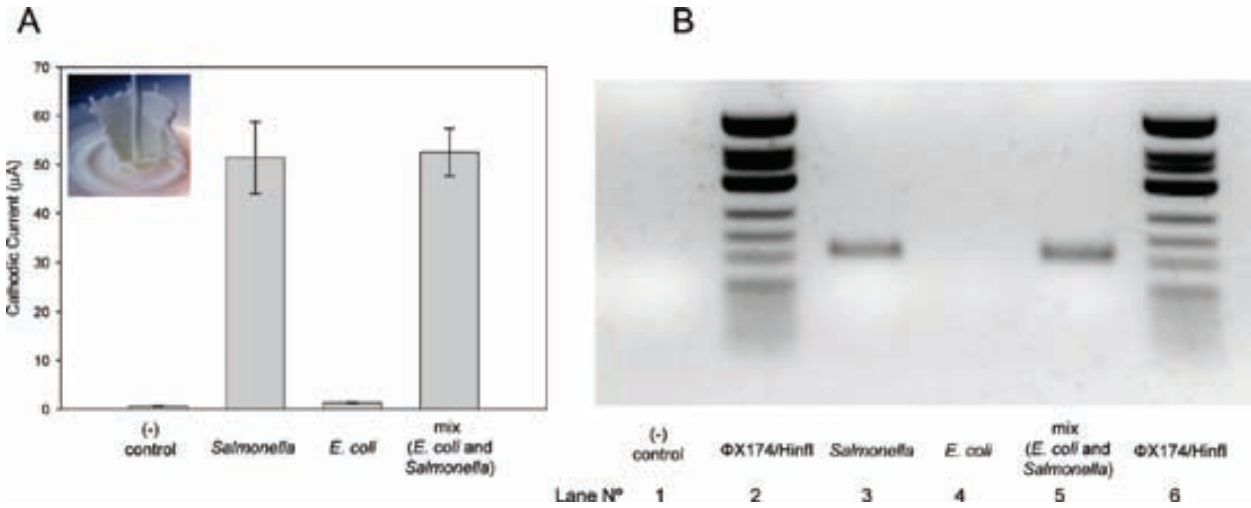

Figure 5. Specificity study for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach. (A) Gray bars show the electrochemical signal for milk diluted 1/10 in LB artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control, $n = 4$); 2.4×10^5 CFU mL^{-1} *Salmonella* ($n = 4$); 1.1×10^5 CFU mL^{-1} of *Escherichia coli* ($n = 5$); 1.1×10^5 CFU mL^{-1} of *E. coli* and 2.4×10^5 CFU mL^{-1} of *Salmonella* spp. ($n = 5$). All other conditions as in Figure 4. (B) Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the IMS/double-tagging PCR/electrophoresis approach. Lane 3: 2.4×10^5 CFU mL^{-1} *Salmonella*; Lane 4: 1.1×10^5 CFU mL^{-1} of *Escherichia coli*; Lane 5: 1.1×10^5 CFU mL^{-1} of *E. coli* and 2.4×10^5 CFU mL^{-1} of *Salmonella* spp. Lane 1 is the negative control. Lanes 2 and 6 are the molecular weight marker (ΦX174-Hinf I genome). Results were obtained for artificially inoculated skim milk diluted 1/10 in LB broth.

genosensing approach compared with LODs reported for IMS/real-time PCR systems.[13] This fact can be ascribed to the sensitivity of the amplicon detection with the m-GEC electrochemical genosensing strategy.

Specificity Study of the IMS/Double-Tagging PCR/m-GEC Electrochemical Genosensing Approach. Figure 5A shows the results of the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach for milk diluted 1/10 in LB artificially inoculated with 1.1×10^5 CFU mL^{-1} of *Escherichia coli*, 2.4×10^5 CFU mL^{-1} *Salmonella* and, finally, a mix containing both bacteria (1.1×10^5 CFU mL^{-1} of *E. coli* as well as 2.4×10^5 CFU mL^{-1} of *Salmonella* spp.), as well as a negative control. Figure 5B shows the corresponding electrophoresis images of the double-tagged amplicon (IMS/double-tagging PCR/electrophoresis approach). As expected, the electrochemical signal obtained for *E. coli* (1.1×10^5 CFU mL^{-1}) is similar that for the negative assay, whereas the mix of both pathogens (1.1×10^5 CFU mL^{-1} *E. coli* and 2.4×10^5 CFU mL^{-1} *Salmonella* spp.) gave a similar signal that the sample spiked just with *Salmonella* (2.4×10^5 CFU mL^{-1}). Similarly, no electrophoresis band was observed for *E. coli* 1.1×10^5 CFU mL^{-1}, (Figure 5B, lane 4) as well as for the negative control (Figure 5B, lane 1), whereas the mix of both pathogens (1.1×10^5 CFU mL^{-1} *E. coli* and 2.4×10^5 CFU mL^{-1} *Salmonella* spp., Figure 5B, lane 5) and the *Salmonella* sample (2.4×10^5 CFU mL^{-1}, Figure 5B, lane 3) give a unique positive electrophoresis band producing only the expected 201 bp fragment, corresponding to the amplification of the IS200 element specific for *Salmonella*.

The same results were obtained by plating and growing the bacteria attached to the magnetic beads in LB agar for 18−24 h at 37 °C. No growing was observed for 1.1×10^5 CFU mL^{-1} of *Escherichia coli*, whereas typical colony features of *Salmonella* were observed for the mix of both pathogens as well as for just *Salmonella*. These results confirm that the specificity of the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach is coming mainly from the IMS step, due to the specific antibody toward *Salmonella* which coated the magnetic beads. According to the suppliers, the magnetic beads reacts with all current *Salmonella* serovars of importance as the cause of human and animal disease occurring in food, feed and environmental samples, comprising the somatic groups from B to Z with variable reactivity depending on the serotype.

Another source of specificity for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach to detect *Salmonella spp.* is coming from the double-tagging PCR. In this case, the chosen set of primers amplified exclusively the IS200 insertion sequence, a transposable element of some 700 bp.[14] A survey of the presence of IS200 among enteric bacteria revealed that more than 90% of the pathogenic or food-poisoning isolates of *Salmonella* spp contained one or more copies of the IS200 insertion sequence.[8] For practical purposes, IS200 has been used as a suitable probe to identify isolates of *Salmonella* with high accuracy.[15] The selection of the IS200 specific set of primers provides an additional source of specificity for the assay, particularly for those bacteria antigenically related to salmonellae genus.

Study of the Limit of Detection with a Pre-Enrichment Step. As cells are injured when exposed to adverse conditions during food processing, a pre-enrichment step is usually included in classical methods to achieve the proliferation of stressed *Salmonella* spp. cells. If this step is not included in

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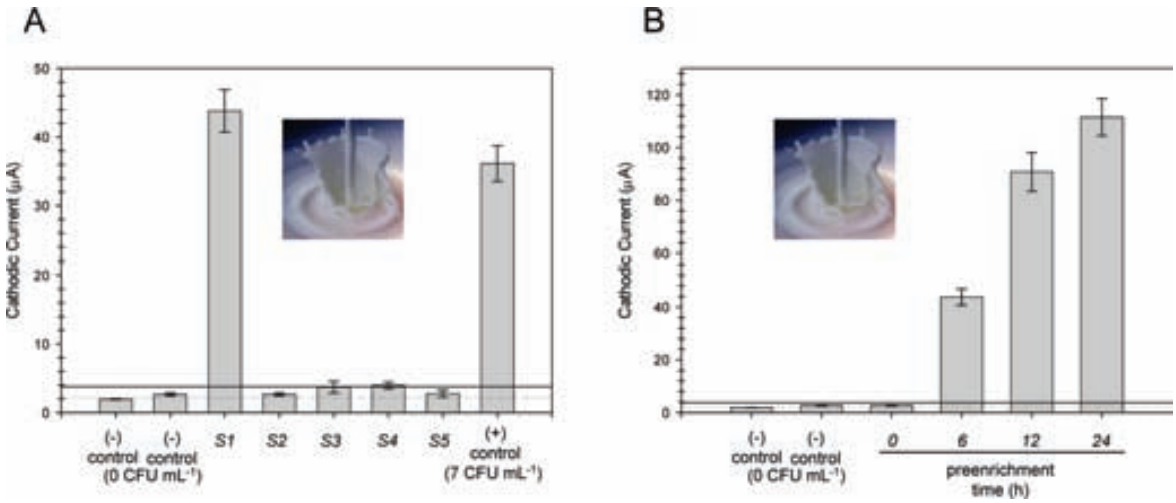

Figure 6. (A) Electrochemical signals for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach with a pre-enrichment step of 6 h for artificially inoculated skim milk. Five 5 mL portions (S1 to S5) of skim milk, two negative controls (0 CFU mL^{-1}), and a positive control (7 CFU mL^{-1}) are shown. (B) Electrochemical signals for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach with a pre-enrichment step for the positive sample at a 0, 6, 12, and 24 h of pre-enrichment. Two negative controls (0 CFU mL^{-1}) are also shown. In all cases, $n = 4$. All other conditions as in Figure 4.

the method, then stressed cells that have not fully repaired cells injury may be missed.[16] A pre-enrichment step was thus performed with a nonselective broth medium, in this case LB broth.

The milk sample (25 mL) were spiked with just 1 CFU, divided in five-5 mL portions and processed as above. The samples were pre-enriched in LB broth and assayed at 0, 6, 12, and 24 h of pre-enrichment in LB at 37 °C. A positive control (7 CFU mL^{-1}) was also evaluated as well as two negative controls (0 CFU mL^{-1}). Just one of the five portions—as expected, the one containing 1 CFU of *Salmonella*—gave positive results after 6 h (as shown in expanded version of Figure 6, Supporting Information). Figure 6A shows the amperometric signal for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach after 6 h of pre-enrichment in LB at 37 °C, for the negative control, as well as for the five-5 mL sample portions and for the positive control. Clearly, just the sample number 1 gave a positive signal due to the presence of as low as 0.04 CFU mL^{-1}. Figure 6B shows the amperometric signal of the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach for the positive sample (N° 1) at 0, 6, 12, and 24 h of pre-enrichment in LB at 37 °C. As a conclusion, after the pre-enrichment in LB, the procedure is able to detect as low as 0.04 CFU mL^{-1}, according to the legislation (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each in different points, Real Decreto 1679/1994, BOE 24-09-94), and with an amperometric signal of above 40 μA and with a signal-to-background ratio of 20.

CONCLUSIONS

A rapid and sensitive assay combining immunomagnetic separation (IMS), double-tagging PCR, and electrochemical magneto genosensing of the double-tagged amplicon for *Salmonella* in milk is presented. The *Salmonella* was captured on magnetic beads modified with an antibody specific for *Salmonella* spp. The confirmation of the attached bacteria on the magnetic beads was performed by amplifying the genomic DNA with a double-tagging set of primers specific for *Salmonella*. The double-tagged amplicon was sensitively detected by and electrochemical magneto genosensor. This strategy was able to detect as low as 1 CFU mL^{-1} of bacteria in LB as well as in milk diluted 1/10 in LB, without showing any matrix effect. As such, the novel methodology was not affected by the food matrix (in this case milk), due to the use of magnetic beads. The magnetic immunoseparation was also able to effectively replace the selective plating while the double-tagging PCR strategy with electrochemical genosensing, the biochemical probes and the serological confirmation. The time of the assay was thus considerably reduced from 3−5 days to 3.5 h. It should be pointed out that the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach is able to give also positive signal with death or injured whole cells, but DNA released during food processing is not detected, as a difference with commercial PCR approaches without IMS. Moreover, PRC inhibitors are also avoided. If the sample is pre-enriched for 6 h in LB, as low as 0.04 CFUs mL^{-1} of *Salmonella* can be feasibly detected with a signal to background ratio of 20, according to the legislation.

The novel procedure presented here is suitable for the rapid and sensitive on-site analysis of *Salmonella* in HACCP. Interestedly, the specificity of this approach is conferred by both the antibody in the IMS and the set of primer during the double-tagging PCR, in this case for detecting *Salmonella* spp. The same approach could be also designed for detecting different *Salmonella* or *E. coli* serotypes by selecting a specific pair of primers or antibody. The sensitivity conferred by the m-GEC electrochemical genosensing in connection with the specificity conferred by the magnetic beads and by the double-

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tagging PCR result in a rapid, robust, and sensitive procedure. Future work will be focused on the possible application of such method to screen printed electrodes allowing the development of disposable electrodes for in-field, low cost and user-friendly detection of multiple food pathogens affecting food safety.

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Financial support from Ministry of Education and Science (MEC), Madrid (Project BIO2007-63300), is acknowledged. MIP also acknowledges the support from the Universidad Nacional del Litoral (Argentina).

SUPPORTING INFORMATION AVAILABLE

Expanded version of Figures 1, 4, and 6, and Table B. This material is available free of charge via the Internet at http://pubs.acs.org.

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Rapid detection of *Salmonella* in milk by electrochemical magneto-immunosensing

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Short communication

Rapid detection of *Salmonella* in milk by electrochemical magneto-immunosensing

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ABSTRACT

A very simple and rapid method for the detection of *Salmonella* in milk is reported. In this approach, the bacteria are captured and preconcentrated from milk samples with magnetic beads through an immunological reaction. A second polyclonal antibody labeled with peroxidase is used as serological confirmation with electrochemical detection based on a magneto-electrode. The 'IMS/m-GEC electrochemical immunosensing' approach shows a limit of detection of 5×10^3 and 7.5×10^3 CFU mL^{-1} in LB and in milk diluted 1/10 in LB broth, respectively, in 50 min without any pretreatment. If the skimmed-milk is preenriched for 6 h, the method is able to detect as low as 1.4 CFU mL^{-1}, while if it is preenriched for 8 h, as low as 0.108 × CFU mL^{-1} (2.7 × CFU in 25 g of milk, in 5 samples of 5 mL) are detected accordingly with the legislation. Moreover, the method is able to clearly distinguish between food pathogenic bacteria such as *Salmonella* and *Escherichia coli*. The features of this approach are discussed and compared with classical culture methods.

1. Introduction

Salmonella has been one of the most frequently occurring foodborne pathogens affecting the microbial safety of foods, including milk (D'Aoust, 1994). Official agencies for food safety, such as US Food and Drug Administration (FDA), US Department of Agriculture (USDA), Association of Official Analytical Chemist International (AOACI), International Organization of Standardization (ISO), recommend classical culture methods for recovering *Salmonella* spp. from food. However, the development of new methodologies with the advantages of rapid response, sensitivity and ease of multiplexing is a challenge for food hygiene inspection for screening-out negative samples (Upmann and Bonaparte, 2004). In recent years, many improvements have been done to replace the time-consuming conventional culture detection for rapid methodologies, mainly polymerase chain reaction (Wan et al., 2000; Settanni and Corsetti, 2007), and immunological assays (Ibrahim, 1986; Rowe et al., 1999; Gehring et al., 2006a, 2008). Moreover, recent advances allow the IAs to be performed on magnetic beads as a support (Paleček and Fojta, 2007; Kuramitz, 2009). The term 'immunomagnetic separation' (IMS), is referred to the use of magnetic beads to capture target bacteria – through an immunological reaction – from contaminating microflora and interfering food components, and to concentrate them into smaller volumes for further testing (Fratamico and Crawford, 2004), IMS procedures has been interestingly combined with optical (Gehring et al., 2006b; Zhao et al., 2009), and electrochemical (Gehring et al., 1996, 1999) detection approaches. In this work, a rapid and sensitive strategy for the detection of *Salmonella* cells in milk is presented. In this approach, the bacteria are captured from food samples and preconcentrated by immunomagnetic separation. During the immunomagnetic separation, the enzymatic labeling of the bacteria is also performed using a polyclonal anti-Salmonella-HRP antibody. Then, the modified magnetic beads are easily captured by a magneto-sensor made of graphite-epoxy composite (m-GEC) which is also used as the transducer for the electrochemical detection. A real shortening of the analytical time is obtained by replacing the time-consuming tandem 'selective enrichment/differential plating culture steps' by the 'immunomagnetic separation', while the classical 'biochemical/serological testing assays' are replaced by a serological confirmation using an electrochemical magneto-immunosensor. This methodology (IMS/m-GEC electrochemical immunosensing) is used for the first time in the sensitive detection of *Salmonella* in skimmed-milk samples.

2. Materials and methods

The Instrumentation, chemicals and methods are described in detail in Supplementary data section. The m-GEC electrodes

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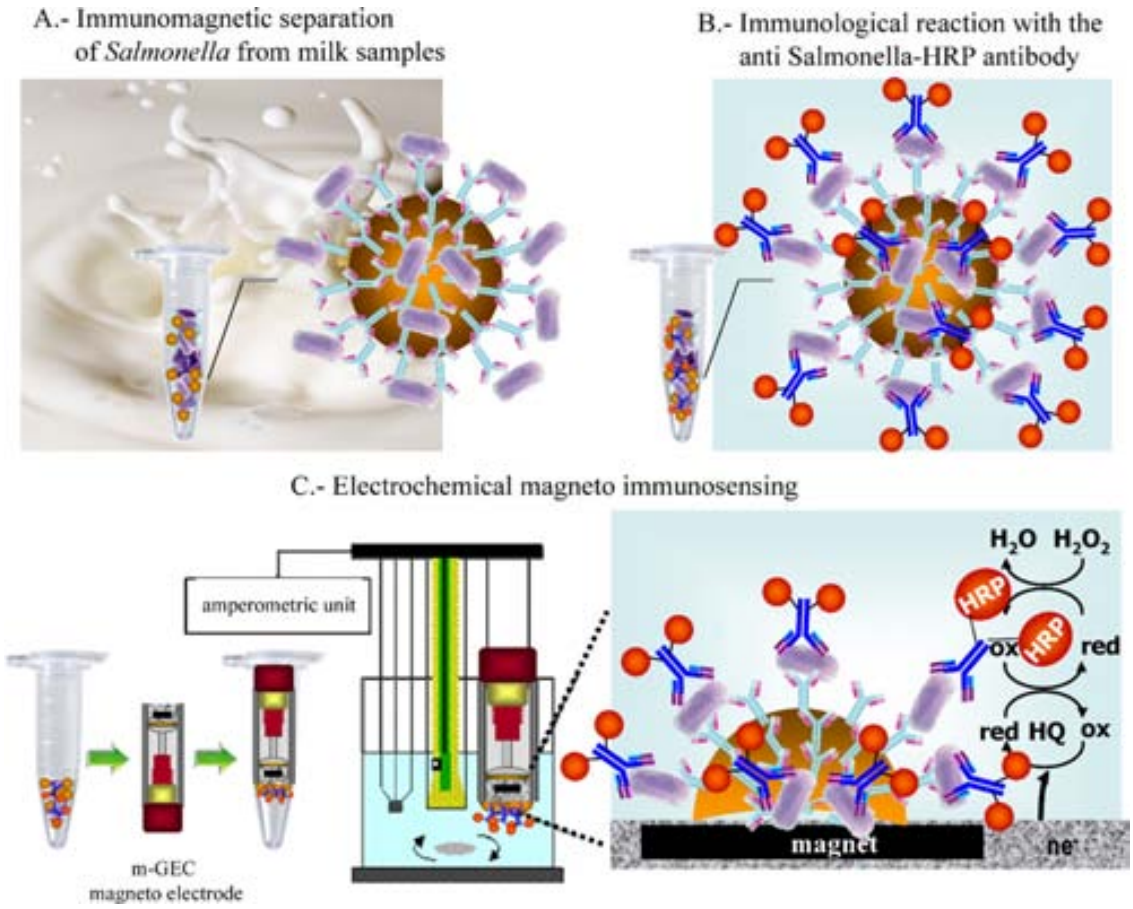

Fig. 1. Schematic representation of the 'IMS/m-GEC electrochemical immunosensing' approach.

were designed in our laboratories for electrochemical genosensing (Pividori and Alegret, 2005; Erdem et al., 2006; Lermo et al., 2008) and immunosensing (Lermo et al., 2009).

The IMS/m-GEC electrochemical immunosensing procedure is schematically outlined in Fig. 1, and it is based on the following steps: (A) immunomagnetic separation (IMS) from milk samples; (B) immunological reaction with the anti-Salmonella-HRP antibody; (C) electrochemical detection. For the optimization of the 'IMS/m-GEC electrochemical immunosensing' approach, four different immunological procedures were evaluated, which vary in the order of the immunological and the washing steps, as detailed explained in Supplementary data and outlined in the expanded version of Fig. 1. The concentration of the anti-Salmonella-HRP antibody was also optimized, as detailed explained in Supplementary data.

