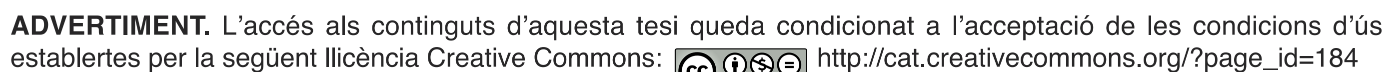
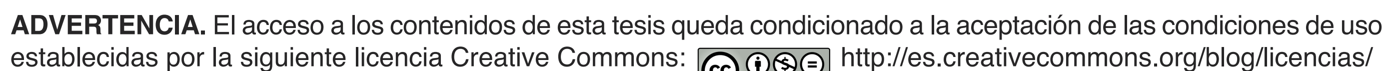
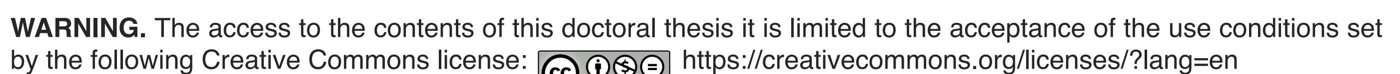
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Simultaneous detection of foodborne bacteria based on magnetic particles

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Doctoral Thesis

Doctoral Studies in Chemistry

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List of abbreviations and symbols

ʎ	Wavelenght
µA	Microamper
Ab	Antibody
AntiDig-HRP	Anti-digoxigenin antibody HRP (labelled)
AntiFlu-HRP	Anti-fluorescein antibody HRP (labeled)
AU	Arbitrary unit
Av	Avidin
BHI	Brain heart infusion broth
Bio	Biotin
bp	Base pair
BSA	Bovine serum albumin
Carboxyl-MNP	Carboxyl magnetic nanoparticles
cDNA	Complementary DNA
CFU	Colony-forming unit
CV	Cyclic voltammetry
Dig	Digoxigenin
DPV	Differential Pulse Voltammetry

DNA	Deoxyribonucleic acid
dsDNA	Double-stranded Deoxyribonucleic acid
E	Potential
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding (Fab) region
Fc	Fragment crystalizable
FITC	Fluorescein isothiocyanate
Flu	Fluorescein
GEC	Graphite-epoxy composite
HACCP	Hazard analysis and critical control points
HRP	Horseradish peroxidase enzyme
i	Intensity current
IAs	Immunoassays
IMS	Immunomagnetic separation
LB	Luria-Bertani broth
LFA	Lateral flow assay
LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharides
mV	Milivolt
m-GEC	Magneto Graphite-epoxy composite
MP	Magnetic particle
mPCR	End-point multiplex PCR

MIPs	Molecularly imprinted polymers
MMPs	Magnetic Microparticles
MNPs	Magnetic nanoparticles
n	Number of replicates
NPs	Nanoparticles
OD	Optical density
PCR	Polymerase chain reaction
QDs	Quantum dots
qPCR	quantitative PCR
rpm	Revolutions per minute
RSD	Relative standard deviation
RT	Room temperature
SEM	Scanning electron microscopy
ssDNA	Single-stranded Deoxyribonucleic acid
Strep-Cy5	Streptavidin Cy5 labelled
Strep-HRP	Streptavidin HRP labelled
Ta	Temperature of annealing
TMB	3,3',5,5'-tetramethylbiphenyl-4,4'-diamine
Tosyl-MP	Tosyl microparticles

Summary

Nowadays, it is widely recognised in Europe that research and innovation are key factors to reinforce the industrial capacities and business perspectives.

We need technology to address world´s problems, but we also need research to develop innovative technologies. Therefore, investing in research and innovation is essential to develop solutions for societal challenges, as for instance Food Safety. In this context, it comes out the term KETs (Key Enabling Technologies) to unify different fields across science, such as Nanotechnologies, Advanced materials, Biotechnology, among others. This indicates a clear convergence of technologies to address new solutions. For instance, analytical chemistry research is not only based anymore on the development of strategies to obtain qualitative and quantitative information about the composition and nature of substances. In order to provide solutions in food safety, analytical chemistry research has been converging into a more applied and multidisciplinary research field, forming alliances between different fields across science. This opens the possibility for the creation of new analytical principles, automated or in-situ detection procedures, as well as specific detection probes or new sensing devices.

This Dissertation is a result of the multidisciplinary character of analytical chemistry. The aim of providing solutions to problems related to food safety bonds different science fields as analytical chemistry, biotechnology and advanced materials for the development of a new sensing device.

Therefore, it is intended to give a general introduction about food safety and its importance worldwide, with special focus on the emerging foodborne pathogens responsible for the main outbreaks and the contribution of biosensors technology as the driver factor for the development

of new methodologies for foodborne bacteria detection with multiplexing capabilities.

Furthermore, the integration of new materials with nano/micrometer dimensions on electrochemical biosensors will be also discussed, highlighting some advantages of the use of magnetic particles: i) preconcentration of the bacteria from complex samples through an immunological reaction, ii) as a platform for the biorecognition element in the biosensing devices iii) as a support for the magnetic immobilisation on the surface of a working electrode under magneto-actuation.

The current state of art for detection methods for food safety shows a significant progress relative to the development rapid and sensitive methods, in which the implementation of bioassays with multiplexing capabilities is one of the emergent trends. However, few approaches based on electrochemical biosensors for the simultaneous detection of foodborne bacteria have been reported. For this reason, it is proposed to develop an electrochemical biosensor for the simultaneous detection of *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli*, based on the use of magnetic particles.

The strategies presented in this Dissertation are based on electrochemical magneto-immuno and genosensing, in which electrochemical magneto-immunosensing provides the detection of whole bacterial cells, whereas the electrochemical magneto-genosensing provides the detection of the bacterial DNA. These two strategies are combined with an immunomagnetic separation step to capture and preconcentrate bacteria from food samples. Hence, a study of different magnetic particles with micro and nanometer sizes will be achieved for the immunomagnetic separation of *S. enterica*, *L. monocytogenes* and *E. coli.* Afterwards, electrochemical magneto-immuno and genosensing will be compared for the detection of *Salmonella* in milk, as a model.

Finally, triple-tagging multiplex PCR combined with an electrochemical magneto-genosensor using silica magnetic particles as a platform will be reported for the simultaneous detection of *S. enterica*, *L. monocytogenes* and *E. coli*.

Resumen

En la actualidad, está ampliamente aceptado en Europa que la investigación y la innovación son factores clave para reforzar la capacidad industrial y las perspectivas de negocios.

La tecnología es necesaria para abordar los problemas del mundo, pero también lo es la investigación, que permite la aparición de tecnologías innovadoras. Por lo tanto, invertir en investigación e innovación es esencial para el desarrollo de soluciones para los desafíos sociales, como por ejemplo la seguridad alimentaria. En este contexto, surge el término KETS (Key Enabling Technologies), que permite unificar diferentes campos de la ciencia, tales como la nanotecnología, los materiales avanzados y la biotecnología, entre otros. Esto indica una clara convergencia de la tecnología en el desarrollo de nuevas soluciones. Por ejemplo, con el fin de ofrecer soluciones en seguridad alimentaria, la investigación en química analítica no está solamente basada en el desarrollo de estrategias para obtener información cualitativa y cuantitativa sobre la composición y naturaleza de las sustancias, sino que ésta ha convergido en un campo de investigación más aplicado y multidisciplinar, formando alianzas entre diferentes áreas de conocimiento a través de la ciencia. Esta nueva concepción abre la posibilidad de crear nuevos principios analíticos, procedimientos de detección automatizados o *in-situ*, así como sondas de detección específica o nuevos dispositivos sensores.

La presente Tesis doctoral es el resultado de dicho carácter multidisciplinar de la química analítica, y con el objetivo de proporcionar soluciones a problemas relacionados con la seguridad alimentaria, se ha desarrollado un nuevo dispositivo sensor en base a conocimientos de química analítica pero también de biotecnología, microbiología y materiales avanzados.

Por ello, el primer apartado de este trabajo pretende ser una introducción general sobre la inocuidad de los alimentos y su importancia a nivel mundial, haciendo especial énfasis en los patógenos alimentarios emergentes responsables de los principales brotes y la contribución de la

tecnología de los biosensores como factor conductor para el desarrollo de nuevas metodologías para la detección de bacterias transmitidas por los alimentos mediante técnicas de multiplexado.

Además, se discutirá también la integración de nuevos materiales con dimensiones nano/micrométricas en biosensores electroquímicos, destacando algunas de las ventajas de las partículas magnéticas: i) su capacidad para preconcentrar las bacterias presentes en muestras complejas mediante una reacción inmunológica ii) us uso como plataforma para el bioreceptor en los dispositivos de biosensores iii) o como soporte para la inmovilización magnética en la superficie de un electrodo de trabajo por atracción magnética.

Los métodos de detección para seguridad alimentaria actuales han permitido un avance significativo en relación al desarrollo de métodos rápidos y sensibles, en los que la implementación de bioensayos con capacidad de multiplexado es una de las tendencias emergentes. Sin embargo, se han descrito pocas estrategias basadas en biosensores electroquímicos para la detección simultánea de bacterias en alimentos. Por esta razón, en el presente trabajo se propone desarrollar un biosensor electroquímico que detecte simultáneamente *Salmonella enterica*, *Listeria monocytogenes* y *Escherichia coli*, basado en el uso de partículas magnéticas.

Las estrategias presentadas en esta Tesis doctoral se basan por un lado en un reconocimiento electroquímico inmunológico, que proporciona la detección de las células bacterianas, y por otro un reconocimiento genético, que permite la detección del ADN bacteriano. Estas dos estrategias se combinan con un paso de separación inmunomagnética que da lugar a la captura y preconcentración de las bacterias a partir de muestras de alimentos. Así, se ha realizado un estudio de las partículas magnéticas de diferentes tamaños (micro y nanométrica), para después efectuarse la separación inmunomagnética de *S. enterica*, *L. monocytogenes* y *E. coli*. Además, se han comparado posteriormente las dos estrategias para la detección de *Salmonella,* usándose como modelo muestras leche.

Finalmente, se describe una estrategia de PCR multiplexada combinada con un magneto-genosensor electroquímico usando partículas magnéticas de sílice para la detección simultánea de *S. enterica*, *L. monocytogenes* y *E. coli*.

CHAPTER 1

Introduction

1.1 Food Safety

1.1.1 Foodborne illnesses and outbreaks

The World Health Organization (WHO) have considered Food Safety as the main topic of World Health´s Day in 2015.[1,2] It is estimated that two million deaths occur every year worldwide from contaminated food or drinking water, of each 200 are related to foodborne diseases, caused by harmful bacteria, viruses or parasites.[3]

The emergence of foodborne infectious diseases in humans worldwide is attributed to several causes, such as the loss of biodiversity due to an intensive agriculture, food industry and land changes, together with the evolution of drug resistance.[4] Moreover, climatic factors related to the increase of the average global temperature and consequently to the raise of CO_2 concentrations, as well as precipitation changes have implications for food production, since they can potentiate a higher growth and survival of pathogenic microorganisms leading to the occurrence of food safety hazards.[2,5] The socioeconomic status impact on food safety has also been studied, showing that low income individuals are more exposed to foodborne illness related to a poor hygiene and proper food handling practices and nutrition.[6]

In Europe, over 320,000 human cases are reported each year being mostly related to the presence of pathogenic microorganisms in meat products, as well as fruits and vegetables. For instance, the notifications of pathogens in meat products are shown in Figure 1.1, being *Salmonella spp.*, *Escherichia coli* and *Listeria monocytogenes* the most common reported pathogenic bacteria.[7]

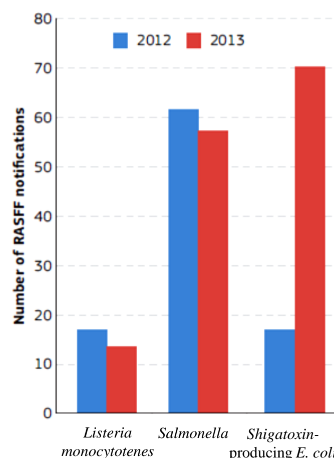


Figure 1.1 Number of notifications for pathogenic bacteria found in meat samples in 2012 and 2013. Reprinted with permission from reference [7].

Some of most virulent foodborne diseases and foodborne pathogens causing diseases include *Salmonella* spp., *E. coli*, *L. monocytogenes*, *Campylobacter jejuni* and *Shigella* spp.. Table 1.1 presents the name of the diseases caused by these pathogens, as well as the source of contamination, infective doses, common symptoms and incubation period time.[8,9]

Table 1.1 Foodborne microorganisms responsible for the main outbreaks. Adapted from reference [8]

Microorganism	Name of the diseases	Common symptoms	Incubation period time	Sample source of contamination	Infectious dosis/ CFU
Salmonella spp.	Salmonellosis	Fever, abdominal pain, diarrhoea, nausea, vomiting	12–72 h	Meat, poultry, eggs and milk	15–20
E. coli, enterohemorragic	Hemorrhagic colitis	Abdominal cramps, diarrhoea, fever, vomiting	Up to 10 days	Water, undercooked meat products and raw milk	2-9
L. monocytogenes	Listeriosis	Fever, muscle aches, nausea or diarrhoea	1 up to 4 weeks	Dairy products, ready-to-eat meat, fish products or transmitted from mother to child during pregnancy and childbirth	<1000
C. jejuni	Campylobacte-riosis	Diarrhoea, abdominal pain, fever, headache, nausea, vomiting	2 up to 5 days	Undercooked poultry or contaminated water	500
Shigella spp.	Shigellosis	abdominal cramps, nausea, vomiting, fever, stool containing blood and mucus	1 up to 7 days	contaminated food or water or through person-to-person contact	10-200

S. enterica sv. Enteritidis and Typhimurium are the most frequent cause of foodborne outbreaks among *Salmonella* serovars (Figure 1.2).[10] One of the most recent cases was reported in August 2014, a multi-country outbreak reported in Austria, France and Luxembourg associated with eggs from Germany.[2,11]

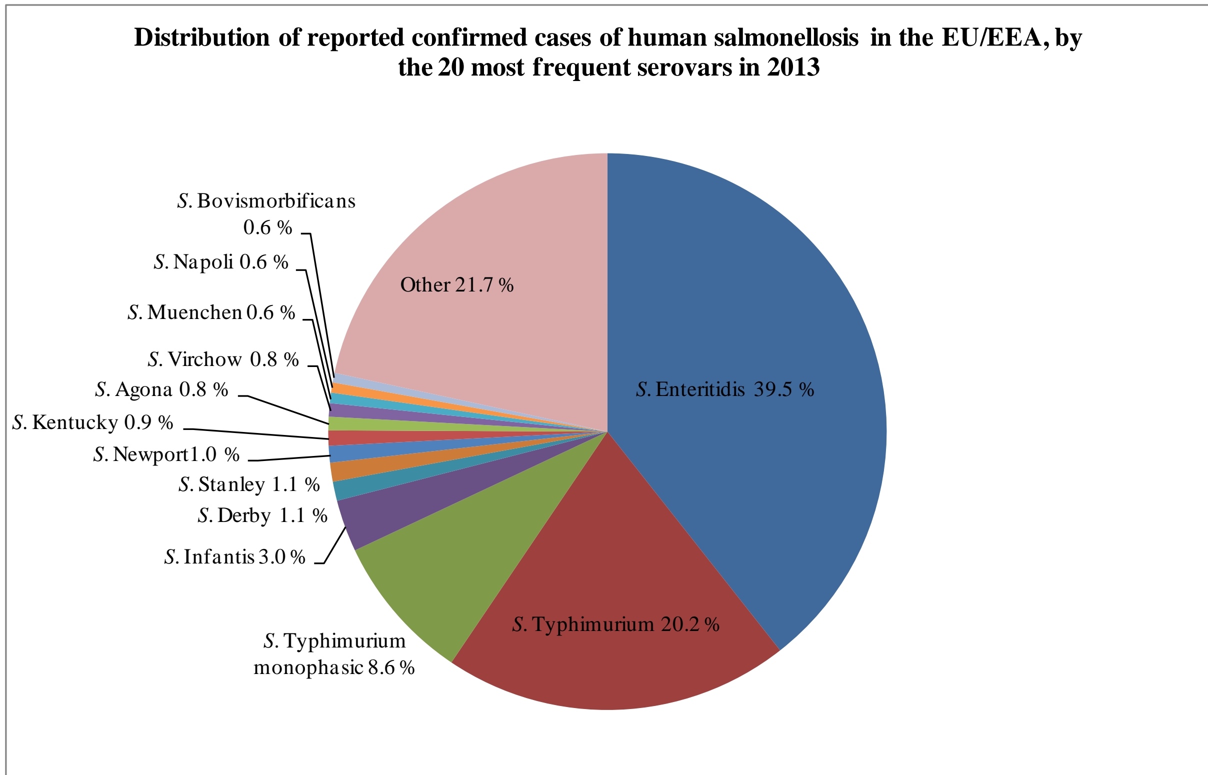


Figure 1.2 Distribution of reported confirmed cases of human salmonellosis in the EU/EEA in 2013, by the 20 most frequent serovars, expressed in percentages. Adapted from reference [10].

One of the most serious outbreaks in Europe related to *E. coli* was reported in Germany, with a total of 3126 cases of diarrheal disease, including 17 deaths in Germany and additional extension to other countries as Norway, USA, Canada and Switzerland. It was caused by a Shiga toxin-producing *E. coli* (STEC) strain found in meat and fenugreek sprouts. This *E. coli* strain belongs to O104:H4 serotype, having the ability to excrete a shiga toxin, which is lethal to humans.[12,13]

L. monocytogenes is a Gram positive pathogen that can survive in different environments, such as low temperatures and pH values or high salt concentrations. In the years of 2008 to 2012 an increase of number of listeriosis cases reported in the EU was registered with a total of 198 deaths in 2012.[14,15]

1.1.2 Preventive approaches

The risk of food contamination is present from farm-to-table, requiring prevention and control related to handling, preparation and storage of food.[8]

"Make food safe" becomes a vital task worldwide, as a result, preventive approaches like Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), Hazard Analysis and Critical Control Point (HACCP) and the food code (*Codex Alimentarius*) have been implemented, which can considerably reduce the survival of pathogens during the process of handling, preparation and storage processing. These approaches include measures for the introduction of methods for decontamination, disinfection and cleaning, the implementation of analytical methods for screening feed and feed ingredients, to provide more inspection and control at industries and farms, as well as the implementation of communication strategies for consumers and the food industry.[16,17]

Examples of regulatory agencies or centers worldwide are the WHO, the US Centers for Disease Control and Prevention (CDC), the US Food and Drug Administration (FDA), the Public Health Agency of Canada (PHAC), the European Food Safety Authority (EFSA), the European Centre for Disease Prevention and Control (ECDC), OzFoodNet, PulseNet International (PNI), National Institute of Public Health, Japan, among many others.

Identification and detection of foodborne bacteria is in general required for routine surveillance and monitoring, evaluation of the most common food sources responsible for specific foodborne, during regulatory actions or from investigation of a foodborne outbreak. In addition, threshold limits for the presence of certain microorganisms in food products have been established in order to ensure that the marketed products available to the consumers are safe. If the food product contains an amount above the required legislation, it must be reject from the market.[16,17]

A wide range of methods are available for foodborne bacteria identification and detection, in connection with these programs, for the prevention and identification of problems related to health and safety. The choice of the method is a key factor for the detection of foodborne pathogens and the intended use of the method, for instance whether for a qualitative or semi-quantitative screening, quantitative and/or confirmatory analysis, must be clearly defined.[18]

Methods can be compared and validated, based on their analytical performance, in detail, the analyte stability, ruggedness or robustness testing, linearity and calibration curve, analytical range, sensitivity, specificity and selectivity, accuracy and recovery, precision including repeatability and reproducibility; measurement uncertainty (MU), sample stability, method comparisons, limit of detection (LOD) and limit of quantification (LOQ). Additionally, an ideal method should be rapid, providing results in a few hours, easy handling, accurate, applicable to several food matrices and foodborne bacteria.[16-18]

Figure 1.3 shows the conventional methods used for the detection of foodborne bacteria, which will be fully detailed in §1.3 of this chapter.

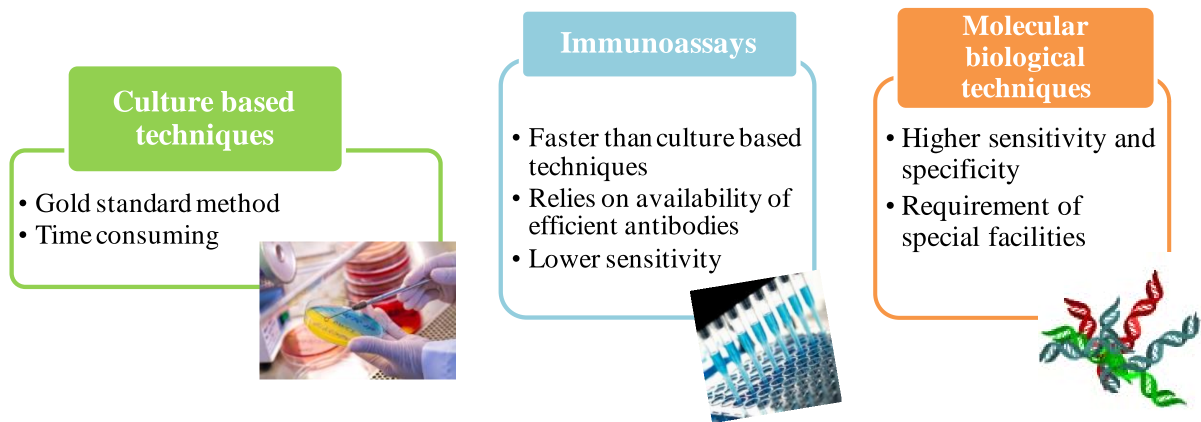


Figure 1.3 Traditional methods for the identification and detection of microorganisms.

1.2 Biosensors technology

The analytical technology based on biosensors has been being widely applied in different areas ranging from agriculture, environment to clinical diagnosis or drug monitoring.[19,20]

Biosensors have been successfully implemented in particular in food industry due to their high specificity and sensitivity, which provides the detection of a broad range of analytes in complex sample matrices as food samples, with minimum sample pretreatment.[20,21]

Based on the IUPAC definition, a biosensor is a self-contained integrated device composed in general by a biological recognition element (bioreceptor) and a physico-chemical transducer, as schematically shown in Figure 1.4. The biological recognition system translates information from the biochemical domain upon a specific biological reaction, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity.[22]

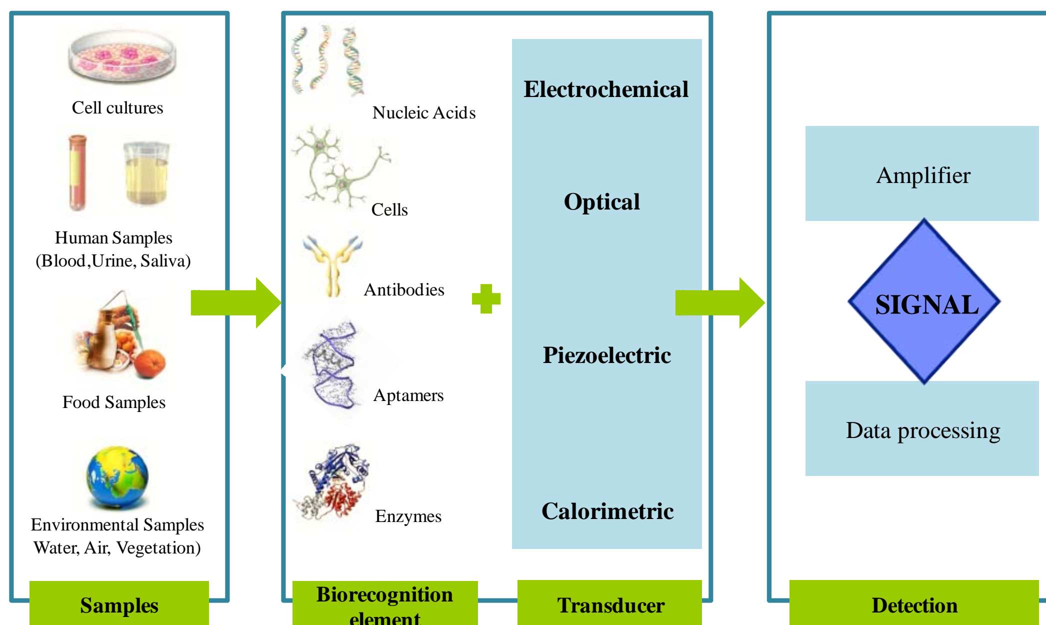


Figure 1.4 Schematic representation of the components of a Biosensor. Adapted from reference [23].

Biosensors can be classified according to the type of biorecognition element (e.g. enzyme, antibody, DNA, cell, aptamers, among many others) and also to the type of transducer (e.g. electrochemical, optical, piezoelectric), as schematically shown in Figure 1.5.

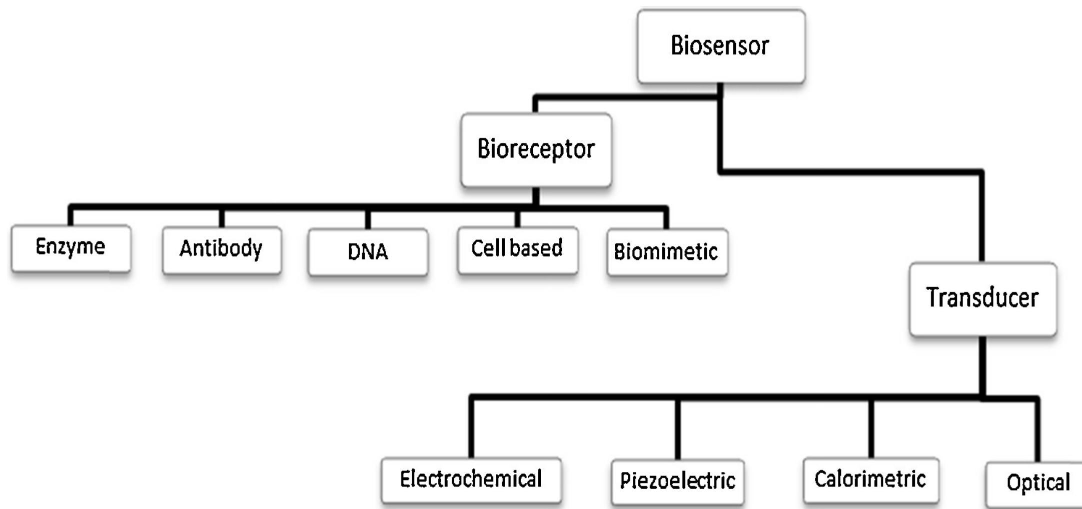


Figure 1.5 Classification of biosensing devices according to the bioreceptor and the transducing element. Adapted from reference [24].

1.2.1 Classification of biosensing devices based on the biorecognition element

1.2.1.1 Enzyme based biosensors

Enzyme based biosensors are very popular, in particular among electrochemical transduction devices, due to their ability to specifically recognise their substrates and to catalyse their transformation. Therefore, in this class of biosensors, the enzyme is the biological recognition element which detects the target analyte from a sample matrix based on its catalytic activity which amplifies the signal response with higher sensitivity and specificity.[24] An example of this device is the popular glucose biosensor for the daily control of glucose levels in whole blood in the diabetic patients, based on the enzyme glucose oxidase.[25]

An important feature in the development of electrochemical enzyme based biosensors is the establishment of a satisfactory electrical communication between the active sites of the enzyme and the electrode surface. In this context, progresses have been done in achieving direct electron transfer by modification of an enzyme or electrode surface with a mediator or a nanoparticle.[26]

Alternatively, enzymes can be used as labels in bioassays, being conjugated with biomolecules, such as antibodies, mainly in electrochemical devices.

1.2.1.2 Antibody based biosensors

Antibodies, also known as immunoglobulins are a family of glycoproteins. They are generally divided into five different classes (IgA, IgG, IgM, IgD, and IgE) based on their molecular characteristics, including the structure of the heavy chain, size and valency (number of antigen binding sites). IgG is the most common class used in immunosensors, their basic structure resembles a "Y"- shaped molecule consisted of two distinct regions: Fragment crystalizable (Fc) region and Fragment antigen-binding (Fab) region, as represented in Figure 1.6. Fc region is composed of two heavy polypeptide chains (approximately 50 000 Da), whereas Fab region is consisted of two light polypeptide chains (approximately 25 000 Da), in which both are linked by disulfide linkages. Fc and Fab regions are also composed of different domains, based on their amino acid sequence variability, such as constant (C) and variable (V), where V domain represents the most important region relatively to the antibody–antigen binding interaction. Fc region is frequently the binding site to immobilise antibodies on solid supports as nano and/or microparticles. The specificity of an antibody towards the binding site or epitope of its antigen, depends on its amino acid sequence.[24,27]

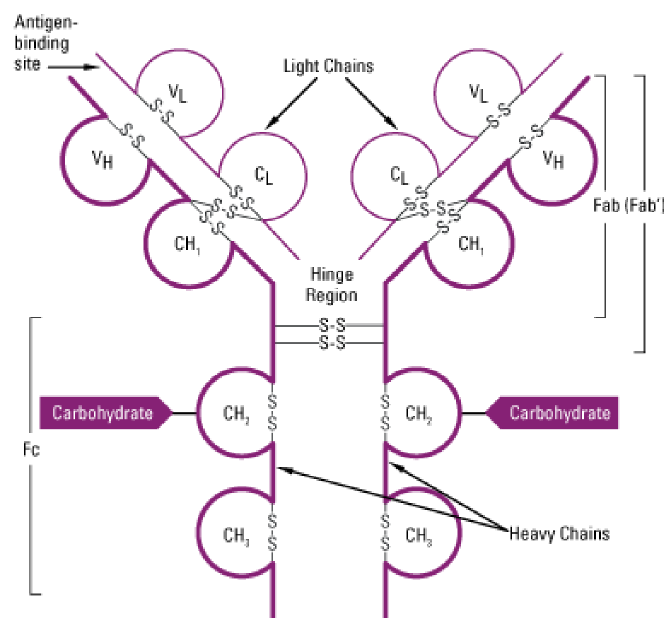


Figure 1.6 Schematic representation of an antibody structure.

Antibodies based biosensors can be also defined as immunosensors, consisting of a biosensing device which principle relies on the antibody-antigen binding. The detection can be achieved either with or without a label, being named as labelled and unlabelled immunosensors respectively.[28]

In labelled immunosensors, the detection is performed based on a reporter, usually an enzyme or fluorescent dye that is coupled with an antibody, being mostly achieved by electrochemical or optical readout platforms, respectively, with notable specificity. For the unlabelled immunosensors, the readout is based on the physical effects, such as optical or piezo transduction or electrode potential change produced during the immunological reaction, as electrochemical transduction.[28]

1.2.1.3 DNA biosensors

DNA based biosensors, also named as genosensors, are biosensing devices which principle is based on the specific DNA hybridisation, directly on the surface of a transducer.[29,30] Similarly to immunosensors, the electrochemical detection of the DNA can be achieved either with or without a label.[31] This definition was also extended to the detection of amplicons coming from DNA amplification techniques, such as PCR in order to increase the sensitivity of the bioassay.[32]

In labelled genosensors, different reporters, such as electroactive hybridisation indicators as heterocyclic dyes (intercalators), ferrocene derivatives and organometallic complexes are among the most widely used redox indicators. Additionally, nanoparticles, such as quantum dots, gold nanoparticles or magnetic particles can be conjugated with DNA, providing high sensitivity and the possibility of multiplexing.[33]

In label free DNA detection, no labels are required, being a simple methodology where the signal is measured upon hybridisation, but with lower sensitivity. The DNA detection can be based on a guanine oxidation signal using carbon, gold, indium tin oxide and polymer coated electrodes.[33] Nevertheless, the main inconvenient of this methodology is the reduction of the guanine signal upon hybridisation,[34] the high background signal and consequently low sensitivity.[35]

1.2.1.4 Cell and biomimetic based sensors

Cell based sensors principle relies on a physiological response of living cells, such as bacteria, fungi, yeast, animal or plant cells, producing a response through the interaction between stimulus and cell. For this reason, cell based sensors are very used to study biological effects induced by analytes such as gene expression, metabolic activity, viability, bioavailability, toxicity and genotoxicity.[20,24]

Biomimetic based sensors principle use the mechanisms found in biological systems as a model for the design of new biosensor techniques. In this context, artificial receptor strategies have been developed for the detection of several target analytes. These strategies include the use of (i) molecularly imprinted polymers (MIPs) for the design of artificial antibodies, (ii) membrane mimicking of natural cell membranes, as well as (iii) peptides or aptamers.[36,37]

Molecularly imprinted polymers (MIPs) are consisted of a synthetic polymer matrix composed of binding sites specific for a target analyte, being for this reason considered as promising candidates to replace enzymes, antibodies and other bioreceptors.[38,39]

The main advantages of MIPs are their high selectivity and affinity for the target molecule used in the imprinting procedure. When compared to other biorecognition elements as proteins or nucleic acids, MIPs provide higher physical robustness, strength, resistance to elevated temperature and pressure and inertness towards acids, bases, metal ions and organic solvents. Moreover, MIPs based recognition elements a longer shelf life, keeping their recognition capacity also for several years at room temperature.[40,41]

Aptamers are consisted of DNA or RNA molecules with the ability to bind to their related molecular targets with comparable affinity and specificity as antibodies.[42] These functional molecules can be synthesised with high reproducibility and purity by *in vitro* selection from libraries of synthetic oligonucleotides known as SELEX (systematic evolution of ligands by exponential enrichment). In addition, aptamers can also be modified with fluorescent probes, electrochemical indicators or other tags, which can significantly improve the biosensor analytical performance.[37,43]

1.2.2 Classification of biosensing devices based on the transduction

1.2.2.1 Optical transduction

Optical transducers are particularly attractive as they can allow, in some approaches, a direct "label-free" and "real-time" detection of several target analytes, being very popular for food safety applications. Optical transducers provide changes in the refractive index or thickness, which occur when a target analyte binds to receptors immobilised on the transducer surface. This method of transduction is available in a wide range of spectroscopic readouts, such as surface plasmon resonance (SPR), absorption, fluorescence, phosphorescence, surface-enhanced Raman, refraction and dispersion spectroscopy.[21,24]

1.2.2.2 Piezoelectric transduction

Piezoelectric transducers are composed of a piezoelectric material as quartz. The readout relies on the principle that an oscillating crystal resonates at a natural resonance frequency. Therefore, the contact between the target analyte and the transducer will create a frequency shift, which will then produce a measurable current signal.[24]

Piezoelectric transducers also provides a direct "label-free" and "real-time" detection of several target analytes and the most common readout platform is quartz crystal microbalance (QCM).[21]

1.2.2.3 Calorimetric transduction

The main principle of calorimetric transduction relies on the concept that many enzyme catalysed reactions or other biochemical reactions are exothermic, generating thus heat which can be correlated to a target analyte concentration. Therefore, calorimetric transduction is based on the measurement of the changes in temperature in the reaction between the biological recognition element and a target analyte.[24]

1.2.2.4 Electrochemical transduction

Electrochemical biosensors have several advantages in comparison with other analytical readouts, such as outstanding sensitivity, possibility of miniaturisation and *in-situ* and point-of-care measurements using hand-held instrumentation operated with batteries. The equipment required for electrochemical analysis is simpler and cheaper.[23,24]

Electrochemical transducers are composed of different readout platforms, briefly described below.[23,44]

- Potentiometry is based on the potential difference measured either between an indicator and a reference electrode, or two reference electrodes separated by a permselective membrane, when there is no significant current flowing between them.
- Conductimetry is based on a two- electrode device designed to measure the conductivity of the thin electrolyte layer adjacent to the electrode surface.
- Surface charge using field-effect transistors is a type of transistor that uses an electric field to control the conductivity of a channel between two electrodes, in a semiconducting material.
- Impedance spectroscopy which combines the analysis of both the resistive and capacitive properties of materials, in some cases without the need of labels.
- Amperometry is based on the measurement of the current obtained from the electrochemical oxidation or reduction of an electroactive species.

Among the different types of electrochemical approaches, amperometry will be further discussed since it is the transduction used in this Dissertation.

Amperometry

Amperometry principle is based on the oxidation and reduction of electroactive species in a (bio)chemical reaction at a working electrode (WE). The potential of the WE is set constant relatively to the reference electrode (RE), as depicted in Figure 1.7. The resulting current is directly correlated to the concentration of the electroactive species or to its production or consumption rate within a catalytic process.[23,44]

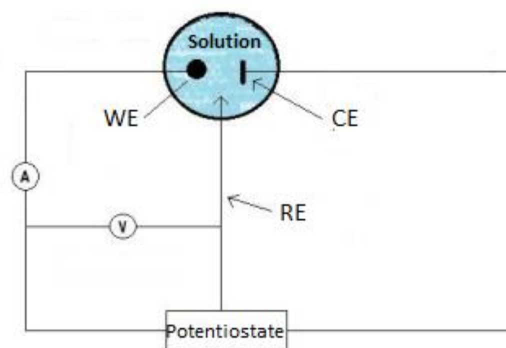


Figure 1.7 Schematic representation of the electrode configuration on an amperometric cell.

In amperometric biosensors, the biorecognition element is usually an enzyme that catalyses an electrochemical reaction of analytes that are electrochemically non-active, into products that can be either oxidised or reduced at a working electrode. [23,44]

Materials used in electrochemical transducers. Composites and biocomposites

Currently, a wide variety of electrochemical transducers are being used for biosensing applications which can differ according to their size, geometry, as well as the kind of material. An ideal material for electrochemical biosensing should allow an effective immobilisation of the biorecognition element on its surface, a robust biological reaction between the target and the biorecognition element, a low non-specific adsorption of the label and a sensitive detection of the biological event.[45]

Examples of classical carbonaceous materials comprise carbon paste, glassy carbon, and pyrolitic graphite, being common choices of electrodes used in biosensing devices. Conducting polymers, such as polypyrrole and polyaniline have been reported for the development of biosensor devices due to their good biocompatibility, conductivity and stability. In the literature different materials have been developed to construct novel transducers for electrochemical based biosensors. In this context, the incorporation of different materials on the transducer has been used to improve the performance of the electrochemical-based device. The resulting material, denominated as modified electrode, can be further modified in order to react with the biorecognition element,[28] as it will be discussed in §1.2.5. For instance, the integration of

nanostructured materials as carbon nanotubes and metal nanoparticles have been extensively reported to generate novel composites with higher sensitivity and stability, improving thus the electrochemical properties of the biosensor.[46] These materials will be further discussed in §1.2.5. When different materials are combined, the properties of the resulting composite material depend on the properties of each component material, such as the size, chemical reactivity and morphology, giving enhanced properties to the composite, as distinctive chemical, mechanical, physical, or biological qualities.[46]

Composite based transducers are generally composed of two or more constituent materials, conductive material, as carbon materials and non-conducting binding material, for instance epoxy resins, polyurethane or other polymers. The main function of the conductive material in the composite is to provide the electrical conductivity needed for the conduction of the signal transduction, whereas the non-conducting binding material is to ensure mechanical consistence in terms of durability and resistance to the electrolytic working medium.[45,46]

In this context, the use of graphite-epoxy composites (GEC) fulfills all these requirements and features, being extensively used for electrochemical biosensing due to their unique physical and electrochemical properties. The central attraction of these composites is their rigidity, providing a high mechanical stability over time. Additionally, the GEC surface can be regenerated by a simple polishing procedure, as well as easily modified with different biological recognitions elements as DNA, oligonucleotides, proteins or antibodies.[47] An additional interesting property of GECs is their biocompatibility. This feature allows not only adsorption but also integration of the biorecognition element into the bulk of the GEC without subsequent loss of the receptor's biological properties, thus generating a rigid and renewable transducing material for biosensing, namely, a graphite–epoxy biocomposite (GEB).[48] These biocomposite transducers will be further discussed in §1.2.3.2.

Electrochemical biosensors based on printing technologies

Another important factor that has been attracting both industry and academia is the miniaturisation of the electrodes and consequently all the electrochemical device. This has led to the application of thick- and thin-film technology, which relies on the deposition of inks on a substrate in a film of controlled pattern and thickness, mainly by screen-printing. Disposable screen-printed electrodes as electrochemical transducers offer the possibility of miniaturisation

for the integration in handheld devices which is very suitable for *in-situ* analysis, being for this reason so much popular in food safety purposes (Figure 1.8).[44,49]

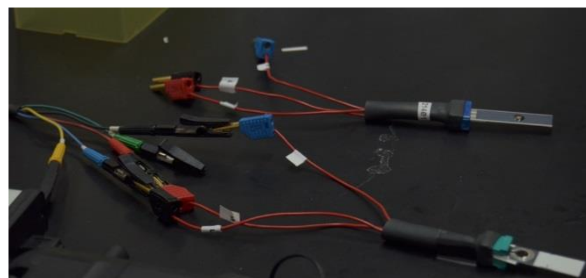


Figure 1.8 Representation of a miniaturised electrochemical device using screen-printed electrodes.

1.2.3 Immobilisation strategies of the biorecognition element on the transducer

An important requirement in biosensors technology is that the biorecognition element should bring physicochemical changes in close proximity of a transducer. In the special case of electrochemical biosensors, the biorecognition element should facilitate, for instance, the electron transfer efficiency of the biosensing process. In this context, several strategies can be used for the immobilisation of the biorecognition element directly on the transducer. Alternatively, the biorecognition element can be also immobilised on membranes in physical contact with the transducer or in nano and micromaterials at the transducer´s surface. In addition, other immobilisation strategies based on the use of polymers or films can be also used for the preparation of novel transducers bringing enhanced sensitivity in the biosensing process, as shown above in §1.2.2.4.[45,46]

1.2.3.1 Classical immobilisation methods

Classical immobilisation methods comprise electrostatic adsorption, entrapment, cross-linking, and covalent bonding procedures, being schematically represented in Figure 1.9. The biological recognition element can be related to enzymes, DNA, peptides, aptamers, antibodies, lectins, as well as bacterial cells or liposomes, among others.[28,50]

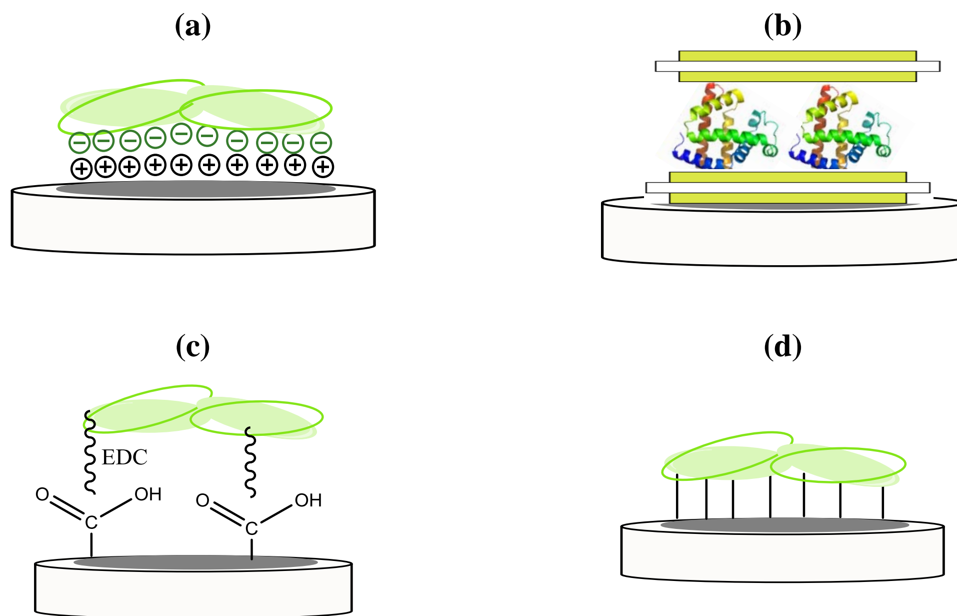


Figure 1.9 Classical strategies for the immobilisation of the biorecognition element: (a) electrostatic adsorption, (b) entrapment, (c) cross-linking and (d) covalent bonding procedures.

Adsorption

The immobilisation of the biorecognition element is based on week forces including hydrophobic interactions, electrostatic interactions, van der Waals forces and hydrogen bonding. As a result, this binding is affected by changes of temperature, pH, and ion strength of solution leading to desorption. Nevertheless, the main advantage of this strategy is its simplicity under mild conditions.[50]

Entrapment

In this procedure, the biorecognition element is physically retained on a polymeric structure. It is a simple and quick procedure in which the main disadvantages are the leakage of the biorecognition element, the permeability to interferents and lost of its biological activity.[50]

Cross-linking

In this methodology, a bifunctional reagent, usually glutaraldehyde, carbodiimide succinimide ester, maleinimide, or periodate creates an intermolecular linkage between the biorecognition element and the transducer. One of the disadvantages of this methodology is the difficulty in controlling the molecular linking.[50]

Covalent bonding

The biological recognition element is directly bound to the transducer though different functional groups with high stability. This strategy is widely used, since it is the strongest bonding to prevent leakage. Nevertheless, it creates difficulties to protect actives sites in binding reactions which might lead to cross- reaction.[50]

Self-assembly monolayers

The formation of self-assembled monolayers (SAMs) on transducer surfaces is a common immobilisation technique which allows an oriented attachment of a wide range of biomolecules based on covalent interactions of thiols, disulphides, sulphides and other related molecules with the surfaces of noble metals, particularly gold, as well as platinum and mercury. Their highly ordered compact layer which significantly reduces the diffusion of electroactive species toward the transducer's surface is point out as one of the main drawbacks of SAMs.[51]

1.2.3.2 Biocomposites

The main advantage of biocomposites is their easy preparation by adding the biorecognition element to the composite formulation through dry-chemistry procedures that can be easily transferred to mass fabrication of thick film devices, avoiding thus laborious, expensive and time-consuming surface immobilisation procedures.[52] Biocompatibility of graphite-epoxy biocomposites (GEB) was previously discussed in §1.2.2.4, in which the biocomposite acts as a support for the attachment biomolecules, keeping the electrochemical and physical properties previously described for GECs §1.2.2.4.

In this context, affinity proteins, such as avidin (Av), Protein A, or Protein G have been incorporated in the biocomposite providing a robust platform for an oriented immobilisation of dsDNA, aptamers or other biomolecules, which enhances the electrochemical biosensing response due to the binding of the biorecognition element to the complementary sites of the target molecule. For instance, the immobilisation of ssDNA using av-GEC electrodes was reported in the literature using streptavidin a linker molecule.[53,54]

1.2.3.3 Magnetic Immobilisation based on magnetic particles Coupled with a Magneto-actuated electrode

One of the most promising materials for biosensor applications are biologically modified magnetic particles, as it will be further discussed in §1.2.5. Magnetic particles (MPs) can be easily functionalised for further immobilisation of biomolecules on their surface and also manipulated by an external magnetic field gradient, enabling thus the possibility of magnetic bioseparation. The integration of magnetic particles and electrochemical biosensing strategies has shown to improve significantly the analytical performance of a bioassay.[47]

Instead of direct modification of the electrode surface, MPs are used as a support for biological reactions (immobilisation, enzymatic labelling, or affinity reactions). For the biosensing process, magnetic particles are attracted to the surface of the surface of GEC electrodes (§1.2.2.4), which contains a small magnet allocated inside the electrodes, being named as magneto graphite epoxy composite (m-GEC). In this way, higher proximity between the electrochemical reporter and the electrochemical transducer is created by magnetic actuation.[47,55]

An additional advantage of the use of m-GEC is the possibility of renewing the sensing surface by a simple polishing procedure. This procedure constitutes a versatile platform for electrochemical biosensing (both genosensors and immunosensors) for a broad range of applications, including the detection of food contaminants, such as pesticides,[56] antibiotic residues,[57] bacteria,[58,59] food additives,[60] allergens,[61] or diseases biomarkers, such as malaria[62] or CD4 cells for AIDS monitoring.[63]

1.2.4 Detection of the biorecognition element

1.2.4.1 Label-free based biosensors

Label-free methods are becoming very popular among biosensors technology due to their simplicity, being the main advantage of this technology, the possibility of a direct detection of the target in real time. The readout platforms commonly used are based on surface plasmon resonance (SPR), electrochemical impedance spectroscopy, quartz crystal microbalance (QCM), or surface-enhanced Raman spectroscopy (SERS).[64,66] The main drawback of these techniques is, in some instances, the lack of sensitivity.

