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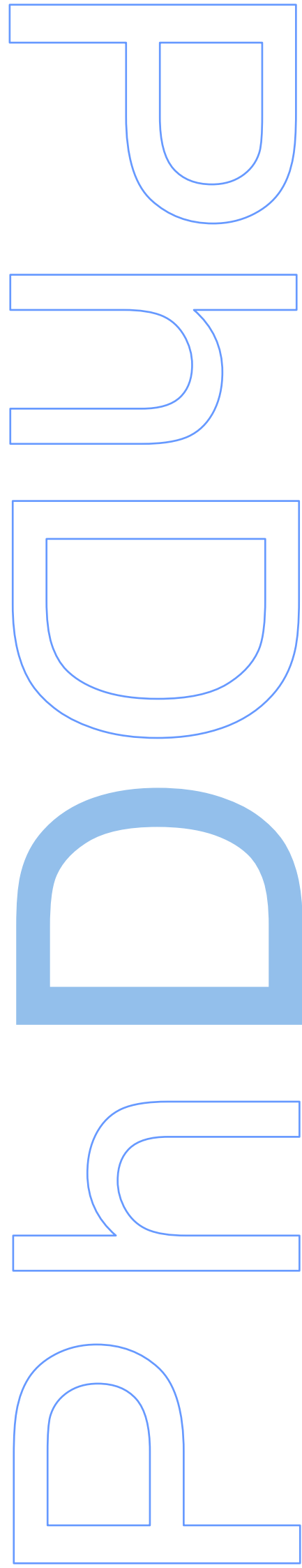CIÊNCIAS E TECNOLOGIA
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Development of Innovative Electrochemical Immunosensing Systems for Foodborne Pathogens Detection
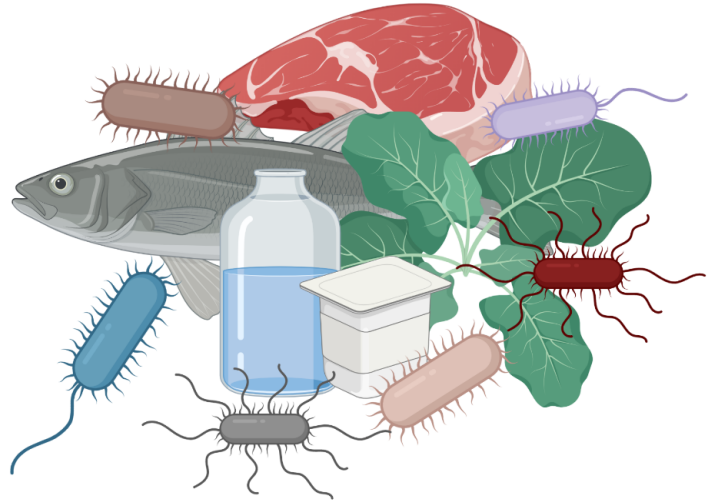
Nádia Filipa Durão da Silva
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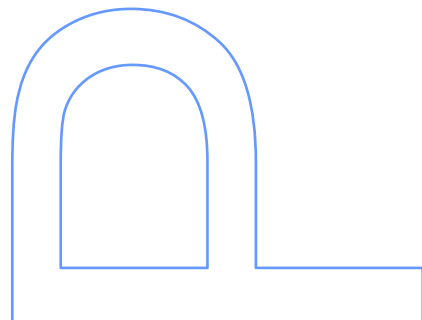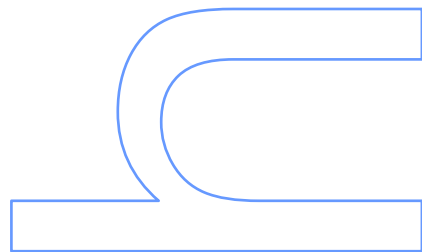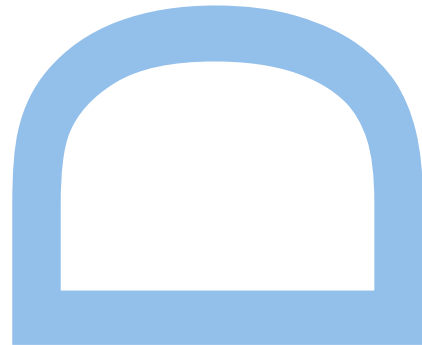
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Development of Innovative Electrochemical Immunosensing Systems for Foodborne Pathogens Detection

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A dissertation presented to the Faculty of Science from Porto University, Faculty of Science and Technology from NOVA Lisboa University and Aveiro University for the degree of Doctor in Sustainable Chemistry
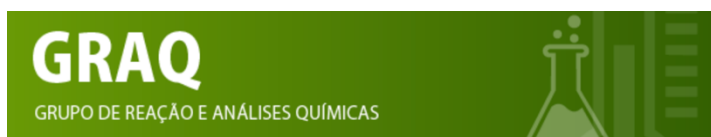
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Only those who attempt the absurd...will achieve the impossible. I think ...I think it's in my basement...Let me go upstairs and check.

M. C. Escher

Resumo

Os microrganismos patogénicos de origem alimentar representam um risco considerável para a saúde humana e animal, originando anualmente um grande número de hospitalizações e mortes. Atualmente, os métodos regulamentados para o controlo e segurança alimentar baseiam-se na cultura e contagem de colónias, métodos estes que se demonstram laboriosos e morosos face às reais necessidades e às normas recentemente impostas.

No decorrer deste trabalho pretendeu-se desenvolver sistemas imunológicos eletroquímicos inovadores para a deteção vestigial de microrganismos patogénicos, que sejam rápidos, simples e económicos face aos métodos existentes no mercado e a outras alternativas recentes reportadas na literatura. O estudo incidiu sobre os microrganismos patogénicos mais frequentemente noticiados na Europa e com repercussões com maior impacto na saúde pública nos últimos anos. Entre outros, a *Salmonella typhimurium* e a *Listeria monocytogenes* ocupam lugares de destaque. Entre as várias técnicas eletroquímicas existentes, a transdução potenciométrica aliada às reações de reconhecimento imunológico, oferecem boas perspetivas para a projeção de dispositivos analíticos sensíveis, específicos e fáceis de usar a um baixo custo. De acordo com esta premissa, foram desenvolvidos três sistemas potenciométricos baseados em anticorpos para deteção descentralizada de *Salmonella typhimurium*. Primeiramente, foi acoplada uma interface imunossensora com um anticorpo imobilizado em nanopartículas de ouro (AuNPs) à superfície de uma membrana de PVC, aplicada numa ponta de pipeta. As AuNPs foram sintetizadas *in situ* através da extração de cloreto de ouro (III) para a membrana polimérica, seguido de uma etapa de redução usando EDTA como agente redutor. O immunosensor otimizado, recorre à capacidade de biorreconhecimento dos anticorpos e à seletividade a iões de uma membrana para a captura e quantificação do analito, através da aplicação do princípio de bloqueio de superfície. Numa segunda abordagem, foi explorado o mesmo princípio, mas o papel foi escolhido como suporte para a construção do elétrodo. Uma fina membrana de PVC flexível, que serve como plataforma de imobilização do biorecetor foi aplicada numa tira de papel de filtro condutora, acoplada na face oposta com uma outra porção de papel que atua como reservatório da solução interna do elétrodo seletivo de iões. Duas interfaces imunossensoras com diferentes áreas superficiais foram aplicadas no elétrodo de papel desenvolvido e o desempenho analítico dos imunossensores resultantes foi comparado. Estes dois trabalhos permitiram explorar as capacidades da deteção potenciométrica, utilizando um método de deteção simples e inovador sem

recurso a marcadores e usando elétrodos de baixo-custo. Um outro imunoensaio magnético em sanduíche que utiliza microeléctrodos seletivos de cádmio foi desenvolvido. Neste trabalho, nanopartículas magnéticas funcionalizadas com anticorpos anti - *Salmonella* são utilizadas para capturar as células de *Salmonella typhimurium* presentes na amostra, seguido de uma segunda ligação a um anticorpo marcado com nanocristais de cádmio (CdS). Posteriormente, foi realizada a dissolução dos CdS, e a concentração de iões cádmio livre foi quantificada e correlacionada com a concentração de *Salmonella typhimurium*. Todos os sistemas potenciométricos desenvolvidos demonstraram ser adequados para deteção rápida (de 60 a 75 min), simples e sensível (limite de deteção de 5 a 20 células mL^{-1}) de *Salmonella typhimurium* em matrizes alimentares complexas, como o leite e sumo de maçã.

A deteção analítica da *Listeria monocytogenes* em amostras alimentares foi projetada através do desenvolvimento de um immunosensor enzimático inovador, que utiliza a p60, - uma proteína associada à invasão celular -, para deteção indireta do microrganismo alvo. Para tal, foram conduzidas várias reações de afinidade imunológica do tipo sanduíche de forma sequencial sobre um elétrodo serigrafado de carbono descartável: um anticorpo monoclonal de captura específico contra uma sequência peptídica específica da proteína p60 proveniente da *Listeria monocytogenes,* a proteína de p60 recombinante, seguida por um anticorpo policlonal específico para a p60 e um anticorpo secundário marcado com fosfatase alcalina. Após a reação enzimática, despoletada pela ação do substrato composto pela mistura de 3-indoxil fosfato e prata, o sinal analítico é adquirido através da remoção voltamétrica da prata depositada enzimaticamente, que por sua vez irá ser proporcional à concentração de p60 na amostra. Em condições otimizadas, um limite de deteção e quantificação de 1.52 e 5.06 ng mL^{-1} foi alcançado em tempo útil (± 3 h). A aplicabilidade do método foi validada através da deteção de p60 em amostras de leite.

Palavras-chave:

Eletroquímica, patogénicos de origem alimentar, Imunoensaio, Imunossensor, *Listeria monocytogenes*, Nanomateriais, *Salmonella typhimurium.*

Abstract

Foodborne pathogens pose a considerable risk to human and animal health, leading to a large number of hospitalizations and deaths every year. Currently, the regulated methods for food control and safety are based on culture and colony counting, which are assumed as laborious and time-consuming methodologies, regarding the real needs and the newly imposed food safety standards.

Along with this work, it was intended to develop innovative electrochemical immunological systems for the trace detection of pathogenic microorganisms in a faster, simpler and economical way compared to the methods currently marketed and other recent alternatives already reported in the literature. This study focused on the most commonly reported pathogenic microorganisms in Europe, with the most significant public health impact in recent years. Herein, *Salmonella typhimurium* and *Listeria monocytogenes* attain to a prominent place. Accordingly, three different potentiometric immunosensing systems for the decentralized detection of *Salmonella typhimurium* have been developed. Firstly, an immunosensing interface with an antibody immobilized on gold nanoparticles (AuNPs) was assembled on the surface of a PVC membrane, previously applied into a pipette tip. The AuNPs were formed in situ through extraction of gold (III) chloride to the polymeric membrane followed by a reduction step using EDTA as a reduction agent. The optimized immunosensor rely solely on antibody biorecognition ability and PVC membrane ionic selectivity for the capture and quantification of the analyte through the application of the surface blocking principle. In a second approach, the same principle was explored, but the paper was chosen as support for the construction of the electrode. A thin flexible PVC membrane, which serves as a platform for biorecognition element loading, was dropped on a conductive paper filter strip integrated into its rear with a filter paper pad which acted as a reservoir of the internal solution. Two different immunosensing interfaces with different superficial areas were assembled on the developed paper-strip electrode and the analytical performance of the resulting immunosensors was compared. These two works have made it possible to exploit the capabilities of potentiometric detection using a simple and innovative detection method without the use of markers resorting to low-cost home-made electrodes. By last, a magnetic sandwich immunoassay using homemade cadmium selective microelectrodes was developed. In those, magnetic nanoparticles functionalized with anti-*Salmonella* antibodies were used to capture *Salmonella* cells present in the sample, followed by a second binding to a cadmium nanocrystal (CdS) labelled antibody. Subsequently, the dissolution of the CdS was performed, and the

concentration of free cadmium ions was quantified and correlated with the concentration of target analyte. All potentiometric systems developed are suitable for fast (60 to 75 min), simple and sensitive detection (detection limit of 5 to 20 cells mL^{-1}) of *Salmonella typhimurium* in complex food matrices such as milk and apple juice.

The analytical detection of *Listeria monocytogenes* in food samples was achieved through the development of an innovative enzymatic immunosensor using the p60, a protein associated with cell invasion, for indirect detection of the target microorganism. To this end, several sequential sandwich-type immunological affinity reactions were conducted on a disposable screen-printed carbon electrode: between a specific capture monoclonal antibody against a p60-specific peptide sequence from *Listeria monocytogenes*, the recombinant p60 protein, followed by a p60-specific polyclonal antibody and the alkaline phosphatase labelled secondary antibody. After the enzymatic reaction, triggered by the addition of the substrate mixture composed by 3-Indoxyl phosphate and silver, the analytical signal is acquired through the voltammetric removal of the enzymatically deposited silver, which in turn is proportional to the p60 concentration in the sample. Under optimized conditions, a limit of detection and quantification of 1.52 and 5.06 ng mL^{-1} was reached within a useful time (± 3 h). Moreover, the applicability of this method was also supported by p60 detection in milk samples.

Keywords:

Electrochemistry, Foodborne pathogens, Immunoassay, Immunosensor, *Listeria monocytogenes*, Nanomaterials, *Salmonella typhimurium.*

List of Publications

Most of the content presented in this thesis was published, accepted or submitted for publication.

Journal articles (with peer review)

Silva, Nádia F.D.; Almeida, Cláudio M.R.; Magalhães, Júlia M.C.S.; Gonçalves, Maria P.; Freire, Cristina; Delerue-Matos, Cristina. 2019. "Development of a Disposable Paper-Based Potentiometric Immunosensor for Real-time Detection of a Foodborne Pathogen". Biosensors and Bioelectronics, 2019: p. 111317.
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Silva, Nádia F.D.; Magalhães, Júlia M.C.S.; Barroso, M. Fátima; Oliva-Teles, Teresa; Freire, Cristina; Delerue-Matos, Cristina. 2019. "In situ formation of gold nanoparticles in polymer inclusion membrane: Application as platform in a label-free potentiometric immunosensor for *Salmonella typhimurium* detection". *Talanta* 194: 134-142.
http://dx.doi.org/10.1016/j.talanta.2018.10.024.

Silva, Nádia F.D.; Magalhães, Júlia M.C.S.; Freire, Cristina; Delerue-Matos, Cristina. 2018. "Electrochemical biosensors for *Salmonella*: State of the art and challenges in food safety assessment". *Biosensors and Bioelectronics* 99: 667-682.
http://dx.doi.org/10.1016/j.bios.2017.08.019.

Silva, Nádia F.D.; Magalhães, Júlia M.C.S.; Oliva-Teles, Teresa; Delerue-Matos, Cristina. A potentiometric magnetic immunoassay for rapid detection of *Salmonella typhimurium.* Analytical Methods, 2015. 7: 4008-4011.
https://doi.org/10.1039/C5AY00053J.

Submitted journal articles

Silva, Nádia F.D.; Neves, M. M. P. S.; Magalhães, Júlia M.C.S.; Freire, Cristina; Delerue-Matos, Cristina. 2019. "Electrochemical immunosensor towards invasion-associated protein p60: an strategy for *Listeria monocytogenes* screening in food". In revision.

Silva, Nádia F.D.; Neves, M. M. P. S.; Magalhães, Júlia M.C.S.; Freire, Cristina; Delerue-Matos, Cristina. 2019. "Emerging electrochemical biosensing approaches for detection of *Listeria monocytogenes* in food samples: an overview". In revision.

International Communications in Scientific Meetings

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N.F.D Silva, J.M.C.S. Magalhães, M.F. Barroso, T. Oliva-Teles, C. Freire, C. Delerue-Matos. "In Situ Formation of Gold Nanoparticles in Polymer Inclusion Membrane: Application as Platform in A Label-Free Immunosensor For *Salmonella Typhimurium* Detection". II Workshop on Electrochemical Devices & II Workshop Electrobionet, 9−10 November 2017, (Asturias, Spain).

Poster Contributions

Nádia F.D. Silva; Júlia M.C.S. Magalhães; Cristina Freire; Cristina Delerue-Matos. "A label-free potentiometric biosensor for the detection of *Salmonella typhimurium*". Work presented in XXI Meeting of Portuguese Society of Electrochemistry and XVIII Iberian Meeting of Electrochemistry, 14−17 September 2016, (Bragança, Portugal).

National Communications in Scientific Meetings

Oral Contributions

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Poster Contributions

Silva, N.F.D.; Almeida, C.M.R.; Magalhães, J.M.C.S.; Gonçalves, M. P.; Freire, C.; Delerue-Matos, C.. "Development of a Disposable Paper-Based Potentiometric Immunosensor for Real-time Detection of a Foodborne Pathogen". Work presented in 4th Meeting of Medicinal Biotechnology, 17 of May 2019, (Porto, Portugal).

Silva, N.F.D.; Almeida, C.M.R.; Magalhães, J.M.C.S.; Freire, C.; Delerue-Matos, C.. "Paper-based Potentiometric Immunosensor for *Salmonella Thyphimurium* Detection". Work presented in 23rd Meeting of the Portuguese Electrochemical Society, 2−4 of May 2018 (Porto, Portugal).

Nádia Silva; Júlia Magalhães; Teresa Oliva-Teles; Cristina Delerue-Matos. 2014. "Potentiometric Magnetic Immunoassay for Rapid Detection of *Salmonella Typhimurium* in Milk". Work presented in XII Encontro de Química dos Alimentos, 10−12 September 2014 (Aveiro, Portugal).

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2.3. Development of a disposable paper-based potentiometric immunosensor for real-time detection of a foodborne pathogen

CHAPTER 3 - Electrochemical Biosensing of *Listeria monocytogenes*

3.2. Electrochemical immunosensor towards invasion-associated protein p60: an alternative strategy for *Listeria monocytogenes* screening in food

List of Abbreviations and Nomenclature

A	Amperometric
Ab	Anti-*Salmonella* monoclonal antibodies
AFNOR	Association Française de Normalisation
AMPs	Antimicrobial peptides
AOAC	Association of Official Analytical Chemists
ASV	Anodic Stripping Voltammetry
ATR	Attenuated Total Reflection
AuNPs	Gold nanoparticles
AuNPs-PIM	Gold nanoparticle polymer inclusion membrane
BSA	Bovine serum albumin
CA	Chronoamperometry
Cd-ISE	Cadmium selective microelectrode
CdS	Cadmium nanocrystals
CFU	Colony Forming Units
CHI	Chitosan
CILE	Carbon Ionic Liquid Electrode
CN or CNT	Carbon Nanotubes
CPE	Constant phase element
CV	Coefficient of variation
CV	Cyclic voltammetry
DOP	di(n-octyl) phthalate
DPV	Differential Pulse Voltammetry
DWCN	Double-walled carbon nanotubes
EC	European Commission Regulation
ECDP	European Centre for Disease Prevention
ECO	European Certification Organization
EDC	N-(3-Dimethylaminopropyl) -N′-ethylcarbodiimide hydrochloride
EDS	Energy Dispersive Spectroscopy
EDT	Lateral secondary electrons detector
EIA	Enzyme Immunoassay
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme Linked Immunosorbent Assay
EMF	Electromotive force

E_{pa}	Anodic peak potential
E_{pc}	Cathodic peak potential
EU	European Union
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
FP	False positives
FTIR	Fourier-Transform Infrared Spectroscopy
GCE	Glass Carbon Electrode
GE	Gold electrode
GO	Graphene Oxide
HACCP	Hazard Analysis and Critical Control Point
HRP	Horseradish peroxidase
I	Impedimetric /Impedimetry
IAME	Interdigitated array microelectrode
IMS	Immunosensing interface
IR	Infrared
ISE	Ion selective electrodes
ISO	International Organization for Standardization
ITOGP	Indium Tin Oxide Glass Plate
LAMP	Loop mediated isothermal amplification
LB	Lysogeny broth
LM	*Listeria monocytogenes*
LOD	Limit of detection
LOQ	Limit of quantification
m-GEC	Graphite-epoxy composite magneto electrode
m-GEC	Magneto-graphite-epoxy composite
MB	Methylene blue
MB's	Magnetic Bed's
MES	4-morpholineethanesulfonic acid hydrate
MNPs	Magnetic nanoparticles
MSNTs	Magnetic silica nanotubes
MSPE	Modified screen-printed electrode
MWCNTs	Multi-walled carbon nanotubes
Na-ISE	Sodium íon selective electrode
Na-ISE	Sodium pseudo-reference microelectrode
NC	Nanocrystal

NGFIS	Netherlands government food inspection service
NHS	N-hydroxy succinimide
NPOE	2-nitrophenyl octyl ether
OFLD	Open finite-length diffusion
P	Potentiometric
PANi	Polyaniline
PB	Phosphate buffer solution
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PEDOT: PSS	Poly(3,4-ethylenedioxythiophene) polystyrene sulfonate
PIM	Polymer inclusion membrane
PNIPAm/IDμEPoly	(N-isopropylacrylamide) polymer Interdigitated micro-electrodes
PSE	Paper-strip electrodes
PVC	Polyvinyl chloride
QD	Quantum dots
RASFF	Rapid Alert System for Food and Feed
Rct	Charge transfer resistance
rGO	Reduced Graphene oxide
RSD	Relative standard deviation
rt-PCR	Real-time PCR
SAM	Self-assembled monolayer
SEM	Scanning electron microscopy
SPCE	Screen-printed carbon electrode
SPE	Screen-printed electrode
SPGE	Screen-printed gold electrode
SPIDME	Screen-printed interdigitated microelectrode
spp.	Species
ss	Single stranded
ST	*Salmonella typhimurium*
STD	Standard deviation values
SWASV	Square wave anodic stripping voltammetry
SWCNT's	Single Walled Carbon nanotubes
TCPB	Tetrakis(4-chlorophenyl) borate
THF	Tetrahydrofuran
TMB	Tetramethylbenzidine

TOMA	Methyltrioctylammonium chloride
TPB	Tetraphenylborate
USA	United States of America
USDA-FSIS	US Department of Agriculture-Food Safety and Inspection Service
WHO	World Health Organization
ΔE	EMF change

CHAPTER 1 Introduction

1.1. Quality, Security and sustainability of Food: a general overview

1.2. Spoilage microorganisms in food

1.3. Legislation in microbial food control

1.4. Challenges in Analytical Methods

1.5. Aim and Organization of the Thesis

References

1.1 Quality, Security and Sustainability of Food: A General Overview

Food security concept emerged in the mid-1970s, at a time of a serious global food crisis, which was scored by an uneven distribution of food around the world. With the resource scarcity and hunger felt in some regions of the world, the need for sustainable production, conservation and transportation of food products beyond the country's barriers also arise. Thereupon, in 1974 emerge the first food security official definition, in which can be seen contextualized and incorporated the social problems at the time, being specially focused into the maintenance of availability and access to food to all people, at all times [1, 2]. Similarly, the following definitions have undergone conceptual changes according to the demographic, economic and social conditions of the period to which the definitions refer [2].

In the last actualization from United Nations' Committee on World Food Security, food security is assumed when "all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life" [3]. This definition is intrinsically connected with other concepts related to the state of food products like quality (nutritional state), safety (stability) and sustainability of food, but also with social and political strategies of each country (social equity) and people individual needs (organoleptic characteristics, special diets). Additionally, this latest review of food security terminology is also in line with recent awareness of world population growth and climate change [4, 5].

In 2050, the global population is expected to reach to 9 billion people [6]. Given this prediction, the amount of food required to meet the needs of the global population will also have dramatically to increase. Moreover, efficient and sustainable methods to produce and distribute food products around the world will have to be developed and improved. However, despite these efforts, a widening gap respecting to food access between rich and poor countries can be anticipated, while the impact of people life-style trends, of harming time conditions and massive productions in food systems is uncertain and difficult to predict [5, 7]. So is being of extreme importance to safeguard the sustainability of the massive agricultural productions that will arise as well ensure the safety of the food that we eat, both to minimize the food losses such to avoid the widespread of foodborne outbreaks trough the increasingly complex food chains that will emerge.

Food safety is ensured when food has no properties capable of impairing well-being or causing illness to the individuals who consume them. Several regulations and

procedures have been developed to assure the proper handle, preparation and stock of the food products at the different levels of food trade, warranting the safety of the food "from the farm to consumer" (see Figure 1) [4, 7, 8].

Individual level:
- Food comsuption, health and nutritional status.

Hausehold level:
- Care and feeding pratices;
- Health and sanitation.

National / Regional level:
- Food Availability and stability;
- Food supplies and access.

Underlying conditions level:
- Demographic, ecconomics, enviromental, political, social and culture conditions;
- Food safety hazards;
- Natural resources.

Fig.1 - Different levels along a food trade. Adapted from Gibson, et al. 2012 [4].

The use of pesticides and herbicides or other chemical compounds, the presence of contaminants (physical, chemical) or biological threats (bacteria, viruses, parasites or biotoxins) in food are some of the topics currently revised by the regulation authorities, by their dangerousness to animal and human health. Despite, biological hazard agents such as foodborne pathogens are the most difficult to control, by its natural presence in media, atmospheric and ambient susceptibility, easy animal/human transmission and rapid dissemination [9]. Contrarily, chemical contaminants or pest control agents are normally introduced by the human being - even if unconsciously - and so can be more easily accessed with the implementation of agricultural and food handling good practices at a primary prevention level [10].

Indeed, in last years, it has been observed significant increases in foodborne diseases, even after the creation of legislation and surveillance methods to avoid contaminations and preserve the nutritional quality of food, still being an important public health theme in the whole world [11, 12]. According to the World Health Organization, the consumption of contaminated food and water by pathogenic microorganisms, originate 1.8 millions of deaths per year worldwide [13]. The European Union (EU), has at the time one of the

best food safety standards of the world, due to the prompt upgrading of the food safety control regulations following the notifications stated by the country food safety authorities of each EU member each year. Commonly, these outbreak notifications are forwarded to the Rapid Alert System for Food and Feed (RASFF), which subsequently ensures the correct flow of information between the EU members, by issuing an alert. This alert tool is especially important in multi-country outbreaks and urgent situations, impelling massive contaminations due to the possibility of an efficient and rapid response from related country members. Beyond, the RASFF is also in closer communication with the European Food Safety Authority and European Centre for Disease Prevention and Control that will also create a prompt and appropriated investigation of the outbreak notification in each affected country, to identify the contamination focus and the dissemination vehicles along the food chain [14-16].

Accordingly, in the last annual RASFF report, in 2017, were observed an increase of 26% and 18% in overall notifications and in notifications on pathogenic micro-organisms compared with 2016, after a decreasing tendency observed since 2005 [15]. Therefore, it is still of extreme importance to create and develop new analytical methods for detection of food spoilage agents, at a lower cost, quickly, simply and accurately, allowing its use expeditiously in different economic regions and scenarios, by different users.

1.2. Spoilage Microorganisms in Food

Spoilage is characterized by any change in a food product making it undesirable or harmful for the consumer. Microbial spoilage is the most common cause of food spoilage since the early days, and by those several techniques to microorganisms detection and control have been developed [17]. Besides, the colony counting methods described in the two parts of ISO 11290 to isolate foodborne pathogens from food, still being assumed as the standard validated method for microbiological food control until today [18, 19]. Although considering the recent needs, they seem already misfit by the multistep laborious protocol and the time required to obtain a final result (5 – 7 days), even after the inclusion of more rapid and modern biochemical tests into the traditional standard protocol [20, 21]. This last assumption has an especial impact in short-shelf term food products, as they may be consumed even before the quality control result is achieved and thus the result will have no impact on the control of possible food contamination to the consumers.

Additionally, it is also known that some food products are more susceptible than others to attain microbial spoilage according to its elemental composition and experienced processing methods. Still, out-product features like temperature, pH and atmosphere, also influence the probability of growing or inhibition of specific foodborne pathogens respecting its optimal survival media conditions. In Europe, the priority vehicles of microorganism's contaminations were mainly animal (meat, eggs, sea products) followed by vegetal products [14, 15]. Along, it was estimated that 30 – 50 % of all human infectious diseases have a zoonotic origin [22]. The infection can be transmitted by the consumption of contaminated food or water such as directly by contact with the animal itself or its sub-products. By these, the foodborne infectious zoonotic diseases are a pressing concern since the emergence of food security by the capacity of going undetected until they cause adverse human symptoms, as some microorganisms are not harmful to animals but infectious to humans. These problems are increased by the crescent consumption of meat verified especially in developing countries in the last decades. It was estimated by FAO that was eat up in 2015, 41.3 kg of meat in media per capita in the world per year. Among, *Salmonella, E. coli* and *Listeria* were the most common pathogens presented in meat [14, 23-25].

Salmonella is a Gram-negative bacteria, from the *Enterobacteriaceae* family, and have several different serotypes [26]. The various *Salmonella* serotypes are the more predominant cause of alimentary infection worldwide [23, 27]. Despite, *Salmonella enterica* serotype *typhi* is a bacterium responsible for most of the foodborne outbreaks

[14, 27-29]. In humans, the major vehicles of contamination are food: meat, eggs, milk, and contaminated water. *Salmonella* infection, named as salmonellosis can cause numerous symptoms with different degrees of health dangerousness like diarrhea, vomiting, gastroenteritis, severe dehydrating and typhoid fever, putting in extreme cases to lead to death [26, 27, 30].