3. Results and discussion

The optimization of the immunological reaction for the 'IMS/m-GEC electrochemical immunosensing' approach, was performed by evaluating different procedures for the detection of 7.2×10^6 CFU mL^{-1}, by varying the order of the immunological and the washing steps, being procedures No. 1–3 one-step assays while, in the Procedure No. 4, two sequential immunological reaction were sequentially performed, as detailed explained in Fig. 1A (expanded version in Supplementary content). The incubation time used for the IMS step and for the immunological reaction with the labeled antibody was recommended by the suppliers, while the concentration of the anti-Salmonella-HRP antibody was optimized, for all the procedures, in 1/1000, as detailed explained in Supplementary data. The results for these experiments are shown in Fig. 1B (expanded version) and discussed in detail in Supplementary data contents. The four proposed methods were useful for the detection of *Salmonella* spp., although better results were achieved with Procedure No. 3 (one-step) and No. 4 (two-steps). Moreover, lower level of non-specific adsorption was achieved when the enzymatic conjugate anti-Salmonella-HRP antibody was diluted with LB broth, which seems to act not only as a growing media for the bacteria, but also as a blocking agent for the immunological assay. Considering that the immunological reaction in Procedure No. 3 is performed in one-step, taking a total time of 50 min including the preincubation step, this procedure demonstrated the best performance in terms of rapidity and simplicity, when the presence of interfering flora is not expected to be an issue. On the contrary, the two-steps Procedure No. 4 should be performed when high level of accompanying microflora is expected, in order to favor the specificity of the assay, by including the in-between washing steps, taking a total time of 60 min. As a consequence, the specificity study was performed by using the two-steps Procedure No. 4 and with milk diluted 1/10 in LB artificially inoculated with 2.8×10^6 CFU mL^{-1} of "interfering" *Escherichia coli*, 5.2×10^6 CFU mL^{-1} *Salmonella* and, finally, a mix containing both bacteria (1.4×10^6 CFU mL^{-1} of *E. coli* and 4.65×10^6 CFU mL^{-1} *Salmonella* spp.) and the results are shown in Fig. 2. As expected, the electrochemical signal obtained for *E. coli* (2.8×10^6 CFU mL^{-1}) was similar to that for the negative assay, while the mix of both pathogens (1.4×10^6 CFU mL^{-1} *E. coli* and 4.65×10^6 CFU mL^{-1} *Salmonella* spp.) gave a similar signal that the sample spiked just with *Salmonella* (5.2×10^6 CFU mL^{-1}). Same results were obtained by plating the magnetic beads with the

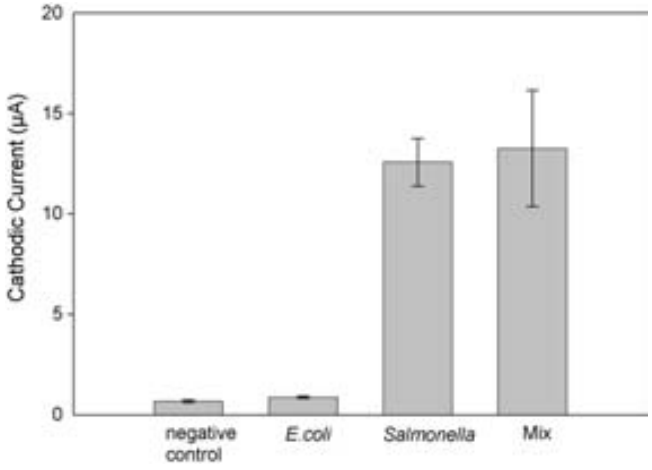

Fig. 2. Specificity study for the 'IMS/m-GEC electrochemical immunosensing' approach. The bars show the electrochemical signal for milk diluted 1/10 in LB artificially inoculated, respectively, with: 0 CFU mL^{-1} (negative control); 2.8×10^6 CFU mL^{-1} of *E. coli*; 5.2×10^6 CFU mL^{-1} of *Salmonella*; and a mix solution containing 1.4×10^6 CFU mL^{-1} of *E. coli* and 4.65×10^6 CFU mL^{-1} of *Salmonella* spp. In all cases, 10 μL of commercial anti-Salmonella magnetic beads were used as well as anti-Salmonella-HRP antibody, diluted 1/1000 in LB broth. The electrochemical detection was performed in PBSE buffer. Mediator: hydroquinone 1.81 mmol L^{-1}. Substrate: H_2O_2 4.90 mmol L^{-1}. Applied potential = −0.100 V (vs. Ag/AgCl). The error bars show the standard deviation for $n = 3$.

attached bacteria in LB agar and grown for 18–24 h at 37 °C (after being modified according to step 1 of Procedure No. 4). No growing were observed for the 2.8×10^6 CFU mL^{-1} of *E. coli*, while typical colony features of *Salmonella* were observed for both the mix of both pathogens as well as just for *Salmonella*. These results confirm that the specificity of the 'IMS/m-GEC electrochemical immunosensing' approach is coming mainly from the IMS step, due to the specific antibody towards *Salmonella* which coated the magnetic beads. However, the selection of a specific enzymatic conjugate anti-Salmonella-HRP antibody could provide not only the electrochemical tag, but also an additional source of specificity for the assay, particularly for those bacteria antigenically related to salmonellae.

The amperometric response of the 'IMS/m-GEC electrochemical immunosensing' approach for artificially inoculated *Salmonella* (from $\times 10^0$ to $\times 10^7$ CFU mL^{-1}) in LB broth is shown in Fig. 3, expanded version (in supplementary data), while Fig. 3 shows the detailed amperometric response at lower concentration, near the LOD, for artificially inoculated *Salmonella* in both LB broth and in skimmed-milk dilute 1/10 in LB. A linear response was obtained in both cases, with a $r^2 = 0.987$ and 0.998, respectively. In the case of the assay performed in LB broth, the amperometric signal corresponding to the LOD was estimated by processing 10 negative control samples (0 CFU mL^{-1}) and performing two different single inter-day assays, and using six batches of magneto-electrode devices, obtaining a mean value of 0.68 μA (Fig. 3A dotted line) with a standard deviation of 0.063 μA. In the case of the skimmed-milk, 15 negative control samples were processed, obtaining a mean value of 0.29 μA (Fig. 3B dotted line) with a standard deviation of 0.088 μA. The amperometric signal corresponding to the LOD value was then extracted with a one-tailed *t*-test at a 99% confidence level, giving a value of 0.85 and 0.52 μA, respectively (shown in Fig. 3A and B as the solid horizontal line). The 'IMS/m-GEC electrochemical immunosensing' approach is able to detect 5.0×10^3 CFU mL^{-1} (in LB broth) and 7.5×10^3 CFU mL^{-1} (in skimmed-milk dilute 1/10 in LB) artificially inoculated with *Salmonella*, in 50 min. Comparing with the classical culture method, as low as 5 CFU mL^{-1} are effectively captured and detected after being modified according to step 1 of Procedure No. 4, followed by plating the magnetic beads with the attached bacteria in LB agar and grown for 18–24 h at 37 °C (expanded version of Fig. 3 in Supplementary data contents). As shown in Fig. 3, a decrease in the slope of the amperometric response in a factor of 0.62 in all the concentration range was observed due to the matrix effect of the milk sample. If this methodology were used for the quantification of *Salmonella* spp. in a sample different from milk, a quantification curve with a negative sample would be performed for each type of food matrix, prior to quantification. However, as the primary use of the 'IMS/m-GEC electrochemical immunosensing' is to screen out negative samples, the most important parameter is the LODs, in order to consider definitive any negative results. Positive test results should be always considered presumptive, and must be confirmed by an approved culture method.

As cells are injured when exposed to adverse conditions during food processing, a preenrichment step is usually included in classical methods to achieve the proliferation of stressed *Salmonella* spp. cells. If this step is not included in the method, small amount of stressed cells that have been not fully repaired may be missed (Amaguaña and Andrews, 2004). Beside this, and accordingly to the results for the LODs obtained in milk (7.5×10^3 CFU mL^{-1}), a preenrichment step should be included to fulfill the legislation

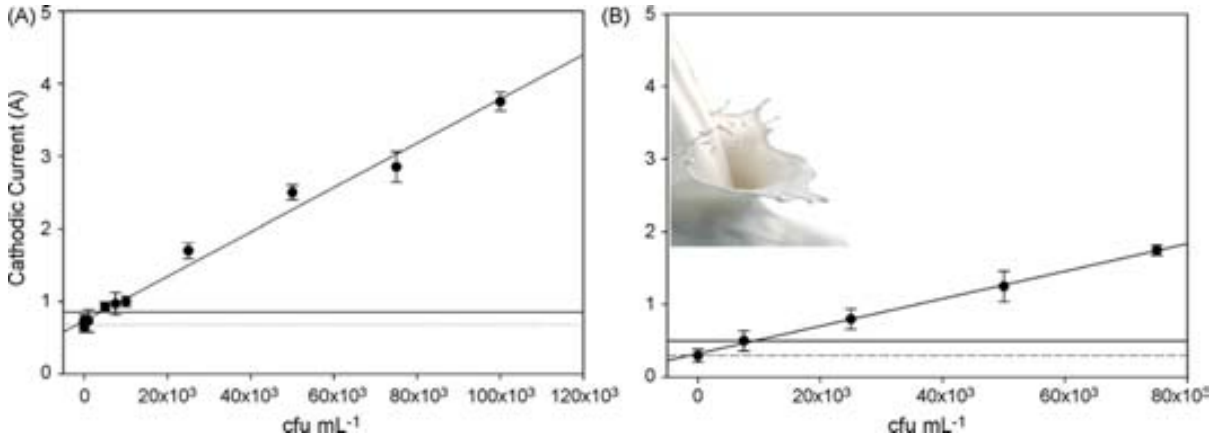

Fig. 3. 'IMS/m-GEC electrochemical immunosensing' approach for the detection from 10^0 to 10^5 CFU mL^{-1} of *Salmonella* cells artificially inoculated in (A) LB broth and (B) skimmed-milk diluted 1/10 in LB, performing the Procedure No. 3, as detailed outlined in Fig. 2. In all cases, anti-Salmonella-HRP antibody, diluted 1/1000 in LB was used. All other experimental conditions as in Fig. 2. The error bars show the standard deviation for $n = 3$, except for the negative control ($n = 10$ for LB broth and $n = 15$ for skimmed-milk diluted 1/10 in LB). The mean value for the negative control (dotted line) and the LOD value extracted with a one-tailed *t*-test at 99% confidence level (solid line) are also displayed in both cases.

requirements for milk (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each in different points, Real Decreto 1679/1994, BOE 24-09-94).

The preenrichment step was study with a nonselective broth medium, in this case LB broth. 25 mL of milk were spiked with just 2.7 CFUs, divided in five portions of 5 mL and processed as above. The samples were preenriched in LB broth at 37 °C, and assayed at 0, 4, 6, 8, 12, and 24 h of preenrichment in LB at 37 °C. A positive control (1.4 CFU mL^{-1}) was also evaluated as well as a negative control (0 CFU mL^{-1}). Fig. 4 (Supplementary data) shows that two of the five portions – as expected, those containing the 1 CFU of *Salmonella* – gave positive results after 8 h, due to the presence of as low as 0.108 CFU mL^{-1}. Moreover, the positive control containing 1.4 CFU mL^{-1} gave a positive signal when the skimmed-milk was preenriched for 6 h. Although the method is able to detect 0.108 CFU mL^{-1} according to the legislation with 8 h of preenrichment, remarkable improvement of the signal is achieved between 8 and 12 h of preenrichment. As a conclusion, after the preenrichment in LB, the procedure is able to detect as low as 1.4 CFU mL^{-1} and 0.108 CFU mL^{-1}, if the milk sample is preenriched for 6 and 8 h, respectively. Comparing with commercial PCR assays for the screening of *Salmonella* without IMS (Wan et al., 2000), the main advantage of this procedure is that free DNA released from death cells during food processing are not detected with this strategy, because of the IMS, which separates and preconcentrates whole bacteria cells – but not DNA –, from food samples. Taking into account that a "positive screen test result" leads to a confirmatory culture assay, this fact is particularly important to avoid unnecessary confirmation testing.

Comparing with other biosensing methodologies for detecting pathogenic bacteria in food (Ivnitski et al., 1999; Leonard et al., 2003; Velasco-Garcia and Mottram, 2003; Terry et al., 2005; Ricci et al., 2007), excellent detection limits were achieved with this procedure in 50 min. Moreover, this method is more rapid and simple than other rapid antibody-based and nucleic acid-based polymerase chain reaction (PCR) methods that have been previously reported (Table B, Supplementary data).

4. Conclusions

A rapid and sensitive assay combining immunomagnetic separation (IMS) and electrochemical magneto-immunosensing for the detection of *Salmonella* in milk was developed. The capturing of *Salmonella* was performed on magnetic beads covered with the specific anti-Salmonella antibody, while the confirmation of the attached bacteria on the magnetic beads was assayed with a second anti-Salmonella antibody labeled with HRP to achieve the electrochemical detection. The magnetic immunoseparation and the detection with a second specific antibody were able to effectively replace the 'selective enrichment/differential plating' and the 'biochemical/serological testing' assays, respectively. As such, the time of the assay was considerably reduced from 4–5 days to 50 min. Although the specificity of the IMS step was enough to isolate *Salmonella* from a mix with *E. coli* in milk, the selection of a specific enzymatic conjugate anti-Salmonella-HRP antibody could provide an additional source of specificity for the assay, particularly for those bacteria closely related to salmonellae. The novel procedure presented here is suitable for the rapid and sensitive on-site screening-out of *Salmonella* in HACCP. Positive test results should be always considered presumptive and must be confirmed by an approved culture method. The sensitivity conferred by the m-GEC electrochemical immunosensing in connection with the specificity conferred by the magnetic beads for the IMS result in a rapid, robust and sensitive procedure. The 'IMS/m-GEC electrochemical immunosensing' approach is able to give positive signal with death or injured whole cells, but DNA released during food processing is not detected, as a difference with commercial PCR approaches without IMS. This fact is particularly important to avoid unnecessary culture confirmation testing. Future work will be focused on the 'in-field', 'low cost' and 'user-friendly' detection of multiple food pathogens affecting food safety.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.07.022.

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A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* on dairy farms by double-tagging PCR and electrochemical genosensing

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A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* on dairy farms by double-tagging PCR and electrochemical genosensing

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Summary. A highly sensitive assay for rapidly screening-out *Mycobacterium bovis* in contaminated samples was developed based on electrochemical genosensing. The assay consists of specific amplification and double-tagging of the IS*6110* fragment, highly related to *M. bovis*, followed by electrochemical detection of the amplified product. PCR amplification was carried out using a labeled set of primers and resulted in a amplicon tagged at each terminus with both biotin and digoxigenin. Two different electrochemical platforms for the detection of the double-tagged amplicon were evaluated: (i) an avidin biocomposite (Av-GEB) and (ii) a magneto sensor (m-GEC) combined with streptavidin magnetic beads. In both cases, the double-tagged amplicon was immobilized through its biotinylated end and electrochemically detected, using an antiDig-HRP conjugate, through its digoxigenin end. The assay was determined to be highly sensitive, based on the detection of 620 and 10 fmol of PCR amplicon using the Av-GEB and m-GEC strategies, respectively. Moreover, the m-GEC assay showed promising features for the detection of *M. bovis* on dairy farms by screening for the presence of the bacterium's DNA in milk samples. The obtained results are discussed and compared with respect to those of inter-laboratory PCR assays and tuberculin skin testing. [**Int Microbiol** 2010; 13(2):91-97]

Keywords: *Mycobacterium bovis* · electrochemical DNA biosensor · avidin · magnetic beads · double-tagging PCR

Introduction

Tuberculosis (TB) in humans and other mammals is usually caused by infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis*. Worldwide, *M. tuberculosis* is the single greatest cause of TB in humans, with the global prevalence of infection involving about one-third of the world's population and expected to increase steadily [2]. In most animals with bovine tuberculosis, *M. bovis* is the infective agent and the disease can be easily transmitted between farm animals. It is also a major zoonosis, mainly involving farm workers on dairy farms and the consumption of contaminated dairy products. Non-pasteurized milk is by far the most probable vehicle for the transmission of pathogenic mycobacteria, especially in developing countries where the prevalence of bovine TB is higher [3], and the isolation of *M. bovis* from milk samples of storage tanks, inadequately pasteurized

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milk, and milk samples from tuberculin non-reactive cattle has been reported [8]. As such, the detection of *M. bovis* in milk samples serves as an indirect diagnostic method—distinguishing infected from non-infected animals and controlling for airborne contamination with the bacilli—in order to prevent further spread of the disease.

To identify cattle infected with *M. bovis,* the intradermal tuberculin test is usually performed, which is based on the inoculation of *M. bovis* antigens called purified protein derivative (PPD). Although the tuberculin skin test is highly sensitive and specific, it requires 48–72 h to process, and veterinarians must be specially trained to perform the assay [4]. The culture of milk samples is another approach to the detection of *M. bovis*, but while it provides acceptable sensitivity and specificity it is labor -intensive, with up to 6 weeks required to detect positive specimens. Moreover, the low sensitivity of cultured milk has been reported, which can be attributed to the drastic pre-culture milk decontamination procedures and to the presence of mammary macrophages able to kill *M. bovis* bacilli [18]. More recent approaches to the rapid detection of *M. bovis* include chromatographic and molecular methods, such as PCR, which have advantages of speed, sensitivity, and specificity; however, they require adequately trained personnel and have high associated costs (reagents and equipment) [17]. Biosensors, by contrast, offer an exciting alternative, allowing the rapid and multiple analyses essential for the detection of bacteria in food [5]. Consequently, they are of particular interest for developing countries, where contaminated milk remains an important issue. Biosensors are devices based on the combination of biological receptors (mainly antibodies, enzymes, nucleic acids, whole cells) and physical or physicochemical transducers. In most cases, they allow "real-time" observations of specific biological events (e.g., antibody-antigen interaction) as well as the detection of a broad spectrum of analytes in complex sample matrices. In the literature, a few assays for *M. bovis* detection have been described that are based on optical and piezoelectric biosensors or on a gas sensor array [1,7,9,15]. These devices, although less robust, are more user-friendly, portable, and cost-effective than electrochemically based transduction devices. Furthermore, electrochemical biosensors can operate in turbid media and of fer enhanced sensitivity.

To our knowledge, the present report is the first description of an electrochemical strategy for the rapid screening-out of raw milk contaminated with *M. bovis*, using a procedure based on electrochemical genosensing. The insertion fragment IS*6110*, highly related to *M. bovis* [16–18], was amplified by double-tagging PCR using a set of primers labeled with biotin and digoxigenin, respectively. During PCR amplification of the *M. bovis* insertion fragment, the amplicon ends were double-tagged with (i) the biotinylated capture primer, to achieve immobilization on the genosensing transducer , and (ii) the digoxigenin signaling primer , to allow enzymatic detection through the antiDigG-horseradish peroxidase (HRP) reporter. The genosensing transducer was immobilized by using a highly specific biocomposite bulk-modified with the protein avidin (Av-GEB) [14] or, alternatively, streptavidin-modified magnetic beads to achieve improved retention of the beads on a highly sensitive magneto sensor (m-GEC) [6,13]. In this report, the features of both electrochemical assays are discussed and compared with respect to inter-laboratory PCR assays and the tuberculin screen-out skin test, the current gold standard for identifying cattle exposed to *M. bovis*.

Materials and methods

Instrumentation. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems, USA). A three-electrode setup was used, comprising a platinum auxiliary electrode (Crison 52-67 1, Spain), a double junction Ag/AgCl reference electrode (Orion 900200) with 0.1 M KCl as the external reference solution, and a working electrode (m-GEC or Av-GEB electrode) [6b]. The amperometric signals were registered using a DUO-18 data recording system (WPI, UK). Temperature-controlled incubations were done in an Eppendorf compact thermomixer PCR was carried out in an Eppendorf Mastercycler Personal thermocycler . Magnetic beads were magnetically separated using a Dynal MPC-S magnetic separator (prod. no. 120.20, Dynal Biotech ASA, Oslo, Norway).

Chemicals and biochemicals. The graphite-epoxy composite and biocomposite were prepared using 50- µm particle size graphite powder (BDH, UK) and Epo-Tek H77 epoxy resin and hardener (both from Epoxy Technology, Billerica, MA, USA). The Av-GEB biocomposite was prepared with avidin (prod. no. A9275, Sigma, S teinheim, Germany). The streptavidin-modified magnetic beads with Dynabeads M-280 and streptavidin (prod. no. 1 12-05D, Dynal Biotech ASA). Fab fragments of anti-digoxigenin-POD (prod. no. 1207733) were used as enzyme reporter and were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Two primers 20-nucleotides long were obtained from TIB-MOLBIOL (Berlin, Germany) and designed for PCR amplification of the insertion sequence IS *6110*, related to *M. bovis* [18]. The primer sequences were: biotinylated IS*6110* up: 5′ bio-GCG TAG GCG TCG GTG ACA AA-3′ and digoxigenated IS*6110* down: 5′ dig-CGT GAG GGC ATC GAG GTG GC-3′. The Expand High Fidelity PCR System Kit (Roche Molecular Biochemicals) was used for the PCR.

All other reagents were of the highest available grade. Aqueous solutions were prepared with Milli-Q water. The compositions of these solutions were as previously described [6b].