1.2.4.2 Label based biosensors

The use of labels is of special importance in biosensors because their use involves improved analytical features, such as an increased sensitivity. During the readout, the amount of label attached to a biomolecule (e. g. antibody, DNA, among others) is detected and correlated to the number of bound targets. Most popular labels are conjugated enzymes and fluorophores. Recently, nanoparticles have been used, as it will be further discussed in §1.2.5. Among the labels used, enzymes are the mostly preferred in electrochemical biosensors, such as horseradish peroxidase (HRP), glucose oxidase (GOx) or alkaline phosphatase (ALP), being often conjugated with an antibody or DNA and providing fast electrochemical responses with high sensitivity due to the high turnover rate of these enzymes. Moreover, in genosensors, intercalators as methylene blue (MB), meldola blue (MDB), can be also used, acting as an electron acceptor in the transduction process.[65,66]

Mediators are used as carriers of electrons from the biorecognition element to the electrode. Thus, an ideal mediator must be rapidly oxidised and/ or reduced at the electrode surface and it should readily react with the biorecognition element. The electron transfer will occur at the mediator´s potential. Therefore, chemical compounds as osmium or ruthenium complexes, ferrocene, quinone or viologen derivatives, as well as small redox proteins as horse heart cytochrome are examples of mediators.[67,68]

HRP enzyme is frequently used as electrochemical reporter for the biosensing process for the detection of many analytes. In this process, mediators can be used to shuttle electrons between the transducer and the biological recognition element.

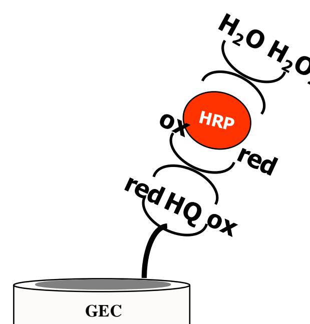


Figure 1.10 Schematic diagram with the biosensing process catalysed by HRP.

In Figure 1.10 a diagram of the biosensing process based on HRP as electrochemical reporter is represented using hydroquinone as mediator. Therefore, the substrate H_2O_2 in the solution is reduced by the enzyme HRP, while the mediator is oxidised in the enzymatic reaction.

Finally, the oxidised mediator is electrochemically reduced on the electrode, resulting on the increase of the cathodic current at lower applied potencial.[35,69]

1.2.5 Integration of nano and micromaterials in biosensing

The integration of novel materials either with nanometer or micrometer dimensions on biosensors, in the transducer or as a label, has been abruptly increased, since it improves significantly the analytical performance of the biosensing process towards high sensitivity and selectivity. In this section, emerging materials, like nanostructured carbon materials, colloidal silver and gold particles, semiconductor particles, liposomes, molecularly imprinted polymers and magnetic particles, schematically represented in Figure 1.11, will be discussed.

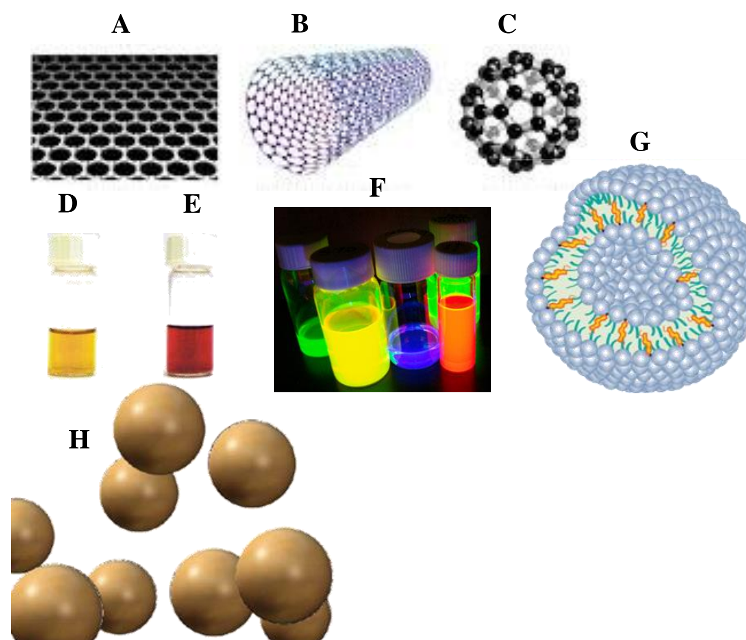


Figure 1.11 Nano and micromaterials used in biosensing. From A to H, graphene, carbon nanotube, fullerene, silver nanoparticles, gold nanoparticles, quantum dots, liposome and magnetic nanoparticles, respectively.

1.2.5.1 Nano and microstructured carbon materials

Carbon materials, which form a variety of allotropes occupy an unique position in electrochemistry. Examples of nano and microstructured carbon materials are carbon nanotubes, activated carbon, carbon black, xerogel carbon, fullerenes or grapheme, among others. Their high surface area-to-volume ratio, excellent mechanical properties and fast electron-transfer capabilities makes them so useful in the preparation of new transducers. In addition, carbon materials can also be modified with biomolecules or with nanoparticles to react with a specific target in the biorecognition process.[70]

Among these materials, carbon nanotubes (CNT) showed to enhance the analytical performance of the biosensors, compared to traditional carbon materials, such as glassy carbon, diamond or carbon black, due to their high specific area, their tubular nanostructure and chemical stability which allows the fabrication of ultrasensitive sensors consisting of only one nanotube, good biocompatibility, easy functionalisation of ends and sidewalls very useful for the fabrication of multifunctional electrochemical sensors, as well as the possibility of miniaturisation.[71,72]

Graphene is a two-dimensional single atom thick nanomaterial, which has been attracting attention in biosensors research due to its remarkable properties as fast electron transfer, transportation, high thermal conductivity, excellent mechanical and flexibility. When combined with metal nanoparticles, the analytical performance of the biosensors is improved.[73,74] However, some improvements need to be done for the development of methodologies for the conjugation of biological molecules such as enzymes, oligonucleotides, antibodies and aptamers.[75]

1.2.5.2 Colloidal gold and silver particles

Metallic nanoparticles (NPs), such as gold and silver NPs have been attracted attention for the design of new biosensors, due to their unique optical and photothermal properties arise from the large density and susceptibility of their free electrons, as well as high surface area that differ significantly from the corresponded microparticles or bulk materials.[76]

These NPs can be modified with thiols, disulfides, amines, nitriles, carboxylic acids, and phosphines. In addition, electron transfer, which is easily promoted by metallic NPs towards conducting polymers, CNTs and redox biomolecules, represents a promising feature for the construction of a broad range of novel nanocomposite materials to be used as transducers in electrochemical biosensors with improved analytical performance.[46,76]

Gold NPs are among the most common choice in biosensing applications due to their biocompatibility, offering the possibility of an easy conjugation with DNA, antibodies, or proteins, leading to the development of high sensitive biosensors. In particular, the conjugation of ssDNA with gold NPs have been extensively reported, in which ssDNA probes are modified with thiol groups.[77]

Moreover, Au NPs, exhibit intense plasmon absorbance bands in the visible spectral region that are controlled by the particles size, reason why they have been being combined with optical or SPR devices or imaging, as labels.[46]

Furthermore, these nanoparticles can be used as electrochemical labels providing improved sensitivity, as well as integrated in microfabrication techniques opening new possibilities for novel devices.[78,79]

1.2.5.3 Inorganic semiconductor nanocrystals

Colloidal nanocrystals with a semiconductor as the inorganic material are consisted of nanoparticles denominated as quantum dots (QDs) when their dimensions are lower than 10 nm. Their distinctive properties are related to the density of their electronic states, presenting size tunable band gaps and luminescence energies due to the quantum size effect.[78] As a result, their emission spectra are symmetric, narrow and tunable according to their size and chemical composition, allowing the use of different probes without substantial spectral overlap, being for this reason widely used as labels in bioassays, especially in optical biosensors.[80] Moreover, inorganic semiconductor particles can be combined with electrochemical biosensors, in detail for the electrochemical analysis of their metal components (CdS, PbS, and ZnS), being commonly detected using electrochemical anodic stripping voltammetry transduction.[81]

Inorganic semiconductor particles can be either conjugated with antibodies and DNA or as detection labels in biorecognition and biosensing process providing signal amplification of the analytical signal.[79]

1.2.5.4 Liposomes

Liposomes are consisted of a lipid bilayer artificially prepared, with sizes ranging from a few nanometers up to micrometer dimensions. The lipid bilayer is composed of a hydrophobic tail of the phospholipids, whereas the hydrophilic head is oriented towards the extravesicular solution and inner cavity. These structures are also considered a good candidate to improve the analytical performance of a biosensor, as a signal amplification tool, offering the possibility of encapsulation of enzymes, DNA, as well as electrochemical or chemiluminescent labels. In addition, their surface can also be modified with several proteins, antibodies, DNA and enzymes.[70,82]

1.2.5.5 Magnetic iron oxide particles

Magnetic iron oxide particles (MPs) can be categorised accordingly to their size in nanoparticles and microparticles for sizes ranging from tens of nm up to 10 µm in diameter.[83]

MPs are composed of one or more magnetic cores with a coating matrix of polymers, silica, inorganic metals or metals oxides, with terminal functionalised groups. The magnetic core is generally composed either of magnetite (Fe_3O_4) or maghemite (γFe_2O_3).[26,84]

For biosensing applications, superparamagnetic particles are the mostly preferred, meaning that particles are only magnetised in the presence of a magnetic field and readily about to homogeneously disperse upon removal of the magnetic field.[85] This led to the magnetic separation concept, which rapidly propagated to a broad variety of fields as environment, diagnostics or agriculture, revolutionising the isolation and separation of many biological components. In this context, the term immunomagnetic separation (IMS) was created, allowing a specific capture and isolation of different targets including cells, proteins, biomarkers, among others targets , directly from a complex matrix through an immunological reaction with an antibody immobilised on the magnetic particle.[86,87]

The main features of magnetic actuation (Figure 1.12) are the reduction of the nonspecific adsorption or interfering biomolecules in a real samples, avoiding centrifugation steps which also shortens the handling time, as well as the concentration of the target from the complex matrixes.[85-87]

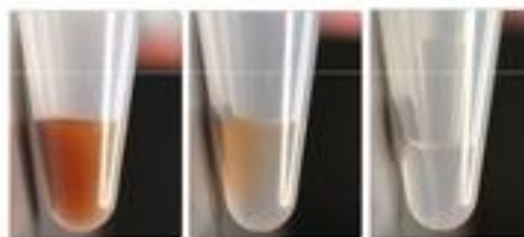


Figure 1.12 Magnetic actuation, representative of one of the main advantages of the use of magnetic particles.

MPs are commercially available for a wide variety of companies, being functionalised with different molecular groups (for instance, tosyl, carboxyl, amine, hydroxyl, aldehyde or thiol), as well as with biomolecules, (such as antibodies, biotin, streptavidin, oligonucleotides, protein A or G), which makes them useful to react with a variety of targets. Table 1.2 shows a

selection of MPs from commercial sources, commonly used in bioassays based on their diameter, functional group, manufacturer and approximate price.[88]

Table 1.2 Magnetic particles from commercial sources classified according to their diameter, functional groups, manufacturer and approximate price.

Product	Diameter (μm)	Functional groups	Manufacturer	Approximate Price per mL/ €
Dynabeads®	2.8	Tosylactivated, amine, epoxy, carboxylic acid,	Life Technologies	80-130
Dynabeads® MyOne™ Silane	1.0	Silanol	Life Technologies	60
Dynabeads® M-280 Streptavidin	2.8	Streptavidin	Life Technologies	150-205
Standard Adembeads	0.30 and 0.50	Carboxylic acid and amine	Ademtech	unknown
PureProteome™ NHS FlexiBind Magnetic Beads	10	NHS	Merck Millipore	unknown
PureProteome™ A/G magnetic beads	10	Mix Protein A and G	Merck Millipore	79
BioMag®	1-2	Amino, Carboxylic acid	Qiagen	35
nanomag®-D-spio	0.020	PEG-COOH, PEG-NH2, PEG 300, Carboxylic acid, amine	micromod Partikeltechnologie GmbH	30
nanomag®-D-spio	0.10	avidin, biotin	micromod Partikeltechnologie GmbH	80

MPs, especially with nanometer dimensions, can also be used as labels in the bioassays to produce an externally observable signal, bringing important advantages, as for instance, unlike chemiluminescent, radioactive or fluorescent labels, MPs are not affected by reagent consumption, or photobleaching.[88]

The development of novel bioassays based on MPs has been fully studied and reported in the literature. The need of miniaturisation and automation of analytical applications, together with the excellent results provided by the use of MPs in bioassays led this technology to microfluidics field, in order to implement new magneto-actuated microfluidic platforms with improved analytical performance.[89,90]

For microfluidic applications, MPs with sizes in the range of 0.2-5 μm are the most commonly used due to the time of magnetic actuation, which is shorter and also the viscous forces that are less dominant for particles with larger size. Manipulations of magnetic particles

in microfluidic systems are achieved using electromagnets, coils or permanent magnets and may involve several steps, such as separation of magnetic particles from a flow by actuation of electromagnets or positioning of magnets, transport of the MPS using an electromagnet or magnet, detection step using a magnetic field sensor, after specific binding of the labelled MP on the sensor surface and the mixing step, using a locally applied alternating magnetic field.[91] As it was previously discussed in §1.2.3.3, these materials have been widely implemented in biosensing approaches for the immobilisation of the biorecognition element, followed by magnetic actuation with a magneto-electrode.

1.2.5.6 Bioconjugation techniques on micro/nanomaterials

In the §1.2.3, the importance of the immobilisation of bioreceptors on the transducers, as well as the advantages of introducing nano and micromaterials in the biosensing process was referred. However, in most of the cases, these materials need to be functionalised with biomolecules to react specifically with a target analyte. Therefore, in this section, the techniques for the bioconjugation of materials with nano and micrometer dimensions will be briefly discussed. In general, bioconjugation of nanomaterials materials involves covalent or non-convalent interactions between the material and a suitable biomolecule.

Non-covalent interactions involve (i) physical adsorption, based on van der Waals, electrostatic interaction, or π– π stacking, (ii) Affinity interaction related to specific interactions such as antibody-antigen, (iii) encapsulation of a biomolecule or a nanoparticle onto micro/nanomaterials.[38]

Covalent interactions are based on (i) direct chemical reaction, (ii) linker strategies, and (iii) click chemistry. In direct chemical reaction, the binding is achieved through molecular groups (e.g., –OH, $–NH_2$, –COOH, –SH, or $–CH=CH_2$) on the material surface and the biomolecule. [38]

Depending on the molecular group at the material surface, an activation step may be necessary for further bioconjugation. Therefore, in Table 1.3, some examples of the molecular groups and coupling agents used in the activation process are presented for further immobilisation of biomolecules.[38,92]

Table 1.3 Activation approaches for the bioconjugation of materials.

Funtional group of the particles	Coupling agent	Reactive sites of the biomolecule
–OH	cyanogen bromide (CNBr)	$–NH_2$
–OH –COOH	*N*-hydroxy Succinimide (NHS) esters	$–NH_2$
–COOH	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide EDC in the presence or absence of NHS	$–NH_2$
$–NH_2$	EDC	–COOH
–CHO	sodium borohydride or Sodium tetrahydridoborate ($NaBH_4$)	$–NH_2$

Additionally, heterobifunctional linkers as *N*-hydroxysuccinimide (NHS), maleimide, pyridyl disulfide, and vinyl sulfone can be added to react with the molecular groups on the material surface for further immobilisation of the biomolecule. Click chemistry is based on an azide–alkyne cycloaddition catalyzed by Cu (I). This reaction occurs under mild conditions with high yield, reason why it is so popular for the functionalisation of surfaces with biomolecules.[38,93]

1.3 State of art in detection methods for Food Safety

This section addresses the novel approaches for the detection of food poisoning bacteria, paying closer attention to three of the most common pathogens involved in food outbreaks: *S. enterica*, *L. monocytogenes* and *E. coli* O157:H7.

End-point and real-time PCR, classical immunological techniques, biosensors, microarrays and microfluidic platforms, as well as commercial kits for detection of food pathogens are discussed, with special focus on the multiplex detection, as well as the role of MPs in these approaches.

Although the immunomagnetic separation for capturing single bacteria from contaminating microbiota and interfering food components has demonstrated to improve the performance on these approaches, the integration of MPs for multiplex detection of bacteria is still in a preliminary stage and requires further studies.

1.3.1 Traditional methods for foodborne pathogens detection

In Europe, threshold limits for the presence of certain important microorganisms, their toxins and metabolites, such as *Salmonella*, *L. monocytogenes*, *Enterobacter sakazakii*, Staphylococcal enterotoxins and histamine have been set down for some food products. The microbiological contamination control is applicable to products placed on the market.[94] Therefore, in this section, the traditional methods commonly used for the microbiological contamination control in foodstuffs will be discussed, with special focus on their advantages and disadvantages.

1.3.1.1 Microbiological culture techniques

Microbiological culture techniques are currently the gold standard for isolation, detection, and identification of microorganisms. However, microbiological analysis of foods, especially for a particular pathogenic has many limitations due to the complex composition of food matrices, the heterogeneous distribution of low levels of pathogens, as well as possible stress or injuries caused to the microorganism or indigenous microbiota that may be present in

some food matrices at very high levels.[1] Therefore, in order to overcome these limitations and to enhance the identification and detection of specific pathogens, several steps were implemented being consisted of preenrichment, selective enrichment, selective plating, as well as biochemical screening and serological confirmation.

In the preenrichment step the food samples are incubated in a nutritious, non-selective medium to restore the injured or stressed bacterial cells to a stable physiological condition. Incubation during preenrichment step is usually performed for 18–24 h. Afterwards, in the selective enrichment the food samples are further enriched in a promoting growth medium, specific for the intended bacterium to improve the detection for low bacterial levels. Then, selective plating is performed on a selective agar to recognize the intended bacteria and restrict the growth of other bacteria. The identification of the bacteria is confirmed using several biochemical tests and serologic typing.[1,8,95]

1.3.1.2 Immunological assays

Immunological assays (IAs) are highly specific *in-vitro* tests that are based on antigen-antibody reaction to detect low concentrations of a broad range of analytes in different types of samples (clinical, environmental, food, among many others). The antigen-antibody reaction is known since the end of the 19th century and the first approaches were based on precipitation in gel, agglutination and turbidimetry assays.

The IAs were then gradually developed until their potential have been fully appreciated since 1960s when higher sensitivity was achieved by the use of labels conjugated with biomolecules involved in the immunological reaction. Immunoassay reactions can be performed in a large variety of formats, being generally classified according to three main criteria:

- Limited or excess of reagents, i.e., competitive (usually performed for small molecules or haptens) or sandwich format (for antigens showing multivalency), respectively.
- Homogeneous (performed in solution) or heterogeneous (one of the reagent is immobilised on a solid support and washing steps are thus required).
- Labelled or unlabelled assay, from which among labelled assays, they can be classified accordingly to the type of the labelling, such as Radioimmunoassays (RIA),[96,97]

Enzyme immunoassays (EIA), Immunofluorescence Assay (IFA), Chemiluminescence immunoassay (CLIA), among others.[98]

The principle of IAs, as previously stated, relies on the specificity of the antigen-antibody recognition, being suitable in the case of the bacteria for the detection of whole bacterial cells or specific cellular components as lipopolysaccharide (LPS) or other biomolecules present on the bacterial outer membrane.[99]

Enzyme Linked ImmunoSorbent Assays

Enzyme Linked ImmunoSorbent Assays (ELISAs) are the most prominent IAs used for bacteria detection. Accordingly to the previous classification, ELISA is a heterogeneous format based on enzyme labelling. In the case of bacteria, as they show multivalent epitopes, they are mostly based on a sandwich approach, as shown in Figure 1.13. In this case, a specific antibody towards the bacteria is immobilised on a solid support, usually a polystyrene microplate. The bacteria in the sample are then incubated with the excess of antibody immobilised on the plate. To detect the amount of bacteria attached to the antibody, a second antibody labelled with an enzyme is added which binds to another epitope of the bacteria. This leads to the formation of a sandwich-complex between the primary antibody, the sample antigen and the second, labelled-antibody, if a direct labelling is employed (as shown in Figure 1.13, panel A). After washing off of any reagent in excess, the sandwich complexes containing the label can be detected. The signal generated is directly related to the amount of bacteria in the sample upon the addition of a chromogenic substrate for the enzyme, which yields a visible colour change, indicating the presence of antigen.

For sandwich-complex formation, at least two binding sites are required on the analyte molecule. The indirect labelling (as shown in Figure 1.13, panel B) is based on the further incubation of the second antibody specific for the bacteria, in these case without any label, with a secondary-labelled and polyclonal antibody. This format shows increased sensitivity and flexibility, although a further incubation step is requiring.[99,100]

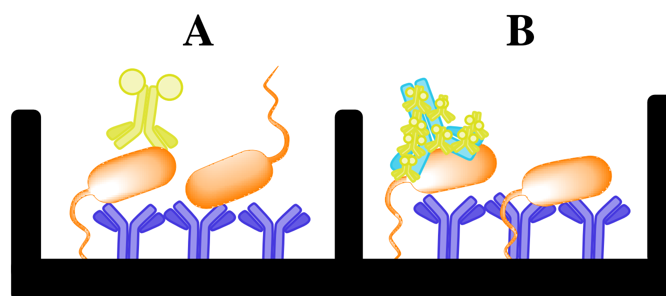


Figure 1.13 Different formats of immunological reactions commonly used for bacteria detection: (A) sandwich and (B) indirect.

ELISA methods have been approved by regulatory agencies, being commercially available. The detection limits for pathogens are normally in the range of 10^3 and 10^5 CFU mL^{-1} and the assay time can take up to 48h, since a preenrichment step is commonly required, in order to achieve the threshold limits for the presence of the bacteria on food samples.[16,101]

Luminescent based immunoassays have gained increasing interest in the field of food safety due to their low cost, rapidity and high sensitivity, consisting of variations of the standard ELISA.[102,103]

Luminescence is described by the emission of light from a substance, as it returns from an electronically excited state to ground state. The various forms of luminescence (bioluminescence, chemiluminescence, photoluminescence) differ in the way the excited state is reached. For instance, photoluminescence is simply fluorescence, in which the excitation is initiated by light at a particular wavelength.[103] Additionally, bioluminescence is characterised by the use of a bioluminescent compound, such as luciferin and firefly luciferase and chemiluminescence is light produced by a chemical reaction.[98]

Enzyme-linked luminescent assay methods based on bioluminescence or chemiluminescence are consisted of another type of immunoassay, in which an enzyme converts a substrate to a reaction product that emits photons of light instead of developing a visible color. Bioluminescent enzyme immunoassay (BLEIA) is characterised by the use of a euglobulin protein, firefly luciferase as the enzyme marker for the detection of bacteria.[102] The LODs can vary from 10^2 to 10^4 CFU mL^{-1}, depending on the type of culture medium or food matrix.[104,105] Chemiluminescent enzyme immunoassay (CEIA) is related to the emission of light (ultraviolet, visible or infra-red radiation) from a molecule or atom as the result of the transition of an electronically excited state during a chemical reaction, using commonly luminol

as substrate. For the detection of foodborne bacteria, CEIA is often combined with an IMS step.[102,106]

ELFA methods are consisted of fluorometric assay by using a substrate that yields a fluorescent product upon enzyme reaction.[107] Alkaline phosphatase, ß-galactosidase, as well as peroxidase are some of the most widely used enzymes for fluorescent applications.[103] For foodborne bacteria application, this approach can be generally more sensitive than colorimetric assays but the differences are not considerable. Alternatively, antibodies labelled with fluorescent probes can also be used in immunoassays, being known as Fluoroimmunoassays (FIA).[98,108] In this context, high sensitivity of the fluorescence measurement is combined with the sensitivity of the probe, resulting on a non-enzymatic process with feasibility of detect at the single bacterium cell detection within 20 min.[109]

Latex Agglutination immunoassays (LAIs)

LAIs have been widely used for foodborne bacteria identification being commercially available by several manufactures due to their simplicity. In this method, polymer colloidal carriers (< 1 μm) are modified with antibodies to attach the target bacteria creating a huge complex, which is visible as a precipitate. Latex particles are used to magnify the antigen-antibody complex, however it still requires a high number of cells ($>10^7$) to obtain visible results, being for this reason combined with a preenrichment step. Additional detection methods can be coupled with LAI, such as turbidimetry or particle counting, in order to improve the LODs.[110,111]

Immunochromatography (ICG) strip test

ICG strip test or lateral flow assay (LFA) that combines both thin-layer chromatograpy with immunological assays principles, being considered a useful tool for the rapid screening of food and raw materials.

A typical LFA format, as depicted in Figure 1.14 is composed of a surface layer to wick the sample through capillary action from the sample application pad, where the sample is introduced and then transported into the conjugate/reagent pad, which contains antibodies specific to the target analyte labelled to coloured particles (for instance gold nanoparticles, latex

microspheres or fluorescent labels). This binding reaction continues as the sample flows in the detection zone. Additionally, the strip contains at least at least two lines: a test line and a control line. At the test line the recognition of the sample analyte and the reporter will result in the required response while the control line shows a successfully processed test.[112]

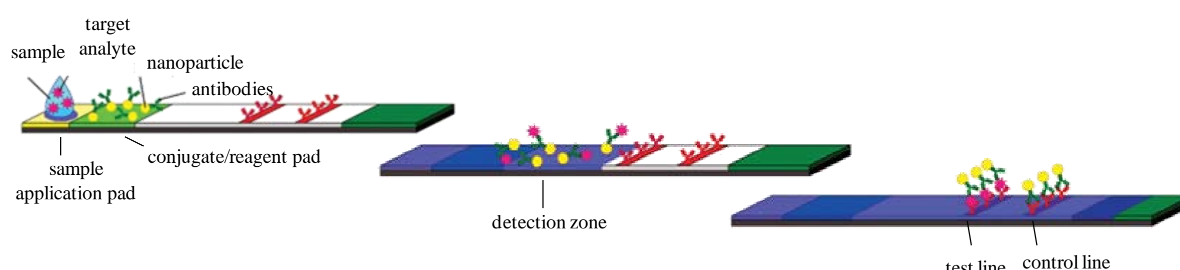


Figure 1.14 Schematic representation of a lateral flow test. Adapted from reference [113] with permission of The Royal Society of Chemistry.

The main advantages of this method are the rapidity, simplicity and low price. Since it is a one-step assay, it avoids the possibility of washing steps, being also less sensitive than other classical IAs. However, when integrating nanomaterials as MPs or gold NPs, respectively for preconcentration and signal amplification, LODs can decrease until 100 CFU mL^{-1}.[114,115] A preenrichment step is generally needed to achieve lower LODs around tens of CFU per 25 g of food sample.[116,117]

Immunomagnetic separation (IMS)

IMS is commonly integrated in immunoassays for bacteria detection. In this technique, magnetic particles (MPs) modified with a variety of molecular groups are coated with antibodies specific to the target bacteria as detailed in §1.2.5.5. Therefore, bacteria will be captured and preconcentrated either from a culture medium or from complex food matrices by simply applying an external magnet.

The use of MPs provides several advantages to IAs, such as (i) preconcentration of the target bacteria into smaller volumes for further testing (ii) reducing and simplifying the preenrichment step (iii) eliminating the matrix effect of the food components.[90,118] In this context, by applying a magnetic field, the particles with the cells attached will be retained, whereas the unbound cells and/or food components will be removed by washing. Afterwards,

the detection of bacteria can be achieved by microbiological culture techniques, ELISA or nucleic-amplification methods, with LODs generally around 10^3 CFU mL^{-1} of culture medium or food homogenate, being lower if IMS is preceded by enrichment step.[87]

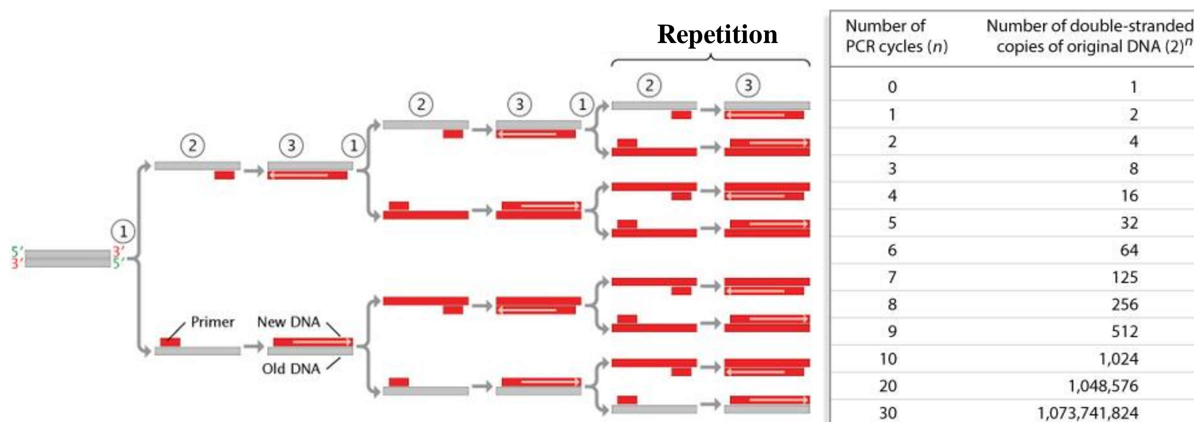
MPs can also be modified with bacteriophages for capturing and preconcentration of bacteria, in an ELISA-like format, named as phagomagnetic immunoassay, decreasing significantly the LOD of a classic immunoassay, up to 19 CFU mL^{-1} in 2.5 h without any preenrichment, in milk samples.[119] In addition, enzyme-linked immunomagnetic chemiluminescence (ELIMCL) has been widely applied for the detection of foodborne pathogens being able to detect around 10^3 CFU mL^{-1} within 1.5 h.[120]

IMS have also been successfully implemented on microfluidic chips, offering additional advantages, as the manipulation outside the microchip and a significantly large surface for immobilisation improving thus the performance of the immunoassays.[121] In magnetic bead-based microfluidic immunoassays, both MPs and bacteria are manipulated through a microchannel by applying a magnetic field.[122] The detection is commonly achieved by microscopy methods or electrochemical methods.

1.3.1.3 Nucleic acid amplification methods

Nucleic acid amplification methods include end-point polymerase chain reaction (PCR) and real-time PCR (qPCR) for single or simultaneous detection of bacteria. PCR allows the production of multiple copies of DNA from the amplification of a single copy or a few copies of a DNA template by applying repeated thermal cycle sets of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA, using thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs, as schematically explained in Figure 1.15.[123]

PCR procedure is consisted of 20-40 repeated cycles based on three steps: denaturation, elongation and extension.



Number of PCR cycles (n)	Number of double-stranded copies of original DNA $(2)^n$
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	125
8	256
9	512
10	1,024
20	1,048,576
30	1,073,741,824

Figure 1.15 Explanation of the PCR methodology: 1 is related to the denaturation step, 2 the annealing and 3 the extension step. Reprinted (adapted) with permission from reference [123]. © 2014 Nature Education.

During the denaturation step, the temperature is usually raised to 93-96 °C, breaking the hydrogen bonds and thus increasing the number of non-paired bases. Consequently, the dsDNA melts, opening up to ssDNA, and all enzymatic reactions stop (i.e. the extension from a previous cycle). Afterwards, the annealing of primers to ssDNA takes place at temperatures closer to their Tm (usually 55-65 °C), known as temperature of annealing (Ta). The oligonucleotides used as primers will line up, flanking the segment of target DNA to be amplified and hybridise into a dsDNA molecule. Finally, extension step takes place by using a heat-stable DNA polymerase (usually 70-74 ºC) in the presence of dNTPs and $MgCl_2$, leading to a duplication of the starting target material.[123,124]

After each cycle, the newly synthesised DNA strands can serve as template in the next cycle, as a result the number of target DNA copies is doubled in each cycle, achieving for instance, about a million (2^{20}) copies after 20 cycles, as shown in Figure 1.15.[123,124]

1.3.1.4 Concluding remarks about traditional methodologies for bacteria detection

Microbiological culture techniques are still considered the gold standard for bacteria detection. Although they are considered sensitive and simple, they are also time-consuming, laborious and might introduce sampling and enumeration errors, due to the low numbers of pathogenic bacteria in food samples.[1,8]

IAs are advantageous for decreasing the assay reaction time, as well as the enrichment step time, in comparison with microbiological culturing techniques, providing also the possibility of being easily integrated in automated equipment, which consists of an important advantage for industrial applications. Nevertheless, the efficiency of an immunoassay is strongly dependent on the antibodies affinity and specificity towards the target bacteria. The risk of antibody cross-reactions brings a disadvantage of immunological assays by increasing the possibility of false positive results or high background signals. Finally, the inability to distinguish between dead or alive cells presents another limitation of these methods.[80,125]

Due to its high sensitivity, PCR methodologies have been widely used for the identification and detection of pathogens in food samples, being considered an alternative confirmation method aside from conventional microbiological culture techniques. As occurred in the immunological assays, PCR methodologies also required an enrichment step, being able to detect, for instance in the case of *Salmonella*, few CFUs in 25 g of food product. The need of a special facilities, the presence of inhibitors for PCR in real samples and the fact that this methodology does not discriminate between live of dead cells are pointed out as the main limitations.[95,124,126]

Hence, it was shown that traditional methodologies can be sensitive for food microbiological control. However, the stricter and increased legislations and controls to implement public food safety lead mostly to a need of the development for novel rapid alternatives to screen-out food samples. In this context, the development of new methodologies with multiplexing capabilities becomes an important advantage presenting a cost effective and time saving strategy.

1.3.1.5 Emerging technologies

Immunosensors DNA biosensors and phagosensors

Over the recent years, a lot of effort has been directed into the study and development of rapid methods for foodborne pathogens, as an alternative methodology to microbiological culturing, IAs and PCR approaches.[127]

Biosensors technology has been revolutionised research conducted in food safety. These devices are available in a wide range of readout platforms, as previously stated in §1.2.2, mostly based on SPR,[128] or other optical biosensors[129] and electrochemical approaches.[130]

Most of the recent developed biosensors for pathogenic bacteria are based on the specific antigen–antibody binding reactions, where the antibody is immobilised on the sensor platform to capture the bacteria. Then, the bacteria detection is measured through electrochemical, optical, or piezoelectric readouts.[21] Moreover, genetic biorecognition is also widely used in biosensing, as well as the biorecognition through bacteriophages. All these approaches have been extensively reviewed.[127,131]

The integration of MPs in immunosensors, genosensors and phagosensors was reported, for instance, in electrochemical based approaches, improving the analytical performance in terms of LODs. A magneto-immunosensor with electrochemical readout was reported for the detection of *Salmonella* in milk. In this approach, the bacteria were captured and preconcentrated from milk samples with magnetic micro or nanoparticles through an immunological reaction. A second polyclonal antibody labelled with peroxidase was used for the electrochemical detection based on a magneto-electrode.[132,133] This strategy was able to detect $1x10^4$ CFU mL^{-1} in 1h. If the sample is pre-enriched for 8h, then, 2.7 CFU would be detected in 25 g of milk, according to the legislation.

Another approach, based on electrochemical magneto-genosensing involves the lysis of the captured bacteria after immuno or phagomagnetic separation, followed by amplification of the genetic material by PCR with a double-tagging set of primers. Then, the double-tagged amplicon was immobilised on streptavidin-modified MPs, based on a high affinity interaction though the biotin tagging the 5`end DNA of the amplicon, while the digoxigenin label was used for the enzymatic reaction. The electrochemical detection was finally achieved by an enzyme marker, such as anti-digoxigenin HRP. A LOD of 1 CFU mL^{-1} was obtained in 3.5 h without

any pretreatment. If the milk is preenriched for 6 h, the method would be able to feasibly detect as low as 1 CFU in 25 g of milk.[134,135]

The integration of the MPs improved the analytical performance of these approaches, providing the preconcentration of the bacteria during the IMS, reducing the time required for the preenrichment step and the LODs, eliminating the matrix effect of the food components and PCR inhibitors, and serving also as a platform for the electrochemical readout based on magneto-actuated electrodes.

1.3.2 Simultaneous detection of pathogenic bacteria

1.3.2.1 Nucleic acid amplification methods

Nucleic acid amplification methods such as end-point multiplex PCR (mPCR), quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) will be reviewed and compared for the simultaneous detection of *Salmonella*, *E. coli* and *Listeria*. In Table 1.4, selected studies published in the literature are shown, summarising and highlighting the most important parameters in terms of the analytical performance, including the food matrix, the time required for the preenrichment step, the total assay time and the LODs.

End-point multiplex PCR

End-point multiplex PCR (mPCR) has been widely applied to detect simultaneously multiple targets in the same amplification reaction. The efficiency of this methodology is strongly dependent on different factors, such as primers specificity, buffer, magnesium chloride and Taq DNA polymerase concentration, thermal cycling conditions and the amount of DNA template. When applied to the detection of foodborne pathogenic bacteria, a preenrichment step is required in order to enhance detection of pathogens in samples.[141,136] Recently, mPCR for the detection of five different pathogens in artificially contaminated pork samples was reported.[140,137] For instance, the simultaneous detection of *E. coli*, *Salmonella* spp. and *L. monocytogenes* by end-point multiplex PCR showed a LOD of 10 CFU in 25 g of sample within 15 h of preenrichment.[139] The detection by end-point mPCR usually requires analysis of the PCR final product on an agarose gel or on capillary electrophoresis-based DNA sequencer, being for this reason time consuming.

Table 1.4 Strategies for the simultaneous detection of foodborne bacteria based on nucleic acid amplification methods.

Assay format	Detection technique	Test matrix	Pre enrichment	Total assay time	LOD	Ref.
mPCR of *Salmonella* spp., *L. monocytogenes*, *E. coli*	Agarose gel	Several meat samples	30h	1h 10 min	1 CFU in 25 g of inoculated sample	[138]
mPCR of *E. coli*, *Salmonella* spp., *L. monocytogenes*	Agarose gel	Liquid whole egg	>15 h	1h 45 min	10 CFU in 25 g sample	[139]
mPCR of *S. aureus*, *L. monocytogenes*, *E. coli*, *S.* Enteritidis, *S. flexneri*	Agarose gel	Meat samples	24 h	~ 1h 35 min	10–17 CFU g^{-1} sample	[140]
mPCR of *S. aureus*, *L. monocytogenes*, *E. coli*, *Salmonella*, *Yersinia Entero Colitica*.	Agarose gel	Pork samples	overnight	1h 40 min	9-670 CFU mL^{-1}	[141]
q PCR of *Salmonella* spp., *E. coli*, *L. monocytogenes*	Fluorescence	Meat samples	20 h	60 min	<18 CFU in 10 g sample	[142]
q PCR of *Salmonella* spp., *E. coli*, *L. monocytogenes*	Fluorescence	Food and environmental samples	30h	55 min	5 CFU in 25 g sample	[143]
Nonaplex q PCR detection of *L. monocytogenes*, *Campylobacter*, *Salmonella*, enteropathogenic *E. coli*	Fluorescence	Yoghurt or parmesan cheese	24 h	60-90 min	2 CFU g^{-1} of food sample	[144]
LAMP amplification of *Salmonella* spp. and *Shigella* spp.	Agarose gel	Milk samples	19h	60 min	5 CFU in 10 mL of food sample	[145]
LAMP amplification of seven *E. coli* serotypes	Turbidimetry	Different food samples	6-8 h	45 min	1-2 CFU in 25 g of food sample	[146]
LAMP amplification of *Shigella*, *Salmonella*, and *Vibrio cholerae*	Colorimetric methods and turbidimetry	Pure culture bacteria	overnight	60 min	< 9.6, 48, 3.2 CFU mL^{-1}, respectively	[147]
IMS-mPCR assay for S. Typhimurium, *E. coli*, *L. monocytogenes*	Agarose gel	Lettuce, tomato and ground beef	-	4.5 h	10^3 CFU g^{-1}	[148]
IMS-qPCR assay for *Salmonella* spp., *Shigella* spp., *S. aureus*	Fluorescence	Fresh pork samples	6 h	~ 50 min	2-9.6 CFU g^{-1}	[149]

Real-time multiplex PCR

Real-time multiplex PCR (multiplex qPCR) provides the detection and quantification during the amplification process in each cycle. In this methodology, the fluorescence intensity of the amplicons is measured by either intercalation of fluorescent dyes in the double-stranded DNA or with dual-labelled fluorescent oligonucleotide probes, among others readout

strategies.[150,151] The application of multiplex qPCR for simultaneous detection has been studied for several years and it requires the use oligonucleotides tagged with different fluorophores specific for the different microorganisms to be detected. Moreover, in some instances, an internal amplification control is recommended to be added into the PCR mixture,[142,150] which consists on a non-target DNA sequence, in order to avoid false-negative results caused by inhibitors, such as phenolic compounds, fats and glycogen, that may affect different steps of the qPCR method.

Multiplex qPCR procedures are in general faster than the related end-point methodology, with similar sensitivity. Nevertheless, expensive bench top equipment and high technical requirements are the main limitation of this methodology.

Loop-mediated isothermal amplification (LAMP)

Another methodology for nucleic acid amplification as loop-mediated isothermal amplification (LAMP) has been emerging in food safety applications, being schematically represented in Figure 1.16. LAMP provides high specificity and sensibility, simple operation and low cost, under isothermal conditions, around 60-65 ºC, avoiding thus the use of thermocyclers. Briefly, a set of four or six primers are used to recognise six distinct sequences of the target DNA together with a *Bst* DNA polymerase large fragment to initiate the DNA amplification resulting on a stem-loop DNA.[152,153]

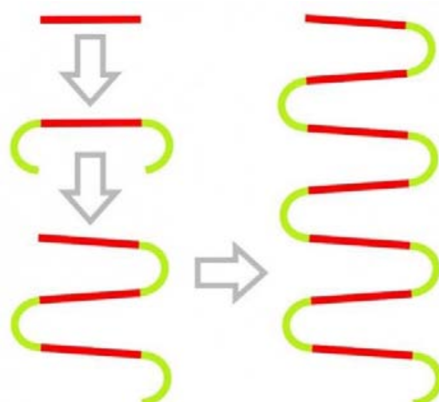


Figure 1.16 Representation of the LAMP reaction workflow. In red the starting-point of a segment of target DNA to be amplified is shown and in green the “loop” structures which are then formed. Adapted from reference [154].

The detection of the LAMP end products can be achieved by several methods, such as agarose gel, turbidity, fluorescence or intercalating dyes.[155] A comparison of LAMP with quantitative and end-point PCR was studied for *Salmonella* detection showing similar sensitivity within the three techniques. Nevertheless LAMP is highlighted for its rapidity and robustness, and for the fact that the thermocycler is avoided. The limitation to control the presence of inhibitors in the samples is pointed out as the main inconvenient.[156,157] This technique has shown to be very effective on the detection of seven different *E. coli* serotypes as O26, O45, O103, O111, O121, O145, and O157 in spiked ground beef, beef trim, lettuce, and spinach samples, being able to detect between 1-2 CFU in 25 g of food sample after 6 to 8 h of enrichment.[146] Although very few studies for the simultaneous detection of different foodborne have been reported, it is suggested that this methodology might be promising for the detection of different serotypes within the same bacteria strain. In Table 1.4, by comparing all the studies, it is suggested that LAMP assay reduces significantly the enrichment, as well as the total assay time.

Integration of MPs in PCR based methodologies

Recently, a high number of studies comprising integration of MPs with PCR based methodologies have been reported. As an example, a rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork was reported, being able to detect 10 CFU g^{-1} of all pathogens, within 6 h.[148,149] In this example, the use of MPs provided a significant decrease of the total assay time, including the preenrichment step. This strategy is already commercially available for single pathogens detection, proving the potential application of MPs in food industry.

1.3.2.2 Microfluidic devices

Microfluidic devices enable the possibility of integration of multi-step bio-assay, such as sample preparation, analyte enrichment, labelling, signal amplification and detection in the same platform device. For this reason, it is a very attractive technology, providing several advantages as portability, lower reagent consumption, rapidity and the possibility for automation.[158]

In Table 1.5, some approaches for the simultaneous detection of bacteria using based on microfluidic systems are presented and compared.

Microfluidic devices for simultaneous detection of foodborne bacteria are mostly based on nucleic acid amplification, being often combined with agarose gel electrophoresis for DNA analysis, but they can also be coupled with other platforms.[161,164] The risk of cross-contamination in the process of sample loading is pointed out as one of the main inconvenient of these systems.[159] Finally, the cost of production of some microfabricated devices still constitute a bottleneck and may put them out of range for end users.

Table 1.5 Nucleic acid amplification based microfluidic systems for the simultaneous detection of foodborne bacteria.

Assay format	Detection technique	Test matrix	Total assay time	LOD	Ref.
Microchip capillary electrophoresis for of *V. parahemolyticus*, *Salmonella*, *E. coli*, *Shigella*	Capillary electrophoresis	Pure culture bacteria	8 min	52-290 CFU mL^{-1}	[160]
Multichannel oscillatory-flow multiplex PCR for *S. enterica*, *E. coli*, *L. monocytogenes*	Agarose gel electrophoresis and Gold-View™	Milk, banana and hotdog	13 min	$(1.79 - 3.72)\times10^4$ gene copies μL^{-1}	[161]
Oscillatory-flow multiplex PCR PCR for *S. enterica*, E. *coli*, *L. monocytogenes*	Agarose gel stained with GoldView™	Pure culture bacteria	35 min	314- 626 gene copies μL^{-1}	[162]
LAMP based microfluidic chic for *Salmonella*, *C. jejuni*, *Shigella*, *V. cholerae*	Light emitting diode (LED) and charge coupled device (CCD)	Pure culture bacteria	< 20 min	10–100 gene copies μL^{-1}	[163]
Segmented continuous-flow multiplex PCR for *S. enterica*, *E. coli*, *L. monocytogenes and S. aureus*	Agarose gel electrophoresis and Gold-View™	Milk, banana, and sausage,	19 min	10^2 gene copies μL^{-1}	[164]
Paper-based microspot assay for E. *coli*, S. Typhimurium, L. *monocytogenes*	Colorimetry	Inoculated ready to eat meat	8-12 h	10^1 CFU cm^{-2}	[165]
Immunological assay for *E. coli, Salmonella, Listeria, and Shigella*	Flow cytometry	Pure culture bacteria	< 20 min	10^2-10^6 CFU mL^{-1}	[166]
Immunological assay for *S. aureus* and *S. enterica*	Fluorescence	Pure culture bacteria	10 min	61 and 800 CFU mL^{-1}	[167]

For instance, the simultaneous detection of *S. enterica*, *E. coli* and *L. monocytogenes* was reported within 35 min, with a LOD of 399, 314, and 626 gene copies per µL, respectively.[162] Another study was also reported using LAMP methodology combined with

microfluidic chip for the simultaneous detection of *Salmonella*, *C. jejuni*, *Shigella*, *V. cholerae*, having shown a LOD of 10-100 gene copies per µL in less than 20 min.[163]

A paper-based analytical device (µPAD) has been developed for the detection of *E. coli*, *S.* Typhimurium and *L. monocytogenes* in food samples. In this strategy, an enzyme−substrate pairs, specific for each bacterium were selected, each one providing a different color when in presence of the bacteria specific for.[165] An integrated system with automated IMS and processing of fluidic samples was proposed for the detection of *E. coli*, *Salmonella*, *Listeria*, and *Shigella*, being able to detect as low as 10^4, 10^2, 10^5 and 10^6 CFU mL^{-1} in less than 20 minutes.[166] Finally, a microfluidic biochip using aptamers for the dual detection of *S. aureus* and *S. enterica* was achieved in approximately 10 min, with a LOD of 61 and 800 CFU mL^{-1}, respectively.[167]

Hence, it was observed that the development of fully integrated microfluidic chips for the simultaneous detection of foodborne bacteria is still in an initial phase, presenting with promising devices for high-throughput assays.