E-coli is normally colonizing the human large intestine and warm-blooded animal's giving in most of the cases no symptoms [13]. Despite that, some strains from *E-coli*, like *enterohemorrhagic*, are very pathogenic and can cause diarrhea, infections in gastrointestinal tracts and immunosuppression, especially in fragile hosts, like children and elderly or sick people [31]. *E-Coli O157:H7* is the *enterohemorrhagic* strain more dangerous and predominant [32, 33]. The infections normally are caused by consumption of meat, vegetables, milk, contaminated juice and non-treated water [25]. By those, the presence of *E-coli* in food products is considered one microbiologic indicator of water quality and is also important to guarantee this control in the food industry.

Listeria is a Gram-positive bacillus that comprises seventeen different species (spp.) [34]. From those, *Listeria monocytogenes* is the one that can cause listeriosis in humans [35]**.** Listeriosis is a worrying medical condition characterized by severe symptoms like meningitis, fetal anomalies, abortion, febrile gastroenteritis even generalized infection. Despite the low incidence that present, listeriosis is associated with high hospitalization and mortality rates (20-30%) [34, 36, 37]. Contrarily, with the other common food-borne diseases (e.g. Salmonellosis or *E-coli spp.* infections), the human listeriosis is often acquired by ingestion of ready-to-eat and processed food products, therefore increasing the need of control. Due to statistical facts abovementioned and the acute danger to public health, *Salmonella* spp. and *Listeria* spp. played a prominent role and were considered as priorities for microbial food control in the development of this work.

1.3. Legislation in Microbial Food Control

To decrease the risk of foodborne diseases, proper food handling and preparation principles should be followed as preventive actions to avoid cross-contaminations and maintain food safety. Among others, Hazard Analysis and Critical Control Point (HACCP) program is the regulatory program established in most of the countries, including the EU and United States of America, to set up controls for the prevention, identification and traceability of the biological and chemical contamination. Additionally, the HACCP program also refers to several procedures to ensure the correct labelling, packaging and the nutritional quality of its content [38].

European Commission creates in 2005, a regulation on microbiological criteria for foodstuffs for relevant foodborne bacteria and their sub-product, with practical applicability since 1 January 2006. The European Commission Regulation No 2073/2005 [39] criteria define the acceptability parameters of a product just placed on the market and also hygiene criteria to be employed in the food production stage, taking into account the risk of the most dangerous foodborne bacteria (see table 1). This regulation merged the information present in previous council publications like the regulation (EC) 2160/2003 or the directive 2003/99/EC, specific for *Salmonella* and all zoonotic agents respectively. According, *Salmonella* was considered a life-threatening pathogen, and so a zero-tolerance policy was associated with the acceptability of the ready-to-eat products. So, only the confirmation of the absence of *Salmonella spp.* in a ready-to-eat food sample (a portion of 25 g) will classify it as satisfactory otherwise, it will have to be rejected.

E-coli spp. are frequently found in foodstuffs, food preparation surfaces and human hands. Therefore, in food industry its presence, such as the level of contamination or absence was considered as a hygiene indicator in food processing and handling, in which some limits in CFU/g per 25 g samples were also taken (Table 1), rely on food category and the point of sample collection [39, 40]. Before the analysis and the application of these limits, if one sample or a batch of samples were considered as unsatisfactory, doesn't mean that this food will be considered as unsafe. The *E. coli* presence can only indicate that the sample has a poor hygiene indicator and the food business has to carry out improvement actions, develop new hygiene procedures and treatments to improve the microbiological contamination of the product and ensure that in the next analysis the contamination levels were lower.

Respecting to *Listeria spp*., namely *Listeria monocytogenes,* the legislation addressed the objective of keeping its concentration in food below the minimum infection dose

reported to humans during the all product shelf-life. By those, the Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs [40], try to make a distinguished between foodstuffs unable to support the *Listeria* monocytogenes growth or just placed on the market from the others categories [36, 39]. Accordingly, a zero-tolerance policy was also attended for *Listeria spp.* presence in ready-to-eat foods intended for infants and special medical purposes, whereas other not specified ready-to-eat foods only may contain <100 CFU/g during their shelf-life. So, the food quality control to these pathogens has to be expanded to food sector enterprises that produce ready-to-eat products that should proceed to the sampling of the producing area and the transformation equipment's avoiding that food pathogens enter in the food supply, reducing the public health risk. Beyond, in 2017 a large scale multi-country outbreak caused by *Listeria monocytogenes* was identified in frozen corn, that usually is consumed after cooking and as so it's not considered consensually as a ready-to-eat product, do not need to follow the criteria imposed by (EC) No 2073/2005 and which subsequently delay the outbreak notice and the contamination control. By those it will be important to make in a closer future a reformulation of food categories covered by the regulation, taking also into account the current people consuming habits.

Table 1. Limits in the legislation according with the Commission regulation (EC) No 2073/2005 and (EC) No 1441/2007 on microbiological criteria for foodstuffs for *Salmonella* spp., *E. coli*. and *Listeria*.

Pathogen	Results in (CFU/g) or absence/presence in 25 g sample		
Classification	Satisfactory	Borderline	Unsatisfactory
Salmonella spp.	Not detected	NA	Detected
E. coli (manufacturing)	<20	$20 <= 10^2$	$>10^2$
E. coli *	$<10^2$	$10^2 - 10^3$	$>10^3$
Listeria monocytogenes	$<10^2$	$<10^2$ -	$>10^2$

*applicable to cheeses made from milk, ready-to-eat pre-cut fruit and vegetables or unpasteurized juices samples collected directly in the point-of-sale (foods category 2.2.2, 2.5.1, 2.5.2).

1.4. Challenges in Analytical Methods

Gold-standard methods for foodborne pathogens detection are based on culture and colony counts, which have proven to be laborious and time-consuming according to current demands in food industry as well as mismatch both by present global food safety hazards and the number of control points required by the legislation. Hereupon, in last years it was seen a demanding for alternative and innovative analytical methods able to overcome the disadvantages of the traditional ones. The emerging methods developed in academic and commercial ambit for *Salmonella spp.* and *Listeria monocytogenes* are mainly based in new chromogenic media, antibodies or nucleic acids with high specificity for the analyte coupled with optical, electrochemical and mass-based transduction. Nevertheless, most of those methods still require specific and expensive equipment and imply a considerable workload to perform the analysis. Moreover, the need for pre-treatment techniques to food analysis, still suppress the transition thought prototypes developed academically to widely accepted commercial equipment. Despite those, antibody-based methods associated with electrochemical transducers have been presented as an excellent alternative among the others [41, 42], since they can address some of the most pressing analytical problems in foodborne pathogen screening, pursuing a rapid, sensitive, decentralized, cost-effective and user-friendly performance [43].

1.5. Aim and Organization of the Thesis

1.5.1. Aims

Pathogenic microorganisms pose a considerable risk to human and animal health, so their rapid, economical and easy-to-carry monitoring is of paramount importance for maintaining public health and ensuring timely compliance with legal food safety standards. To meet this need, we sought to develop innovative immunosensing systems to assess the foodborne pathogens contamination degree of food, with high sensitivity, selectivity and specificity, of fast, simple and economical execution, expecting that may become an alternative to the conventional techniques. Thus, it was also intended that the liquid samples could be directly quantified and that the preparation of the remaining samples would be simpler than necessary when using standard techniques.
Starting from these premises, several smaller intermediate objectives were set to reach the ultimate aim:

i) develop low-cost miniaturized potentiometric and/or voltammetric cells that enable to achieved to very low detection limits;

ii) develop and implement new nanostructured interfaces using biocompatible and high surface area nanometric metallic and non-metallic materials for direct capture of microorganisms in label-free immunosensors;

iii) optimize the developed label-free nanostructured immunosensors and immunoassays performances to be applied in real-food matrices, resorting if necessary, to immunomagnetic separation methods;

iv) integrate enzyme labelled immunoassays into stable electrochemical immunosensors resorting to a low-step and short preparation protocols.

1.5.2. Organization of the Thesis

This thesis compiles all the work developed in the scope of the doctoral plan project in sustainable chemistry. The experimental works were organized along to the literature reviews in four different chapters, concerning the related theme and the specific objectives of each one. For all the articles, the original content, formatting and structures organization were maintained in agreement with the journal in which they were published or submitted.

Firstly, was introduced the problem that urged this investigation, the present challenges and its theoretical background. Chapter 1 starts with a general contextualization of the developed work and its relevance (Section 1.1.), such as an overview of the thesis scope (Section 1.2), associated regulations (Section 1.3) and pressing challenges covered by the thesis theme (Section 1.4). Here, it was presented the motivation, main objectives as well as the thesis outline (Section 1.5).

The development work was divided into two main chapters, according to the target foodborne pathogen: *Salmonella* (Chapter 2) or *Listeria* (Chapter 3). In those, a general overview of the literature about electrochemical biosensing of each microorganism was firstly introduced, next to the related experimental work. Chapter 2 was focused into the development of innovative potentiometric immunosensing systems for detection of *Salmonella* in food. In Section 2.1 it was presented an exhaustive critical overview about electrochemical biosensors and commercial options just available for *Salmonella* detection, such as the challenges presents in this type of methodologies. Then, three experimental works were included. Section 2.2 and 2.3 look over to the development of new, simple and low-cost immunosensing platforms to the development of simpler and more sustainable electrochemical devices for control and quantification of *Salmonella* in food products. Section 2.2. focused the integration of the biorecognition element into a gold nanoparticles polymer inclusion membrane, formed in a greener way comparing with the common conjugation protocols. Moreover, this construction methodology allows both to use a "labeless" detection mechanism based on the surface blocking principle, as amplify the analytical signal recorded. To explore even more this simple principle of detection, the same mechanism was employed in the work presented in Section 2.3, however an innovative transducing platform was introduced. Hereupon, a paper-based strip electrode was developed to act as a transducer of the biorecognition event. The inherent objective of this sensor platform upgrade was to achieve a decentralized immunosensing system, by using a low-cost and widespread material such as the paper. This prototype allowed the detection of *Salmonella typhimurium* at low levels of

contamination using for the first time, a paper-based platform combined with electrochemical transducing. In order to increase the recoveries rates and the effectiveness of the developed methods in the detection of *Salmonella* in real samples, a potentiometric magnetic immunoassay was also designed (Section 2.4). In this work, the potentialities of the immunomagnetic separation protocols associated with the specificity of a sandwich format assay and the ion selective electrodes high sensibility were explored.

Chapter 3 accessed to electrochemical biosensing approaches for *Listeria monocytogenes* detection. Section 3.1 included a critical review of the emerging topics related to electrochemical biosensing of *Listeria* according the type of bioreceptor, such as new emerging alternatives and hot topics in the field. In Section 3.2, was shown the last experimental work, in which a voltammetric immunosensor towards invasion-associated protein p60 was constructed. This target protein was chosen in detriment of *Listeria* cell epitopes, to attain to a more specific an accurate immunoassay. This promising strategy was the first that use electrochemical detection for this specific invasion associated protein.

Finally, Chapter 4 embrace the major conclusions reached along with development of the thesis. Future perspectives about the developed and future work were also predicted.

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CHAPTER 2 Potentiometric Immunosensing of *Salmonella* in Food

2.1. Electrochemical biosensors for *Salmonella*: State of the art and challenges in food safety assessment

2.2. In situ formation of gold nanoparticles in polymer inclusion membrane: Application as platform in a label-free potentiometric immunosensor for *Salmonella typhimurium* detection

2.3. Development of a disposable paper-based potentiometric immunosensor for real-time detection of a foodborne pathogen

2.4. A potentiometric magnetic immunoassay for rapid detection of *Salmonella typhimurium*

2.1. Electrochemical biosensors for *Salmonella*: State of the art and challenges in food safety assessment

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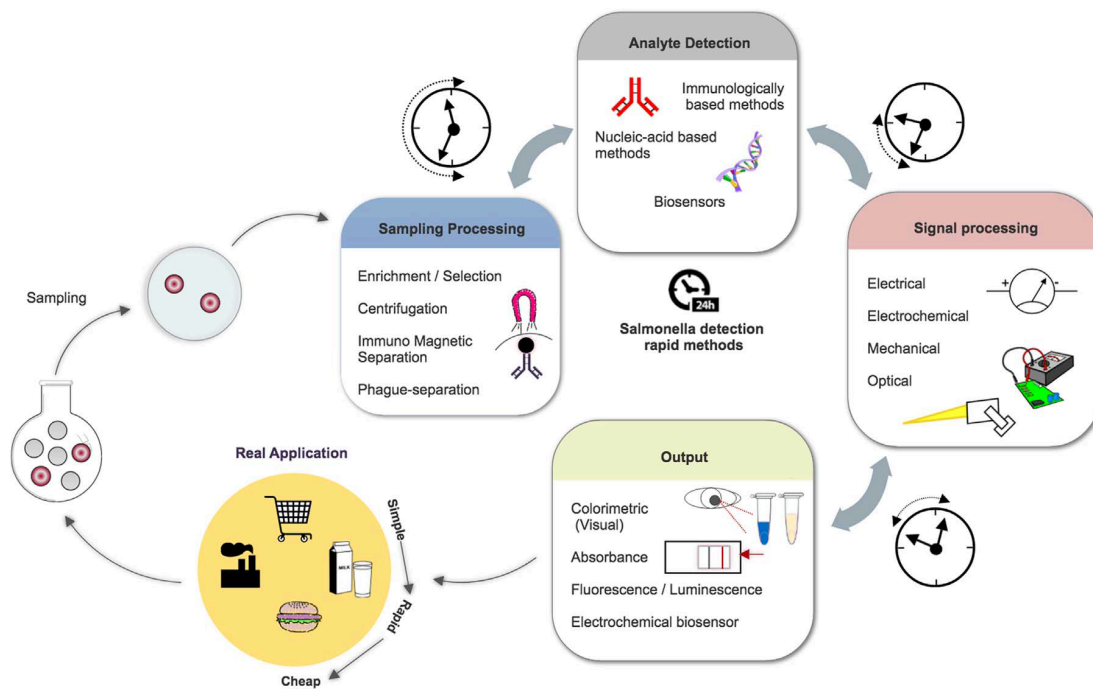
Statement of contribution

The contribution of the candidate, Nádia F.D. Silva, in this work includes the literature review on promising analytical techniques for *Salmonella spp.* with focus on developed electrochemical biosensors and the written of the original draft.

CrossMark

Electrochemical biosensors for *Salmonella*: State of the art and challenges in food safety assessment

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ABSTRACT

According to the recent statistics, *Salmonella* is still an important public health issue in the whole world. Legislated reference methods, based on counting plate methods, are sensitive enough but are inadequate as an effective emergency response tool, and are far from a rapid device, simple to use out of lab. An overview of the commercially available rapid methods for *Salmonella* detection is provided along with a critical discussion of their limitations, benefits and potential use in a real context. The distinguished potentialities of electrochemical biosensors for the development of rapid devices are highlighted. The state-of-art and the newest technologic approaches in electrochemical biosensors for *Salmonella* detection are presented and a critical analysis of the literature is made in an attempt to identify the current challenges towards a complete solution for *Salmonella* detection in microbial food control based on electrochemical biosensors.

1. Introduction

Foodborne diseases are caused by ingestion of water or food contaminated by pathogenic microorganisms, like bacteria and virus, pesticides residues or other toxins(Xihong Zhao et al., 2014). Despite the legislation and control methods developed to preserve food nutritional quality and prevent contamination, a significant increase in foodborne diseases has been observed since 1980 and it continues to be an emerging public health theme in whole world(2009; Brandão et al., 2015; Thakur and Ragavan, 2013). According to World Health Organization (WHO) the consumption of food and water contaminated by pathogenic microorganisms causes 1.8 millions of deaths per year worldwide (Shen et al., 2014), and the various *Salmonella* serotypes are the more predominant cause of alimentary infection (Dong et al., 2013; Lee et al., 2015).

In Europe, as reported in the Rapid Alert System for Food and Feed (RASFF) in 2013, the priority vehicles of contaminations were animal products (meet, eggs, milk, and sea products), vegetables and water. *Salmonella* is one of the most common pathogens in meat (Chemburu et al., 2005; Farabullini et al., 2007; Lee et al., 2015). *Salmonella* is a Gram-negative bacterium, from *Enterobacteriaceae* family. *S. (Salmonella) enterica* and *S. bongori* are the species that can cause illness in humans producing numerous symptoms like diarrhea, vomiting, gastroenteritis, severe dehydrating (Bula-Rudas et al., 2015; Dong et al., 2013; Yang et al., 2009) and other sickness stages as typhoid fever. These two species were divided into 2500 known serotypes based on the Kaufmann-White typing scheme (Brenner et al., 2000; Bula-Rudas et al., 2015). The *S. enterica* serotype *typhi* is the bacteria responsible for most of the foodborne diseases and along with serotype *paratyphi,* it can be found only in humans. The *S. paratyphi* causes typhoid salmonellosis, which according to the Food and Drug Administration (FDA)**,** if not treated can result in a mortality rate of 10%. In this case, the infection dose is 1000 Colony Forming Unit (CFU), which is much higher than the infection dose required to occur the symptoms associated with a non-typhoid salmonellosis - which are as low as 1 CFU - although the dangerousness of the side-effects is higher for typhoid salmonellosis (Administration, 2012; Dong et al., 2013; Dungchai et al., 2008).

Due to the extremely low infection limits, 1 CFU, the associated side effects and the high *Salmonella* susceptibility for dissemination in perishable and semi-perishable products, the limits imposed by law have been tightened over the years. In the European Commission (EC) regulation No 2073/2005 on microbiological criteria for foodstuffs, the *Salmonella spp.* are considered a group of pathogens which its presence by itself in ready-to-eat food (portion of 25 g), is enough to be considered a risk factor for human health. Consequently, if this pathogen is detected the food product is classified as unsatisfactory. The absence of *Salmonella* spp. is a figurative quantification, since

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"zero" in analytical measures is unreal, because each method has a limit of detection and there are always errors associated. Even the conventional culture methods recommended by International Organization for Standardization (ISO) (ISO standard 6579:2002) due to their exceptional sensitivity (Melo et al., 2016) are only capable to detect 1 CFU/ 25 g of foodstuffs.

These regulations are compatible with the Hazard Analysis and Critical Control Point (HACCP) approach, which are used in most of the countries, including the European Union (EU) and United States of America (USA), to establish adequate controls for the identification of *Salmonella* in ready-to-eat foods to assure that it is absent when it is taken by the consumers(Lawley, 2012). Additionally, some countries have specific rules for products like eggs and fresh daily products. For instance, the FDA has a specific rule to prevent S. *enteritis* in eggs, because it is one of the largest contamination vehicles for infection dissemination in the country. This rule is a set of measures which are implemented in the production (for example, the pasteurization implementation), storage and transportation of shell eggs(Lawley, 2012). The effect of more control and the sanctions for non-compliant producers has recently shown positive effects in the statistics of salmonellosis outbreaks in EU. Indeed, between 2004 and 2009 the human cases reduced almost for one-half (EFSA 2014). Counterbalancing these encouraging statistics from the European Food Safety Authority (EFSA), in the USA it was estimated from 2 to 4 million cases of salmonellosis annually, being already considered one of the major causes of hospitalization and dead (Elaine et al., 2011; Oliver et al., 2005; Xihong Zhao et al., 2014).

Because of these alarming statistics, it is still necessary to develop new simple methods and technologies for *Salmonella spp.* detection with the ability to provide valid results at the time of consumption of perishable foods, thus avoiding mass contaminations. Nowadays there are several methods purposely designed to accelerate the pathogen detection but most of them have difficulties to get validated and enter to the market, because they have a high probability of false negative results, sometimes are restricted to a specific type of food or considered expensive by the food industries (Valderrama et al., 2016). In the future, the best approaches for rapid *Salmonella* detection in food control will be designed for application outwards the laboratory and may involve disruptive innovations to minimize the pre-enrichment and sample preparation steps.

The purpose of this review is to give an overview of current methods for *Salmonella* detection in microbial food control and to present the authors view about the most promising route to develop new rapid methods. A critical survey of rapid commercial methods is presented aiming to identify current needs for further development in rapid practical food control. Among several existing methods, which have already been recently reviewed (Lee et al., 2015; Rahman et al., 2016; Su et al., 2011; Valderrama et al., 2016), the biosensors were chosen as an emerging tool for *Salmonella spp.* control due to the increasing interest in the scientific community, as shown by the increasing number of publications using this technology, and their characteristics, namely the operational simplicity, sensitivity, readiness and real-time analysis potential. Among all existing biosensors for *Salmonella spp.*, the electrochemical biosensors are reviewed because they show distinguished advantages like the low cost of the equipment, miniaturization capacity and inherent sustainability, due to the use of a few solvents and low sample volumes, both in its development and application. Considering that the acceptance by the industry for novel rapid methods depends not only on speed but also on the initial investment, cost, technical support, and ease of use, electrochemical biosensors are specially well suited to fulfill these requirements[1].

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[1] This review is not intended to endorse or recommend any commercial product, and any omission of a commercial product is not intentional.

2. Commercial rapid methods for *Salmonella spp.* detection in food products

Conventional methods for bacteria detection rely on standard culture methods that involve the use of different enrichment and selective broths for the isolation of each bacteria, in which large amounts of sample are used in a complex sequencing of steps (Lee et al., 2015). Beyond their sensitivity and high accuracy, the conventional methods require at least 1 week for trusted results (2–3 days for results and 7–10 days for confirmation)(Farabullini et al., 2007; Yang et al., 2009). Besides these time consuming methods recommended by ISO, it is already possible to obtain similar results in 24–48 h using nucleic acid-based assays or even in less than 24 h with some immunologically-based methods like Enzyme Linked Immunosorbent Assay (ELISA), which together with the biosensors belongs to the rapid methods for pathogen detection in food samples (Valderrama et al., 2016).

In the last years, various devices for rapid detection of *Salmonella spp.* were developed, tested and commercialized (Brandão et al., 2015; Law et al., 2015; Lee et al., 2015; Melo et al., 2016; Valderrama et al., 2016). According to current regulation for food control parameters, commercial methods should accomplish several requirements: the devices have to be able to detect a single *Salmonella* CFU in 25 g of food; they must have a sensitivity and specificity of at least 99%; and operational personnel ideally must need no special skills to perform the analysis (Eijkelkamp et al., 2009). Besides these general requirements, the analysis time of rapid methods preferably must be in the range of hours to a limit of 24 h (Valderrama et al., 2016).

Commercial rapid detection methods should be validated by the competent authorities for example the HACCP, the FDA and the Association of Official Analytical Chemists (AOAC) in the United States of America, and the European Certification Organization (ECO) for the validation and approval of alternative methods for the microbiological analysis of food and beverages (MicroVal) in the EU. The validity of a method depends upon its sensitivity and specificity. Sensitivity is the probability of the test to detect a true positive, while specificity is the probability of the test to detect a true negative. A schematic overview of current rapid methods for salmonella detection in food products is provided in Fig. 1. They can be divided into several categories including miniaturized culture assays - modified or adapted from conventional procedures, but using new selective culture media - immunologically-based assays, nucleic acid-based assays and biosensors. It is difficult to make an accurate comparative analysis about the performance of commercial rapid methods because it depends on several experimental factors, such as sampling, sample matrix, enrichment processes and it lacks normalization of the evaluation schemes (Lee et al., 2015). Comparative studies for the test kits should be set up under identical test conditions to better compare and evaluate the test results from different laboratories. Information about the performance (sensitivity, analysis time, advantages and limitations) of validated commercial methods were obtained from the producer's brochures and websites, or scientific papers (Barthelmebs et al., 2010; Cheung et al., 2007; Eijkelkamp et al., 2009; Oxoid Limited; RomerLabs 2013b; SM, 2004/, 2005a) and it is organized in Tables 1–4, according to their methodology.

2.1. Immunologically based methods

The immunologically based methods for *Salmonella* spp. detection explore the specificity of the antibodies (monoclonal or polyclonal) for specific antigens, normally located at *Salmonella* cellular membrane surface. There are several formats for these assays but the commercially available methods are mainly based on agglutination, immunoprecipitation, immunodiffusion and enzyme immunoassay (EIA) / ELISA, which includes several lateral flow devices.

The agglutination and immunoprecipitation methods use particles
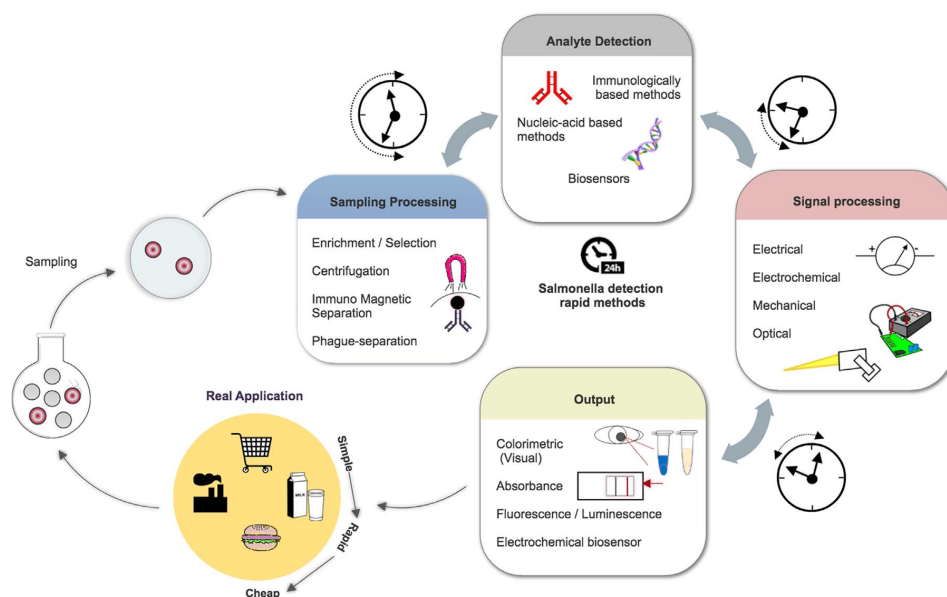
Fig. 1. Schematic overview of current rapid methods for Salmonella spp detection in food products.

coated with antibodies that react with antigens and form clusters visible to the naked eye. This approach gives simplicity and rapidity to the analysis but the methods show a high limit of detection (LOD) that is incompatible with the regulated limits. It is worth to note that all the analyzed commercial methods (Table 1) need an enrichment step prior to the test. For this reason, these tests are frequently used as a confirmatory analysis technique or for serotyping previous positive findings. Among the latex agglutination tests, the Spectate test from May & Baker diagnostics Ltd. and the color *Salmonella* from Wellcolex allow simultaneously the *Salmonella* spp. detection and serotyping. From the user point of view, it is very simple to make and to interpret the analysis, because the positive presumptive result is visually identified by the clusters formed in the presence of the antigen or by a color change attributed according to the serotype, making them attractive devices to the food industry despite the impossibility of quantification.

The EIA/ELISA are the most promising methods for rapid detection, because they combine the specificity of the antibodies with the sensitivity of the enzymatic assays by coupling easily assayed enzymes to antibodies or antigens. Usually, they are more selective and sensitive than agglutination or immunoprecipitation assays but need a longer time to obtain quantitative results, although still shorter than most of the nucleic acid-based methods.

There are three ELISA formats schematized in Fig. 2: the direct, indirect and sandwich or capture assay. In the direct approach, it is used an enzyme labeled antibody that recognizes the antigen which is previously bound to a solid matrix. In the indirect formats two antibodies are used, a primary non-labeled antibody that recognizes the immobilized antigen and a secondary enzyme labeled antibody that binds to the primary antibody. In the sandwich formats, a capture antibody immobilized on a solid matrix binds to the antigen and then an enzyme labeled antibody also binds to the captured antigen. The sandwich ELISA formats benefit in terms of selectivity due the use of a second labeled antibody after the capture of the antigen and attain low detection limits due to enzymatic amplification. For these reasons, it is the most used formats in immunologically-based methods. Apart of the formats, the ELISA assays can be competitive or non-competitive, if the

measured signal is inversely or directly proportional to the amount of antigen present in the sample.

Almost all ELISA commercial rapid methods (TRANSIA® Plate *Salmonella* Gold, the 3M[TM] Tecra[TM] *Salmonella* VIA, Ridascreen[R] *Salmonella* R-Biopharm, assurance GOLD and Assurance *Salmonella* from Biocontrol, *Salmonella* Tek from Organon TeKnika, BacTrace from KPL and BioLine from HardyDiagnostics) uses a sandwich format, where a peroxidase enzyme like Horseradish Peroxidase (HRP) was used as enzymatic label. This design is very attractive both from the analytical and practical application point of view, due to the capacity of these enzymes to catalyze chromogenic substrates like tetramethylbenzidine (TMB) in the presence of a substrate solution (H_2O_2 or urea and H_2O_2). After adding a stop solution, a color change is observed and the results can be read visually or more precisely with an automatic micro plate reader.