DNA amplification and double tagging for the electrochemical detection of *M. bovis*. Raw milk samples were collected from local dairy farm tanks and transported refrigerated to the laboratory . The samples were deactivated at 70°C for 70 min and stored at –20°C until they were used. A 125-ml volume of the sample was centrifuged at 3000 rpm for 15 min, and the cell layer obtained was washed in PBS and resuspended in 1 ml of PBS. The cellular suspension was diluted 1:2 in NTE buf fer with 10% SDS, incubated at 37°C for 1 h, and then overnight at 37°C with 1% proteinase K. DNA was purified by two extractions with phenol:chloro-

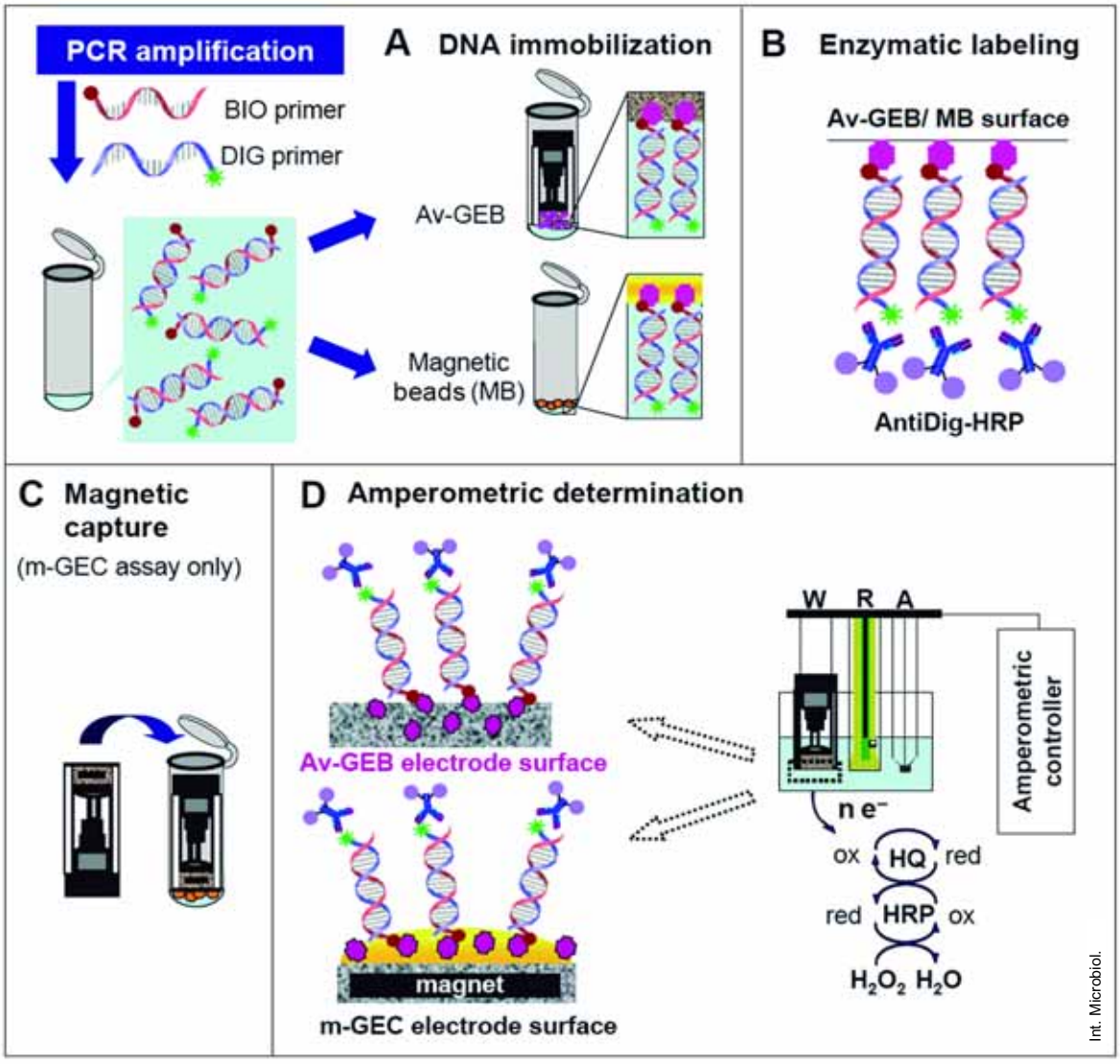

Fig. 1. Schematic representation of the electrochemical strategy for the detection of *Mycobacterium bovis.* For details, see text.

form:isoamyl alcohol (25:24:1) and one with chloroform:isoamyl alcohol (24:1), collected by precipitation with 5 M NaCl and isopropanol, and kept overnight at –20°C. The precipitated DNA was then washed with 1 ml of ethanol 70° and resuspended in 40 µl of RNase-free water.

As shown in Fig. 1A, a primer pair tagged with biotin and digoxigenin, respectively, was used for amplification and double-tagging of the PCR amplicon. PCR was performed in a 100-µl reaction mixture containing 8 µl of purified DNA isolated from *M. bovis*. Each reaction contained 100 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 0.1 µM of the double-tagged set of primers (biotinylated IS *6110* up and digoxigenated IS*6110* down), and 5.6 U of polymerase. The reaction was carried out in Expand High Fidelity 1× buffer (Roche), containing 1.5 mM $MgCl_2$ and 5% v/v dimethyl sulfoxide (DMSO). The amplification mixtures were exposed to an initial step at 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 64°C for 30 s, and 72 ºC for 30 s, and a last step of 7 min at 72°CThe resulting samples were stored at 4°C.

All of the amplifications included a negative control, which contained all reagents, except *M. bovis* template, in the PCR mixture. The amplification products were analyzed by electrophoresis on 2% agarose gel in TAE buffer (0.04 M Tris, 0.1% v/v acetic acid, 1 mM EDTA, pH 8.0), containing 0.5 µg ethidum bromide/ml. A *Hin*fI-digested ϕX174 genome consisting of DNA fragments ranging in size from 24 to 726 bp was used as a DNA size marker. The DNA bands were visualized by UV trans-illumination. As the primers were labeled with biotin and digoxigenin, the amplicon was expected to be double-tagged with both biotin and digoxigenin at each terminus.

Construction of the magneto graphite-epoxy composite (m-GEC) and avidin graphite-epoxy composite (Av-GEB) electrodes. The m-GEC and Av-GEB electrodes were designed in our laboratories. The detailed preparation was extensively described by Pividori et al. [12] and was based on a rigid graphite-epoxy composite [10,1 1]. For the Av-GEB electrode, avidin was hand mixed with the graphite power and epoxy resin paste, resulting in a 1% (w/w) bulk-modified biocomposite. The magneto electrodes based on GEC as well as those based on the biocomposite material were cured at 40°C for one week. Prior to each use, the electrode surface was renewed by a simple polishing procedure, i.e., wetted with doubly distilled water, and then thoroughly smoothed with abrasive paper and finally with alumina paper [6].

Electrochemical genosensing of the double-tagged *M. bovis* amplicon using Av-GEB electrodes. Electrochemical detection based on the Av-GEB electrodes consisted briefly of the following steps (Fig. 1): (i) immobilization of the double-tagged amplicon on the Av-GEB electrode (Fig. 1A), with the biotin-tagged terminus of the dsDNA amplicon attached to the surface of the electrode; (ii) enzymatic labeling using antiDig-HRP, which attaches to the 3′ digoxigenin end of the amplicon (Fig. 1B); (iii) amperometric determination (Fig. 1D).

After the amplification, the PCR amplicon was diluted in Milli-Q water and 10 µl were incubated in 5× SSC for 15 min at 42°C. The double-tagged amplicon was then immobilized by dipping the Av-GEB electrode into an Eppendorf tube containing the diluted amplicon. Immobilization was carried out in 5× SSC solution at a final volume of 140 µl for 30 min at 42°C. The prepared electrode was then washed twice with 140 µl of 5× SSC for 10 min at 42°C. In the next step, the immobilized amplicon was enzymatically labeled for 30 min at 42°C using antiDig-HRP (60 µg) in a reaction containing Tris blocking buf fer and a final volume of 140 µl. The immobilized, enzymatically labeled amplicon was then washed twice for 10 min at 42°C in 140 µl of Tris buffer. Electrochemical determination was carried out using the modified Av-GEB electrode as working electrode and by dipping the three-electrode setup (described in Materials and methods) in 20 ml of phosphate buf fer. The response was determined by polarizing the electrodes at –0.150 V (vs. Ag/AgCl). Amperometric detection was based on the activity of the HRP enzyme as electrochemical reporter , using 1.81 mM hydroquinone as the mediator and 4.90 mMhydrogen peroxide as the substrate for the enzyme HRP.

Electrochemical genosensing of the double-tagged *M. bovis* amplicon using m-GEC electrodes. Electrochemical detection based on m-GEC electrodes consisted briefly of the following steps (Fig. 1): (i) immobilization of the double-tagged amplicon on streptavidin magnetic beads, with the 5′ biotin end immobilized on the beads (Fig. 1A); (ii) enzymatic labeling using antiDig-HRP, which attaches to the 3′ digoxigenin end of the amplicon (Fig. 1B); (iii) magnetic capture of the modified magnetic particles by the m-GEC electrode (Fig. 1C); (iv) amperometric determination (Fig. 1D).

As was done in the Av-GEB procedure, following amplification, the PCR amplicon was diluted in Milli-Q water and 10 µl were incubated in 5× SSC for 15 min at 42°C. The double-tagged amplicon was then immobilized by adding 6.5×10^6 streptavidin magnetic beads to an Eppendorf tube containing the diluted amplicon. Immobilization was carried out in 5× SSC solution at a final volume of 140 µl for 30 min at 42°C. The subsequent washing steps and electrochemical detection were the same as described for the Av-GEB platform.

Results

DNA amplification and double tagging for electrochemical detection of *M. bovis*. As shown in Fig. 2, under the PCR conditions used here, the double-tagged set of primers exclusively amplified IS*6110*. Figure 2 also shows no bands in the negative PCR control sample, which included all reagents except the DNA template.

Electrochemical genosensing of the double-tagged *M. bovis* amplicon using Av-GEB and m-GEC electrodes. In Fig. 3A, the responses obtained with the different dilutions of double-tagged IS*6110* PCR amplicon using the Av-GEB (1/15, 1/8, 1/4, and 1/2) and m-GEC (1/960, 1/480, 1/240, 1/120, 1/60, 1/30, 1/15, 1/8, and 1/4) electrodes are plotted against the PCR amplicon concentration determined spectrophotometrically at 260 nm. The electrochemical signals were obtained under conditions at which the enzyme was saturated with the substrate. For each measurement, a steady-state current was obtained after the addition of hydroquinone and hydrogen peroxide (normally after 1 min of addition of the latter), as shown in Fig. 3B. This steady-state current was also used for the electrochemical signal plotted in Fig. 4.As shown in Fig. 3A, the analytical response of both electrodes increased quantitatively with the amount of double-tagged amplicon, but the sensitivity of the assay based on the m-GEC electrodes (black line) was higher than that obtained with the Av-GEB electrode (dotted line). The inset in Fig. 3A shows in detail the responses obtained with the two electrodes at the lowest concentration range. The lowest amount of analyte producing a meaningful analytical signal was 620 fmol for the Av-GEB electrode and 10 fmol for the m-GEC electrode.

Figure 4 shows the electrochemical response provided by five different milk samples from dairy farms using double-tagging PCR combined with either the Av-GEB or the m-GEC strategy, as an indicator of infected cattle. In order to screen-out negative samples, a cut-off value was established by using both electrochemical genosensing strategies to analyze a negative milk sample (as confirmed by two inter-laboratory PCR assays and by the tuberculin skin test). Accordingly, four replicates of the negative control were

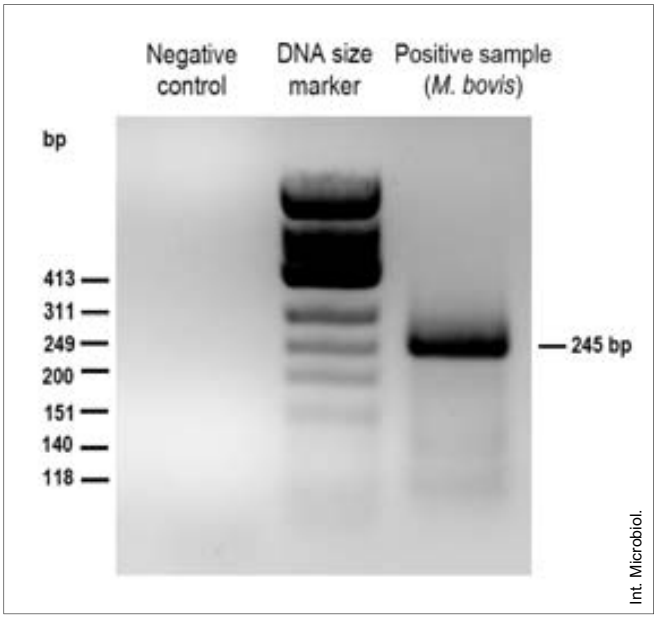

Fig. 2. Gel electrophoresis of the DNA amplification product using the double-tagging set of primers.

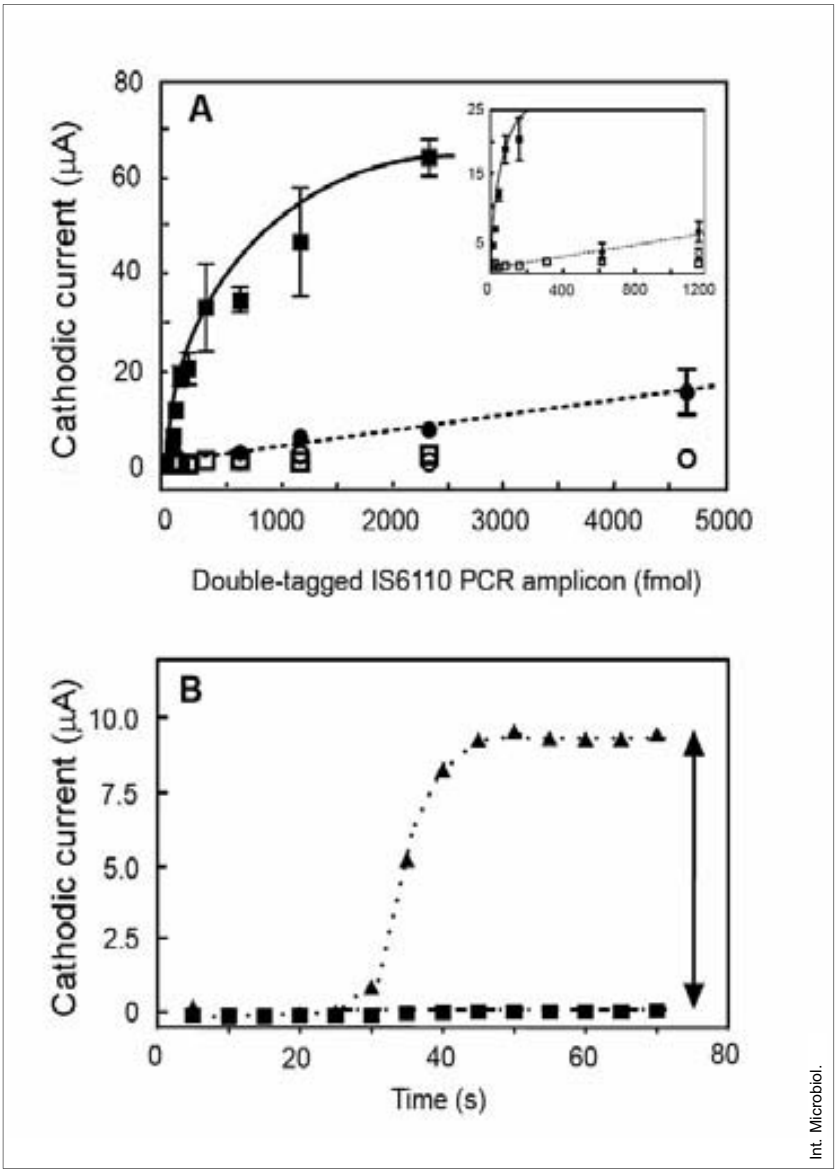

Fig. 3. (**A**) Electrochemical detection of the *Mycobacterium bovis* PCR product based on the Av-GEB and m-GEC strategies. Closed circles, positive sample Av-GEB; open circles, negative control Av-GEB; closed squares, positive *M. bovis* sample m-GEC; open squares, negative control m-GEC. (**B**) The typical amperometric curve, showing the enzyme saturation signal. Closed triangles, positive *M. bovis* sample; closed squares, negative control. The dotted line shows the cut-off value. In all cases (A and B), n = 3.

processed, obtaining a mean value of 1.4 μA with a standard deviation of 0.8 μA for the Av-GEB assay and a mean value of 3.3 μA with a standard deviation of 0.3 μA for the m-GEC assay. The cut-off value was then extracted using a one-tailed *t* test at a 95% confidence level, giving a value of 3.9 μA and 4.2 μA for the Av-GEB and m-GEC strategies, respectively (shown in Fig. 4 as a dotted line). The assay based on the m-GEC electrode showed the presence of *M. bovis* in samples 3, 4, and 5, whereas only sample 5 was positive in the Av-GEB assay (3 and 4 gave negative results). The inset of Fig. 4 shows in detail the responses obtained for samples 3 and 4.

These results were compared with those obtained in analyses of the milk samples by inter-laboratory PCR assays and administering the tuberculin skin test to the animals (Table 1).

Discussion

DNA amplification and double tagging for the electrochemical detection of *M. bovis*. To our knowledge, this is the first report in which double-tagging PCR was carried out for the detection of *M. bovis*. Both the biotin and the digoxigenin moieties could be successfully incorporated into the PCR product using a set of 5′ labeled primers, as confirmed in Fig. 2. After annealing of both 5′ labeled primers with the template, a new DNA strand was enzymatically assembled by the Taq polymerase, by the addition of nucleotides to the 3′ end of both primers. The primers, and thus their tags, were included in the amplicon.

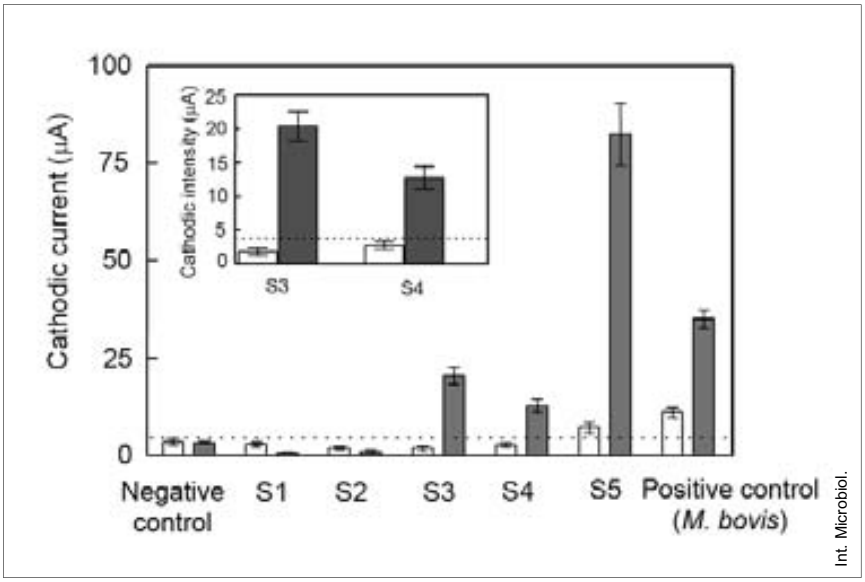

Fig. 4. Detection of *Mycobacterium bovis* in milk samples using the electrochemical detection based on the Av-GEB (white bars) and m-GEC (gray bars) strategies. The dotted line shows the cut-of f value, n = 3. (S1, sample 1; S2, sample 2; S3, sample 3; S4, sample 4; S5, sample 5.)

Electrochemical genosensing of the double-tagged amplicon of *M. bovis* using Av-GEB and m-GEC electrodes. In the electrochemical genosensing procedure based on the Av-GEB electrodes, the double-tagged amplicon was immobilized on the surface of the avidin biocomposite transducer , while in the electrochemical genosensing procedure based on the m-GEC electrodes, the double-tagged amplicon was immobilized on streptavidin magnetic beads and then captured on the surface of the magneto electrode (m-GEC). In both cases, the electrochemical response of the double-tagged product was due to the enzymatic reporter antiDig-HRP. As shown in Fig. 3, the electrochemical genosensing strategy based on m-GEC had a higher sensitivity, obtained by using streptavidin magnetic beads, which immobilized the biotinylated amplified material on the m-GEC surface and permitted rapid magnetic separation of the unbound components. However , non-specific adsorption for both electrodes, as determined with the negative PCR control, was low and almost the same throughout the evaluated concentration range. The results showed that both strategies, using Av-GEB and m-GEC electrodes, were suitable for the detection of amplified PCR amplicon, although a better limit of detection (LOD) was achieved with (strept)avidin magnetic beads coupled with m-GEC electrodes.

The high specificity of the tuberculin skin test (96%, i.e., the proportion of negatives that are correctly identified) ensures the correct identification of a negative sample. Nevertheless, the test is unable to ensure the total absence of *M. bovis* in milk samples, as its sensitivity is only 86% [4]. Since screening assays are used on large sample populations, often with the aim of determining which samples require further investigation, false-positives are not as problematic as false-negatives, since the former will be further examined.