1.3.2.3 DNA microarrays

DNA microarrays were reported for the detection and identification of several bacteria. In these arrays, DNA probes or short oligonucleotides are immobilised at fixed positions on a substrate and used to capture the target molecule through hybridisation of the amplified DNA, offering in this way higher capacity for multiplexing, as well as the possibility of miniaturisation and automation.[168,169]

Several examples of DNA microarrays for the simultaneous detection of foodborne bacteria are summarised on Table 1.6.

Table 1.6 DNA microarrays for the simultaneous detection of foodborne pathogenic bacteria.

Assay format	Detection technique	Test matrix	Total assay time	LOD	Ref
DNA microarray based PCR for 14 different bacterial strains	Chemiluminescence	Pure culture bacteria and foodborne mock samples	~ 7-8 h	10 CFU mL^{-1}	[170]
DNA microarray for 4 different bacterial strains	Fluorescence	Meat samples	~ 7-8 h + 5h enrichment	25 CFU/25 g	[171]
DNA microarray for 10 different enteropathogenic bacteria	Fluorescence	Human stool samples	10 h	10 CFU mL^{-1}	[172]
DNA microarray for 7 different bacterial strains using MPs	CCD camera	Bacterial suspension	6 h	10^3 CFU mL^{-1}	[173]
DNA microarray for 3 different bacterial strains using MPs	Chemiluminescence combined with a CCD camera	Water samples	3.5 h	< 500 CFU mL^{-1}	[174]
DNA microarray for 7 different bacterial strains	visible light-assisted signal using a CCD camera	Fishery, environment and human samples	~ 4 h	10^3 CFU mL^{-1}	[175]
DNA microarray for 4 different bacterial strains	Chemiluminescence	Chicken meat samples	7 h	10^5 CFU mL^{-1}	[176]
DNA microarray for 12 different bacterial strains	Fluorescence	Milk and meat samples	3 h + 2h enrichment	10 CFU mL^{-1}	[177]

In this context, it is emphasised that DNA microarrays provide an important advantage of bacteria screening in a high number of food samples. The detection is achieved by measuring the fluorescence intensity, which presents the limitation of being expensive and non-portable. Alternatively, colorimetric methods and biochips combined with chemiluminescent labels can be used. Therefore, a DNA microarray based on a colorimetric detection was reported using digoxigenin and biotin labelled on the DNA.[178] The simultaneous detection of *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, and *E. coli* was achieved with a LOD of 10^5 CFU mL^{-1}, without any enrichment step. However, after a preenrichment, this methodology could detect 10 and 3 CFU of *L. monocytogenes* and *Shigella* spp. respectively in 25 g of food sample.[176]

A microarray for bacteria detection combining PCR methodologies with fluorescence readout was developed for the simultaneous detection of *E. coli*, *S. enterica*, *L. monocytogenes* and *C. jejuni*, being able to detect as low as 103 CFU mL^{-1} of culture medium or food sample and 25 CFU of all pathogens in 25 g after an enrichment step.[171]

The combination of DNA microarrays with nanomaterials are also being explored as an alternative to overcome problems related to photo bleaching caused by fluorescent the organic

dyes. In this context, the identification of twelve bacterial strains, using quantum dots coated with streptavidin as fluorescent labels was achieved with a LOD of 10 CFU mL^{-1} in pure culture, without any enrichment step.[177] Furthermore, MPs can also be integrated on DNA microarrays opening the possibility of final detection based on a digital camera or a light microscopy.[174] In this context, it was reported the detection of *E. coli*, *S. enterica*, *C. jejuni*, presenting LODs of 136, 500, and 1 CFU mL^{-1}, respectively, without any enrichment procedures, using MPs as a preconcentration tool for DNA attachment (Figure 1.17).[174]

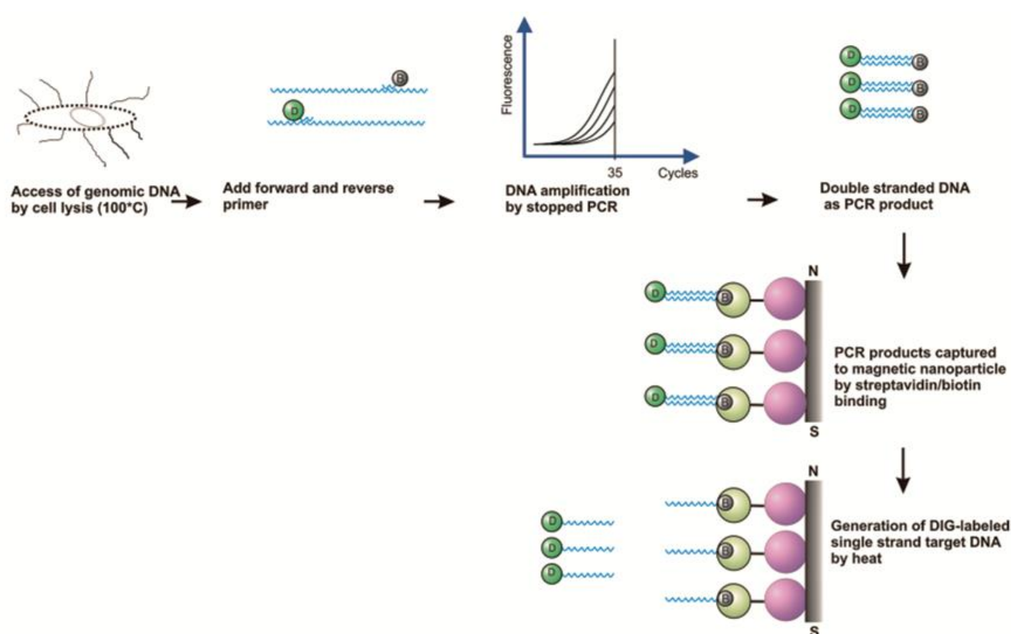


Figure 1.17 Generation of digoxigenin (D)-labelled single target DNA using MPs for the DNA Microarray Analysis. Reprinted (adapted) with permission from reference [174]. Copyright 2015 American Chemical Society.

In this study, prior to the detection, bacterial DNA was extracted and amplified by PCR reaction using a set of primers labelled with biotin (B) and digoxigenin (D). The PCR reaction was stopped at a defined cycle step and MPs modified with streptavidin were added to bind the PCR products for the strand separation, as schematically detailed in Figure 1.17. Afterwards, digoxigenin labelled to the single strain DNA target were loaded in the chemiluminescence flow-through DNA microarray readout system, in which the target DNA was covalently immobilised using specific DNA probes on the modified glass substrates in a microarray arrangement. Finally, an anti-digoxigenin HRP antibody was added for the readout in the presence of luminol and hydrogen peroxide. The generated light emission was recorded by a CCD camera.[174]

In this context, it is emphasised that DNA microarrays provide an important advantage of bacteria screening in a high number of food samples. Introduction of nanomaterials may also increase the sensitivity. Nevertheless, the microarray fabrication and also the hybridisation procedure can be time consuming, presenting the main disadvantage of this methodology.[171-178]

1.3.3 Biosensors for the multiplex detection of bacteria

1.3.3.1 Biosensors based on the integration of nanomaterials

Over the past years, a new challenge has been attracting researchers in this field, the design of novel biosensors with multiplexing capabilities, where the integration of nanomaterials plays an important role. These novel bionanomaterials, including nanostructured carbon materials, inorganic nanoparticles (i.e., semi-conducting, noble metal and magnetic nanoparticles), among others, appears to be a key factor in bacteria multiplex detection in biosensors, enhancing the biological reactions, providing high selectivity and improving the LODs.[70,179,180,181]

In this section, different biosensor strategies will be discussed. These strategies are summarised in Table 1.7, with special focus on the assay type, detection technique, food matrix, LODs and assay times, being classified according to the type of nanomaterial integrated in each approach, as metallic nanomaterials, quantum dots and magnetic particles.

Table 1.7 Biosensor platforms for the simultaneous detection of bacteria based on the integration of nanomaterials.

Assay format	Type of nanomaterial	Detection technique	Test matrix	Total assay time	LOD	Ref.
Sandwich Immunoassay for *E. coli*, *S.* Typhimurium, *L. monocytogenes*, *C. jejuni*	Gold nanolayer	Multi-channel SPR	apple juice	50 min	3.4×10^{3} to 1.2×10^{5} CFU mL^{-1}	[182]
Immunological assay for *S.* Typhimurium and *E. coli*	Au, Ag and Au-Ag core-shell NPs	SERS	Bacterial suspension	45 min	10^{2} CFU mL^{-1}	[183]
Sandwich immunoassay for the detection of *S.* Typhimurium and *E. coli*	MPs and gold NPs	Colorimetry	Milk and pineapple juice	~1 h	3-15 CFU mL^{-1}	[184]
DNA hybridisation for *L. monocytogenes* and *S. enterica*	Gold nanoparticles	Colorimetry	Milk samples	3-4 h	0.013-0.015 ng mL^{-1}	[185]
Sandwich immunoassay for *E. coli Campylobacter* and *Salmonella*	QDs	Square wave anodic stripping voltammetry	Milk samples	1 h	400 – 800 CFU mL^{-1}	[186]
Sandwich immunoassay for *V. parahaemolyticus, S.* Typhimurium	QDs and carbon NPs	FRET	Shrimp and chicken	2h 20 min	25, 35 CFU mL^{-1}	[187]
Sandwich immunoassay for *E. coli* and *S.* Typhimurium	QDs and MPs	Fluorescence	Pure bacteria culture	2 h	10^{4} CFU mL^{-1}	[188]
Sandwich immunoassay for *S.* Typhimurium, *Shigella flexneri*, and *E. coli*	QDs and MPs	Fluorescence	Apple juice and milk	2h	10^{3} CFU mL^{-1}	[189]
Sandwich immunoassay for *Listeria, E. coli* and *Salmonella*	QDs and MPs	Fluorescence	Meat and vegetables	1 h 30 min	20 –50 CFU mL^{-1}	[190]
Sandwich immunoassay for *E. coli* , *S.* Typhimurium, *L. monocytogenes*	MPs and denatured BSA labelled with fluorophores	Fluorescence	Spinach, chicken, milk	2 h	< 5 CFU mL^{-1}	[191]
DNA hybridisation for *S. aureus, V. parahemolyticus*, *S.* Typhimurium	MPs and multicolour upconversion NPs	Luminescence	Milk and shrimp samples	~1h	25, 10 and 15 CFU mL^{-1} respectively	[192]

Biosensing based on metallic nanomaterials

Metallic nanomaterials, such as gold or silver nanoparticles, as well as gold films are the most common selection for the immobilisation of biomolecules and/or signal amplification. In this context, a biosensor for the detection of *Salmonella* and *E. coli* based on Raman spectroscopy was reported, in which gold, silver and core–shell nanoprobes were coated with biomolecules specific for the bacteria and also a Raman reporter molecule to improve the LOD of the assay.[183] Afterwards, the probes with the bacteria were passed through a nanoporous membrane in order to retain the bacteria on the filter membrane. This membrane, containing probes attached to the captured bacteria were then used for Raman spectral analysis, as depicted in Figure 1.18.[183] This strategy was able to detect both bacteria with a LOD of 10^2 CFU mL^{-1} in 45 min. A multi-channel SPR biosensor for the simultaneous detection of *S.* Typhimurium, *L. monocytogenes*, *C. jejuni*, and *E. coli* based on a sandwich immunoassay was also reported, presenting LODs of from 3.4×10^3 to 1.2×10^5 CFU mL^{-1} in 50 min.[182]

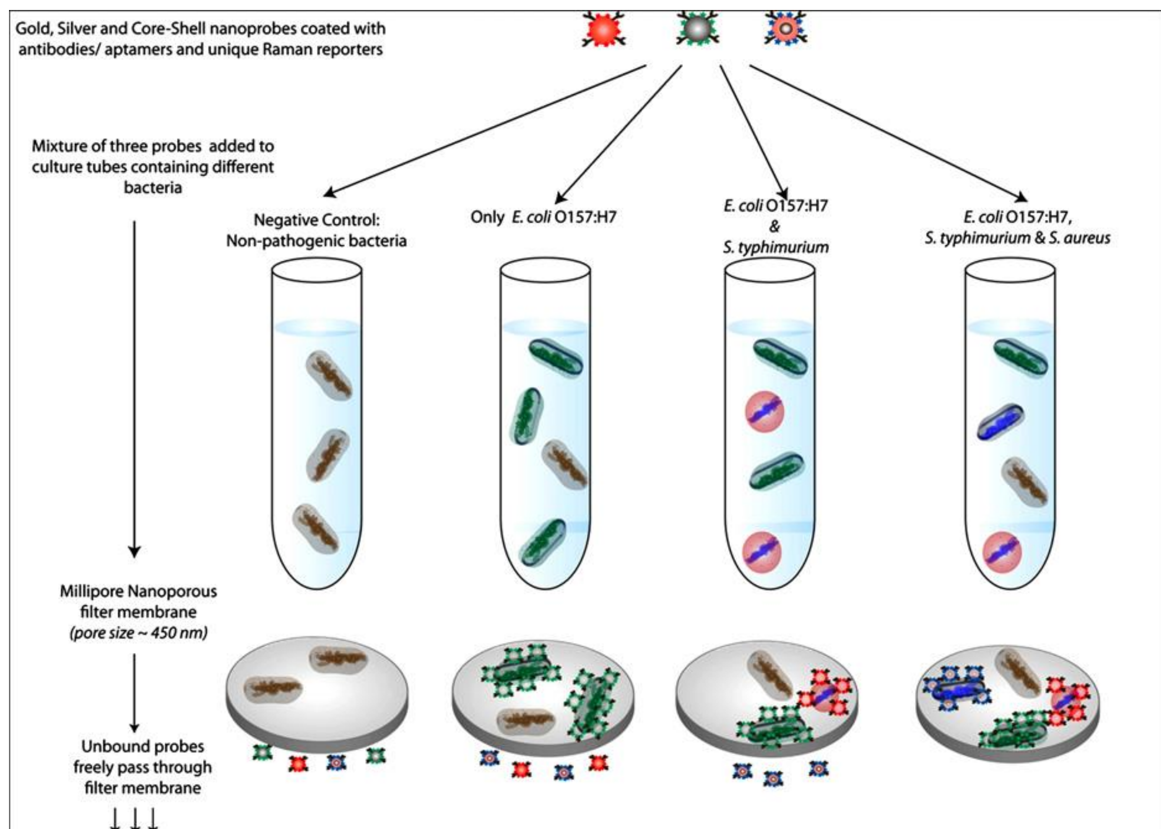


Figure 1.18 Schematic representation of the approach for the simultaneous detection of foodborne bacteria based on Raman spectroscopy. Reprinted from reference [183]. Copyright (2015), with permission from Elsevier.

Biosensing based on Quantum Dots

Semiconductor particles as quantum dots (QDs) are also a common choice in the strategies for multiplexing, either as a support for the target analyte or as a label to enhance the optical or electrochemical readout. In this context, an electrochemical immunosensor for the multiplex detection of *E. coli*, *Campylobacter* and *Salmonella* based on a sandwich immunoassay with QDs modified with three antibodies specific for each bacterium was reported with a LOD of 400 CFU mL^{-1} for *Salmonella* and *Campylobacter* and 800 CFU mL^{-1} for *E. coli* in 1h.[186] Another strategy was reported recently using aptamers specific for *Vibrio parahaemolyticus* and *S.* Typhimurium immobilised on quantum dots. The detection of bacteria was based on dual fluorescence resonance energy transfer (FRET) between QDs (QDs-apts) and carbon nanoparticles (CNPs), being able to detect 25 and 35 CFU mL^{-1} of *V. parahaemolyticus* and *S.* Typhimurium respectively within 2h 20 min. As observed in Figure 1.19, in the absence of bacterium, the interaction between QDs-apts and CNPs is very high, leading to a FRET quenching of the fluorescence from the QDs-apts and consequently providing the lowest fluorescence signal intensity. Therefore, this signal increases with the increase of the bacteria concentration.[187]

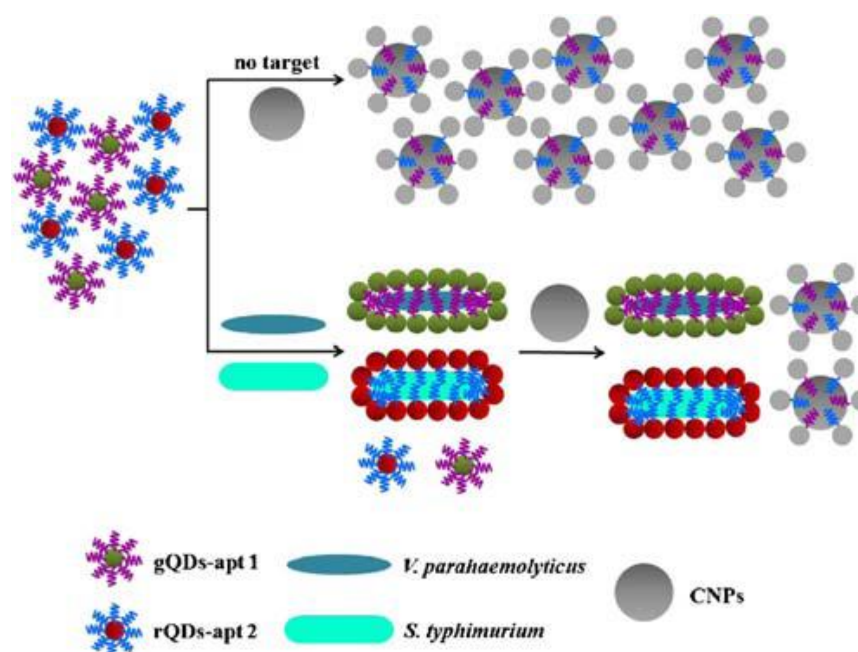


Figure 1.19 Representation of the strategy based on dual fluorescence resonance energy transfer from QDs modified with aptamers (QDs-apts) and carbon nanoparticles (CNPs). Reprinted from reference [187]. Copyright (2015), with permission from Springer.

Biosensing based magnetic particles

MPs have also been extensively combined with several biosensors platforms, especially biosensors with optical readout. In this context, some studies were reported using both MPs and quantum dots in a sandwich immunoassay. For one side, IMS of bacteria was achieved with further fluorescence detection, using different quantum dots modified with antibodies specific for each bacterium.[188,189]

Recently, an aptasensor with optical readout for the simultaneous detection of *S. aureus*, *V. parahemolyticus* and *S.* Typhimurium was reported, using MPs and multicolour upconversion nanoparticles (UCNPs), as luminescence labels (Figure 1.20).[192]

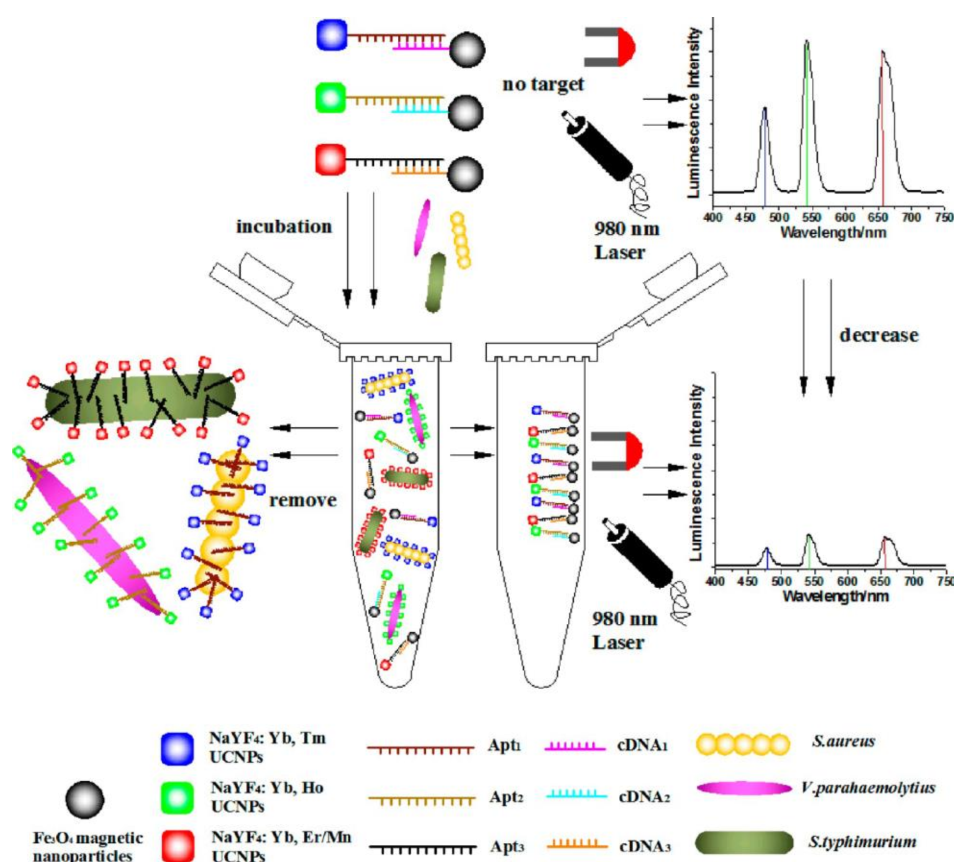


Figure 1.20 Strategy used for the simultaneous detection of *S. aureus*, *V. parahemolyticus*, and *S.* Typhimurium, using MPs and multicolour upconversion nanoparticles (UCNPs), as luminescence labels. Reprinted with permission from reference [192]. Copyright (2014) American Chemical Society.

In this strategy, multicolour UCNPs were conjugated with aptamers specific for each bacterium and hybridised with the complementary DNA sequence, which was coupled to MPs, these last providing an important advantage of improving the washing steps. These conjugates were capable of emitting strong visible luminescence with the excitation of Near-infrared (NIR) light (typically 980 nm), using a 980 nm laser, giving three independent peaks at different wavelengths for each of the three bacteria. Upon addition of the bacteria, these signals were proportionally reduced, since the multicolour UCNPs conjugated with the aptamers react with their specific bacterial target being then eliminated as a supernatant after applying a magnetic field, as shown in Figure 1.20. The remaining UCNPs-MNPs were then separated and washed three times, and the luminescence was measured with a 980 nm excitation laser.[192]

The concentration of the three bacteria was related to the corresponding emission peak of the multicolour UCNPs. The basic principle of the strategy was that aptamers could form a defined conformation when binding to the targets and were also able to hybridise to the complementary DNA sequences attached to the MPs to form a duplex structure.[192] When the targets and the complementary oligonucleotides were introduced, the aptamers preferentially bound to the targets, resulting in the specific recognition of the targets. Therefore, < 25 CFU mL^{-1} of all pathogens were detected in approximately 1h with this approach.[192]

1.3.3.2 Electrochemical Biosensors

Electrochemical immuno and genosensors have been extensively explored for food safety applications due to their high sensitivity, rapidity, low cost and possibility of being a hand-held platform for field applications.[127] Examples of multiplexed electrochemical biosensing of pathogenic bacteria with different detection platforms are showed in Table 1.8.

Few methodologies were reported using screen printed electrode based arrays, especially for the design of electrochemical genosensors. Examples of electrochemical biosensors reported in the literature include a bio-barcoded DNA assay based on gold and magnetic nanoparticles in a screen-printed carbon electrode chip was able to detect as low as 0.5 ng mL^{-1} of *S. enteritidis* and 50 pg mL^{-1} of *Bacillus anthracis* in 2.5 h.[195] Screen printed carbon arrays were also coated with multi-walled carbon nanotubes, sodium alginate and carboxymethyl chitosan composite films to enhance the sensitisation of the electrode being able

to detect 4.57×10^3 and 3.27×10^3 CFU mL^{-1} respectively for *E. sakazakii* and *E. coli* O157:H7 in 2-3 h.[197]

Table 1.8 Main features of rapid approaches for simultaneous electrochemical biosensing of foodborne bacteria.

Target	Assay format	Detection technique	Test matrix	Total assay time	LOD	Ref.
Salmonella spp., *L. monocytogenes*, *E. coli, S. aureus*	Screen-printed gold electrode arrays, PCR, hybridisation	Differential pulse voltammetry	Pure bacteria culture	1 h	5 nmol L^{-1}	[193]
S. aureus and *Salmonella choleraesuis*	Glucose determination	Flow injection amperometry	Pure bacteria culture	7 h	6.5 CFU mL^{-1}	[194]
Protective antigen A (*pagA*) gene of *B. anthracis* and the insertion element (*Iel*) gene of *S.* Enteritidis	Nanoparticle-based, bio-barcoded electrochemical biosensor	Square wave anodic stripping voltammetry	Pure bacteria culture	2 h 30 min	50 pg mL^{-1} (B. *anthracis)* 0.5 ng mL^{-1} (S. *enteritidis*)	[195]
E. coli, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Serratia*, *Providencia*, *Morganella* and *Staphylococcus* spp.	Integrated nucleic acid and protein biosensor assay	Amperometry	Urine samples	1 h	10^4 CFU mL^{-1}	[196]
E. coli, *Campylobacter* and *Salmonella*	Sandwich immunoassay	Square wave anodic stripping voltammetry	Food samples	1 h	400 – 800 CFU mL^{-1}	[186]
E. coli and *E. sakazakii*	Immunosensor arrays	Cyclic voltammetry	Pure bacteria culture	2 – 3 h	10^3 – 10^4 CFU mL^{-1}	[197]

Hence, some studies have been published in the literature about strategies based on electrochemical biosensing for the simultaneous detection of bacteria, suggesting that those remain in a preliminary stage.[127,131]

1.3.4 Commercial approaches for the simultaneous detection of foodborne bacteria

The commercialisation of novel devices for the detection of food microorganisms is based on a set of specific standards in food microbiology, such as quality control of culture media, preparation of test samples, uncertainty estimation, method validation and proficiency testing.[198]

Currently, there are an increasing number of kits commercially available for a rapid simple and reliable detection of pathogenic bacteria in food samples. In Table 1.9, selected kits, mostly for single bacteria detection are compared in terms of assay format, target pathogens, preenrichment step time, total assay time and LODs.

Table 1.9 Commercial kits for the detection of pathogenic bacteria.

Commercial kit	Assay format	Target pathogens	Preenrichment	Total assay time	LOD	Company
Assurance® EIA	Solid-phase enzyme immunoassay	*E. coli, Listeria*	18-48 h	2 h	-	Biocontrol
DuPont™ BAX® System	Quantitative PCR methodology	*E. coli*, *Salmonella*, *Listeria*, *Vibrio*, *Campylobacter*	24 h	~ 1 h	1 CFU per sample, from 25 g up to 375 g	DuPont
GeneQuence	DNA hybridisation assay	Simultaneous detection of *Salmonella*, *Listeria* spp., *L. monocytogenes*	24 h	2 h	1 CFU in 25 g	Neogen
Mericon DNA Bacteria Kit and *Mericon* DNA Bacteria Plus Kit	Quantitative PCR methodology	S. *aureus Camplylobacter* spp., *Cronobacter* spp., *Shigella* spp.	20 h	73 min	10 copies per reaction	Quiagen
Assurance GDS® MPX	IMS+ multiplex PCR methodology	*E. coli* O157:H7, O26, O45, O103, O111, O121, O145	10-18 h	~1h	-	Biocontrol
DuPontTM Lateral Flow System	Sandwich immunoassay	*Salmonella*, *Listeria*, *E. coli*	20-40 h	10 min	1 CFU in 25 g	DuPont
VIP® Gold	Sandwich immunoassay	EHEC, *Listeria*, *Salmonella*	8-18 h	10 min	-	Bicontrol
Atlas® System	Transcription-Mediated Amplification	*Listeria*, *Salmonella*, *E. coli*	24 h	2 h	-	Roka Bioscience
Pathatrix®	IMS+ PCR methodology	*E. coli*, *Salmonella*, *Listeria*	7-10 h	15 min	-	Life technologies

Commercial kits are based on different assays formats either immunological assays or PCR methodologies for the detection of several foodborne pathogens, being able to detect in general 1 CFU in 25 g of food sample as required for the legislations, after a preenrichment culturing step. Most recent kits make use of a fully automated system, which reduces the assay time, increasing the number of samples per test until 300 samples in one assay (Atlas® System, Assurance® EIA or GeneQuence). Rapid nucleic acid amplification and detection technologies have been increasingly applied to pathogen detection in food industry. In DuPont™ BAX® System, a qPCR methodology is used for the detection of several pathogens with LODs of one CFU per food sample ranged from 25 g to 375 g.

The use of nano/microsized particles on bioassays has been successfully introduced into food industry. Lateral Flow System has been commercialised by Dupont (DuPontTM Lateral Flow System) and Biocontrol (VIP® Gold), where food samples are combined with gold-colloid particles coated with antibodies specific for the target bacteria. The results are produced in only 10 min, being for this reason a suitable tool for pathogens screening.

Finally, MPs modified with antibodies specific to the target bacteria have been used for IMS as a preconcentration tool from food samples, being able to reduce substantially the time of enrichment step. IMS is usually combined with PCR methodologies, as occurred in Assurance GDS® MPX and Pathatrix®. Currently, the commercialisation of kits for the simultaneous detection of foodborne pathogens is available in a reduced number.

1.4 Conclusions

The increasing incidence of foodborne illnesses caused by pathogenic bacteria represents a serious public health concern, resulting in stricter legislation for the food safety control. *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 are among the most dangerous food-borne bacteria in terms of human illnesses, since numerous food types are susceptible to contamination by these microorganisms.[1,8,10]

Rapid detection and identification of foodborne pathogens remains an important goal for food safety resulting in loss of investments from both public and private sector annually worldwide. A considerable progress regarding food safety in EU has been done in terms of rapid and multiplexed approaches for detecting bacteria outbreaks. However, food

contamination caused by pathogenic bacteria is still a serious threat for the consumers.[10] The common strategies for the detection of pathogenic microorganisms are consisted of the gold standard conventional microbiological culturing techniques, IAs and PCR methodologies. The development of novel strategies with multiplexing capabilities is highlighted as a rapid and cost effective alternative for the detection of bacteria.[8]

Therefore, in the current state of art based on the detection methods for foodborne bacteria showed that mPCR based methodologies could detect below 10 CFU in 25 g of sample after a preenrichment step up to 30 h.[139,143] The integration of MPs with PCR based methodologies leads to a LOD of less than 10 CFU g^{-1} within 6 h.[148,149] DNA microarrays showed LODs below 500 CFU mL^{-1} when using MPs, in approximately 3.5 h, without the need of a preenrichment step.[174] In this context the development of biosensors are very promising due to their high specificity and sensitivity, which provides the detection of a broad range of analytes in complex sample matrices as food samples, with minimum sample pretreatment, based on a broad range of readout platforms.[8,21,24]

In addition, nano and micromaterials, such as nanostructured carbon materials, gold NPs, QDs, magnetic particles, among others can also be integrated with biosensors to increase the sensitivity. Therefore, nano and micromaterials can be used as a solid support for the attachment of the target analyte, as labels for the detection and/or immobilised onto tranducers.[76,78,79]

When used as a platform to immobilise biomolecules, these nano and micromaterials may need to be functionalised with suitable molecular groups to react with an intended biomolecules. Biosensors combined with nanomaterials have led to a significant improvement of the assay time and LOD. In this context, no significant differences were noticed between the different nanomaterials, however it was observed that the integration of MPs allows a significant decrease of the enrichment times, reducing thus the total assay time.[190-192]

Finally, the integration of magnetic carriers and IMS step in food safety applications for capturing the bacteria through an immunological reaction from contaminating microbiota and interfering food components introduces advantages on the analytical performance due to the preconcentration upon magnetic actuation for further testing or readout. For instance, the matrix effect is eliminated and the PCR inhibitors are avoided which leads to a decrease of the background signals. The improvement of the washing steps and the decrease of the time required for enrichment steps are also other advantages. Finally, MPs offers an attractive

support to be incorporated in magnetic actuated biosensors, microfluidic platforms or other devices.

MPs have also been successfully integrated in kits commercially available for a rapid, simple and reliable detection of single pathogenic bacteria. However, there are still only a few commercial kits available for simultaneous detection, with integrated magnetic nanoparticle functionality. As a conclusion the integration of MPs for the multiplex detection of bacteria is still in a preliminary stage, requiring further studies due to their promising features.

1.5 References

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CHAPTER 2

Objectives of this Dissertation

The main objective of this Dissertation is the design of novel electrochemical biosensing approaches for the detection of the three most relevant foodborne bacteria, in detail *S.* Typhimurium, *L. monocytogenes* and *E. coli* by the integration of magnetic particles (MPs). In this context, it was intended to improve the current state of art in biosensors with multiplex capabilities by developing a novel strategy with promising applicability for bacteria screening.

To achieve this task, during this dissertation work, developed over the last 3 years, the following specific objectives were proposed, as follows:

- Tailored bioconjugation of magnetic particles with specific antibodies towards the three aforementioned bacteria.
- Assessment of the antibodies immobilisation procedure on different magnetic platforms.
- Optimisation of the immunomagnetic separation for *S.* Typhimurium, *E. coli* and *L. monocytogenes* using tailored nano and microsized MPs and comparison with magnetic particles from commercial sources (Dynabeads®).
- Evaluation of the immunomagnetic separation by different methodologies, including microbiological culture techniques and microscopy techniques, such as Scanning Electron Microscopy (SEM) and Confocal Microscopy.
- Comparison of the performance of different MPs for the detection of foodborne bacteria in terms of actuation time, matrix effect and binding pattern.
- Comparison of magneto-actuated electrochemical immuno and genosensing biosensors for the detection of *S.* Typhimurium in milk samples in terms of the analytical performance.
- Design of a triple-tagging set of primers for the multiplex PCR amplification of the *S.* Typhimurium, *L. monocytogenes* and *E. coli*.
- Optimisation of the immobilisation of triple-tagged primers on silica magnetic particles (silica-MPs).
- Design and optimisation of a simultaneous electrochemical magneto genosensing approach based on triple-tagging multiplex amplification for the detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli*.

Chapter 3 describes the strategies for the tailored bioconjugation of magnetic particles with antibodies specific for *S.* Typhimurium, *L. monocytogenes* and *E. coli*.

A study of the IMS procedure for *S.* Typhimurium, *L. monocytogenes* and *E. coli* was discussed by comparing the performance of commercial and tailored-modified magnetic micro and nanoparticles. These studies were based on the immunological reaction time, determination of the bacteria attached on the MPs, type of MPs in the capture of the three bacteria, type of *E. coli* strain, binding pattern and bacteria concentration. Finally, matrix effect and specificity studies were also evaluated for the capture of *S.* Typhimurium.

In Chapter 4, a comparison between two strategies based on electrochemical magneto-immunosensing and genosensing for the detection of *S.* Typhimurium in milk was fully discussed, regarding the type of magnetic carrier, LODs and assay time.

In the electrochemical magneto-immunosensing approach, nano (300 nm) and micro (2.8 μm)-sized MPs, modified with anti-*Salmonella* antibody were used to capture and preconcentrate bacteria from milk samples based on an immunological reaction. Then, a second polyclonal antibody labelled with peroxidase was used for the electrochemical detection based on a magneto-electrode.

The electrochemical magneto-genosensing approach was also combined with an IMS step, being designated as IMS/Single-Tagging PCR/Electrochemical magneto-genosensing. Afterwards, the captured bacteria were lysed, followed by amplification of the genetic material by single-tagging PCR with a set of primers specific for *S.* Typhimurium *yfiR* (375 bp), labelled with fluorescein. The amplicons tagged with fluorescein amplicons in the 5´end DNA were then immobilised on silica-MPs. Finally, the electrochemical detection was achieved by an enzyme marker, such as anti-fluorescein (HRP).

In Chapter 5, the simultaneous detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* based on triple-tagging multiplex PCR and electrochemical magneto genosensing on silica-MPs was performed. A set of tagging primers were selected for the specific amplification of *yfiR* (375 bp), *hlyA* (234 bp) and *eaeA* (151 bp), being one of the primers for each set labelled

with fluorescein, biotin and digoxigenin coding for *S.* Typhimurium, *L. monocytogenes* and *E. coli*, respectively. Afterwards, electrochemical magneto-genosensing of the bacteria was achieved by using silica-MPs as a carrier and three different electrochemical reporters, specific for each pathogen.

Chapter 6 summarises the work presented in this Dissertation with relevant considerations about the improvement of the strategies presented, as well as the application of MPs on microfluidic devices as future perspectives.

Finally, the Chapter 7 is related to science communication activities, in which the publications in international journals, communications in scientific meetings, as well as the participation in workshops, organisational events and teaching activities were detailed.

CHAPTER 3

Study of immunomagnetic separation of *Salmonella, E. coli* and *Listeria* based on commercial and tailored-magnetic particles

3.1 *Introduction*

In the previous chapter (§1.2.5.5), the importance of the use of magnetic particles (MPs) for food related applications has been highlighted, especially focused on foodborne pathogens. It was also shown that these particles can be easily functionalised with different molecular groups to be conjugated with a broad range of biomolecules for the specific interaction with the target. Thus, several strategies can be used, depending on the functionalisation at the MPs surface and the biorecognition element that it is intended to be attached on the MPs.[1,2]

Covalent immobilisation of biomolecules is the most common strategy, enabling, for instance, the attachment of antibodies on the surface of MPs to bind a variety of targets as cells, bacteria, proteins, small molecules (toxins, antibiotics, pesticides) or other molecules from complex samples. After the immobilisation reaction of an antibody, it is important to evaluate both orientation of the antibodies, as well as the amount of antibody attached to the MPs.[2,3,4]

The magnetic actuation on MPs to enhance the separation and preconcentration of the target from the complex sample has been widely emphasised in this Dissertation. If the biorecognition element is an antibody which reacts with the target, this procedure is called immunomagnetic separation (IMS). In order to study the IMS of the bacteria as a target, it is very important to understand their structure, especially their cell membrane.

A bacterial cell is consisted of structural components shown in Figure 3.1, as the cell wall, which is surrounded by the cell membrane and it may contain an additional surface layer, a cytoplasmic region that contains the DNA (Chromosome and extrachromosomal elements such plasmids), ribosomes and in some cases it may also contain inclusions as granules and/or vesicles, and finally the external structures, such as capsules, flagella, and pili (or fimbriae).[5]

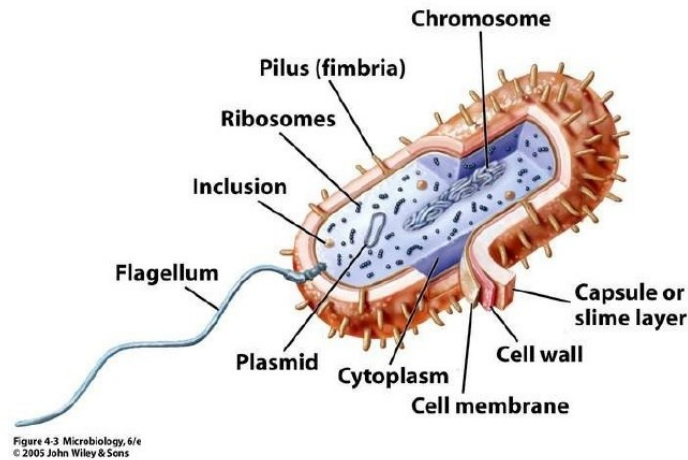

Figure 3.1 Structural components of a bacterial cell. Taken from reference [5].

Bacterial cell walls are particularly emphasised in this chapter, since they can provide many antigens of which the antibodies currently available are specific for.

Figure 3.2 shows a comparison of the bacterial cell walls for both Gram positive and Gram negative. For instance, one of the main characteristics that differentiate both Gram negative and Gram positive bacteria is the presence of an outer membrane layer in the former and consequently the thickness of peptidoglycan layer for both bacterial cells.[5,6]

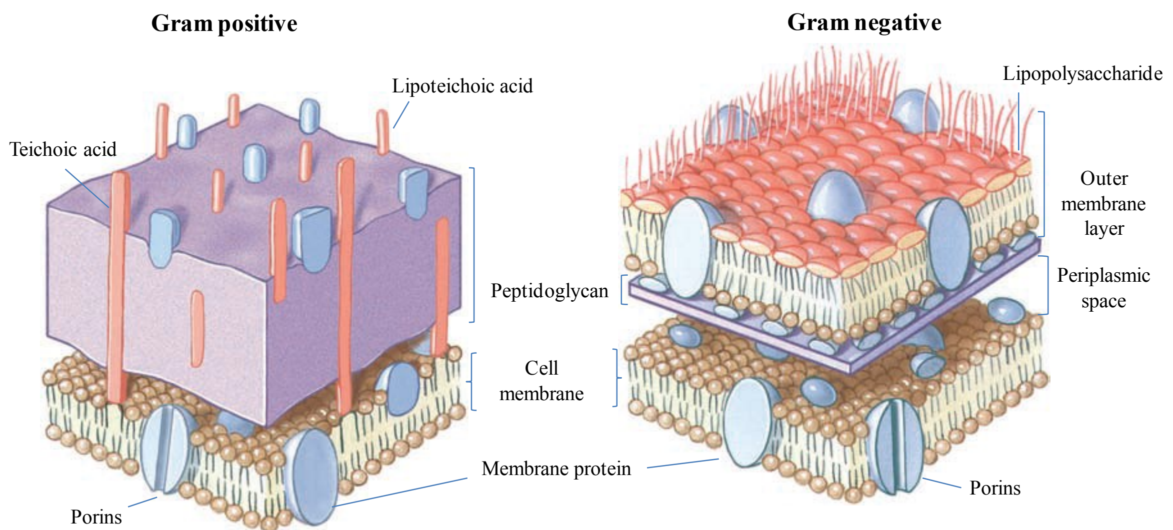

Figure 3.2 Components of Gram positive and Gram negative bacterial cells. Adapted from reference [5].

Another important feature of Gram negative bacteria, such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *V. cholerae* or *Brucella* is the presence of a complex macromolecular lipopolysaccharide (LPS) in their outer membrane, being in general absent in Gram positive bacteria with exception for *L. monocytogenes*.[7] LPS are consisted of three regions, schematically represented in Figure 3.3: i) the Lipid A structure which is inserted in the outer membrane of the bacterial cell and responsible of the endotoxic properties of LPS; ii) a polysaccharide core, covalently attached to the lipid A structure and composed of 2-keto-3deoxyoctonic acid (KDO) and heptose. Additional sugars as N-acetylglucosamine, glucose, and galactose may also be found in the core and finally iii) the O-antigen, a somatic (O) polysaccharide chains bounded to the core, responsible for the LPS immunogenicity and antigenicity.[7,8,9]

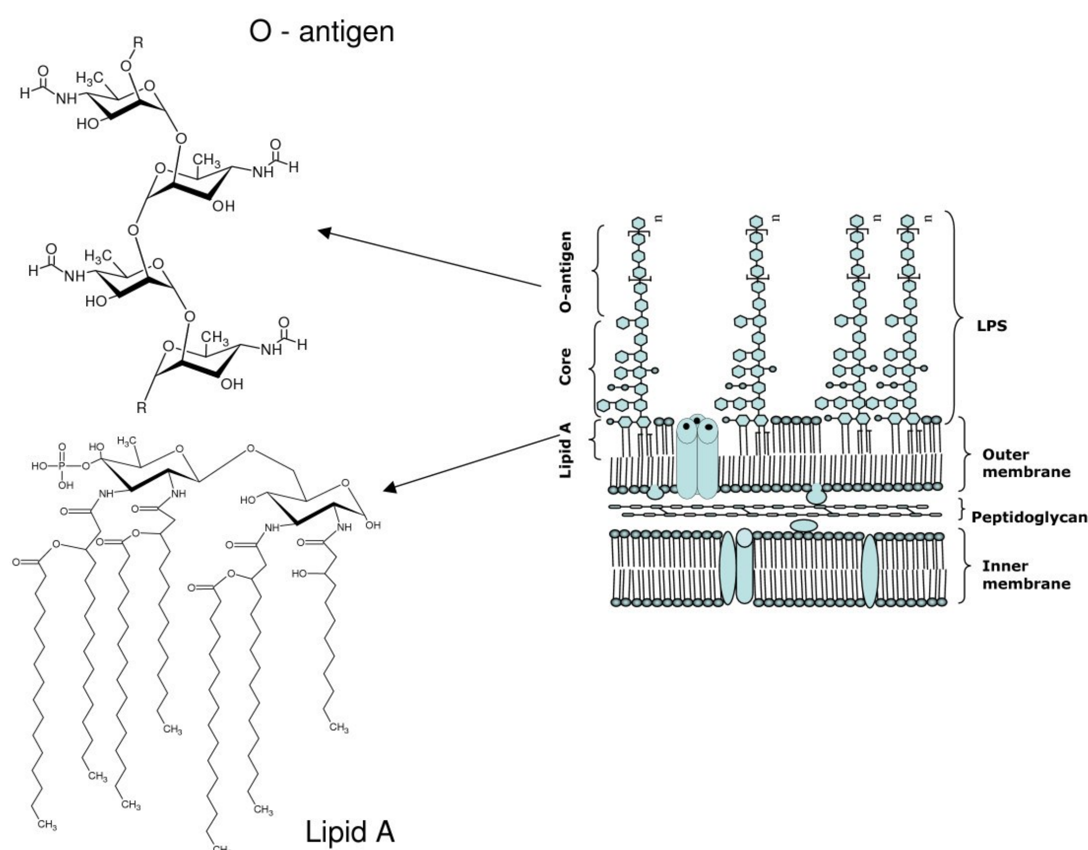

Figure 3.3 Schematic structure of lipopolysaccharide (LPS) from *Brucella* spp.

Additionally, flagella are also consisted of immunogenic proteins as flagellins, comprising a group of protein antigens named as H antigens for certain bacterial strains and serotypes, as *S.* Typhimurium, *E. coli* and *L. monocytogenes*. Moreover, capsular antigens, known as K antigens are composed of polysaccharides and can also be found on several bacteria, as *E. coli*.[7,10]

In order to perform an IMS step, the most important fact is the careful selection and immobilisation of specific antibodies towards the bacteria. In this chapter, the covalent immobilisation of different commercial antibodies specific for *S.* Typhimurium*, E. coli* and *L. monocytogenes* on the MPs will be studied. Tosylactived magnetic microparticles (tosyl-MMP) and carboxyl magnetic nanoparticles (carboxyl-MNP) from commercial sources were reacted with amine groups present in the antibody structure, as shown in Figure 1.6 (§1.2.1.2). A preactivation step of the carboxyl groups on carboxyl-MNP was needed to further bioconjugation via carbodiimide coupling. Afterwards, ELISA was performed in order to quantify the total amount of antibody immobilised on the magnetic particles.[11]

The specificity of the antibodies, as well as their orientation on the MPs after immobilisation was studied for *S.* Typhimurium, *E. coli* and *L. monocytogenes* by the IMS procedure. The IMS was comparatively evaluated on commercial and tailored-modified magnetic micro and nanoparticles (tailored-MMPs and MNPs), in this last case using different MPs modified with commercial antibodies. The binding pattern on different magnetic carriers was analysed by Scanning Electron Microscopy (SEM) and Confocal Microscopy, while the IMS performance, expressed as percentages of captured bacteria, was comparatively studied using classical culture techniques based on two methodologies. In addition, the IMS was determined upon the increase of bacteria concentration, the time of reaction and also the effect of milk samples. The specificity of the magnetic carriers was also studied in milk samples.