The ELISA methods are in a rather advanced development stage and has already application in the food industry (Lee et al., 2015; Valderrama et al., 2016). The major challenge for these methods is to achieve good sensitivity and specificity values in complex matrixes containing inhibitory substances, like fats or proteins present in food, and background microflora, because these substances inhibit the immunological response and mask the presence of target bacteria, increasing the probability of false negatives (Valderrama et al., 2016). The detection limit of the ELISA methods under optimized conditions is typically in the range 10^4 to 10^5 CFU mL^{-1} (Lee et al., 2015; López-Campos et al., 2012). Considering the regulated limit all ELISA methods for food control may involve selection/enrichment steps to attain the required limits of detection and to minimize sample matrix interference. In fact, a brief analysis of data in Table 1 shows that enrichment/selection step is necessary for most of the commercial ELISA based methods, thus increasing the total analysis time, typically between 18 and 48 h for presumptive results, despite of the short analysis time for the ELISA process, which usually is complete in less than 2 h. Furthermore, most of the commercial devices can only be used in specific food types, like for example the Assurance test for *Salmonella* from Biocontrol, or need extra time for sample pre-enrichment/selection proportional to the matrix complexity. Indeed, the Assurance Gold

Table 1
Immunologically-based commercial methods for Salmonella detection.

Method	Assay/Manufacturer	Analysis time	Sensitivity	Advantages	Disadvantages
Latex Agglutination	Spectate (May & Baker Diagnostics Ltd.)	3-5 min. for test only (after enrichment)	NR	- Specific and simple; - Used as a confirmatory analysis technique;	- Allows the detection and the serotyping/ grouping
	Wellcolex color *Salmonella* (Wellcolex)	3 min. for test only (after enrichment)	NR	- High positive and negative predictive values (PPV > 98.7%); - Easy interpretation.	- Only for screening proposes (presence/ absence); - Need storage at 2 - 8º C; - Allows the detection and the serotyping/ grouping.
	Salmonella Latex test (Oxoid)	3-5 min. for only test (after enrichment) Total time 24 h	NR	- Easy interpretation; -Ssensitivity of 100% and a specificity of 98.7% (Oxoid Limited).	- Effective only in some *Salmonella* serotypes; - Not validated for non-motile specie; - Store all reagents at 2 -8º C.
	Bactigen (Wampole Laboratories) Slidex (biomerieux)	3-5 min for test only (after enrichment) NR	NR	- Reliable results; - Easy interpretation.	- Only applicable to pure culture or animal Specimens.
Immunomagnetic Precipitation	VIP for *Salmonella* (BioControl)	Total 24 h	NR	- Room Temperature storage; - Suitable for testing all food products - lateral flow assay	- Only positive or negative result; - Need confirmative tests for quantification; - 81.9% and 98.8% (relative sensitivity to reference method OMA, depending the contamination level of poultry). (Eijkelkamp et al. 2009).
	Salmonella enteritidis	Total analysis time 22h	NR	- Can be integated in analytical detection procedure; - latex agglutination for positive samples.	- Relatively expensive cost; - Need confirmative tests.
ELISA	TRANSIA® Plate *Salmonella* Gold (Raiso Diagnostics Ltd.)	Enrichment/Selection 36 to 46 h. ELISA assay – 1.5 h	1 CFU/25 g (Eijkelkamp et al. 2009)	- Easy interpretation: based on a simple color change; - Results in 24h with TAG 24 supplement.	- High LOD; - Long analysis time.
	TRANSIA® Card (Raiso Diagnostics	Enrichment/Selection	Transia Card:	- Simplicity;	- The Transia Card is less selective in food samples;
		18 to 24h. ELISA assay –10 min.	10^5-10^6 cells/mL	- Shorter enrichment and detection time.	- High LOD.
	3M™ Tecra™ *Salmonella* VIA (Tecra)	Enrichment/Selection 18 to 24h. ELISA assay –less than 2 h.	1-5 CFU/25 g	- Good sensitivity; - Simultaneous detection of various pathogens in a single analysis.	- Long analysis time.
	3M™ Tecra™ *Salmonella* Unique Plus™ (Tecra)	Results in < 22h	1-5 CFU/25 g	- Convenient in medium and small scale samples; - Simultaneous analysis of different - Simple results interpretation; - All food application; - Satisfactory sensitivity; - Automation	- Need of pre-enrichment; - Validated for: Salmonella spp. in food and environmental samples; - Relatively expensive cost.
	Ridascreen® *Salmonella* (R-Biopharm)	Presumptive results in less than 23h	1 cell/25 g ≈ 10^4 cells/mL after enrichment	- Approved for AFNOR EN/ISO16140, FDA and for ISO EN/ISO 16140; -Simplicity in results analysis, based in a simple color changes; - Good sensitivity; - Approved for food, feed and	- Long analysis time. - Laborious; - Only screening result (presumptive presence /absence).

(continued on next page)

Table 1 *(continued)*

Method	Assay/Manufacturer	Analysis time	Sensitivity	Advantages	Disadvantages
	Assurance EIA GOLD *Salmonella* (BioControl)	Total time Analysis: - Processed food (24h) - Raw foods (28h); ELISA - 2 hours		environmental samples - High throughput efficiency; - AOAC Official Method 999.08; - All food approval	- Need storage 2 - 8° C
	Assurance Salmonella) (BioControl	Total analysis time 48h		- Affordable cost;	- Need storage 2 - 8° C - Only applicable to some foods - Several equipment requirements: microplate washer, microplate reader; - Only presumptive results;
	Salmonella Tek (Organon Teknika) BacTrace (KPL, Inc.)	Total 42–52 h	1–5 CFU/25 g ≈10000 -50000 cells/mL	- Simplicity; - Easy interpretation. - Easy interpretation	. - Long analysis time
	BioLine (Hardy Diagnostics) Assurance Enzyme Immunoassay (EIA)	Total 42–52 h	1 CFU/25 g	- Approved for HACCP and AOAC - Already tested by some authors in alfalfa sprouts and chicken meat	- Long analysis time
Enzyme-linked	MicroELISA (Dynatech Lab.) EIA Foss (Foss Electric)	Total 48h Total time Analysis < 24 h	NR NR	- Semi-automation - Automated process; - Applicable to all foods and feedsproducts; - Immunomagnetic separation of the target - Give next day results	- Need to buy equipment relatively expensive; - Relatively expensive cost
Fluorescence assay	VIDAS SLM + ICS (bioMerieux Vitek)	Total time Analysis: 24 h (IMS) - 48 h	1 CFU/ 25 g (producer) 5–50 CFU/25 g (Eijkelkamp et al. 2009; Uyttendaele et al. 2003)	- Allows to perform 30 tests simultaneously; - AOAC official method no. 2001.09; - All foods application;	- Long time to results; - Sensitivity 93% and specificity 96% (relative reference method DIASALM) (Torlak et al. 2012)
Lateral Flow	RapidChek® Select™ (Romer Labs®)	Total: 22-30h	1 CFU/25 g (Torlak et al. 2012)	- Simple use; - Validated by AFNOR, AOAC and FDA; - High selectivity and selectivity	- Not applicable to all foodstuffs; - Only for Salmonella Enteritidis specie; - Available two different modules: for presumptive detection and identification(extra 24h)
	Reveal® 2.0 for *Salmonella* (Neogen)	Total time Analysis:<24h	1-10 CFU \25 g	- Provide next day results; - Simplicity; - IMS sample isolation; - Good sensibility for emergency applications.	- Not validated for all foods; - Sensitivity of 52–71% and specificity of 58–78% relative to reference method (SM 2004/2005).

NR- Not reported.

Table 2
Nucleic acid-based commercial methods for Salmonella detection.

Method	Assay/Manufacturer	Analysis time	Sensitivity	Advantages	Disadvantages
PCR	7500 Fast system (Applied Biosystems)	Less than 30min	1 cell/20 μL 99,7% sensitivity	- Very Good sensitivity; - Fast in PCR Analysis	- Need of sample preparation; - Long period to achieve a quantitative result.
	BAXR System Real-Time	Enrichment/Selection:	10^4 CFU/mL	- High specificity	- Less sensitivity relatively with others PCR techniques;
	PCR Assay for *Salmonella* (Dupont)	10 to 24h; PCR 70 min	after enrichment	- Automated procedure; - Sensitivity 98%;	- Long analysis time; - High limit of detection
	TAQMAN (PE Applied Biosystems)	10 to 24h; PCR 70 min Enrichment/Selection: 16 h. PCR - 4 hours	1-10 copies of the target DNA per reaction;1 CFU/ 25 g of food.	-100% inclusivity for 51 strains of *Salmonella* enterica – and 100%. exclusivity for 24 other non-Salmonella strains; - Certified by AOAC and AFNOR;	- Long analysis time; - Can be only used for screening proposes.
DNA Hybridization	Gene – Trak® (Neogen)	Total assay: 27 – 48 h	1-5 CFU/25 g $\approx 10^4$ cells/mL	- Simple, reliable, and rapid procedure. -Simultaneous detection of various food pathogens in a single analysis; - Good sensitivity 99,2% and specificity 98.5–99.75% (Eijkelkamp et al. 2009)	- Long analysis time; - Expensive

Table 3
Commercial miniaturized biochemical tests for Salmonella detection.

Method	Assay/Manufacturer	Analysis time	Sensitivity	Advantages	Disadvantages
Miniaturized Biochemical tests	API 20E (BioMérieux sa.)	Enrichment/Selection; 18 to 24h PCR – 70 min.	100% True Positive	- Good sensitivity; - High confidence test: 99,9% correlation with conventional tests results.	- Expensive, relatively with other biochemical test; - Can be necessary more 24h of incubation for confirmation; - Matrixes: only pure cultures.
	1-2 Test kit (BioControl) Motility/Immunodiffusion	36-58h (14 hours after enrichment);	NR	- Single-use test; - Easy of use; - High specificity; - Low cross-reaction. - No equipment is required to read results	- Relatively expensive cost. - Need storage at 2 - 8° C
	Salmonella Rapid Test (Oxoid) Motility/Selective and indicator media	42 h	NR	- Suitable for testing all food products - Sensitivity: 96.8%; - Approved by FDA and HACCP authorities;	- Application in all food materials and finished food products; - Only presumptive detection. - Relatively expensive cost.
	S.P.R.I.N.T. *Salmonella* (Oxoid) Enrichment Selective and Identification	Enrichment/Selection:	NR	- Approved by FDA and HACCP authorities;	- Only for screening;
	Salmonella Rapid Test (Unipath) Motility, Enrichment/biochemical detection	< 24 h 42 h	NR	- Detects motile and non-motile *Salmonella*; - Saves on unnecessary confirmations - Low false positive rate (<0.3% for all matrices)	- Relatively expensive cost

NR- Not reported.

Table 4
Commercial biosensors for Salmonella detection.

Method	Assay/Manufacturer	Analysis time	Sensitivity	Advantages	Disadvantages
Biosensors	RBD 3000 Micro PRO™ (AATI) Flow cytometry method	Enrichment/Selection: 18 to 24h Measurement 3-5 min	10^1-10^6 CFU/mL	- Simultaneous detection of various food pathogens in a single analysis; - Reduce the human errors; - Good sensitivity - Simplicity; - Allows to choose for a qualitative or quantitative analysis; - Similar results compared to counting plate methods;	- Long total analysis time
	RAPID-B ™ Vivione (Biosciences Company) Flow cytometry method		1 CFU/25 g	- Able to provide living bacteria counts within 15 min; - Can be coupled with a bacterial destroy system;	- Quantitative data

version for *Salmonella* from the same company distinguishes the total time necessary to perform the analysis in processed or raw food, and the last type of food needs extra 4 h, perhaps due the presence of bacteria from their normal microflora. In a rapid approach, these ELISA give only a presumptive result and need confirmatory tests. Both presented fluorescence ELISA assays (EIA from Foss Electric and VIDAS SLM plus ICS from bioMérieux Vitek), involve immuno-separation and probably for this reason are classified as "all food application" without significant change in total time of the test.

Most of the lateral flow assays is an adaptation of ELISA method involving more simple procedures, although present higher rate of false positives (FP) due to matrix effects comparatively to other methods. Therefore, testing for each foodstuff is necessary before utilization. The lateral flow device Singlepath® *Salmonella* from Merck Millipore (Table 1) shows a considerable FP rate of 7.3%. On the other hand, the Reveal system from Neogen fulfills the time and LOD requirements for rapid tests but lacks full validation and application to all food samples because presents low values of sensibility and specificity when compared with reference methods (SM, 2004/, 2005b). The RapidChek® Select™ from Romer Labs® bypassed this lack introducing a patented phage-based enrichment step that increases the sensitivity and selectivity of the method to 100%, receiving the validation from FDA and AOAC(RomerLabs 2013).

2.2. Nucleic acid-based assays

Nucleic acid-based assays detect a specific nucleic acid sequence within the target organism. Several PCR methods have already been validated and standardized by ISO to be used in the industry and in a screening context (Valderrama et al., 2016). There are many nucleic acid-based assay formats for foodborne pathogens but direct hybridization (DNA probe) and nucleic acid amplification techniques as polymerase chain reaction (PCR) are the most popular and have already been developed commercially. The performance characteristics of some representative nucleic acid-based commercial methods for

Salmonella control in food is summarized in Table 2.

Direct hybridization assay use a labeled DNA probe with an oligonucleotide sequence highly complementary to the target sequence of a DNA or RNA molecule present in *Salmonella*, with the intention of using the hybridization phenomenon between them as a mediator to DNA quantification, by correlation of labels or labeled substrates or, sub-products correlation (Lee et al., 2015; Mozola, 2006). The analytical detection technique used depends on the DNA labels characteristics (enzymatic, radioisotope, fluorescence, etc.), but the colorimetric assays are the most common. The simple concept of the probe methods can hide the complexity of the steps necessary to perform before the DNA probe test: lysis of *Salmonella* cells, DNA probes purification, DNA labelling and several washing steps made to reject unbound DNA probes (Lee et al., 2015). A key factor for the success of the hybridization-based tests is the amount of DNA present in the final culture used for detection, which can extend the time of pre-enrichment of the samples depending on their complexity. In fact, the Gene-Track ® from Neogen presents a higher analysis time comparing to the PCR methods presented in Table 2, where the DNA amplification is performed, although it can be an interesting device to make an intensive and detailed analysis of several serotypes or pathogens in the same sample, due its multiplexing detection potential.

The conventional PCR methods are based on the isolation, amplification and quantification of a small portion of DNA genetic material of the bacteria under study. Therefore, have a unique pathogen detection potential (Lazcka et al., 2007; Pestana et al., 2010). The real-time PCR (rt-PCR) has an added value because it amplifies, detects and quantifies the target labeled-DNA sequence in the sample after each PCR cycle in "real-time" exploring the correlation of the label signal intensity with the number or DNA copies (amplicons)(Pestana et al., 2010).

Several commercial kits based on real-time PCR technique are already available in the market for the detection and characterization of foodborne pathogens (Table 2). They are faster than colony counting methods, with LOD typically of 10^4 CFU mL^{-1} and involve analysis time comparable to the ELISA kits (Cheung and Kam, 2012; López-Campos
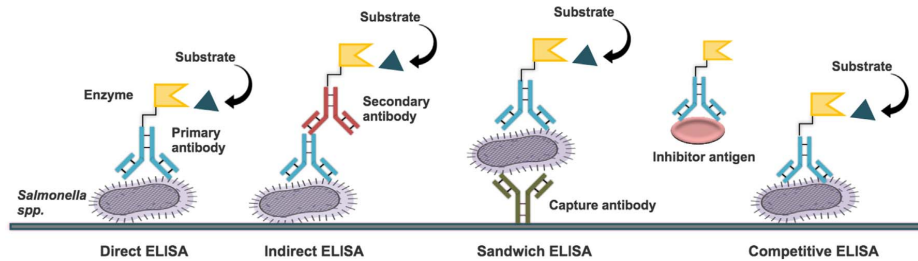
Fig. 2. Schematic representation of the ELISA formats.

et al., 2012). On the other hand, as they are based on DNA detection it is impossible to distinguishing between viable and non-viable cells as in the immunologically-based methods. In a brief analysis it can be clearly observed that rt-PCR kits have been developed in an attempt to eliminate the need or to decrease the time necessary for the preprocessing steps (Wilson and Gifford, 2005), but have not yet achieved the level of sensitivity required for a quantitative method with validation for foodstuffs. Therefore, most rt-PCR kits are used as qualitative methods (López-Campos et al., 2012). Among the rapid nucleic acid-based methods (Table 2), only the TAQMAN and the BAX® System Real time PCR assay for *Salmonella* are capable to give results in less than 24 h. Although, only the TAQMAN achieved to a LOD of 1 CFU per 25 g with the disadvantage that it can only be used for screening purposes and for the detection of only one of *Salmonella* species (*S. enterica*). Other limitations of commercial devices with PCR technology is that normally involve the acquisition of expensive equipments in addition to the detection kits. As output, experimental results are not of simple visual interpretation (fluorescence and absorbance signals) as immunological and need trained specialists to conduct the analysis and treat the data. These requirements limit the possibilities to use nucleic acid-based methods to achieve the lab-on-chip reality or a cheap method to implement in mass scale by the food industries or services.

2.3. Miniaturized culture assays

The miniaturized culture assays come from conventional counting plate methods showing 90–99% accuracy in comparison with those, but with higher sample throughput. This is achieved by the reduction of the plaques to reduced vessels, that need less amounts of reagents and sample volumes, resulting in an economical saving, especially if automatic methods are used (Lee et al., 2015; Lindström and Andersson-Svahn, 2011). These miniaturized tests for *Salmonella* consist mainly in devices containing 15–30 media or substrates selected specifically to identify a target serotype or a *Salmonella* species. The selection of the media is based on the identification of compositional or metabolic intrinsic properties of the target *Salmonella* serotype or of the entire specie. The detection is made using chromogenic reagents in the substrate in which the color change can be directly correlated with the sample colony density, by a simple visual chromogenic evaluation, after an incubation period that normally is in the range of 18–24 h (Feng, 2001). In immunodiffusion techniques, like the 1–2 Test kit from Biocontrol, a positive result is identified visually by the appearance of a track line indicative of immunocomplex conjugation.

Various miniaturized kits for rapid biochemical characterization of *Salmonella* are commercially available (Table 3), including the *Salmonella* Rapid test (Oxoid), S.P.R.I.N.T. *Salmonella* (Oxoid) and *Salmonella* Rapid Test (Unipath), that are all validated by both FDA and HACCP authorities. These tests have a maintained interest in routine lab because they can be used in all food materials, have similar sensitivity to reference methods, can distinguish motile and non-motile *Salmonella* and can be performed in a large-scale sampling cases, still they show a relatively high cost and slow analysis. API 20E (bioMerieux sa.), apply a rt-PCR technique to perform the final detection, achieving an excellent sensitivity (100% true positives) with improved time of analysis, although limiting the test applicability only for pure cultures and the presumed positive results need confirmation tests. The other commercial kits have superior total time analysis, even less than conventional methods, but generally can be applied to all food materials and avoid unnecessary confirmative tests.

2.4. Biosensors

A biosensor is an analytical device able to perform chemical or biological analysis theoretically with no considerable sample pre-processing. A biosensor comprises a bio-receptor integrated with a

signal transducer. The different types of biosensors can be classified according with the mechanism that confers biological specificity, the type of signal transduction or combining both criteria (Thévenot et al., 2001). In the first case, biosensors can be classified by their bio-receptor (which recognizes the target analyte) as bio-catalytic or bio-affinity biosensors. The catalytic biosensors are based on macromolecules that catalyze reactions, in which the biological components can be enzymes (the most widely used), whole cells, particles (microorganisms, bacteria) or portions of animal or vegetal tissues (Thévenot et al., 2001). The bio-affinity biosensors explore the binding events through specific proteins, like membrane receptors, antibodies or their fragments, nucleic acids or related substances with bio-molecular recognition capacity. Biosensors that use antibodies as bio-receptor, are denominated immunosensors (Piro et al., 2016). They use the same concept of the immunoassays and the high specificity of the antibody-antigen complex, with the advantage that normally biosensors are portable, need a reduced sample volume per analysis and have an elevated potential for automation (Afonso, 2012). Transducers have the capability to translate the biological reaction detected by de bio-receptor, in a measurable signal, proportional to the target analyte concentration (Alonso-Lomillo et al., 2010; Sharma and Mutharasan, 2013; Su et al., 2011). Relatively to the transducing methods, most of the biosensors can be classified as electrochemical, optical or piezo-electric/mass sensors (Leonard et al., 2003). Electrochemical transducing seems to be the most promising in terms of autonomy, applicability and output read by the ordinary user, both in screening as quantitative goals.

From all commercial devices analyzed, about 55% allow visual identification of a positive result to *Salmonella,* showing the significance of this design in the food control specific market. Although, the most rapid ones only can be used as screening of contaminated samples and need extra confirmative tests. The remaining are mainly represented by nucleic acid-based methods and enzyme linked fluorescence assays, that contrarious to the methods presented before comprise the quantification of the target at low levels of contamination, but a more laborious interpretation of results and more equipment resources are necessary. In this point of view, the biosensors are the ones capable to comprise high sensibility, real-time analysis and lab-on-a-chip concept, in a friendly physical support capable to give an intuitive user interface and easy interpretation of the results.

Many commercial devices for rapid foodborne pathogen detection were developed recently (Bahadır and Sezgintürk, 2015; Barthelmebs et al., 2010; Law et al., 2015; Lee et al., 2015; Melo et al., 2016; Pashazadeh et al., 2017; Valderrama et al., 2016) but, to the best of our knowledge, the examples of commercial biosensors currently available in the market are limited. The RBD 3000 Micro PRO™ and the Rapid B (Valderrama et al., 2016) biosensors requires flow cytometry equipment and trained personal to perform the analysis and treat the output data. Therefore, the commercially available biosensors for *Salmonella* detection are still far from a simple to use out of lab device. In this context, it is worth to analyze the literature in the field of electrochemical biosensors for *Salmonella* detection to identify the current challenges towards a complete solution for rapid detection of this pathogen in microbial food control.

3. Electrochemical biosensors in *Salmonella* control in food products

Electrochemical transducers stand out because the electroanalytical techniques incorporate essential proper characteristics for biosensors analytical applications such as high versatility, sensitivity, instrumental simplicity and miniaturization potential. The analysis of the literature (Table 5) shows that the best electrochemical biosensors developed for *Salmonella* detection incorporated nanomaterials in the biosensor architecture. These materials are employed in attempt to improve detection limits. As the LOD are just very good, the trend is maintain-

Table 5
Electrochemical biosensors for Salmonella detection.

Serotype	Bioreceptor /Design	Transducer	Nanomaterial /Label	Detected analyte	Detection Technique	Working Range	LOD	Analysis Time	Sample	Refs.
S. entérica (no serotype)	Immunosensor (Sandwich)	SPE	HRP	H_2O_2 TMB	A	5×10^6 to 5×10^8 CFU/mL	2×10^6 CFU/Ml in Buffer	3h	PBS	Delibato et al. (2006)
S. pullorum & S. gallinarum	Immunosensor (Sandwich)	MSPE	rGO HRP	H_2O_2	CV	$10^1 - 10^9$ CFU/mL	1.61×10^1 CFU/mL		Buffer	Wang et al. (2014)
S. typhi	Immunosensor (Sandwich)	GCE	copper-enhanced Au@NP's	Cu^{2+}	ASV	$130 – 2600$ CFU/mL in PBS 260-2600 CFU/mL in human serum	98.9 CFU/mL in PBS	About 6.5h	PBS Human serum	Dungchai et al. (2008)
S. typhi	Immunoassay (Sandwich)	SPCE	Au@NP's, MB's	NA	DPV	$10^3 - 10^6$ cells/mL in PBS 143 cells/mL in PBS	1.5×10^3 cells/mL in milk	1.5h	PBS Milk	Afonso et al. (2013)
S. spp (no serotype)	Immunoassay (Sandwich)	MSPE	CuS NC	Cu^{2+}	SWASV	1×10^3-5×10^5 cells /mL in buffer	400 cells /mL in buffer	About. 63 min	Tris-HCl and Milk	Viswanathan et al. (2012)
S. typhi	Immunoassay (Sandwich)	SPCE	Fe_3O_4 NP's	Fe^{3+}	ASV	10^3-10^8 CFU/mL of bacteria and Antigen	8.18 and 1.51 CFU/ml of bacteria and antigen respectively		Acidic solution	Brainina et al. (2010)
S. pullorum & S. gallinarum	Immunosensor (Sandwich)	SPCE	Au@NP's HRP	Thionine (red)	CV	$10^4 - 10^9$ CFU/mL	3.0×10^3 CFU/ mL in PBS for both species	About 24h	PBS Eggs Chicken meat	(Fei et al. 2015)
S. typhi	Immunosensor (Sandwich)	SPE	Au@NP's CdTe QD	Cd^{2+} Gold Ions	ASV	1 ng - 625 ng of Vi antigen			Buffer Solutions	Pandey et al. (2015)
S. spp (no serotype)	Immunosensor (Sandwich)	m-GEC	HRP	H_2O_2	A	$10 – 10 \times 10^7$ CFU/mL	$5 \times 10^3 / 7.5 \times 10^3$ CFU/ mL in LB / milk LB	About 50 min.	LB Milk	Liébana et al. (2009b)
S. typhi	Immunoassay (Label – free)	Double Walled Electrode	CNT's	NA	CA CFU/mL	$10^2 - 10^7$	8.9 CFU/mL	Total ≈6h	PBS	Punbusayakul et al. (2013)
S. typhi	Immunosensor (Sandwich)	SP-IDME	Glucose oxidase Streptavidin- Biotin	Gluconic acid	I	$10^2 - 10^6$ CFU/mL chicken /pure culture	$1.04 \times 10^3/10^2$ CFU/ml in water	<2 h water	Pure culture Chicken rinse	Xu et al. (2016)
S. spp (no serotype)	Immunosensor (Direct)	GCE	Au@NP's	NA	EIS	$1.0 \times 10^2 - 1.0 \times 10^5$ CFU/mL in PBS	1.0×10^2 CFU/ mL	40 min.	PBS Pork meat	Yang et al. (2009)
S. typhi	Immunosensor (Label – free)	GCE	MSNT's	NA	I	$10^3 – 10^7$ CFU/mL	5×10^2 CFU in PBS	30 min.	PBS	Nguyen et al. (2014)
S. typhi	Immunosensor (Label – free)	GE	NA	NA	EIS		500 CFU/mL	Total of 6 min	PBS	Nandakumar et al. (2008)
S. typhi	Immunoassay (Sandwich)	Cd^{2+} ISE	Fe_3O_4@Au CdS NC	Cd^{2+}	P	$10 - 10^8$ CFU/mL	20 cells / mL in PBS	75 min	PBS Milk	Silva et al. (2015)
S. typhi	Immunoassay (Indirect)	SPE's	Alkaline phosphatase	1-naphthol	A	$2.5 - 25.0 \times 10^{-6}$ M		1h 15min	Human Serum	Rao et al. (2005)
S. typhi	Immunoassay (Sandwich)	SPGE	HRP	H_2O_2	CA	$10 - 10^7$ CFU/mL	20 cells/mL		Buffer solutions	Salam and Tothill (2009)
S. typhi	Immunosensor (Sandwich)	GCE	Au@NP's MWCNT's	NA	I	$10^3 - 10^7$ CFU/mL in PBS PBS/milk	500/1000 CFU/mL in	About 1h	PBS Milk	Dong et al. (2013)
S. typhi	Genosensor (Label – free)	MSPE	Au@NP's methylene blue	NA	DPV	0.01 - 5 nM in PBS	0.05 nM in PBS	About 2h (2 min. for Blood Serum)	PBS Human	Das et al. (2014)
S. typhi	Genosensor	(ITO)GPE	Graphene oxide (GO) methylene blue	NA	CV/DPV	$10 \times 10^{-6} – 50$ nM in PBS 100 fM in serum Samples	10×10^{-6} nM in PBS	Preparation ≈20 h; 60 s for hybridization	PBS Human Blood Serum	Singh et al. (2013)
S. enteritidis	Genosensor	SPCE	Au@NP's, PbS CdS NC	Pb^{2+}, Cd^{2+}	SWASV	50pg/mL – 50ng/mL	0.5 ng/mL	Assay (≈2,5h) 10 min. for detection	PBS	Zhang et al. (2010)
S. typhi (Label – free	Genosensor	GCE	SWCNTs	NA	EIS		1 nM	Total of ≈26h	Phosphate buffer	Weber et al. (2011)
S. typhi	Aptasensor (Label – free)	ISE	SWCNT	NA	P	0.2 to 10^6 CFU/mL	6 /26 CFU / mL in PBS/ apple Juice	60 s after inoculation	PBS Milk Fruit juice	Zelada-Guillén et al. (2013)

(continued on next page)