Due to the high specificity of the tuberculin skin test (96%), samples 3 and 4 (Fig. 4), with positive tuberculin skin test results (Table 1), in all likelihood came from infected animals. However , inter -laboratory PCR assays as well as

Table 1. Results of inter-laboratory PCR assays and tuberculin skin tests of milk samples screened for *Mycobacterium bovis*

	Inter-laboratory PCR assays			Electrochemical assays	
Sample	Lab1	Lab2	Tuberculin skin test	m-GEC	Av-GEB
1	Positive	Positive	Negative PPD	Negative	Negative
2	Positive	Positive	Negative PPD	Negative	Negative
3	Negative	Positive	Positive PPD	Positive	Negative
4	Negative	Negative	Positive PPD	Positive	Negative
5	Positive	Negative	Negative PPD	Positive	Positive

electrochemical genosensing of the double-tagged amplicon based on the Av-GEB platform gave false-negative results for these samples. As a false-negative can be the source of misdiagnoses, with severe consequences, the poor analytical performance in screening-out negative samples is noteworthy. By contrast, positive results, consistent with the tuberculin skin tests (Table 1), were obtained for samples 3 and 4 by electrochemical genosensing of the double-tagged amplicon using the m-GEC platform (Fig. 4).

The discrepancy in the electrochemical genosensing results was likely due to the fact that the m-GEC approach has a better LOD than the Av-GEB approach (10 vs. 620 fmol of double-tagged amplicon, respectively), allowing the identification of samples 3 and 4 as positive with higher sensitivity.

The negative results obtained for sample 5 with the tuberculin skin test but not by electrochemical genosensing with either the m-GEC or the Av-GEB electrodes (as displayed in Fig. 4 and Table 1) could be ascribed to the lower sensitivity of the traditional test. Accordingly, sample 5 should be further investigated. However, as the primary use of electrochemical genosensing of the double-tagged amplicon based on m-GEC is to screen-out negative samples, the most important parameter is the LOD, and thus to consider any negative results as definitive. By contrast, positive test results always should be considered presumptive and must be confirmed by an approved culture method.

Electrochemical genosensing with m-GEC electrodes shows interesting analytical features suggesting this approach as a promising strategy to screen-out negative dairy samples and thereby to isolate negative cattle from presumptive infected animals. The combination of genome amplification by double-tagging PCR, capture of the double-tagged amplicon, and electrochemical genosensing detection using the sensitive m-GEC electrode provides a rapid, cheap, and sensitive assay for the screening-out of samples contaminated with *M. bovis*.

Future work will be focused on the analytical validation of this promising electrochemical genosensor by processing a higher number of dairy samples. In addition, the modification of this methodology to include disposable, low-cost screen-printed electrodes is of great interest.

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Phagomagnetic Separation and Electrochemical Magneto-Genosensing of Pathogenic Bacteria

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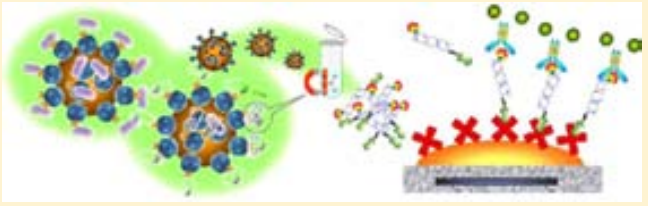
Supporting Information

ABSTRACT: This paper addresses the use of bacteriophages immobilized on magnetic particles for the biorecognition of the pathogenic bacteria, followed by electrochemical magneto-genosensing of the bacteria. The P22 bacteriophage specific to *Salmonella* (serotypes A, B, and D_1) is used as a model. The bacteria are captured and preconcentrated by the bacteriophage-modified magnetic particles through the host interaction with high specificity and efficiency. DNA amplification of the captured bacteria is then performed by double-tagging polymerase chain reaction (PCR). Further detection of the double-tagged amplicon is achieved by electrochemical magneto-genosensing. The strategy is able to detect in 4 h as low as 3 CFU mL^{-1} of *Salmonella* in Luria−Bertani (LB) media. This approach is compared with conventional culture methods and PCR-based assay, as well as with immunological screening assays for bacteria detection, highlighting the outstanding stability and cost-efficient and animal-free production of bacteriophages as biorecognition element in biosensing devices.



Bacteriophages (or phages) are natural host-specific, self-reproducing, and self-assembling nanostructured particles, with both structure and function encrypted in the genomic DNA. Bacteriophages bind to specific receptors on the bacterial surface in order to inject the genetic material inside the bacteria, using the host's own replication machinery for multiplication. The replicated virions are eventually released, killing the bacteria and allowing the infection of other host cells. Beside the promising features of phage therapy,[1] bacteriophage-based diagnostic is attracting much interest[2] due to the high specificity of phages, which makes them ideal agents not only for the detection of bacteria, but also for the detection of almost all kinds of targets, ranging from small molecules to proteins and even cells, by using the phage display technique.[3] As phages have the ability to display peptides or proteins on their surface, those showing a very high affinity and specificity for a target can be selected out of a library. Unfortunately, as the use of phages as biorecognition elements is in its infancy, the range of commercially available bacteriophages is still limited. Another important advantage is the fast, cheap, and animal-friendly phage production, which is achieved by just infecting the host bacteria.[3] Moreover, phages are stable in a range of harsh conditions including pH and temperature.[3] Phages can even be used in the presence of nucleases or proteolytic enzymes, without degradation. The high stability of phages in a variety of environmental conditions makes them suitable for in situ monitoring of food and environmental contaminants. These naturally occurring nanoparticles have other interesting properties in comparison with synthetic nanoparticles: all bacteriophages are nearly identical, being monodisperse in shape and size, a fact difficult to achieve by laboratory synthesis. On the contrary, these nanoparticles are self-synthesized in their specific host, by producing a large amount of viral coat proteins with a large surface for further chemical modification.

The reported methods for bacteria detection using bacteriophages include (i) expression of bacteriophage-encoded bioluminescent genes which produce visible products within the specific target cells (lux-bacteriophage strategy),[4] (ii) fluorescence-labeled phage, which can be combined with immunomagnetic separation (labeled phage strategy),[5] (iii) detection of bacteria by the intracellular replication of specific bacteriophages (named "phage amplification" strategy),[6,7] and the (iv) detection of the phage-mediated bacterial lysis and release of host enzymes (e.g., adenylate kinase) or ATP (termed "lysin-release ATP bioluminescence strategy").[8]

Bacteriophages recognize the bacterial receptors through their tail spike proteins. This biorecognition is highly specific and has been employed for the typing of bacteria. This level of specificity and selectivity opens avenues for the development of specific pathogen detection technologies and for the creation of biosensing platforms. Biosensing approaches based on quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) as transduction platform were reported.[9−11] These early reports relied on physical adsorption of the bacteriophage on the sensor surface. Single-point, oriented, covalent attachment of the bacteriophages on different surfaces and transducers was

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also reported in order to yield better coverage and to improve the performance of these devices. Streptavidin-mediated attachment of bacteriophages that were genetically modified to directly express biotin on their capsid was reported.[12,13] Covalent immobilization of bacteriophages on gold surface,[14] screen-printed carbon electrode,[15] and glass substrates[16] for biosensor application was also reported.

This paper addresses the use of bacteriophage nanoparticles as a highly specific biorecognition element for the capture and preconcentration of pathogenic bacteria by using "phagomagnetic separation" (PMS), followed by electrochemical magneto-genosensing detection. The main advantage of using bacteriophages relies on cost-efficient and animal-free production, as well as their outstanding stability, overcoming thus the main challenges of the biorecognition elements in biosensing devices. The icosahedral-shaped bacteriophage (P22) specific to the pathogenic bacteria *Salmonella* was studied as a model.[17,18] The immobilization of the native, non-modified, P22 phage nanoparticles on tosylated magnetic particles was achieved throughout the amine moieties of the lysine residues in the main capsid monomeric protein (gp5)[19] by covalent amine linkage. After preconcentration of the bacteria on the magnetic particles by PMS, the bacteria were easily detected by double-tagging polymerase chain reaction (PCR) amplification of the DNA of the captured bacteria followed by electrochemical magneto-genosensing (PMS/double-tagging PCR/m-GEC genosensing).[20]

The main features of the PMS/double-tagging PCR/m-GEC electrochemical genosensing approach are compared with conventional culture methods and PCR-based assay.

EXPERIMENTAL SECTION

Instrumentation. Temperature-controlled incubations were performed in an Eppendorf Thermomixer compact. The magnetic separation during the washing steps was performed using a magnetic separator Dynal MPC-S (product no. 120.20D, Dynal Biotech ASA, Norway). The PCR reaction was carried out in an Eppendorf Mastercycler personal thermocycler. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems Inc., U.S.A.). A three-electrode setup was used comprising a platinum auxiliary electrode (Crison 52-67 1), a double-junction Ag/AgCl reference electrode (Orion 900200) with 0.1 mol L^{-1} KCl as the external reference solution, and a working electrode (the magneto-electrode, m-GEC). The detailed preparation of the m-GEC electrodes has been extensively described by Pividori and co-workers[21,22] (Figure i, Supporting Information). The scanning electron microscopy (SEM) images were taken with the scanning electron microscope Hitachi LTD S-570 (Hitachi LTD, Tokyo, Japan).

Chemicals, Biochemicals, and Materials. Tosylactivated magnetic particles (MP-Tosyl) (Dynabeads M-280, product no. 142.03) as well as the streptavidin magnetic particles (Dynabeads M-280 Streptavidin, product no. 112.05) were purchased from Life Technologies, Invitrogen Dynal AS (Oslo, Norway). AntiDig−HRP (antidigoxigenin−POD, product no. 11.207.733.910) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Bradford assay was performed with the Coomasie Bradford protein assay kit, ref. 23200, Pierce, U.S.A.

The Expand High Fidelity PCR System kit (Roche Molecular Biochemicals) was used for performing the PCR. The primers for the double-tagging PCR amplification in the genosensing strategy were obtained from TIB-MOLBIOL (Berlin, Germany). These primers were selected for the specific amplification of the *IS200* insertion sequence[23] related to *Salmonella* spp. The primer sequences were biotin-*IS200* up: 5′ BIO-ATG GGG GAC GAA AAG AGC TTA GC 3′, digoxigenin-*IS200* down: 5′ DIG-CTC CAG AAG CAT GTG AAT ATG 3′.

The buffer solutions were prepared with Milli-Q water. All other reagents were analytical reagent grade supplied from Sigma and Merck. The composition of the solutions is detailed in the Supporting Information.

Bacterial Strains and P22 Bacteriophage. The P22 bacteriophage (ATCC 19585-B1), *Salmonella enterica* serovar Typhimurium LT2, and *Escherichia coli* K12 strains were used in this work. The bacteriophage lysate was obtained by infecting exponential cultures of *Salmonella* Typhimurium LT2 (10^8 CFU mL^{-1}) with the P22 bacteriophage, and by further purification with cesium chloride gradient,[24] as detailed in the Supporting Information. The bacteriophage titer was determined by plating them using double agar layered conventional method (Figure iv, part A, Supporting Information). The phage stock solutions were maintained in $MgSO_4$ 10 mM in Milli-Q water solution at 4 °C retaining a constant titer for several months. When specified, the P22 bacteriophages were inactivated by exposure to a UV-C (254 nm) germicidal lamp to avoid the lytic cycle.

Covalent Immobilization of P22 Bacteriophage on Magnetic Particles and Coupling Efficiency Study. The native P22 phage nanoparticles were covalently coupled for the first time to tosyl-activated magnetic particles by the reaction of aminated aminoacidic lysine moieties of the main capsid monomeric protein (gp5)[18] (as schematically outlined in Figure ii, Supporting Information), by an amine linkage, in order to obtain the P22 phage-modified magnetic particle conjugate (P22-MP). The binding was performed using purified P22 phage stock solution (200 μL) at a concentration level of 2×10^{12} PFU mL^{-1}, reaching a concentration in the immobilization solution of 4×10^{11} PFU mL^{-1} as explained in detail in the Supporting Information.

The coupling efficiency was evaluated by the Coomassie Bradford protein assay,[25] analyzing the protein concentration of the P22 phage capsid in the supernatant after the covalent attachment, and performing the calibration curve with the purified P22 phage solution from 2×10^{10} to 5×10^{11} PFU mL^{-1}, as described in the Supporting Information, Figures ii and iii.

A similar approach was performed by the double agar layered conventional method for counting active phages. In this approach, 10-fold dilutions of the supernatant after the covalent attachment were plated through the double agar layered method as described in the Supporting Information (Figure iv).

Evaluation of the Immobilized P22 Bacteriophage on Magnetic Particles by SEM and Conventional Culture Methods. The evaluation of the immobilized P22 bacteriophages was performed by microscopic techniques (SEM) as well as by conventional culture methods. For the microscopic evaluation by SEM, 10 μL of the P22 phage-modified magnetic particles (P22-MPs) in 5 mL of Milli-Q water (1924 PFU/MP) was filtered through a Nucleopore membrane (25 mm Ø, 0.2 μm pore size). The filters were then fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydratated with ethanol, and dried by CO_2 critical point before gold metallization and observation.[26]

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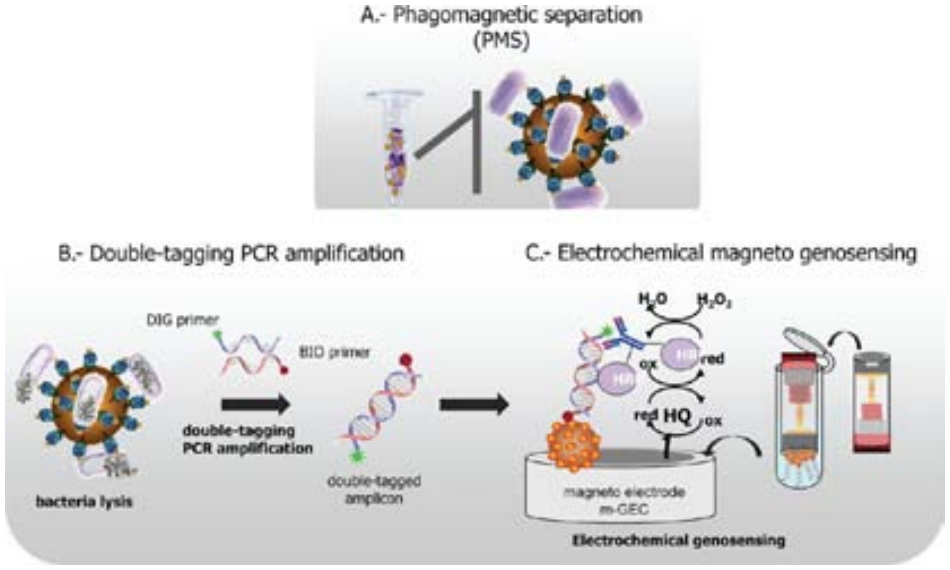

Figure 1. Schematic representation of the phagomagnetic separation (PMS) (A) of the bacteria followed by the double-tagging PCR (B) and the electrochemical magneto-genosensing (C) of the attached bacteria (PMS/double-tagging PCR/m-GEC electrochemical genosensing).

As previously addressed, the orientation of the bacteriophages on the solid support is an important issue to be considered. This orientation was studied by the double agar layered method and enumeration of plaques by culturing the P22-MPs, since oriented phages immobilized on magnetic particles will produce bacteria attachment and further infection of viable bacteria, producing the plaques (Figure v, Supporting Information). A 10-fold dilution of the P22-MPs was plated through the double agar layered conventional method as previously explained (Figure iv, Supporting Information).

Phagomagnetic Separation of *Salmonella*. Evaluation by SEM and Conventional Culture Methods. The procedure for the PMS of the bacteria is schematically outlined in Figure 1A. Inactivated bacteriophages by UV radiation were used for the phagomagmetic separation to avoid the lytic cycle in order to keep the attached bacteria as a whole cell while being captured, preconcentrated, and cultured since both SEM and culturing require non-infected bacteria.

Bacterial solutions that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1} in Luria–Bertani (LB) broth were performed for the PMS of *Salmonella* Typhimurium LT2. A negative control of LB broth was also processed. The culture in LB (500 μL) was mixed with 50 μL of P22-MPs (Figure 1A). An incubation step was performed for 30 min at 37 °C without agitation. After that, the magnetic particles with the attached bacteria were separated with a magnet and then washed with PBST for 5 min (3×) at room temperature. Finally, the modified magnetic particles were resuspended in 80 μL of Milli-Q water.

The evaluation of the PMS (Figure 1A) was performed by SEM and conventional culture methods. For the SEM study, the PMS was performed with a concentration of bacteria of 2.9×10^7 CFU mL^{-1}, and the filters were treated as above-described. In order to study the efficiency of the PMS step by conventional culture method, 10 μL of modified magnetic particles of each solution that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1} in LB broth including LB broth as negative control was plated in LB agar and grown for 18–24 h at 37 °C.

Phagomagnetic Separation, Double-Tagging PCR Amplification, and Electrochemical Magneto-Genosensing. The procedure for the PMS of the bacteria followed by the double-tagging PCR and the electrochemical magneto-genosensing of the attached bacteria (PMS/double-tagging PCR/m-GEC electrochemical genosensing) is schematically outlined in Figure 1. In this case, UV-inactivated P22 phage nanoparticles were also used for the phagomagmetic separation to avoid the lytic cycle and the release of the genomic DNA of the bacteria while being captured and preconcentrated, since DNA is required for the double-tagging PCR amplification. For each concentration of bacteria in LB (from 3.2×10^6 to 3.2×10^0 CFU mL^{-1}), the lysis of the bacteria attached on the inactivated P22-MPs was performed at 99 °C for 20 min in order to break the cells and to achieve the releasing of the genomic DNA and the cellular debris to the solution for the PCR amplification (Figure 1B). The amplification of the specific *IS200* insertion sequence related to *Salmonella* spp. was thus performed (Figure 1B) by a double-tagging PCR using two labeled primers with biotin and digoxigenin[27] (Figure vii, expanded version of Figure 1B, Supporting Information). During the PCR, not only the amplification of pathogenic bacteria genome was achieved, but also the double tagging of the amplicon ending with (i) the biotinylated capture primer to achieve the immobilization on streptavidin-modified magnetic particles and (ii) the digoxigenin signaling primer to achieve the enzymatic detection through antiDig–HRP reporter.

All of these amplifications included not only a positive control, but also a blank as a negative control, which contained LB broth without *Salmonella* spp. template. The double-tagged amplicon was analyzed by electrochemical genosensing with the m-GEC electrodes as well as with the conventional gel electrophoresis.

The electrochemical genosensing strategy of the double-tagged amplicon (Figure 1C) comprises the following steps, as outlined in the Supporting Information and the Figure viii, expanded version of Figure 1C: (a) immobilization of the double-tagged amplicon in which the 5′ biotin end was immobilized on the streptavidin magnetic particles; (b) enzymatic labeling with the antibody antiDig–HRP able to bond the 5′ digoxigenin end of the ds-DNA amplicon; (c) magnetic capture of the modified magnetic particles by the m-GEC electrode; (d) amperometric determination.

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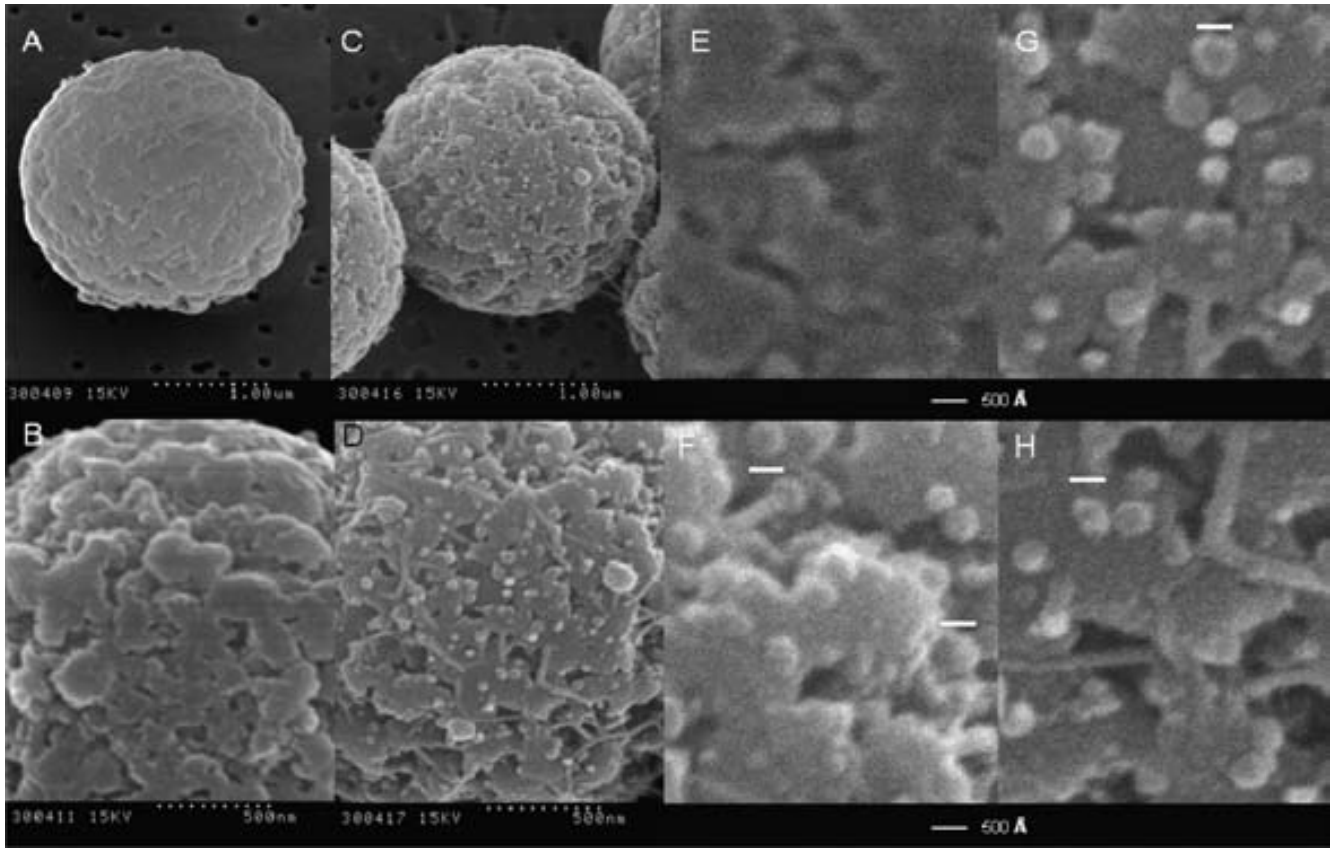

Figure 2. Evaluation of the immobilized P22 bacteriophage on magnetic particles by SEM (1924 PFU/MP). Images C, D, and F–H show, at different resolution levels, the P22 bacteriophages attached to the magnetic particles. Panels A, B, and E show the magnetic particle without modification as a negative control. In all cases, identical acceleration voltage (15 kV) was used.