3.2 *Experimental Section*

3.2.1 Chemicals and Biochemicals

Dynabeads M-280 Tosylactivated (tosyl-MMP, 2.8 µm, Product Nº 142.04), Dynabeads® anti-*Salmonella* (2.8 µm, Product Nº 710.02), Dynabeads® anti-E. *coli* O157 (2.8 µm, Product Nº 710.03) and Dynabeads® anti-*Listeria* (2.8 µm, Product Nº 710.06) were purchased from Dynal Biotech ASA (Oslo, Norway). Carboxyl-Adembeads (carboxyl-MNP, size, Product Nº 0213) were kindly supplied by Ademtech (Pessac, France). Primary antibodies as mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against LPS of *S.* Typhimurium (Product Nº ab8274), rabbit polyclonal anti-*Salmonella* (biotin) (Product Nº ab 69255), goat polyclonal to *E. coli* (Product Nº ab 25823), goat polyclonal to *L. monocytogenes* (Product Nº ab 30747), rabbit polyclonal to *L. monocytogenes* (Product Nº ab 35132) were used. Goat polyclonal anti-mouse IgG HRP-conjugated antibody (Product Nº ab 20043) and rabbit polyclonal anti-Goat IgG-Heavy + Light chains HRP-conjugated antibody (Product Nº ab 6741) were used as secondary antibodies. All antibodies were purchased from Abcam (Cambridge, UK). Hydrogen Peroxide and TMB (3,3',5,5'- tetrametylbenzidine) solutions used for optical measurements (TMB Substrate Kit, Reference no. 4834021) were purchased from Pierce (Rockford, USA). The nucleic acid stain Hoechst 33342 and the streptavidin labelled with cyanine 5 (Strep-Cy5) dye used in confocal microscopy were purchased from Life Technologies (product no. H-3570 and SA-1011, respectively). Table 3.1 summarises the different MPs tested from commercial sources, the antibodies used for the tailored-MMPs and MNPs, as well as the bacterial strains referred in this chapter.

Table 3.1 Bacterial strains and reagents from commercial sources, including MPs and antibodies used for the IMS studies based on microbiological culture techniques.

Target (bacterial strain)	MMP from commercial sources (2.8 µm)	Specific antibody (host / clonality / immunogen / commercial source) immobilised on tailored-MMs and MNPs	
		tailored-MNPs (300 nm) performed on carboxyl-MNP (Ademtech, Nº 0213)	**tailored-MMPs (2.8 µm)** performed on Tosyl-MMP (Dynal, Nº 142.04)
S. Typhimurium	Dynabeads® anti-*Salmonella* (Dynal, Nº 710.02)	mouse / monoclonal IgG1/LPS of *S.* Typhimurium / Abcam, Nº ab 8274	
E. coli DH5α	Dynabeads® anti-*E. coli* O157 (Dynal, Nº 710.03)	goat / polyclonal / *E. coli* from K12, DH5α and other *E. coli* strains / Abcam, Nº ab 25823	
L. monocytogenes	Dynabeads® anti-*Listeria* (Dynal, Nº 710.06)	goat / polyclonal / *L. monocytogenes* whole cells / Abcam, Nº ab 30747	
		rabbit / polyclonal / *L. monocytogenes* whole cells / Abcam, Nº ab 35132	

All buffer solutions were prepared with Milli-Q water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions was: MES buffer (0.1 mol L^{-1} [2-(N-(morpholino)ethanesulfonic acid)], 0.9 % (w/v) sodium chloride, pH 4.7); borate buffer (0.1 mol L^{-1}, pH 8.5); ammonium sulphate buffer (3 mol L^{-1} in borate buffer); phosphate blocking buffer (10 mmol L^{-1} sodium phosphate, 0.5% w/v BSA, pH 7.4); phosphate storage buffer (10 mmol L^{-1} sodium phosphate, 0.1% w/v BSA, 0.01 % (w/v) sodium azide, pH 7.4); PBS (10 mmol L^{-1} sodium phosphate, pH 7.4); PBS casein solution (10 mmol L^{-1} sodium phosphate, 1 % casein, pH 7.0); PBS 3% BSA (0.01 mol L^{-1} sodium phosphate, 3 % w/v BSA, pH 7.4); sulphuric acid solution (2 mol L^{-1}); PBST (10 mmol L^{-1} sodium phosphate, 0.8 % w/v NaCl, 0.05 % v/v of Tween 20, pH 7.4), PBST 2 % BSA (10 mmol L^{-1} sodium phosphate, 0.8 % w/v NaCl, , 0.05 % v/v of Tween 20, 2 % w/v BSA, pH 7.4), PBST 0.1 % casein (10 mmol L^{-1} sodium phosphate, 0.8 % w/v NaCl, pH 7.4, 0.05 % v/v of Tween 20, 0.1 % casein).

3.2.2 Bacterial strains

The bacterial strains used in this work were *S.* Typhimurium LT2 (ATCC® 700720™), *E. coli* DH5α (Clontech), *E. coli* K12 (MC4100, MG1655 from The Coli Genetic Stock Center) and *L. monocytogenes* ATCC 15313 (DSMZ). In addition, an *E. coli* DH5α strain carrying a pGEMT vector containing the *eaeA* gene was used to perform the specificity studies. *S.* Typhimurium and *E. coli* were grown at 37°C in Luria-Bertani (LB) agar plates and *L. monocytogenes* in BHI agar plates (Oxoid). All bacterial suspensions were performed in Brain Heart Infusion (BHI) (Oxoid) from freshly grown plates and its turbidity was adjusted at OD_{600} of 0.2 A.U. The exact concentration of each bacterial suspension was quantified by plating serial dilutions onto LB plates for *S.* Typhimurium and *E. coli* and BHI plates for *L. monocytogenes*.

3.2.3 Instrumentation

All incubations and washing steps were performed on microtiter plates under shaking conditions using a Minishaker MS1 (IKA, Germany). Temperature-controlled incubations with Eppendorf tubes were performed in an Eppendorf Thermomixer compact. Magnetic separation during the washing steps was performed using a magnetic separator Dynal MPC-S (Product Nº 120.20D, Dynal Biotech ASA, Norway). Polystyrene MaxiSorp microplates were purchased from Nunc (Catalogue no. 442404, Roskilde, DK). Optical measurements were performed on a TECAN Sunrise microplate reader operated with the Magellan v4.0 software.

3.2.4 Tailored covalent immobilisation of antibodies on magnetic micro and nanoparticles

Different antibodies specific for *S.* Typhimurium, *E. coli* and *L. monocytogenes* were covalently coupled on both MPs, tosyl-MMP (2.8 µm, 30 mg mL^{-1}, 2 x 10^9 MP mL^{-1}) and carboxyl-MNP (300 nm, 30 mg mL^{-1}, 1 x 10^{12} MP mL^{-1}), as described in Table 3.1. The outline of the procedure is schematically represented in Figure 3.4. After the immobilisation, the

supernatant was collected for the determination of the total amount of antibody immobilised on the magnetic particles by ELISA, as it will be further explained in §3.2.5. The tailored-MPs were used for downstream studies, including the performance of the IMS of the bacteria strains upon different parameters by classical culture, SEM and Confocal Microscopy.

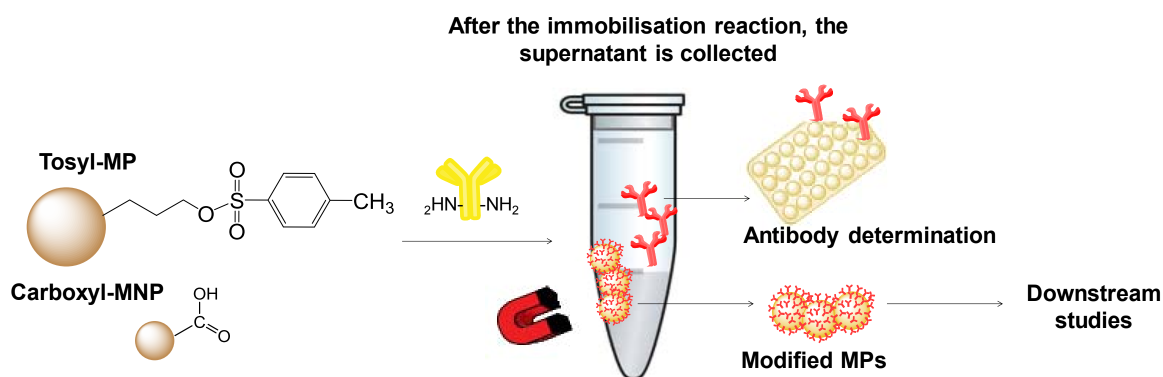

Figure 3.4 Schematic representation of the tailored covalent immobilisation of antibodies on magnetic micro and nanoparticles.

3.2.4.1 Covalent immobilisation of antibodies on tosyl-MMP

A volume of 35 µL of tosyl-MMP was washed twice with 1 mL of borate buffer, avoiding foaming. Afterwards, 20 µg of antibody was added in borate buffer performing a total volume of 140 µL. Then, 100 µL of ammonium sulphate buffer were added to the antibody solution.

MPs were incubated with stirring for a total reaction time of 18 h at 37 ºC. After incubation, the supernatant was collected to perform the quantification of the remaining protein by ELISA, as shown in Figure 3.4. Then, 1 mL of phosphate blocking buffer was added to tosyl-MMP and incubated under shaking for 2 h at 37 ºC, in order to block the remaining tosyl groups.

Finally, the tailored-magnetic microparticles (tailored-MMPs) were washed and resuspended in phosphate storage buffer to reach a concentration of 5.0 mg mL^{-1} and were stored at 4 ºC for further use. Before each assay, the tailored-MMPs were washed twice and

resuspended in PBST buffer. Afterwards, the modified MPs were plated in BHI agar plates to evaluate possible microbiological contamination.

3.2.4.2 Covalent immobilisation of antibodies on carboxyl-MNP

Before antibody immobilisation, carboxyl-MNPs were activated as follows: 35 μL of carboxyl-MNP were resuspended and washed twice in 1 mL of borate buffer.

Then, 4 mg mL^{-1} of 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC) and 8 mg mL^{-1} of *N*-hydroxy-sulfosuccinimide (sulfo-NHS) were added at a final volume of 80 μL in MES buffer and incubated under shaking at room temperature (RT) during 8 h.

For the covalent immobilisation, a volume of 35 μL activated carboxyl-MNP following the same experiment conditions described in §3.2.4.1. The resulting particles were named as tailored-magnetic nanoparticles (tailored-MNPs).

3.2.5 Determination of the total amount of antibody immobilised on tailored-magnetic particles by ELISA

In order to evaluate the total amount of antibody towards *S.* Typhimurium, *L.* monocytogenes and *E. coli* attached on the MPs, the concentration of antibody in the supernatant before and after the immobilisation step was determined by Enzyme-linked immunosorbent assay (ELISA). In this context, serial dilutions of the supernatant collected during the covalent immobilisation (as shown in Figure 3.4) were adsorbed on a 96-well microplate overnight at 4 ºC. The outline of the procedure is schematically represented in Figure 3.5.

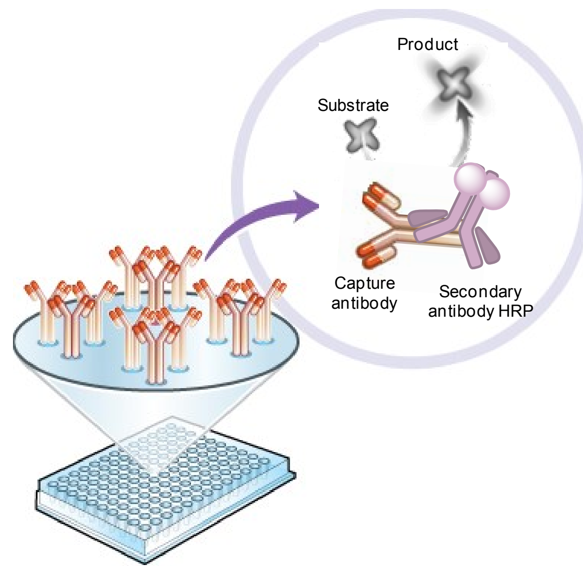

Figure 3.5 Schematic representation of the ELISA for the determination of the total antibody coupled on the modified MPs. (Adapted from Abcam website, www.abcam.com)

As an example, Table 3.2 shows in detail the experimental procedure for the determination of the total amount of antibody towards *Salmonella* immobilised on tailored-MPs by ELISA.

Table 3.2 Experimental procedure of the ELISA for the determination of the total amount of antibody (towards *Salmonella*) immobilised on MPs.

Experimental procedure	**mouse / monoclonal IgG1/LPS of *S.* Typhimurium / Abcam, Nº ab 8274**
Coating of the specific antibody	0 to 0.10 µg mL^{-1} in 1.25 mol L^{-1} ammonium sulphate dissolved in 54.2 mmol L^{-1} borate buffer.
Washing step (3X)	200 µL of phosphate buffer, shaking, RT, 5 min
Blocking step	200 µL of PBS 3% BSA, shaking 450 rpm, RT
Washing step (3X)	200 µL of PBS, shaking, RT, 5 min
Enzymatic labelling	100 µL of anti-mouse (HRP), diluted 1/3000 in PBST 2% BSA, shaking, RT, 30 min
Washing Steps	200 µL of PBS, shaking, RT, 5 min
Colorimetric reaction	100 µL TMB/H_2O_2 in the proportion of 1:1, shaking, RT, 30 min
Reaction stop	100 µL H_2SO_4 (2 mol L^{-1})

Therefore, a calibration curve, obtained by plotting Abs vs. concentration (µg mL^{-1}), was prepared with serial dilutions of mouse monoclonal to *S.* Typhimurium from 0 to 0.10 µg mL^{-1} in 1.25 mol L^{-1} ammonium sulphate dissolved in 54.2 mmol L^{-1} borate buffer.

A similar procedure was performed for the determination of the total amount of antibodies specific for *E. coli* and *L. monocytogenes*, with the exception of the blocking agent used in the buffer solution, which in this case was casein blocking buffer, due to cross-reactions observed between anti-goat HRP-conjugated antibody and BSA. Being both goat antibodies, the same calibration curve was applied for the calculation of the total antibody amount immobilised on the tailored-MPs specific for *E. coli* and *L. monocytogenes*.

3.2.6 Immunomagnetic separation of the bacteria on tailored-micro and nano sized magnetic particles

The IMS involves the reaction of the magnetic carriers with the bacteria, throughout an immunological reaction, performed in solution. After the reaction, the separation and preconcentration of the bacteria is achieved under magnetic actuation, as schematically shown in Figure 3.6.

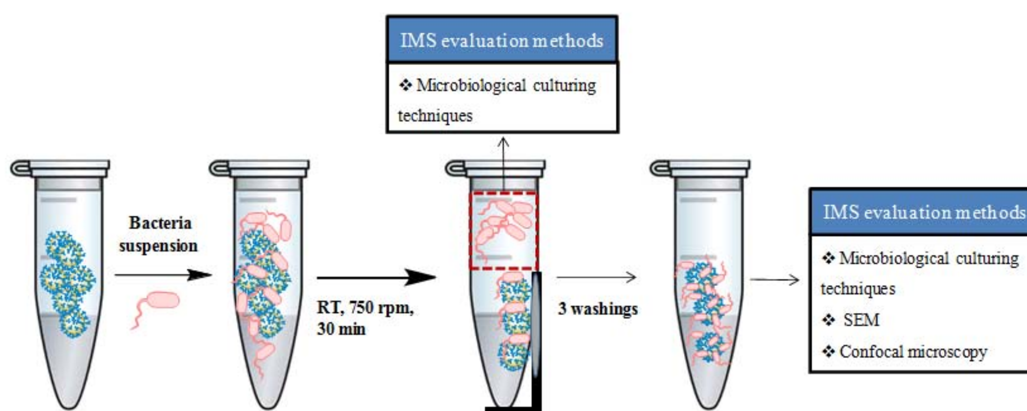

Figure 3.6 Representation of the IMS procedure and downstream evaluation methods.

The general procedure for the IMS was performed as follows: a volume of 10 μL of MPs (particles stock concentration of 5 mg mL^{-1}, including commercial and tailored-MMPs and MNPs) was added to 500 μL of a bacterial suspension and reacted for 30 min at RT and 700 rpm. Afterwards, the bacteria attached to the MPs were separated with a magnet and the supernatant was collected for further studies, as depicted in Figure 3.6. Then, MPs were washed with PBST (X3) under shaking for 1 min at RT and 700 rpm. Finally, the collected modified MPs were resuspended in 110 μL of PBST for further studies.

The IMS was fully studied and characterised based on microbiological culturing techniques, SEM and confocal microscopy.

Different parameters of the IMS were evaluated by conventional culture methods, including the reaction time, type of magnetic carrier, size effect of tailored-MPs on the capture of bacteria, as well as bacteria concentration. Finally, the IMS was also study in complex matrixes including milk samples. SEM and confocal microscopy were also performed in order to study and characterise comparatively the tailored-MMP and MNPs in the IMS, as outlined in Figure 3.6.

3.2.7 Evaluation of the IMS by microbiological culture techniques

The quantification of the bacteria attached to the tailored-MPs was performed by microbiological culture techniques. After the IMS procedure described in §3.2.6, the supernatant was collected for plating on LB for *S.* Typhimurium and BHI agar for both *E. coli* and *L. monocytogenes* and grown for 18-24 h at 37 °C, as depicted in Figure 3.6. Then, the tailored-MPs, with the bacteria attached, were washed and resuspended for plating, on LB or BHI agar for 18-24 h at 37 °C.

Table 3.1 summarises the bacterial strains, tailored-MPs and capture antibodies studied in the IMS studies based on microbiological culture techniques.

First of all, preliminary studies were performed by comparing two different methods for the determination of the IMS efficiency, either by plating directly the MPs with the bacteria attached or the unbound cells remaining in the supernatant, using an amount of 3.33 x 10^6 and

1.83 x 10^9 of tailored-MMPs and MNPs, respectively and a *S.* Typhimurium concentration of approximately 10^4 CFU mL^{-1}.

Afterwards, different parameters of the IMS were studied by microbiological culture techniques. The optimisation of the reaction time was firstly performed, using tailored-MMPs for *S.* Typhimurium as a model. To achieve this, the IMS step was performed at different reaction times, including 10, 20, 30, 45 and 60 min for a fixed *S.* Typhimurium concentration in the range of 10^4 CFU mL^{-1}. Afterwards, the supernatant was collected and plated as described above, to evaluate the IMS efficiencies obtained at different reaction times.

Another study shown in this section, was the comparison of the IMS efficiency of *S.* Typhimurium, *E. coli* and *L. monocytogenes* using commercial and tailored-MMPs and MNPs. The IMS of *E. coli* was achieved for different bacterial strains as K12 and DH5α strains. Thus, in this study, fixed concentrations of bacteria and MPs were used. For instance, the IMS was studied for concentrations of approximately 10^4 CFU mL^{-1} for both *S.* Typhimurium and *L. monocytogenes* and 10^5 CFU mL^{-1} for *E. coli* (K12 and DH5α strains). Relatively to the amount of MPs, a volume of 10 μL of all MPs, including commercial and tailored-MMPs and MNPs) (stock concentration of 5 mg mL^{-1}). After the IMS reaction, the supernatants were plated as described above. In this context, a comparison of the IMS efficiencies given by the different MPs was evaluated and the results are shown separately for each bacterium.

3.2.8 Evaluation of the IMS by scanning electron microscopy and confocal microscopy

SEM was used for evaluation of the IMS efficiency and the binding pattern of the bacteria. Therefore, the IMS procedure, as described in §3.2.6, was performed at a bacteria concentration of 10^6 CFU mL^{-1} in BHI broth (as well as a negative control).

In this context, a volume of 10 μL of MPs (particles stock concentration of 5 mg mL^{-1}, including commercial and tailored-MMPs and MNPs) was added to 500 μL of a bacterial suspension and BHI broth and reacted for 30 min at RT and 700 rpm. Afterwards, MPs were washed with PBST (X3) under shaking for 1 min at RT and 700 rpm. Then, the tailored-MPs, with the bacteria attached, were collected under magnetic actuation to be resuspended in 4 mL

of Milli-Q water and then filtered through a Nucleopore membrane as a support for the SEM microscopy. Afterwards, the filters were fixed by immersing them in 3 mL of a 3 % glutaraldehyde buffer for 2 hours at 4 ºC. Four washing steps were performed for 10 min, in 0.1 mol L^{-1} phosphate buffer pH 7.4. Then, the filters were post-fixed in 1 % w/v OsO_4, 0.1 mol L^{-1} phosphate buffer, pH 7.4 for 2 hours at 4 ºC. Four washing steps in phosphate buffer for 10 min each were then performed. Subsequently, 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 metallization with gold in order to improve the electric conductivity and the electron emission.

SEM images were recorded with the scanning electron microscope Hitachi LTD S-570 (Hitachi LTD, Tokyo, Japan) and MERLIN FE-SEM (ZEISS, Germany). The samples were coated with a thin gold film using an E5000 Sputter Coater Polaron Equipment Limited (Watford, UK) and processed by the K850 Critical Point Drier Emitech (Ashford, UK).

Confocal microscopy imaging was used for the study of the binding pattern of *S.* Typhimurium on tailored-MMPs and MNPs. The procedure was based on the following steps: 1 mL of 2.29 x 10^6 CFU mL^{-1} of *S.* Typhimurium (dilutions prepared in PBS buffer) were incubated with 4.5 µL Hoechst dye, for 20 min at 500 rpm and RT. Afterwards, 200 µL from the previous solution were mixed with 10 µL of tailored-MPs (5 mg mL^{-1}), for the IMS during 30 min at RT and 750 rpm. The particles were then washed twice and collected for further reaction with 200 µL of anti-*Salmonella*/Biotin (diluted 1/4000 in PBST 2% BSA) for 30 min at RT. After that, MPs were washed twice and further reacted with 200 µL of Strep-Cy5 (diluted 1/500 in PBST 2% BSA) for the enzymatic labelling during 30 min at RT. Finally, after two washing steps, the MPs were resuspended in 200 µL of PBST.

The confocal images were collected on the microscope Leica, TCS SP5. The laser lines used were for Hoechst (405 nm excitation, 415–570 nm emission) and for cy5 (633 nm excitation, 650-785 nm emission).

3.2.9 Study of the IMS efficiency at different bacteria concentrations by microbiological culture techniques

In this study, a comparison the IMS efficiencies of the three bacteria, *S.* Typhimurium, *E. coli* and *L. monocytogenes* was reported for different bacteria concentrations, using the MPs that provided higher IMS efficiencies based on the previous studies. A volume of 10 μL (5 mg mL^{-1}) of commercial and tailored-MMPs and MNPs specific for *S.* Typhimurium, *E. coli* and *L. monocytogenes* were added to 500 μL of several serial dilutions of bacterial suspensions ranged from 0 to 10^5 CFU mL^{-1}. The IMS was performed as optimised for 30 min at 700 rpm. All supernatants collected after the IMS were plated on LB for *S.* Typhimurium and *E. coli* and BHI agar for *L. monocytogenes* and grown for 18-24 h at 37 °C.

3.2.10 Study of the IMS efficiency in milk samples by microbiological culture techniques

The study of IMS efficiency in milk samples was evaluated for *S.* Typhimurium in three different media, such as i) BHI, ii) whole milk diluted 1/10 in BHI broth and iii) plain whole milk. In this context, a volume of 10 µL (5 mg mL^{-1}) tailored-MMPs and MNPs were incubated with suspensions of *S.* Typhimurium for concentrations in the range of 10^5 and 10^3 CFU mL^{-1}, as well as negative controls prepared in the aforementioned media. The IMS was performed for 30 min at 700 rpm. Afterwards, the bacteria attached to the particles were separated with a magnet and the supernatant was collected for plating on LB or BHI agar for 18-24 h at 37 °C.

3.2.11 Specificity study in milk samples by microbiological culture techniques

In this study, a volume of 10 μL (5 mg mL^{-1}) tailored-MNPs were incubated with suspensions prepared in whole milk diluted 1/10 in BHI broth of (i) *E. coli* (2.28 x 10^5 CFU mL^{-1}), (ii) *L. monocytogenes* (5.61 x 10^5 CFU mL^{-1}) and (iii) *S.* Typhimurium (6.28 x 10^5 CFU mL^{-1}, as a positive control), as well as mix solutions containing (iv) 3.14 x 10^5 CFU mL^{-1} of *S.*

Typhimurium and 1.14 x 10^4 CFU mL^{-1} of *E. coli* and (v) 3.14 x 10^5 CFU mL^{-1} of *S.* Typhimurium and 2.80 x10^5 CFU mL^{-1} *L. monocytogenes*. The IMS was performed for 30 min at 700 rpm. Afterwards, the bacteria attached to the MPs were separated with a magnet and the supernatant was collected for plating. Then, MPs were washed with PBST (X3) shaking for 1 min at RT. Finally, the collected tailored-MPs were resuspended in 30 μL of Milli-Q water for PCR studies that will be further discussed in §4.2.8.4. All the supernatants collected after the immunological reaction were plated on different media, such as BHI, XLD and Palcam agar and grown for 18-24 h at 37 °C.

3.3 *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.[12] According to these guidelines, the experiments involving *S.* Typhimurium, *E. coli* and *L. monocytogenes* 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.

3.4 *Results and Discussion*

3.4.1 Tailored covalent immobilisation of antibodies on magnetic micro and nanoparticles

The immobilisation procedure of the antibodies on the magnetic carriers is a critical step, since both the orientation and the coupling efficiency of the antibodies will determine the analytical performance of the IMS of the bacteria. In this context, antibodies specific for *S.* Typhimurium, *E. coli* and *L. monocytogenes* were covalently immobilised on both magnetic carriers, tailored-MMPs and MNPs, as detailed in Table 3.1, based on the amine groups present in the antibody structure, as shown in Figure 1.6 (§1.2.1.2). A solution of EDC and sulfo-NHS were used to activate the carboxyl groups on the surface of carboxyl-MNP. This reaction creates an active ester, which binds to the primary amine groups of the native antibody. Tosyl groups present on the surface of Tosyl-MMP reacted directly throughout the amine groups on the native antibody.

Although a different chemistry was used for the attachment of the antibodies on tailored-MMPs and MNPs, the same addressable moieties on the antibodies were used for the covalent immobilisation, in particular primary amine groups from basic amino acids residues (mostly lysine and arginine). As a result, no significant differences in the orientation of the antibodies are expected for the same antibody immobilised on the different MPs. On the contrary, the coupling efficiency should be determined to evaluate the amount of antibody immobilised the MPs, as it will be explained in the next section.

3.4.2 Determination of the total amount of antibody immobilised on tailored magnetic particles by ELISA

After the covalent coupling of the antibodies on tosyl-MMP and carboxyl-MNP, the determination of the total amount of antibody immobilised on the tailored-MPs was performed by ELISA. Furthermore, the coupling efficiency was then calculated, by comparing the concentration of the antibody presented in the supernatant before (Ab_i) and after the immobilisation step (Ab_S).

The absorbance values obtained for each supernatant dilution were fitted in the dynamic range of the calibration curve, for the calculation of the antibody concentration (μg/mL) of each sample. Afterwards, the total immobilisation was calculated in percentages based on the following formula:

$$Immobilisation\ (\%)\ \frac{|Ab_i| - |Ab_s|}{|Ab_i|} \times 100 \qquad \text{Equation 3.1}$$

An example of calibration curve is shown in Figure 3.7 for the mouse monoclonal IgG1 antibody against *S.* Typhimurium (Abcam, Nº ab 8274). Similar calibration curves were obtained with known concentrations of the each specific antibodies for *E. coli* and *L. monocytogenes*.

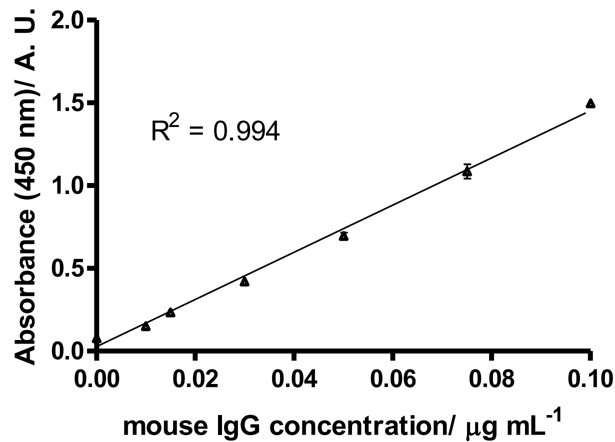

Figure 3.7 Calibration curve performed in 1.25 mol L^{-1} ammonium sulphate dissolved in 0.0542 mol L^{-1} borate buffer for ELISA using mouse monoclonal IgG1 antibody against *S.* Typhimurium, ranging from 0 to 0.10 μg mL^{-1}. The error bars show the standard deviation for n = 3.

The calculation of the total amount of antibody immobilised on tosyl-MMP and carboxyl-MNP, expressed in percentages and relative standard deviation values (RSD) are presented in Table 3.3.

Table 3.3 Total amount of antibody immobilised on tailored-MMPs and MNPs, expressed in percentages and RSD values.

Tailored-MPs	Specific antibody (host / clonality / immunogen / commercial source)	Target bacteria	Number of tailored-MPs	Concentration of antibody found in SPN /μg mL^{-1}	Total antibody immobilised % (RSD)
MMPs	mouse / monoclonal IgG1/LPS of S. Typhimurium / Abcam, Nº ab 8274	*S.* Typhimurium	7.0x10^{7}	0.46	99.4 (0.2)
MNPs			3.8x10^{10}	3.7	94.0 (1.8)
MMPs	goat / polyclonal / *E. coli* from K12, DH5α and other *E. coli* strains / Abcam, Nº ab 25823	*E. coli* (DH5α, MC 4100 and MG 1655)	7.0x10^{7}	0.65	96.9 (4.2)
MNPs			3.8x10^{10}	0.075	99.1 (0.9)
MNPs	goat / polyclonal / *L. monocytogenes* whole cells / Abcam, Nº ab 30747	*L. monocytogenes*	3.8x10^{10}	11	88.4 (2.6)

A mean percentage of 99.4 (RSD = 0.2) and 94.0 (RSD = 1.8) of mouse monoclonal anti-*Salmonella* antibody immobilised on tailored-MMPs and MNPs were respectively obtained. Goat polyclonal antibodies to *E. coli* were immobilised on tailored-MMPs and MNPs with a mean percentage of 96.9 (RSD = 4.2) and 99.1 (RSD = 0.9) and finally, tailored-MNPs modified with goat polyclonal anti-*L. monocytogenes* antibodies presented a mean immobilisation percentage of 88.4 (2.6). Hence, immobilisation of antibody specific for *S.* Typhimurium, *E. coli* and *L. monocytogenes* was achieved high efficacy and the tailored-MPs were then used for the IMS of the three bacteria.

3.4.3 Evaluation of the IMS by microbiological culture techniques

The IMS step, as it was previously explained, involves the reaction of the magnetic carriers with the bacteria, throughout an immunological reaction, performed in solution. After the reaction, the separation and preconcentration of the bacteria is achieved under magnetic actuation, as schematically shown in Figure 3.6. However, in order to evaluate the IMS efficiencies, preliminary studies were performed by using two different methodologies in order to define which methodology allows a more accurate calculation of the IMS efficiency.

The first one was based on the direct plating of the tailored-MPs with the bacteria attached, while the second one was consisted of the plating of unbound cells remaining in the supernatant, as it will be discussed in §3.4.3.1.

After that, different parameters of the IMS were studied by microbiological culture, including the time of the immunological reaction (§3.4.3.2) and a comparative study of the IMS efficiency given by commercial and tailored-MMPs and MNPs (§3.4.3.3). In both cases, the efficiency of the IMS for the different parameters was expressed as a percentage (% IMS) and calculated by comparing the amount of the bacteria counted in the initial bacterial suspension and in the supernatant collected after the IMS step, as it will be explained in §3.4.3.1, Equations 3.2 and 3.3.

3.4.3.1 Preliminary study of the IMS efficiencies calculated by platting the supernatants or the magnetic carriers

The determination of the IMS efficiencies based on microbiological plating can be achieved either by plating directly the particles with the bacteria attached or the unbound cells remaining in the supernatant, as explained in Figure 3.8.

In this section, it is intended to evaluate the most accurate method for the calculation of the IMS efficiency. To achieve this, two different methodologies were preliminary performed and compared, as outlined in Figure 3.8.

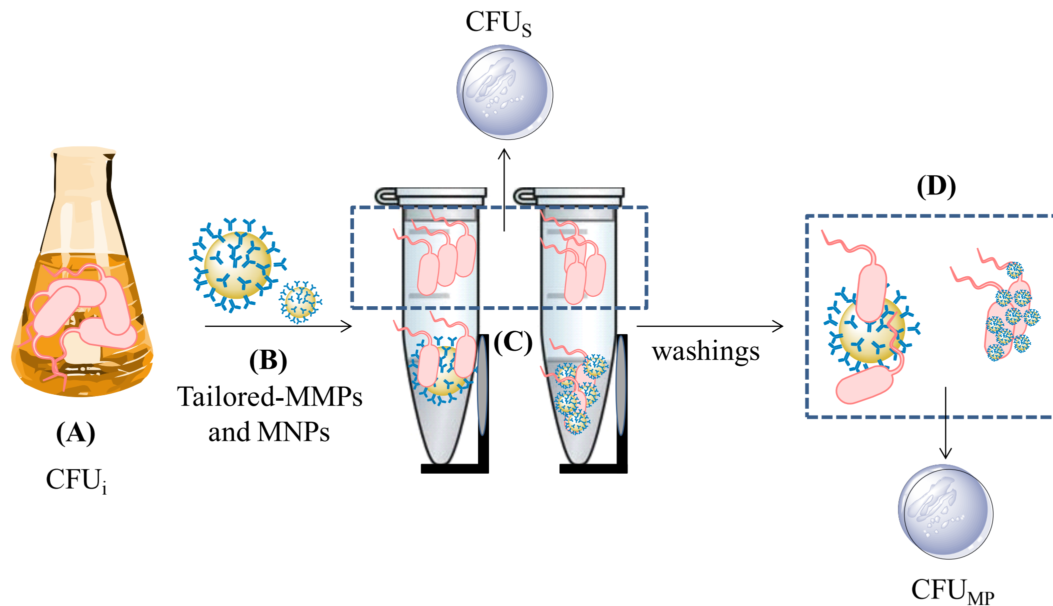

Figure 3.8 Schematic procedure for the determination of the IMS efficiencies of *S.* Typhimurium by microbiological culture technique: (A) artificially contaminated milk. The CFU$_i$ parameter is described as the number of CFU$_S$ in the initial suspension; (B) IMS using both tailored-MMPs and MNPs; (C) Collection of supernatant. The CFU$_S$ parameter is described as the number of CFU in the supernatant, after the IMS, which is obtained by plating this fraction; (D) Resuspension of the MPs in PBST. The parameter CFU$_{MP}$ is obtained by plating this fraction.

The first methodology was relied on the direct plating of the tailored-MPs with the bacteria attached (CFU$_{MP}$, Figure 3.8, panel D). The efficiency of the IMS was then expressed as a percentage (% IMS) and calculated by comparing the amount of the bacteria presented in the initial bacterial suspension before the IMS, (CFU$_i$, Figure 3.8, panel A) and the amount of bacteria attached on the tailored-MPs after the IMS (CFU$_{MP}$, Figure 3.8, panel D). Hence, the number of CFU was obtained by plating the initial supernatant solution, as well as the solution containing the bacteria attached on tailored-MPs. IMS (%) was then calculated based on the following formula:

$$IMS\ efficiency\ (Method\ 1) = \frac{CFU_{MP}}{CFU_i} \times 100 \qquad \text{Equation 3.2}$$

Thus, in the formula, CFU_{MP} is defined as number of CFU counted in the solution containing the bacteria attached on tailored-MPs and CFU_i as number of CFU counted in the initial bacterial suspension.

The second methodology was performed by comparing the amount of the bacteria from the initial suspension (CFU_i, Figure 3.8, panel A) and in the supernatant collected the after the IMS (CFU_s, Figure 3.8, panel C). The number of CFU was thus obtained by plating the initial suspension and the supernatant (before and after the IMS) in order to calculate the IMS (%) based on the following formula:

$$IMS\ efficiency\ (Method\ 2) = \frac{(CFU_i - CFU_S)}{CFU_i} \times 100 \qquad \text{Equation 3.3}$$

Thus, in the formula, CFU_S is defined as number of CFU counted in supernatant collected after the IMS, while CFU_i are related to the number of CFU counted in the initial bacterial suspension.

This study was performed for *S.* Typhimurium as a model and by using tailored-MMPs and MNPs for the IMS. The results are shown in Table 3.4, in which the IMS efficiency obtained by plating both tailored-MMPs and MNPs using both methodologies are compared.

Table 3.4 Efficiencies for the IMS of *Salmonella* in BHI broth using tailored-MMPs and MNPs based on microbiological culturing of MPs (CFU_{MP}) and supernatant (CFU_S). Data is given as the mean and the RSD values obtained with replicates (n=3).

***S.* Typhimurium concentration/ CFU mL^{-1}**	**IMS efficiencies (%) and RSD (%)**			
	Method 1 Tailored-MMPs	**Method 2 Tailored-MMPs**	**Method 1 MNPs**	**Method 2 Tailored-MNPs**
10^5	63 (37)	98 (1)	45 (21)	97 (2)
10^4	100 (8)	98 (1)	52 (26)	100 (1)
10^3	85 (25)	96 (1)	69 (7)	98 (4)
10^2	100 (49)	97 (3)	100 (37)	93 (4)

In this experiment a fixed amount of the both tailored-MPs was used to react with the serial dilutions of bacteria, being 3.33 x 10^6 and 1.83 x 10^9 MPs, respectively for tailored-MMPs

and MNPs, in both cases at higher concentration than the bacteria. It was observed that the IMS efficiencies of *S.* Typhimurium at the concentration range of 10^2 up to 10^5 CFU mL^{-1} were significantly lower when MPs were plated, showing also poorer reproducibility. This efficiency decrease can be attributed to bacteria aggregation caused by the multivalency in both magnetic carriers and bacteria, which was clearly confirmed by the SEM and confocal images, as it will further discussed in §3.4.4.

Comparing the IMS efficiencies obtained for both type of MPs, the results suggested that the plating of the MPs (Method 1), might introduce errors in the estimation of the IMS efficiency, with underestimation especially relevant at high concentration of bacteria[13], as well as a poor reproducibility. However, when plating the supernatant (Method 2), consistent results and excellent reproducibility were obtained in all the study range of concentration, showing comparable IMS efficiencies for the bacteria, ranging from 90 to 100 %, regardless the magnetic carrier. These results confirmed that good orientation and coupling efficiency of the antibody were achieved during the immobilisation procedure in both types of magnetic carriers. As a result, the subsequent IMS studies based on microbiological culture technique were performed by platting the supernatants to avoid underestimations, following the Method 2. In all these cases, the efficiency of the IMS under the different parameters was expressed as a percentage (% IMS), and calculated as shown in Equation 3.3.

3.4.3.2 Optimisation of the reaction time for the IMS

The optimisation of the IMS reaction time was performed by classical microbiological plating, by using tailored-MMPs for *S.* Typhimurium as a model. The reaction time was studied ranged from 0 to 60 min, for a fixed *S.* Typhimurium concentration of approximately 10^4 CFU mL^{-1}.

The results shown in Figure 3.9, in which the IMS efficiency was calculated by the Equation 3.3, are represented along the time of reaction.

As shown in Figure 3.9, the IMS efficiency significantly raised until 30 min to a value of 90 %, following by a slight increase of captured *S.* Typhimurium until 1 h to a value of 99 %. Based on these results, the reaction time of 30 min was selected as the optimal experimental condition for further studies, since it showed an excellent IMS performance in lower time.

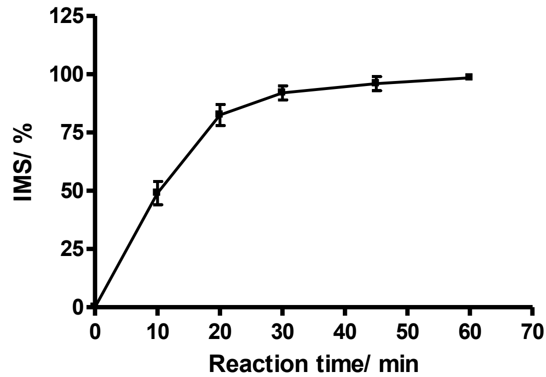

Figure 3.9 IMS efficiency of 3.01×10^4 CFU mL^{-1} *S.* Typhimurium, for different reaction times of 0, 10, 20, 30, 45 and 60 min using 0.05 mg of tailored-MMPs. The error bars presented in this graph show the standard deviation for n = 3.

3.4.3.3 Comparative study of the IMS efficiency performed with commercial and tailored magnetic micro and nanoparticles

In this section, different types of MPs, such as commercial Dynabeads®, tailored-MNPs and MMPs were used to capture a fixed concentration of *S.* Typhimurium (panel A), *E. coli* (panel B) and *L. monocytogenes* (panel C). The IMS efficiencies were studied by platting the supernatant (as shown in Figure 3.8, panel C) and calculated accordingly to Equation 3.3. The results are presented and compared in Figure 3.10 and detailed in Table 3.1.

The IMS of the three bacteria was achieved with different efficiencies for each bacterium and results will be discussed separately for each bacterium in the next section.

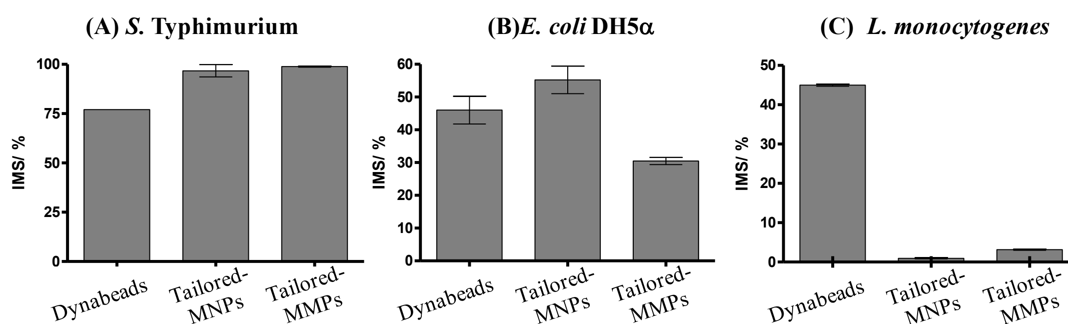

Figure 3.10 Comparative study of the IMS performed with commercial and tailored-MNPs and MMPs by microbiological culture technique upon different bacteria: (A) *S.* Typhimurium (4.42 x10^4 CFU mL^{-1}), (B) *E. coli* DH5α (1.75 x10^5 CFU mL^{-1}), (C) *L. monocytogenes* (3.39 x10^4 CFU mL^{-1}), using 0.05 mg of Dynabeads®, tailored-MNPs and MMPs. The error bars show the SD for n = 3.

IMS performed with commercial and tailored magnetic micro and nanoparticles for S. Typhimurium

No significant differences in the IMS efficiencies were observed between the tailored-MPs for *S.* Typhimurium regardless the size of the magnetic carrier, presenting IMS efficiencies in all cases of around 99 %, as shown in Figure 3.10, panel (A). Furthermore, the IMS efficiency of *S.* Typhimurium using Dynabeads® was achieved with a percentage of 75 %, being in agreement with the work previously published in the group using these MPs, which IMS efficiencies ranged from 55 to 79 %.[14] It is important to highlight that the use of tailored-MMPs and MNPs improved the efficiency when compared with the commercial Dynabeads®. This can be ascribed to the performance of the mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against LPS of *S.* Typhimurium antibody, used in this study.

IMS performed with performed with commercial and tailored magnetic micro and nanoparticles for E. coli

The IMS efficiencies for *E. coli* were obtained with average percentages of 46, 55 and 30 %, respectively for Dynabeads®, tailored-MNPs and MMPs, as shown in Figure 3.10, panel (B). In order to improve the IMS efficiency, further studies were performed by using tailored-MMPs containing higher amount of antibody, presenting an IMS efficiency of about 55 %,

suggesting not only low IMS efficiency but also poor reproducibility due to the RSD value of 41%.

An additional study was performed with other *E. coli* K12 strains, such as MC 4100 and MG 1655. In this study, 0.05 mg of the tailored-MNPs were added to comparatively capture DH5α, MC 4100 and MG 1655 at a concentration range of 10^5 CFU mL^{-1}, following the procedure described in §3.2.6. The results, presented in Table 3.5, showed higher IMS efficiencies for DH5α (55 %), while IMS percentages obtained for both K12 strains were similar (44 and 41 %, respectively for MC 4100 and MG 1655 strains). Regarding the RSD values, the differences are not so significant between the different strains. As a result of the low IMS efficiencies obtained for the different *E. coli* strains, even using commercial MPs, it is concluded that further studies should be done to obtain a good commercial antibody, in order to improve this important parameter.

Table 3.5 IMS efficiencies obtained with different *E. coli* strains (DH5α, K12 MC 4100 and MG 1655 using tailored-MNPs. Data is given as the mean and the RSD values obtained with replicates (n=3).

Samples	IMS efficiencies (%) and RSD (%)
DH5α	55 (11)
MC 4100	44 (15)
MG 1655	41 (18)
(-) control	0.00

IMS performed with commercial and tailored-magnetic micro and nanoparticles for L. monocytogenes

The IMS efficiencies for *L. monocytogenes* were found to be about 45 % using Dynabeads® and with percentages lower than 5 % for the tailored-MPs obtained with a goat polyclonal anti-*Listeria* antibody, as shown in Figure 3.10, panel (C). The poor antibody affinity towards this bacteria strain is pointed out as the main reason for the obtained result.

Another commercial antibody against *L. monocytogenes,* rabbit polyclonal anti-*Listeria* antibody, was used to obtain tailored-MMPs. The percentages of IMS were found to be as low

as 15 %, presenting still lower efficiencies than Dynabeads®. Characterisation of the IMS efficiency using these MPs was also evaluated by SEM, as it will be further shown in §3.4.4.

In the literature, the detection of this bacterium was mostly achieved by nucleic acid amplification methods than immunoassays.[15,16] This fact might anticipate the existence of a lower number of good commercial candidates antibodies, which could also be explained due to the structural differences of *L. monocytogenes*, being a Gram positive bacterium, presenting a thick peptidoglycan layer, that may hinder the access and recognition binding sites to which the antibody used is specific for.[17] Therefore, as a result of the low IMS efficiencies obtained even using commercial MPs, further studies should be done to obtain a good commercial antibody in order to improve this important parameter.

3.4.4 Evaluation of the IMS by Scanning Electron and Confocal Microscopy

The possibility of formation of clusters between particles and bacteria was previously proposed in (§3.4.3.1), due to the discrepancy found between the IMS efficiencies obtained by plating directly the MPs with the bacteria attached (Method 1) and the remaining unbound bacteria in the supernatant (Method 2). Therefore, the binding pattern of both tailored-MMPs and MNPs for the three bacteria was characterised by SEM microscopy.

Figure 3.11 shows the binding pattern obtained with tailored-MNPs (A, B) and MMPs (C, D) for the IMS of *S.* Typhimurium at a concentration of 10^6 CFU mL^{-1}. The binding pattern obtained with commercial Dynabeads for *S.* Typhimurium was previously reported by our research Group, showing a similar binding pattern that the tailored-MMPs.[14] Confocal microscopy is shown in Figure 3.12 for *S.* Typhimurium with tailored-MNPs (A) and MMPs (B).