Table 5 (*continued*)

Serotype	Bioreceptor /Design	Transducer	Nanomaterial /Label	Detected analyte	Detection Technique	Working Range	LOD	Analysis Time	Sample	Refs.
S. typhi	Genosensor	m-GEC	HRP	H_2O_2	A	1 CFU/mL		3.5 h Milk	PBS	Liébana et al. (2009a)
S. typhi	Immunosensor (Label Free)	SPE		EIS		10^3 to 10^8 CFU /mL	10^3 CFU /mL	Total ≈16.5h Analysis: 20 min	PBS Milk	Farka et al. (2016)
S. typhi	Aptasensor (Label Free)	GE			EIS (non-faradic)	10^2 to 10^8 CFU /mL	3 CFU /mL	45 min.	Apple Juice	Sheikhzadeh et al. (2016)
S. spp (no serotype)	Immunosensor (Sandwich)	GE	Magnetic Beads Alkaline phosphatase	L-Ascorbic Acid	A		7.6×10^2 CFU /mL in PBS 6×10^2 CFU /mL agricultural water	3h	PBS Agricultural water	Wang et al. (2016)
S. pullorum	Immunoassay (Sandwich)	4-SPCE	MBeads rGox AuNP' s	AuNP's	DPV	10^2 to 10^6 CFU /mL	89 CFU /mL	80 min. preparation 120 s analysis	PBS Chicken liver	Fei et al. (2016)
S. ATCC 50761	Aptasensor (Label Free)	GCE	rGO MWCNT's		I	75 to 7.5 x10^5 CFU /mL	25 CFU /mL	60 min. Chicken	Physiological saline	Jia et al. (2016)
S. typhi	Immunosensor (Sandwich)	GCE	Au@NP's HRP	H_2O_2	CV EIS	10 to 10^5 CFU /mL	25 CFU /mL	Total 4h Tap water Milk	PBS	Xiang et al. (2015)
S. spp (no serotype)	Genosensor (Label free)	GCE	Nanoporous glassy carbon		DPV EIS	10 to 400 pM in DVP 1 to 400 pM in EIS	2.1 pM in DVP 0.15 pM in EIS	Total ≈4.2h	PBS	Amouzadeh Tabrizi and Shamsipur (2015)
S. typhi	Genosensor	GC			DPV EIS	$10^{-10} - 10^{-15}$ M 1000 copies of *Salmonella* in genomic DNA extracts	0.97 fm in reaction mixture	Total time >56h	DNA extracts	Yan et al. (2016)

Anodic Stripping Voltammetry ASV; Carbon Nanotubes (CN); Chronoamperometry (CA); Differential Pulse Voltammetry (DPV); Glass Carbon Electrode (GCE); horseradish peroxidase(HRP); Impedimetry (I); Indium Tin Oxide Glass Plate Electrode (ITO-GPE); Lysogeny broth (LB); Magnetic Bed's (MB's); magnetic silica nanotubes (MSNTs); magneto-graphite-epoxy composite (m-GEC); Modified Screen Printed Electrode (MSPE); Multi- walled carbon nanotubes; Nanocristal (NC); Phosphate Buffer Solution(PBS); Potentiometry (P);Quantum Dots (QD); reduced Graphene oxide (rGO); Screen-Printed Carbon Electrode (SPCE); Screen-printed gold electrode (SPGE); Screen-Printed Interdigitated Microelectrode (SP-IDME); Single Walled Carbon nanotubes (SWCNT's);Square Wave Anodic Stripping Voltammetry (SWASV); graphite-epoxy composite magneto electrode(m-GEC).
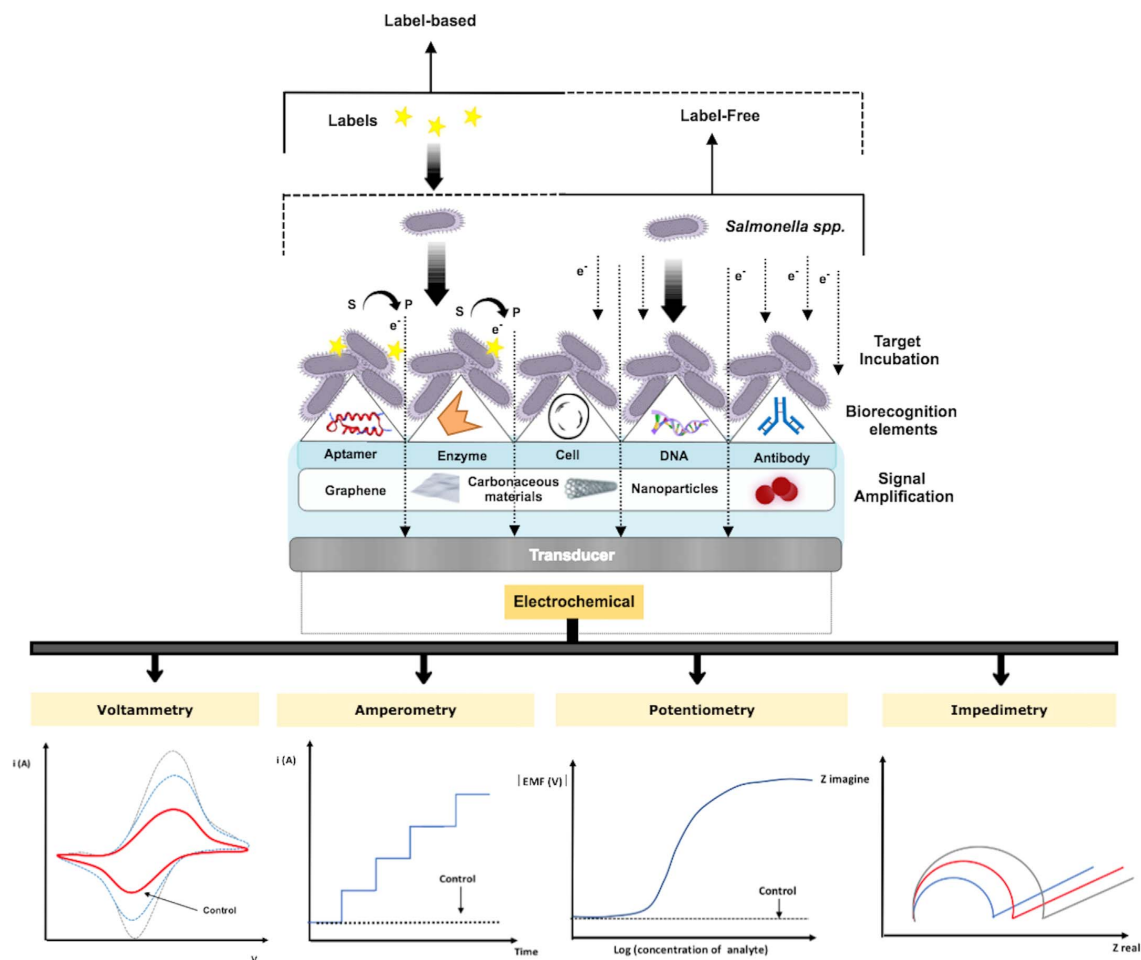
Fig. 3. Schematic representation of typical biosensor elements (transducer, amplification layers, bioreceptor), with different detection modes (label-based or label-free) and electrochemical transducing techniques (voltammetry, amperometry, potentiometry and impedimetry).

ing or increasing the sensitivity already achieved in parallel with the development of label-free biosensors or magneto-capture assays adding value to the biosensors in terms of assay simplicity, pre-enrichment step elimination, sample pre-treatment and selectivity. A schematic representation of different types of electrochemical biosensors is presented in Fig. 3.

3.1. Bio-receptor immobilization and biosensor design

Normally the nanoscale materials are used in biosensors design to enhance sensor characteristics like surface reactivity and electrical conductivity, and in some cases, they also add interesting features like paramagnetic and biocompatible properties. So they can be used to play different roles, as is shown in Fig. 3, for example as like support materials for aptamer, DNA, enzyme or antibody immobilization, or as labels for electrochemical signal amplification(Stephen Inbaraj and Chen, 2016). Nanocrystals are the most frequently used for the last purpose because they easily solubilize to ionic species like heavy metals (ex. Cd^{2+}, Pb^{2+}), that are unusual and residual in target samples of microbial analysis (food, ambient samples) (Pashazadeh et al., 2017; Stephen Inbaraj and Chen, 2016).

Graphene-based composites are the nanomaterials most chosen in electrochemical biosensors to improve the bio-receptor immobilization and for signal amplification. In 2008, graphene was employed for the first time as an electrode material for electrochemical biosensing(Bo et al., 2017). Since then, its use has been modeled per the type of biomolecule to detect in each application area. Although, according to the literature reviews(Bo et al., 2017; Chen et al., 2011; Kuila et al., 2011; Atta et al., 2015; Park et al., 2016; Zhao, 2015), graphene has been seldom explored in biosensors for food safety. There are only a few studies involving graphene-based electrochemical biosensors for *Salmonella* detection (Fei et al., 2016; Jia et al., 2016; Singh et al., 2013). Considering the current trend of electrochemical biosensors, there is a gap in graphene application in *Salmonella* electrochemical sensing, although sensing of this pathogen using carbonaceous materials like carbon nanotubes conjugated with others materials have been reported since 2004 (Dong et al., 2013; Jia et al., 2016; Nguyen et al., 2014; Punbusayakul et al., 2013; Zelada-Guillén et al., 2013). These materials were mainly used in label-free approaches where the amplification was made by the improving the electric conductance of the biosensor or increasing the active surface area for biomolecules immobilization, which in most of the transducing techniques are

proportional to the signal intensity (Table 5*)*. In labeled approaches, the nanomaterials most chosen are magnetic nanoparticles with a ferrite core – used both to concentrate the sample, decreasing the enrichment times and for bio-receptor immobilization (Brandão et al., 2015).

3.2. Label-free electrochemical biosensors

Label-free biosensors use a receptor molecule connected to the biosensor transducer to recognize a specific analyte in a sample. The bio-complex formation is enough to trigger a measurable electrochemical signal correlated in some way with the analyte concentration. These sensors are capable to give a direct and in real-time measurement, with no requirement of labels or intermediaries, making the assay simplest, with less variables to control and resources needs.

Recently this assay format has awakened large attention and there are many just developed label-free biosensors to detect *Salmonella*(Amouzadeh Tabrizi and Shamsipur, 2015; Dhand et al., 2013; Farka et al., 2016; Jia et al., 2016; Nandakumar et al., 2008; Nguyen et al., 2014; Punbusayakul et al., 2013; Sheikhzadeh et al., 2016; Weber et al., 2011; Zelada-Guillén et al., 2013). The most widely used transducing technique in the emerging electrochemical label-free biosensors for detection of bacteria is impedimetry. Using this technique very good results were obtained in complex matrix samples like fruit juice, chicken or milk (Amouzadeh Tabrizi and Shamsipur, 2015; Dhand et al., 2013; Farka et al., 2016; Jia et al., 2016; Nandakumar et al., 2008; Nguyen et al., 2014; Punbusayakul et al., 2013; Sheikhzadeh et al., 2016; Weber et al., 2011; Zelada-Guillén et al., 2013). Recently, Sheikhzadeh et al. (2016), developed an aptasensor capable to detect S. *Typhimurium* selectively in real samples (spiked apple juice) with a limit of quantification (LOQ) of 100 CFU mL^{-1} and a LOD of 3 CFU mL^{-1} in a 45 min assay.

Punbusayakul et al. (2013), developed a double-walled carbon nanotubes (DWCN) electrode to detect *S. typhimurium* in a label-free immunoassay, in which they used chronoamperometry as a transduction technique. They explored the influence of carbon nanotubes architecture in electrochemical signal amplification when they are used as an immunosensor platform. With the conjugation of the nanomaterials and the specificity of antibody anti-*Salmonella* (attached to DWCN), they obtained in about 6 h a very good LOD of 8.9 CFU mL^{-1} in a citrate-phosphate buffer matrix.

3.3. ELISA-based electrochemical biosensors

Most of the electrochemical biosensors for *Salmonella* detection are immunosensors based on lock and key binding event between the antibody (usually fixed at the transducer) and the antigen (that are in the sample) which recently have been specifically reviewed(Kokkinos et al., 2016; Melo et al., 2016; Ricci et al., 2007). Antigens usually are peptides, polysaccharides or lipid molecules. The antigens present in microorganism's surface normally are composed by oligosaccharides associated with lipopolysaccharide and flagellar proteins, which are the biomolecules recognized by bio-receptor of the biosensor. Apart from these, the most investigated are DNA-based and enzymatic biosensors, which are characterized by their bio-recognition elements.

As mentioned, ELISA exist in three principal formats in non-competitive version (antibody reacts proportionally to the amount of analyte), that are currently adapted to electrochemical biosensors: sandwich, indirect and direct.

In the various formats, it can be necessary the separation of the bound immunocomplexes from the solution, in solution or in transducer. These assays are known as heterogeneous immunoassays, and normally magnetic beads or nanoparticles are used to enable the preconcentration or complex separation by simply using a magnet. These procedures normally increase the LOD of the assay or the signal intensity, at same time that reduce the length of the assay.

Sandwich electrochemical immunosensors are the most studied, because they just demonstrated high sensitivity, principally when are developed in simultaneous with new electroactive materials as labels that had reduced costs and greater possibility of reuse compared to the use of enzymatic markers (Piro et al., 2016; Yang et al., 2015). This format give a high efficiency in the antigen capture and at same time allows to use labels in a secondary specific antibody, (but their utilization isn't mandatory). Salam, F. and I.E. Tothill (2009), developed a biosensor for *Salmonella* in sandwich ELISA format, that use a HRP as enzyme and experimented two different antibody immobilization methods on transducer. With a covalent immobilization and amperometry as transducing technique, they achieved to a LOD of 20 cells mL^{-1} in a linear range of 10–10^7 CFU mL^{-1}, by a simple correlation of the enzyme HRP reaction products with the number off cells that are connected with the specific antibody anti *S. typhimurium*.

Wang et al., proposed a voltammetric immunosensor for *S. pullorum*&*S. gallinarum* with the same enzyme (HRP) based on rGO electrochemical properties to enhance the electric conductivity and a polyvinyl alcohol (PVA)-multilayer polydimethylsiloxane (PDMS) that works as a biocompatibility enhancer for de rGO, increasing the sensitivity of the sensor. The modifying layer showed a good linear response range from 10^1–10^9 CFU mL^{-1} and LOD of 1.61×10^1 CFU mL^{-1}(Wang et al., 2014). In fact, due the facility of detection of sub-products of HRP, this is one of the enzymes choose for pure ELISA format (Delibato et al., 2006; Fei et al., 2015; Liébana et al., 2009b; Salam and Tothill, 2009; Xiang et al., 2015b).

The direct and indirect formats are very little used in biosensors for *Salmonella* (Table 5), perhaps due to the possible loss of selectivity and sensitivity of the assay, due the connection of only one antibody to the bacteria. In indirect ELISA biosensors, the antigen is immobilized directly in the transducer and use one primary antibody and a secondary one (conjugated with the primary) allowing the detection of the antigen trough a label. In direct format are used only one antibody as recognition and labeled element. The antigen can be immobilized directly on the assay plate or in the form of a capture assay.

3.4. Electrochemical detection

In biosensors for microbial detection, the most common transducing methods are the optical and electrochemical. The optical detection uses optical signals like chemiluminescence, color or fluorescence to quantify the concentration of the target compound. The electrochemical biosensors measure de current or/and potential changes that occurred in the interface between de working electrode and the sample matrix (Sharma and Mutharasan, 2013). Normally, in microbial biosensors, this signals translate the interaction of the microorganisms with one specific target (Su et al., 2011).

Recently, some excellent works on microbial detection with optical transduction (Cho et al., 2014; Duan et al., 2015; Duan et al., 2016; Kim et al., 2015; Koba et al., 2016; Rios-Corripio et al., 2016; Wu et al., 2014; Zhang et al., 2016), reporting lower or similar detection limits as compared to most of the electrochemical biosensors, aroused in the literature. Still, in our view electrochemical transducers show significant advantages over their optical counterparts, as they allow developing more versatile detection schemes, their miniaturization is simple and allow for real-time quantification. Moreover, if the aim of the developed application is the food industry and the final consumers, then the lower price and minimal electrochemical equipment requirement also makes them more attractive (Wang et al., 2016).

According to the specific transduction technique employed and type of recorded signal, electrochemical biosensors can be classified into other basic groups as: amperometric (current), potentiometric (potential), voltammetric (current and potential), condutimetric and impedimetric (impedance) biosensors (Su et al., 2011).

3.4.1. Amperometric biosensors

Amperometric biosensors operate at a given value of potential difference applied between the working and reference electrodes. This potential difference will trigger an oxidation-reduction reaction on electrode surface involving a metabolic product or an electroactive species in the sample (Luppa et al., 2001) and, consequently, a change in electric current intensity is observed. After current intensity measurement, the values are correlated with concentration of the target analyte (Su et al., 2011).

According to the literature, this type of transduction has been widely explored in biosensors for the detection of proteins but it is not the most widely used transduction technique in microbial biosensors. Liébana et al. (2009b), developed an immunosensor for the detection of *Salmonella* spp. in milk. They used a sandwich format with two polyclonal anti-*Salmonella* antibodies, one was labeled with a HPR enzyme and the other one was used in association with magnetic particles to concentrate and separate the formed immune-complexes from the sample matrix, directly, on a magnetic work sensor. With this methodology they reached to poor limit of detection of 7.5×10^3 CFU mL^{-1} in 1/10 diluted milk, and a fast response time of 50 min per analysis (Liébana et al., 2009b). In another approach, they used the same enzyme but a different bioreceptor, that was DNA instead antibodies. In this work the specificity of the immunological reaction with the specific antibody against *Salmonella* was used only to capture the bacteria and perform their magnetic immunoseparation from skim milk samples resort to use of magnetic beads with no matrix adulteration. Posteriorly to capture the bacteria, the DNA were amplified by PCR techniques insuring a correct serotype identification (Liébana et al., 2009a). With this labeled design and complementary molecular techniques, in 1.5 h, in PBS, they achieved an incredible LOD of 1 CFU mL^{-1} in milk. The authors proved that immunoseparation can substitute the selective culture media in conventional methods, and the genosensing with electrochemical transducing is a good option to reduce the time to obtain confirmative results (Liébana et al., 2009a, b).

In turn, Punbusayakul et al. (2013), created a label-free immunoassay for *S. thypi*, by covalent immobilization of antibodies onto the double walled carbon nanotubes modified electrode, in which chronoamperometry was used as transducing technique. They achieved a very good LOD of 8.9 CFU mL^{-1}, in a linear range from 10^2–10^7 CFU mL^{-1}, in a simple assay structure without labels or sample preconcentration.

3.4.2. Potentiometric biosensors

Potentiometry consist on measuring the potential difference, between a reference electrode and a working electrode with a current level almost zero. There is a lot of types of potentiometric electrodes, but the most commonly used are the Ion Selective Electrodes (ISE). This type of transducers consists of membranes with selective permeability and high affinity to certain ionic species generated or consumed in a target biological process (Eggins, 2002; Leonard et al., 2003; Luppa et al., 2001).

Potentiometric biosensors show some advantages over other electrochemical transducers due their recognized capacity of miniaturization plus that in comparison with voltammetric techniques, the signal isn't dependent on the electrode surface area. The synergic combination of these features with the notable sensitivity and selectivity levels that ISE can achieve, prompts to think that potentiometric biosensors have a high potential in the field of microbial food and environment control (Hassan et al., 2016). Although this type of biosensors is not the most studied, probably because a lot of work is necessary to optimize the experimental conditions to use the biosensor and the reference electrode stabilization, that according to IUPAC rules must be rigorous (± 0,1 mV/min.) poses several challenges specially in miniaturized potentiometric cells.

Despite these limitations, homemade pipette tips electrodes were used in a potentiometric assay, which can detect 20 cells of *Salmonella thyphimurium* in a linear range of $10^1 \times 10^8$ cells mL^{-1}, through a capture sandwich assay format, magnetic sample pre-concentration and CdS nanocrystals as labels (Silva et al., 2015). Zelada-Guillén et. al. (2013), developed one aptasensor to detect *Salmonella* using an ISE and a single walled carbon nanotubes (SWCNT). They achieved to a LOD of 6 CFU mL^{-1} and 26 CFU mL^{-1} in PBS and apple juice, respectively.

3.4.3. Voltammetric biosensors

Voltammetry is a transducing technique where the current is measured in function to the applied potential, and because of that it is the most versatile electrochemical technique. The position of top of the current peak depends on the chemical species or the target analyte and peak current intensity is proportional to their concentration, allowing in this way the simultaneous detection of multiples analytes (Freitas et al., 2014; Luppa et al., 2001; Su et al., 2011). Among electrochemical techniques, voltammetry is the less to prone to noise, it is the most widely used in microbial analysis by biosensors, and was already applied to all types of bioreceptors (Amouzadeh Tabrizi and Shamsipur, 2015; Das et al., 2014; Freitas et al., 2014; Xu et al., 2016).

Freitas et al. (2014), developed a magnetic immunoassay in a sandwich format for *Salmonella typhimurium*, where Fe@Au nanoparticles was used to increase sample pre-concentration efficiency and, CdS nanocrystals to amplify the obtained electrochemical signal by stripping voltammetry. In this approach, it was possible to obtain the results in 1 h (in PBS matrix) with a LOD of 13 cells mL^{-1} and a linear range of 1×10^1-1×10^6 cells mL^{-1}. In a similar approach, Afonso et al. (2013) used a permanent magnet underneath a Screen-Printed Carbon Electrode (SPCE) to do the sample concentration and gold nanoparticles as secondary antibody labels. But in this work the analyze time it is more long (1.3 h), and the LOD higher (143 cells mL^{-1}).

Singh et al. (2013), applied for the first time a GO (Graphene Oxide)-Chitosan (CHI) nano-composite in the design of a DNA based electrochemical biosensor for detection for detection of *Salmonella Thypi*. The modification was based on the enhance electrochemical activity and electrons transferring offered by the GO, the bio-affinity of the CHI, and the specificity of 5-amine labeled single stranded (ss) DNA probe. These characteristics united synergistically originate an extremely sensitive biosensor that can detect 10×10^{-15}M of DNA probe within 60 s hybridization times in a concentration range of 10×10^{-15}M to 50×10^{-9}M, able to successfully distinguish between complementary and non-complementary sequences, even in real samples like human serum (Singh et al., 2013).

In recent work, Fei et al., (2015, 2016) showed clearly the effect of nanomaterials as label in sensitivity of voltammetric immunosensors. In a first work, they used a 4-SPCE modified with an ionic liquid, gold nanoparticles and antibody anti-*Salmonella pullorum* to capture de *Salmonella* from one spiked sample, and before in a sandwich format they incubate the immunocomplex with a secondary antibody labeled with HRP, reaching to a LOD of 3×10^3 CFU mL^{-1}(Fei et al., 2015). With the same biosensor design and electrode, using silica modified immunomagnetic beads for capture and reduced graphene oxide coated with gold nanoparticles instead an enzyme as label, they succeeded at amplifying the electrochemical signal and attained a LOD as low as 89 CFU mL^{-1}(Fei et al., 2016).

3.4.4. Impedimetric biosensors

Impedimmetry is frequently associated with immunosensors. In this technique the changes in an electric field caused by the antibody/ antigen interaction and resultant by the change in the electric conductance or capacitance that happens at the electrode surface or in solution in a constant potential (condutimetry) is detected (Eggins, 2002; Jiang et al., 2008).

This technique is the one of the most used in the microbial electrochemical analysis, because it allows the miniaturization and a

fast response; although even combined with nanomaterials, when it is used in samples with low conductance (Su et al., 2011) it can't achieve the other techniques sensitivity. These implies higher LOD's and in last instance a weaker potential use in real samples, demonstrating that it will need more optimizations in future. Despite of these general considerations,(Yang et al., 2009) developed an immunosensor with high sensitivity towards for *Salmonella spp*, based on grafted ethylene diamine and self-assembled gold nanoparticle monolayer. The developed biosensor showed a good LOD of 100 CFU mL^{-1} in a complex matrix (pork meat), in a 40 min analysis time, with a simple and direct detection methodology based in conductance changes on the immunosensor surface, probing that is too a capable technique for pathogens screening, although more complex and laborious than the others electrochemical techniques.

Yan et al. (2016) proposed the coupling of a homogeneous target-initiated transcription amplification (HTITA) method directly into sensing interface without resorting to nanomaterials. Although the simplification of the procedures and resources needs, important towards point-of-care screening, the biosensor developed don't show be appropriate in real contamination scenarios due the long time needed to achieved to results, still its good LOD reached of 9.7×10^{-16} M.

3.5. Biosensors with developed food application

To compare the different electrochemical transducers used in the literature on *Salmonella spp* analysis some application in foodstuffs were selected (Table 6) although the complexity of the samples wasn't taken into account. The critical analysis was based on increasing complexity of the biosensor design where the simplest assay is one that doesn't require labels and allows a direct measure, followed by assays that require labels but are made through a direct measure and the most complex design involves using labels and the detection is based on indirect measures. Other parameters considered in the analysis were the LOD; the time spent to perform the analysis and their potential application in real samples considering the EC legislated limits. Taking into account these marks the study by (Delibato et al., 2006) is the worst classified because has a LOD that is far from zero *Salmonella* spp. CFU's (presence or absence). Among the methods (Table 6) the study with best evaluation is terms of LOD is from (Liébana et al., 2009a) and it is the only one with potential for application in real samples because its LOD of 1 CFU/25 g covers the imposed limits in EU regulations for food products. Although the analysis time was increased in 6 h (pre-enrichment) and complementary molecular techniques were used to achieve this detection limit in skimmed milk (dilution factor 1/10).

Beyond this finding almost two thirds of the works can detect infection contamination levels of *Salmonella spp*. Among this group, the study by Sheikhzadeh et al. (2016), stands out because the results can be achieved in less than 1 h and it combines the simplicity of conception with an excellent LOD (3 CFU mL^{-1}) and adequate % recoveries obtained in food samples.

This analysis shows that the label-free biosensors, beyond simplifying the procedures of detection, have a high potential for application in real samples, due to their sensitivity and reproducibility. Although an equilibrium between the endeavor to reduce the time of analysis and to improve the simplicity of the assay is a key issue.

4. Conclusions and prospects

Many scientific studies on biosensors for *Salmonella* detection are still being carried out, emphasizing the importance of its accurate and rapid detection in foodstuffs, which is reinforced by the recent alarming statistics. At the same time, many commercial rapid methods are just available. In this paper, was presented a perspective in which were highlight the development stage and relative value for food industry of both. Most of the commercial rapid methods derive from technologies already in use in the biochemistry or microbiological labs. The development of methods in microplates allowed performing many tests almost simultaneously whereas most biosensors pose challenges at the base sensors development and most of them incorporate the schemes and knowledge from bioassays.

Sample preparation, enrichment and selection are critical steps in the performance of all detection methods, including the electrochemical biosensors. The ideal methods shall be as simple as possible, rapid, low cost and with minimal sample preparation requirements, applicable to all foodstuffs and materials, especially where the rapid method is developed in a lab-on-chip concept or for out-of-lab usage. Additionally, a correct and specific separation of all *Salmonella* target cells from the samples matrix is a crucial step to improve the sensibility and specificity of methods based on electrochemical detection due to the elimination of inhibitory substances, microflora or physical interferences from the analytical detection.

It is difficult to compare the sensitivity and specificity of different electrochemical biosensors and bioassays in the literature because some of them were only applied on ideal optimized conditions (phosphate buffers), and others were applied to different types of food samples which follow different steps of sample preparation. The validation of the methods sensibility should attend to its detection limit, but also to the probably of heterogeneous distribution of *Salmonella* in foodstuffs when this pathogen is present at low levels. Consequently, the methods used for sampling and the stress and alterations suffered by the bacteria during sample processing

Table 6

Comparison of developed electrochemical methods in the literature with food application.