Specificity Study of the "PMS/Double-Tagging PCR/m-GEC Electrochemical Genosensing" Approach. In order to verify the specificity of this approach, the above procedure was also performed with 4.5×10^5 CFU mL^{-1} of *E. coli*, *Salmonella*, and finally, a sample containing both bacterial species (4.3×10^5 CFU mL^{-1} of each bacterial specie) artificially inoculated in LB, as well as a negative control.

Safety Considerations. All the procedures involving the manipulation of potentially infectious materials or cultures were performed following the guidelines for safe handling and containment of infectious microorganism.[28] Strict compliance with BSL-2 practices was followed in all experiments involving *Salmonella* Typhimurium LT2, *E. coli* K12, and active P22 bacteriophage, and proper containment equipment and facilities were used. The ultimate disposal was performed according to local regulations.

RESULTS AND DISCUSSION

Covalent Immobilization of P22 Bacteriophage on Magnetic Particles and Coupling Efficiency Study. The native P22 phage nanoparticles were covalently coupled for the first time to tosyl-activated magnetic particles by the reaction of aminated aminoacidic moieties of the main capsid monomeric protein (gp5).[18] The amount of viral protein present in the supernatant before and after the immobilization step was determined by the Bradford test to quantify the coupling efficieny of the capsid protein to the magnetic particle. As shown in Figure iii, Supporting Information, a good calibration curve was obtained with the Bradford method of the P22 phage nanoparticles showing good reproducibility at each concentration level ($n = 3$) and a linear range from 2×10^{10} to 5×10^{11} PFU mL^{-1} ($r = 0.968$). By comparing the phage concentration before and after immobilization, the coupling efficiency of nonmodified P22 bacteriophages (4×10^{11} PFU mL^{-1}) on tosyl-activated magnetic particles on both 7×10^8 and 7×10^7 magnetic particle units was found to be 100.4% and 23.6%, respectively, with ratios of 626 and 1924 P22 phage nanoparticles (PFU) immobilized per magnetic particle, respectively. Moreover, the immobilization of an increased amount of P22 phage nanoparticles (1×10^{12} PFU) on the same amount of magnetic particles (7×10^7) showed a similar coupling efficiency (25.6%), with a ratio of 2163 P22 phage nanoparticles (PFU) per each magnetic particle, indicating a plateau in the immobilization efficiency in approximately 2000 PFU/MP. A similar approach for coupling efficiency study was performed by quantifying the plaque forming units (PFU) in the supernatant before and after the immobilization step by the double agar layered conventional method for counting active phages (as explained in detail in the Supporting Information, Figure iv). After comparing the bacteriophage counting (PFU) before and after the immobilization, the coupling efficiency for 1.44×10^{11} PFU on 7×10^7 magnetic particle units was found to be 37%, with a ratio of 757 phage nanoparticles (PFU) immobilized in each magnetic particle. The results are comparable to those obtained by the Bradford method, considering that, in this last case, the starting amount of phage for immobilization (1.44×10^{11} PFU) was around 35% of the amount used for Bradford (4×10^{11} PFU, the saturating

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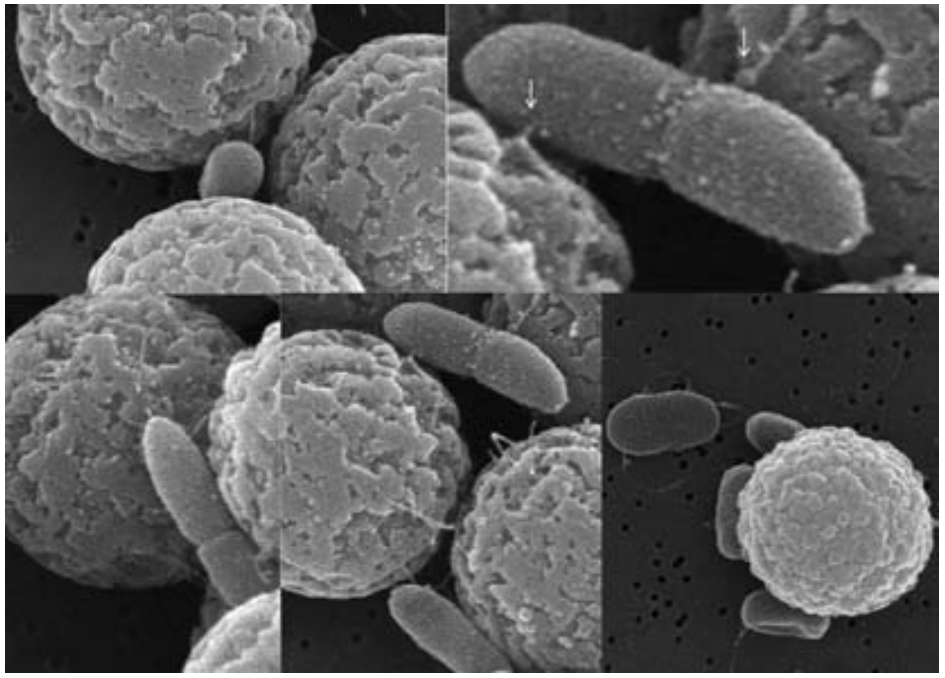

Figure 3. Evaluation of the PMS by SEM at a *Salmonella* concentration of 2.9×10^7 CFU mL^{-1}. The images show the *Salmonella* cells attached to the magnetic particles through the tail spikes. In all cases, identical acceleration voltage (15 kV) was used.

phage concentration), the immobilized phages on 7×10^7 MP being thus also approximately 35% (757 PFU per MP) of the saturated value (2000 PFU phage nanoparticles per magnetic particle) obtained by Bradford. The Bradford method showed thus good performance as a rapid alternative for the time-consuming microbiological methods in order to estimate the coupling efficiency of phage nanoparticles, not only on magnetic particles, but also in other supports. Finally, the optimum ratio to achieve the higher covering of P22 bacteriophages on 7×10^7 magnetic particles was found to be 4×10^{11} PFU mL^{-1}, reaching a coupling efficiency of around 25% with approximately 2000 PFU per MPs.

Evaluation of the Immobilized P22 Bacteriophage on Magnetic Particles by SEM and Conventional Culture Methods. Figure 2 shows the microscopic characterization by SEM of non-modified (Figure 2, parts A, B, and E) and modified (Figure 2, parts C, D, and F–H) magnetic particles with P22 phages nanoparticles. Figure 2 shows the spherical structures of P22 bacteriophages (~600–700 Å in diameter[19]) (Figure 2F–H) uniformly distributed on the surface of the magnetic particles (Figure 2, parts C and D).

Although the successful in the immobilization of P22 phage nanoparticles on magnetic particles was demonstrated by different methodologies (Bradford, phage counting on the supernatant by the double agar layered conventional method, and SEM), none of these methods can ensure the orientation of the tail spikes away from the solid support. This orientation was studied by the double agar layered method and enumeration of plaques by culturing the P22 phage-modified magnetic particles (P22-MPs). The quantification of the number of bacteriophages per magnetic particle is not possible by plating the P22-MPs conjugates, due to the fact that all the bacteriophages attached on the same magnetic particle will produce a unique plaque, as explained in Figure v (Supporting Information). However, the phage counting for the immobilization of 1.44×10^{11} PFU on 7×10^7 magnetic particle units was found to be 5.3×10^7, which demonstrated lytic activity in at least 75% of the magnetic particles and, as such, confirmation of the oriented immobilization of the phages on the magnetic particles.

Phagomagnetic Separation of *Salmonella*. Evaluation by SEM and Conventional Culture Methods. The microscopic characterization by SEM was also performed for the evaluation of the PMS, i.e., the bacteria attachment to the magnetic particles throughout the interaction between the tail spikes and the O-antigen polysaccharide receptor on the bacteria.[17] In this case, instead of active P22 phages, UV-inactivated P22 phage nanoparticles were used for the phagomagnetic separation to avoid the lytic cycle in order to keep the attached bacteria as a whole cell while being captured. The procedure for the PMS of the bacteria is schematically outlined in Figure 1A.

Figure 3 shows that the binding was achieved with more than one specific binding site of the bacteria to the magnetic particle. Single-point attachment of the bacteria to the magnetic particle was mostly observed. Moreover, a unique magnetic particle was able to attach more than one bacterium. Finally, some aggregates were observed due to the binding of two or more different magnetic particles by a unique bacterium cell.

Conventional culture method was also performed by growing the bacteria attached on magnetic particle for 18–24 h at 37 °C, as schematically outlined in Figure vi, part A (Supporting Information). Colony counting was clearly decreasing from 3.2×10^6 to 3.2×10^0 CFU mL^{-1}. The corresponding plates are also shown, displaying the characteristic colony features of *Salmonella* in LB media. However, an underestimation of the expected amount of bacteria was observed in all the concentration range. The counted colony number was found to be in all cases under 10% of the expected amount, perhaps due to the formation of the aggregates observed by SEM, formed by several bacterium cells but growing at a unique colony point in the agar plate or, for instance, due to infection

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of remaining active bacteriophages and, thus, under growing of the attached bacteria.

Phagomagnetic Separation, Double-Tagging PCR Amplification, and Electrochemical Magneto-Genosensing. The second step in the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is the double-tagging PCR for the amplification of the *Salmonella* spp. genome for the final genosensing detection.[20,27] The chosen set of primers amplified exclusively the *IS200* insertion sequence, producing only the expected 201 bp fragments, according to the agarose gel electrophoresis shown in Figure 4, for the

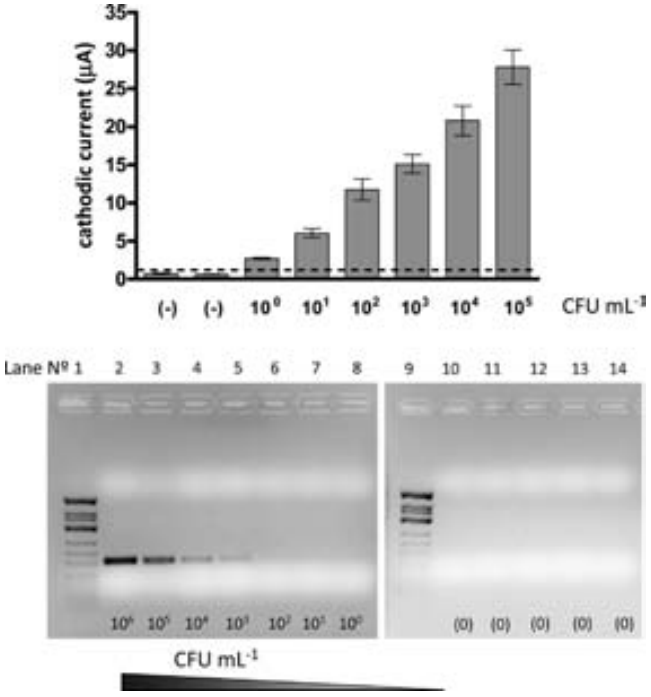

Figure 4. Top: Electrochemical signals for the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach. Gray bars show the signal by increasing the amount of *Salmonella* ranged from 3.2×10^0 to 3.2×10^5 CFU mL^{-1} artificially inoculated in LB broth. Two negative controls (0 CFU mL^{-1} and PCR negative control) are also shown, respectively. In all cases, $n = 4$, except for the 0 CFU mL^{-1} negative control ($n = 8$). Bottom: Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "PMS/double-tagging PCR/electrophoresis" approach. Lanes 2−8 are 10-fold dilutions that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1}. Lanes 10−13 are 0 CFU mL^{-1} negative controls, while lane 14 is the PCR negative control. Lanes 1 and 9 are the molecular weight marker (ΦX174-Hinf I genome).

concentration that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1} (lanes 2−8) in LB broth artificially inoculated with *Salmonella*. As shown in Figure 4, the limit of detection (LOD) for the PMS/double-tagging PCR/electrophoresis was found to be 3.2×10^3 CFU mL^{-1} (lane 5 in the gel electrophoresis). No bands were observed for the negative controls (0 CFU mL^{-1}) performed in LB broth (Figure 4, lanes 10−13). In order to increase the sensitivity of the assay, instead of the "PMS/double-tagging PCR/electrophoresis" approach, the proposed methodology is based on the "PMS/double-tagging PCR/m-GEC electrochemical genosensing", by replacing the electrophoresis detection for the electrochemical magneto-genosensing of the double-tagged amplicon[20,27] (Figure viii, expanded version of Figure 1C, Supporting Information). The amperometric response of the doubly labeled product was evaluated for artificially inoculated bacteria in LB (Figure 4). The amperometric signal corresponding to the LOD was estimated by processing the negative control samples of 0 CFU mL^{-1} in LB and performing three different single interday assays, using six magneto-electrode devices from different batches, obtaining a mean value of 0.75 μA with a standard deviation of 0.20 μA. The amperometric signal corresponding to the LOD value was then extracted with a one-tailed *t* test at a 99% confidence level, giving a value of 1.33 μA, respectively (shown in Figure 4 as the dotted horizontal line).

As shown in Figure 4, the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is able to give a clear positive signal (15.1 μA with a standard deviation of 2.08 μA) and a signal-to-background ratio value of 20 for 3.2×10^3 CFU mL^{-1}, while the electrophoresis for the same concentration shows a weak positive band (Figure 4, lane 5). On the other hand, as low as 3 CFU mL^{-1} was clearly detected with a total assay time of 4 h for the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach, with an amperometric signal of 2.7 μA, a standard deviation of 0.20 μA, and a signal-to-background ratio value of 3.6. Compared with other biosensing methodologies for detecting pathogenic bacteria (ref 27 and references therein, Table A, Supporting Information), excellent detection limits were achieved with this procedure. In addition, this method is more rapid and sensitive than other rapid antibody-based and nucleic acid-based PCR methods that have been previously reported (ref 27 and references therein, Table A, Supporting Information). Moreover, the procedure is able to detect at least 3 CFU mL^{-1} in 4 h without the use of any culturing pre-enrichment or selective plating enrichment steps, with higher sensitivity than PCR followed by electrophoresis or plating by conventional culture method. Other rapid approaches based on immunological recognition coupled with electrochemical impedance spectroscopy or fluorescence detection are able to detect the bacteria faster (ranging from 6 min to 2.5 h) but with significantly higher LODs (from 10^2 to 10^5 CFU mL^{-1}) (Table A, Supporting Information). Regarding other rapid approaches based on genetic recognition, most of them are demonstrated with synthetic oligonucleotides, and only few procedures are based on inoculated bacteria detection obtaining LODs ranged from 10 to 10^4 CFU mL^{-1} (Table A, Supporting Information). To the best of our knowledge, only detection techniques based on fluorescence are able to obtain similar features to the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach.

Comparing the procedure for the bacteriophage-based and the immunological magnetic separation[27] coupled with double-tagging PCR/m-GEC electrochemical genosensing, the PMS approach gave significantly lower background values for the negative control (0.75 vs 2.2 μA, respectively), better standard deviation values (0.2 $n = 8$ vs 0.65 $n = 35$), and thus lower amperometric signal corresponding to the LOD value (1.33 vs 3.78 μA) allowing better discrimination at lower concentration levels. It should be also pointed out that remarkably improved LOD was also achieved with the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach compared with LODs reported for other biosensing approaches using bacteriophages (Table A, Supporting Information). This fact can be ascribed to the combined used of the magnetic separation and the sensitivity of the amplicon detection with the m-GEC electrochemical genosensing strategy.

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Specificity Study of the "PMS/Double-Tagging PCR/m-GEC Electrochemical Genosensing" Approach. Figure 5A shows the results of the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach for LB artificially inoculated, in all cases, with 4×10^5 CFU mL^{-1} of *E. coli*, *Salmonella*, and both *E. coli* and *Salmonella*, as well as a negative control. Figure 5B shows the corresponding electrophoresis images of the double-tagged amplicon ("PMS/double-tagging PCR/electrophoresis" approach). As expected, the electrochemical signal obtained for *E. coli* is similar to the negative control signal, while the solution of both pathogens gave a similar signal to that of the sample spiked just with *Salmonella*. Similarly, no electrophoresis band was observed for *E. coli* (Figure 5B, lane 4), while the mix of both pathogens (Figure 5B, lane 2) and the *Salmonella* (Figure 5B, lane 5) gave a unique positive electrophoresis band producing only the expected 201 bp fragments, corresponding to the amplification of the *IS200* insertion sequence specific for *Salmonella*, as confirmed for the positive PCR control (lane 3). The same results were obtained by plating the bacteria attached to the magnetic particles in LB agar for 18−24 h at 37 °C. No growing was observed for *E. coli*, while typical colony features of *Salmonella* were observed for the mix of both pathogens as well as for just *Salmonella*. These results confirm that the specificity of the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is coming mainly from the PMS step, due to the P22 bacteriophage specific to *Salmonella* which coated the magnetic particles whose tail-spike proteins specifically recognize the repetitive O-antigen part present in the lipopolysaccharides (LPS) of *Salmonella* serotypes A, B, and D_1 outer membrane.[29] The selection of the *IS200* specific set of primers for *Salmonella* spp.[20,23,27] in the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach provides an additional source of specificity, coming from the double-tagging PCR. This fact could be especially useful in other applications when bacteriophages with low specificity are involved in the PMS step.

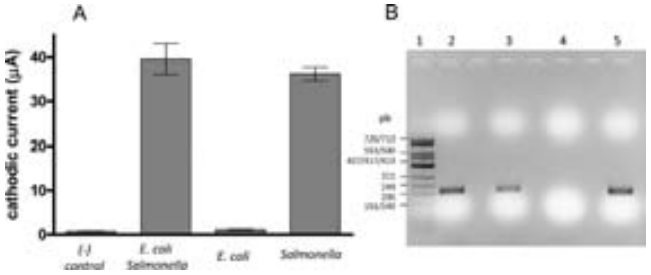

Figure 5. (A) Specificity study for the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach. Gray bars show the electrochemical signal for LB artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control, $n = 4$), 4.3×10^5 CFU mL^{-1} *E. coli* and *Salmonella* spp. ($n = 4$), 4.5×10^5 CFU mL^{-1} *E. coli* ($n = 4$), and 4.5×10^5 CFU mL^{-1} of *Salmonella* ($n = 5$). (B) Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "PMS/double-tagging PCR/electrophoresis" approach: lane 2, 4.3×10^5 CFU mL^{-1} *E. coli* and *Salmonella* spp.; lane 4, 4.5×10^5 CFU mL^{-1} *E. coli*; lane 5, 4.5×10^5 CFU mL^{-1} of *Salmonella*. Lane 1 is the molecular weight marker (ΦX174-Hinf I genome) while lane 3 is a PCR positive control.

CONCLUSIONS

A rapid and sensitive assay combining PMS, double-tagging PCR, and electrochemical magneto-genosensing of the double-tagged amplicon for *Salmonella* is presented. This is the first time that native, whole bacteriophages are used as a biorecognition element for magnetic separation and bacteria preconcentration. The main advantages of using phagomagnetic instead of the immunomagnetic separation rely on the use of the bacteriophages for biorecognition. Contrary to antibody generation, phages are animal-free, cost-efficiently produced by bacteria infection, taking only few hours. Another feature which makes them suitable as a biorecognition element is their outstanding stability. The specificity is mainly conferred by the P22 bacteriophage specific to serotypes A, B, and D_1 during the PMS, being thus an extremely useful tool to trace the source of outbreaks by phage typing. A phage cocktail can be employed by using the same strategy to increase the host range of this assay or for multiplexing the bacteria detection toward others food-borne pathogens, such as *Listeria* or *E. coli*.