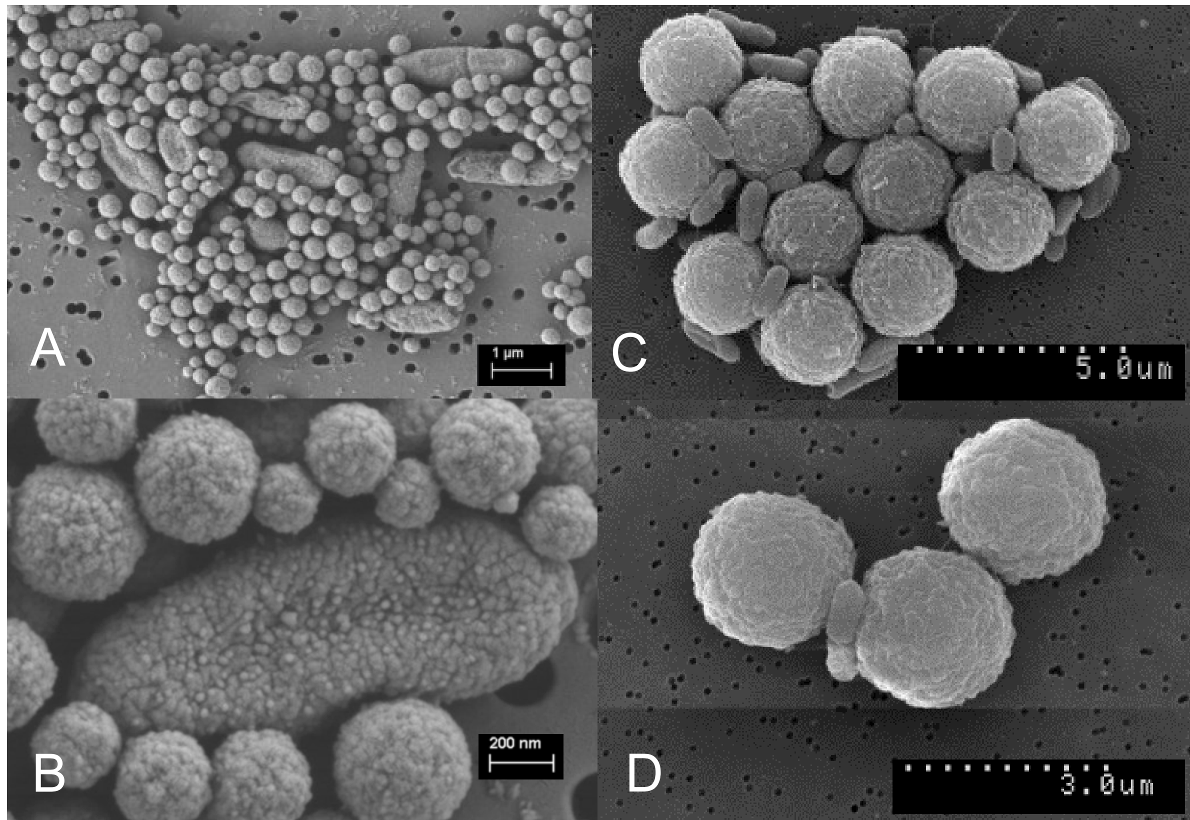

Figure 3.11 Microscopic characterisation of tailored-MNPs (A and B) and tailored-MMPs (C and D) by SEM for *S.* Typhimurium concentrations of 10^6 CFU mL^{-1}.

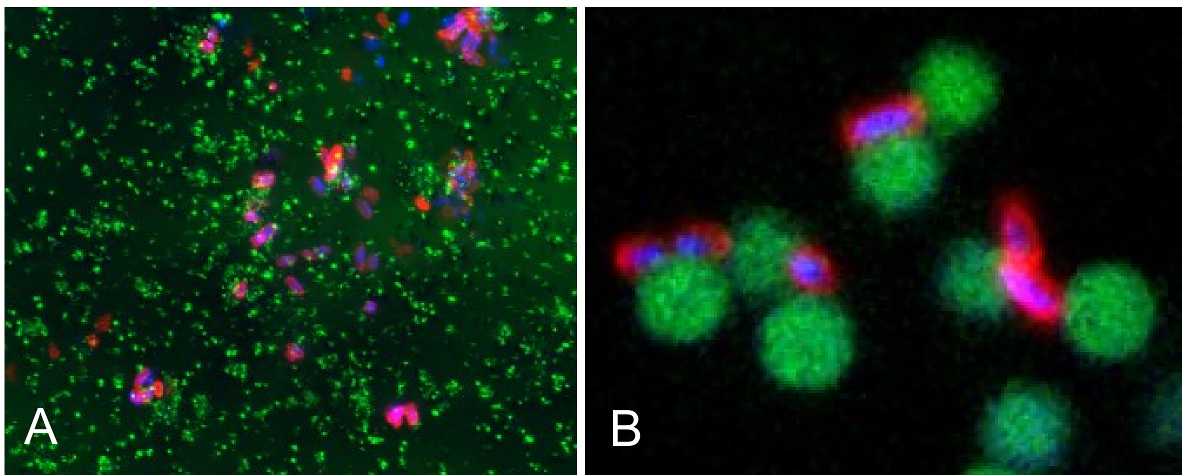

Figure 3.12 Microscopic characterisation of tailored-MNPs (A) and tailored-MMPs (B) by Confocal Microscopy for *S.* Typhimurium concentrations of 10^6 CFU mL^{-1}.

The formation of aggregates is undoubtedly observed for the tailored-MPs in the SEM and Confocal images presented in Figures 3.11 and 3.12, being in agreement with the results obtained by microbiological culturing shown in §3.4.3.1. In this context, it was previously discussed in §3.4.3.1 that the capture efficiencies of *S.* Typhimurium at the concentration range of 10^2 up to 10^5 CFU mL^{-1} were significantly lower when tailored-MNPs were plated, showing also poor reproducibility. This efficiency decrease can be attributed to bacteria aggregation caused by the multivalency in both magnetic carrier and bacteria, which was clearly confirmed by the SEM and confocal images corresponding respectively to Figures 3.11 and 3.12.

In addition, confocal microscopy (Figure 3.12) also shows the binding pattern of the anti-*Salmonella* antibody used for the fluorescence readout, directed against *Salmonella* "O" & "H" antigens. A consistent labelling (shown in red) of the cell membrane can be observed using both tailored-MPs, shown in green due to the autofluorescence of the MPs shell composed of polystyrene.

Figure 3.13 shows the binding pattern obtained by SEM with tailored-MNPs (panels A, B) and commercial Dynabeads® (panels C and D) for the IMS of *E. coli* at a concentration of 10^6 CFU mL^{-1}. As in the case of *Salmonella*, aggregation of tailored-MPs (panels A, B) and bacteria (panels C and D) was observed. However, a high amount of non-captured bacteria can also be observed, in agreement with the poor IMS efficiency obtained for *E. coli*, as discussed in §3.4.3.3.

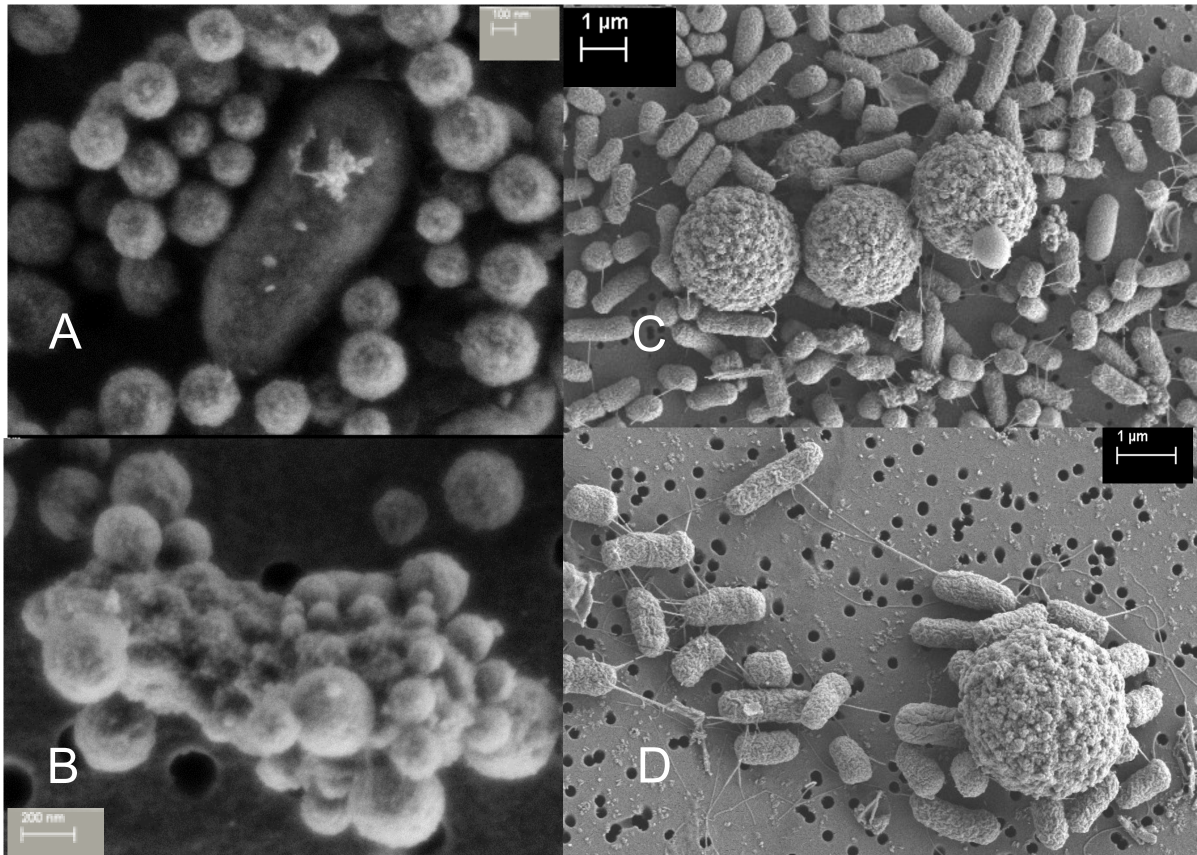

Figure 3.13 Microscopic characterisation of IMS of *E. coli* using tailored-MNPs (A and B) and Dynabeads® (C and D) by SEM for concentrations of 10^6 CFU mL^{-1}.

Therefore, comparing the SEM images corresponded to the IMS of *S.* Typhimurium and *E. coli*, as observed in Figures (3.11 and 3.13), the multivalency in both magnetic carriers and bacteria is confirmed, in which tailored-MMPs and Dynabeads® can bind to one or more bacterial cells, forming clusters. The same effect was observed for tailored-MNPs.[18] However, due to their size which is smaller than the bacteria, the binding pattern was different, being the bacteria covered with several tailored-MNPs. Clustering and aggregation are responsible for the lower IMS efficiency when platting the tailored-MPs with the bacteria attached (Method 1). Since one cluster is composed of bacteria that are aggregated, which is clearly observed for instance in Figure 3.13 (C, D), this leads to an underestimation of the IMS efficiency. For this reason, it is confirmed that the estimation of the IMS efficiency should be based on the remaining unbound bacteria from the supernatant, and calculated accordingly to Equation 3.3.[13]

IMS of *L. monocytogenes* was studied by SEM for a concentration of 10^6 CFU mL^{-1} using Dynabeads® (A and B) and tailored-MMPs modified with rabbit polyclonal anti-*Listeria* antibody (C and D), being represented in Figure 3.14.

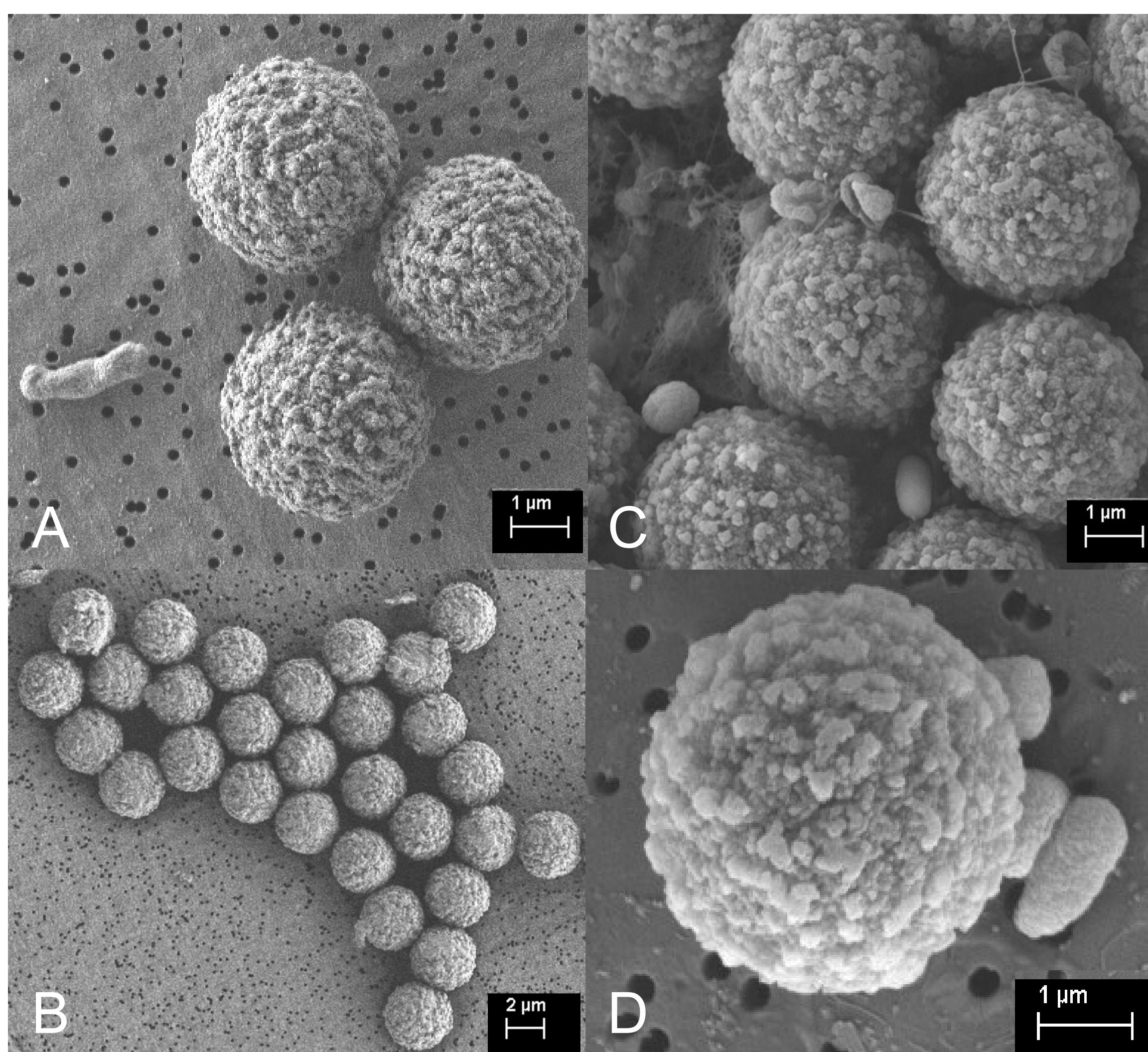

Figure 3.14 IMS of *L. monocytogenes* using Dynabeads® (A and B) and tailored-MMPs modified with rabbit polyclonal anti-Listeria (C and D) antibody by SEM for concentrations of 10^6 CFU mL^{-1}.

The capture of *L. monocytogenes* was observed using Dynabeads®. However, no bacteria attached to the MPs were observed using tailored-MMPs modified with rabbit polyclonal anti-*Listeria* antibody. These results showed to be in agreement with the results obtained by conventional culture methods discussed in §3.4.3.3, in which tailored-MMPs presented lower IMS efficiencies than Dynabeads®.

3.4.5 Study of the IMS efficiency at different bacteria concentrations by microbiological culture techniques

In the study, tenfold bacterial dilutions ranged from 10 to 10^5 CFU mL^{-1} in BHI broth were reacted with the MPs presenting higher IMS efficiencies in the previous studies. Therefore, for *S.* Typhimurium, the three types of MPs presented in this chapter were used, tailored-MNPs and Dynabeads® were both selected for the capture of *E. coli*, and finally, Dynabeads® were only chosen for the capture of *L. monocytogenes*. The IMS efficiencies were evaluated by classical microbiological platting of the supernatants, being shown in Figure 3.15.

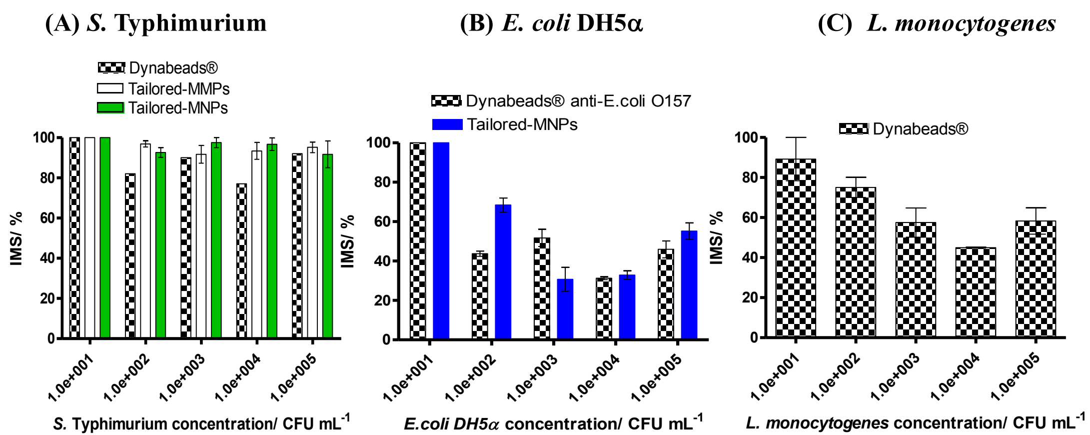

Figure 3.15 Study of bacteria concentration: Serial dilutions of *S.* Typhimurium, *E. coli* and *L. monocytogenes* ranged from 10 to 10^5 CFU mL^{-1} using 0.05 mg of MPs. The error bars show the standard deviation for n=3.

Along this chapter, it was demonstrated that the IMS of *S.* Typhimurium was achieved with excellent efficiencies, in which both tailored-MMPs and MNPs provided higher performance than the commercial Dynabeads® MPs. In Figure 3.15, it is demonstrated that the IMS efficiency is high and near the 100 % for all the concentration range studied for *S.* Typhimurium. However, the same results were not observed for the IMS of *E. coli* nor *L. monocytogenes*. As shown in Figure 3.15, the IMS efficiencies for both *E. coli* and *L. monocytogenes* were lower than for *S.* Typhimurium and tended to decrease by increasing the bacteria concentration, suggesting a poorer IMS efficiency due to lower efficacy on the recognition of the antibodies. This fact leads to a central question related to immunoassays, which efficiency is strongly dependent on the antibodies efficiencies in the immunological reaction.

Table 3.6 shows a summary of selected IMS results from the literature for *Salmonella*, *E. coli* and *Listeria*.

Table 3.6 IMS of *Salmonella*, *E. coli* and *L. monocytogenes* in pure bacterial suspensions.

Bacterial strain	Modified MPs	Antibody	Working range/ CFU mL^{-1}	IMS efficiency/ %	Ref
Salmonella spp.	Modified magnetic microparticles	Polyclonal to *Salmonella*	10^5-10^7	> 80	[19]
S. Typhimurium	Dynabeads®	-	10^6	84	[20]
S. Enteritidis	Dynabeads®	-	10^2-10^5	~54	[21]
S. Typhimurium	CELLection Dynabeads	Rabbit Biotinylated polyclonal to *Salmonella* spp.	10^3-10^7	74-86	[22]
E. coli O157	Dynabeads®	-	$1.6x10^2 – 1.6x10^8$	43-100	[13]
E. coli O157	Protein-A-conjugated MPs	Monoclonal to O 157	10^4	50	[23]
E. coli O157	Modified magnetic microparticles	Antibody specific to O 157	50-10^5	14-23	[24]
E. coli O157	Modified Tosyl-MP	Monoclonal to O 157	1-10^6	85-100	[25]
L. monocytogenes	Modified magnetic nanoparticles	Rabbit polyclonal	$5x10^1 – 5x10^5$	54-60	[26]
L. monocytogenes	Modified Tosyl-MMP (My One)	Monoclonal, produced specifically for this strain	10^3–10^8	5-9	[16]
L. monocytogenes	Dynabeads®	-	10^1–10^4	5-15	[27]
L. monocytogenes	Protein-A-conjugated MPs	Monoclonal to *L. monocytogenes*	10^5	~ 22	[28]

It is observed that the results obtained for the IMS efficiencies of *Salmonella* using Dynabeads® are lower than tailored-MPs, similarly to the results presented in this study.[19-22] This can be explained due to the specificity of the antibodies immobilised on these particles, related to several *Salmonella* strains. In this context, modification of MPs can be advantageous since the immobilisation procedure is very simple and it offers the possibility of choosing an antibody for a specific bacteria strain.

Based on the bibliography summarised in Table 3.6, it is also shown that IMS of *E. coli* was mostly studied for *E. coli* O157 strain, using monoclonal antibodies, being also lower than the efficiencies obtained for the capture of *Salmonella*.[23-25]

Comparing the IMS efficiencies obtained for the three bacteria, it is shown that the IMS of *L. monocytogenes* was achieved with the lowest percentages.[26-28] For instance, IMS of *L. monocytogenes* using Dynabeads® was achieved with percentages ranging from 5 to 15 %, even lower than the efficiencies obtained in this Dissertation.[16,27]

3.4.6 Study of the IMS efficiency in milk samples by microbiological culture techniques

The study of the IMS in food samples taking milk as a model was evaluated for *S.* Typhimurium by culturing the supernatant in three different media, such as i) BHI, ii) whole milk diluted 1/10 in BHI broth and iii) plain whole milk, for both type of magnetic carriers. The IMS efficiency was calculated accordingly to Equation 3.3. The results are presented in Table 3.7.

Table 3.7 IMS efficiencies for *Salmonella* in BHI broth, whole milk diluted 1/10 in BHI broth and pure whole milk based on microbiological culturing of the supernatant. Data is given as the mean and the RSD values obtained with replicates (n=3).

***Salmonella* concentration**	**10^5 CFU mL^{-1}**		**10^3 CFU mL^{-1}**	
Magnetic carriers / Media	**MNPs**	**MMPs**	**MNPs**	**MMPs**
	IMS efficiencies (%) and RSD (%)			
BHI broth	97 (2)	98 (1)	98 (4)	96 (1)
Diluted whole milk	96 (1)	97 (1)	100 (1)	100 (1)
Whole milk	92 (1)	97 (1)	94 (1)	100 (1)

No matrix effect was observed if the IMS was performed in the different media using tailored-MMPs. However, the capture efficiency of tailored-MNPs at a bacteria concentration of 10^5 CFU mL^{-1} decreases from BHI to whole milk diluted 1/10 in BHI broth and plain whole milk ranging from 98 to 92 %, suggesting a slight matrix effect. This discussion brings up a question about which type of MPs are more suitable for the capture of bacteria, especially in complex food samples.

Magnetic nanoparticles have larger surface -to- volume ratio providing more chemical reactive sites for the attachment of higher amounts of biomolecules at their surface.[29] However, for the attachment of bacterial cells, which sizes are about 1 µm, no advantages are seen by this effect, since the bacteria is surrounded by the particles. Moreover, the IMS percentages obtained in whole milk for tailored-MNPs suggest that these particles might be more affected to matrix effects.

In the literature, no conclusive results were found about the influence of the size of the MPs on the IMS of *S.* Typhimurium to support this discussion, as a result, more studies are needed to be performed in order to understand and justify these results.

3.4.7 Specificity study in milk samples by microbiological culture techniques

The specificity of the IMS step was evaluated using the tailored-MNPs modified with anti-*S.* Typhimurium antibodies based on the procedure described in §3.2.11. In this study, tailored-MNPs towards *Salmonella* were incubated with a suspension of *E. coli*, *L. monocytogenes* and *S.* Typhimurium, as well as mix solutions containing *S.* Typhimurium each one of *E. coli* and *L. monocytogenes*. All bacterial suspensions and negative controls were prepared in whole milk diluted 1/10 in BHI broth. Since all bacteria can grow in BHI broth, different media were chosen in order to differentiate the bacteria found in the mix solutions as XLD agar to differentiate *Salmonella* and *E. coli* colonies and Palcam agar specific to *L. monocytogenes*. In Figure 3.16, the agar plates related to XLD (panel A), Palcam (panel B), BHI (panels C and D) media are presented.

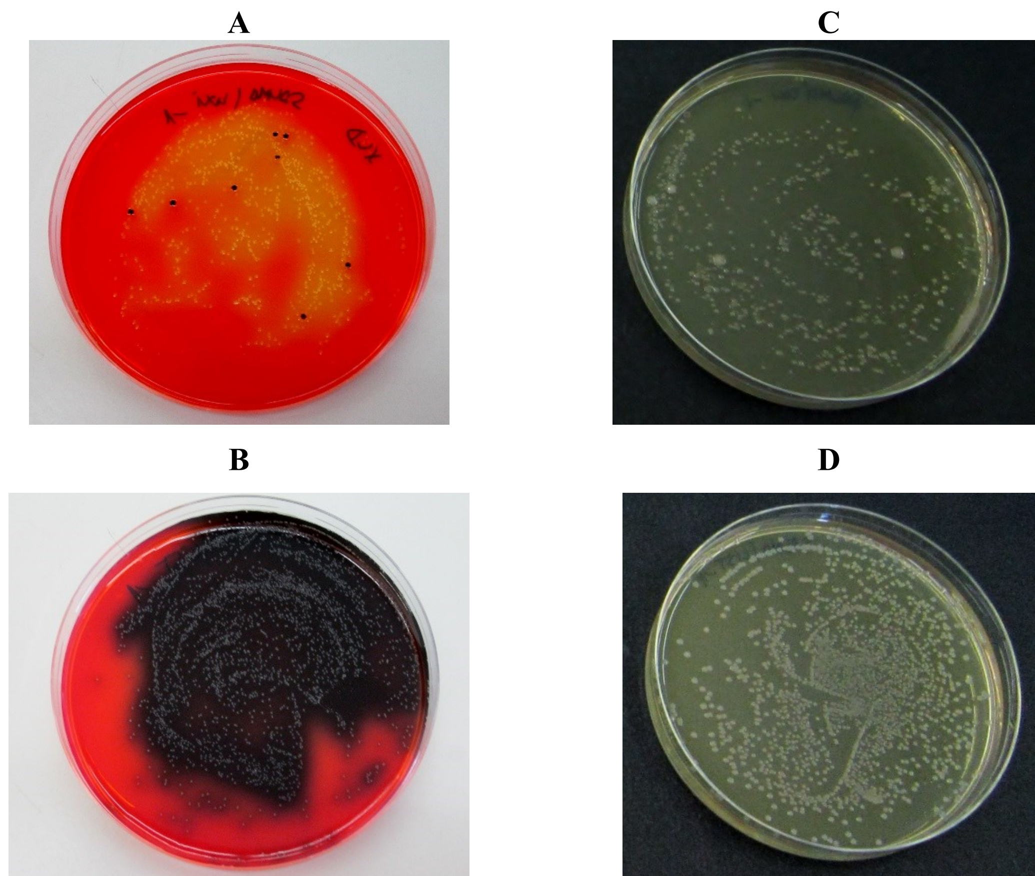

Figure 3.16 IMS of the mixed bacterial strains in different media using tailored-MNPs: (A) *S.* Typhimurium/*E. coli* in XLD agar, (B) *S.* Typhimurium/ *L. monocytogenes* in Palcam agar, (C) *S.* Typhimurium/*E. coli* in BHI broth and (D) *S.* Typhimurium/*L. monocytogenes* in BHI broth.

Direct plating using selective media as XLD was found to be successful in detecting and isolating *Salmonella* and *E. coli* strains.[30] The differentiation of these two bacteria is based on the pH variation during the fermentation process. One of the components of this medium is lysine. Lysine decarboxylation, caused by *Salmonella*, changes the pH of the medium to an alkaline condition which leads to the formation of red colonies, some with black centres (Figure 3.16, panel A). Microorganisms, such as *E. coli*, which ferment xylose, are lysine decarboxylase-negative, being for this reason enable to ferment lactose or sucrose. As a result, the pH of the medium will be in the acidic range, leading to the formation yellow colonies, as observed in Figure 3.16, panel A.[30]

The mix containing *S.* Typhimurium/ *L. monocytogenes* was plated in Palcam media, consisted of a selective and differential medium for the detection of *L. monocytogenes* (Figure 3.16, panel B). Thus, only *L. monocytogenes* can grow in this plate and consequently the IMS efficiency can only be calculated for the non-specific attachment of this bacterium on tailored-MNP modified with anti-*Salmonella* antibodies.

Figure 3.16 also shows the mix containing *S.* Typhimurium/ *E. coli* and *S.* Typhimurium/ *L. monocytogenes* plated in BHI (panels C and D, respectively). However, since all bacteria can grow in BHI, it enables the possibility to calculate the corresponded IMS efficiency in the mix samples.

The IMS efficiencies, shown in Table 3.8 were calculated by platting the supernatant after the IMS, as discussed above in §3.4.3.1 and calculated accordingly to Equation 3.3.

Table 3.8 Specificity study for *Salmonella* based on the IMS of milk samples contaminated with *E. coli* and *L. monocytogenes*, using tailored-MNPs towards *Salmonella*

Contaminated milk samples	**Medium**	**IMS/ %**
Salmonella	BHI	97.3
Listeria	BHI	0.0
E. coli	BHI	0.0
***Salmo**/E. coli*	XLD	99.6
*Salmo/**E. coli***	XLD	0.0
Salmo/Listeria	Palcam	0.0
(-) control	BHI	0.0
(-) control	XLD	0.0
(-) control	Palcam	0.0

As observed in the results shown in Table 3.8, no IMS of *E. coli* and *L. monocytogenes* was obtained by using the tailored-MNPs towards *Salmonella*, either in the suspensions containing one of the aforementioned microorganisms or in the mix solution, as expected, suggesting high specificity of the IMS performed on the tailored-MNPs towards *S.* Typhimurium from the contaminated milk samples.

The capture of *S.* Typhimurium in XLD agar in the mix solution containing *E. coli* was achieved with a percentage of 99.6 %. It was not possible to calculate the IMS capture of this bacterium in the Palcam agar, since only *L. monocytogenes* can grow in this medium. Nevertheless, in the supernatant, a number of *L. monocytogenes* colonies were found to be in the same range as the initial suspension, which suggests that no *L. monocytogenes* were attached to tailored-MNPs towards *Salmonella.* Additionally, similar numbers of colonies were found in the BHI plate of the supernatant the mix solutions of *S.* Typhimurium/ *L. monocytogenes*, indicating also that no *L. monocytogenes* were attached to tailored-MNPs towards *Salmonella* and consequently the *S.* Typhimurium cells were attached to the tailored-MNPs.

In order to demonstrate these results, additional experiments based on PCR amplification was performed, which will be further presented in §4.2.8.4.

3.5 *Conclusions and final remarks*

In this chapter, it was demonstrated that MPs can be easily modified with antibodies from different sources based on covalent immobilisation strategies. Thus, different antibodies specific for *S.* Typhimurium, *E. coli* and *L. monocytogenes* were covalently coupled on both MPs: tosyl-MMP and carboxyl-MNP.

The methodology used to evaluate the total amount of antibodies immobilised on the magnetic particles surface was based on ELISA. The total amount of antibody immobilised on tosyl-MMP and carboxyl-MNP was calculated by measuring the difference of the antibody amount present in the supernatant before and after the immobilisation step, having shown that more than 87 % of the antibody initial amount was immobilised on the MPs.

The IMS was fully studied and characterised based on microbiological culturing techniques, SEM and confocal microscopy. Different parameters of the IMS were evaluated by conventional culture methods, including the reaction time, type of magnetic carrier, size effect

of MPs on the capture and bacteria concentration. Finally, the IMS was also study in complex matrixes including milk samples. In this context, it was shown that 30 min was the optimised experimental condition for the IMS based on the efficiency obtained and taking as a model *S.* Typhimurium. Although the determination of IMS efficiency can be theoretically achieved by plating the MPs with the bacteria attached (Method 1) or plating the supernatant (Method 2), it was shown that the first one presented more consistent results, since poor reproducibility and underestimated values were achieved when MPs were directly plated.

The study of the IMS with commercial and tailored-MPs for *S.* Typhimurium showed excellent efficiencies about 99 %, with similar results using tailored-MMPs and MNPs, and 75 % using Dynabeads®, based on the work previously published by our Group.[14]

The IMS efficiencies obtained for *E. coli* and *L. monocytogenes* were in all cases lower than for *S.* Typhimurium, which main reason might be associated to the antibody efficiencies in the recognition of these bacterial strains.

The IMS efficiency of *E. coli* was obtained with the percentages of 46, 55 and 30 %, respectively for Dynabeads®, as well as tailored-MMPs and MNPs. Afterwards, another study was performed with two *E. coli* K12 strains using tailored-MNPs. However, the efficiencies in the capture of *E. coli* K12 were lower than *E. coli* DH5α strain. The lower efficiencies of Dynabeads® may be justified due to the recognition of antibodies attached on these particles, related to O157 antigen. In the literature, the IMS studies reported were mostly related to the capture of *E. coli* O157 with efficiencies in general lower than the efficiencies obtained for the capture of *Salmonella*.[23-25]

The IMS of *L. monocytogenes* was achieved with efficiencies of 45 % using Dynabeads® and percentages lower than 5 % using the tailored-MPs. An additional experiment was performed with a different antibody attached to the tailored-MMPs for the capture *L. monocytogenes.* The IMS, evaluated by microbiological culture methods showed an efficiency of 15 % suggesting poor antibody affinity which was further confirmed by SEM, where no bacteria attached to the particles were observed. Nevertheless, the capture of *L. monocytogenes* using Dynabeads® was achieved with improved results in comparison with the studies reported in the literature.[16,27]

SEM and confocal microscopy were also performed in order to study and characterise comparatively the tailored-MMPs and MNPs in the IMS, confirming the presence of aggregates for the three bacteria studied. This fact was attributed to multivalency in both magnetic carriers and bacteria, mostly observed at high concentration range. Moreover, while tailored-MMPs and Dynabeads® were able to attach more than one bacterium, with tailored-MNPs was the opposite, in which bacteria were surrounded with more than one tailored-MNP, creating clusters between both particles and bacteria. This may lead to errors in the estimation of the capture efficiency and for this reason, it is concluded that the estimation of the capture efficiency should be based on the remaining unbound bacteria from the supernatant after the IMS.[13]

The study of the IMS efficiency at different bacteria concentrations was evaluated for *S.* Typhimurium using the three types of MPs, in detail, commercial and tailored-MPs, for *E. coli* using tailored-MNPs and Dynabeads® and finally for *L. monocytogenes* using Dynabeads®. It was concluded that the IMS efficiency is near the 100 % for all the concentration range studied for *S.* Typhimurium. However, the IMS efficiencies for both *E. coli* and *L. monocytogenes* were lower than for S. Typhimurium and tended to decrease by increasing the bacteria concentration, suggesting a poorer IMS efficiency due to lower recognition of the antibodies. This fact leads to a central question related to immunoassays, which efficiency is strongly dependent on the antibodies efficiencies in the immunological reaction. The recognition of *S.* Typhimurium is based on a monoclonal antibody directed to LPS of *S.* Typhimurium, having shown to be very efficient. Due to the results obtained with *S.* Typhimurium, it is suggested that a monoclonal antibody directed to the LPS of *E. coli* could be a suitable option for the capture of *E. coli* bacterial cells. However, *E. coli* DH5α strain does not express a long-chain LPS, lacking the O-antigen, on the contrary to pathogenic *E. coli* strains that express a long-chain LPS, which increases the difficulties to find an effective antibody.[31]

As mentioned above, *L. monocytogenes* is composed of a highly cross-linked peptidoglycan layer, much thicker than Gram negative as *Salmonella* or *E. coli* bacteria that consequently may hamper the access and recognition of the binding sites to which the antibody used is specific for.[17] Studies based on the proteins binding secreted by *L. monocytogenes* to its cell surface showed that InlB and p60 are the proteins to which this bacteria strain present more affinity.[15,32] These proteins are coded for specific genes as *inlB* and/or *iap*, widely used in PCR

based assays for the detection of *L. monocytogenes*. This fact supports the above explanation about the higher number of PCR based assays in comparison with the number of immunoassays.

The last study of this chapter was based on the evaluation of the IMS of *S.* Typhimurium in different media, such as BHI broth, diluted whole milk and whole milk for both tailored-MMPs and MNPs. In this study, no noticeable matrix effect was observed for the different sized magnetic carriers, although the efficiencies were slightly lower when using tailored-MNPs in whole milk. In order to understand these differences obtained for tailored-MNPs in milk, the detection of *S.* Typhimurium will be achieved by IMS, followed by electrochemical magneto-immunosensing, and the results presented in chapter 4. In addition, high specificity of the IMS performed on the tailored-MNPs towards *S.* Typhimurium from the contaminated milk samples was shown, when reacted with suspensions of *E. coli* and *L. monocytogenes* with no capture.

It is worth noting that there are no conclusive results in the literature about the influence of the size of the MPs on the IMS of *Salmonella*. Therefore, in the next chapter, a comparison of both magnetic carriers in terms of performance for the detection of *S.* Typhimurium based on electrochemical magneto-immunosensing will be fully discussed. Moreover, electrochemical magneto-immunosensing and also genosensing procedures combined with IMS will also be compared for the detection *S.* Typhimurium in milk samples.

To conclude, IMS step has been demonstrated to be applicable with MPs with different types and sizes. Magnetic nanoparticles are advantageous because of their larger surface areas, but require stronger magnetic forces or more magnetic actuation time on a conventional magnet in comparison with magnetic microparticles. Magnetic microparticles require only conventional magnets and theoretically allow several cells to bind to each bead.

Another important aspect to highlight is the limitation of microbiological quantification for lower bacteria concentration, since the general ranges in common acceptance for countable numbers of colonies on a plate are 15–300 CFU mL^{-1}.[33,34] For this reason, IMS should be coupled with other techniques such as PCR amplification or electrochemical biosensors.[35] In addition, a preenrichment step would be also needed for directly counting the colony-forming unit in a reliable manner.

3.6 *References*

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CHAPTER 4

Immunomagnetic separation coupled with electrochemical immunosensing and genosensing of *Salmonella* on tailored-magnetic particles

4.1 Introduction

In chapter 3, the immunomagnetic separation (IMS) was demonstrated to be successfully performed when using magnetic particles (MPs) of different types and sizes, providing several advantages in food safety. However, it was demonstrated that the IMS procedure coupled with microbiological culturing techniques introduces sampling and enumeration errors.[1] For this reason, IMS are usually coupled with other detection techniques, mostly PCR,[2] immunoassays,[3] as it was previously concluded in §3.5. In this way, microbiological plating could be shortened or eliminated, which consequently leads to the reduction of the assay time, simplifying thus the analytical procedure.

In this chapter, MPs are integrated in electrochemical biosensors for the detection of *Salmonella* in milk samples, using different approaches based on both immunological and genetic biorecognition.

The integration of the MPs on biosensors brings two main advantages. Instead of the direct modification of the electrode surface, MPs act as a particulate support to improve the performance of the biological reactions (for instance, the attachment of bacteria or bacterial DNA, as well as the enzymatic labelling). On the other hand, the immobilisation is easily performed under magnetic actuation on a magneto electrode designed in our laboratories, named as magneto graphite epoxy composite (m-GEC), as explained in chapter 1, §1.2.3.4.[4,5]

In this chapter, it is proposed to compare two different strategies based on electrochemical magneto-immunosensing and genosensing for the detection of *Salmonella* in milk, using tailored micro and nano sized magnetic carriers (tailored-MMPs and MNPs, respectively).

In electrochemical magneto-immunosensing approach, nano (300 nm) and micro (2.8 μm)-sized MPs, modified with anti-*Salmonella* antibody (mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against LPS of *S.* Typhimurium presented in Table 3.1, §3.2.1, were used to capture and preconcentrate bacteria from milk samples based on an immunological reaction. Then, a second polyclonal antibody labelled with peroxidase (rabbit polyclonal to *Salmonella* (HRP), polyvalent for *Salmonella* "O" & "H" antigens) was used for the electrochemical detection based on a m-GEC electrode.[6] The performance of the tailored-MMPs and MNPs in terms of LOD and specificity was discussed and compared.

The second approach for the detection of *S.* Typhimurium was based on IMS combined with electrochemical magneto-genosensing. This approach involved the lysis of the captured bacteria after the IMS step, followed by the amplification of the genetic material by a single-tagging PCR with a set of primers specific for *S.* Typhimurium *yfiR* (375 bp), labelled with fluorescein.[7] The amplicons tagged with fluorescein in the 5´end were then adsorbed for the first time on silica magnetic particles (silica-MPs) based on the nucleic acid-binding properties of silica particles in the presence of the chaotropic agent guanidinium thiocyanate. Finally, the electrochemical detection was achieved by an enzyme marker, such as anti-fluorescein antibody labelled with horseradish peroxidase (HRP). The performance of both methods in terms of LOD was discussed and compared with the classical techniques, as microbiological culturing and gel electrophoresis. In terms of specificity, this strategy combined both immunological and the genetic biorecognition of the bacteria, due to the IMS and the single-tagging PCR/electrochemical genosensing, respectively.

4.2 Experimental Section

4.2.1 Chemicals and Biochemicals

Dynabeads MyOne Silane, Product N° 37002D were purchased from Dynal Biotech ASA (Oslo, Norway). Rabbit polyclonal Anti-*Salmonella* (HRP) polyvalent for *Salmonella* "O" & "H" antigens (Product N° ab 20771) was purchased from Abcam (Cambridge, UK). AntiFlu-HRP (Anti-Fluorescein-POD Fab fragments, 11426346910) was purchased from Roche Diagnostics S. L. The graphite-epoxy composite was prepared with graphite thin powder (1.04206.2500, Merck, Darmstadt, Germany) and Epo-Tek H77 (epoxy resin and hardener both from Epoxy Technology, USA). The hydroquinone used as electroactive species for the electrochemical characterisation was acquired from Sigma-Aldrich (product no. H9003).

All buffer solutions were prepared with milli-Q water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). PBST (10 mmol L^{-1} sodium phosphate, 0.8 % w/v NaCl, pH 7.4, 0.05 % v/v of Tween 20); PBST 2 % BSA (10 mmol L^{-1} sodium phosphate, 0.8 % w/v NaCl, pH 7.4, 0.05 % v/v of Tween 20, 2 % w/v BSA); Binding buffer (0.1 mol L^{-1} Tris, 5 mol L^{-1} GuSCN, pH 6.4); Washing buffer (10 mmol L^{-1} Tris, pH

4.5); 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) and PBSE (0.1 mol L^{-1} sodium phosphate, 0.1 mol L^{-1} KCl, pH 7.0).

The bacterial strains used in this work were *S.* Typhimurium LT2 (ATCC® 700720™), *L. monocytogenes* ATC 15313 (DSMZ) and *E. coli* DH5α strain carrying a pGEMT vector that contains the *eaeA* amplified plasmid. *S.* Typhimurium and *E. coli* were grown at 37°C in Luria-Bertani (LB) agar plates and *L. monocytogenes* in BHI agar plates (Oxoid). All bacterial suspensions were performed in Brain Heart Infusion (BHI) (Oxoid) from freshly grown plates and its turbidity was adjusted at OD_{600} of 0.2 A.U. The exact concentration of each bacterial suspension was quantified by plating serial dilutions onto LB for both *S.* Typhimurium and *E. coli* and BHI plates for *L. monocytogenes*.

4.2.2 Oligonucleotides sequences

The oligonucleotide sequences for the electrochemical magneto-genosensing of *S.* Typhimurium were obtained from TIB-Molbiol GmbH (Berlin, Germany). These primers were selected for the specific amplification of *yfiR* gene coding for *S.* Typhimurium.[7,8] Fluorescein (FLU) was used as label and it was inserted in 5' end of the oligonucleotide sequence. The primer sequences, as well as the tag used for the single-tagging PCR amplification are shown in Table 4.1. In addition, primers specific for *L. monocytogenes* and *E. coli* were also used for the specificity study which will be further explained in §4.2.8.4.

Table 4.1 Primer pair and label selected for the PCR amplification.

STRAIN	GENE	PRIMER SEQUENCE (5'-3')	TYPE	5'-LABELS	SIZE (bp)
S. Typhimurium	*yfiR*	GTCACGGAAGAAGAGAAATCCGTACG	Forward	Fluorescein	375
		GGGAGTCCAGGTTGACGGAAAATTT	Reverse	Non-labelled	
L. monocytogenes	*hly*A	CGGAGGTTCCGCAAAAGATG	Forward	Biotin	234
		CCTCCAGAGTGATCGATGTT	Reverse	Non-labelled	
E. coli	*eae*A	GGCGGATAAGACTTCGGCTA	Forward	Digoxigenin	151
		CGTTTTGGCACTATTTGCCC	Reverse	Non-labelled	

4.2.3 Instrumentation

The PCR reaction was carried out in a thermal cycler (Product Nº 2720, Applied Biosystems, Life Technologies Corporation). The voltammetric characterisations were carried out using an Autolab PGSTAT EcoChemie. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems Inc, West Lafayette, USA). 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 magneto electrode (m-GEC), as working electrode.

4.2.4 Construction procedure of electrodes based on graphite epoxy composite

The detailed preparation of the m-GEC electrodes has been extensively described by Pividori *et al.*[9] and it is explained in Figure 4.1. The procedure is based on the following steps: After the removal of the metal screw (A), a copper disk with a diameter of 6 mm was welded to the metal end (2 mm diameter) of the female electric connector using a solder wire (B-C). Then, the female electric connector was inserted on a cylindrical PVC tube (D) (6 mm id, 8 mm

od, 21.5 mm long), using a hammer. Thus, a gap with a depth of 3 mm was obtained in the end of the body electrode. Meanwhile, a paste was prepared at a 20:3 (w/w) ratio, by mixing of Epo-Tek H77 (epoxy resin) and its hardener (both from Epoxy Technology, USA), according to previous optimisations.[9] Afterwards, the graphite powder (particle size 50 µm, BDH, UK) was added in a 1:4 (w/w) ratio. The graphite-epoxy composite paste was mixed for approximately 30 min and then inserted in the PVC cylindrical body, within the 3 mm of depth (E). Then, a neodymium magnet with 3 mm diameter was added in the centre of the tube (F) with final covering with the remaining soft GEC paste. Finally, m-GEC electrodes were cured at 80 ºC for 1 week until the paste becomes completely rigid (G).

Prior to each use, the m-GEC electrodes surface were renewed by a simple polishing procedure and stored in a dried place at RT.

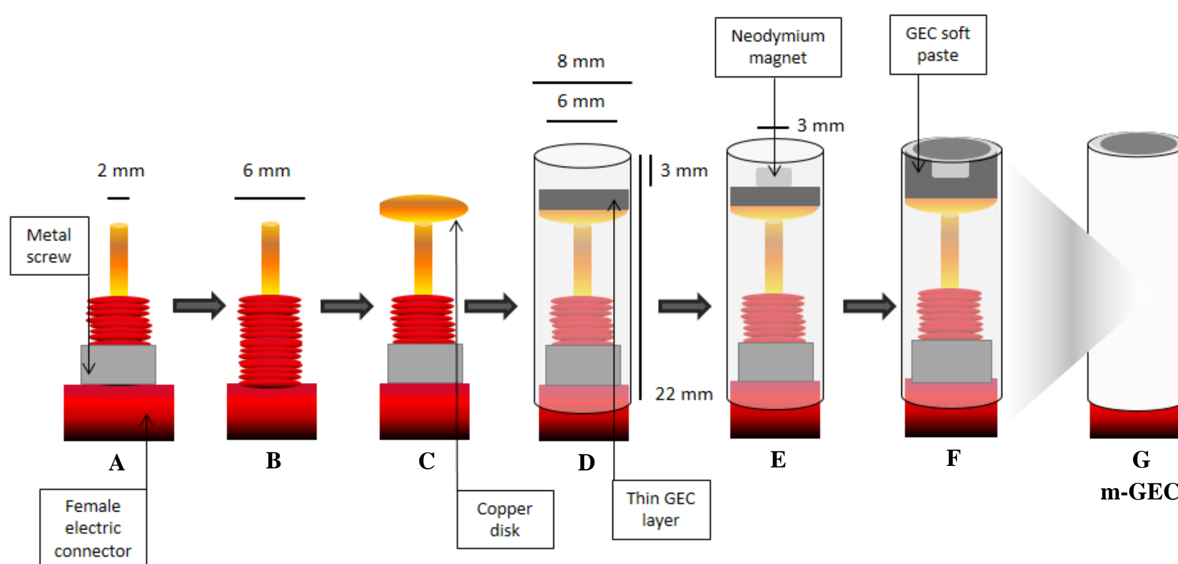

Figure 4.1 Schematic representation of the electrode construction based on graphite epoxy composite.