Serotype	Simplicity	LOD	Sample	Time	in Food Samples	Refs.
S. spp (no serotype)	+	1 CFU/mL in milk	Milk	+++	Yes	Liébana et al. (2009a)
S. typhimurium	+++	3 CFU/mL	Apple juice	+	infection leve	Sheikhzadeh et al. (2016)
S. typhimurium	+	13 cells/mL	PBS	+	infection level	Freitas et al. (2014)
S. typhimurium	+	20 cells/mL	PBS/Milk	++	infection level	Silva et al. (2015)
S. typhimurium	+	25 CFU/mL	Tap water	+++	infection level	Xiang et al. (2015)
S. typhimurium	++	26 CFU/mL 1	Apple juice	+	infection level	Zelada-Guillén et al. (2013)
S. pullorum	+	89 CFU/mL	PBS Chicken liver	++	infection level	Fei et al. (2016)
S. typhimurium	++	1.5×10^3 and 143 cells/mL	PBS Milk	++	infection level	Afonso et al. (2013)
S. spp (no serotype)	++	1.0×10^2 CFU/mL	PBS pork meet	+	infection level	Yang et al. (2009)
S. spp (no serotype)	+	4×10^2 cells/mL	Buffer	++	infection level	Viswanathan et al. (2012)
S. typhimurium	+++	10^3 CFU/mL	PBS /milk	+++	No	Farka et al. (2016)
S. typhimurium	+++	10^3 CFU/mL	Milk	+	No	Dong et al. (2013)
S. typhimurium	+	$1.04 \times 10^{3\backslash}$ CFU/mL	Chicken	++	No	Xu et al. (2016)
S. pullorum and *S. gallinarum*	+	3.0×10^3 CFU/mL PBS for both species		+++	No	Fei et al. (2015)
S. spp (no serotype)	+	7.5×10^3	Skim milk (1/10)	+	No	Liébana et al. (2009b)
S. enterica	+++	5×10^7 CFU/mL		+++	No	Delibato et al. (2006)

Simplicity: +++ no labels and direct measure; ++ label, direct measure; +labels, indirect measure; Analysis time: + ≤ 1 h; ++ ≤ 2 h; +++ > 2 h.

are important parameters to optimize but these issues have been poorly explored in the literature on rapid methods. Therefore, besides ensuring that the analyzed sample is representative of the complete foodstuff it is also necessary to consider the level of pathogenicity for humans according salmonella cells viability. The DNA-based and immnunosensors are unable to discriminate among living or death *Salmonella* cells. This is a key point as bacterial cell pathogenicity is related to their grown potential in foodstuffs and in humans after their ingestion.

Very good results have been accomplished with immnunomagnetic and phage-based separation techniques in both commercial devices and academic works. They are highly effective in accurate selection of the target bacteria and in reducing the time needed for sample preparation, due the elimination of conventional enrichment steps, thus allowing next day results for a better food emergence response preventing spreading of microbial contamination. Their effectiveness is more visible in biosensors that incorporate simultaneously a very sensitive and selective bioreceptor platform, for example antibodies or DNA-based nanomaterials, and a simple and direct transduction technique such as electrochemical techniques. The simplicity and cost efficiency of the biosensors can also be increased, with apparent no loss of accuracy, using label-free electrochemical biosensors.

Despite of the great advances in the technologies and in line with previous studies in the literature, none of the current rapid detection methods for *Salmonella,* both the commercially available or the ones yet at a development stage, do not meet all the requirements for food application, considering the regulation limit. Therefore, further improvements in terms of validation parameters, time of analysis, portability and autonomy are necessary. Furthermore, electrochemical biosensors seem to be the most acceptable and reliable technology to achieve the regulation requirements and overcome industrial implementation barriers, because it is possible to achieve a lab-on-chip device, with the desired analytical properties and adapted to specific industrial needs, at a potentially low cost.

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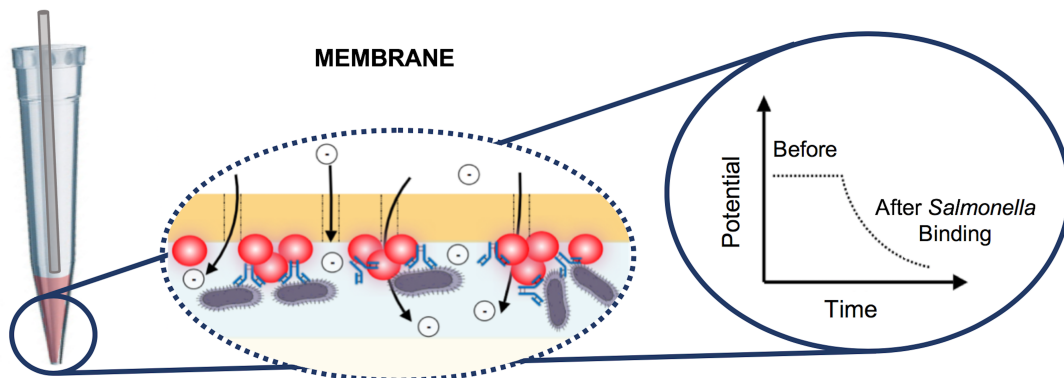
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2.2. In situ formation of gold nanoparticles in polymer inclusion membrane: Application as platform in a label-free potentiometric immunosensor for *Salmonella typhimurium* detection

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Statement of contribution

The contribution of the candidate, Nádia F.D. Silva, in this work includes the literature review, investigation and written of the original draft.

In situ formation of gold nanoparticles in polymer inclusion membrane: Application as platform in a label-free potentiometric immunosensor for Salmonella typhimurium detection

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ARTICLE INFO

ABSTRACT

Polymeric ion selective electrodes are highly sensitive to changes in zero current ion flow and this offers a route to signal amplification in label-free potentiometric immunosensors. In this work, a label-free potentiometric immunosensor toward Salmonella typhimurium (ST) assembled in a home-made pipette-tip electrode is described. The signal-output amplification was implemented on a gold nanoparticle polymer inclusion membrane (AuNPs-PIM) which was used as sensing platform and for antibody immobilization. Additionally, a marker ion was used to detect the antibody-antigen binding event at the electrode surface. The immunosensor construction was performed in several steps: i) gold salt ions extraction in PVC membrane; ii) AuNPs formation using Na_2EDTA as reduction agent; iii) antibody anti-*Salmonella* conjugation on AuNPs-PIM in pipette-tip electrodes. The potential shift observed in potentiometric measurements was derived simply from the blocking effect in the ionic flux caused by antigen-antibody conjugation, without no extra steps, mimetizing the ion-channel sensors. A detection limit of 6 cells mL^{-1} was attained. As proof-of-concept, recovery studies were performed in spiked commercial apple juice samples with success. Due to the simplicity of use, the appealing cost of equipment and sensor production and being able to provide a quick analytical response (less than 1 h for a complete assay, including sample preparation for analysis), this scheme represents a good prototype device for the detection of foodborne pathogens like ST or other immune-responsive bacteria.

1. Introduction

Salmonella is a Gram-negative bacterium from Enterobacteriaceae family and is one of the most important foodborne pathogens that affects human health. Normally, the infection is acquired by ingestion of contaminated food and water [1–3] with rapid dissemination. Several rules and legislation have been applied in most of the developed countries to control the infection prevalence, although the number of cases in some of them is still in a worrying level at a public health scale [4].

The standard methods for S. spp. detection are based on colony counting plate methods which have high sensitivity, but only can give results in 3–7 days after analysis. Other methods, such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) commercial kits, have been used in food safety and can reduce the time to obtain definitive results to less than 24 h. Although, to achieve the sensitivity of conventional methods, long pre-concentration and enrichment steps are frequently necessary [5]. Furthermore, some of the commercial rapid methods also require expensive equipment, advanced knowledge and laboratory expertise to conduct the analysis and treat the data. Therefore, simple, rapid and reliable methods for *Salmonella* control out-of-lab are still necessary in aid to ensure food safety.

In past decades, electrochemical biosensors have drawn increased attention, due to their capability to perform chemical or biological analysis with no considerable sample processing and using simple procedures in a user-friendly interface [6,7]. Additionally, electrochemical transducing techniques enable out-of-lab analysis with high sensitivity. Among electroanalytical techniques, potentiometry with ion selective electrodes (ISE) has recognized merits in the detection of small ionic analytes at low cost and using simple instrumentation [8,9].

However, for the quantification of large biological species it frequently resorts to nanomaterials to enable and/or to amplify the signal readout, increasing the analysis time and the complexity of the assay [10]. Label-free potentiometric biosensors for bacteria detection are appealing [11,12] but it is necessary to work at low ionic strength because, charged species in the samples may lead to a potentiometric response producing false positive results. Furthermore, these biosensors frequently present a low signal to noise ratio, that makes it impossible to detect very low concentration levels in samples with complex matrix composition as desired in food safety issues [8]. On the other hand, polymeric ion selective electrodes are highly sensitive to changes in zero current ion fluxes across the sensor membrane [13,14]. This characteristic combined with highly specific recognition reactions at the sensor surface has been explored in a few potentiometric sensors based on the blocking surface principle [8,15,16] and offers a route to signal amplification in label-free potentiometric biosensors which has seldom been explored [17,18].

In this work, it is reported the development of a biocompatible platform to be applied as interface in a biosensor device, capable to detect directly antibody-antigen interactions based only on blocking surface principle. The proposed strategy schematized in Fig. 1 integrates an immunosensing interface (IMS), constituted by the bio-receptor immobilized on gold nanoparticles (AuNPs), in a miniaturized ISE responsive to a steady-state concentration of a selected marker ion (not redox active) the sensing membrane vicinity. The application of this label-free potentiometric sensing principle aims to achieve to amplification capabilities close to labelled-based approaches pursuing their sensitivity more simply.

The AuNPs were selected to assemble the IMS due to the well-known biocompatibility, electrical conducting properties and large surface area [19], which are key factors in label-free designs. Additionally, the possibility of an oriented immobilization of antibodies is also an interesting feature for the development of this immunosensor [20]. The transducer in the proposed biosensor is a ISE with a polymer inclusion membrane (PIM) [21] which is also the support for IMS assembling with the incorporation of AuNPs formed in situ. The basic concept of using PIMs to synthetize metallic nanoparticles monolayers, with a low spent of solvents, is supported in two main steps: the extraction of the interesting ion from a solvent trough a counter ion reaction - mediated by a ion selective membrane - and a reduction step based simply on adding a reducing agent to react with the extracted metal ion or complex [22,23]. The ease of AuNPs synthesis and functionalization and the possibility to control particle size and membrane coverage simply changing the extraction and reduction conditions makes this procedure a versatile approach for IMS assembling and optimization. Additionally, enhanced stability is usually observed for in situ formed nanoparticles [24–26]

Taking this innovative IMS platform, the sensor working mechanism was optimized starting from a zero-current outward flux of marker ion established from the backside of the modified ISE membrane to the sample solution (Fig. 1). After a steady-state condition is reached, the antibody-antigen affinity reaction that occurs at IMS surface will partially block the ion mass transfer to the bulk solution. Upon, this flux is retarded, and the increasing marker ion concentration in aqueous layer unleash a change in the measured electromotive force (EMF) *vs*. an Ag/AgCl reference electrode. This effect was accessed over stir studies, during step-by-step IMS construction.

This innovative procedure was successfully tested as a biosensing platform in a pseudo label-free potentiometric immunosensor for *Salmonella typhimurium* (ST) detection and the analytical performance of several support membrane configurations was evaluated. The IMS were characterized by Scanning Electron Microscopy (SEM), Fourier-Transform Infrared Spectroscopy-Attenuated Total Reflection (FTIR-ATR) and its architecture was confirmed with Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS). As proof-of-concept, recovery studies were performed in spiked commercial apple juice samples.

2. Experimental

2.1. Chemicals and solutions

S. typhimurium positive control, containing 5×10^9 cell mL^{-1}, was obtained from Kirkegaard & Perry Laboratories. Anti-*Salmonella* monoclonal antibodies (Ab), specific for *S. typhimurium* [27,28], were purchased from Santa Cruz Biotechnology.

2-nitrophenyl octyl ether (NPOE), di(n-octyl) phthalate (DOP), methyltrioctylammonium chloride (TOMA), bovine serum albumin (BSA), sodium dihydrogenphosphate monohydrate, potassium dihydrogen phosphate, hydrochloric acid, sodium tetraphenylborate (TPB) and Na$_2$EDTA were from Sigma Aldrich. Polyvinyl chloride (PVC) was purchased from Fluka and hydrogen tetrachloroaurate (III) trihydrate 99.9% was obtained from Alfa Aesar. All other reagents were pro-analysis quality (pa) or equivalent and were used as received.

The sensor cocktails were prepared by mixing the ion-exchanger (TOMA, 1 wt%), the plasticizer (DOP or NPOE, 66 wt%) and PVC (33 wt%) dissolved in tetrahydrofuran (THF), that after dried forming the polymer inclusion membrane (PIM) [22].

Phosphate buffered saline (PBS, 0.1 mol L^{-1} phosphate buffer, pH 7.4) was prepared with Na$_2$HPO$_4$ and KH$_2$PO$_4$ with or without 25 mmol L^{-1} NaCl. A pH 7.0 phosphate buffer solution (PB) was also prepared adjusting the pH of a 0.01 mol L^{-1} NaH$_2$PO$_4$ with 1 mol L^{-1} NaOH.
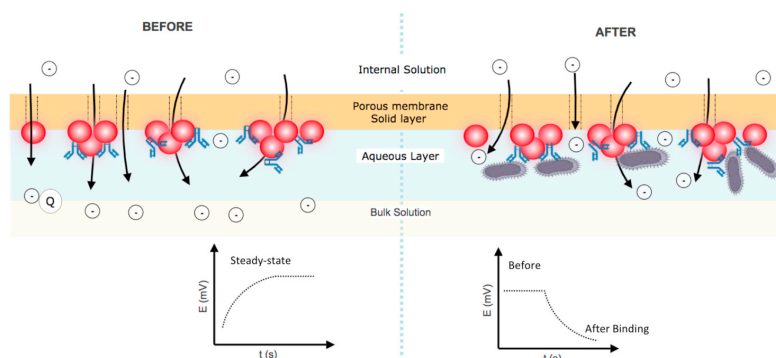
Fig. 1. Schematic representation of surface blocking effect detection mechanism in the developed immunosensing interface.

2.2. Fabrication of the immunosensor

The PVC membrane electrodes ($\varnothing$ 1.5 mm) were constructed in 5000 μL pipette tips, cut to a final height c.a. 45 mm, and cleaned by dipping twice in THF. After solvent removal, they were immersed in the sensor cocktail, which due to intrinsic capillarity filled the tips until approximately the same height ($\approx$ 5 mm). The membranes were obtained upon THF evaporation at room temperature, shielded from light in a laboratory closet (SI Fig, S1.1).

The AuNPs-PIM formation procedure was adapted from [22,23,29] and optimized to develop a biocompatible platform. The extraction of Au(III) to the just dried PVC membrane was performed in 5 mL eppendorf tubes containing 2.5 mL of $HAuCl_4 \cdot 3H_2O$ 100 mg L^{-1} in a 2.5 mol L^{-1} HCl solution. During the extraction step, the electrodes were shaken on an orbital mixer at 150 rpm under controlled temperature (at 25 °C). Then the electrodes were immersed in 2.5 mL of 0.1 mol L^{-1} EDTA with pH adjusted to 6, 7, or 8, and allowed to reduce the extracted Au(III) under the same previous extraction conditions during 24 h (SI Fig. S1.2). Between gold complex extraction and reduction steps, the electrodes were washed twice with distilled water (SI Fig. S1.2). All glassware used in the procedures was cleaned with aqua-regia and rinsed three times in ultrapure water.

Antibody assembling on AuNPs-PIM was performed by drop-casting 10 μL of antibody solution (diluted 1/100 in PBS with 2.5 mmM NaCl) directly to the washed and dried AuNPs-PIM electrode surface. It was left in contact in a humid atmosphere overnight at 4 °C. After, the obtained immunosensors (Ab/AuNPs-PIM) were rinsed with PBS and water to remove the not immobilized antibodies and the sensors were stored at 4 °C in a humid atmosphere until further use. After antibody immobilization some biosensors were also treated with 10 μL of BSA solution (10 mg mL^{-1}; 1 h) (SI Fig. S7.1).

Finally, the electrodes were filled with a TPB solution (10 mg mL^{-1} in PB) and placed in a moist atmosphere at 4 °C during the conditioning treatment and when they were not in use. For the potentiometric measurements, homemade Ag/AgCl electrodes [30] were used as inner reference in the pipette tip biosensors. A schematic representation of the procedure was detailed in Supporting information (SI Fig. S1.1).

The optimization of sensor and internal solution composition to achieve a reliable and reproducible passive ion flow is described in the Supplementary material S2.

2.3. Immunosensing interface characterization

2.3.1. Scanning electron microscopy

SEM studies were carried out at CEMUP (Centro de Materiais da Universidade do Porto), Porto, Portugal. SEM images were obtained with a scanning electron microscope Quanta 400 FEG scanning electron microscope, (SEM, FEI, Hillsboro, OR), operated in high vacuum/secondary electron imaging mode using an accelerating voltage of 15 kV and working distances between 10.3 and 12.4 mm. The AuNPs-PIM films were assembled on aluminum stubs covered with carbon adhesive tabs (Electron Microscopy Sciences, Hatfield, PA). The AuNPs penetration into the PIM was analyzed using fracture images, surface images at both sides of the PIM and lateral secondary electron detector (ETD) configured to backscattered electrons (without bias voltage applied) to give a shadow effect over the polymer surface. ImageJ software was used to study the size distribution and surface morphology of AuNPs-PIM.

2.3.2. Cyclic voltammetry and faradaic impedance measurements

Cyclic voltammetry (CV) measurements were performed using an electrochemical system (PGSTAT12, Metrohm Autolab) and the potential was swept between − 0.2 and 0.6 V at different scan rates (5–100 mV/s) in PBS solution without NaCl (pH 7.4) using [Fe (CN)$_6$]$^{4-/3-}$ (1 mmol L^{-1} in 0.1 mol L^{-1} KNO$_3$) as redox marker.

Electrochemical impedance spectroscopy (EIS) experiments were performed with an Autolab Electrochemical Analyzer (PGSTAT128N, Metrohm). All tests were conducted at an open circuit, recorded for 75 data points, at a single modulated AC potential of + 0.375 V with frequency ranging between 10 mHz and 100 kHz.

The General Purpose Electrochemical System (GPES, version 4.9) software from Metrohm was used to control the system and process the CVs. To obtain and process the EIS spectra the NOVA (version 1.7) software also from Metrohm Autolab was used.

All these experiments were made in a one-compartment three-electrode cell system comprising a bare or modified glassy carbon (GCE, $\varnothing$ 3 mm) working electrode, an Ag/AgCl (KCl 3 mol L^{-1}) reference electrode and a platinum wire counter electrode, all from Metrohm.

2.4. Potentiometric measurements towards Salmonella typhimurium

The EMF was measured with an EMF16 Interface from Lawsons Labs and the potentiometric cell was in a faraday cage. A double junction reference electrode (Orion 90-02-00) from Thermo Scientific Orion and a magnetic micro-stirrer from Velp Scientifica were used. All measurements were made in PB buffer, stirred at 150 rpm and at room temperature.

The potentiometric response of the immunosensor towards ST was based on the change of EMF before and after antigen–antibody reaction [18]. Control and real sample evaluation were performed subtracting the steady-state EMF value measured in a blank solution (PB, pH 7) to the EMF value in the presence of a certain amount of ST cells or non-spiked solutions (positive control or negative controls). The calibration curves were obtained by standard addition method, and the EMF was calculated subtracting the steady-state value of EMF in PB to the values registered after each addition and presented as average responses associated to the respective standard deviation of three different intra-assay replicas.

Fig. 1 is a schematic representation of the mechanism of detection of ST were the shift of EMF is originated by the resistance to mass transport of the marker ion provoked by the immunobinding on the membrane surface.

2.5. Analysis of apple juice samples

As a proof of concept artificially contaminated apple juice samples, purchased in a local supermarket, were examined with the developed biosensor. The samples were diluted 1:10 in water and then spiked with different amounts of ST cells, to achieve to a final concentration in the range from 0 (no-spiked sample) to 100 cells mL^{-1}.

A pretreatment protocol, based on simple filtration and elution steps was taken [11]. Briefly, a sterilizing syringe filter (0.2 μm pore size) was used to pull and separate the ST cells from the juice matrix. Then the filter was turned down-side and a controlled volume of PB was injected to elute the cells retained in the filter. Finally, potentiometric detection of the eluate was taken.

3. Results and discussion

3.1. Characterization AuNPs-PIM platform

The possibility of assembling a stable AuNPs layer on the PIM membrane is a necessary condition for biosensor development. A characteristic of the selected method for in situ AuNPs synthesis is the possibility to control the shape, size, location (within or on the membrane surface) and the extent of membrane coverage simply changing the experimental conditions for AuNP synthesis. Different reducing agents have been used for AuNPs preparation [23,31] but in this work it was used Na$_2$EDTA because it gave rise to membrane surface coverage by the newly formed nanoparticle [29]. As Na$_2$EDTA oxidation depends on the presence of water, PVC was chosen as polymeric matrix because it is not hydrated and due the polar characteristics of its C–Cl functional
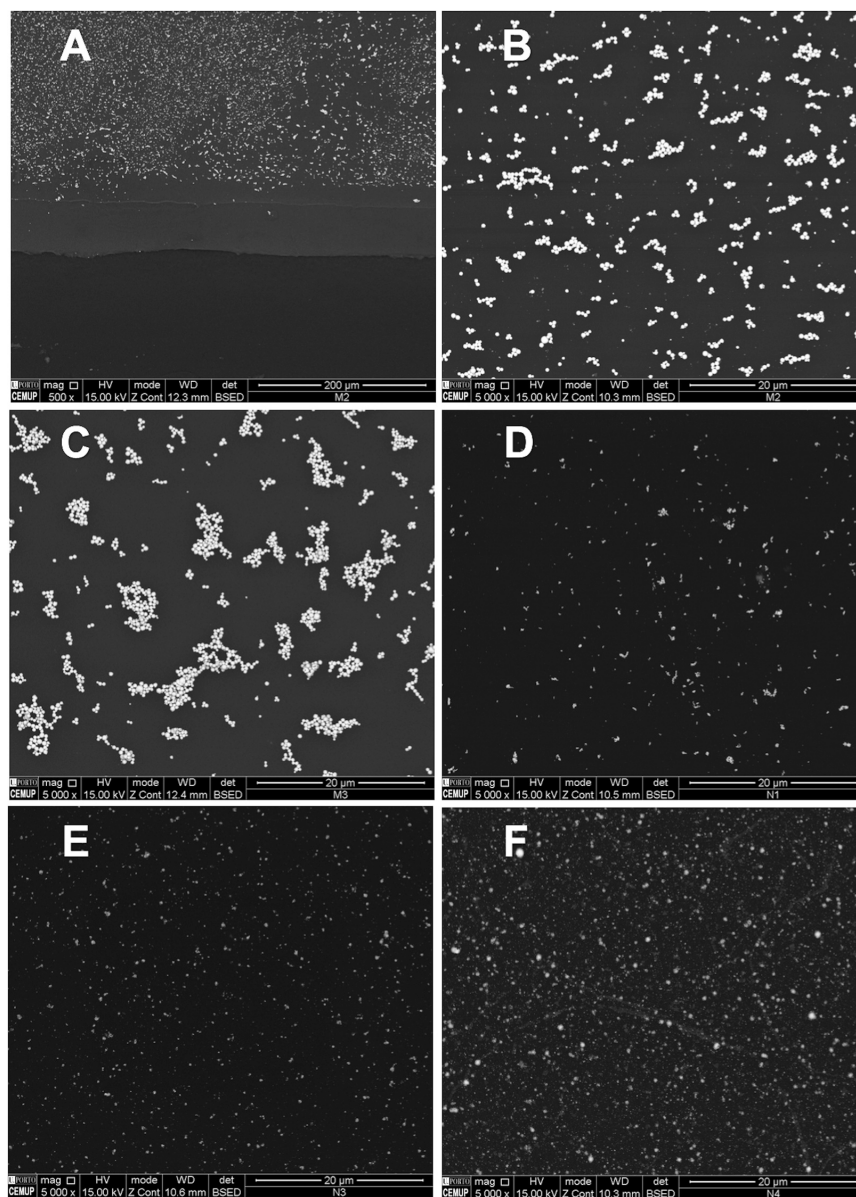
Fig. 2. SEM images of gold nanoparticles formed on polymer inclusion membranes (AuNPs-PIM) with different protocols using 0.1 mol L^{-1} Na$_2$EDTA for Au(III) reduction:(A and B) cross section and surface of a PIM membrane with AuNPs obtained with 1 h extraction of tetrachloroaurate(III) and 30 h of reduction at pH 6; (B) magnification of image A; (C) 2 h of extraction and 30 h reduction at pH 6. (D, E and F) Surface images of AuNPs-PIM for 1 h of extraction and 24 h of reduction at pH 6 (D), pH 7 (E); and pH 8 (F).

group [22]. The cross-section image in Fig. 2 A shows that using Na$_2$EDTA as the reducing reagent the AuNPs were formed on the PIM surface.

The effect of extraction and reduction time as well as the composition (pH and concentration) of the Na$_2$EDTA solution on the morphology, topology and size of the formed AuNPs were also assessed. The images in Fig. 2 show the effect of experimental conditions on the quantity and quality of the AuNPs produced. Regarding extraction (Figs. 2, 1 h (B) and 2 h (C)) or reduction time (Fig. 2, 24 h (D) to 30 h

(B)) it was observed that as the reaction time increases, the amount of grown spherical AuNPs on PIM membrane also increases. A similar effect was observed with increasing Na$_2$EDTA concentrations (0.1 and 0.2 mol L^{-1}) at pH 6 (Fig. 2 and SI Fig. S3.1).

Employing short reduction time periods (1.5 h and 3 h), AuNPs were not formed (data not shown). From SEM images in Fig. 2(D, E and F) it can be observed that the best AuNPs coverage was reached using 0.1 mol L^{-1} Na$_2$EDTA solutions at pH 8 (using an extraction and reduction time of 1 h and 24 h respectively). Although the AuNPs
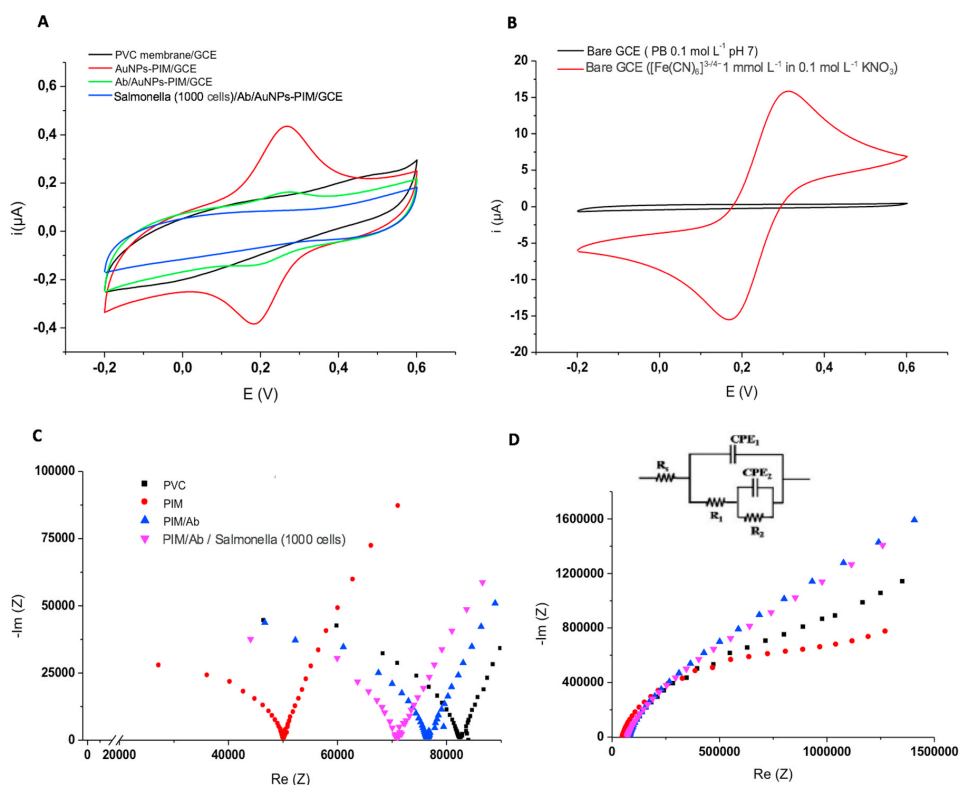
Fig. 3. (A) Cyclic voltammograms of different modified electrodes and; (B) bare electrode in PBS (0.1 mol L^{-1} pH 7.4, without NaCl) and in 1 mmol L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ solution probe with KNO$_3$ 0.1 mol L^{-1} as support electrolyte. (C and D) Nyquist plots of EIS at high (C) and low frequencies (D): PVC membrane/GCE (black square); AuNPs polymer inclusion membrane PIM/GCE (red circle); Antibody anti-Salmonella loaded into AuNPs-PIM (Ab/PIM/GCE) (blue triangle) and; after incubation of Ab/PIM/GCE with Salmonella (1000 cells) (pink triangle); all in 0.1 mol L^{-1} KNO$_3$ containing 2.5 mmol L^{-1} of [Fe(CN)$_6$]$^{3-/4-}$ probe; electric circuit representative of the developed IMS into a GCE electrode (inset in the figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presented as clusters of closely packed particles having a highly irregular shape comparing to pH's 6 and 7.

Particle size was also evaluated processing the SEM images with ImageJ software and the corresponding histograms (SI Fig. S3.2) showed that with Na$_2$EDTA 0.1 mol L^{-1} at pH 6 the obtained AuNPs size were 229 $\pm$ 87 nm and 357 $\pm$ 89 nm for 24 h and 30 h reduction time respectively. These particle sizes are clearly beyond the nanoscale materials, with properties closer to metallic gold. Increasing the concentration of Na$_2$EDTA to 0.2 mol L^{-1} the particles diameter diminished considerably to 41 $\pm$ 14 nm of diameter but increasing polydispersity was also observed. The increase of pH of the reduction reaction also produced nanoscale particles with diameters of 44 $\pm$ 13 nm and 28.5 $\pm$ 8 nm at pH 7 and 8 respectively.