This strategy is able to detect in 4 h as low as 3 CFU mL^{-1} of bacteria in LB media. As in the case of other rapid methods, such as PCR and immunological assays, the primary use of this approach is focused on screening out negative samples. As such, positive test results should be always considered presumptive and must be confirmed by an approved culture method. The high sensitivity of the approach conferred by the m-GEC electrochemical genosensing coupled with magnetic separation results in an extremely specific, rapid, robust, and sensitive procedure, all of them promising features for being implemented as a microfluidic system mainly for food industry applications.

Future work will focus on further validation of this assay in artificially inoculated as well as in naturally contaminated meats, poultry, dairy products, and environmental samples by assaying in parallel with standard plating techniques. Moreover, and in order to reach the LODs according to the legislation (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each in different points, Real Decreto 1679/1994, BOE 24-09-94), a pre-enrichment step of the sample in LB will be implemented. Other approaches based on PMS followed by electrochemical immunosensing, as well as the use of phage as tags to increase the sensitivity of the detection, are currently being studied.

ASSOCIATED CONTENT

Supporting Information

Figures i−viii, expanded version of Figure 1, parts B and C, and Table A. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Magneto-capillary valve for integrated purification and enrichment of nucleic acids and proteins

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Magneto-capillary valve for integrated purification and enrichment of nucleic acids and proteins†

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We describe the magneto-capillary valve (MCV) technology, a flexible approach for integrated biological sample preparation within the concept of stationary microfluidics. Rather than moving liquids in a microfluidic device, discrete units of liquid are present at fixed positions in the device and magnetic particles are actuated between the fluids. The MCV concept is characterized by the use of two planar surfaces at a capillary mutual distance, with specific features to confine the fluids by capillary forces, and the use of a gas or a phase-change material separating the stationary aqueous liquids. We have studied the physics of magneto-capillary valving by quantifying the magnetic force as a function of time and position, which reveals the balance of magnetic, capillary and frictional forces in the system. By purification experiments with a fluorescent tracer we have measured the amount of co-transported liquid, which is a key parameter for efficient purification. To demonstrate the versatility of the technology, several MCV device architectures were tested in a series of biological assays, showing the purification and enrichment of nucleic acids and proteins. Target recovery comparable to non-miniaturized commercial kits was observed for the extraction of DNA from human cells in buffer, using a device architecture with patterned air valves. Experiments using an enrichment module and patterned air valves demonstrate a 40-fold effective enrichment of DNA in buffer. DNA was also successfully purified from blood plasma using paraffin phase-change valves. Finally, the enrichment of a protein biomarker (prostate-specific antigen) using geometrical air valves resulted in a 7-fold increase of detection signal. The MCV technology is versatile, offers extensive freedom for the design of fully integrated systems, and is expected to be manufacturable in a cost-effective way. We conclude that the MCV technology can become an important enabling technology for point-of-care systems with *sample in–result out* performance.

Introduction

The integration and miniaturization of laboratory procedures into lab-on-chip devices is an important technological trend in *in vitro* diagnostics. The aim is to achieve a seamless use of diagnostics in the medical workflow by providing compact systems that can analyze patient samples at the point-of-care, close to the patient.[1] Ease-of-use is an important characteristic of point-of-care diagnostics, ideally with *sample in–result out* performance. An important problem is that raw biological samples are often not directly suitable for analysis and that elaborate multi-step sample preparation processes are required before actual analysis of the sample can take place.[2–4] Several detection technologies have been successfully miniaturized and integrated into lab-on-chip formats, but the integration of sample preparation has been more problematic. Sample preparation often still demands a substantial amount of additional manual handling by a trained operator and is an important bottleneck in the process from sample to result. Sample handling can be automated using pipetting robots, however, pipetting mechanisms are fundamentally difficult to integrate and miniaturize in a lab-on-chip format. One approach is to use pressure-driven microfluidic flows, but a disadvantage of pressure-driven microfluidics is that the external equipment required to operate a miniaturized lab-on-chip device is often large and complex. Such systems may be suited for decentralized laboratory settings, but for real point-of-care diagnostics novel solutions are necessary that miniaturize the total system and make it rapid, easy-to-use and cost-effective.

A novel approach in the search for less complicated methods for integrated sample preparation is the concept of stationary microfluidics.[5–12] Instead of moving liquids in a microfluidic device, discrete units of liquid are present at fixed

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positions in a device and magnetic particles are displaced between the liquids. Stationary microfluidics offers a great potential for integration and miniaturization, since no bulky external equipment is required to operate the miniaturized lab-on-chip device. The fluids are stationary and for the actuation of the magnetic particles a small movable permanent magnet is sufficient. The literature on the moving of magnetic particles between stationary fluids describes different approaches. Pipper *et al.*[5] have demonstrated an open device with droplets on a hydrophobic surface. Lehmann *et al.*[6] have used aqueous liquids immobilized on hydrophilic spots surrounded by a bulk volume of oil. Shikida *et al.*[7] have shown a device with aqueous droplets separated by a small constriction and surrounded by a bulk volume of oil. Berry *et al.*[8] have used the same concept and applied it in a miniaturized well plate format to eliminate the gravity dependence. Sur *et al.*[9] have used a layer of oil floating on top of the aqueous liquids. These systems have been applied for various biological sample preparation processes, such as the purification of nucleic acids from cells in buffer,[6,8] blood[5,9] or urine,[9] and also for an enzymatic immunoassay.[7]

A common element in the approaches listed above is that all use an oil phase between the aqueous phases. The oil has two functions: (i) it forms an immiscible barrier between the aqueous phases and (ii) the oil lowers the interfacial tension and makes it possible to extract the magnetic particles from the aqueous phases with a relatively small force. However, the presence of an oil phase has important drawbacks. A first drawback lies in the complications for system integration. The goal of a completely integrated system is to require only the insertion of the sample and no other fluids. Pipetting steps should be eliminated as much as possible. In the reported devices, the oil is inserted by an additional pipetting step and moreover, the sequence of sample pipetting *versus* oil pipetting steps is subtle and needs to be carefully obeyed to achieve successful operation. In the system of Lehmann the aqueous fluids need to be pipetted through the volume of oil, and in the systems of Sur and Berry the oil needs to be pipetted after the aqueous fluids have been applied. The oil application processes thus put constraints on the system and limit the number of suitable technical solutions, which is an important drawback from the perspective of full system integration. A second drawback of using an oil phase is that bio-assay incompatibilities arise. Some assay processes are compatible with oil, *e.g.* nucleic acid purification by the Boom method,[2] but other processes like the purification and enrichment of proteins can be strongly disturbed by the presence of an oil phase. Non-specific interactions are promoted at the aqueous/oil interface, the aqueous/oil interface can become unstable, and the interface may change the properties of the biomaterials. Surfactants may help to reduce such non-specific interactions, but surfactants have a strong influence on the physical properties of the aqueous/oil interface and thereby decrease the window of stable and reproducible operation. Recently, the first oil-free devices have been reported.[10,11] Bordelon *et al.*[10] used a one-dimensional tube with a millimeter-sized inner diameter. Disadvantages of this large-diameter tube concept are that the miniaturization possibilities are limited, and that the concept lacks the fabrication and scaling advantages of planar lab-on-a-chip technologies. Building on earlier developed open-surface droplet concepts,[5,6] Zhang *et al.*[11] have demonstrated a technology with surface elevations which improves the positional control of the droplets. However, a general disadvantage from an application perspective is that open droplet technologies require accurate pipetting steps and have risks of contamination and evaporation.

In this paper we describe the magneto-capillary valve (MCV) technology, a stationary microfluidic concept that allows enrichment and purification of nucleic acids and proteins without the need for an oil phase. The uniqueness of the MCV approach lies in the use of two planar surfaces at a capillary mutual distance, with specific features to confine the fluids by capillary forces, and the use of a gas or a phase-change material separating the stationary aqueous liquids. The designs intrinsically have high liquid confinement forces and low amounts of co-transported liquid upon transfer of particles through the magneto-capillary valve. Fig. 1A depicts how magnetic particles are transported from one stationary liquid to another by magnetic forces originating from a small movable permanent magnet. Since the operation of the device is based on the balance between magnetic and capillary forces, we have named it the magneto-capillary valve.[12] A number of device architectures are depicted in Fig. 1B. The first one is the patterned air valve, in which the aqueous solutions are confined in well-separated volumes by a pattern of hydrophilic and hydrophobic regions. The second is the geometrical air valve, in which the aqueous solutions are confined by sharp geometrical transitions. The third is the patterned paraffin valve, in which the aqueous solutions are separated by a plug of solid paraffin that can be briefly melted when magnetic particles need to cross the valve. Fig. 1B also shows the concept of an enrichment module, a device with fluid volumes of different sizes in order to allow enrichment of the sample. The enrichment module provides a practical and cost-effective way to accommodate a sample volume in the milliliter range combined with an elution volume in the microliter range.

The MCV technology provides a platform for solid phase extraction, which is a common type of sample preparation.[2,13,14] Analytes are coupled to magnetic particles in the sample matrix and are transported through one or more washing buffers to be finally eluted from the particles in a buffer that is appropriate for detection of the analyte. Intrinsic differences exist between sample preparation methods for nucleic acids, cells and proteins. Therefore, it is challenging to conceive a platform that suits such widely different purposes. In this paper we will demonstrate that the MCV technology is able to handle nucleic acids, cells and proteins. First, a physical characterization of the MCV technology is presented. We describe the behavior of the valve in a quasi-static model that balances magnetic forces, capillary forces and friction forces, we quantify the amount of co-transported liquid during

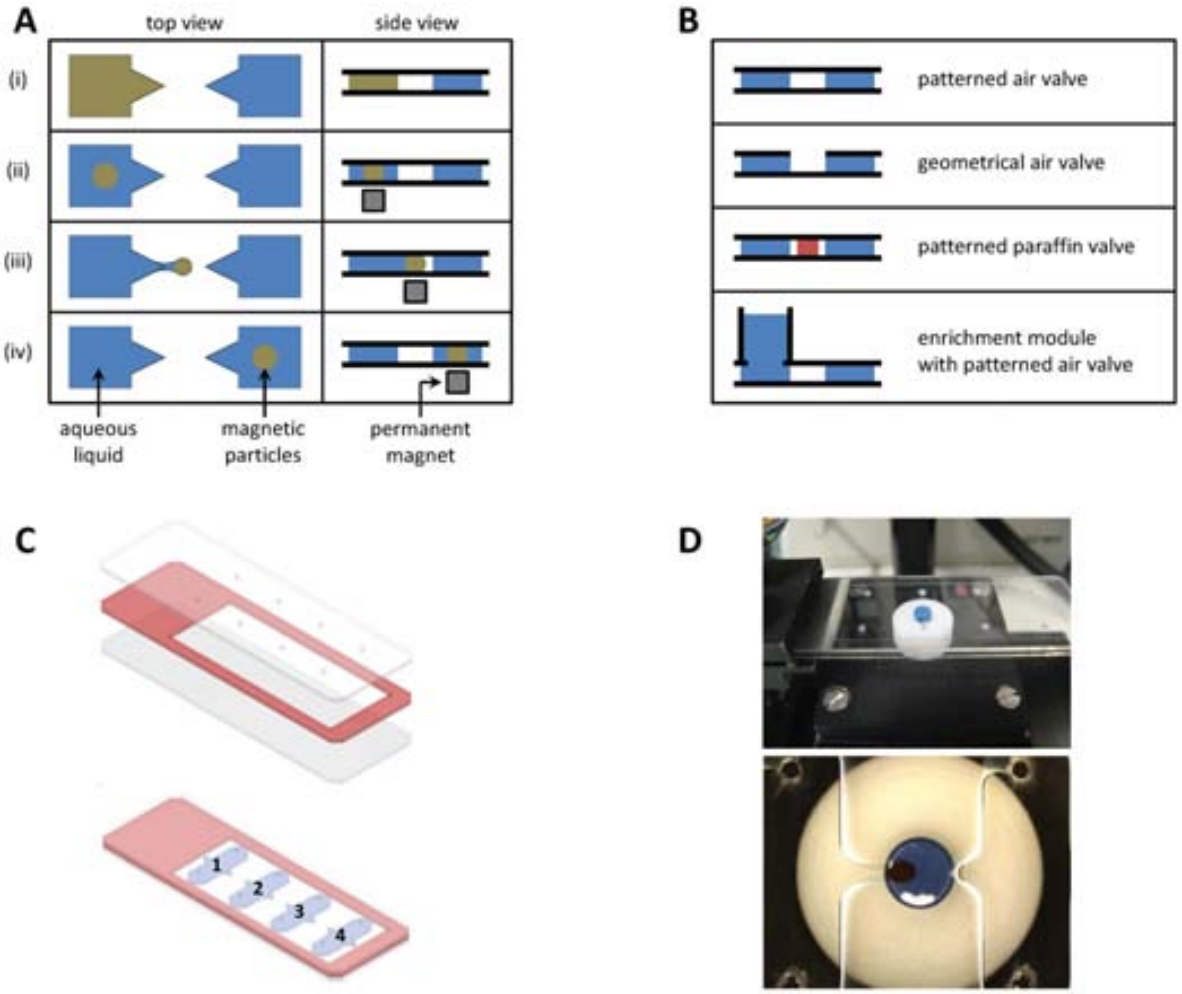

Fig. 1 Overview of the magneto-capillary valve technology. (A) Principle of the magneto-capillary valve: (i) magnetic particles are dispersed in the liquid in chamber 1. (ii) The particles are collected above the magnet in chamber 1 and transported towards the valve region. (iii) The cloud of particles is pulled into the valve region by deforming the meniscus. (iv) The particles arrive in chamber 2 and the magneto-capillary valve closes by capillary forces. (B) Schematic drawings of MCV device architectures: three distinct valve types and an enrichment module. (C) Schematic drawing of a patterned air valve cartridge in exploded view (top) and assembled view (bottom), showing double-sided tape (red) that joins the transparant planar top and bottom parts. Aqueous liquids (blue) with a typical volume of 15 μl are confined in four chambers by a pattern of hydrophilic and hydrophobic regions. (D) Top: photo of an MCV cartridge in the experimental setup, showing the permanent magnet (blue, 4 mm Ø) embedded in a white background, gently touching the bottom of the cartridge. Bottom: top view microscope image, showing two translucent aqueous chambers and the magnet in the valve region between the chambers. The magnet (blue) draws a cloud of magnetic particles (black/brown) from the left chamber to the right chamber.

the magnetic valving process, and we describe a number of parameters that define the window of operation for the device. Subsequently, we demonstrate a wide range of sample preparation processes, namely the extraction of DNA from cells in buffer, the enrichment of DNA in buffer, the purification of DNA from blood plasma and the enrichment of a biomarker protein (prostate-specific antigen, PSA).

Experimental methods

Cartridge fabrication

As depicted in Fig. 1C, the MCV cartridges studied in this paper consist of a planar bottom part, a planar top part, and a layer of double-sided adhesive tape that joins the two parts together. The top part is a standard microscope glass slide (25 × 75 mm^2) of 1 mm thickness, in which small filling holes have been fabricated by laser machining. The bottom part is a thin microscope glass slide of 0.5 mm thickness. The two glass slides are joined together by a double-sided adhesive tape with a thickness in the order of 100 μm, in order to form a planar capillary microfluidic device. The experiments were performed with MCV devices of various architectures, as illustrated in Fig. 1B. In all cases the bottom part is homogeneously hydrophobic and the top part has specific features. In the case of patterned air valves, the top part is patterned into hydrophilic and hydrophobic regions, which confines the liquid into separate chambers with a volume of typically 15 μl each. The double-sided adhesive tape is used in this case only

as a spacer and is not in direct contact with the liquids. The glass slides are rendered hydrophobic by depositing a self-assembled monolayer (SAM) of 'fluorosilane' (1*H*,1*H*,2*H*,2*H*-perfluorodecyldimethylchlorosilane 97%, ABCR) by chemical vapor deposition. The pattern of hydrophilic and hydrophobic regions is created by locally removing the SAM with an atmospheric oxygen plasma applied through a metal mask. In the case of geometrical air valves, the top part is homogeneously hydrophilic, and the liquids are confined by the double-sided adhesive tape and by holes in the top part that are located at the valve region. In the case of paraffin valves, the top part is patterned into hydrophilic and hydrophobic regions, and the liquid is confined by the double-sided adhesive tape and by a plug of paraffin wax (docosane, $C_{22}H_{46}$, T_m = 44.4 °C) that is located at the valve region. The paraffin is deposited into the valve region after joining the top and bottom parts together with the double-sided adhesive tape. The cartridge is heated to approximately 50 °C and melted paraffin is injected into the cartridge. By the design of the double-sided adhesive tape and the pattern of hydrophilic and hydrophobic regions, the paraffin fills exclusively the valve region. The bottom part of a paraffin cartridge is equipped with thin-film resistive heaters of Indium Tin Oxide (ITO) to be able to locally melt the paraffin.

The enrichment of target molecules requires a sample volume at the input that is significantly larger than the sample volume at the output. Since the typical volume for real-time PCR detection is in the order of 10 μl, an input volume in the order of a milliliter is required to realize a volumetric enrichment of 100 times. To keep the footprint of the MCV cartridge small, the large input volume is implemented by placing an extension module of acrylic glass on top of the cartridge. The height of the module is 10 mm, which results in a maximum input volume of 2.88 ml. With a typical elution volume of 15 μl in the MCV cartridges, this results in a volume ratio of 190 times.

Quantification of the magnetic force

The magnetic force that is applied to the cloud of particles is determined by three parameters: 1) the magnetic field of the permanent magnet, 2) the magnetic response of the superparamagnetic particles to the magnetic field, and 3) the position of the particles relative to the magnetic field. The magnetic flux density of the permanent magnet was calculated numerically (Comsol) using the parameters of the actual magnet used for the experiments: a neodynium iron boron cylinder of 4 mm diameter, 5 mm length and a remanent magnetization of 1200 mT. The magnetic response of the superparamagnetic particles was determined by measuring their magnetic susceptibility in a Vibrating Sample Magnetometer (VSM). The VSM data was fitted to a Langevin function and corrected for demagnetization effects that arise due to the large aspect ratio of the cloud of particles in an MCV cartridge. In every experiment the time evolution of the position of the particles relative to the magnet was determined by a series of top-view microscopic images (as in the bottom panel of Fig. 1D). An image processing algorithm (Matlab) processes the recorded movie and stores for each movie frame the position of the particle cloud in a Boolean matrix. Subsequently, the magnetic force that is applied to the cloud of particles is determined by numerically evaluating,

$$\vec{F} = \mu_0 \int_V \left(\vec{M}\cdot\vec{\nabla}\right)\vec{H}\,dV \quad (1)$$

in which $\vec{H}$ represents the magnetic field of the permanent magnet, $\vec{M}$ the magnetic response of the superparamagnetic particles to the magnetic field, and dV the position of the particles relative to the magnetic field. In this approach it is assumed that the number of particles in the cloud is known and constant during an experiment and that the particles are homogeneously dispersed in the cloud. Under these assumptions, the numerical evaluation yields the magnetic force that is applied to the cloud of particles as a function of time.

In the numerical evaluation, the magnetic force is decomposed into the *x*-, *y*- and *z*-component. The largest force is in the *z*-direction, which is directed perpendicular to the bottom surface of the cartridge. The force in the *y*-direction is nearly zero, since the movement of the magnet is linear in the *x*-direction. Most interesting is therefore the force in the *x*-direction, because this is the driving force of the cloud which is counteracted by capillary force and friction.

Buffers and particles

The following buffers[2,15] were used in the biological model assays: lysis/binding buffer LB1 (100 mM Tris-HCl, 5 M GuSCN, 100 mM Triton X-100, pH 7.6), lysis/binding buffer LB2 (100 mM Tris-HCl, 5 M GuSCN, pH 6.4), wash buffer WB (100 mM Glycine-HCl, pH 3.5), and elution buffer EB (10 mM Tris-HCl, pH 8.5).

The following superparamagnetic particles were used in the experiments: magnetic particles (Dynabeads M-270 Carboxylic Acid, Invitrogen) with a diameter of 2.8 μm at a concentration of 30 mg ml^{-1} (2×10^9 particles ml^{-1}) and magnetic silica particles (Dynabeads MyOne Silane, Invitrogen) with a diameter of 1 μm at a concentration of 40 mg ml^{-1}.

Measurement of co-transported liquid

Magnetic particles (5 μl of M-270 particles) were washed in water (Milli-Q purified). The particles were separated from their storage buffer using a magnetic rack, the supernatant was replaced by water, the solution was vortexed to mix well, and the same steps were repeated again once. Subsequently, 9 μl fluorescent dye solution (9.25 μM ATTO-532 stock) was added to the 5 μl of particles, resulting in 14 μl solution of 5.95 μM fluorescent dye containing 10^7 magnetic particles. The solution was injected into the first chamber of an MCV cartridge and the other chambers were filled with pure water. The particles were magnetically transferred from chamber 1 to chamber 4 at a velocity of 0.5 mm s^{-1}, with a 30 s linear mixing motion (50 mm s^{-1}) in each of the four chambers, all effectuated by an automatic stage. For each chamber, the concentration of the fluorescent dye was measured off-chip in a Raman Systems R-3000 spectrometer in fluorescence mode, using 532 nm laser excitation. By diluting the highest concentrations and by using a longer integration time for the lower concentrations, it was possible to measure over a range of five orders of magnitude, from 10 μM down to 1 nM.