4.2.5 Characterisation procedure of magneto-electrodes based on graphite epoxy composite

The characterisation and reproducibility studies of m-GEC electrodes were performed by cyclic voltammetry (CV). This method provides significant information about the thermodynamics of redox processes, the kinetics of electron-transfer reactions between the electroactive species and the electrode surface, as well as the coupled chemical reactions or adsorption processes.[10]

During the potential sweep, the potentiostat measures the current between two limiting potentials E1 and E2, as represented in Figure 4.2, panel A. The same sweep rate is normally chosen for the forward and reverse sweep and the current response is recorded as a function of the applied potential, named as voltammogram. Figure 4.2, panel B, shows a theoretical cyclic voltammogram for a reversible system, where the potential (E) and current (i) of the anodic (Epa, ipa) and cathodic (Epc, ipc) peaks are shown, corresponding to the oxidation and reduction reactions respectively.[11]

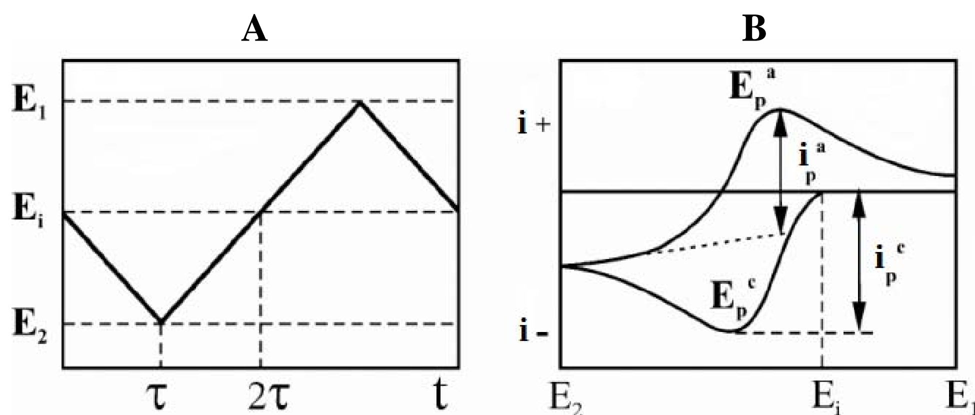

Figure 4.2 (A) Potential sweep during cyclic voltammetric measurement; E_i is the initial potential, E_1 and E_2 correspond to the range of potentials applied. (B) A typical cyclic voltammogram of a reversible reaction showing the oxidation or anodic (a) and the reduction or cathodic (c) processes and Ep as the potential at the current peak i_p.

For the characterisation of the m-GEC electrodes by CV, benzoquinone/hydroquinone redox couple was used as electroactive species, since this pair will be used as a mediator for the enzyme peroxidase, employed as an electrochemical reporter in this Dissertation. To achieve this task, each m-GEC electrode was immersed into the electrochemical cell containing 20 mL

of PBSE buffer. Then, 100 µL of the 0.4 mol L^{-1} hydroquinone solution was added under stirring for a few seconds before starting the measurement. The electrochemical behaviour of all electrodes was recorded in the range of potentials from -700 to +1200 mV, using a scan rate of 100 mVs^{-1}.

4.2.6 Immunomagnetic separation coupled with electrochemical immunosensing and genosensing of *Salmonella* on tailored magnetic micro and nanoparticles

A comparison of electrochemical magneto-immuno and genosensing was fully studied and compared in terms of LOD and assay time using tailored-MMPs and MNPs, being schematically described in Figure 4.3.

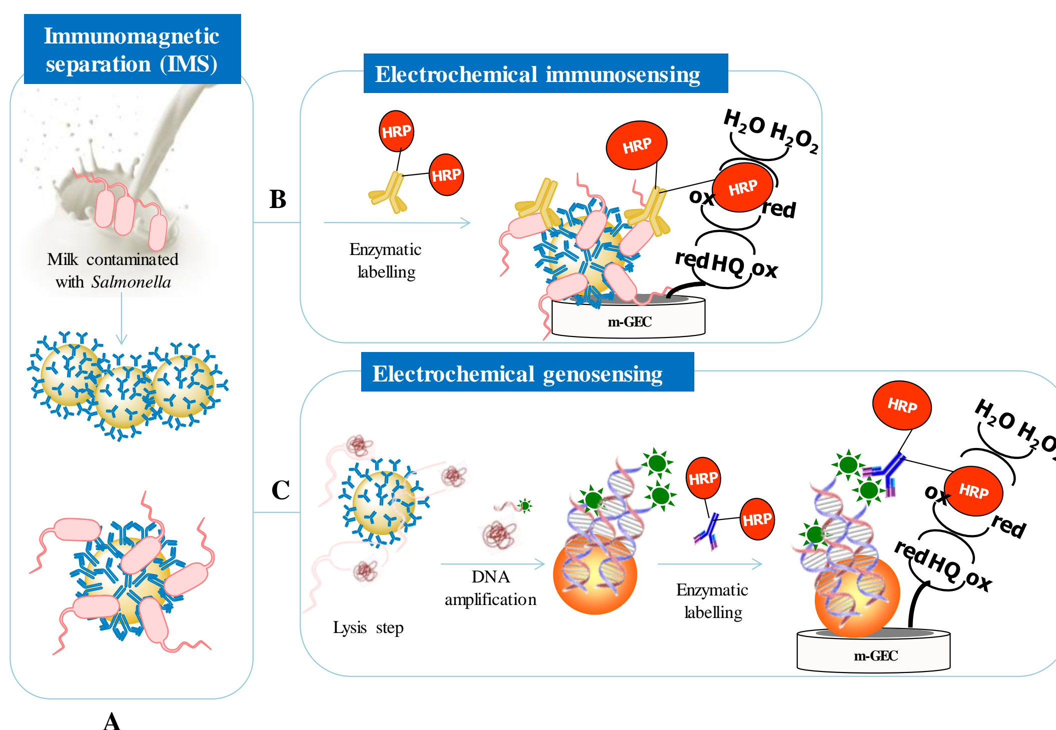

Figure 4.3 Integration of MPs in immunosensors and genosensors. After an IMS step (A) a magneto-immunosensor (B) and a magneto-genosensor (C) with electrochemical readout were performed for the detection of *S.* Typhimurium in milk.

Both strategies were combined with an IMS step based on a micro or nanosized MPs modified with a specific antibody towards S. Typhimurium (Figure 4.3, panel A), in which electrochemical magneto-immunosensing provides the detection of the whole bacterial cells based on a immunological recognition (Figure 4.3, panel B), whereas the electrochemical magneto-genosensing provides the detection of the tagged-PCR amplicons, based thus on a genetic recognition after the lysis and also the amplification of the genetic material (Figure 4.3, panel C). In this first approach, as it can be noticed from Figure 4.3, the same magnetic carrier (tailored-MMPs and MNPs) is used for IMS and electrochemical magneto-immunosensing (Figure 4.3, panel A and B). In the second approach, two different magnetic carriers were used: the first one for the IMS (tailored-MNPs) and the second one (silica-MPs), in which the single-tagged amplicons were adsorbed for the electrochemical magneto-genosensing (Figure 4.3, panel A and C).

The electrochemical readout in both approaches relies on the same principle, providing the detection of the HRP-modified electrochemical reporter on the magneto-actuated electrodes. Therefore, the electrochemical readout is based on amperometry, in the presence of hydrogen peroxide (H_2O_2) as a substrate of the enzyme and hydroquinone (HQ) as mediator to shuttle electrons between the m-GEC electrode and the horseradish peroxidase enzyme (HRP) used as electrochemical reporter. 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 (H_2O_2) conditions were used, as shown in Figure 4.4.

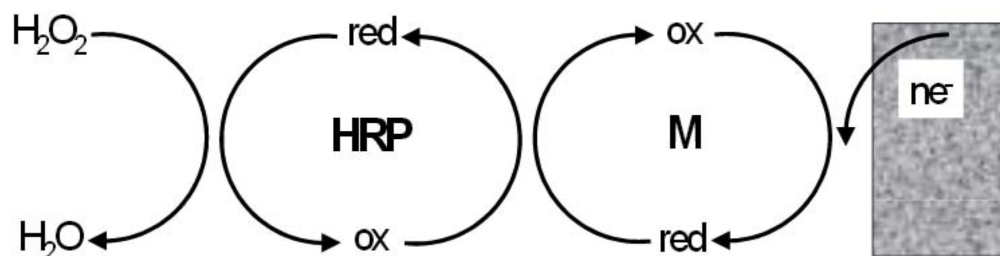

Figure 4.4 Enzymatic mechanism of the HRP enzyme in the surface of the m-GEC electrode, using H_2O_2 as a substrate of the enzyme and hydroquinone as a mediator.

In each measurement a steady-state current was obtained normally after 1 min of hydroquinone (1.81 mM) and hydrogen peroxide (4.90 mM) addition in phosphate buffer, as mediator and substrate for the enzyme HRP, respectively. The electrochemical cell contained 20 mL of PBSE buffer with 1.81 mmol L^{-1} hydroquinone as mediator and under continuous magnetic stirring, for an applied potential of -150 mV vs Ag/AgCl. When a stable baseline was reached, 500 µL 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 labelling procedure) and the current was measured until the steady state current was reached.

4.2.7 Electrochemical magneto-immunosensor on tailored-magnetic micro and nanoparticles

The electrochemical magneto-immunosensing procedure is based on an IMS step, followed by the immunological reaction with the anti-*Salmonella*-HRP antibody, upon magnetic actuation on the m-GEC electrode and the electrochemical readout, as depicted in Figure 4.3, panels A and B.

The IMS step was performed as previously described in the Chapter 3, §3.2.6, based on tailored-MMPs and MNPs for the comparison of the performance of both magnetic carriers. Serial dilutions of *S.* Typhimurium were prepared in BHI broth ranged from 0 to 10^5 CFU mL^{-1} for both tailored-MPs. The same procedure was also performed in whole milk diluted 1/10 in BHI a set of concentrations of 0 to 10^5 CFU mL^{-1} for both tailored-MMPs and MNPs. After discarding the supernatant, the collected MPs were further reacted with 140 µL of anti-*Salmonella* HRP antibody (diluted 1/1000 in PBST 2% BSA) for 30 min at RT. Finally, the modified MPs were washed twice and resuspended in 140 µL of PBST.

The modified MPs were then captured by dipping the magneto electrode (m-GEC) inside the reaction tube for the electrochemical measurements, as explained in §4.2.6.

4.2.7.1 Specificity study of the electrochemical magneto-immunosensor

The specificity of this strategy was studied for both tailored-MMPs and MNPs modified with the anti-*Salmonella* antibody. Therefore, the tailored-MPs were incubated with an *E. coli* suspension, as well as with a mixture of *E. coli* and *S.* Typhimurium, in whole milk diluted 1/10 in BHI, based on the protocol described above.

Positive and negative controls, respectively with and without *S.* Typhimurium suspensions were also studied. In detail, for tailored-MMPs, the bacteria concentrations used in the experiment were 2.08×10^5 CFU mL^{-1} of *E. coli*, 2.50×10^5 CFU mL^{-1} of *S.* Typhimurium and a mix solution containing 2.08×10^5 CFU mL^{-1} and 2.50×10^5 CFU mL^{-1} of *E. coli* and *S.* Typhimurium, respectively. For tailored-MNPs, the concentrations were 9.75×10^4 CFU mL^{-1} of *E. coli*, 2.95×10^5 CFU mL^{-1} of *S.* Typhimurium, and a mix solution containing 9.75×10^4 CFU mL^{-1} and 2.95×10^5 CFU mL^{-1} of *E. coli* and *S.* Typhimurium, respectively.

4.2.7.2 Electrochemical magneto-immunosensor for the detection of *Salmonella* in preenriched milk

A preenrichment step of *S.* Typhimurium was performed in BHI broth as nonselective broth medium using tailored-MNPs, in order to evaluate the time required to detect lower amounts of *S.* Typhimurium, according to the legislation requirements (Real Decreto 1679/1994, BOE 24-09-94).

The description of this procedure is outlined in Figure 4.5. Thus, 250 mL of whole milk were spiked with a *S.* Typhimurium concentration of 28.0 CFU mL^{-1}. The milk samples were preenriched in BHI broth at 37 ºC and assayed at 0, 4, 6, 8 and 10 h. A positive control was prepared by artificially contamination of 25 mL of whole milk with 2.80×10^2 CFU mL^{-1} of *S.* Typhimurium, as well as a negative control (0 CFU mL^{-1}).

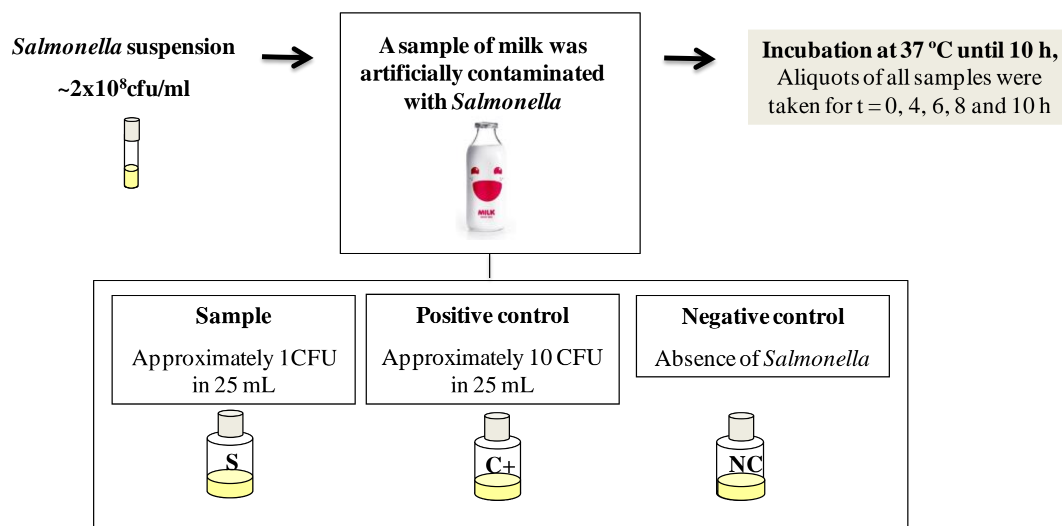

Figure 4.5 Description of the procedure for the preenrichment of milk samples spiked with approximately 1 CFU *Salmonella* in 25 mL of sample.

4.2.8 Immunomagnetic separation/Single-Tagging PCR amplification/Electrochemical Magneto-Genosensing based on silica magnetic particles

The electrochemical magneto-genosensing of *S.* Typhimurium was firstly combined with an IMS step, followed by a lysis step of the captured bacteria and further amplification of the genetic material by single-tagging PCR, as depicted in Figure 4.3, panels A and C. The electrochemical detection was achieved after an enzymatic labelling with anti-fluorescein HRP, as electrochemical reporter.

4.2.8.1 IMS efficiency with tailored-MNPs for *Salmonella* in whole milk

The IMS step was performed according to the procedure described in §3.2.10, in which tailored-MNPs were reacted with serial concentrations of a *S.* Typhimurium suspension prepared in whole milk diluted 1/10 in BHI broth, ranged from 10^0 to 10^6 CFU mL^{-1}. The IMS efficiencies were performed by comparing the amount of the bacteria existing in the supernatant

before and after the IMS. The CFU obtained by plating each supernatant solution (before and after the IMS) was thus used to calculate the IMS (%) based on the Equation 3.3, as previously described in §3.4.3.1.

4.2.8.2 Single-Tagging PCR Amplification

After the IMS, the tailored-MPs with the bacteria attached, were resuspended in 30 µL of Milli-Q water. The bacterial cells were then heated at 99 ºC for 20 min and centrifuged at 13000 rpm for 2 min, for the extraction of the bacterial DNA. The amplification of *S.* Typhimurium was performed with a single-tagging PCR procedure, as depicted in Figure 4.6.

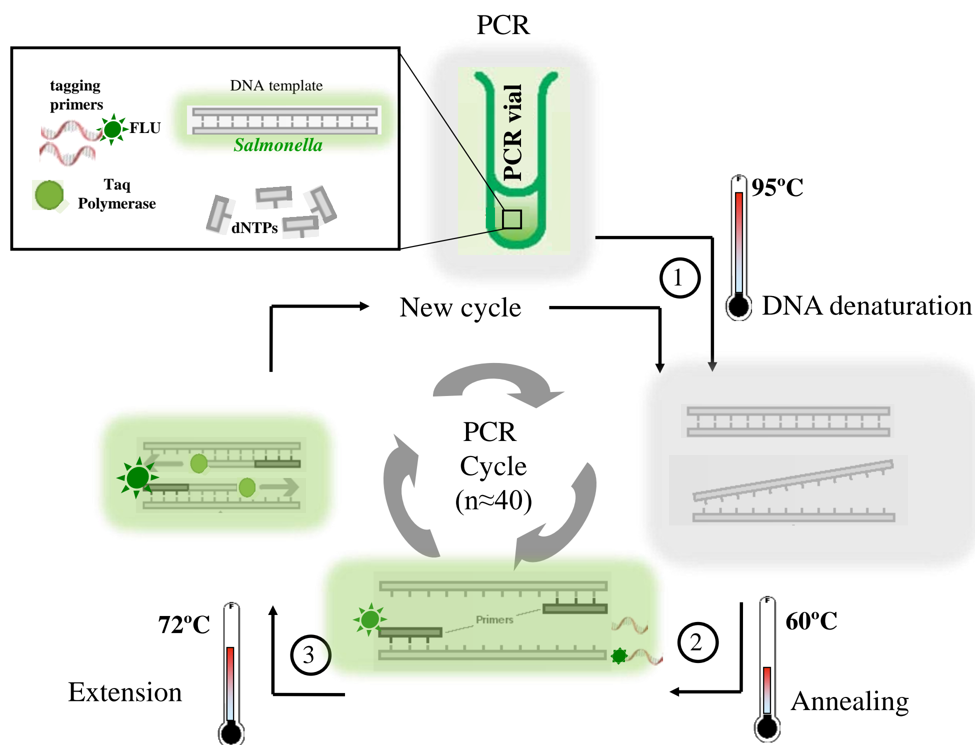

Figure 4.6 Representation of the single-tagging PCR procedure.

All PCR reactions were performed in a final volume of 100 µL, using 25 µL of extracted DNA obtained from each bacterial dilution of the IMS step, as a template. The Mastermix composition was prepared with the following products: 200 µmol L^{-1} of each deoxynucleotide

triphosphate (dATP, dGTP, and dCTP), 120 μmol L^{-1} of each *S.* Typhimurium detection primer (Table 4.1), in 5.0 mmol L^{-1} of $MgCl_2$. Thermal cycler conditions (summarised in Table 4.2) were as follows: pre-incubation at 95 °C for 10 min; 40 cycles consisting of dsDNA denaturation at 95 °C for 20 s, primer annealing at 60 °C for 30 s, primer extension at 72 °C for 30 s; final elongation at 72 °C for 7 min. The resulting samples were stored at 4 ºC.[7]

Several negative controls were included: (i) from the PCR mixture, where no DNA template was added and also (ii) from the IMS, where MPs were reacted with BHI broth, instead of *S.* Typhimurium. The amplicons were analysed by electrophoresis on 2 % agarose gel in TAE buffer containing 0.5 μg mL^{-1} ethidium bromide.

Table 4.2 Thermal cycler conditions for the PCR amplification.

	Initial step	**DNA denaturation**	**Annealing**	**Extension**	**Last step**
	1 cycle		40 cycles		1 cycle
Temperature (°C)	95	95	60	72	72
Time (sec)	600	20	30	30	420

4.2.8.3 Electrochemical magneto-genosensing on silica magnetic particles

After PCR, the electrochemical magneto-genosensing procedure for the detection of *S.* Typhimurium was based on the following steps: (i) Immobilisation of the tagged PCR amplicons on silica-MPs, 5 μL of silica-MPs (10 mg mL^{-1}, washed and diluted 1/4 in binding buffer) were diluted in 45 μL of binding buffer and mixed with 30 μL of the diluted amplicons (1/10) in milli-Q water. The mixture was incubated for 10 min at 55 ºC without shaking. Afterwards, two washing steps were performed in 80 μL of washing buffer; (ii) Incubation with the electrochemical reporter, AntiFlu-HRP (10 μg), prepared in blocking Tris buffer and added at a final volume of 140 μL for 30 min at RT and 700 rpm. Two washing steps were then performed for 5 min at room temperature in 140 μL of Tris buffer; (iii) Magnetic actuation

using m-GEC electrodes for the amperometric detection of *S.* Typhimurium, as previously explained in §4.2.6.

4.2.8.4 Specificity study of the electrochemical magneto-genosensor based on silica magnetic particles

The specificity study was performed in order to evaluate if the specificity of the procedure was mainly achieved by the immunological recognition during the IMS procedure or by the genetic recognition during the PCR.

Firstly, the specificity was evaluated by electrophoresis on 2 % agarose gel in TAE buffer containing 0.5 μg mL^{-1} ethidium bromide. In the IMS step, tailored-MNPs against *S.* Typhimurium were incubated with suspensions of the bacteria in whole milk diluted 1/10 in BHI of (i) *E. coli* (2.28 x 10^5 CFU mL^{-1}), (ii) *L. monocytogenes* (5.61 x 10^5 CFU mL^{-1}) and (iii) *S.* Typhimurium (6.28 x 10^5 CFU mL^{-1}, as a positive control), as well as mix solutions containing (iv) 3.14 x 10^5 CFU mL^{-1} of *S.* Typhimurium and 1.14 x 10^5 CFU mL^{-1} of *E. coli* and (v) 3.14 x 10^5 CFU mL^{-1} of *S.* Typhimurium and 2.80 x10^5 CFU mL^{-1} *L. monocytogenes,* as fully explained in §3.2.11. After the IMS, the bacterial DNA was extracted. In this instance, the PCR was performed at the same conditions described in Table 4.2, but in this case, by adding not only the set of primers specific for *Salmonella*, but also for *L. monocytogenes* and, *E. coli* as shown in Table 4.1, at concentrations of 120, 100 and 80 nmol L^{-1} respectively. After that, the gel electrophoresis was performed.

Finally, the same specificity study was then performed with the IMS/PCR/ Electrochemical magneto-genosensing procedure based on different magnetic carriers, as tailored-MNPs and silica-MPs, at a concentration range of bacterial suspension near the LOD (1 x 10^1 CFU mL^{-1}), in whole milk diluted 1/10 in BHI containing, as shown above, the single combination of the bacteria, as well as the binary combinations.

4.3 Safety Considerations

All procedures involving the manipulation of potentially infectious materials or cultures were performed following the safe handling and containment of infectious microorganism's

guidelines.[12] According to these guidelines, the experiments involving *S.* Typhimurium, *E. coli* and *L. monocytogenes* 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.

4.4 Results and Discussion

4.4.1 Characterisation procedure of magneto-electrodes based on graphite epoxy composite

Prior to the amperometric studies, the m-GEC electrodes were characterised by CV, commonly used to provide rapid and qualitative information about an electrochemical reaction, in which a range of fixed potentials were applied over a working electrode. During the potential sweep, the potentiostat measured the current and the resulting voltammogram contained the location of the redox potentials of the electroactive species.

In this characterisation study, hydroquinone/benzoquinone redox couple was selected as electroactive species, since hydroquinone was the mediator used in the amperometric detection strategies (Figure 4.4, §4.2.6). Thus, the electrochemical behaviour of the electrodes was recorded in the range of potentials from -700 to +1100 mV, to ensure a total oxidation and reduction of all species and also to compare the redox potential obtained for all m-GEC electrodes.

In Figure 4.7, the voltammograms of all m-GEC electrodes of the same batch were shown with similar responses, suggesting high reproducibility in the preparation of the electrodes. Moreover, a reduction potential of -150 mV was chosen for the amperometric readout in further experiments shown in this Dissertation.

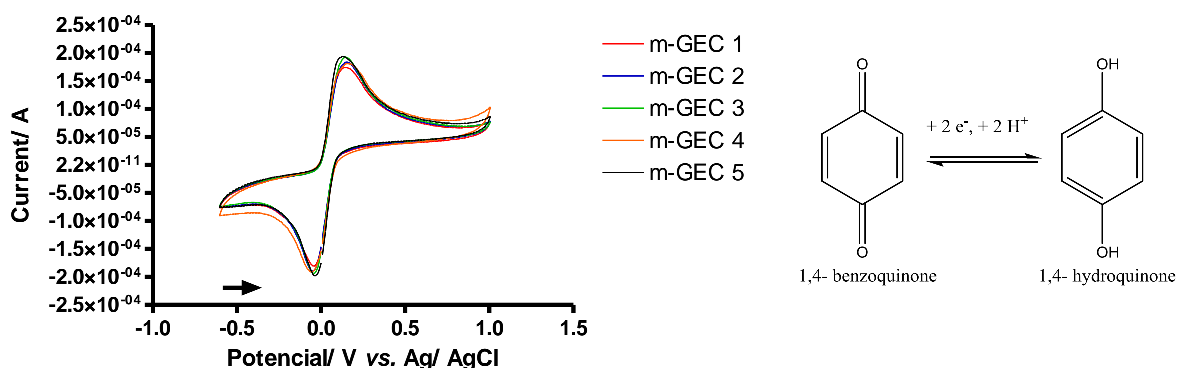

Figure 4.7 Cyclic voltammograms for the characterisation of m-GEC electrodes using 1.81 mmol L^{-1} hydroquinone in phosphate buffer.

4.4.2 Electrochemical magneto-immunosensor on tailored-magnetic micro and nanoparticles

The IMS of *S.* Typhimurium using both tailored-MPs was achieved with excellent efficiencies, as discussed in §3.4.5. However, a slight matrix effect was observed using tailored-MNPs for the capture of this bacteria in plain whole milk (as previously discussed in §3.4.6). In this context, and in order to evaluate the analytical performance in terms of LOD and the matrix effect, the electrochemical magneto-immunosensing procedure was performed for a range of concentrations from 0 to 10^5 CFU mL^{-1}, in both BHI broth and whole milk diluted 1/10 in BHI broth. The results were comparatively presented in Figure 4.8, for both tailored-MMPs (Figure 4.8, panel A) and tailored-MNPs (Figure 4.8, panel B).

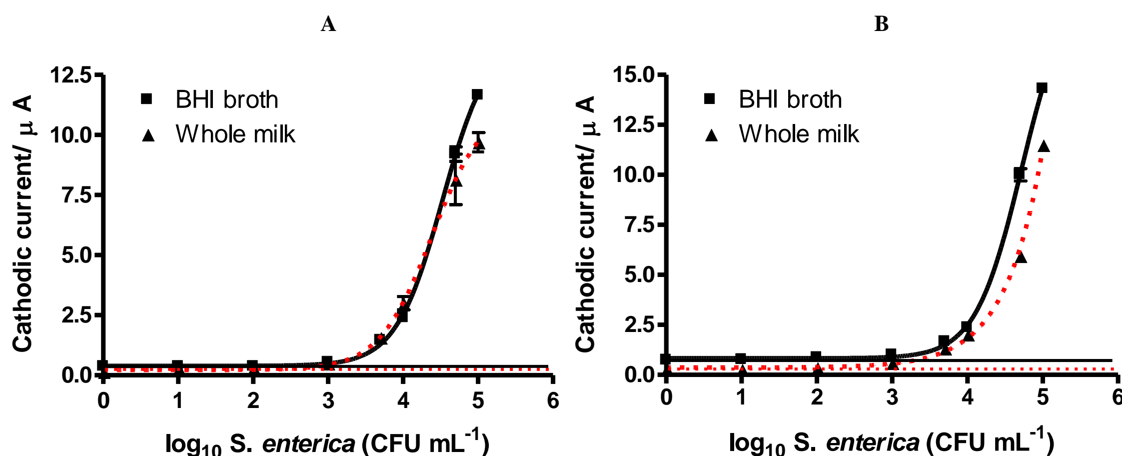

Figure 4.8 Electrochemical magneto-immunosensor for the detection of *S.* Typhimurium from 0 to 10^5 CFU mL^{-1} in BHI broth and in artificially inoculated whole milk diluted 1/10 in BHI broth (in dotted red) performed in (A) tailored-MMPs and (B) tailored-MNPs. The error bars show the standard deviation for n=3, except for the negative controls (n=7). Solid black and dotted red lines represent the cut-off limit values respectively in BHI broth and in whole milk, extracted with a one-tailed t-test at 95% confidence level.

The amperometric signal corresponding to the LOD (cut-off values) in BHI broth was estimated by processing the negative control samples (n=7, 0 CFU mL^{-1}) for tailored-MMPs and MNPs. The cut-off values were then determined by processing the negative control samples with a one-tailed t test at a 95% confidence level, giving values of 0.47 and 0.85 µA for tailored-MMPs and MNPs in BHI broth, respectively (shown in Figure 4.8, as solid lines).

Similarly, the amperometric signal corresponding to the LOD (cut-off values) were also estimated for whole milk in both kinds of magnetic carriers. The cut-off values were then determined by processing the negative control samples (n=7, 0 CFU mL^{-1}) with a one-tailed t test at a 95% confidence level, giving values of 0.32 and 0.43 µA for tailored-MMPs and MNPs in whole milk, respectively (shown in Figure 4.8, as dotted red lines).

The results based on the mean value, standard deviation, cut-off limit and LOD in both media are summarised in Table 4.3.

Table 4.3 Comparison of the statistical parameters for the determination of the cut-off limits and LOD for both tailored-MPs, in BHI broth and whole milk media.

Sample	Media	mean values for the negative control/µA	SD(95%)/ µA	Cut-off/µA	LOD/CFU mL^{-1}
Tailored-MMPs	BHI broth	0.371	0.048	0.47	835
	Whole milk	0.153	0.084	0.32	538
Tailored-MNPs	BHI broth	0.740	0.055	0.85	462
	Whole milk	0.306	0.062	0.43	291

In all cases (BHI broth and whole milk in tailored-MMPs and MNPs), the data obtained from 0 to 10^5 CFU mL^{-1} were then adjusted to a nonlinear regression (sigmoidal dose-response with variable slope – GraphPad Prism Software), as shown in Figure 4.8. By interpolating the cut-off values (represented as black solid and red dot line, correspondingly in BHI and whole milk), the LODs obtained in BHI broth matrix were 835 CFU mL^{-1} (R^2= 0.9980) and 462 CFU mL^{-1} (R^2=0.9983) for tailored-MMPs and MNPs, respectively, while in whole milk, the LODs values were found to be 538 CFU mL^{-1} (R^2= 0.9862) and 291 CFU mL^{-1} (R^2= 0.9946) for tailored-MMPs and MNPs, respectively.[13]

Figure 4.9 shows the same data, but at concentrations near the LOD. From the comparison between the cut-off values obtained in BHI and whole milk, a significant decrease was observed for both tailored-MPs in whole milk, suggesting a matrix effect.

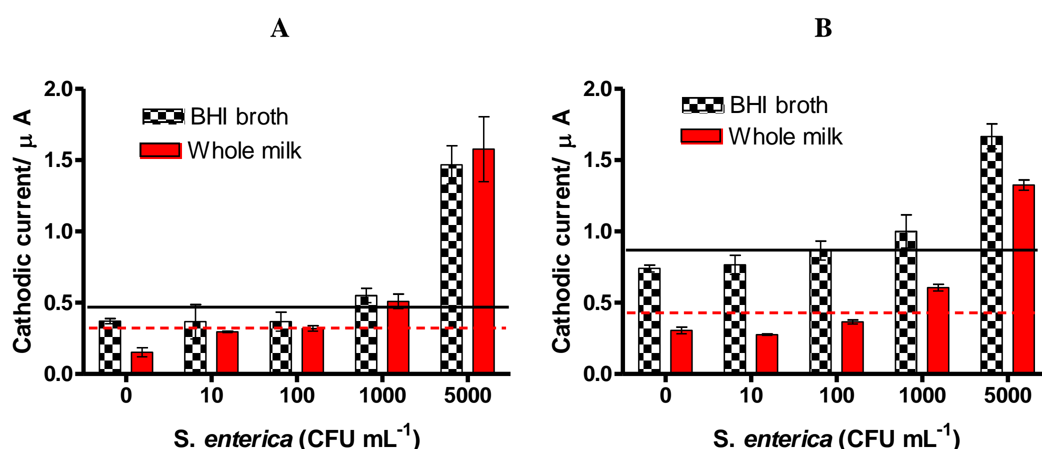

Figure 4.9 Detailed information near the LODs for the detection of *S.* Typhimurium from 0 to 10^5 CFU mL^{-1} in in BHI broth and in artificially inoculated whole milk diluted 1/10 in BHI broth (in red) performed in (A) tailored-MMPs and B) tailored-MNPs. The error bars show the standard deviation for n=3, except for the negative controls (n=7). Solid black and dotted red lines represent the cut-off limit values respectively in BHI broth and in whole milk, extracted with a one-tailed t-test at 95% confidence level.

As this matrix effect is even noticeable in the negative control, this fact can be attributed to the proteins from the whole milk, including casein, which acts as a blocking reagent for the labelled antibody and consequently produces the reduced background value observed with whole milk when compared with BHI media. This effect is more significant in tailored-MNPs, possibly due to their higher surface area -to- volume ratio given by their smaller size, leading to an increased non-specific adsorption.

In addition, a significant matrix effect at higher bacteria concentration is observed in Figure 4.9, especially when tailored-MNPs were used as a magnetic carrier (Figure 4.9, panel B). However, the LODs obtained with tailored-MNPs were slightly lower regardless the media.

4.4.2.1 Specificity study of the electrochemical magneto-immunosensor

Figure 4.10 shows the results obtained for the specificity study in whole milk diluted 1/10 in BHI broth. As expected, the electrochemical signal obtained for *E. coli* was similar to the value obtained when processing the negative control, while the mix of both bacteria (*E. coli*

and *S.* Typhimurium) gave a similar signal than the sample spiked just with *S.* Typhimurium for both tailored-MNPs and MMPs.

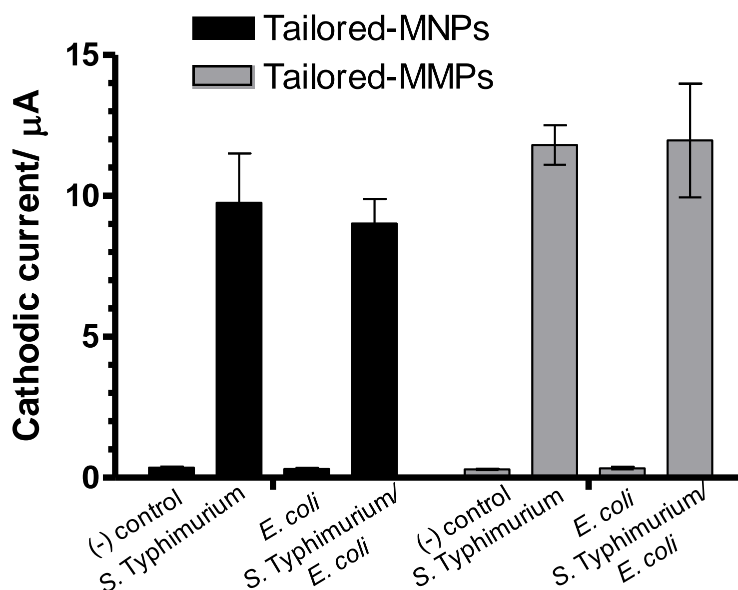

Figure 4.10 Specificity study for the electrochemical magneto-immunosensor in tailored-MNPs (left bars) and tailored-MMPs (right bars). The bars show the electrochemical signal in whole milk diluted 1/10 in BHI broth with. For tailored-MNPs: negative control (0 CFU mL^{-1}); *S.* Typhimurium (2.95×10^{5} CFU mL^{-1}); *E. coli* (9.75×10^{4} CFU mL^{-1}); *S.* Typhimurium (2.95×10^{5} CFU mL^{-1}) and *E.* coli mix (9.75×104 CFU mL^{-1}). For tailored-MMPs: negative control (0 CFU mL^{-1}); *S.* Typhimurium (2.50×10^{5} CFU mL^{-1}); *E. coli* (2.08×10^{5} CFU mL^{-1}); *S.* Typhimurium (2.50×10^{5} CFU mL^{-1}) and *E. coli* mix (2.08×10^{5} CFU mL^{-1}). The error bars show the standard deviation for n=3.

The differentiation of bacterial strains and serotypes among Enterobacteriaceae is dependent on the different specificities of the thermostable somatic O antigens and for some bacteria the thermolabile flagellar H antigens.[14,15] In this context, several studies were reported about possible cross-reactions between O-antigens of close related Enterobacteriaceae as *E. coli* with *Salmonella*.[16]

The electrochemical magneto-immunosensing is based on a double immunological recognition provided by the combination of a monoclonal antibody directed against bacterial lipopolisaccharides for the IMS and a polyclonal antibody towards "O" & "H" antigens for the electrochemical readout, confirming the excellent specificity of the electrochemical magneto-immunosensor, although further studies should be done using other related Enterobacteriaceae.

4.4.2.2 Electrochemical magneto-immunosensor for the detection of *Salmonella* in preenriched milk

Accordingly to the results for the LODs obtained in milk, a preenrichment step should be included to fulfill the legislation 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).

In order to evaluate the preenrichment time to achieve the legislation requirement, a sample of whole milk was artificially contaminated with *S.* Typhimurium, following the proportion of 1 CFU of *Salmonella* in 25 mL of milk. Moreover, a positive (~10 CFU in 25 mL) and a negative samples (absence of *Salmonella*), were also prepared. All milk samples were preenriched in BHI broth at 37 ºC and assayed at 0, 4, 6, 8 and 10 h. The amperometric responses were measured using tailored-MNPs, being presented in Figure 4.11.

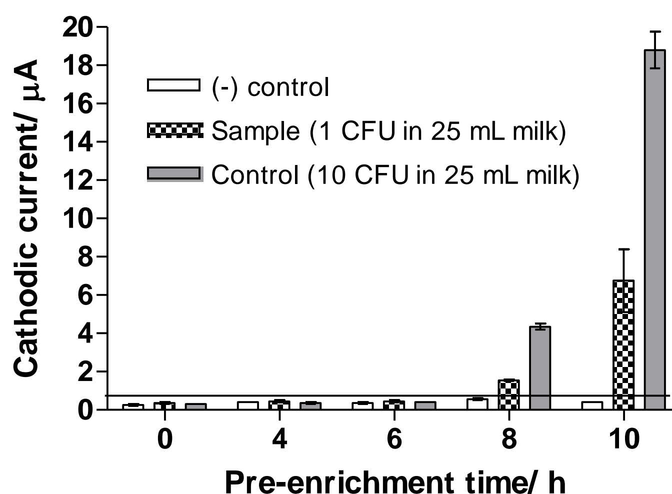

Figure 4.11 Electrochemical signals for the electrochemical magneto-immunosensor after a preenrichment step of 0, 4, 6, 8 and 10 hours for whole-milk artificially inoculated with *Salmonella*. In all cases tailored-MNPs were used. The error bars show the standard deviation for n = 3 and the solid line represents the cut-off signal at the LOD.

It was observed that after 8 h of enrichment, the spiked-milk sample showed signals above the cut-off signal at the LOD (1.55 vs 0.49 μA, respectively), suggesting that this system

was able to detect as low as 1 CFU of *S.* Typhimurium in 8 h of enrichment in BHI broth. The same result was achieved when commercial magnetic microparticles were used.[5]

Hence, electrochemical magneto-immunosensing of *S.* Typhimurium was achieved with tailored-MMPs and MNPs with similar performance in terms of LODs, although a slightly higher matrix effect was observed when using tailored-MNPs. A total assay time of around 9 h, including 50 min of assay time are needed to detect 1 CFU in 25 mL of milk.[13]

Few strategies containing preenrichment studies for the detection of *Salmonella* were reported in the literature. For instance, an immunosensor array based on a 96-well electrochemical plate coupled with IMS step was able to detect from 1 up to 10 CFU of *Salmonella* in 25 g of food sample within 8 h (6 h preenrichment and 2 h of assay time), being more rapid but with higher LOD than in the methodology presented in this Dissertation.[17] Commercial kits available for the detection of *Salmonella* require more time of preenrichment ranging from 16 to 24 h in order to achieve comparable LODs.[18]

4.4.3 Immunomagnetic Separation/Single-Tagging PCR Amplification/Electrochemical Magneto-Genosensing based on silica magnetic particles

The electrochemical magneto-genosensing procedure was firstly combined with an IMS step, followed by a lysis step of the captured bacteria and further amplification of the genetic material by single-tagging PCR. The electrochemical detection was achieved after an enzymatic labelling with anti-fluorescein HRP electrochemical reporter. In the next sections, each of these steps involving this procedure will be fully studied, for the detection of *S.* Typhimurium in whole milk, using tailored-MNPs for the IMS and silica-MPs for the electrochemical magneto-genosensing.

4.4.3.1 IMS efficiency with tailored-MNPs for *Salmonella* in diluted whole milk

The IMS of *S.* Typhimurium using tailored-MPs was achieved with excellent efficiencies, as discussed in §3.4.6. In this section, the IMS efficiency was studied in a whole concentration range from 0 to $1x10^6$ CFU mL^{-1} in milk samples diluted 1/10 in BHI broth, by using tailored-MNPs and the results are presented in Table 4.4.

Table 4.4 IMS efficiencies for *S.* Typhimurium using tailored-MNPs in diluted whole milk.

Correspondent lanes in electrophoresis gel in Figure 4.12	**Approximate *S.* Typhimurium concentration/CFU mL^{-1}**	**IMS/ %**
1	10^6	99.4
2	10^5	95.4
3	10^4	100
4	10^3	100
5	10^2	100
6	10^1	100
7	10^0	100
8	Negative control	0.00
9	Negative control	0.00

The IMS efficiencies were performed by comparing the amount of the bacteria found in the supernatant before and after the IMS. The CFU obtained by plating each supernatant solution (Method 2) was thus used to calculate the IMS (%) based on the Equation 3.3 (§3.4.3.1).

The results showed that more than 95 % of *S.* Typhimurium was attached to the particles for all range of concentrations, confirming the outstanding performance of the tailored-MNPs for the IMS of *Salmonella* in whole milk.

4.4.3.2 Single-Tagging PCR Amplification

After the IMS separation step, a lysis treatment was applied to release the DNA by heating all the samples in a water bath at 99 ºC for 20 min. Amplification of the bacterial DNA obtained after the IMS was performed by single-tagging PCR methodology, as depicted above in Figure 4.3.

The electrophoresis gel containing the bands related to the amplification of the captured bacteria ranged from 10^6 (lane 1) until 10^0 CFU mL^{-1} (lane 7) is presented in Figure 4.12. Additionally, negative controls from the IMS (lanes 8 and 9), obtained by incubating the tailored-MNPs with milk samples diluted 1/10 in BHI broth, as well as the negative controls from the PCR (lane 10), performed without DNA template reaction, are also shown. The results based on agarose electrophoresis gel showed a specific detection of *S.* Typhimurium *yfiR* gene (375 bp) obtained with a LOD of 10 CFU mL^{-1}, as shown in lane 6. Moreover, the chosen set of primers amplified exclusively the *yfiR* gene, producing only the expected 375 bp fragments, according to the agarose gel electrophoresis shown in Figure 4.12, for serial concentrations ranging from 10^0 to 10^6 CFU mL^{-1} (lanes 1−7) in milk artificially inoculated with *Salmonella*. No bands were observed for the negative controls (0 CFU mL^{-1}) performed in diluted whole milk, with no bacteria attached to the particles (Figure 4.12 lanes 8−10).

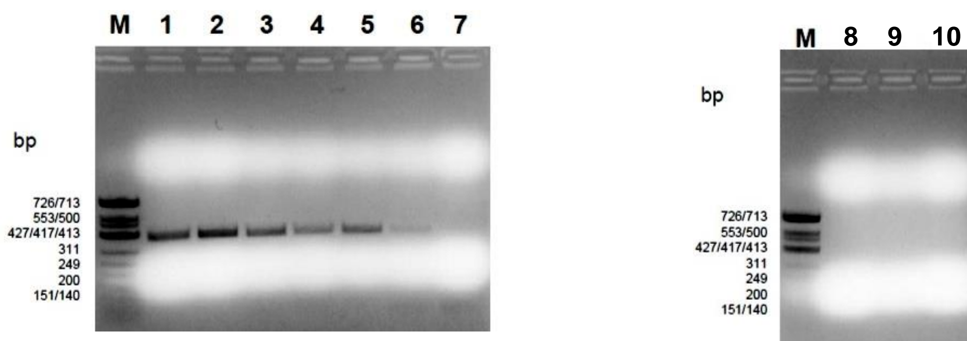

Figure 4.12 Electrophoresis of the specific detection of *S.* Typhimurium *yfiR* gene. A PCR was performed with serial dilutions of *S.* Typhimurium captured with tailored-MNPs ranged from 10^6 until 10^0 CFU mL^{-1} (lanes 1-7). The amplification bands correspond to *S.* Typhimurium *yfiR* gene fragment (375 bp). The lanes 8 and 9 correspond to the negative controls from the IMS and the lane 10 the negative control from the PCR, all performed without DNA template. In all cases, a molecular weight marker *Hinf*I digested ϕ174 DNA was used.

4.4.3.3 Electrochemical magneto-genosensing on silica magnetic particles

After the amplification of the *yfiR* gene fragment from the captured *S.* Typhimurium samples on tailored-MNPs, all fluorescein single-tagged amplicons were immobilised on silica-MPs upon enzymatic labelling using anti-fluorescein HRP for the electrochemical magneto-genosensing.

It is worth noting that, silica-MPs were used the first time as a platform for DNA immobilisation followed by electrochemical magneto-genosensing. The DNA adsorption on the silica shell is based on the negative charge density of the sugar-phosphate backbone, as well as the silica contact layer, and it has been attributed to different driving forces, such as intermolecular electrostatic interactions, dehydration and hydrogen bond formation.[19] The silica surface is negatively charged, due to the weakly acidic silanol groups.[20] On the other hand, phosphate diesters on the backbone of DNA carry two univalent negative charges per base pair.[21] This leads to a strong electrostatic repulsion, due to the negatively charged silica surface and the negative charge of the DNA phosphate backbone. In order to compensate the electrostatic repulsion and promote the attachment of DNA, the chaotropic agent guanidinium thiocyanate was used in the binding buffer.[22] Therefore, the amount of DNA adsorbed on a silica surface and the conformation of the DNA mostly depends on the solution pH, ionic strength and electrolyte type.[19-22]

The amperometric signal corresponding to the LOD was estimated by processing the negative control samples (n=6, 0 CFU mL^{-1}, from the IMS procedure and single-tagging PCR amplification) and the amplicons from the captured bacteria in two different single inter-day assays, obtaining a mean value of 0.36 μA with a standard deviation of 0.052 μA using silica-MPs. The cut-off values were then determined by processing the negative control samples with a one-tailed t test at a 95% confidence level, giving a value of 0.46 μA, as depicted in Figure 4.13, as a solid line.