3.2. Optimization of IMS

The response towards *Salmonella* was evaluated to assess the effect of different IMS configurations with the AuNP monolayer on the sensor surface. The experimental results show that the amplitude of the immunosensor response to ST decreased (SI Fig S4.1A) as extraction time of Au(III) to the PVC membrane increased (0.5–3 h), suggesting that increasing this experimental parameter produced a higher amount of AuNPs and a more effective barrier to the marker ion diffusion from the membrane to the bulk solution (Fig. 2B and D).

A reduction time of 24 h was already assumed by other authors as optimal to obtain good surface coverage by AuNPs [29]. Indeed, no improvement in potentiometric response was observed when longer reduction times were employed. Although an amplification of the signal was observed but the reproducibility between electrodes response was poor (SI Fig. S4.1B). Correlating these results with the SEM images an increase in the number of AuNPs in large agglomerates (Fig. 2C and D) and an increase in average AuNPs diameter (SI Fig. S3.2) and polydispersity was observed upon increasing reduction time.

Similarly, in the potentiometric study of the effect of the Na$_2$EDTA solution pH, the electrodes subjected to a reduction step at pH 7 showed the best performance as compared with similar sensors involving the Au (III) reduction at pH 6 or 8 (SI Fig. S4.2).

Based on these observations we can assume that the presence of irregular and agglomerated distribution of AuNPs were decisive in the effectiveness degree of antibody load and consequently antigen conjugation.

Considering the antibody size [32], usually 10–15 nm, an AuNPs size of 52 $\pm$ 14 nm was chosen to reduce the immobilized antibody steric hindrance and AuNPs aggregation. This optimum particle size corresponded to extraction during 1.5 h and reduction with 0.1 mol L^{-1} Na$_2$EDTA at pH 7 for 24 h < Furthermore, particles prepared under these conditions showed moderate polydispersity and good coverage of the polymeric membrane (c and d in SI Fig. S3.1).

After antibody immobilization some electrodes were treated with BSA. The signal of the BSA modified electrodes was amplified at least four times, although the response relative standard deviation between electrodes was worse (SI Fig. S7.1). The oriented conjugation of BSA and its negative charge contribution to membrane potential at neutral pH can explain the signal amplification. Therefore, its presence also can hide some antibody active spots and increase the steric hindrance between them [33]. Because of the duality of the effect of the BSA, this modification step was excluded in further experiments, considering the pretreatment protocol used (see Section 2.5), which excludes in advance the presence of common interferers, and consequently non-specific interactions with AuNPs.

3.3. IMS characterization

3.3.1. Cyclic voltammetry

An electrochemical study using the $[Fe(CN)_6]^{3-/4-}$ redox couple as molecular probe was performed to observe and characterize the surface changes of different layers immobilized onto GCE. Fig. 3(A and B) shows CVs of $[Fe(CN)_6]^{3-/4-}$ after each assembly step. As it is possible to verify, when it was used a bare GCE a redox peak was observed whose anodic peak potential (E_{pa}) was $+0.32\,V$ with a peak intensity of $15.83\,\mu A$ and a cathodic peak potential (E_{pc}) at $+0.17\,V$ with a peak intensity of $-15.5\,\mu A$. A good reversibility with a peak to peak separation of $0.15\,V$ was observed. The same experiment was performed using a PIM/GCE and a displacement of the $[Fe(CN)_6]^{3-/4-}$ redox potential to more positive values was observed (E_{pa} at $+0.60\,V$), furthermore a 98% reduction of electrochemical signal (i_{pa} decreased to $0.3\,\mu A$) was obtained, exhibiting an irreversible response. These results clearly demonstrate that the PIM was effectively immobilized onto GCE surface and it blocks the diffusion of the redox couple towards the GCE surface and drastically decreased the current response. The slight diffusion rate of the $[Fe(CN)_6]^{3-/4-}$ from the solution to the electrode surface was probably promoted by the anionic exchanger in the PVC membrane.

However, when AuNPs were assembled onto PVC/GCE (AuNPs-PIM/GCE), the E_{pa} of $[Fe(CN)_6]^{3-/4-}$ shifted to $+0.27\,V$ with an i_{pa} of $0.435\,\mu A$. The observed increase of the i_{pa} as compared to the PVC/GCE electrode was attributed to an increase in surface area, promoted by the AuNPs, which enhance the electron transference rate and promoted a slight increase of the diffusion rate of the redox couple from the electrolyte to the electrode surface. In AuNPs-PIM configuration the kinetics of charge transfer was independent of the thickness of the modification layer on the GCE. In the cases of partially or almost complete coverage/blockage of the electrode surface like Ab/AuNPs-PIM/GCE or *Salmonella*/Ab/AuNPs-PIM/GCE, this effect is significant, and the maximum current intensity decreases proportionally to the thickness increasing of the modification layer. Specifically, after antibodies adsorption the anodic peak intensity reduce almost 63% comparing with AuNPs-PIM/GCE, corresponding to almost 99% of electrochemical signal reduction (bare GCE), confirming the antibody assembling.

3.3.2. Electrochemical impedance spectroscopy

EIS technique employing $[Fe(CN)_6]^{3-/4-}$ redox couple as probe (2.5 mM) was used to characterize and verify layer by layer the efficiency of the electrode modification, such as some information about electrode surface electron transfer kinetics. These electrochemical responses were obtained through the differences in electron-transfer rate, diffusion limited process and double-layer capacitance in electrode/redox probe solution interface between the different assembling steps.

Fig. 3(C and D) represents the real and imaginary impedances plotted against each other in a Nyquist plot representation. The several figures correspond to the different electrode modification steps, showing two distinct frequency regions. The semicircle portion, which was observed at higher frequencies Fig. 3(C), was associated with a

process that was limited by electron transfer at the electrodes surface. The linear features observed at lower frequencies Fig. 3(D) were attributed to diffusion-limited electron transfer [34]. It was observed a high increase in the resistance to the electron transfer, due to the PIM layer (a non-conductive hydrophobic polymer) onto GCE [35]. The drop-cast of the plasticized membrane creates an insulation layer on GCE interface, although as it was observed in the CV only partial blocking of electron transference was verified. A small redox peak can be seen in this specific modification step plot after some time immersed in the probe solution. In this case the electron transfer rate is limited at first by the slow diffusion of the electrolyte solution trough the PIM plus next step modifications and by the decreasing of active spots on GCE surface. Some PIM membrane show micropores which act as small double capacitive layers in parallel with expected kinetically controlled charge-transfer reaction [36,37] (SI Fig. S3.1g).

In the high frequency region Fig. 3C, it can be noted too that the initial part of semicircle disappears when the PIM was introduced on the GCE surface and can be attributed to a combined effect of the charge-transfer resistance at GCE electrode interface and the double-layer capacitance [38]. This is characteristic of the presence of a non-perfect capacitor and another constant phase element (CPE) like the Open Finite-Length Diffusion (OFLD) in the electric circuit model (inset of Fig. 3 D) [36,37].

So, as the modification layers' thickness increases, the time required to obtain the first visible register in our circuit model (AC voltage = + 0.375 V), should also increase if the electrode reaction occurs only at the bare spots on the electrode surface. In this model, the charge-transfer resistance is intrinsically connected with the resistance of ion-conducting (pore resistance). Although when AuNPs were assembled, the resistance decreases comparing to the previous configuration. The phenomenon can be explained by the capacity of nanoparticles create electron-conducting tunnels and their electrocatalytically properties, enhancing the transference of electrons [16]. Parallel to this, the diffusional part of the plot (straight line), presents a squeezed depressed semicircle in the PIM configuration, which was associated to the negative net charge of AuNPs (see SI section S6) that increases the $[Fe(CN)_6]^{3-/4-}$ solution charge repulsion [39,40]. The immobilization of antibody IgG anti-ST results in a significant increase of total resistance due the formation of an additional insulating layer on the electrode surface. With the insertion of the ST antigen the resistance decreases, contrariwise what was expected, hypothetically due the more oriented and surface detached position of the *ST*-Ab immune-complexes that can facilitate the diffusion of the ions to the GCE [41]. This phenomenon is according to the literature, for other biomolecules specially the ones conjugated by physical adsorption [42,43]. Additionally, in these two last approaches the diffusion limited process is represented mostly as a straight line. According to previous results, it can represent the neutralization of carboxylic group's negative charges presents at the AuNPs-PIM membrane and homogenization of electrode surface by antibody adsorption.

3.4. Immunosensor performance

It was clear that the hydrophilic AuNPs layer formed on the PVC membrane electrode enhances the potentiometric signal, probably by the enlargement of the electrode surface area, ordered antibody density and tunneling effect [16]. As suggested from electrochemical characterization of the IMS, the AuNPs grow preferably close to the membrane pores [29,44], so the antibody load and the immunocomplex formation occurs mainly close to the electrode active spots, amplifying the blocking effect caused by the phenomena, and consequently the immunosensor sensitivity [15,16].

The dynamic potentiometric response curves of the immunosensors presented in Fig. 4 A were obtained upon successive additions of ST standard solutions (10^4, 10^6, 10^8 cells mL^{-1}) to a stirred PB matrix, after a steady-state potential was achieved. These curves illustrate the
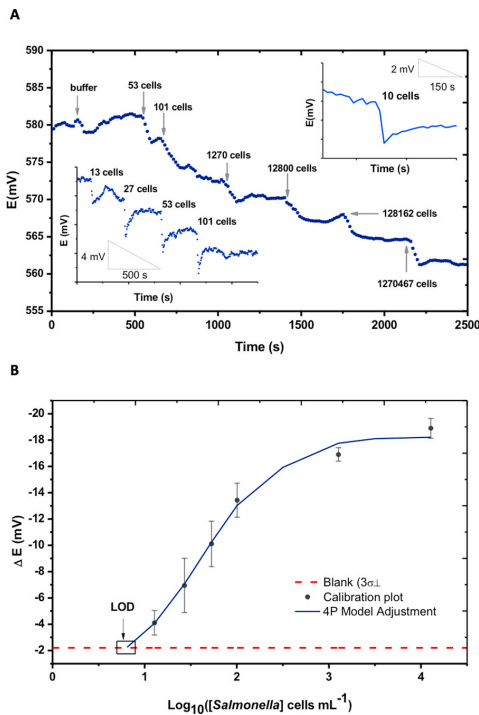
Fig. 4. A) Typical dynamic response curves of the immunosensors obtained in PB 0.01 M pH 7 for *ST* in response to the indicated concentrations expressed in cells mL^{-1}; B) Calibration plot (potential difference in reason of the log of *ST* concentration) in PB 0.01 M pH 7 showing the response of three different electrodes constructed in the same conditions (n = 3). Baseline and standard deviation (solid and dashed red lines. Error bars indicate the standard deviation of the three immunosensors assayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect of increasing surface blocking upon ST binding to the IMS on the TPB flow from the internal to the bulk solution. A extrapolating from Fick's first law, shows that once steady-state was achieved, -that is the outward flux trough ISE membrane, the diffusional layer closer to IMS surface and thought the aqueous are the same, - any potential changes

that arises is due to differences in diffusion and binding layer (named all as aqueous layer, Fig. 1) or surface-confined layer thickness and respective diffusion coefficients [17]. Taking this appointment, it was expected that the signal attenuation that coming from convective stirring was compensated by large *Salmonella* size (lengths from 2 to 5 μm). The immunosensors show a rapid response and upon addition of the ST cells a stable response is observed in less than 10 min.

In Fig. 4B the relation of EMF difference and the logarithm of ST concentration was represented. The response curve shows a sigmoidal shape as is frequently observed in biological processes. A calibration curve (black dashed line in Fig. 4B) was calculated using the four-parameter (4 P) regression model [45], quite used in biological receptor-ligand binding assays [46,47]. The calculated detection limit (set out by Eq. 1 (n = 3, 6 replica each)) of the developed immunosensor was 6 cells mL^{-1}.

$$LOD = x\left(\frac{a - d}{(a - d) - 3\sigma} - 1\right)^{-1/k} \tag{1}$$

Comparing with other electrochemical label-free immunosensors in the literature (Table 1) we achieved to a very good LOD, even some of them accomplished lower values [11,48,49]. Specifically, Zelada-Guillén et al. (2009) and Ranjbar et al. (2018) works reported LODs as low as 1 cell mL^{-1} that was aligned to the legislated limits. Although, in Zelada-Guillén et al. (2009) work a low ionic strength media was needed to perform the analysis and the method applicability to real samples was not stated. Instead, Ranjbar et al. (2018) presented the applicability test in a complex sample, despite the multi-step sample preparation and the high contamination levels tested. Likewise, the assumed LOD was quite far from the lower limit of the linear range, increasing the uncertainty at low contamination levels. Nevertheless, biosensors presenting a LOD much lower than the infection levels for ST (1000 cells mL^{-1}), may be useful for screening purposes or as confirmation test of infection in human.

3.4.1. Regeneration and stability of the immunosensor

Reusing of an immunosensor is very appealing due the possibility of application to many samples in a short period at a low cost relatively to the disposable ones [54]. Accordingly, several regeneration reagents which have already been successfully used by Park et al. [55].in 2000 were tested. After the regeneration step, the immunosensor response was evaluated with a ST concentration of 12800 CFU mL^{-1}. The best results were achieved using glycine-HCl 0.1 M pH 2 but the response of the immunosensor after the third cycle of regeneration drop to 53% of initial value.

Table 1
Label-free electrochemical biosensors for *Salmonella* spp. detection.

Transducer	Nanomaterial	Detection Technique	Working range	LOD	Analysis Time	Sample	Refs.
Double-Walled Electrode	CNTs	CA	10^2–10^7 cells mL^{-1}	8.9 cells mL^{-1}	Total ≅ 6 h	PBS	[48]
GCE	MSNTs	I	10^3–10^7 cells mL^{-1}	5×10^2 cells mL^{-1} in PBS	30 min	PBS	[50]
GE	–	EIS	–	500 cells mL^{-1}	Total of 6 min	PBS	[51]
GCE	AuNPs MWCNTs	I	10^3–10^7 cells mL^{-1} in PBS	500/1000 cells mL^{-1} in PBS/milk	About 1 h	PBS Milk	[52]
SPE	–	EIS	10^3–10^8 cells mL^{-1}	10^3 cells mL^{-1}	Total ≅ 16.5 h Analysis- 20 min	PBS Milk	[53]
GCE	SWCNT	P	0.2–10^3 cells mL^{-1}	1 cell mL^{-1}	1 min	PBS	[11]
GCE	Nanoporous gold	EIS	6.5×10^2–6.5×10^8 cells mL^{-1}	1 cell mL^{-1}	About 40 min	PBS Eggshell	[49]
ISE	AuNPs	P	13–1.3×10^6 cells mL^{-1}	6 cells mL^{-1}	About 1 h for a complete assay	PB Apple juice	This work

Glassy Carbon Electrode (GCE); Gold Electrode (GE) Impedimetry (I); Magnetic silica nanotubes; (MSNTs); Single-walled.
Carbon nanotubes(SWCNT); Multi-walled carbon nanotubes (MWCNTs); Potentiometry (P); Chronoamperommetry (CA).
Screen-printed electrode (SPE).

Table 2

Recovery (%) of *Salmonella typhimurium* (10, 75 and 100 cells mL^{-1}), in commercial apple juice samples.

Theoretical concentration	Recovery (%)		
(cells mL^{-1})	n	Mean $\pm$ SV	Coef. V (%)
10	3	72.9 $\pm$ 1.0	1.3%
75	3	80.6 $\pm$ 1.6	2%
100	3	40.4 $\pm$ 2.0	5%
Mean	–	64.6 $\pm$ 0.5	2.8%

Mean $\pm$ SV = mean $\pm$ standard deviation of three replicates.

Coef. V = coefficient of variation; n = number of samples.

The immunosensor stability was tested along the time by the calibration of immunosensors with different lifetime after construction. The storage was made without internal solution, at 4 °C in hydrated atmosphere until use. After 10 days storage a decay to a third of the initial response (34%) was observed.

3.5. Apple juice sample analysis

The results obtained in the analysis of samples with 10, 75 and 100 CFU mL^{-1} of ST were correlated with the calibration curve performed in PB. The respective recoveries % are shown in Table 2.

The average recovery of ST was consistent in the range of concentrations 10–100 CFU mL^{-1} with an average value of 64.6. Despite the low recovery rates achieved, the method reveals a good inter-assay precision with a Coef.V (%) $\leq$ 5, demonstrating the applicability of the developed immunosensor for screening purposes at low ST concentration. As prospects to optimize the quantification potential of the developed immunosensor in foodstuffs with complex matrix, without a significant increase of the total time of analysis, it is worth couple the immunosensor to immunomagnetic separation methods.

4. Conclusions

AuNPs formed in-situ on a PIM has been successfully used as a biocompatible sensing platform for bioreceptor conjugation and the developed IMS also promoted the amplification of the measured potentiometric signal in a label free biosensor with sensitivity close to labelled approaches. The proposed label-free potentiometric immunosensor shows potential for on-site food control owing to the easiness of the experimental procedure and the simplicity and portability of the potentiometric instrumentation. A more effective separation method, such as an immunomagnetic separation can be applied to magnify the recovery of the immunological assay, to be applied to complex matrixes. At this point the developed immunosensor can be a simple, low-cost, disposable and useful tool for screening of ST in food and water samples. The proposed potentiometric immunosensor stands out from its conventional counterparts because it combines the feasibility of miniaturized polymer membrane ISE and the specificity of surface confined immune reactions to assemble a robust and sensitive immunosensor without labels and resorting to a simple instrumentation.

Since the signal amplification was obtained through the developed IMS, without resorting to redox labels or enzymatic amplification, this reliable method can be easily applied to different bacteria-antibody couple, simply changing the specific antibody and optimizing the AuNP-PIM. Furthermore, the strategy presented in this work shows potential for application to other bioreceptors as it allows to control, particle size distribution and membrane surface coverage and the selectivity of the PIM sensor towards the selected marker ion allows working in several biological pH buffers.

Associated content

Supporting information. Detailed information about the fabrication optimization and characterization of the developed immunosensor.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.10.024.

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Supplementary Material

In Situ Formation of Gold Nanoparticles in Polymer Inclusion Membrane: Application as Platform in a Label-Free Potentiometric Immunosensor for *Salmonella Typhimurium* Detection

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S1. Fabrication of the Immunosensor

Fig.S.1.1 − Final structure of the biosensor and representation of the ion-selective membrane assembling experimental procedure.
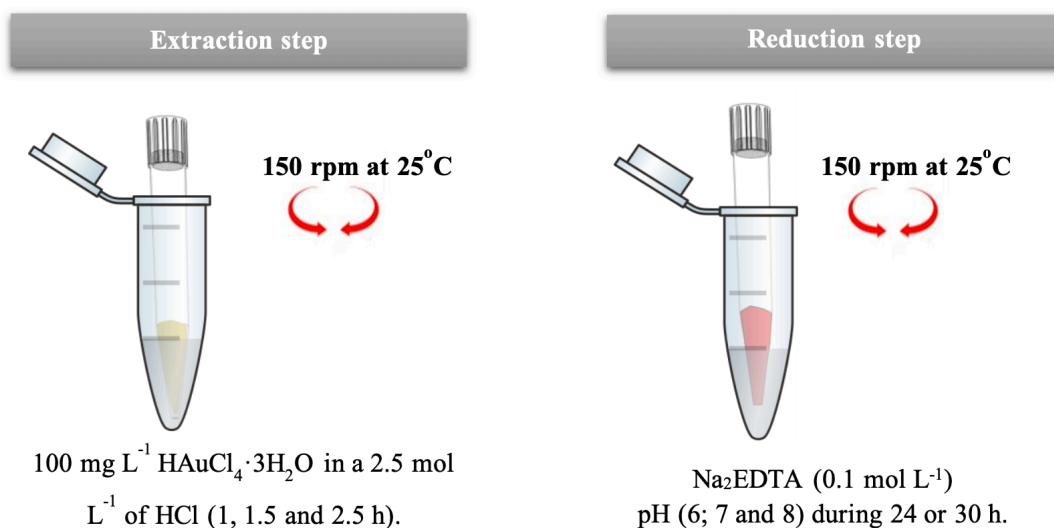
Fig.S1.2 − Schematic representation of the AuNPs-PIM formation experimental procedure: extraction and reduction steps.

S2. Ion diffusion optimization

Considering that the passive ion diffusion of the system is representative of a situation where the ionic concentration in diffusional layer (aqueous boundary) are different from the sample bulk (non-equilibrium mode), to minimize irreproducibility among different electrodes and to avoid drifting responses [44, 45] it is necessary to adjust several factors which modulate the ion fluxes. In this work sensor membrane composition, membrane conditioning time, internal solution composition and the buffer used (concentration and background electrolytes) were optimized. Analyzing the experimental results obtained in preliminary stirring studies as well as the final potentiometric response to *ST*, it was clear that the potential variation was influenced by these variables.

S2.1. Sensor composition

Different modified PVC membrane electrodes were developed. Firstly, blind membranes, constituted only by a mixture of PVC and the plasticizer (di(n-octyl) phthalate (DOP), or 2-nitrophenyl octyl ether (NPOE)) were tested. For comparison, the same membrane was doped with different concentrations of methyltrioctylammonium chloride (TOMA) (1, 3 and 5% (m/m)) (maintaining the other assay conditions), to adjust the optimal membrane composition. Tetraphenylborate (TPB) was chosen as a marker ion due its hydrophobicity, size and relative position in Hofmeister series. The membrane doped with 1% of TOMA, which uses NPOE as a plasticizer revealed the best results in terms passive flux control.

S2.2. Internal Solution and Internal Conditioning, membrane modifications

The effect of concentration (2, 4, 6 or 10 mg/mL) internal filling solution and solvent matrix, (ultra-pure water or phosphate buffer), internal conditioning treatment time (4 ,12,18, 24 and 120 h) and the effect of pH (pH's equal to 6,7 and 8 performed in PB) and the presence of interfering ions in the control solution (ultra-pure water and phosphate buffer) in the final potentiometric response to *ST* was also considered and optimized.
Performing the preliminary stirring experiments, the data showed a typical anionic response after inducing abrupt changes in stirring speed under optimized conditions through an Ab/AuNPs-PIM electrode, simulating - at minimum stirring - the blocking

effect caused by ST-antibody complexation. At maximum stirring the potential goes up, due to the dissipation of TPB close to the membrane (Fig S2.1. A).

This effect was observed in all experimental conditions tested, although the tests performed in PB matrix showed a baseline membrane potential lower (but well-defined) than the tests performed in water matrix (Fig. S2.1. B).

Hereupon, after the experiments a 10 mg/mL of TPB prepared in PB was chosen as internal filing solution with optimal conditioning time of 4 h. PB 0.01 mol L^{-1} (pH 7) was used as control matrix in the next experiments.
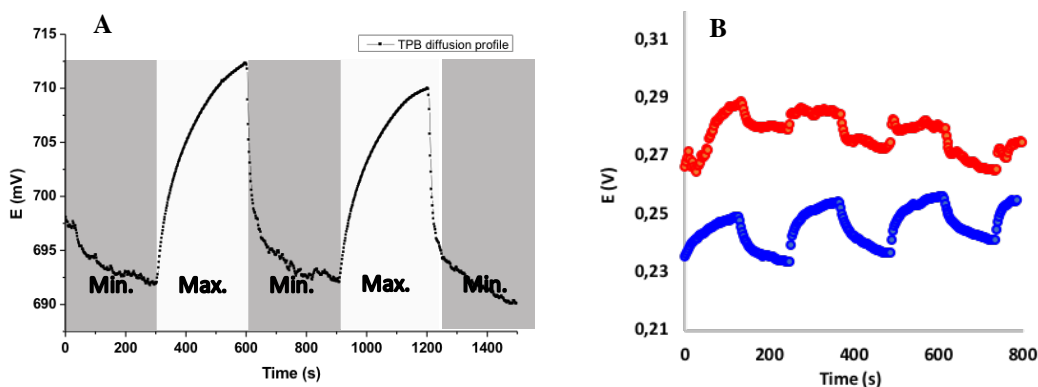
Fig.S2.1 – A - Stirring effect in a PVC Membrane, with NPOE as plasticizer and doped with 1% of TOMA, in different sample matrices: in a water (red) and PB (blue) sample; B - Stir-effect observed for the Ab/AuNPs-PIM biosensors, at minimum (light green) and maximum (light grey) stirring.

S3. Scanning electron microscopy (SEM) and particle size analysis
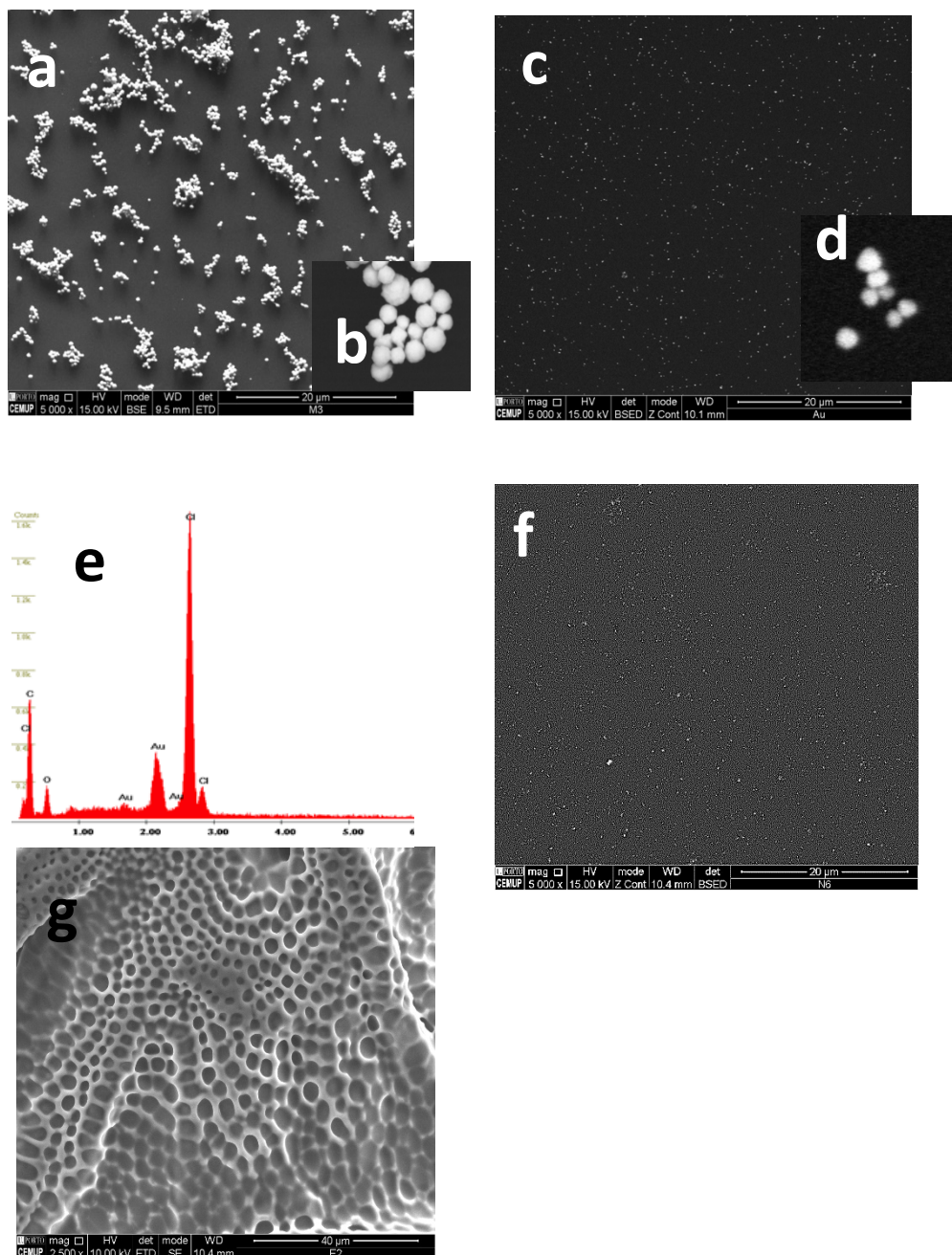
Fig.S3.1 – SEM images of AuNPs-PIM surface obtained with 2 h extraction of tetrachloroaurate(III) and 24 h of reduction with EDTA 0.1 mol L^{-1} at pH 6 (a) showing a shadow effect over the polymer surface, obtained with the lateral secondary electrons detector (ETD) configured to backscattered electrons (without bias voltage applied); and (b) near-spherical AuNPs formed on PIM surface. Surface images obtained (c) with 1.5 h extraction and 24 h of reduction with EDTA 0.1 mol L^{-1} at pH 7; and (d) near-spherical AuNPs formed on PIM surface under these conditions. (e) Energy Dispersive Spectroscopy (EDS) analysis, with the peak of gold from the grown AuNPs at 2.2 KeV. AuNPs-PIM surface obtained with 1 h extraction and 24 h of reduction with EDTA 0.2 mol L^{-1} at pH 6. SEM image of an ISE polymeric membrane surface casted on filter paper modified with PEDOT:PSS (g).
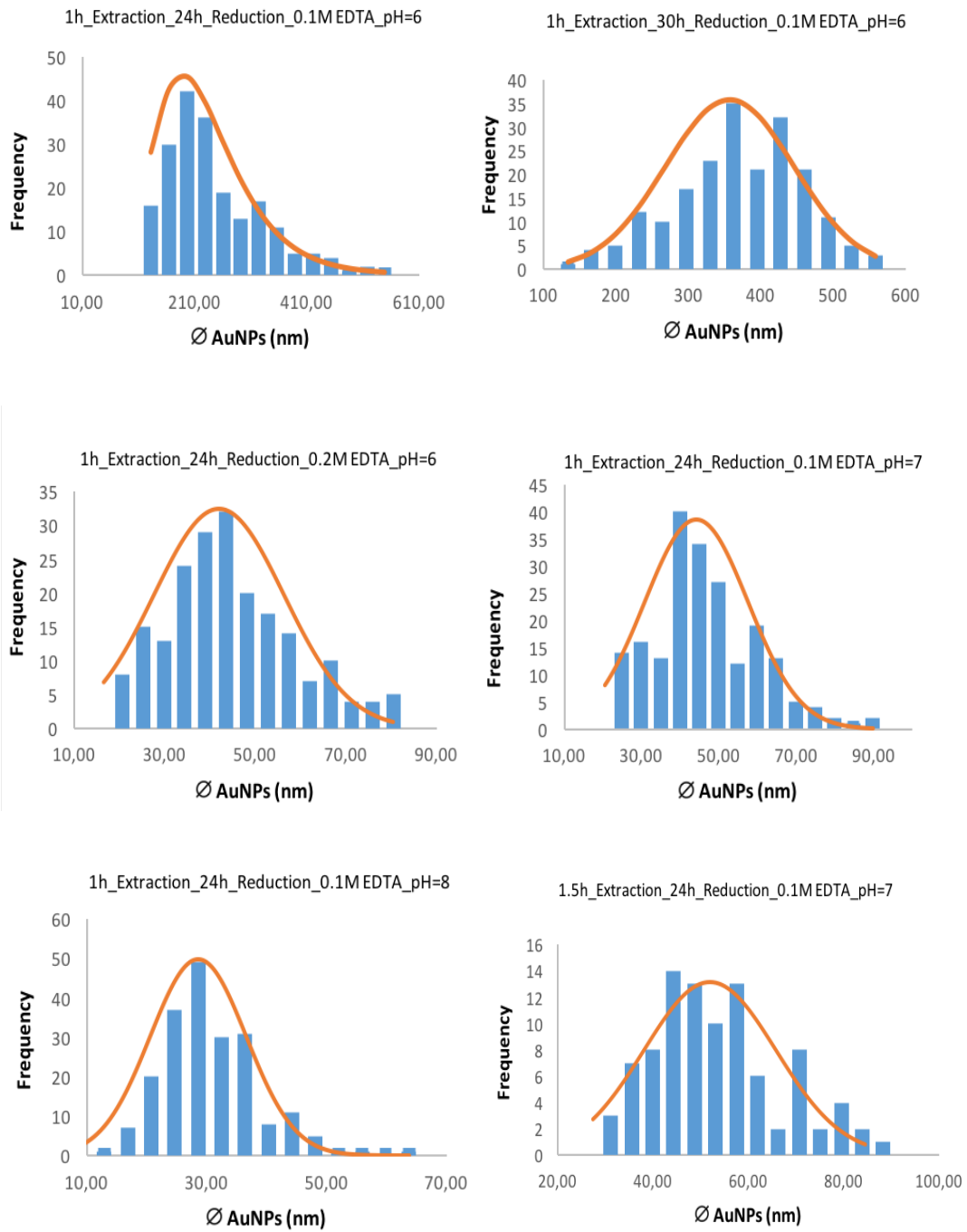
Fig. S3.2 – Histograms for AuNPs sizes obtained at the indicated conditions.