Extraction of DNA from human cells in buffer

Human genomic DNA (gDNA) was purified from samples with THP-1 cells in MCV devices with patterned air valves. The THP-1 cells, which originate from a human acute monocytic leukemia cell line, were cultured in RPMI growth medium (RPMI 1640, Pen-Strep, Glutamax and fetal bovine serum) and transferred into phosphate buffered saline (PBS) buffer. The number of cells was determined by cell counting under a microscope using a Burker Turk counting chamber. A volume of 5.6 μl sample, containing on average 5.6, 56 or 560 cells, was incubated for 10 min in a tube with 3.4 μl of binding buffer LB2 and 5 μl of magnetic silica particles washed in LB2. THP-1 cells lyse easily in the LB2 buffer due to the high concentration of GuSCN, which at the same time inactivates nucleases to protect the DNA from degradation. During incubation of the lysis/binding step, the cartridge was filled with 14 μl wash buffer WB in chamber 2 and 14 μl elution buffer EB in chamber 3. After the lysis/binding step, the liquid with the DNA binding magnetic silica particles was injected into chamber 1 of the cartridge. The particles were magnetically transferred to WB and to EB. The elution in EB was enhanced by a magnetic mixing motion for 5 min. Finally, the particles were removed from the eluate by transporting them to chamber 4. The complete process was performed at room temperature within a total time of 16 min (10 min binding, 1 min magnetic particle transport and 5 min magnetically actuated elution). The eluate was pipetted out of the cartridge and a volume of 5 μl was analyzed by real-time PCR (polymerase chain reaction), using a commercial assay targeting RNase P (TaqMan RNase P detection reagents, Applied Biosystems). A reference assay was performed using the QIAamp Blood Mini kit (Qiagen). The QIA kit provides extraction of nucleic acids using a silica filter membrane in a standard Eppendorf tube (spin column). The instructions of the manufacturer were followed, which involves a sample volume of 200 μl (containing on average 5.6, 56 or 560 cells) incubated with 200 μl lysis/binding buffer and an elution volume of 100 μl, of which 5 μl was analyzed by real-time PCR.

Enrichment of DNA in buffer

Integrated enrichment of DNA was performed in MCV devices with patterned air valves and an enrichment module. 800 μl of Milli-Q water was spiked with plasmid DNA containing a *Staphylococcus aureus* gene fragment (SA pDNA) and incubated for 10 min in a tube with 1200 μl binding buffer LB2 and 5 μl of magnetic silica particles washed in LB2. The quantity of DNA was 10^4 or 10^5 copies per sample, corresponding to a sample concentration of 12.5 or 125 cps μl^{-1}, respectively. During incubation of the binding step, the cartridge was filled with 14 μl WB in chamber 2 and 14 μl EB in chamber 3. After the binding step, the 2 ml of liquid with the DNA binding magnetic silica particles were injected into chamber 1 of the cartridge and a large magnet (8 mm diameter, 10 mm length) was performing an automated motion under the cartridge for about 1 min to collect the particles at the bottom of the chamber. Subsequently, the magnet was exchanged for a smaller one (4 mm diameter, 5 mm length) and the particles were magnetically transferred to WB and to EB. In EB the elution was enhanced by a magnetic mixing motion of 5 min. Finally, the particles were removed from the eluate by transporting them into the hydrophobic valve region. The complete process was performed at room temperature within a total time of 17 min (10 min binding, 2 min magnetic particle transport and 5 min magnetically actuated elution). The eluate was pipetted out of the cartridge and analyzed by real-time PCR, using a Taqman[16] assay targeting the 442 gene.[17]

Purification of DNA from blood plasma

Plasmid DNA containing a *Staphylococcus aureus* gene fragment (SA pDNA) was purified from plasma samples in MCV devices with patterned paraffin valves. A volume of 3.4 μl binding buffer LB1 was spiked with SA pDNA and incubated for 10 min in a tube with the sample matrix (5.6 μl of defibrinated plasma) and 5 μl of magnetic silica particles washed in LB1. During incubation of the binding step, the cartridge was filled with 14 μl of LB2 in chamber 2 and 14 μl of EB in chamber 3. After the binding step, the liquid with the DNA binding magnetic silica particles was injected into chamber 1 of the cartridge. The particles were magnetically transferred to LB2 and to EB. Just before each transfer, the paraffin valve was heated just above the melting temperature (44 °C) by an integrated thin film heater. Right after each transfer, the heater was switched off and the paraffin resolidified, thus closing the valve. In EB the elution was enhanced by a magnetic mixing motion of 5 min. Finally, the particles were removed from the eluate by transporting them to chamber 4. The complete process was performed at room temperature within a total time of 16 min (10 min binding, 1 min magnetic particle transport and 5 min magnetically actuated elution). The eluate was pipetted out of the cartridge and analyzed by real-time PCR, using a Taqman[16] assay targeting the 442 gene.[17]

Protein enrichment in buffer

The protein enrichment assay was performed in MCV devices with geometrical air valves using an assay for prostate-specific antigen (PSA), a protein that is associated with prostate disorders. The details of this assay have been described previously by Sabatte *et al.*[18] 5 pM PSA was prepared in 1200 μl of 5% bovine serum albumin (BSA) in PBS. PSA was captured by magnetic particles coated with αPSA-10 *via* a DNA linker for 15 min in a tube. After the capture step, the particles were magnetically separated from the liquid, resuspended in 15 μl of 0.05% Triton X-100 in PBS, and injected into chamber 1 of an MCV cartridge with geometrical air valves. The particles were magnetically transferred to chamber 2, containing 15 μl of NEB EcoR1 buffer (New England Biolabs) with DNase, in which elution was performed by degrading the DNA linker between particle and antibody. The elution was enhanced by a magnetic mixing motion of 10 min. Finally, the particles were removed from the eluate by transporting them to chamber 3. The complete process was performed at room temperature within a total time of 26 min (15 min capture, 1 min magnetic particle transport and 10 min magnetically actuated elution). The eluate was pipetted out of the cartridge and analyzed directly in a sandwich immunoassay (IA) detection system

based on frustrated total internal reflection.[19] As a reference, the same enrichment assay was also performed in tubes.

Results and discussion

1. Physical characterization of the MCV technology

To understand the working principles of the MCV technology, we investigated the physical mechanisms that determine the behavior of the magneto-capillary valve by (1) measuring the magnetic force during a crossing of particles through the valve, (2) measuring the amount of co-transported liquid and (3) defining a window of operation.

1.1 Magnetic force profile during crossing. The transport of an ensemble of magnetic particles from one chamber to another can be described by a quasi-static model that balances magnetic, capillary and friction forces.[20] The magnetic force is the driving force of the ensemble, which is counteracted by capillary and friction forces. The capillary force is described by

$$F_{cap} = w\,\gamma_{lv}\,(\cos\,\theta_t + \cos\,\theta_b) + 2\,t\,\gamma_{lv} \qquad (2)$$

in which w is the lateral width of the displaced contact line, γ_{lv} is the surface tension of the liquid, θ_t and θ_b are the contact angles of the liquid at the top and bottom parts respectively, and t the capillary thickness, defined as the mutual distance between the top and bottom parts of the cartridge. In this equation, the first term accounts for the displacement of the contact line onto the hydrophobic valve region,[21] while the second term accounts for the increase in surface area of the water–air interface. With typical values, such as a width of 1 mm, a surface tension of 70 mN m^{-1}, contact angles of 120° and a capillary thickness of 100 μm, the capillary force is in the order of 100 μN.

Fig. 2 shows the magnetic force of an ensemble of 10^7 superparamagnetic particles of 2.8 μm diameter in an MCV cartridge. The cartridge has a capillary thickness of 100 μm and contains two chambers with aqueous fluid, separated by a 4 mm hydrophobic region. The ensemble of magnetic particles is concentrated into a dense cloud by a small neodynium permanent magnet, resulting in a cloud of particles with a diameter of about 1 to 1.5 mm. The particles were magnetically transferred at a velocity of 0.5 mm s^{-1} between the two chambers. By combining recorded images of the magnetic particle cloud with the measured susceptibility of the particles and the calculated magnetic field of the magnet, the magnetic force that is applied to the particles during the transfer process can be determined. The results are shown in Fig. 2.

The particle transfer process can be characterized by several phases. Phase A represents intra-chamber transport, where the cloud of particles moves within the liquid of a chamber. In phase B, the cloud is compressed against the meniscus, leading to a smaller cloud with a larger particle density. In phase C, the cloud moves into the hydrophobic valve region. In phase D, necking of the liquid thread occurs until pinch-off separates the dense cloud of particles from the bulk of the liquid in the chamber. In phase F, the cloud of particles is transported through the valve region towards the next chamber. In phase G, the cloud touches the liquid in the next chamber and the particles flow into the chamber, which concludes the crossing of particles through the valve.

The forces in these different phases are as follows. Before phase A, the system is at rest and the magnetic force equals the static surface friction. In phase A, the magnet is set into motion and the cloud accelerates to follow the movement of the magnet. The force of 19 μN thus represents the sum of surface and viscous friction. In phase B, the cloud is compressed against the meniscus in the protrusion. The magnetic force increases until it equals the sum of capillary

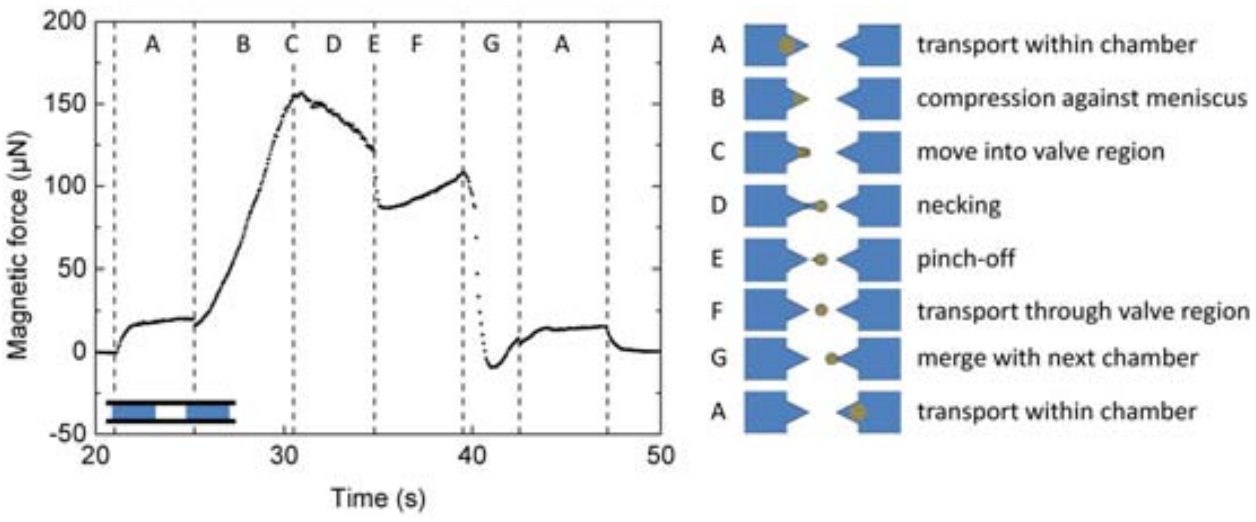

Fig. 2 Magnetic force profile during an MCV crossing. The force has been determined from video images recorded during the crossing of an ensemble of magnetic particles through a magneto-capillary valve (see Experimental methods for details). The force reflects the variations in capillary and frictional forces that the ensemble of particles experiences. The different phases of the crossing process (A to G) correspond to clearly distinguishable features in the magnetic force profile. The small discontinuities in the magnetic force profile between phase A and B and between phase G and A are due to an artifact in the image processing.

and frictional forces of 157 μN, marked by phase C. The cloud then moves into the hydrophobic region and almost immediately necking (phase D) starts to occur. Necking lowers the force to 121 μN at which point the liquid neck breaks and pinch-off occurs (phase E). In phase F, the cloud moves as a separate droplet through the hydrophobic region, which apparently requires a slightly increasing force as the droplet travels. At the transition between phase F and G, the droplet with particles first touches the hydrophilic region and shortly thereafter (at t = 40 s) the droplet touches the liquid in the next chamber. This pulls the droplet forward and lowers the magnetic force to −9 μN. When the cloud is moving into the liquid of the second chamber, the system is again in phase A, experiencing a friction force of 15 μN during the intra-chamber transport. At the end of phase A, the magnet stops moving and after deceleration of the cloud the system is at rest again.

1.2 Co-transported liquid. The core function of the magneto-capillary valve is to transport magnetic particles with a minimum of co-transported liquid from one chamber to another. We have quantified the MCV co-transported fluid volume in two independent ways, namely from fluorescence measurements and from the cloud density. In the fluorescence experiment, the concentration of a fluorescent tracer dye was monitored in a purification procedure. A solution with 10^7 magnetic particles and a high concentration of fluorescent dye was injected in the first chamber of a cartridge with a capillary thickness of 180 μm, while the other chambers were filled with pure water. The particles were magnetically transferred from chamber 1 to chamber 4 at a velocity of 0.5 mm s^{-1} having a 30 s magnetic mixing motion (50 mm s^{-1}) in each of the four chambers. Fig. 3 shows for each chamber the concentration of fluorescent dye, which was measured off-chip in a fluorescence spectrometer. As expected, the concentration in chamber 1 was equal to the initial dye concentration. On the other side of the device, the concentration in chamber 4 was so low that it could not be distinguished from the background fluorescence of water. A linear curve fit of the average concentrations in chamber 1, 2 and 3 results in a dilution factor of about 100 for each crossing of particles through a magneto-capillary valve. Given that the volume of each chamber is 14 μl, the experiment quantifies the MCV co-transported fluid volume to be 0.13 ± 0.03 μl.

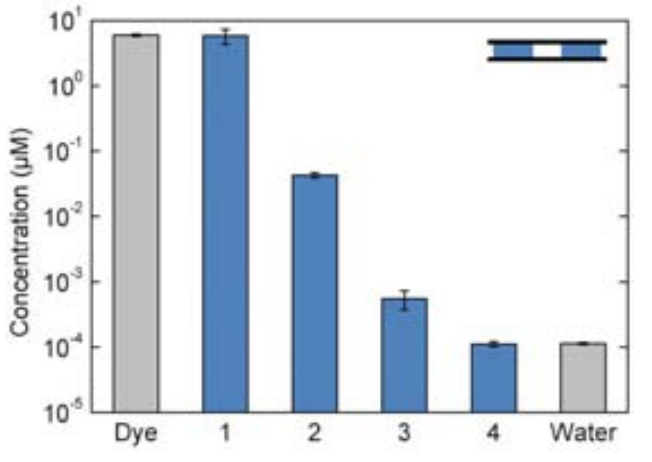

Fig. 3 Concentration of a fluorescent tracer dye in the four chambers of an MCV cartridge after a purification procedure. Each chamber contained a volume of 14 μl. The dye concentration decreases by two orders of magnitude for each crossing of magnetic particles through a magneto-capillary valve. The concentration in chamber 4 was not distinguishable from the background fluorescence of water. Each bar represents the average of three independent experiments. The error bars indicate the standard deviation.

In the cloud density method, the co-transported liquid volume was estimated from the recorded top view images. Using the observed cloud area, the known capillary thickness of the device, and the known total amount of magnetic particles in the cloud (*i.e.* the volume occupied by the particles), the volume of interstitial liquid can be determined. An average co-transported volume of 0.14 ± 0.01 μl was found, which corresponds very well to the results of the fluorescence measurements. The results demonstrate a very low amount of co-transported liquid, which is a key requirement for efficient purification.

The compression of the cloud, as described in phase B of Fig. 2, is an important parameter, since it determines how much interstitial liquid will be co-transported with the cloud. A typical cloud of particles that is magnetically confined, but not compressed (phase A) has a total volume of 0.89 μl, which consists of 0.12 μl solid particle material and 0.77 μl interstitial liquid. A compressed cloud (phase C) has a total volume of 0.27 μl, which means that typically only 0.15 μl of interstitial liquid is present. This points to the importance of having high capillary forces in the device. A high capillary force strongly confines the liquid and demands a high magnetic force for the particle cloud to exit from the fluid, which results in strong compression of the cloud and a low amount of co-transported liquid.

1.3 Window of operation. In section 1.1 the behavior of the valve has been investigated with a particle load of 10^7 particles and a capillary thickness of 100 μm. Variation in these parameters changes the behavior of the valve. Increasing the particle load, for example, increases the maximum magnetic force that can be applied to the cloud, while at the same time it also increases the diameter of the cloud. A minimum amount of magnetic particles is needed to be able to overcome the capillary forces, but if the particle load is too high not all magnetic particles will be transported across the magneto-capillary valve. Fig. 4 shows experimental results for the parameter space of particle load and capillary thickness. In this diagram, the center region indicates a window in which successful operation of the MCV is observed, *i.e.* the magnetic particles are successfully transported between the two chambers. The upper, lower and left boundaries indicate observed failure mechanisms. The boundary at the left side indicates the observed critical particle load that is required for successful crossing. The theoretically estimated critical particle load at which the magnetic force equals the capillary force is about 10^6 particles at a capillary thickness of 180 μm. In the experiments a somewhat higher value is observed, possibly caused by additional friction forces and/or inaccuracy in the quantification of the magnetic force. For a capillary thickness of 50 μm, successful crossings were incidentally also observed for particle loads below the observed critical particle load. This

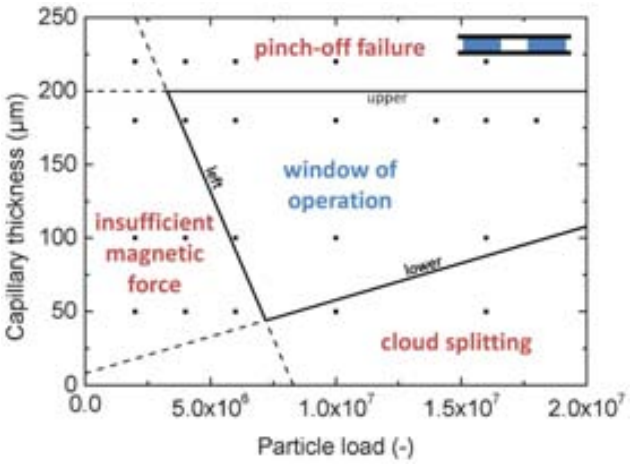

Fig. 4 Parameter space of capillary thickness and particle load. The squares indicate the experiments that were performed. The areas in the diagram describe the behavior of the magneto-capillary valve. In the central region successful MCV crossing is observed. In the three outer regions non-ideal operation is observed.

may be attributed to the larger cloud compression that was observed for a smaller capillary thickness in combination with variabilities in the surface tension of the device materials. To determine the critical particle load more precisely, more experiments should be carried out for combinations close to the boundary line. Overall, the standard operating conditions that were used in the biological assays (10^7 particles and a capillary thickness of 180 μm) are well within the region of successful crossing.

The lower boundary indicates the occurrence of cloud splitting, which means that only a part of the magnetic particle ensemble is pulled into the second fluid chamber. Cloud splitting occurs when the necking and pinch-off processes (phases D and E in Fig. 2) divide the magnetic particle cloud into two parts. Cloud splitting is observed for large cloud diameters in combination with strong pinch-off forces, which is the case for a large particle load in combination with a small capillary thickness.

The upper boundary indicates the limit for successful pinch-off, an important event in the process of crossing, which closes the valve after the cloud of particles has crossed the hydrophobic valve region. Pinch-off is driven by the capillary pressure difference between the hydrophobic valve region and the hydrophilic chamber. The capillary pressure originates from the curvature of the meniscus, so the distance t between the top and bottom part of the MCV device is a critical parameter. The capillary pressure difference that drives the pinch-off can be described by

$$\Delta p = -\gamma_{lv}\left(\frac{2\cos\theta_{phob}}{t} - \frac{\cos\theta_{phob} + \cos\theta_{phil}}{t}\right) \qquad (3)$$

with γ_{lv} the surface tension of the liquid, θ_{phob} and θ_{phil} the contact angles of the liquid at the hydrophobic and hydrophilic regions respectively, and t the capillary thickness. The first term describes the overpressure on the hydrophobic valve region where both top and bottom are hydrophobic, the second term describes the underpressure on the hydrophilic chamber region where the top part is hydrophilic and the bottom part hydrophobic. Indeed, the results in Fig. 4 show that successful pinch-off is determined by the capillary thickness and not by the particle load.

Since pinch-off is a very important process in the MCV, several other parameters that influence the processes of necking and pinch-off were investigated: (i) shape of the protrusion, (ii) distance between the chambers, and (iii) hydrostatic pressure in the chamber. The parameters of surface tension and surface energy were not varied.