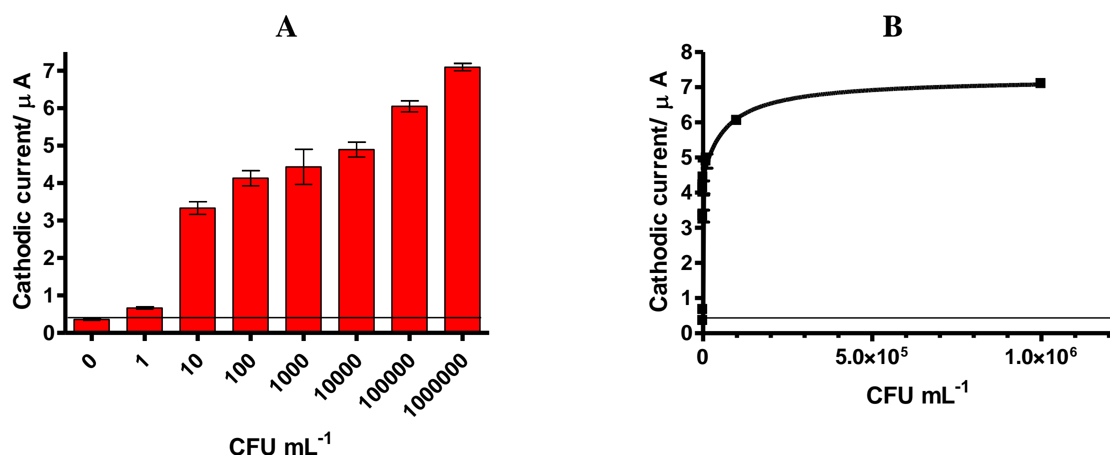

Figure 4.13 (A) Electrochemical signals for IMS, Single-Tagging PCR amplification, and electrochemical magneto-genosensing based on silica-MPs. Red bars show the signal by increasing the amount of *Salmonella* ranged from 10^0 to 10^6 CFU mL^{-1} artificially inoculated in in whole milk diluted 1/10 in BHI broth. The negative control is also shown. In all cases, 10 µg AntiFluo-HRP and 5×10^7 silica-MPs 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 -0.150 V (vs Ag/AgCl). In all cases, n=3, except for the negative control (n=6). Solid lines represent the cut-off limit values, extracted with a one-tailed t-test at 95% confidence level. (B) Data fitted with a nonlinear regression (Two site binding (hyperbola) Y=Bmax1*X/(Kd1+X) + Bmax2*X/(Kd2+X), $R^2 = 0.9801$.

As shown in Figure 4.13, panel A, IMS/Single-Tagging PCR amplification /electrochemical magneto-genosensor based on silica-MPs in milk samples, was able to give a clear positive signal (3.3 µA with a standard deviation of 0.29 µA) and a signal-to-background ratio value of 9.3 for 10 CFU mL^{-1}, while the electrophoresis for the same concentration shows a weak positive band (Figure 4.12, lane 6). In addition, the electrochemical magneto-genosensor based on silica-MPs was able to clearly detect as low as 1 CFU mL^{-1} with a total assay time of 4 h, and also an amperometric signal of 0.67 µA, a standard deviation of 0.06 µA and a signal-to-background ratio value of 1.9.

Lastly, the data were adjusted to a nonlinear regression (Two site binding (hyperbola) Y=Bmax1*X/(Kd1+X) + Bmax2*X/(Kd2+X), as shown in Figure 4.13, panel B. Thus, the binding of the single-tagged amplicon to the tailored-MNPs is described, being Bmax the maximal binding and Kd the concentration of amplicon required to reach half-maximal binding, obtaining a R^2= 0.9801.

Hence, this method showed to be more sensitive than other rapid antibody-based and nucleic acid-based PCR methods that have been previously reported and extensively

reviewed.[18] Moreover, this procedure was able to detect at least 1 CFU mL^{-1} in 4 h without the use of any culturing preenrichment or selective plating enrichment steps, with higher sensitivity than gel electrophoresis of PCR products or conventional microbiological plating techniques.

In addition, a comparison of this methodology was evaluated with other biosensing methodologies for the detection of *Salmonella*, such as bacteriophage-based genosensor[23] and IMS coupled with double tagging PCR/m-GEC electrochemical genosensing,[24] using commercial magnetic microparticles for both methodologies. Thus, it was observed that the use of tailored-MNPs for the IMS combined with silica-MPs for the electrochemical magneto-genosensing improves the analytical performance of the bioassay. In this context, significantly lower background values for the negative control were achieved (0.35 vs 0.75 and 2.2 μA, respectively for the electrochemical magneto-genosensor based on silica-MPs, the bacteriophage-based genosensor[23] and the double tagging PCR/m-GEC electrochemical genosensing[24], as well as improved standard deviation values (0.05 n=6; vs 0.2 n = 8 and 0.65 n = 35, respectively). As a result, lower amperometric signal corresponding to the LOD value was achieved (0.46 vs 1.33 and 3.78 μA), allowing higher sensitivity and better discrimination at lower bacteria concentration levels.

This fact can be ascribed to the combination of the IMS step using tailored-MNPs and the sensitivity of the amplicon detection with the m-GEC electrochemical magneto-genosensing strategy based on silica-MPs, being an effective tool for the microbiological control in food samples.

4.4.3.4 Specificity study of the electrochemical magneto-genosensor based on silica magnetic particles

The IMS/Single-Tagging PCR/Electrochemical magneto-genosensing procedure for the detection of *S.* Typhimurium is based on the IMS combined with the electrochemical magneto-genosensing. This approach involves the lysis of the captured bacteria after the IMS step, followed by the amplification of the genetic material by a single-tagging PCR with a set of primers specific for *S.* Typhimurium *yfiR* (375 bp), labelled with fluorescein.[25] In terms of the

specificity, this strategy combines the immunological and the genetic biorecognition of the bacteria, due to the IMS and the single-tagging PCR/electrochemical genosensing, respectively.

Preliminary IMS results obtained by classical culturing were previously shown in §3.4.7, suggesting an outstanding specificity of the IMS procedure performed with tailored-MNPs, based on the immunological recognition of *S.* Typhimurium, throughout the mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against LPS, since no attachment of *E. coli* and *L. monocytogenes* occurred.

This study was performed in order to confirm if the specificity of this procedure is mainly provided by the immunological recognition during the IMS step or by the genetic recognition during the PCR.

In this context, tailored-MNPs against *S.* Typhimurium were incubated with suspensions of the bacteria in whole milk diluted 1/10 in BHI of (i) *E. coli*, (ii) *L. monocytogenes* and (iii) *S.* Typhimurium, as well as binary combinations containing (iv) *S.* Typhimurium and *E. coli* and (v) *S.* Typhimurium and *L. monocytogenes*. The IMS study was fully explained in §3.2.11.

After the IMS, the bacterial DNA was extracted and the specificity was evaluated by electrophoresis on 2 % agarose gel in TAE buffer containing 0.5 μg mL^{-1} ethidium bromide and the results are shown in Figure 4.14. In this instance, the PCR was performed by adding not only the set of primers specific for *Salmonella*, but also for *E. coli* and *L. monocytogenes*. Therefore, if any cross reaction occured during the IMS between the tailored-MNPs and *E. coli* and/or *L. monocytogenes*, bands at 151 and 234 bp should be observed, respectively, since the specific primers for the amplification of these bacteria were added during the PCR, as shown in Figure 4.14, lanes 8 and 9. In order to show the amplification efficiency of these primers, genomic DNA of *L. monocytogenes* and *E. coli* were added. In Figure 4.14, the amplification of *hlyA* and *eaeA* gene fragments of *L. monocytogenes* and *E. coli*, respectively are shown in the Lanes 8 and 9.

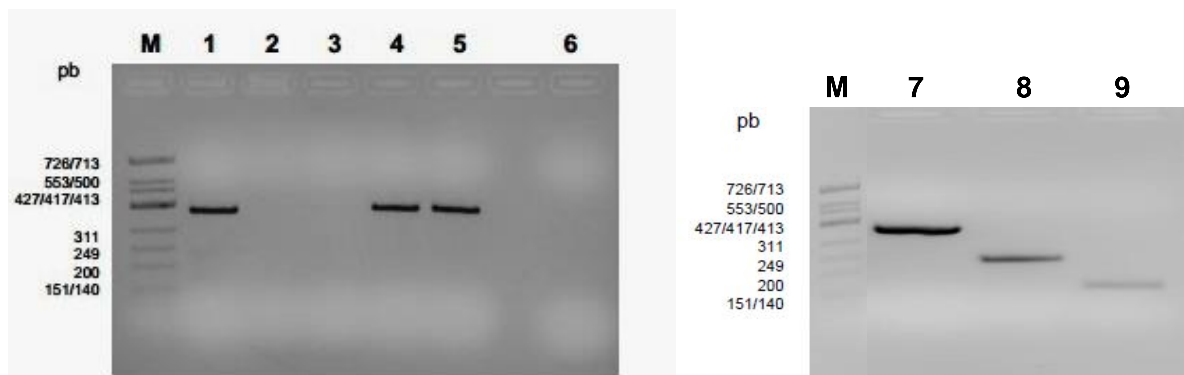

Figure 4.14 Specificity study based on IMS, followed by PCR and agarose gel electrophoresis of PCR amplicon. Lane 1: *S.* Typhimurium; Lane 2: *E. coli*; Lane 3: *L. monocytogenes*, Lane 4: Mix containing *S.* Typhimurium and *E. coli*, Lane 5: Mix containing *S.* Typhimurium and *L. monocytogenes* and Lane 6: PCR negative control. The amplification bands correspond to *yfiR* gene fragment of *S.* Typhimurium (375 pb). In addition, genomic DNA of each bacterium was also added to show specificity of each primer, in which the three amplification bands correspond to Lane 7: *S.* Typhimurium *yfiR* gene fragment (375 pb), Lane 8: *L. monocytogenes hlyA* gene fragment (234 pb) and Lane 9: *E. coli eaeA* gene fragment (151 bp). In all cases, a molecular weight marker *Hinf*I digested ϕ174 DNA was used. Results shown in lanes 1-5 were obtained for artificially inoculated whole milk diluted 1/10 in BHI broth.

As observed in Figure 4.14, in the Lanes 1 to 6, only the *yfiR* gene fragment of 375 pb specific for *S.* Typhimurium is observed, in the positive control containing only *Salmonella* (lane 1), as well as in the binary combinations containing *S.* Typhimurium/*E. coli* (lane 4) and *S*. Typhimurium/*L. monocytogenes* (lane 5), while no amplification were observed in the negative controls (lane 6), as well as in the suspensions containing only *E. coli* (lane 2) or *L. monocytogenes* (lane 3). With this experiment, the outstanding specificity of the IMS procedure performed with tailored-MNPs towards *Salmonella* based on the immunological recognition throughout the mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against the LPS is confirmed.

Finally, the same specificity study was then performed with the IMS/PCR/ electrochemical magneto-genosensing procedure based on silica-MPs, but at a concentration range of bacterial suspension near de LOD of 1 x 10^1 CFU mL^{-1} in whole milk diluted 1/10 in BHI containing, as above, the single combination of the bacteria, as well as the binary combinations.

The results are shown in Figure 4.15. As expected, the electrochemical signals obtained for both *E. coli* and *L. monocytogenes* are similar that for the negative control, whereas the mix

of *Salmonella* with both bacteria (*E. coli* and *L. monocytogenes*) gave similar signals to the sample spiked with *Salmonella.*

These results confirmed the outstanding specificity of the IMS/Single-Tagging PCR/Electrochemical magneto-genosensing procedure, combining tailored-MNPs for the IMS and silica-MPs for the electrochemical magneto-genosensing.

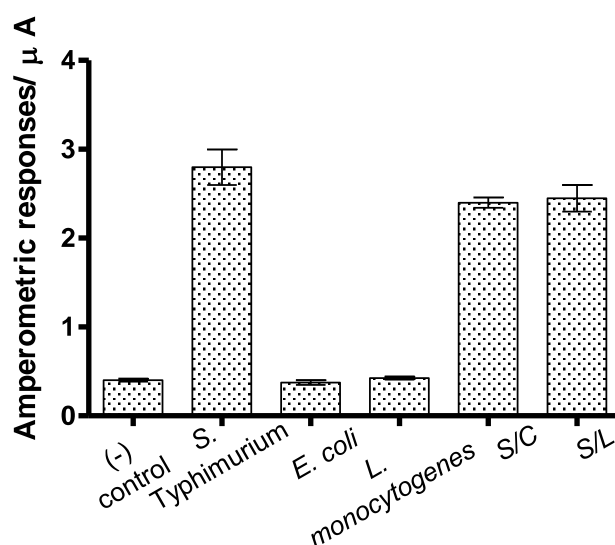

Figure 4.15 Specificity study for the IMS/ single-tagging PCR/electrochemical magneto-genosensing approach. Gray bars show the electrochemical signal for milk diluted 1/10 in LB artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control), and 1×10^1 CFU mL^{-1} *S.* Typhimurium and 1×10^5 CFU mL^{-1} of *E. coli* and *L. monocytogenes*, mix containing *S.* Typhimurium and *E. coli* (S/C) and mix containing *S.* Typhimurium and *L. monocytogenes* (S/L). All other conditions as depicted in Figure 4.13. The error bars show the standard deviation for n = 3.

High specificity of the IMS/Single-tagging PCR/Electrochemical magneto-genosensing on silica-MPs approach is explained mainly from the IMS step, due to the use of a specific antibody towards *Salmonella* which is immobilised on the tailored-MNPs. According to the suppliers, a mouse monoclonal Isotype IgG1 antibody to *S.* Typhimurium 0-4, being directed against the LPS of *S.* Typhimurium. Moreover, the selection of the set of primers specific for *S.* Typhimurium *yfiR* gene fragment provides an additional source of specificity of the approach, particularly for those bacteria antigenically related to Salmonellae genus. The chosen set of primers amplified exclusively the *S.* Typhimurium *yfiR* gene fragment specific for *Salmonella* will be further discussed in the next chapter, §5.2.4.

Hence, it was shown that the IMS/Single-Tagging PCR/Electrochemical magneto-genosensing procedure based on silica-MPs approach provides high specificity in the detection of *S.* Typhimurium in whole milk, being able to distinguish between *S.* Typhimurium and *E. coli* and/or *L. monocytogenes*.

4.5 Conclusions and final remarks

This chapter addressed a comparison of two strategies, electrochemical magneto-immunosensing and genosensing for the detection of *S.* Typhimurium in milk samples combining tailored-MPs for the IMS and silica-MPs, used for the first time, as a platform for DNA immobilisation in electrochemical magneto-genosensing approach. These strategies are presented and compared in Table 4.5 based on total assay time, enrichment step (if required) and LODs.

Table 4.5 Comparison of the electrochemical magneto-immunosensor based on tailored-MNPs and MMPs and the IMS/Single-Tagging PCR/Electrochemical magneto-genosensing strategies, for the detection of *S.* Typhimurium in milk samples reported in this chapter.

Strategy	Magnetic carriers	Total assay time	Enrichment step	LOD
Electrochemical magneto-immunosensor based on tailored-MNPs	Tailored-MNPs	50 min	n.a.	291 CFU mL^{-1}
		~9 h	8h	0.04 CFU mL^{-1} with preenrichment
Electrochemical magneto-immunosensor based on tailored-MMPs	Tailored-MMPs	50 min	n.a.	538 CFU mL^{-1}
		~9 h	8h	0.04 CFU mL^{-1} with preenrichment
IMS/Single-Tagging PCR/Electrochemical magneto-genosensing combining tailored-MNPs and silica-MPs	Tailored-MNPs (for the IMS) and silica-MPs (for the electrochemical genosensing)	4 h	n.a.	1 CFU mL^{-1}

The electrochemical detection was based on amperometry using m-GEC electrodes. In this way, tailored-MPs played a dual function as a support for the immobilisation of biological recognition element, improving the incubation and the washing steps of the biological assay, but also for the magnetic immobilisation on the surface of a m-GEC electrode under magneto-actuation (§1.2.3.4).[4,5]

Prior to the amperometric studies, the m-GEC working electrodes of the same batch used in this study were characterised by CV. In this study, hydroquinone/benzoquinone redox couple was selected as electroactive species, since hydroquinone is the mediator for the electrochemical reporter HRP used in the amperometric detection strategies.[9] All m-GEC electrodes showed similar electrochemical behaviour, suggesting high reproducibility in the construction. Finally, based on this study, a potential of -0.150 V was the condition selected for the amperometric measurements.

A comparison in the analytical performance of both tailored-MMPs and MNPs for the detection of *S.* Typhimurium in whole milk was firstly studied using a magneto-actuated electrochemical immunosensor (Table 4.5).

The rapid detection was based on double antibody recognition, in detail, a mouse monoclonal Isotype IgG1 antibody to *S.* Typhimurium 0-4, immobilised on both tailored-MMPs and MNPs for the IMS, and also a polyclonal antibody towards "O" & "H" antigens labelled with HRP. In this instance, the same immunological biorecognition reaction is used for the IMS and detection of the bacteria. However, the bacteria were recognised with two antibodies with different clonalities towards different epitopes, increasing the specificity of the approach. In this context, LODs of 538 CFU mL^{-1} for tailored-MMPs and 291 CFU mL^{-1} for tailored-MNPs were achieved in 50 min without the need of a preenrichment step and at least 0.04 CFU mL^{-1} (1 CFU in 25 mL of whole milk) if the sample is preenriched until 8 h. Moreover, it was also shown that this strategy was able to distinguish between *S.* Typhimurium and *E. coli*.[13]

In the literature, no conclusive results were found about the influence of the size of the MPs on the IMS and electrochemical detection of *Salmonella*. In this Chapter, a comparison of the performance of both tailored-MMPs and MNPs showed similar analytical performance for the detection of *Salmonella*, although with slightly improved LODs for tailored-MNPs. In addition, tailored-MNPs showed a slightly higher matrix effect, higher degree of aggregation,

requiring also longer time for magnetic actuation. Furthermore, a decrease of the amperometric responses was also observed in whole milk, especially when using tailored-MNPs as a carrier, suggesting a matrix effect possibly attributed to the higher surface area -to- volume ratio due to their smaller size, which could also increase the non-specific adsorption, in the presence of complex media as whole milk, raising thus the influence of the matrix components during the assay.[26]

A sensitive assay combining IMS on tailored-MNPs, single-tagging PCR and electrochemical magneto-genosensing using silica-MPs for the detection of *Salmonella* in milk was also presented (Table 4.5). This strategy was able to detect 1 CFU mL^{-1} of *S.* Typhimurium in milk diluted 1/10 in BHI, within 4 h, being also able to distinguish between *S.* Typhimurium and *E. coli* and *L. monocytogenes*.

S. Typhimurium was captured on tailored-MNPs, then the attached bacteria was released and the genomic DNA amplified based on single-tagging PCR using a set of primers specific for *S.* Typhimurium. Afterwards, the single-tagged amplicon was detected by electrochemical magneto-genosensing, using silica-MPs, reported for the first time in the literature. Therefore, the assay time of this assay was thus considerably reduced from 3-5 days when conventional microbiological culture techniques are used to 4 h.

It is important to highlight that the specificity of this approach is conferred by the IMS, which showed an outstanding specific towards *Salmonella* based on the immunological recognition throughout the mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against LPS of *S.* Typhimurium. However, the selection of the set of primers specific for *S.* Typhimurium *yfiR* gene fragment provides an additional source of specificity of the approach, particularly for those bacteria antigenically related to Salmonellae genus. The chosen set of primers amplified exclusively the *S.* Typhimurium *yfiR* gene fragment specific for *Salmonella* will be further discussed in the next chapter. On the other hand, the sensitivity conferred by electrochemical magneto-genosensing is given by the combination of different magnetic carriers and also by the single-tagging PCR resulting in a robust and sensitive procedure.

Hence, the integration of the MPs showed to be a determinant factor to improve the analytical performance of both electrochemical magneto-immuno and genosensors, providing the preconcentration of the bacteria during the IMS, reducing the time required for the

preenrichment step and the LODs, reducing the effect of the food components and PCR inhibitors, and serving also as a platform for the electrochemical readout based on magneto-actuated electrodes.

Another aspect to highlight is related to silica-MPs which have been extensively used for the isolation of PCR products and genomic DNAs.[27] However, it is the first time that these particles are reported 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.[19] Interestingly, the silica magnetic particles showed differential adsorption properties, based on the negative charge density, for longer dsDNA amplicon incorporating the tagged-primers over shorter ssDNA tagging-primers, showing to be not only a robust platform for the electrochemical detection of PCR products but also a promising magnetic carrier for fluorescence or other detection approaches.

Over the last years, several approaches for *Salmonella* detection in food samples were reported and recently reviewed by our research group.[18] A PCR based assay was presented for the detection if *Salmonella* in milk, ice-cream and fruit juice, being able to detect 5 to 10 CFU mL^{-1} in 3-4h by agarose gel under the UV transilluminator.[28] A q-PCR assay using aptamers was developed showing a LOD of 10^2 –10^3 CFU of *Salmonella* per 290 µL sample volume.[29] A surface modified with polyacrylonitrile (PAN) fibres as a novel matrix of sandwich immunoassay was shown for the detection of *Salmonella* in milk samples, presenting a LOD of 10 CFU mL^{-1} within 4h.[30] Finally, an *in-situ* immuno-gold nanoparticle network ELISA biosensor was presented, being able to detect 15 CFU mL^{-1} of *Salmonella* in food samples, within 2 h of inoculation.[31] Therefore, comparing the LOD and assay time of the aforementioned strategies, it was observed that IMS/Single-Tagging PCR/Electrochemical magneto-genosensing procedure showed improved results in comparison with the recent studies published in the literature for the detection of *Salmonella*.

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CHAPTER 5

Simultaneous electrochemical magneto-genosensing of foodborne bacteria based on triple-tagging multiplex amplification

5.1 Introduction

As it was discussed in the Introduction (§1.1.1), the increase of foodborne infections has become an important public health concern worldwide, being bacteria the main cause of food-borne illness.[1] Among all foodborne pathogens, *S. enterica*, *L.monocytogenes* and *E. coli* O157:H7 are considered the most dangerous in terms of human illnesses and food poisoning.[2] In fact, the number of reported confirmed cases of foodborne illnesses in humans in 2013 in 32 the European countries, was 82,694, 6,043 and 1,763 presenting a case-fatality rate of 0.14, 15.6 and 0.36 respectively for *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7. In recent years, many improvements have been made to replace time-consuming conventional culture detection methods by rapid methodologies, such as polymerase chain reaction, immunological assays and biosensing approaches. However, the development of new methods with the advantages of rapid response, sensitivity, specificity and ease of multiplexing for food hygiene inspection, in order to achieve the threshold limits set by the Food Regulatory agencies is still a challenge.[3]

The development of novel strategies for simultaneous detection of different foodborne pathogens presents a cost effective and time saving strategy, reducing substantially the assay times and costs.[1] Based on the results presented in the previous chapters, electrochemical magneto-genosensing showed to be a powerful strategy in terms of specificity and sensitivity for the detection, of *S.* Typhimurium at 1 CFU mL^{-1} in whole milk. Hence, it is proposed to develop a magneto-actuated electrochemical genosensor for the simultaneous detection of *S.* Typhimurium, *E. coli* and *L. monocytogenes* combining for the first time, triple-tagging multiplex PCR methodology and electrochemical magneto-genosensing based on silica magnetic particles (silica-MPs).

In this context, a multiplex triple-tagging PCR procedure was developed. Some studies based on multiplex PCR have been reported for the simultaneous detection of different *S. enterica* genes, such as *invA, hilA, ttr, spvC*, of *L. monocytogenes* genes as *hlyA* (the most common target), *prfA*, *inlA*, *inlB*, *iap* or of *E. coli* genes, such as *rfbE*, *eaeA*, *vt1*, *vt2*, *hlyA*, *lt*, *st*, *bfpA*, *ipaH*, *aaF/I*.[4,5,6]

In this approach, a set of tagging primers for the triple-tagging multiplex PCR were selected for the amplification of *yfiR* (375 bp), *hlyA* (234 bp) and *eaeA* (151 bp), being one of the primer for each set tagged with fluorescein, biotin and digoxigenin coding for *S.* Typhimurium, *L. monocytogenes* and E. *coli*, respectively.[7] Afterwards, electrochemical

magneto-genosensing of the bacteria was achieved by using silica-MPs as a carrier and three different electrochemical reporters, specific for each bacteria.

Electrochemical magneto-genosensing combined with double-tagging PCR have shown to be a very efficient strategy for the sensitive single bacteria detection.[8] Nevertheless, the simultaneous electrochemical detection of foodborne bacteria based on triple-tagging multiplex amplification is presented for the first time, as promising approach for bacteria screening of the most relevant pathogens in food, i.e. *S.* Typhimurium, *L. monocytogenes* and *E. coli*.

5.2 Experimental Section

5.2.1 Chemicals and Biochemicals

Dynabeads MyOne Silane, Product Nº 37002D were purchased from Dynal Biotech ASA (Oslo, Norway). AntiFlu-HRP (Anti-Fluorescein-POD Fab fragments, 11426346910), Strep-HRP (Streptavidin-POD conjugate, 1089153001) and AntiDig-HRP (Anti-Digoxigenin-POD Fab fragments, 11207733910) were purchased from Roche Diagnostics. The graphite-epoxy composite was prepared with graphite thin powder (1.04206.2500, Merck, Darmstadt, Germany) and Epo-Tek H77 (epoxy resin and hardener both from Epoxy Technology, USA).

All buffer solutions were prepared with milli-Q water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions were: Binding buffer (0.1 mol L^{-1} Tris, 5 mol L^{-1} GuSCN, pH 6.4); Washing buffer (10 mmol L^{-1} Tris, pH 4.5); 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); Phosphate buffer (0.1 mol L^{-1} sodium phosphate, 0.1 mol L^{-1} KCl, pH 7.0).

5.2.2 Bacterial strains, growth conditions and DNA extraction

Bacterial strains used in this work were *S.* Typhimurium, *L. monocytogenes* and *E. coli* O157:H7. The detailed information about *S.* Typhimurium and *L. monocytogenes* is shown in Chapter 3 (§3.2.2). *E. coli* O157:H7 clinical isolate strain was supplied by Hospital of Bellvitge, Barcelona, Spain.

All bacterial strains were grown in Luria Bertani (LB) broth or agar plates for 18 h at 37 ºC. The lysis of the bacteria, 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.

5.2.3 Oligonucleotide sequences

Oligonucleotide for the simultaneous detection of *S.* Typhimurium *L. monocytogenes* and *E. coli* were obtained from TIB-Molbiol GmbH (Berlin, Germany). These primers were selected for the amplification of *yfiR* (375 bp), *hlyA* (234 bp) and *eaeA* (151 bp) gene fragments specific to *S.* Typhimurium, *L. monocytogenes* and *E. coli*, respectively.[7] Each oligonucleotide sequence was tagged at 5' end with different tags, such as fluorescein, biotin and digoxigenin, for further coding between the different bacteria in the electrochemical detection. The primer sequences, as well as the tags used for the triple-tagging multiplex PCR are shown in Table 5.1.

Table 5.1 Triple-tagging set of primers for the multiplex PCR amplification.

STRAIN	GENE	PRIMER SEQUENCE (5'-3')	TYPE	5'-LABELS	SIZE (bp)
S. Typhimurium	*yfiR*	GTCACGGAAGAAGAGAAATCCGTACG	Forward	Fluorescein	375
		GGGAGTCCAGGTTGACGGAAAATTT	Reverse	Non-labelled	
L. monocytogenes	*hly*A	CGGAGGTTCCGCAAAAGATG	Forward	Biotin	234
		CCTCCAGAGTGATCGATGTT	Reverse	Non-labelled	
E. coli	*eae*A	GGCGGATAAGACTTCGGCTA	Forward	Digoxigenin	151
		CGTTTTGGCACTATTTGCCC	Reverse	Non-labelled	

5.2.4 Triple-tagging multiplex PCR

The triple-tagging multiplex PCR was achieved by a set of three tagging primers for the amplification of the *yfiR* (375 bp), *hlyA* (234 bp) and *eaeA* (151 bp),[7] being one of the primer for each set labelled with fluorescein, biotin and digoxigenin coding for *S.* Typhimurium (S), *L. monocytogenes* (L), and *E. coli* (C), respectively (Table 5.1).

The PCR reaction was carried out in a thermal cycler (Product N° 2720, Applied Biosystems, Life Technologies Corporation). The procedure for the triple-tagging PCR is schematically represented in Figure 5.1.

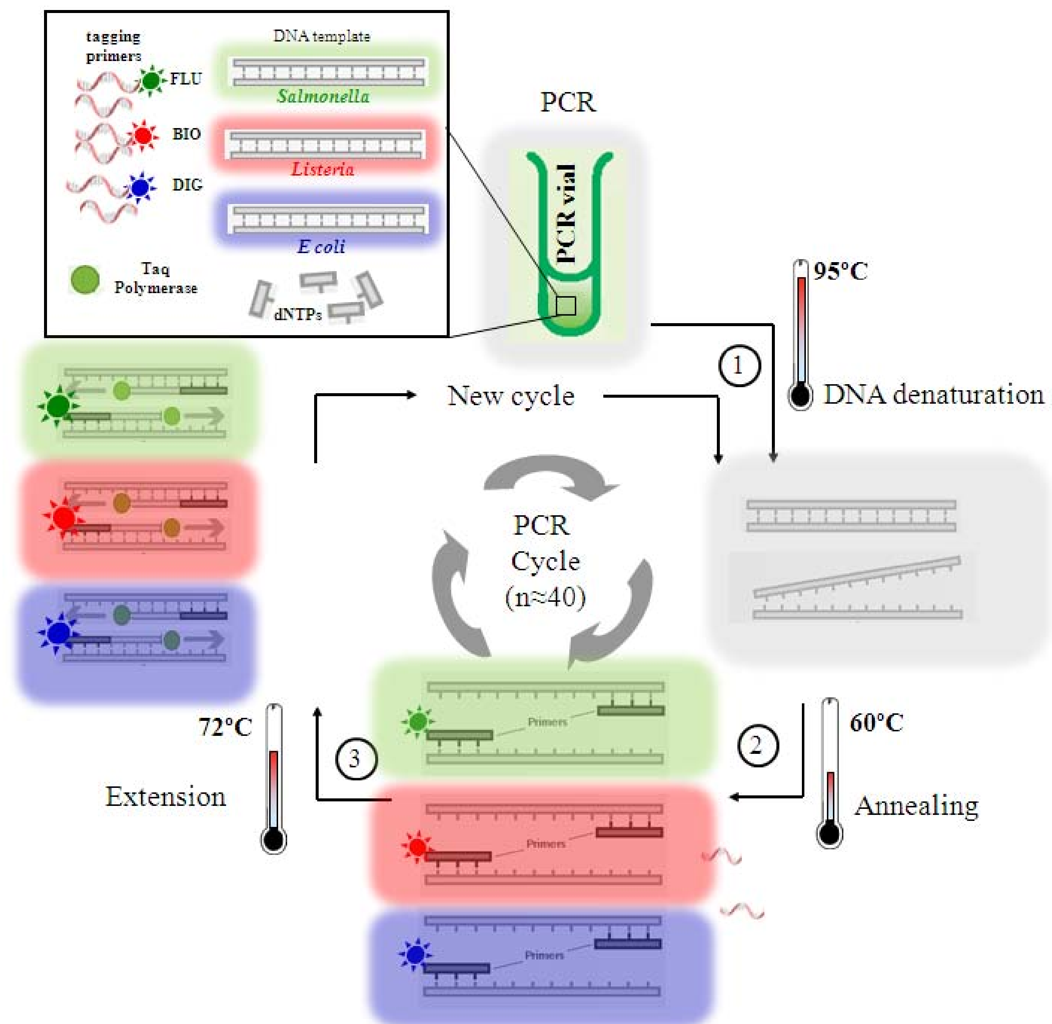

Figure 5.1 Schematic representation of the triple-tagging PCR amplification, in order to obtain the triple-tagged amplicons labelled with fluorescein, biotin and digoxigenin from chromosomal DNA of *S.* Typhimurium, *L. monocytogenes* and *E. coli*, respectively.

All PCR reactions were performed in a final volume of 100 μL, using 100 ng of each genomic DNA as template. The Mastermix composition was prepared with the following products: 200 μmol L^{-1} of each deoxynucleotide triphosphate (dATP, dGTP, and dCTP), 120,100, 80 nmol L^{-1} of each primer, respectively for *S.* Typhimurium, *L. monocytogenes*, and *E. coli* (C), in 5.0 mmol L^{-1} of $MgCl_2$. Thermal cycler conditions are in Table 5.2.

Table 5.2 Thermal cycler conditions for the PCR amplification.

	Initial step	**DNA denaturation**	**Annealing**	**Extension**	**Last step**
	1 cycle		40 cycles		1 cycle
Temperature (°C)	95	95	60	72	72
Time (sec)	600	20	30	30	420

The specificity of the triple-tagging multiplex PCR amplification for the simultaneous detection of the target microorganisms was evaluated. Thus, triple-tagging multiplex amplification was carried out in the presence of all possible combinations including i) the three bacterial DNA templates (S/L/C), ii) the binary combinations (S/C; S/L; C/L), and iii) the single combinations (S; L; C). Moreover, the negative controls were also included, in which no DNA template was added to the PCR mixture.

The performance of the triple-tagging multiplex PCR were analysed with conventional agarose gel electrophoresis on 4% agarose gel containing 0.5X Tris-acetate-EDTA (TAE) and ethidium bromide staining, due to the different size of the amplicons, ranging from 121 to 375 bp.

5.2.5 Confocal microscopy of the tagged amplicon immobilised on silica magnetic particles

The evaluation of the immobilisation procedure of the tagged amplicons on silica-MPs was performed with fluorescence readout by confocal microscopy with the biotinylated amplicons coding for *L. monocytogenes* as a model and compared with the negative control containing the set of tagging primers labelled with biotin.

In this approach, *L. monocytogenes* amplicons and the PCR negative control diluted 1/10 were immobilised on silica-MPs, 5 µL of silica-MPs (10 mg mL^{-1}, washed and diluted 1/4 in binding buffer) were diluted in 45 µL of binding buffer and mixed with 30 µL of the diluted amplicons (1/10) in milli-Q water. The mixture was incubated for 10 min at 55 ºC without shaking. Afterwards, two washing steps were performed in 80 µL of washing buffer. The labelling step was performed using 3 µg of Strep-Cy5. After two washing steps in Tris buffer, the modified silica-MPs were resuspended in 200 µL of Tris buffer. The labelling was performed using the fluorescence reporter Strep-Cy5.

5.2.6 Triple-tagging electrochemical magneto-genosensing on silica magnetic particles

The procedure for the simultaneous detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli*, schematically represented in Figure 5.2 is based on the following steps:

(A) Triple-tagging-PCR, as detailed explained in §5.2.4; (B) Immobilisation of the triple-tagged amplicons coding for three microorganisms on silica magnetic particles, 5 µL of silica-MPs (10 mg mL^{-1}, washed and diluted 1/4 in binding buffer) were diluted in 45 µL of binding buffer and mixed with 30 µL of the diluted amplicons (1/10) in milli-Q water. The mixture was incubated for 10 min at 55 ºC without shaking. Afterwards, two washing steps were performed in 80 µL of washing buffer; (C) Enzymatic labelling with the electrochemical reporters, in detail AntiFlu-HRP (10 µg), Strep-HRP (3 µg) and AntiDig–HRP (3 µg) coding for *S.* Typhimurium, *L. monocytogenes* and *E. coli*, respectively, in three different incubation chambers; the electrochemical reporters were prepared in blocking Tris buffer and added at a final volume of 140 µL for 30 min at room temperature and 700 rpm. Two washing steps were then performed for 5 min at room temperature in 140 µL of Tris buffer; (D) Magnetic actuation by an array of three modified working electrodes, which contain a small magnet, named as magneto-graphite epoxy composite (m-GEC), as detailed in chapter 4 (§4.2.6); (E) Amperometric detection using the m-GEC electrodes which surface was polarised at –0.150 V (vs. Ag/AgCl), under enzyme saturation conditions with the substrate for the simultaneous amperometric detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli*.

The explanation of the amperometric detection using m-GEC electrodes, as well as the selection of the cathodic current for the measurements is fully detailed in §4.2.6. In each measurement a steady-state current was obtained after the addition of hydroquinone (1.81 mM) and hydrogen peroxide (4.90 mM), respectively as mediator and substrate for the enzyme HRP, in phosphate buffer solution, normally after 1 min of hydrogen peroxide addition. This steady-state current was also used for the electrochemical signal plotted in the further results shown in Figures 5.5 to 5.9.

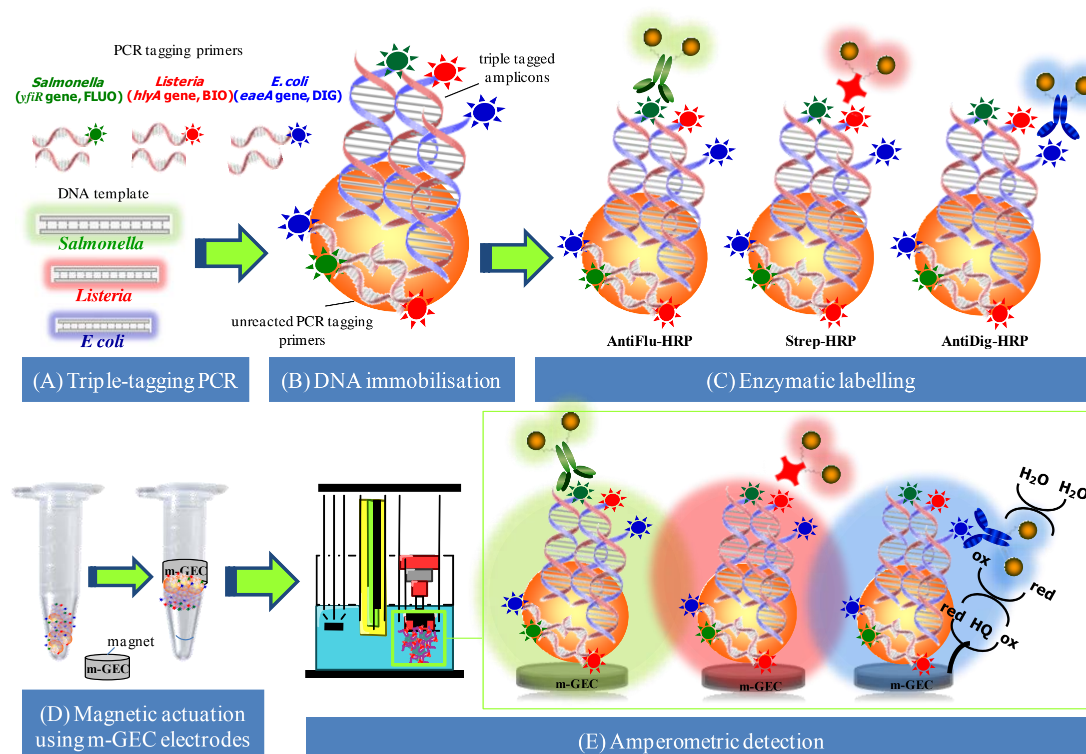

Figure 5.2 Schematic representation of simultaneous detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* based on triple-tagging multiplex PCR and electrochemical magneto-genosensing on silica.MPs.

In all cases, this procedure was followed for all possible combinations, including i) the three bacterial DNA templates (S/L/C), ii) the binary combinations (S/C; S/L; C/L), and iii) the single combinations (S; L; C).

5.2.6.1 Optimisation of the amount of the electrochemical reporter

The amount of electrochemical reporter was firstly optimised on the basis of the signal-to background ratio. In this study, the triple-tagged amplicons obtained from the triple-tagging

multiplex amplification in the presence of the three bacterial DNA templates (S/L/C) were challenged towards 3, 10, 30 µg of AntiFlu-HRP coding for *S.* Typhimurium and 1, 3, 10, 30 µg of both Strep-HRP and AntiDig–HRP, coding for *L. monocytogenes* and *E. coli*, respectively. The positive signals, containing the triple-tagging amplicons were compared with the negative controls, from the multiplex amplification, as signal-to-background ratio.

5.2.6.2 Specificity study

The specificity of the electrochemical reporters coding for each bacterium, *S.* Typhimurium (S), *L. monocytogenes* (L) and *E. coli* (C) was also studied, by challenging the three electrochemical reporters (10 µg AntiFlu-HRP coding for *S.* Typhimurium, 3 µg Strep-HRP coding for *L. monocytogenes* and 3 µg AntiDig–HRP coding for *E. coli*), towards the amplicons obtained from the triple-tagging multiplex PCR, in the presence of all possible combinations, including i) the three bacterial DNA templates (S/L/C), ii) the binary combinations (S/C; S/L; C/L), and iii) the single combinations (S; L; C).

5.2.6.3 Determination of the limit of detection for the triple-tagging electrochemical magneto-genosensing on silica magnetic particles

Electrochemical responses were evaluated for a range of concentrations corresponding to the end-point amplicon dilutions until 1/400, for the detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* towards 10 µg AntiFlu-HRP coding for *S.* Typhimurium, 3 µg Strep-HRP coding for *L. monocytogenes* and 3 µg AntiDig–HRP coding for *E. coli*.

5.3 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.[9] According to these guidelines, the experiments involving *S.* Typhimurium, *E. coli* and *L. monocytogenes* 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.

5.4 Results and Discussion

5.4.1 Triple-tagging multiplex PCR

Since the specificity of the triple-tagging multiplex amplification is a key parameter for the performance of the electrochemical magneto-genosensing, the end-point amplicons were studied by agarose gel electrophoresis. The electrophoresis gel for the simultaneous amplification of the three bacterial DNA is shown in Figure 5.3.

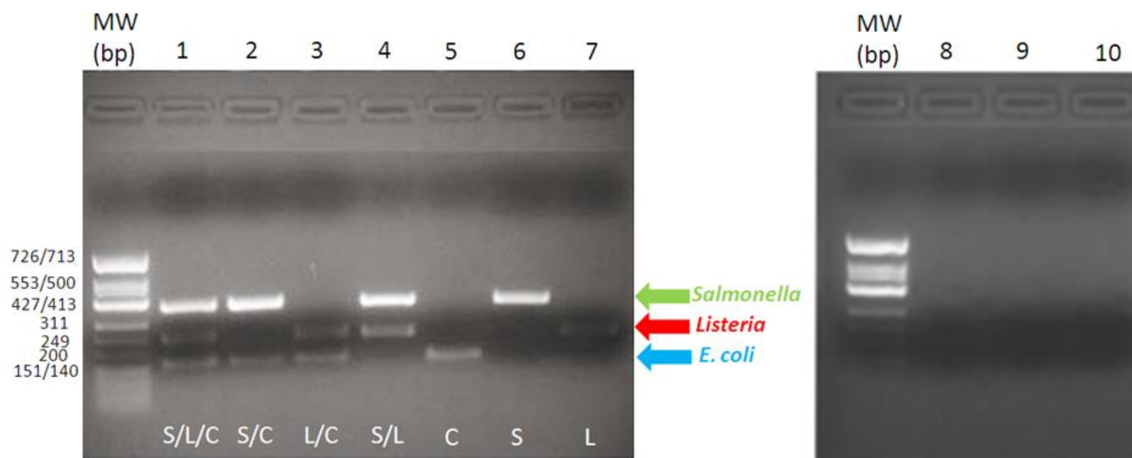

Figure 5.3 Gel electrophoresis of the triple-tagging multiplex PCR. The three amplification bands correspond to *S.* Typhimurium *yfiR* gene fragment (375 pb), *L. monocytogenes hlyA* gene fragment (234 pb) and *E. coli eaeA* gene fragment (151 bp). Lane 1 is the amplification of the three gene fragments for *S.* Typhimurium, *E. coli,* and *L. monocytogenes*; Lane 2 for *S.* Typhimurium and *E. coli*; Lane 3 for *L. monocytogenes* and *E. coli*. Lane 4 for *S.* Typhimurium and *L. monocytogenes*; Lanes 5- 7 are single amplification for *E. coli*, *S.* Typhimurium and *L. monocytogenes,* respectively. The negative controls are shown in the lanes 8 to 10, in which no DNA template has been added to the PCR mixture. In all cases, *Hinf*I digested ϕ174 DNA was used as a molecular weight marker, ranging from 726 to 140 bp.

Three separated bands, related to each fragment target gene, in detail *S.* Typhimurium *yfiR* gene fragment (S, 375 pb), *L. monocytogenes hlyA* gene fragment (L, 234 pb) and *E. coli eaeA* gene fragment (C, 151 bp) were observed in lane 1, corresponded to the expected amplicon size when compared with the molecular weight markers (in base pairs) indicated on the left side of Figure 5.3. Aside from the simultaneous amplification of the three bacterial DNA double and single amplification of each bacterium is also shown in Figure 5.3. Binary combinations are shown in Lanes 2, 3 and 4 for S/C, L/C and S/L, respectively, while the single combinations are shown in Lanes 5 to 7 for C, S and L. Negative controls with no DNA template are also shown in Figure 5.3, lanes 8 to 10. As observed, each target bacteria produced a specific band relative to its correspondent amplicon, suggesting no cross-reactivity and a high specificity for the selected set of tagging primers. The relative intensities of the bands can be correlated with the individual performance of each set of primers, and the amplicons length, since as higher the amplicons length is, greater the signal will be, due to ethidium bromide staining.[7,10]

5.4.2 Confocal microscopy of the tagged amplicon immobilised on silica magnetic particles

The attachment of DNA on the silica-MPs is based on intermolecular electrostatic interactions, dehydration and hydrogen bond formation between the negative charge density of the sugar-phosphate backbone and the silica contact layer, in the presence of a chaotropic agent guanidinium thiocyanate and it was fully explained in the Chapter 4 (§4.4.3.3). Thus, it is expected that both dsDNA tagged amplicons and ssDNA tagging primers will be attached on the silica-MPs surface, however, in higher extension for dsDNA tagged amplicons because of their higher negative charge density towards ssDNA primers.

In order to confirm this, the immobilisation procedure of the tagged amplicons on silica-MPs was performed with fluorescence readout by confocal microscopy with the biotinylated amplicons coding for *L. monocytogenes* as a model and compared with the negative control containing the set of the biotinylated set of tagging primers. In this study, *L. monocytogenes* amplicons and the PCR negative control were immobilised on silica-MPs. The labelling was performed using the fluorescent reporter Strep-Cy5.

As shown in Figure 5.4, negative controls without DNA template are shown in panel A.

Although negative controls contain biotinylated tagging primers of *L. monocytogenes*, no signals from the fluorescent reporter Strep-Cy5 were observed and consequently only silica-MPs are observed in green as a result of their autofluorescence.

In Panels B to F, the binding pattern of the biotinylated amplicons from *L. monocytogenes* attached on the silica-MPs is shown, confirming the increased binding patterns of the biotinylated amplicons compared with the biotinylated tagging primers, revealed in red due to the fluorescent reporter Strep-Cy5.

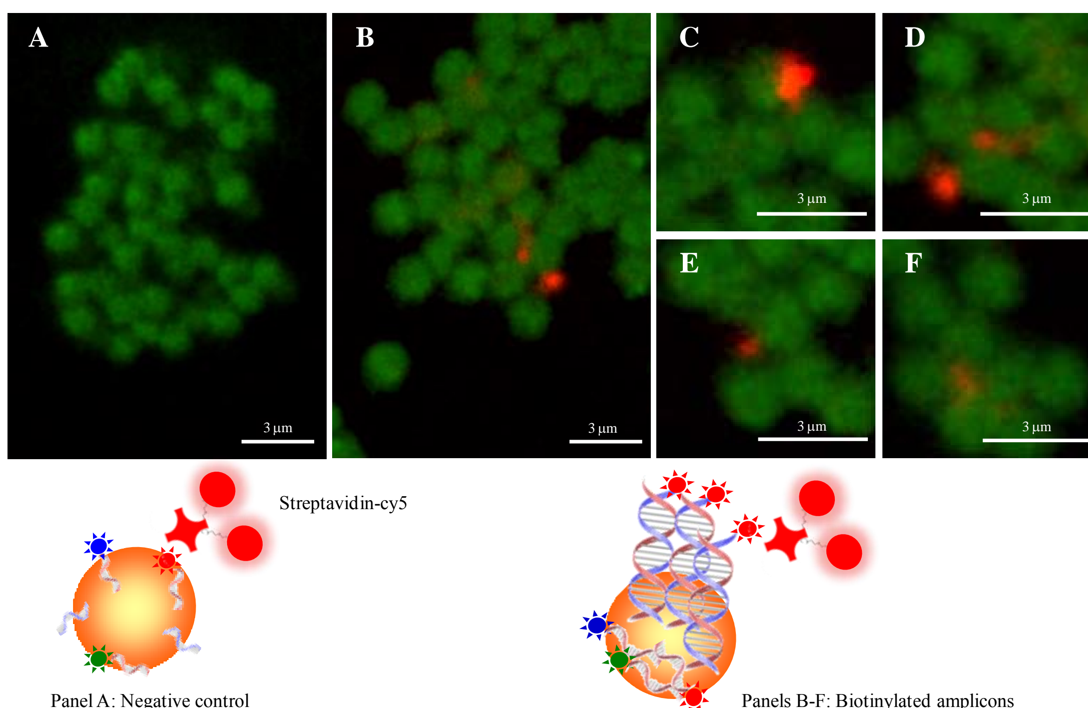

Figure 5.4 Confocal microscopy of the tagged (biotin) amplicon for *L. monocytogenes* immobilised on silica-MPs, using Strep-Cy5 as fluorescent reporter. Panel A shows the background signal, while panels B to F show the binding pattern of the tagged amplicon on the silica-MPs.