S4. IMS Characterization

The time spent in extracting the Au(III) for the PVC membrane and its subsequent reduction to Au (0), gone mediate the distribution and density of the grown AuNPs into the PIM (Fig S4.1). The pH and concentration of the Na_2EDTA was also studied in an attempt of controlling also the size, the surface groups and the electrostatic interactions between them (Fig. S4.2).
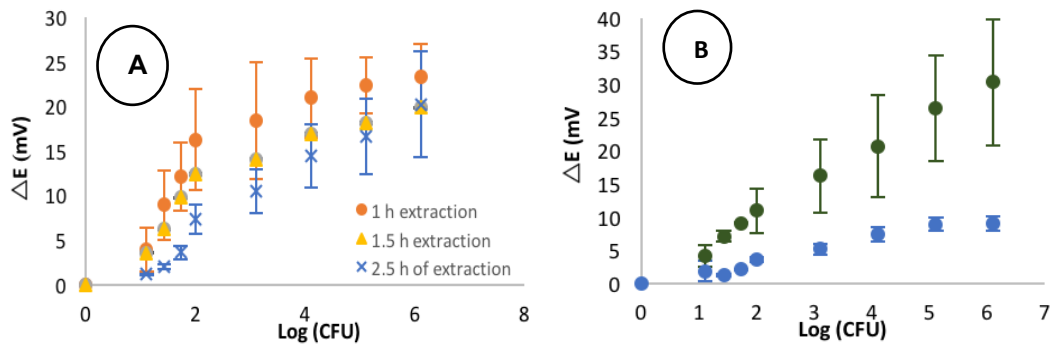
Fig.S4.1 − Examples of extraction time (A) and reduction times effect, comparing 24 h (blue) with 30h (green) of reduction step using 0.1 mol L^{-1} Na_2EDTA solution pH's 6 before an extraction time of 1 h (B), in an immunosensor response to ST. Electrodes conditioned with TPB 10 mg mL^{-1} in PB (n = 2).
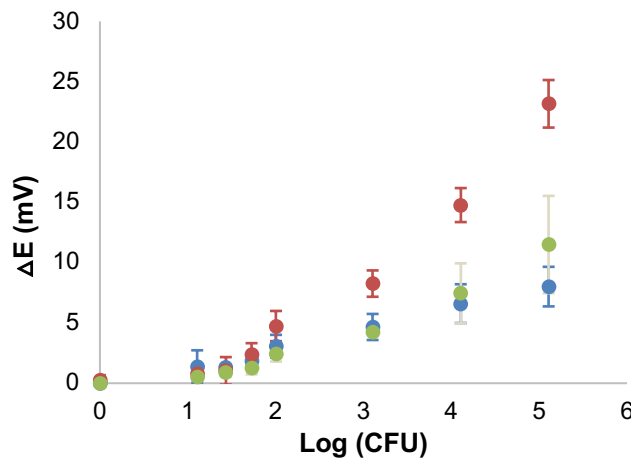
Fig.S4.2 − Response of the Ab/AuNPs-PIM electrode to different ST concentrations (n=3) using 0.1 mol L^{-1} Na_2EDTA solution pH's 6 (blue),7 (orange) and 8 (grey) in reduction step performed during 24h, with a fixed extraction time of 1h. The matrix used was PB 0.01 mol L^{-1} pH=7.

S5. Antibody immobilization

To study the effect of the presence of AuNPs into PVC membrane, it was compared two different antibody immobilization approaches: a capture antibody was immobilized on a PVC membrane trough glutaraldehyde click chemistry (1% v/v ;120 min) and into an AuNPs-PIM electrode (Fig. S5.1). According, immobilization of the antibody were performed by physical adsorption. Potentiometric responses of both electrodes towards *ST* were recorded and correlated with its concentration in sample. We concluded that the AuNPs-PIM enhances the biocompatibility of the platform and the antibody load in a one-step modification, without cross-linkers.
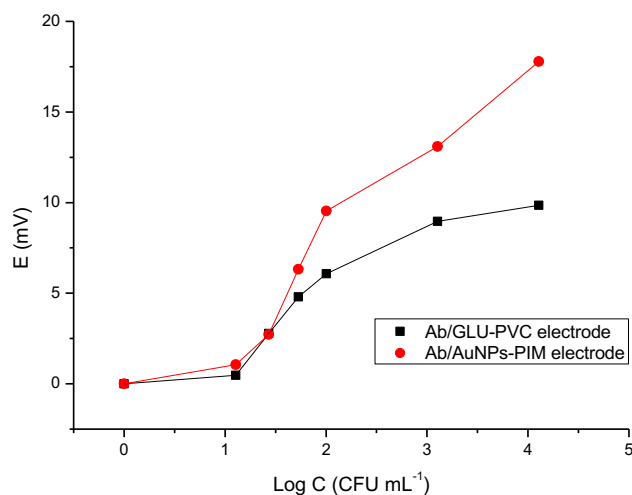
Fig.S5.1 − Calibration plot of two immunosensors with different IMS platforms: an antibody immobilization troughs glutaraldehyde click biochemistry on a PVC membrane platform and; by physical adsorption in a AuNPs polymer inclusion membrane.

S6. FTIR studies

Figure S6.1 shows the absorbance IR spectra obtained after the subtraction of the spectrum for the complete formation of the AuNPs-PIM under optimized conditions with the spectrum corresponding only to the procedure until the Au(III) extraction step. So, the bands can be assigned to the presence of Au (0), instead Au(III) or to the presence of the Na_2EDTA, which was only used in the reduction step.

An excess of Na_2EDTA will promote the functionalization of the formed nanoparticles by the anchorage of Na_2EDTA into the amine groups presents at AuNPs surface and

consequent exposing of its four carboxylic groups [46, 47]. So, surface analysis of the different steps of the AuNPs-PIM formation and the presence of the Na_2EDTA was determined by infrared (IR) spectrometry (Nicolet 6700 FT-IR, from Thermo Electron Corporation) with the Smart Orbit accessory and equipped with Omnic 7.3 software. The IR spectra were obtained after air background subtraction, directly on the just dried membranes without additional treatments, with 96 scans resolution.

By the subtraction of final membrane stage with the extraction stage (with and without Na_2EDTA), was denoted that the Na_2EDTA characteristic band (N-H$^+$) at 3300-3500 cm^{-1} was not present, but the C-N bending appears at 1091cm^{-1} and N-H bending at 1541, 1527, 1518, 1508 cm $^{-1}$, confirming the complexation of the amino groups of Na_2EDTA with AuNPs gold metal ions [48].

The free ionized COO$^-$ absorption band arisen at 1603 cm^{-1} that is characteristic of IR spectrum of Na_2EDTA. The four COO$^-$ ions can connect into surface Au ions either by monodentate or bidentate connection way [48]. These can be proved based on location of COO$^-$ vibration frequencies. Main vibration modes i.e. asymmetric stretching υas (COO$^-$) and symmetric stretching υs (COO$^-$) are present at 1583 cm^{-1} and 1422 cm^{-1}, respectively [48]. Due the matrix complexity, only the asymmetrical C=O stretching band in carboxylate, usually near 1650–1550 cm^{-1}, is clearly observed in the IR spectrum, at 1581 cm^{-1}. Although, the symmetrical C=O stretching it was assigned to the band at 1457 cm^{-1}. It was observed that the υas (COO$^-$) has been decreased and υs (COO$^-$) increased indicating the monodentate coordination [47]. Additionally, other vibrations were assigned to COO$^-$ groups (1347, 1277 and 1255 cm^{-1}), in accordance with the absence of the un-ionized and uncoordinated COO stretching bands that normally occur at 1750–1700 cm^{-1}.
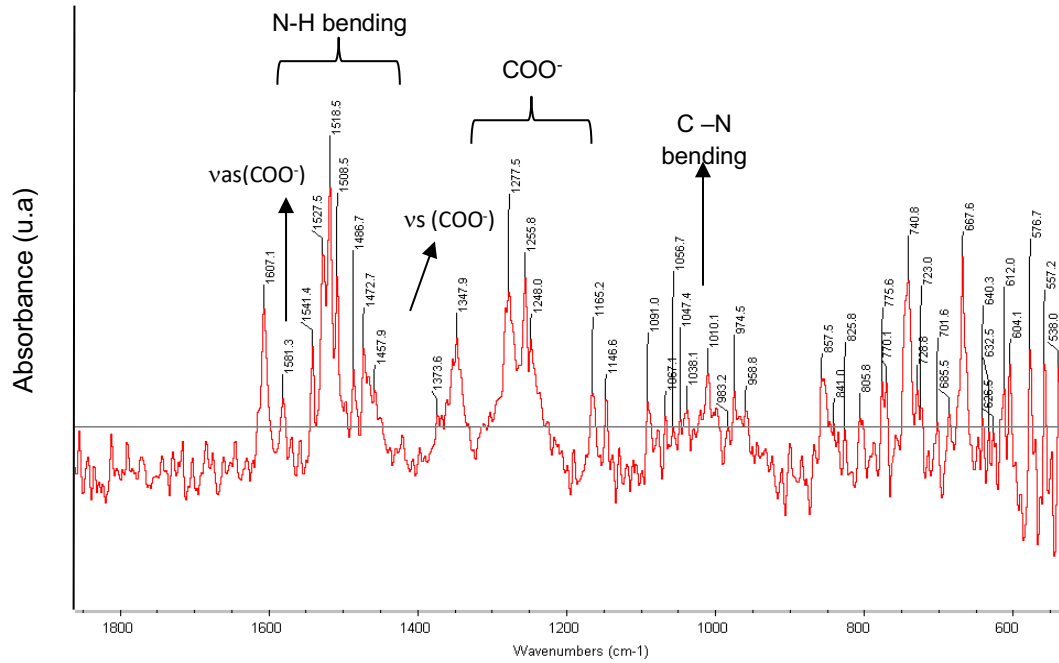
Fig.S.6.1 − Absorbance FTIR-ATR spectra resultant from the subtraction of representative Au extraction step to final AuNPs-PIM spectra.

S7. BSA Effect
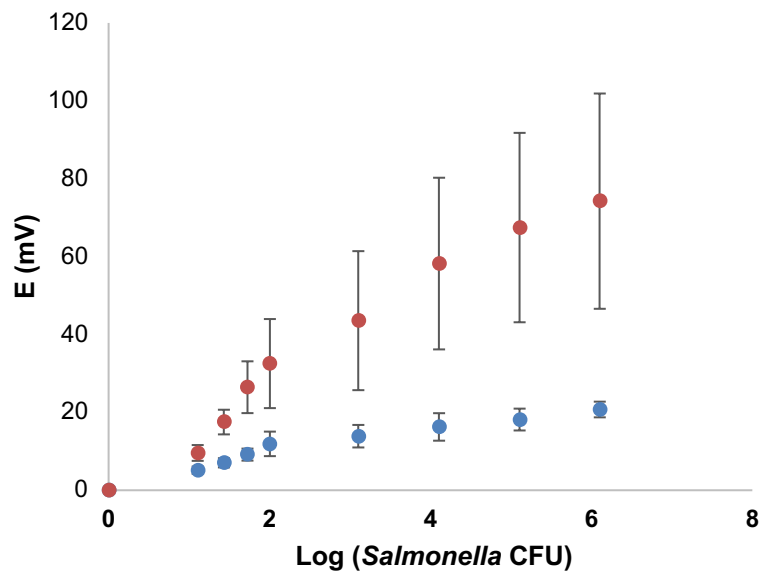
Fig. S7.1 − BSA effect in the potentiometric response to *ST*. Average ±standard deviation of the calibration curves obtained for three immunosensors with (orange) and without BSA treatment.

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2.3. Development of a disposable paper-based potentiometric immunosensor for real-time detection of a foodborne pathogen

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Statement of contribution

The contribution of the candidate, Nádia F.D. Silva, in this work includes the literature review, investigation and writing of the original draft.

Development of a disposable paper-based potentiometric immunosensor for real-time detection of a foodborne pathogen

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ABSTRACT

This work reports a new paper-based sensing platform and its application in a label-free potentiometric immunosensor for *Salmonella typhimurium* detection based on the blocking surface principle. A paper-based strip electrode was integrated with a filter paper pad which acted as a reservoir of the internal solution. The design offers a convenient platform for antibody immobilization and sampling, proving also that is a simple and affordable methodology to control an ionic flux through a polymer membrane.

Two different immunosensing interfaces were assembled on the developed paper-strip electrode. The simplest interface relied on direct conjugation of the antibody to the polymer membrane and the second one resorted to an intermediate layer of a polyamidoamine dendrimer, with an ethylenediamine core from the fourth generation. Electrochemical impedance spectroscopy was used to assess the successive interface modification steps and the resulting analytical performance of both immunosensors was compared. For such, the potential shift derived from the blocking effect of the ionic flux caused by antigen-antibody conjugation was correlated with the logarithm of the *Salmonella typhimurium* concentration in the sample. In optimized conditions, a limit of detection of 5 cells mL^{-1} was achieved. As a proof-of-concept, the proposed method was applied to apple juice samples, demonstrating to be a suitable prototype to be used in real scenarios in useful time (< 1 h assay).

1. Introduction

The detection and effective prevention of foodborne illnesses caused by bacteria still stand today a worldwide public health issue. Despite this topic being of great interest to public health and the constant demanding to create analytical tools that achieve the analytical performance of conventional plate count techniques, those remain along the years as the gold standard methods since they fulfil the limits of detection imposed by the European Union (EU) regulation 2073/2005 (European Commission , 2005) and the standard ISO 6579–1 (Standardization 2017) to food quality assessment. Among other bacterial pathogens, *Salmonella spp.* was one of the most common causes of foodborne outbreaks, originating many hospitalizations and deaths every year (Silva et al., 2018; Srisa-Art et al., 2018). According to the summary of foodborne outbreaks in EU occurred in 2016 reported by the European Food Safety Agency (EFSA) and the European Centre for

Disease Prevention (ECDP) an unexpected increase of 11.5% compared to 2015 data (EFSA and ECDP 2017) was observed and the last summary steps up the increasing tendency of the last years (EFSA and ECDP 2018). By the dangerousness of salmonellosis side-effects (especially in typhoid salmonellosis), a tight limit of a single bacterial cell in a 10–25 g of the sample was imposed, meaning that the presence of a single cell will determine the rejection of the product (EC No, 20173/2005, 2005).

Although, besides the need to attain to a sensitive method, other features like the speed of analysis, cost, portability, the complexity of the procedures and equipment accessibility must be considered given that most of the infection outbreaks occur in developing countries and the cost-effectiveness of the methods is the first barrier to industrial acceptance. Accordingly, in last years a great demand for lab-on-chip infield testing devices for real-time detection of foodborne pathogens has been observed, in which electrochemical biosensors reached a

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prominent place by rapidness, sensitivity, portability, low-cost and user-friendly interface which have shown. Despite this, a perfect method that can be used onsite in different matrices is still needed (Silva et al., 2018).

Important goals have been achieved with the developed electrochemical biosensors. Recently, some potentiometric (Hernández et al., 2014; Zelada-Guillén et al. 2009, 2013) and impedimetric (Ranjbar et al., 2018) label-free aptasensors such as a amperometric genosensor (Liébana et al., 2009) presented capabilities to detect a single cell in a small buffered sample volume in a few minutes. Despite this important outcoming in rapid zero tolerance electrochemical biosensing systems, the final product is still expensive due to the high cost of the electrodes and bioreceptors. So, in our view, the upcoming developments must be directed to using low-cost materials for electrode construction and sensors miniaturization for a low-scale use of chemical and biological reagents.

Filter paper has been extensively used as a substrate or mechanical support of analytical platforms due to its flexibility, biodegradability, biocompatibility, portability, low-cost and decentralized accessibility (Ruecha et al., 2017). Recent paper-based biosensors have been used mainly together to colorimetric or electrochemical detection in test strips format (Hossain et al., 2012), microchips (Khan et al., 2018) or microfluidics devices (Altintas et al., 2018), with obvious focus on point-of-care disposable sensors (Narang et al., 2017; Pavithra et al., 2018; Teengam et al., 2017; Tian et al., 2017; Wang et al., 2016) and environmental monitoring (Apilux et al., 2010). Although, this type of devices for foodborne pathogens detection are scarce in the literature (Bhardwaj et al., 2017; Srisa-Art et al., 2018). Despite the appealing output of the existing colorimetric methods, the paper-based electrochemical biosensors for pathogen detection also offer the possibility to quantify the target analyte in useful time, with results similar to the conventional electrochemical methods (Bhardwaj et al., 2017; Liu et al., 2014). Additionally, potentiometric techniques demonstrated an unparalleled simplicity, portability and low-cost instrumentation (Novell et al., 2012), but paper-based platforms for foodborne pathogens detection with potentiometric detection is still to be explored. Hereupon, the possible interferences from matrix components and the need for a low ionic-strength media is still a big challenge of the electrochemical detection techniques applied to foodstuffs.

In this work, a simple and cost-effective paper-based potentiometric immunosensing platform was developed and a label-free potentiometric immunosensor for *Salmonella thyphimurium* (ST) detection, based on surface blocking principle (Gyurcsanyi et al., 2003; Pawlak et al., 2015; Silva et al., 2019; Xu et al., 2005) is presented as a proof-of-concept. This detection principle comprises the regulation of a marker ion flux from or into an ion selective membrane mediated by a specific biorecognition event that takes place near or on its surface, which is able to induce a potentiometric response, which can be used as analytical signal. In the present case, the disruption of internal solution flux to through the sensing membrane towards the bulk solution (sample side) is disrupted by the binding of ST cells to the immunosensing interface (Ozdemir et al., 2013; Pawlak et al., 2015). Thereby, inspired in the newest paper-based ion-selective potentiometric sensors (Armas et al., 2018; Hu et al., 2016; Rius-Ruiz et al., 2011; Ruecha et al., 2017; Yoon et al., 2017), a common filter paper strip soaked with a poly(3, 4-ethylenedioxythiophene)polystyrene sulfonate (PEDOT:PSS) conducting polymer was used as ion to electron transducer in the indicator electrode, on which it was dropped a thin flexible polymeric film which serves both as ion selective membrane and as platform for bio-recognition element loading.

The detection of biomolecules or larger analytes by paper-based analytical devices frequently involves elaborated construction schemes, resorting to self-assembled monolayers (SAMs) or nanomaterials for bioreceptor anchoring, concerted with enzyme or nanoparticle labeling to reach to desired low detection limits, increasing the cost and complexity of the methods (Canovas et al., 2017; Parrilla et al., 2017; Srisa-

Art et al., 2018). Here, the polymer membrane casting step allowed the introduction of a cation selective membrane with available carboxylic acid groups allowing the covalent attachment of the antibodies without the need of extra elaborated chemical steps for cellulose paper modification (Cao et al., 2017). In turn, the materialization of surface blocking concept as a detection method of the proposed paper-based immunosensor was made resorting to a passive transmembrane flow of a reporter ion from the back of the polymer membrane to the place where the desired confined surface immune reaction happens (Pawlak et al., 2015). For that, a small filter-paper pad which serves as a reservoir of reporter ion solution was placed on the back of the electrode, mimetizing and transforming it in a "vertical ion flow" device.

Considering the effect on the biosensor performance of a stable, strong and oriented immobilization of antibodies (Fu et al., 2005; Yang et al., 2018) two immunosensing interfaces (IMS) were studied. The simplest IMS relies on direct conjugation of the antibody to the carboxyl groups present on polymer membrane backbone by an EDC/NHS covalent coupling chemistry. The second one resorts to a poly-amidoamine dendrimer intermediate layer, with an ethylenediamine core from fourth generation (PAMAM(NH$_2$)64dendrimer) in an attempt to increase the available area to antibody immobilization.

The proposed immunosensor was successfully applied, and the results show that it can accurately predict low levels of ST with high specificity. Some limitations and prospects for use of this electrode prototype in mass-scale and real scenarios are discussed.

2. Experimental

2.1. Chemicals and solutions

ST positive control (5×10^9 cell mL^{-1}) was obtained from Kirkegaard & Perry Laboratories. The *Salmonella* monoclonal antibody (100 µg mL^{-1}), highly specific for surface liposaccharides of the *typhimurium* serotype (Freitas et al., 2014; Viswanathan et al., 2012), were purchased from Santa Cruz Biotechnology.

Bovine Serum Albumin (BSA), sodium dihydrogen phosphate dihydrate (NaH$_2$PO$_4$·2H$_2$O), potassium hydrogen phosphate (KH$_2$PO$_4$·xH$_2$O), 4-morpholineethanesulfonic acid hydrate (MES), hydrochloric acid (HCl), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and PAMAM (NH$_2$)$_{64}$ dendrimer (10 wt% in methanol) were from Sigma Aldrich. Poly(vinylchloride) carboxylated (PVC–COOH, 1.8%) and 2-nitrophenyl octyl ether (2-NPOE) were Selectophore™ grade from Sigma Aldrich. All other reagents used throughout this work were pro-analysis quality (pa) or equivalent and were used as received.

The polymer membrane sensor cocktail was obtained simply by mixing the plasticizer 2-NPOE ($\approx$66.6 wt %) and PVC-COOH ($\approx$33.4 wt %) dissolved in tetrahydrofuran (THF).

Phosphate buffer saline (PBS) 0.01 mol L^{-1} pH 7.4, prepared with 0.1 mol L^{-1} Na$_2$HPO$_4$ and 0.1 mol L^{-1} KH$_2$PO$_4$, was used for ST positive control dilutions. For antibody dilution, PBS 0.1 mol L^{-1} pH 7.4 with a background of 25 mmol L^{-1} of sodium was used. Phosphate buffer (PB) 0.01 mol L^{-1} pH 7 was prepared with NaH$_2$PO$_4$·2H$_2$O and the pH was adjusted with NaOH 4 mol L^{-1}.

2.2. Fabrication of paper-based immunosensor

The general procedure for immunosensor fabrication involves the construction of the paper-strip electrodes (PSE) (Fig. 1), followed by the modification of the PSE with the capture antibody (Fig. 2).

2.3. Construction of paper-strip electrodes

The filter paper was coated with PEDOT: PSS conductive polymer doped with 10% (v/v) of ethylene glycol and was placed in the oven, at 105 °C for about 7–8 min to promote solvent evaporation and to

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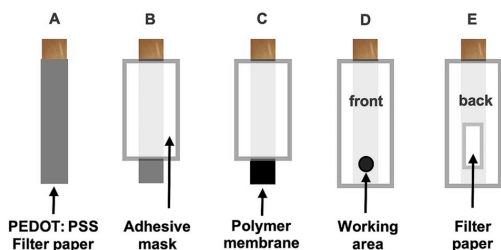
Fig. 1. Schematic representation of the paper-strip micro-electrode construction.

improve the electrical conductivity properties. The amount of conductive ink applied was 35 $\mu L\,cm^{-2}$ and the obtained conductive paper showed a resistivity around 6 $kW\,cm^{-1}$. Then, 5 mm wide × 40 mm long strips were cut (Fig. 1A) and were sandwiched between two adhesive tape masks, leaving an end of the PEDOT: PSS filter paper (5 × 5 mm) and the wire connection zone previously covered with copper tape (Fig. 1B). Then the PVC-COOH based membrane was dropcast of the on the non-covered area (C). For that, 35 μL of membrane cocktail were dropped in five successive additions, 7 μL at once, three in the front and two in the back of the conductive paper strip. A waterproof material was obtained following this procedure because the conductive paper microfibers were embedded by a PVC-COOH polymer membrane. The last two steps were performed to insulate the electrode body. A circular window with 2 mm diameter, which was the active surface area of the biosensor, was exposed on the front of the electrode (D). A small strip of filter paper, which served as a reservoir for the internal solution, was placed on the backside of the sensor membrane and the PSE was sealed with insulating tape (E).