Two different protrusion shapes were investigated in particular: a triangular protrusion with a sharp tip (as in Fig. 1 and Fig. 2) and a straight protrusion with a rounded tip. The rounded tip resulted in successful pinch-off for a capillary thickness of 100 μm or below, but not with 100% success rate. The sharp tip showed successful pinch-off in all cases for a capillary thickness of 180 μm, which has considerably enlarged the window of operation. From visual observations it appears that the triangular shape of the protrusion allows the contact line to flow smoothly along the boundary between the hydrophilic and hydrophobic region. In this way, the necking process is very reproducible and creates a pinch-off always at the same place, exactly at the sharp tip. An additional advantage of the triangular protrusion is that the compression of the cloud against the meniscus is focused, resulting in a smaller width of the cloud and thus minimizing the force required to cross. The focused compression is quite insensitive to the diameter of the cloud, which makes the design very robust for varying particle load.

When the distance between the chambers is too small, pinch-off occurs after the cloud has reached the other chamber. This means that a temporary fluid connection is established between the chambers, which may decrease the purification efficiency. It has been experimentally determined that for typical MCV operation (within the window of operation) a minimal distance of 2 mm is required between the chambers to ensure reliable and reproducible operation.

Finally, also the hydrostatic pressure in the chamber is of influence to the pinch-off process, since pinch-off is driven by the pressure difference between the valve region and the chamber. It has been observed that an underpressure in the chamber enhances the process of necking and pinch-off considerably (see Suplementary Information, Movie 3†). In our experiments, such underpressure was realized by slightly underfilling the hydrophilic chamber.

2. Purification and enrichment of DNA and proteins

To demonstrate the versatility of the MCV technology, four examples of biological sample preparation are presented using the various MCV device architectures as shown in Fig. 1B: (1) extraction of DNA from human cells in buffer using a device with patterned air valves, (2) enrichment of DNA in buffer using an enrichment module and patterned air valves, (3) purification of DNA from blood plasma using patterned paraffin valves, and (4) enrichment of a biomarker protein (prostate-specific antigen, PSA) in buffer using geometrical air

valves. The results are valued on the basis of target recovery, which covers the combined effect of purification efficiency (indicating the suppression of inhibitors) and yield (accounting for capture and elution efficiency and other losses that may occur in the process).

2.1 Extraction of DNA from human cells in buffer. The extraction of DNA from cells is important for infectious disease diagnostics (*e.g.* detection of bacterial DNA from a nasal swab) and cancer diagnostics (*e.g.* circulating tumor cell analysis). As a model system we have used monocyte-like cells from which we extracted DNA using the following process steps (see Experimental methods for details). The cells are lysed in a tube and DNA is captured onto magnetic silica particles. Subsequently, the lysate is injected into an MCV cartridge, in which the particles are transferred to an elution buffer. Finally, the eluate is analyzed by real-time PCR. Important challenges are to efficiently capture and elute the DNA even for low numbers of input cells, and to avoid contamination of the elution buffer by the lysis buffer components that inhibit the PCR reaction.

Fig. 5 shows the real-time PCR results after DNA extraction from three different quantities of THP-1 cells. The cycle threshold (C_t) values are shown for extraction in the MCV cartridge and for extraction by the QIAamp Blood Mini kit (see Experimental methods for details). The elution volume is 14 μl in the MCV cartridge and 100 μl in the QIA kit. In both cases a 5 μl aliquot of the eluate was analyzed in the PCR reaction. We observe that the C_t values from the MCV purification ('MCV raw') are much smaller than the C_t values from the QIA kit ('QIA raw'). This is mainly caused by the much smaller elution volume in the MCV cartridge. C_t values have also been calculated for the total target quantities in the full elution volumes, *i.e.* respectively shifted by a factor of $\log_2(14/5)$ for 'MCV corr' data and a factor of $\log_2(100/5)$ for 'QIA corr' data. The resulting C_t values are very similar for the two methods, showing that the overall process (*i.e.* the binding and elution of DNA and the suppression of PCR inhibitors) is very similar for both methods. The line represents a linear fit through the MCV data points, showing a reproducible target recovery. Finally, the results show that low numbers of cells can be detected with both purification methods. One should however be careful in interpreting the absolute cell numbers, because some free DNA from accidentally lysed cells (not detected in the cell counting procedure) may have contributed to the real-time PCR signal.

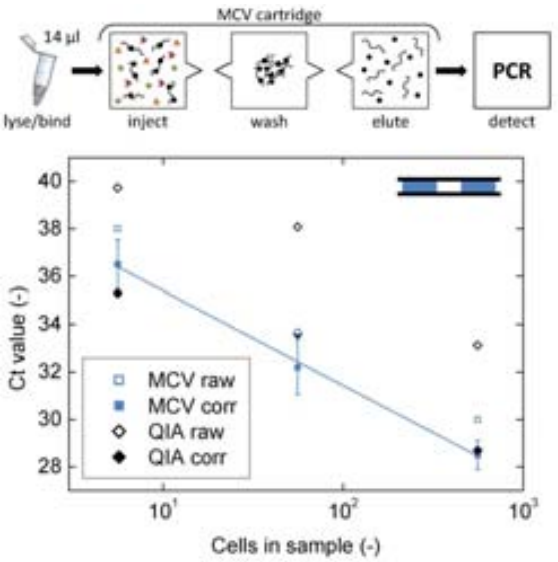

Fig. 5 DNA extraction assay. The top panel shows the assay format, the bottom panel shows the real-time PCR results for the extraction of genomic DNA from THP-1 cells in buffer. The cycle threshold (C_t) value is shown as a function of the average number of input cells. Extraction was performed by MCV cartridge (MCV) or by QIAamp Blood Mini kit (QIA). The open symbols represent the raw C_t data, the closed symbols represent the C_t values after correction for the different volumes of the eluates. The line represents a linear fit through the 'MCV corr' data. For the MCV purifications, each data point represents the average of three independent experiments. The error bars indicate the standard deviation.

2.2 Enrichment of DNA in buffer. An attractive way to increase the sensitivity of a molecular test is by including enrichment in the nucleic-acid purification procedure, *i.e.* by starting the purification procedure with a large sample volume so that more DNA material can be transferred into the eluate. The challenge of an enrichment procedure is to achieve a high DNA capture efficiency in the large sample volume, while realizing a high elution efficiency as well. Furthermore, it is challenging to achieve sufficient purification efficiency, because a large starting volume also implies that more PCR inhibiting material can be transferred into the eluate.

DNA enrichment was performed in MCV devices with patterned air valves and an enrichment module. To enable enrichment, the capture volume needs to be significantly larger than the elution volume. Since the cartridge is a planar capillary device, a much larger volume would require a much larger footprint. Therefore, the enlarged capture volume is extended perpendicular to the surface of the substrates, as illustrated in Fig. 1B. The height of the extension is limited due to the rapid non-linear decrease of the magnetic force over the distance between magnet and particles. The maximum volume is therefore a trade-off between the footprint of the capture chamber and the waiting time before all particles are collected at the bottom of the capture chamber. The height of the module was chosen 10 mm, which results in a maximum capture volume of 2.88 ml. With a typical elution volume of 15 μl, a volumetric enrichment of 190 times can be obtained. Although the enrichment module is combined with patterned air valves in this particular experiment, the enrichment concept can be used in combination with any type of magneto-capillary valve.

Fig. 6 shows the PCR results of the enriched purifications compared to the PCR results of 5 μl aliquots that were taken directly from the 800 μl samples. The C_t values of the direct aliquots are high due to the low DNA concentrations, and as expected the C_t values in the enriched samples are much lower. The volumetric enrichment factor in the MCV device is 57, since the volume is reduced from a sample volume of 800 μl to an elution volume of 14 μl. The total starting volume, however, is 2 ml due to the 1200 μl of lysis/binding buffer that is added to the sample to achieve reliable DNA capture. In the best case, target recovery is 100% and the DNA concentration

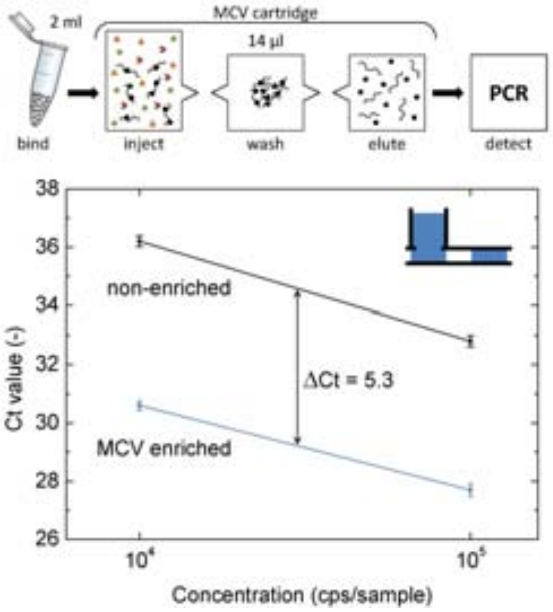

Fig. 6 DNA enrichment assay. The top panel shows the assay format, the bottom panel shows the real-time PCR results for the enrichment of two concentrations of plasmid DNA in buffer. The cycle threshold (C_t) value is shown as a function of the DNA concentration. Data marked 'MCV enriched' refers to MCV cartridges with an enrichment module (2 ml input volume) and patterned air valves. Data marked 'non-enriched' represents PCR results of 5 µl aliquots taken directly from the input sample. Each data point represents the average of three independent experiments. The error bars indicate the standard deviation.

in the eluate would be 57 times higher than the DNA concentration in the 800 µl sample. In theory, the enrichment would therefore result in a ΔC_t of 5.8 compared to the direct aliquots. For the enriched purifications, an average ΔC_t of 5.3 was found, indicating a target recovery of about $2^{-0.5} = 70\%$. As a result, the effective enrichment factor is 40. Table 1 summarizes the performance of the integrated enrichment in terms of ΔC_t and enrichment factor.

The recovery of 70% indicates that the binding step is not negatively influenced by the large volume in which the binding takes place. Since the amount of particles is the same as used for binding in small volumes, we conclude that the capture and binding process is very efficient and apparently not limited by diffusion.

2.3 Purification of DNA from blood plasma. Blood plasma is a sample matrix that contains a wide variety of proteins in high concentrations, of which some are strong PCR inhibitors. In this experiment we demonstrate the purification of DNA molecules from plasma, showing that the MCV can separate DNA from the multitude of other biomolecules that are present in the sample matrix. Examples of clinically relevant tests on plasma are numerous and include for example infectious diseases such as hepatitis and HIV. In MCV devices with patterned air valves the handling of blood plasma is not very robust, because the plasma constituents can adsorb on the pattern of hydrophilic and hydrophobic regions and thereby compromise the valving mechanism. We have therefore used MCV devices with paraffin valves (see Fig. 1B) for the purification of DNA from plasma. Paraffin is an attractive valve medium because it is highly stable in the device and can be reversibly changed from a solid to a liquid at temperatures that are easy to control and that are not harmful to biological material. We have selected a paraffin that is solid at room temperature and which can be rapidly melted at 44 °C to allow the passage of magnetic particles. In our experimental setup the heating was realized by thin-film resistive heaters (ITO) that were integrated in the MCV cartridge. Fig. 7 shows the PCR results of purification with three concentrations of DNA. The results show a recovery between 35 and 70% for concentrations ranging from 10^4 to 10^2 copies of DNA per sample. The results show that the analytical performance of DNA purification in MCV cartridges is on a par with non-miniaturized commercial solutions, but now in a microtechnology format dedicated for miniaturization and integration.

2.4 Enrichment of a protein biomaker in buffer. The enrichment of proteins is more subtle than the enrichment

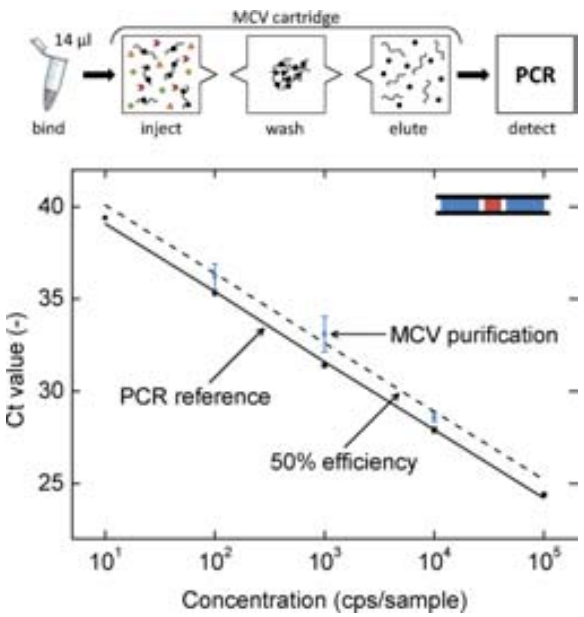

Fig. 7 DNA purification assay. The top panel shows the assay format, the bottom panel shows the real-time PCR results for the purification of different concentrations of plasmid DNA from plasma samples in MCV cartridges with patterned paraffin valves. The cycle threshold (C_t) value is shown as a function of the DNA concentration. The solid line is a linear curve fit to the reference samples for the PCR. The dashed line indicates a recovery of 50%. Each data point represents the average of three independent experiments. The error bars indicate the standard deviation.

Table 1 Analysis of integrated enrichment of DNA in buffer using MCV cartridges with patterned air valves. Data are taken from Fig. 6

		ΔC_t	Enrichment factor
No enrichment	(direct)	0.0	$2^{0.0} = 1$
Volumetric enrichment	(theory)	−5.8	$2^{5.8} = 57$
Target recovery		+0.5	$2^{-0.5} = 0.7$
Effective enrichment	(enriched)	−5.3	$2^{5.3} = 40$

of nucleic acids, since the subsequent method of detection is intrinsically different. Nucleic acids are biologically amplified (in a PCR the DNA template is duplicated many times) while protein detection occurs without target amplification and relies on specific recognition of the 3D structure of the protein. Keeping the protein intact is a major challenge for an enrichment assay, since the conformation of the protein is very sensitive to pH, temperature, buffer composition, *etc.* An assay for the enrichment of proteins has been recently described by Sabatte *et al.*[18] The assay format consists of three steps: 1) capture, 2) elution and 3) immunoassay detection. In the first step, the target proteins are captured by antibody-coated magnetic particles. In the second step, the target proteins are eluted from the particles by cleaving the bond between particle and antibody. In the third step, the complex of target protein and cleaved antibody is detected in a sandwich immunoassay.[19]

The main challenge of integrating the protein enrichment assay into an MCV device was reliable actuation of antibody-coated particles in the cartridge. In various experiments, antibody-coated particles were observed to stick to the surface of the device. It appeared to be important to add a detergent to the solution in order to avoid non-specific sticking of the particles. By using an MCV device with geometrical air valves, the valve operation was not negatively influenced by the reagents used in the protein enrichment assay. In Fig. 8 the result of the protein enrichment assay performed in MCV cartridges is compared to the result of the same enrichment assay performed manually in tubes, showing a comparable performance. Moreover, the results of the enriched samples are compared to direct immunoassay detection of a non-enriched sample of 5 pM PSA, showing that the enrichment assay provides a 7-fold increase in signal. The immunoassay detection of a blank sample (a sample without PSA) gave for all three methods an optical signal of about 1%. The results demonstrate that proteins can be successfully enriched using MCV devices, leading to a significant increase of signal in sandwich immunoassay detection.

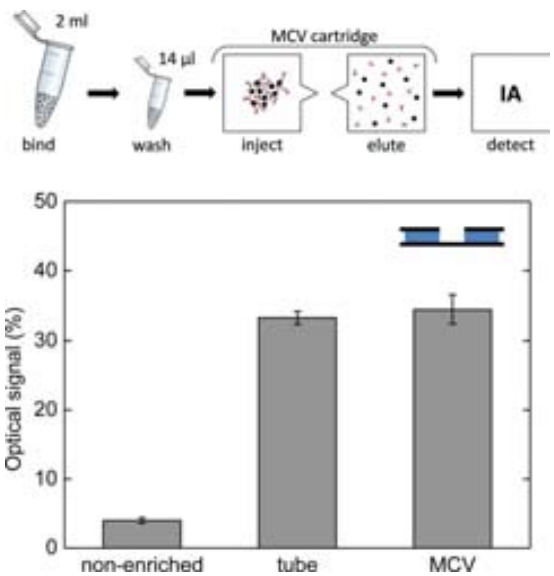

Fig. 8 Protein enrichment assay. The top panel shows the assay format, the bottom panel shows the sandwich immunoassay (IA) data measured after the enrichment of a protein biomarker (PSA) in buffer. The 5 pM PSA samples were either enriched in MCV cartridges with geometrical air valves (MCV), or enriched manually in tubes (tube) or not enriched (non-enriched). Each bar represents the average of three independent experiments. The error bars indicate the standard deviation.

Conclusions

We have presented the magneto-capillary valve (MCV) technology, a novel microfluidic concept for enrichment and purification of nucleic acids and proteins. The technology is based on stationary microfluidics, *i.e.* discrete units of aqueous liquid are present at fixed positions in a microfluidic device and magnetic particles are actuated between the fluids. The uniqueness of the MCV approach lies in the use of two planar surfaces at a capillary mutual distance, with specific features to confine the fluids by capillary forces, and the use of a gas or a phase-change material to separate the stationary aqueous liquids (see Fig. 1). We have demonstrated MCV devices with patterned air valves, paraffin phase-change valves and geometrical air valves. The physics of magneto-capillary valving was investigated by quantifying the magnetic force as a function of time and position from video recordings. The data reveals the magneto-capillary force balance and provides a detailed understanding of the magnetic, capillary and frictional forces that determine the behavior of the system. The results show a large window in which the MCV can be operated successfully, thus providing ample freedom in system design.

A key requirement for efficient purification is a low amount of co-transported liquid. The amount of co-transported liquid was experimentally determined in a device architecture with patterned air valves. An average co-transported volume of 0.14 µl per magnetic transfer was found, which indicates the high potential for efficient purification.

We have demonstrated the wide applicability of the MCV technology in four biological model assays, showing purification and enrichment of nucleic acids and proteins from various sample types. The extraction of DNA from human cells in buffer was demonstrated in MCV devices with patterned air valves. Good target recovery was observed, comparable to the performance of a commercial kit (Qiagen). Enrichment of DNA in buffer using MCV devices with patterned air valves and an enrichment module resulted in a 40-fold effective enrichment, corresponding to a 40 times increase in sensitivity. Successful purification of DNA from blood plasma was demonstrated in MCV devices with patterned paraffin valves. With a target recovery ranging from 35 to 70%, the performance is on a par with commercial solutions, but now in a microtechnology format dedicated for miniaturization and integration. Finally, successful enrichment of a protein biomarker (PSA) was demonstrated using MCV devices with geometrical air valves. A 7-fold increase of detection signal was observed as a result of the enrichment procedure. We can therefore conclude that the MCV technology is very versatile, allowing purification and

enrichment of nucleic acids, proteins and potentially also cells. With its ample freedom in system design, which is essential for further system development, the MCV technology is a valuable building block for integrated point-of-care devices.

Earlier publications on stationary microfluidics have shown efficient purification in various model assays, varying in complexity, analyte and sample matrix.[5–11] However, none of the reports was based on a co-planar capillary device technology, and nearly all used liquid oil as a medium to separate the different stationary fluids.[5–9] In our work we have focused on a concept suited for miniaturization, integration and industrial manufacturing. This has resulted in the MCV technology which is based on the use of two planar surfaces at a capillary mutual distance, with specific features to confine the fluids by capillary forces, and the use of a gas or a robust phase-change material to separate the stationary aqueous liquids in the device. The designs intrinsically have high liquid confinement forces and low amounts of co-transported liquid upon transfer of particles through the magneto-capillary valve.

The future perspective of the MCV technology involves scientific, engineering and integration topics. An interesting scientific topic concerns the structure and fluid mechanics of the cloud of magnetic particles. In this study, we have considered the cloud of particles as a magnetizable body without internal structure. However, in reality the ensemble of magnetized particles contains strings of particles aligned with the magnetic field lines. The magnetic displacement of the particle cloud thus creates flow patterns in which strings are broken and reformed. A detailed study of the magneto-hydrodynamics inside the cloud might provide new insights into phenomena such as viscous friction, surface friction, cloud stability and cloud relaxation. This will also help to understand the pinch-off process and the resulting efficiency of particle transfer for different valve parameters. An interesting engineering topic is to evaluate different base materials and valve designs. The current MCV devices were fabricated from glass slides. It is interesting to also study injection molded plastic parts and the effect of various coatings on particle-surface interactions. In this paper we have reported the patterned air valve, the paraffin phase-change valve, and the geometrical air valve. The paraffin valve has the advantage of a robust solid structure, but disadvantages are that the pinch-off is less strong due to the reduced interfacial tension, and that biomaterials may interact with the paraffin in liquid state. The device architecture with geometrical air valves is very attractive due to its low complexity and good pinch-off properties, so it merits further device and assay studies. Finally, the development of an integrated point-of-care instrument requires a clear application focus.[22] It will be particularly interesting to further develop the technology with focus on applications that will benefit from the MCV enrichment function and thereby enable rapid and highly sensitive bio-assays that are fully integrated.

In summary, the MCV technology adds a new and promising building block to the library of lab-on-a-chip technologies. It offers ample freedom in design, is versatile in terms of biological application, and is expected to be manufacturable in a cost-effective way. We therefore conclude that the MCV technology has the potential to become an important enabling technology for point-of-care devices with *sample in–result out* performance.

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"Uno no es lo que es por lo que escribe sino por lo que ha leído"

Jorge Luis Borges