5.4.3 Triple-tagging electrochemical magneto-genosensing on silica magnetic particles

5.4.3.1 Optimisation of the amount of the electrochemical reporter

The triple-tagging electrochemical magneto-genosensing for the simultaneous detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* was firstly evaluated with different amounts

of each electrochemical reporter, ranging from 3 to 30 µg of AntiFlu-HRP and from 1 to 30 µg of both Strep-HRP and AntiDig–HRP.

In this study, the triple-tagged amplicons obtained from the triple-tagging multiplex amplification in the presence of the three bacterial DNA templates (S/L/C) were compared with the negative controls containing the triple-tagging amplicons.

Silica-MPs were used as a support for biological reactions. Both immobilisation of the triple-tagged amplicons and the enzymatic labelling steps took place on the silica-MPs. For the amperometric readout, the modified silica-MPs were attracted to the surface of the m-GEC electrodes by the magnet allocated inside the electrodes, and thus the electrochemical reporters were put in close contact with the electrochemical transducer by magnetic actuation.[11] The amperometric responses are presented in Figure 5.5.

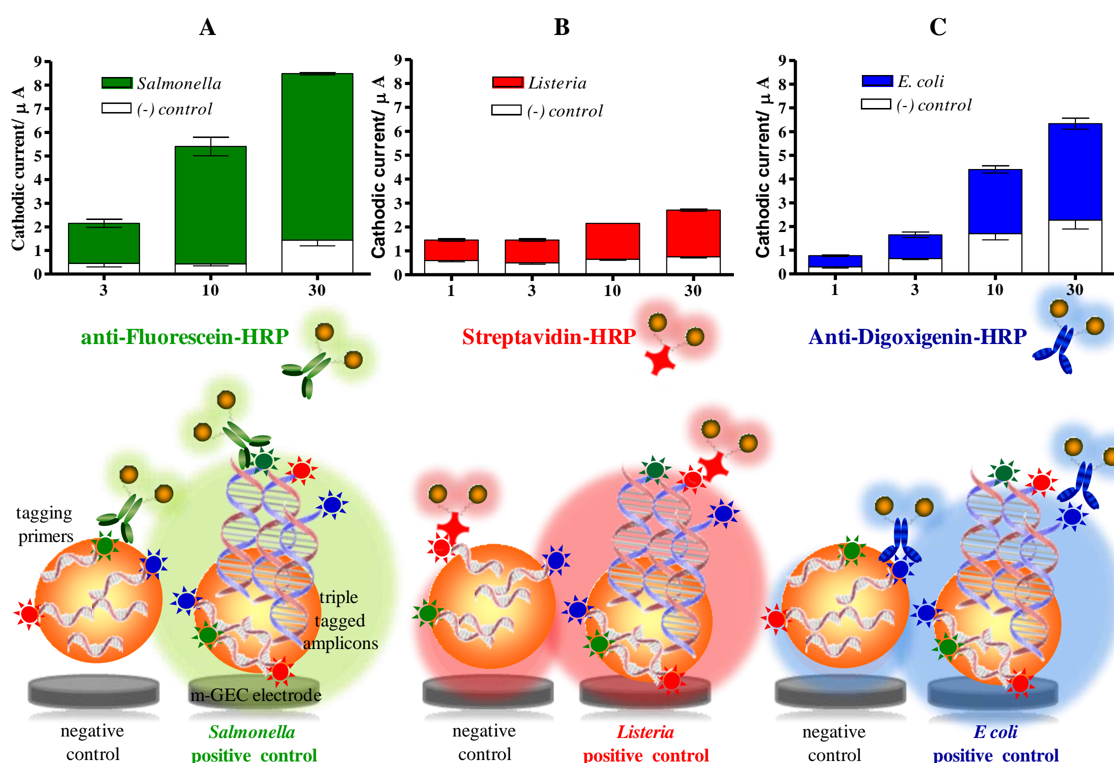

Figure 5.5 Electrochemical responses for the triple-tagging electrochemical magneto-genosensing on silica-MPs by increasing the amount of electrochemical reporter (AntiFlu-HRP, Strep-HRP and AntiDig-HRP from 1 to 30 µg per assay). The triple-tagged amplicons coming from the triple-tagging multiplex amplification in the presence of the three bacterial DNA templates (S/L/C) were challenged towards (A) AntiFlu-HRP coding for *S.* Typhimurium (B) Strep-HRP coding for *L. monocytogenes* and (C) AntiDig–HRP coding for *E. coli*. In all cases, 0.05 mg of silica-MPs was used, with an amplicon concentration of 4.15, 2.52 and 1.03 ng µL^{-1} respectively for *S.* Typhimurium, *L. monocytogenes* and *E. coli*. The error bars show the standard deviation for n = 3. The negative controls containing the triple-tagging amplicons are also shown (n=8).

In all cases, the positive signal for each bacteria is significantly higher than the corresponding negative controls. The cathodic current observed in the negative control is a result of the signal provided from ssDNA tagging primers attached on the silica-MPs surface, whereas the binding of target bacteria amplicons on the silica surface results from the contribution of both dsDNA amplicons and remaining ssDNA primers. The different values of cathodic currents for each pathogen are related with the individual performance of each set of primers, as it was previously discussed for the gel electrophoresis. The increased amount of the electrochemical reports produced, in all cases, increased cathodic currents, not only for the positive signals, but also for the negative controls.

Therefore, the optimal amount of each electrochemical reporter was found to be 10 µg for AntiFlu-HRP, 3 µg for Strep-HRP and 3 µg for AntiDig–HRP, producing both a higher signal to background radio, as well as lower non-specific adsorption of the tagging primers (as shown in Figure 5.6).

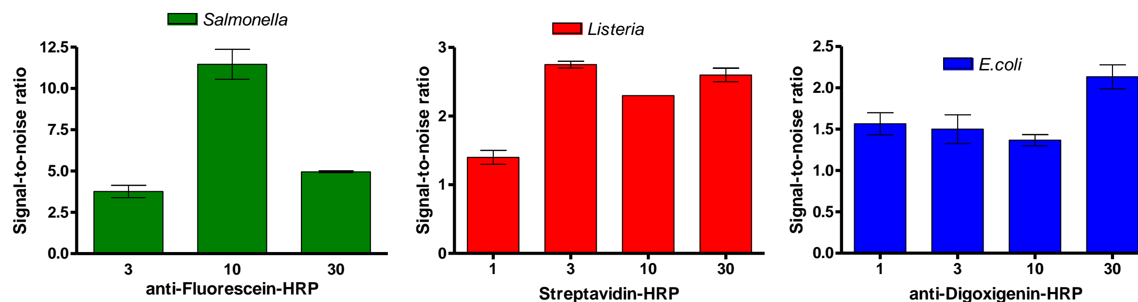

Figure 5.6 Signal-to-background ratio for the magneto-genosensing of the multiplex triple-tagged amplicons by increasing the amount of electrochemical reporter (AntiFlu-HRP, Strep-HRP and AntiDig-HRP from 1 to 30 µg per assay. In all cases, 0.05 mg of silica-MPs with an amplicon concentration of 4.15, 2.52 and 1.03 ng μL^{-1} , respectively, for *S.* Typhimurium, *L. monocytogenes* and *E. coli* (n=3).

5.4.3.2 Specificity study

Aside from the performance of the triple-tagging multiplex PCR, it is also important to study the specificity of the electrochemical reporters for coding the different pathogens. The specificity of the electrochemical reporters coding each bacteria, *S.* Typhimurium (S), *L. monocytogenes* (L) and *E. coli* (C), was studied by challenging the three electrochemical reporters (AntiFlu-HRP, Strep-HRP and AntiDig–HRP, respectively) towards the amplicons

coming from the triple-tagging multiplex amplification in the presence of all possible combinations including i) the three bacterial DNA templates (S/L/C), ii) the binary combinations (S/C; S/L; C/L), and iii) the single combinations (S; L; E).

Figure 5.7 shows the normalised amperometric responses, for all the combinations of amplicons with AntiFlu-HRP, Strep-HRP, AntiDig–HRP coding for *S.* Typhimurium, *L. monocytogenes* and *E. coli*, respectively.

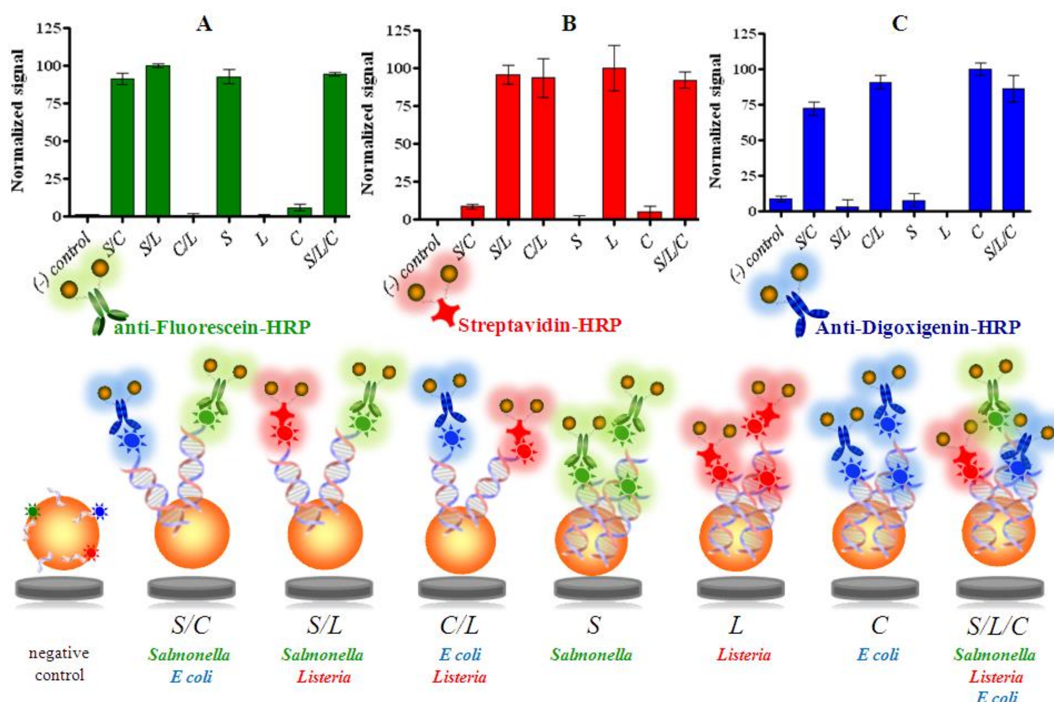

Figure 5.7 Electrochemical responses for the triple-tagging electrochemical magneto-genosensing on silica magnetic particles. The triple, double and? single-tagged amplicons coming from the triple-tagging multiplex amplification in the presence of all possible combinations including i) the three bacterial DNA templates (S/L/C), ii) the binary combinations (S/C; S/L; C/L), and iii) the single combinations (S; L; C), were challenged towards (A) 10 µg AntiFlu-HRP coding for *S.* Typhimurium, (B) 3 µg Strep-HRP coding for *L. monocytogenes* and (C) 3 µg AntiDig–HRP coding for *E. coli*. In all cases, 0.05 mg of silica-MPs was used, with an amplicon concentration of 4.15, 2.52 and 1.03 ng μL^{-1} respectively for *S.* Typhimurium, *L. monocytogenes* and *E. coli*. The error bars show the standard deviation for n = 3. The negative controls containing the triple-tagging amplicons are also shown (n=8).

Thus, it is possible to observe that the samples containing *S.* Typhimurium amplicons only provide signals in the presence of AntiFlu-HRP while the samples containing *L. monocytogenes* provide signal only with Strep-HRP as electrochemical reporter and the samples containing *E. coli* provide higher cathodic currents using AntiDig–HRP. Hence, the

results suggest that this approach was able to clearly distinguish between the different bacteria and their single and binary combinations.

5.4.3.3 Determination of the limit of detection for the triple-tagging electrochemical magneto-genosensing on silica magnetic particles

Figure 5.8 (panel A, B and C) shows the electrochemical signal for the triple-tagging electrochemical magneto-genosensing on silica-MPs for a range of concentrations near de LODs, ranging from 0.0 to 1.04 ng μL^{-1}, using 10 μg AntiFlu-HRP, for the detection of *S.* Typhimurium); from 0.0 to 0.630 ng μL^{-1}, using 3μg Strep-HRP, for the detection of *L. monocytogenes* and from 0.0 to 0.516 ng μL^{-1}, using 3 μg AntiDig–HRP, for the detection of *E. coli*, as well as the negative controls.

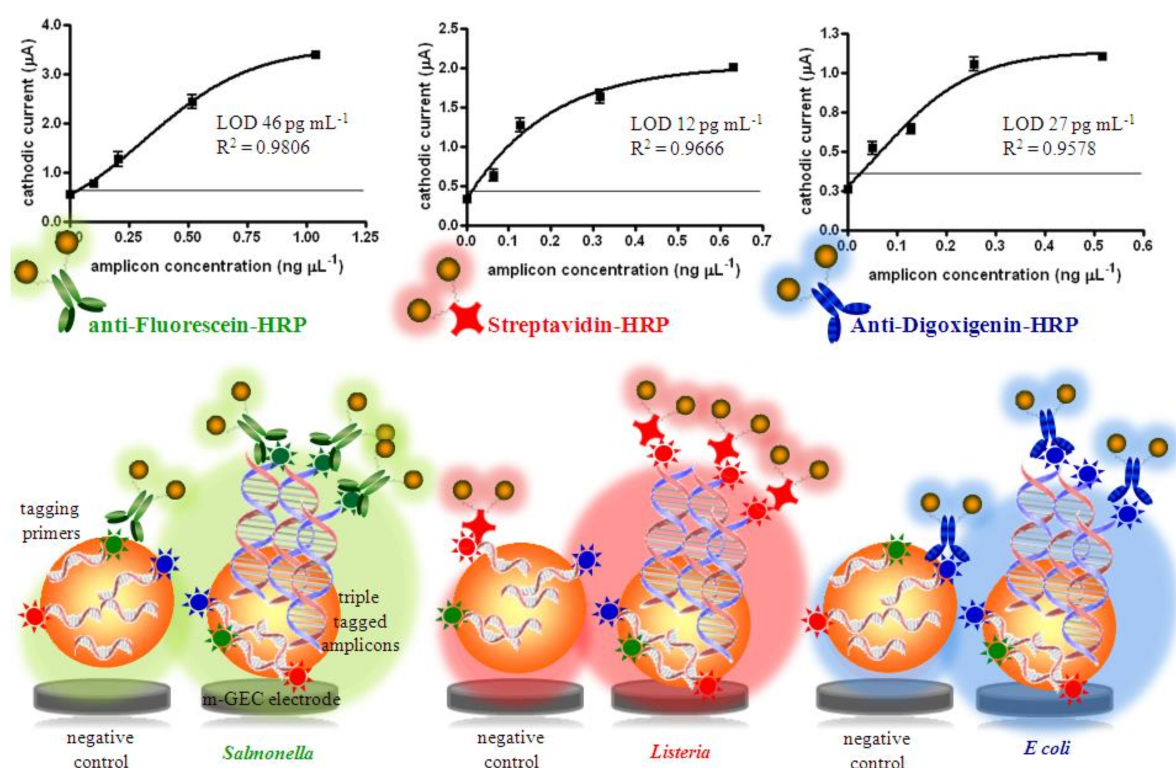

Figure 5.8 Electrochemical responses for the triple tagging electrochemical magneto-genosensing on silica-MPs at concentrations near the LOD, ranging from (A) 0.0 to 1.04 ng μL^{-1} using 10 μg AntiFlu-HRP (for *S.* Typhimurium); (B) 0.0 to 0.630 ng μL^{-1} using 3 μg Strep-HRP (for *L. monocytogenes* and 0.0 to 0. 516 ng μL^{-1} using 3 μg AntiDig–HRP (for *E. coli*) of the amplicons obtained from the single combinations (S; L; E) of the triple-tagging multiplex amplification, as well as the negative controls. The error bars show the standard deviation for n = 3. The negative controls containing the triple-tagging amplicons are also shown (n=8).

The cut-off values were then determined by processing the negative control samples (n=8) with a one-tailed t test at a 95% confidence level, giving values of 0.66, 0.43 and 0.36 µA for *S.* Typhimurium, *L. monocytogenes* and *E. coli* respectively (shown in Figure 5.8, as the solid lines). The data were adjusted to a nonlinear regression (sigmoidal dose-response with variable slope – GraphPad Prism Software) and the LODs obtained were 46 (R^2=0.9806), 12 (R^2=0.9666) and 27 (R^2=0.9578) pg µL^{-1} for *S.* Typhimurium, *L. monocytogenes* and *E. coli* respectively. Figure 5.9 shows the concentrations near the LOD for the single combinations (S; L; E) of the triple-tagging multiplex amplification.

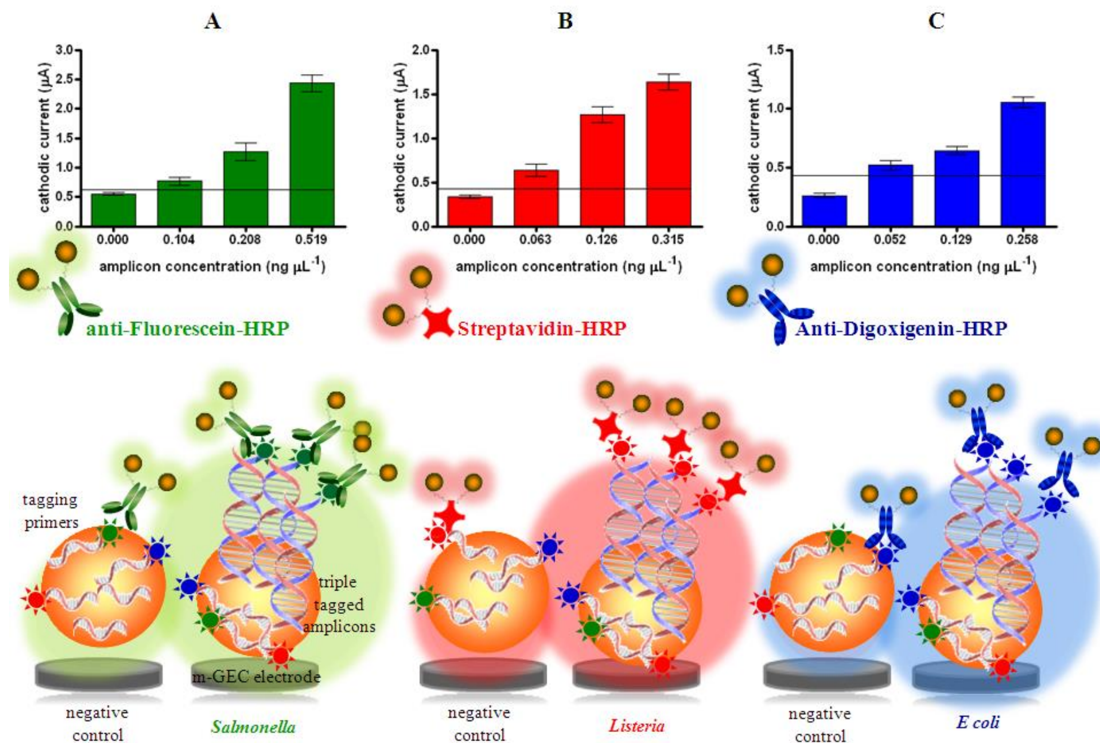

Figure 5.9 Electrochemical responses for the triple tagging electrochemical magneto-genosensing on silica MPs at concentrations near the LOD, ranging from (A) 0.0 to 0.5 ng µL^{-1} using 10 µg AntiFlu-HRP (for *S.* Typhimurium); (B) 0.0 to 0.3 ng µL^{-1} using 3 µg Strep-HRP (for *L. monocytogenes* and 0.0 to 0.3 ng µL^{-1} using 3 µg AntiDig–HRP (for *E. coli*) of the amplicons obtained from the single combinations (S; L; E) of the triple-tagging multiplex amplification, as well as the negative controls. The error bars show the standard deviation for n = 3. The negative controls containing the triple-tagging amplicons are also shown (n=8).

5.5 Conclusions and final remarks

A considerable progress regarding food safety has been done in terms of rapid and multiplexed approaches for detecting bacteria outbreaks, which have been recently reviewed by our research group.[12]

In this Dissertation, the current state of art based on the detection methods for foodborne bacteria was also fully discussed in §1.3. In this context, it was shown that mPCR based methodologies could detect below 10 CFU in 25 g of sample after a pre-enrichment step up to 30 h.[13] The integration of MPs with PCR based methodologies leads to a LOD of less than 10 CFU g^{-1} within 6 h.[14] DNA microarrays showed LODs below 500 CFU mL^{-1} when using MPs, in approximately 3.5 h, without the need of a pre-enrichment step.[15] In addition, in the integration of nano and micromaterials as metallic nanoparticles, quantum dots and magnetic particles in biosensors have been extensively reported in the literature, being available in a wide range of readout platforms, as for instance SPR, or other optical biosensors and electrochemical approaches.

Multiplexing provides a cost-effective assay for simultaneous detection of foodborne pathogens by reducing assay times and consumption of samples and reagents. The most important features of multiplex approaches previously reported for the simultaneous detection of foodborne pathogens were reported previously along Chapter 1, §1.4.2. In addition, Table 5.3 summarises some of the biosensing strategies for the simultaneous detection of foodborne bacteria comprising *S.* Typhimurium, *L. monocytogenes* and *E. coli*.

Table 5.3 Multiplex approaches for the simultaneous detection of foodborne pathogens

Target	Assay format	Detection technique	Test matrix	Total assay time	LOD	Ref.
E. coli, *S.* Typhimurium, *L. monocytogenes*, *C. jejuni*	Sandwich Immunoassay	Multi-channel SPR	Apple juice	50 min	3.4×10^{3} – 1.2×10^{5} CFU mL^{-1}	[16]
E. coli and *S.* Typhimurium	Sandwich immunoassay	Fluorescence	Bacterial suspension	2 h	10^{4} CFU mL^{-1}	[17]
S. Typhimurium, *Shigella flexneri*, *E. coli*	Sandwich immunoassay	Fluorescence	Apple juice and milk	2h	10^{3} CFU mL^{-1}	[18]
Protective antigen A (*pagA*) gene of *B. Anthracis,* the insertion element (*Iel*) gene of *S.* Enteritidis	Nanoparticle-based, bio-barcoded electrochemical biosensor	Square wave anodic stripping voltammetry	Pure bacteria	2 h 30 min	50 pg mL^{-1} (B. *anthracis)* 0.5 ng mL^{-1} (S. *enteritidis*)	[19]
Listeria, E. coli, *Salmonella*	Sandwich immunoassay	Fluorescence	Meat and vegetable	1 h 30 min	20 –50 CFU mL^{-1}	[20]
E. coli, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Serratia*, *Providencia*, *Morganella*, *Staphylococcus* spp.	Integrated nucleic acid and protein biosensor assay	Amperometry	Urine samples	1 h	10^{4} CFU mL^{-1}	[21]
S. Typhimurium, *E. coli*	Immunological assay	SERS	Bacterial suspension	45 min	10^{2} CFU mL^{-1}	[22]
E. coli, *Campylobacter*, *Salmonella*	Sandwich immunoassay	Square wave anodic stripping voltammetry	Milk	1 h	400 – 800 CFU mL^{-1}	[23]
S. Typhimurium, *E. coli*	Sandwich Immunoassay	Colorimetry	Milk and pineapple juice	1 h	3-15 CFU mL^{-1}	[24]
E. coli, *Enterobacter sakazakii*	Immunosensor arrays	Cyclic voltammetry	Pure bacteria	2 – 3 h	10^{3} – 10^{4} CFU mL^{-1}	[25]
L. monocytogenes, S. enterica	DNA hybridisation	Colorimetry	Milk	3-4 h	0.013-0.015 ng mL^{-1}	[26]
E. coli, *S.* Typhimurium, *L. monocytogenes*	Sandwich immunoassay	Fluorescence	Spinach, chicken, milk	2 h	< 5 CFU mL^{-1}	[27]
S. aureus, V. parahemolyticus, *S.* Typhimurium	DNA hybridisation	Luminescence	Milk and shrimp	1h	25, 10 and 15 CFU mL^{-1} respectively	[28]
V. parahaemolyticus, S. Typhimurium	Sandwich immunoassay	FRET	Shrimp and chicken	2h 20 min	25, 35 CFU mL^{-1}	[29]

Among the emerging technologies, biosensors based on fluorescence readout are the most prominent approach for multiplexing capabilities. However, this readout presents the limitation of requiring costly bench top equipments. Electrochemical biosensors are affordable devices which show any loss in the analytical performance, although the application of both electrochemical immuno and/or genosensors for simultaneous detection is still in a preliminary stage.[30,31] For this reason, in this Dissertation it was intended to development of an

electrochemical biosensor for the simultaneous detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* and thus to improve the current state of art.

In this context, a novel procedure for the simultaneous detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* based on triple-tagging electrochemical magneto-genosensing is reported in this Dissertation. This strategy demonstrated to be a useful method to determine tagged PCR products without the interference of remaining primers and by using the same magnetic platforms, such as silica-MPs. Moreover, it is reported for the first time the combination of triple-tagging multiplex PCR amplification with electrochemical magneto-genosensing using silica-MPs as a platform for DNA immobilisation providing an overall analysis time of 3 h, including end-point PCR assay time. In this format, MPs are used not only to preconcentrate the amplicons, but also to immobilise them on the surface of the transducer, increasing thus the sensitivity of the assay.[32]

The triple-tagging multiplex PCR was successfully performed by introducing different tagging primers to amplify DNA regions coding for specific genes of each bacterial strain targeted, having shown to be highly specific. Each amplicon was labelled with fluorescein, biotin and digoxigenin, coding for *S.* Typhimurium, *L. monocytogenes* and *E. coli* respectively, using antiFlu-HRP, Strep-HRP and AntiDig-HRP as the electrochemical reporters for the amperometric readout. The triple-tagging electrochemical magneto-genosensing strategy was able to simultaneously detect *S.* Typhimurium, *L. monocytogenes* and *E. coli*, presenting feasibility to discriminate the different pathogens at lower concentrations, until 12 pg uL^{-1}. Moreover, this approach was able to clearly distinguish between the different bacteria, which can be considered as rapid alternative to the time consuming classical methodology. This strategy showed feasibility to detect simultaneously different pathogens within 50 min and for this reason it might be considered as a promising application tool for simultaneous bacteria screening. Moreover, it is a very versatile approach, since it can to be implemented with other readouts including lateral flow devices or other microfluidic platforms.

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CHAPTER 6

Concluding remarks

6.1 Final considerations

Many factors have been contributed to food safety problems over recent decades, such as intensive agriculture, globalisation of the food industry and changes in food production and consumer demands. Among all foodborne pathogens, *S. enterica*, *L. monocytogenes* and *E. coli* are considered the most dangerous in terms of human illnesses and food poisoning.

The common strategies for the detection of pathogenic microorganisms, reviewed in chapter 1, comprise conventional microbiological culturing techniques, immunological assays and nucleic acid amplification methods. Moreover, an increased number of kits are commercially available for a rapid, simple and reliable detection of foodborne bacteria. However, the development of new methods with the advantages of a rapid response, high sensitivity and specificity, as well as an ease of multiplexing for food hygiene inspection, in order to achieve the threshold limits set by the Food Regulatory agencies is still a challenge.

In chapter 1, novel methodologies for the simultaneous detection of *Salmonella*, *Listeria* and *E. coli* were also reviewed and discussed. The role of biosensors technology was emphasised as a promising application to monitor and control foodborne bacteria from farm to fork. The integration of nanomaterials has been widely highlighted as the key factor to achieve higher sensitivity and selectivity in biosensing either connected to the biorecognition element or to the transducer.

Superparamagnetic iron oxide particles, with sizes ranging from a few nanometers up to micrometer dimensions have the capability of being only magnetised in the presence of a magnetic field and readily about to resuspend upon removal of the magnetic field. For this reason, these particles are a promising choice to be integrated in bioassays for food safety applications, due to their capability of magnetic separation for the isolation and separation of a broad range of biological components. Moreover, their easy functionalisation with different molecular groups (for instance, tosyl, carboxyl, amine, hydroxyl, aldehyde or thiol), makes them very useful for the immobilisation of biomolecules, such as antibodies, streptavidin, oligonucleotides, protein A or G, among many others.

In this context, in chapter 3, nano (300 nm) and micro (2.8 µm)-sized magnetic particles were covalently modified with antibodies specific for *S.* Typhimurium, *L. monocytogenes* and *E. coli*, with outstanding performance. Afterwards, the IMS of *S.* Typhimurium, *L.*

monocytogenes and *E. coli* was comparatively studied for commercial and tailored-modified magnetic micro and nanoparticles. IMS efficiency, expressed as percentages of captured bacteria, was evaluated using classical culture methods. Moreover, the binding pattern of the bacteria on tailored-MMPs and MNP were also studied by microscopy (SEM and confocal microscopy).

It was concluded that the IMS efficiency is strongly dependent on the antibodies efficiency towards recognition of the bacterial outer membrane, in which IMS of *Salmonella* was achieved with higher efficiencies and reproducibility, because of the excellent recognition of LPS from *S.* Typhimurium. Relatively to the capture of *E. coli*, the IMS studies previously reported were mostly related to the capture of *E. coli* O157 with efficiencies, in general, lower than those obtained for *S.* Typhimurium. Finally, the capture of *L. monocytogenes* using Dynabeads® was achieved with improved results in comparison with the studies reported in the literature.

Additionally, the performance of the magnetic actuation provided by the MPs to enhance the separation and preconcentration of *S.* Typhimurium, as a model from complex samples, such as whole milk samples was also shown in chapter 3, offering an attractive technology by the integration of MPs in biosensors, microfluidic platforms or other devices, with high specificity.

Electrochemical immuno and genosensors remain very popular in biosensor technology for food safety applications, since it is not necessary the acquisition of expensive signal transduction equipment and also due to their high accuracy, rapidity and possibility of miniaturisation.

The integration of MPs in magneto-actuated electrochemical immuno and genosensors improved the analytical performance of these devices. In this Dissertation, MPs played a dual function either as a support for the immobilisation of the biorecognition element, improving thus the incubation and the washing steps of the biological assay, but also for the magnetic immobilisation of the MPs on the surface of the electrode under magnetic actuation. The use of MPs also allowed the application of an IMS step, which leads to a decrease in the background signals. In this way, the matrix effects are reduced and the LODS improved due to preconcentration.

In this context, in chapter 4, a comparison of two strategies, such as electrochemical magneto-immunosensing and genosensing were discussed for the detection of S. Typhimurium in milk samples.

Electrochemical magneto-immunosensing was based on double antibody recognition, in detail, a mouse monoclonal Isotype IgG1 antibody to *S.* Typhimurium 0-4, immobilised on both tailored-MNPs and MMPs for the IMS and also a polyclonal antibody towards "O" & "H" antigens labelled with HRP. In this instance, the same immunological biorecognition reaction was used for the IMS and detection of the bacteria. Thus, the bacteria were recognised with two antibodies with different clonalities towards different epitopes, increasing the specificity of the approach. The specificity of electrochemical magneto-immunosensing was shown by using suspensions of *E. coli*, as well as mix solutions containing *S.* Typhimurium and *E. coli*.

Hence, a comparison of both magnetic carriers as tailored- MMPs and MNPs in terms of analytical performance and specificity for a rapid detection of *S.* Typhimurium was presented in whole milk. It was concluded that both magnetic carriers showed similar analytical performance for the detection of *S.* Typhimurium in food samples, although with slightly improved LODs for tailored-MNPs. In addition, tailored-MNPs showed a slightly higher matrix effect, as well as higher degree of aggregation, requiring also longer time for magnetic actuation.

On the other hand, a sensitive assay, in detail IMS/Single-Tagging PCR amplification/Electrochemical magneto-genosensing was also presented in chapter 4, combining IMS on tailored-MNPs, single-tagging PCR and electrochemical magneto-genosensing using silica-MPs for the detection of *Salmonella* in milk.

S. Typhimurium was captured on tailored-MNPs, then the attached bacteria on tailored-MNPs was released and the genomic DNA amplified based on single-tagging PCR using a set of primers specific for *S.* Typhimurium. Afterwards, single-tagged amplicon was detected by electrochemical magneto genosensing, using silica-MPs, reported for the first time. Therefore, IMS/Single-Tagging PCR amplification/Electrochemical magneto-genosensing showed to be a very sensitive approach, being capable to reduce the assay time from 3-5 days (obtained by conventional microbiological culture techniques) to 4 h.

Another aspect to highlight is the use of silica-MPs, which were reported for the first time as a platform for DNA immobilisation, followed by electrochemical magneto-genosensing. Interestingly, the silica-MPs showed differential adsorption properties, based on

the negative charge density, for longer dsDNA amplicon incorporating the tagging-primers over shorter ssDNA tagged-primers, showing to be not only a robust platform for the electrochemical detection of PCR products, but also a promising magnetic carrier for fluorescence or other detection approaches.

In addition, the high sensitivity and specificity of this approach was explained due to the IMS, which showed an outstanding specific towards *Salmonella* based on the immunological recognition throughout the mouse monoclonal Isotype IgG1 to *S.* Typhimurium 0-4 directed against LPS of *S.* Typhimurium, as also described above. Another source that confers high specificity is given by the selection of the set of primers specific for *S.* Typhimurium *yfiR* gene fragment. Hence, it was concluded the high sensitivity and specificity of IMS/Single-Tagging PCR amplification/Electrochemical magneto-genosensing is given by the combination of IMS and also by the single-tagging PCR. The readout is lastly achieved based on electrochemical magneto-genosensor resulting in a robust and sensitive procedure.

Finally, in chapter 5, the simultaneous electrochemical magneto-genosensing of foodborne bacteria, in detail *S.* Typhimurium, *E. coli* and *L. monocytogenes* based on triple-tagging multiplex amplification was achieved. This strategy demonstrated to be a useful method to determine tagged PCR products without the interference of remaining primers and by using the same magnetic platforms, such as silica-MPs. Moreover, silica-MPs were used as platform for the immobilisation of triple-tagged amplicons, being able to detect simultaneously the three bacteria and presenting feasibility to discriminate the different pathogens at low concentrations. In this format, MPs are used not only to preconcentrate the amplicons, but also to immobilise them on the surface of the transducer, increasing thus the sensitivity of the assay.

Among emerging technologies applied to biosensors, readout platforms based on fluorescence are currently the most prominent approach for multiplexing capabilities. However, this readout presents the limitation of requiring costly bench top equipments. Electrochemical biosensors are affordable devices with high analytical performance, but the application of both electrochemical immuno and/or genosensors for simultaneous detection is still in a preliminary stage counting with few studies reported in the literature. In this context, a progress of the current state of art of electrochemical biosensors for the simultaneous detection of foodborne bacteria is shown in this Dissertation.

6.2 Future perspectives

To sum up, in this Dissertation it was shown how MPs can improve the biosensing process for both electrochemical immuno and genosensors. In addition, triple-tagging multiplex PCR/ electrochemical magneto-genosensing strategy was shown for the simultaneous detection of *S.* Typhimurium, *E. coli* and *L. monocytogenes*. Thus, future work should be developed in order to test this method in real samples. Thus, to achieve this task, an IMS step should be combined with triple-tagging multiplex amplification, in which several tailored-MPs modified with antibodies specific for the three bacteria can be mixed for IMS of the three bacteria. Alternatively, antibodies specific for *S.* Typhimurium, *E. coli* and *L. monocytogenes* can be immobilised on the same MPs. In this way, MPs act as a hybrid-like tailored structure for the IMS of the three bacteria at the same time.

As it was concluded in chapter 3, the efficiency of IMS is strongly dependent on the efficiency of the antibody. In this context, further studies should be done for the selection of commercial antibodies candidates for highly efficient IMS. Thus, the immobilisation of different antibodies against the different bacterial strains is suggested in order to increase the recognition sites of the bacteria, improving the IMS efficiencies and offering the capability to detect more bacterial strains. Another suggestion could be the immobilisation of aptamers or bacteriophages on the MPs, as an alternative to antibodies. In addition, since IMS is combined with triple-tagging multiplex amplification which is highly specific, it is also suggested that the IMS of the three bacteria could be non-specific, as an alternative to the use of antibodies. For instance, polysaccharides as chitosan or lectins can be immobilised on MPs to non-specifically react with foodborne bacteria affording the preconcentration of bacteria from food samples and the decrease of the matrix effect under magnetic actuation. Afterwards, triple-tagging multiplex amplification, would only amplify the intended gene sequences for further electrochemical detection.

The advantages of combining MPs with microfluidic techniques have been highlighted among this Dissertation for miniaturisation, integration and automation. As a result, the implementation of the strategies reported in this Dissertation in microfluidic platforms are also presented as a future perspective. In this context, triple-tagging multiplex PCR/ magneto electrochemical genosensing is considered a very versatile approach, since it can to be implemented with other readouts including lateral flow devices or other microfluidic platforms.

In this context, the use of fluorescent tags in the triple-tagging multiplex amplification could be a suggestion for further application on devices with optical readouts.

Hence, this Dissertation opens up new possibilities for future work, in which novel kinds of materials either with nano or micrometer dimensions can be explored, as well as other readouts for a rapid detection of foodborne bacteria in addition to electrochemical platform.

As a final conclusion, the multidisciplinary of this research work is emphasised, bringing Science to another level where different disciplines as analytical chemistry, biotechnology and materials chemistry become closer to provide solutions to problems related to social and industrial needs.

CHAPTER 7

Science communication

“Nothing in Science has any value to Society if it is not communicated”

(Anne Roe)

Cultural integration of science is becoming an important issue nowadays for the Society. In this context, science communication plays a vital role not only to unify scientist's community in both academia and industry, but also to bring and disseminate science and technology to the society.

The research work presented in this Dissertation obtained over these latest 3 years originated a total of six research articles in top journals of our specialty, with two more in progress. In addition, the participation in national and international conferences, with a total of eleven presentations from which three were oral communications and eight poster communications, are also reported.

In this Dissertation, formation in complementary fields, such as Microfluidics and Molecular Medicine was also an important asset. In this context, the participation on 3 workshops was achieved in the framework of BioMax, a Marie Curie ITN project.

The participation in the organisation of scientific events are also emphasised as a result from the collaboration of the summer school "Magnetic Particle based Platforms and Bioassays (Barcelona, June 2014), in the framework of BioMax, as well as in the 3rd Bioanalytical Nanotechnology School (Philippines, January 2014). During these events, teaching activities were also done, being also presented and highlighted in this Dissertation.

Finally, two research trainings were done within BioMax ITN project, in Katholieke Universiteit Leuven (KUL) provided the opportunity of learning about microfluidic lab-on-a-chip techniques, as well as the advantages of combining magnetic particles with microfluidic techniques for miniaturisation, integration and automation. The second training was done in Ademtech, which provided the opportunity to obtain specific skills of an industrial Research and Development environment, as well as to help identifying exploitable research directions with potential for future valorisation and commercialisation.

7.1 Publications in international journals

7.1.1 Published works

1) S. Liébana, D. Brandão, S. Campoy, P. Cortés, S. Alegret, M.I. Pividori, Electrochemical genosensing of *Salmonella*, *Listeri*a and *E. coli* on silica magnetic particles, *Anal Chim Acta*, 904 (2016), 1-9. Selected as a "featured article". Published on the front cover of the issue. 10.1016/j.aca.2015.09.044

2) D. Brandão, S. Liébana, S. Campoy, P. Cortés, S. Alegret, M.I. Pividori, Simultaneous electrochemical magneto genosensing of foodborne bacteria based on triple-tagging multiplex amplification, *Biosens Bioelectron*, 74 (2015), 652-659. 10.1016/j.bios.2015.07.008

3) D. Brandão, S. Liébana, S. Campoy, P. Cortés, S. Alegret, M.I. Pividori, Immunomagnetic separation of *Salmonella* with tailored magnetic micro and nanocarriers. A comparative study, *Talanta*, 143 (2015), 198-204. 10.1016/j.talanta.2015.05.035

4) D. Brandão, S. Liébana, M.I. Pividori, Multiplexed detection of foodborne pathogens based on magnetic particles, *N Biotechnol*, 32(5) (2015), 511-250. (review). 10.1016/j.nbt.2015.03.011

5) S. Liébana, D. Brandão, S. Alegret, M.I. Pividori, Electrochemical immunosensors, genosensors and phagosensors for bacteria detection, *Anal Methods*, 6, (2014), 8856 – 8874 (review). 10.1039/C4AY01373E

6) D. Brandão, S. Liébana, S. Campoy, P. Cortés, S. Alegret, M.I. Pividori, Electrochemical magneto-immunosensing of *Salmonella* based on nano and micro-sized magnetic particles, *J Phys Conf Ser*, 413, (2013), 012020. 10.1088/1742-6596/421/1/012020

7.1.2 In preparation

7) D. Brandão, S. Campoy, P. Cortés, M.I. Pividori, Immunomagnetic separation of *Salmonella* on tailored magnetic nanoparticles combined with electrochemical magneto-genosensing based on silica magnetic particles. In preparation.

8) D. Brandão, S. Campoy, P. Cortés, M.I. Pividori, Immunomagnetic separation of *Salmonella*, *Listeria* and *E. coli* based on commercial and tailored magnetic particles. In preparation.

7.2 Communications in scientific meetings

7.2.1 Oral communications

1) Detection of foodborne pathogens based on immunomagnetic separation and electrochemical magneto-genosensing. D. Brandão, Susana Campoy and M.I. Pividori, 19th Transfrontier Meeting of Sensors and Biosensors, Bellaterra, Spain, 25-26th September 2014. **Oral presentation**.

2) Rapid Detection of Salmonella in Milk with Nano- and Micro-sized Magnetic Particles. D. Brandão, S. Liébana, S. Campoy, S. Alegret, M.I. Pividori, XVIII Transfrontier Meeting of Sensors and Biosensors, Ales, France, 19-20th September 2013. **Oral presentation**.

3) Multiplexed Electrochemical Magneto-Immunosensing of Food Pathogenic Bacteria. D. Brandão, S. Liébana, S. Campoy, S. Alegret, M.I. Pividori, “VII International Workshop on Sensors and Molecular Recognition”, Valencia, Spain, 4-5th July 2013. **Oral presentation**.

7.2.2 Poster communications

1) Simultaneous electrochemical magneto genosensing of foodborne bacteria based on triple-tagging multiplex amplification. D. Brandão, S. Liébana, S. Campoy, S. Alegret, M.I. Pividori, XX SIBEE, Uberlândia, Brazil, 19-21st August 2015. **Poster presentation**.

2) Detection of foodborne pathogens based on immunomagnetic separation and electrochemical magneto-genosensing. D. Brandão, Susana Campoy and M.I. Pividori, Annual Conference Brno 2014: Frontiers in Material and Life Sciences, Brno, Czech Republic, 21-24th October 2014. **Poster presentation**.

3) Detection of foodborne pathogens based on immunomagnetic separation and electrochemical magneto-genosensing. D. Brandão, Susana Campoy and M.I. Pividori, Diatech: Novel technologies for in vitro diagnostics, Leuven, Belgium, 6 - 8th October 2014. **Poster presentation**.

4) Multiplex Electrochemical immunosensing of food Pathogenic Bacteria. D. Brandão, S. Liébana, S. Campoy, S. Alegret, M.I. Pividori, 3rd International Conference on Bio-Sensing Technology, Sitges, Spain, 12-15th May, 2013. **Poster presentation**.

5) Multiplex immunosensing detection of pathogenic bacteria. D. Brandão, S. Liébana, S. Campoy, S. Alegret, M.I. Pividori, Ibersensor 2012, Puerto Rico, 16 - 19th October 2012. **Poster presentation**.

6) Multiplex Immunosensing Detection of Pathogenic Bacteria. D. Brandão, S. Liébana, S. Campoy, M.I. Pividori, 17th edition of Tranfrontier Meeting of Sensors and Biosensors, 20th-21st September 2012, Tarragona, Spain. **Poster presentation**.

7) Multiplex Immunosensing Detection of Pathogenic Bacteria. D. Brandão, S. Liébana, S. Campoy, M.I. Pividori, First Workshop on Nanomedicine UAB- CEI, 6th June 2012, Barcelona, Spain. **Poster presentation**.

8) Immunomagnetic Separation of Pathogenic Bacteria for Multiplex Electrochemical Magneto Biosensing. D. Brandão, S. Liébana, S. Campoy, M.I. Pividori, 9th

International Conference on the Scientific and Clinical applications of magnetic carriers, Minneapolis, 22 - 26th May 2012. **Poster presentation**.

7.3 Participation in workshops

1) "VII International Workshop on Sensors and Molecular Recognition", Valencia, Spain, 4-5th July 2013.

2) "Microfabrication/Microfluidics, EPFL, Lausanne, Switzerland, 24-26th June 2013.

3) "Ionophore-based sensors" UAB, Barcelona, Spain, 8-10th April 2013.

4) "Advanced Techniques in Molecular Medicine", Uppsala, Sweden, 24-26th October 2012.

5) "First Workshop on Nanomedicine UAB- CEI", Barcelona, Spain, 6th June 2012.

6) "System Architecture of Integrated Biosensors", Eindhoven, Netherlands, 12th -13th, February 2012.

7.4 Organizational events

1) Summer school "Magnetic Particle based Platforms and Bioassays, 30th June- 3rd July 2014, Autonomous University of Barcelona, Barcelona, Spain.

2) 3rd Bioanalytical Nanotechnology School, 29th January- 1st February 2014, University of Santo Tomas, Manila, Philippines.

7.5 Teaching activities

1) Practical classes in of the summer school "Magnetic particles based platforms and bioassays", 30th June- 3rd July 2014, Autonomous University of Barcelona, Barcelona, Spain.

2) Practical classes in 3rd Bioanalytical nanotechnology school, 29th January- 1st February 2014, University of Santo Tomas, Manila, Philippines.

3) Laboratory classes of Analytical Chemistry (Gas chromatography and UV-VIS spectroscopy), from February until March 2013, Autonomous University of Barcelona, Barcelona, Spain.

7.6 Secondments

1) Secondment in the department of Biosystems, Division of Mechatronics, Biostatistics and Sensors of KUL, under supervision of Prof. Jeroen Lammertyn from April until June 2014. The work developed was mostly focused on the following activities:

- Immunomagnetic separation of bacteria
- Integration of magnetic particles and bacteria onto a digital microfluidic chip
- Observation on the inverted microscope

2) Secondment in Ademtech, Pessac, France, under supervision of Manuel Gaboyard in July 2014. The work developed was mostly focused on the following activities:

- Coupling of antibodies on magnetic particles, assessment of different coupling strategies
- Binding capacities measurements by enzymatic and fluorescent assays
- Immunocapture of bacteria