2.4. Biofunctionalization of the paper-strip electrodes

The functionalization of PSE with the capture antibody was made resorting to two different IMS configurations, represented in Fig. 2. In IMS1, the covalent attachment of ST antibodies was made using the carboxylic groups in the polymeric membrane exposed in the front of the bare PBE. As the *O*-acylisourea formed by the reaction of EDC alone is very unstable, a carbodiimide coupling protocol using EDC along with NHS reagent was used, whereupon the NHS-ester is more stable at physiological pH values. A two-step protocol was used to prevent self-conjugation between the antibodies (Booth et al., 2015). The mixture was prepared in a molar ratio of 1:7 (EDC: NHS) in 0.1 $mol\,L^{-1}$ MES buffer at pH 6 and used just after its preparation in a 20 min activation protocol. The excess of EDC was eliminated with two washing steps, with MES and water respectively. Next, the antibody loading was promoted by the incubation of the antibody against ST (Ab) trough dropcast of an Ab solution (diluted 1:100 in PBS pH 7.4, 1 $\mu g\,mL^{-1}$, 20 μL) for 2 h, at 4 °C in a moist atmosphere.
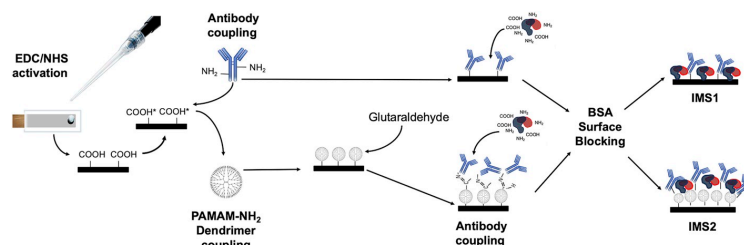
In a second approach (IMS2), a PAMAM$(NH_2)_{64}$ dendrimer was also used in the immunosensing interface. Herein, the PAMAM$(NH_2)_{64}$ dendrimer (0.5 wt% in MES buffer) was coupled with carboxylic groups in the polymer membrane through EDC/NHS in a two steps reaction, in similar conditions previously used for antibodies load. The second modification step was performed resorting to glutaraldehyde, which is a bidirectional crosslinker, can react with the terminal primary amines from a PAMAM$(NH_2)_{64}$ dendrimer and the amine moieties from antibodies. At optimized conditions, 20 μL of glutaraldehyde solution (1 wt %, in 0.01 M PBS pH 7.4) was cast on the sensor membrane and reacted for 10 min Following a washing step with MES and incubation with the Ab (diluted 1:100 in PBS 0.1 M pH 7.4, 1 $\mu g\,mL^{-1}$, 20 μL), during 2 h, at 4 °C in a moist atmosphere. Afterwards, to avoid non-specific interactions in both configurations, a BSA solution (1% in 0.01M PBS pH 7.4, 20 μL) was cast on the sensor surface and allowed to react for 30 min. A washing step with PBS at the end of these two final steps was taken.

Since the modification steps were performed, the electrodes were filled (in the backside) with a tetrabutylammonium chloride (TBACl) solution (14.4 mM in PB pH 7, ≈100 μL) until the paper filter stays bridged by the solution and were kept at 4 °C in a moist atmosphere until use. These parameters were chosen upon experimental optimization of the sensor response towards TBACl using both conventional and paper-based sampling, in parallel with stir and conditioning effect studies (Supporting Information, S1).

2.5. Immunosensing interface characterization

The structural and morphological features of the developed PSE were characterized by Scanning Electron Microscopy (SEM). The SEM studies were carried out at CEMUP (Centro de Materiais da Universidade do Porto), Porto, Portugal. The SEM images were obtained with a scanning electron microscope Quanta 400 FEG scanning electron microscope, (SEM, FEI, Hillsboro, OR), operated in high vacuum/secondary electron imaging mode using an accelerating voltage of 10 kV and working distances of 9.8 mm. Elemental analysis was performed using the same scanning electron microscope coupled with an energy dispersive spectroscopy (EDS) operated at 15 KV with a detector type SUTW SAPHIRE analysis system of resolution 132.19.

The electrochemical behaviour and step-by-step modification protocol were accessed by faradaic electrochemical impedance spectroscopy (EIS). Experimental details were presented in section S.3 in Supporting Information.

2.6. Potentiometric measurements

The electromotive force (EMF) was measured with an EMF 16 Interface (Lawsons Labs Inc., Malvern) and recorded automatically with the provided software. A double junction commercial reference electrode (Orion 90-02-00) from Thermo Scientific Orion with 0.01 M PB pH 7 solution in the outer compartment was used. All measurements

Fig. 2. Schematic representation of the two immunosensing interfaces (IMS) implemented (IMS1 and IMS2), showing the different modification steps performed to antibody coupling and electrode surface blocking.

were performed at room temperature ($\approx 23\,°C$) in a faraday cage. A magnetic micro-stirrer from Velp was used (150 rpm).

Potentiometric response of the immunosensor towards IgG antigen is based on the EMF change (ΔE) induced by the antigen-antibody surface confined reaction. Control and real sample evaluations were performed measuring the difference between the steady-state potential obtained in a blank solution (PB, pH 7) – named as baseline potential - and the potential shift obtained in the presence of a certain amount of ST cells or non-spiked solutions (positive control or negative controls). Calibration curves were obtained by the standard addition method and presented as the average of three different inter-assay replicas with the respective standard deviation. The analytical limit of detection (LOD) was calculated as 3σ of the baseline noise of at least three replicates experiments. Additionally, the "smallest amount of analyte in the sample that can be reliably distinguished from zero" was experimentally attained, according to the IUPAC guidelines (Currie, 1995) and ISO 11843 ((ISO) 1997, 2003, 2008).

2.7. Juice sample analysis

Apple juice samples purchased in a local supermarket were tenfold diluted with water and artificially contaminated with different amounts of ST cells. Then, a protocol based on filtration through a sterilizing syringe filter ($0.2\,\mu m$ pore size) followed by one washing (100 mL of PB, once) and elution step (5 mL of PB) were taken (Zelada-Guillén et al. 2010, 2013). Finally, the potentiometric immunosensors were used for ST cells detection and quantification in the eluate. Following this procedure, three samples (2 replicas each) with ST cells concentration up to critical infection dose level (50, 100 and 1000 cells mL^{-1}) and control samples (non-spiked) were analyzed.

3. Results and discussion

3.1. Immunosensor development

The development of this label-free immunosensor involves several challenges related to immunosensor design, the development of a reproducible construction procedure of the PSE base sensor, the biofunctionalization of the sensor membrane with an appropriate immunosensing interface and the general assay conditions. All these parameters must be in tune in order to achieve a sensitive and robust analytical method.

3.2. Paper-based cation-selective electrode construction

Most of the common zero-current potentiometric techniques resort to highly specific ion selective electrodes (ISE) able to detect changes in ion activities between their phase boundaries (Xu et al., 2005). In these simplest cases, the response follows the Nernst equation independently of the adsorption phenomena at the membrane surface. Conversely, this work intends to use a label-free sensing mechanism based on the blocking surface principle whereupon a controlled ionic flux is disturbed by a biorecognition event at an ISE polymer membrane surface, triggering an EMF change (Gyurcsanyi et al., 2003; Pawlak et al., 2015). Hence, the PSE development faces an extra challenge of maintaining the practical and functional design of all solid state ISE (De Marco et al., 2008; Ozdemir et al., 2013), despite the need of developing a controlled passive ion flow from the inner side of the sensing membrane towards the sample solution (Supporting Information, S1). To the best of our knowledge, this detection method has never been explored using a paper-based platform.

The foundation of the developed immunosensor is the transducing layer formed by the PVC-COOH polymer membrane and the PEDOT: PSS conductive ink. Fig. 3A shows that after membrane casting, the cellulose paper microfibers were completely and homogeneously coated by the polymer membrane. So, the chosen cocktail volume of $35\,\mu L$,
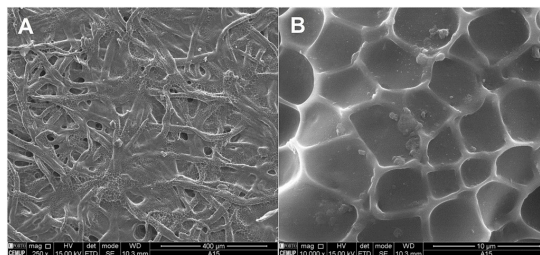
Fig. 3. SEM image of a working area of a PSE coated with the polymer membrane, using two different magnification levels: $250 \times$ (A) and $10000 \times$ (B).

such as the adopted enforcement procedure appears to be enough to waterproof the conductive paper (Ozdemir et al., 2013) and also to maintain the ion permeation capacity (Supporting Information, Fig. S1.2, S1.3). Besides, it was clear that polymer membrane was not only on the paper surface but also embedded into the pores and inter-cellulose fiber spaces, which will protect it from cracking (Fig. 3B) (Knoll et al., 1994). These features were observed in both sides of the conductive paper strip confirming the formation of a triple matrix ion sensitive membrane (Borchardt et al., 1995) and the possibility to use the developed PSE in combination with the surface blocking detection method. In further work, this PSE was used as the base sensor to assemble the two IMS represented in Fig. 2.

Regarding that the sensor membrane has to be suitable for bio-receptor immobilization, PVC-COOH was chosen because it provides free carboxylic groups to further modifications despite the known possible drawbacks in ion-sensor specificity (Xu and Bakker, 2009). Indeed PVC-COOH has a hydrophilic surface by the carboxylic groups present, but its backbone is hydrophobic (Belegrinou et al., 2011; Pawlak et al., 2015). Therefore, these selectivity issues were thoughtfully minimized choosing a lipophilic marker ion with a privileged position in the Hofmeister series.

3.3. Working sensing mechanism: proofing the theory

The key point to establish and apply the proposed sensing mechanism is to reach a stable steady-state potential. In this specific condition, by mathematical deduction from Fick's first law, any changes in EMF caused by an impediment in marker ion transfer kinetics can be correlated with a specific biorecognition event at aqueous layer (De Marco et al., 2008; Ozdemir et al., 2013). To explore this mechanism, it is necessary to promote a controlled passive flow of a marker ion through the sensing membrane. In conventional ISE operated in zero current potentiometry, the inner reference solution is the source of this ion flow towards the sample solution, but this electrode design poses several practical limitations (Cui et al., 2014; Ding et al., 2017; Lisak et al., 2015). Lisak, G et al. (Lisak et al., 2015) studied the possibility of using filter paper sampling coupled to potentiometric detection with all-solid-state and the sampling filter paper matrix may influence the response of the sensors towards some positive ions. Therefore, the response characteristics of the developed sensors to TBA^+ using this sampling strategy were evaluated (Supporting Information, Fig. S1.1) and the results showed that it is feasible to use a filter paper pad on the backside of the sensor to promote a TBA^+ flow. Fig. S1.3 in Supporting Information shows the changes in electrochemical signal triggered by the outwards flow surface disturbance caused by stir-effect on PSE, using two different TBA^+ concentrations. As expected, the accumulation of the marker ion next to sensor membrane amplifies the EMF step signal obtained, implying a subsequent improvement in immunosensor sensitivity. It was also verified, that the EMF step obtained due stir effect was correlated with the membrane conditioning time and the TBA^+ concentration in the PSE inner

solution. Upon experimental optimization of these parameters, at least 1-day conditioning and a 14.4 mM TBACl were selected for further work (Supporting Information, Fig. S4.1).

Additionally, diffusion profile studies suggests that the solution injected into the back of the PSE acts as the conventional electrode internal solution, in a manner assembled to a microfluidic or flow injection device, creating a passive diffusion profile established from the paper filter reservoir through the polymer membrane/PEDOT:PSS/ polymer membrane interfaces to the blank solution, since 20 min was enough to stabilization of EMF in a PB matrix (Supporting Information, Fig. S1.2). Besides the inherent increasing of the steps in the PSE construction, this procedure allowed us to use the zero-current potentiometry technique, that uses unparalleled instrumentation in terms of cost, complexity, and portability when compared with other techniques - like the pulstrode technique - capable to explore ion fluxes through an ESI membrane(Ding et al., 2013; Shvarev and Bakker, 2003).

3.4. Immunosensing interface development

In order to maximize the functionalization degree of the PSE transducing layer, some parameters of the two chosen IMS configurations were optimized: the EDC/NHS concentration used for coupling the –COOH groups of the polymer membrane to the capture antibody (IMS1); the $PAMAM(NH_2)_{64}$ dendrimer concentration (0.5; 2 and 5%, in IMS2); the glutaraldehyde crosslinker concentration and activation time (10 or 20 min, IMS2) with such as the activation method used (two steps or one step, IMS2); the time used for antibody incubation (2 h or "overnight", both IMS) and its concentration (1/50 or 1/100 of Ab 100 µg mL^{-1}, IMS2); and BSA surface blocking step effect (both IMS) (Supporting Information, S2).

The results for IMS1 show that a slight increase of immunosensor sensibility was verified for longer overnight incubation comparing to 2 h, although the benefit doesn't justify the increase in immunosensor preparation time. In IMS2, no significant advantage was verified using a higher antibody concentration, besides the increased surface area. The implementation of the BSA surface blocking step in both IMS studied increases the reproducibility between different modified electrodes. Although, decreased the amplitude of the signal obtained in correlation with the amount of ST added, confirming the surface blocking, greater uniformity of electrode modification despite the slight deceleration of the diffusion gradient through the modified membrane (Supporting Information Fig. S2.1). The optimized functionalization methods were fully described in section 2.2 above.

3.5. Electrochemical impedance spectroscopy characterization

Faradaic EIS characterization was conducted to monitor the impedance and resistance changes of the polymer surface along each modification step. All EIS data were modelled considering the Randle's equivalent circuit and fitted resorting to the electrochemical circle fit analysis tool from NOVA 1.7 in which charge transfer resistance (Rct) values were calculated and used as an indicator of modified electrode interface capability to transfer ions of the redox probe to the electrode surface.

The EIS studies performed allowed to confirm the success in the implementation of successive modification steps in both interfacial architectures (Supporting Information, Fig. S3.1A, B), herein an acceptable inter-electrode modification reproducibility was observed in all steps (RSV < 22.05%). Additionally, for IMS1 was possible to confirm the successful antigen-antibody binging (Supporting Information Fig. S3.1A). This data was later corroborated by SEM images (Supporting Information, Fig. S3.1C).

Regarding IMS2 it was verified a higher change in Rct after the antibody and BSA immobilization steps comparing with results obtained for IMS1, suggesting an effective increase in the electrode area by the incorporation of the dendrimer in the IMS and a higher antibody
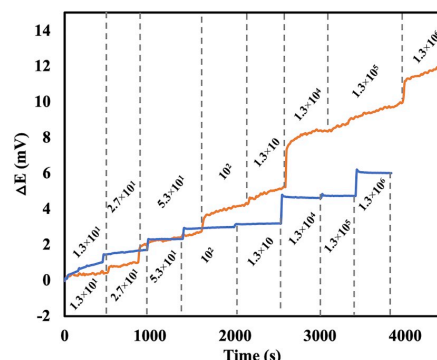
Fig. 4. Dynamic potentiometric measurements showing the response towards the indicated ST concentrations (cells mL^{-1}) in 0.01 M PB buffer. PBE modified with IMS1 (orange) and IMS2 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

load. Despite those, a bad inter-electrode reproducibility of the antibody immobilization step (RSV = 52.64%) was observed.

3.6. Immunosensor performance

The analytical performance of the proposed label-free immunosensor depends on the effectiveness of the biofunctionalization achieved and the integration efficiency of the proposed detection method in the modified PSE. Therefore, to look for the best functionalization method considering the detection method used, preliminary stir-effect experiments with PSE modified with IMS 1 and IMS2 were performed. Fig. S4.1 in Supporting Information shows that both IMS are sensitive to stir-effect after at least 4 h of internal conditioning, demonstrating that they are promising designs to develop a detection method based only on the surface blocking effect, but a lower potential difference was observed for IMS2, due to a denser barrier formed, creating a higher resistance to ionic flow comparing with IMS1 (see the previous section). Herein, successive additions of ST standard solutions (10^4, 10^6, 10^8 cells mL^{-1}) in a stirred PB matrix was carried out and typical dynamic potentiometric response curves of the immunosensors modified with IMS1 and IMS2 are presented in Fig. 4. After each addition, it was clear that the increase in TBA$^+$ concentration in the immunosensor surface vicinity caused by the passive flow blockage was electrochemically translated by an evident and rapid signal increase followed by a potential stabilization plateau.

Comparing the dynamic responses for the two IMS, lower noise level and sensitivity towards ST was observed for the IMS2 modified PSE. This observation can be attributed to changes in IMS structure, caused by the repulsion operated by non-connected dendrimer amine groups (that stays protonated at pH 7), that may transform the dendrimer in a flatter and dislike shape over the analysis time. As $PAMAM(NH_2)_{64}$ dendrimer presents a relative flexibility "container properties", this setback is usually overcome simply by the use of a phosphate ionic background that gone into dendrimer pores creating an inert and stable conformation (Banyai et al., 2013; Scott et al., 2005; Taghavi Pourianazar et al., 2014). Although, should be noted that in this work was used a low concentration of ions in PB (0.01 mol L^{-1}) in that may not be sufficient to block the positive charge (Lee et al., 2005).

Besides the inherent irreproducibility of IMS2 electrodes response anticipated by the EIS studies, a low sensitivity towards ST (slope of 1.0136 mV per decade of cells concentration) was observed (Fig. 5 and Supporting Information, Fig. S4.3). Hereupon, by the need of a sensitive, feasible and accurate method, the IMS 1 was selected to integrate the optimized immunosensor design. Furthermore, experiments with a
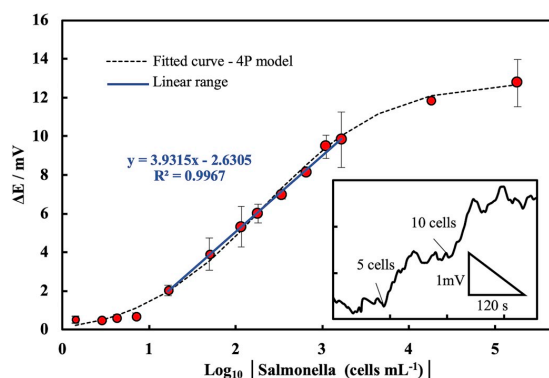
Fig. 5. Calibration curve representing the average values of $\triangle$E (measured potential – baseline potential ($3\sigma = \pm 0.115$ mV)) with the standard deviation values (STD) of 3 different assays (n = 3); Dynamic potentiometric measure at low-level contamination of 5 and 10 cells (insert).

filtered matrix of *Salmonella* dilutions were made, to exclude possible non-specific interactions effects (Supporting Information, Fig.S4.2). None of the tested electrodes reacted to ST additions, confirming that the obtained EMF response is only derived by the biorecognition event between the antibody immobilized on the PSE surface and the ST cells added.

Fig. 5 represents the relation of EMF difference and the logarithm of ST concentration from 1 to 1.28×10^5 cells mL^{-1}. The experimental data were fitted to a 4P sigmoidal model (dashed line in Fig. 5). In the concentration range from 12 to 12×10^3 cell mL^{-1} a linear response translated by equation EMF (mV) = 3.93×-2.63, R^2 = 99.70% was achieved. Under these optimized experimental conditions, the calculated instrumental limit of detection was 115 μV ($3\sigma = 3 \times 38.20$ μV), meaning that higher changes in EMF can be resolved. Nevertheless, even at low-level contamination experiments (n = 3), an unambiguous response was only obtained after inoculation of 5 cells mL^{-1} in a 5 mL sample volume, that corresponds to a Δ EMF = 296.0 μV (inset of Fig. 5).

Considering the disposable character of the developed immunosensor it can be assumed that the obtained reproducibility (RSV = 12.10%, n = 3) was satisfactory. However, it was tried to maximize the sustainability of the method, through the possibility of reusing of the electrode in several measures. Different chemical regeneration agents typically used for chemical regeneration (NaOH, Urea and Glycine-HCl) were employed after first time-use and the remaining immunosensor sensitivity after several regeneration cycles was evaluated (Supporting Information, S4.4) (Goode et al., 2015; Park et al., 2000). Urea solution successfully dissociates the antibody-antigen bond, although, after the second regeneration cycle, the response reduces to 46.00% and 50.49% of the initial response for 10^4 and 10^6 ST cells, respectively.

Long-term stability of the method was tested in ready-to-use immunosensors that were stored at 4 °C in a moist atmosphere until use. After 3 weeks, the response falls to 64.20% of the initial (no longer times were tested).

Table S4.3 in Supporting Information compares the emerging methods in the literature - including other paper-based devices - for foodborne pathogens detection. Comparing with other electrochemical immunosensors (Altintas et al., 2018; Bhardwaj et al., 2017; Burrs et al., 2016; Khan et al., 2018) we can assume similar or higher time resolution of method, such as a higher range of quantification. Lower LOD values were attained with potentiometric aptamer resorting to the SWCNTs and graphene as ion-to-electron transducers (Hernández et al., 2014; Zelada-Guillén et al. 2009, 2010, 2013) and nonporous gold

Table 1
Recovery (%) of *Salmonella typhimurium* (50, 100 and 1000 cells mL^{-1}), in commercial apple juice samples.

Theoretical concentration	Recovery (%)		
(cells mL^{-1})	n	Mean	Coef.V (%)
50	2	45.9	3.7%
100	2	63.0	29.6%
1000	2	53.0	11.0%
Mean	–	54.0	15.0%

Coef.V (%) = coefficient of variation; n = number of samples.

modified platforms (Ranjbar et al., 2018), although this is the first time that such low LOD is reached for foodborne pathogens resorting to a potentiometric paper-based strip electrode. Some colorimetric methods have been used for the range reported, although the quantification at this level of infection can't be addressed (Hossain et al., 2012; Li et al., 2017; Srisa-Art et al., 2018; Suaifan et al., 2017a, b).

3.7. Real sample analysis: proof of concept

To guarantee a low strength ionic background, an efficient separation/purification technique was employed prophylactically prior to the analysis (see section 2.5 above). Next, the results of apple juice commercial real samples analysis were correlated with the calibration curve performed in PB. The recoveries % obtained in samples inoculated with 50, 100 and 1000 cells mL^{-1} of ST are shown in Table 1. The recovery of ST in the range of concentrations 50 to 1000 cells mL^{-1} has an average value of 54.00%, with a coefficient of variation of 15.00% like observed in the buffer (12.00%). Besides the effectiveness of the pre-treatment in sample purification, the modified protocol allows a poor recovery % according to the previous values reported for (80–90%) (Zelada-Guillén et al., 2010). Likely, the use of immunomagnetic separation coupled to the developed immunosensor may improve the recovery % in a simpler way, without increase total time of analysis.

4. Conclusion and future prospects

In this study, we present a label-free potentiometric immunosensor based on surface blocking principle and a zero current passive ion flux developed on a paper-based platform. The device consists of a paper strip ion selective electrode with a carboxylated PVC membrane integrated with a filter paper pad that acts as a reservoir for the internal solution. This design simultaneously provides a platform for antibody immobilization and is a simple and affordable methodology for controlling ionic flow through the polymer membrane.

As proof of concept, an immunosensor for *Salmonella typhimurium* was assembled using this platform and a limit of detection of 5 cells mL^{-1} was achieved in phosphate buffer. This prototype was applied in the quantification *Salmonella* in apple juice with an average recovery value of 54.00% and a coefficient of variation of 15.00%, close to the value obtained in the phosphate buffer (12.00%). Probably, the use of the immunomagnetic separation coupled to the developed immunosensor can improve the % recovery more simply, without increasing the total time of analysis (< 1 h).

The strategy presented in this work is a simple and accessible methodology to control ionic flow through the polymer membrane and shows potential for application to other bio-receptors. In addition to the optimization of the interface architecture with the immobilized bioreceptor, it is possible to design and optimize other devices inspired by the presented prototype, using different types of paper pad, selecting different ion selective polymer sensors and marker ions.

CRediT authorship contribution statement

Nádia F.D. Silva: Investigation, Writing - original draft. **Cláudio**

M.R. Almeida: Investigation. **Júlia M.C.S. Magalhães:** Conceptualization, Supervision, Writing - review & editing. **Maria P. Gonçalves:** Resources. **Cristina Freire:** Funding acquisition, Supervision. **Cristina Delerue-Matos:** Funding acquisition, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111317.

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Supporting Information

Disposable Paper-Based Potentiometric Immunosensor for Real-time Detection of Foodborne Pathogens

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S1. PSE development

Sensor response towards tetrabutylammonium. Carboxylated PVC membranes plasticized with 2-NPOE show potentiometric response towards several cations (i.e. ammonium, sodium and potassium ions) [49]. In this work, the response of carboxylated PVC sensor towards TBACl was also evaluated as this a lipophilic ion well suited to develop gradient based potentiometric immunosensors [50]. A typical calibration curve (n=3) of a pipette tip ISE (33 % wt. polymer with 60 % wt. of plasticizer) against a conventional Ag/AgCl microelectrode in optimized conditions is presented in Fig. S.1.1B(blue). The electrode exhibited a Nernstian response with a slope of 63.2 mV per decade over a concentration range (1.0×10^{-4} −1.0×10^{-1} M) with a limit of detection of 1.28×10^{-5} M. Based on these results, it was decided to explore the potentialities of developing a controlled TBA^+ gradient using the PSE as platform.

Working the sensing mechanism. In order to demonstrate the feasibility of using the filter paper pad to promote the TBA^+ flow from the back of the PSE to its confined layer surface, it was performed an experiment using an adaptation of the methodology developed by Lisak, G *et.al* , in 2015 [51]. For that, a pipette tip ISE was constructed using the same PSE polymer membrane. After, the pipette tip ISE previously optimized using a common sampling were used as working electrode along a conventional Ag/AgCl microelectrode in a two-electrode configuration (Fig. S1.1A), using a filter paper-based sampling (Fig. S1.1B).

Herein, it was observed that pipette tip ISE presented a higher slope and a faster response using the common sampling – 63.2 mV/decade compared with 38.2 mV/decade in paper-based sampling – in the same linear range (1.0×10^{-4}−1.0×10^{-1} M).

B
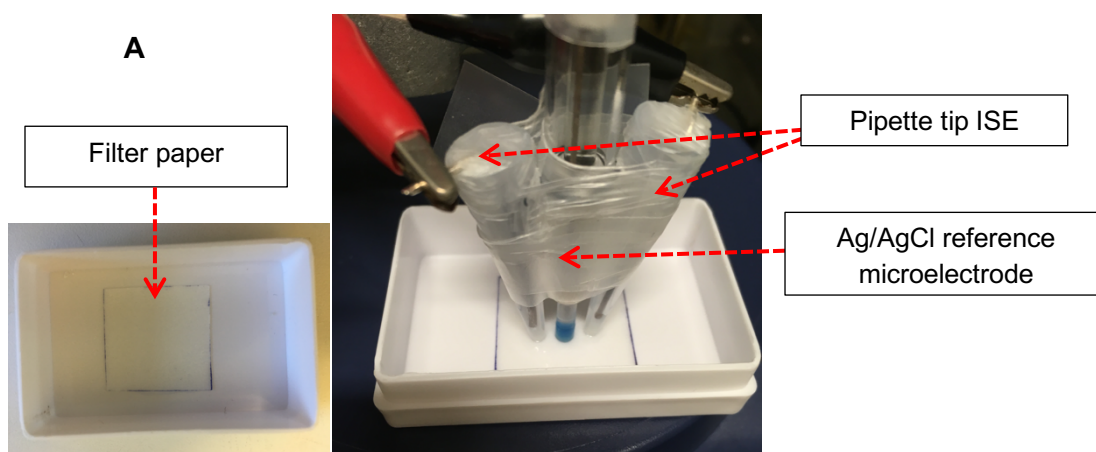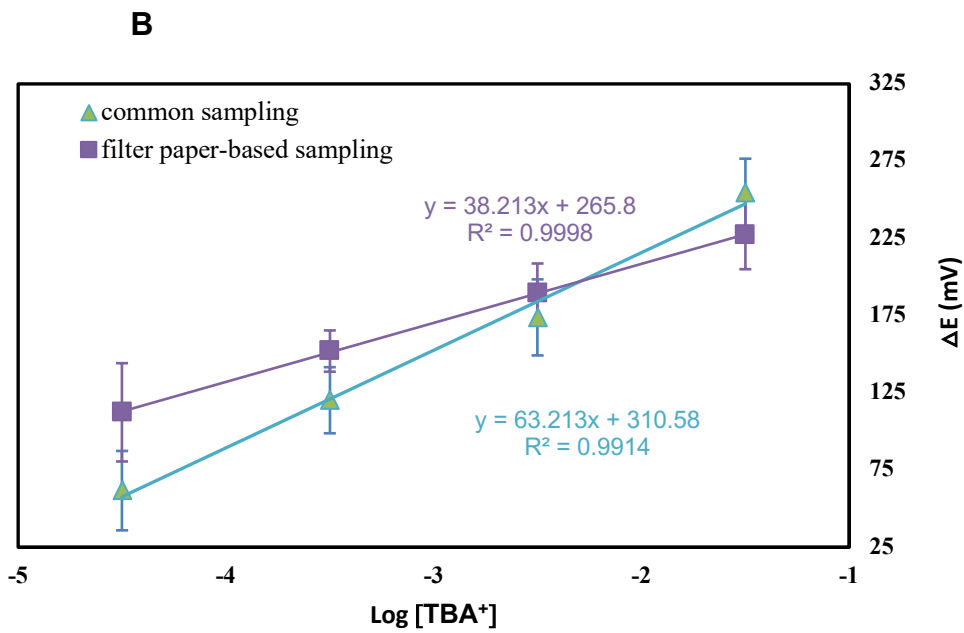
Fig.S1.1 – (A) Assembly used to couple the filter paper-based sampling with potentiometric measurements; (B) Calibration plots obtained resorting to TBACl standards solution made in 0.01 M PB; optimized pipette tip ISE (conditioned in 0.4 mg mL^{-1} TBACl in PB; n=3) using common sampling(blue) or; a filter paper-based sampling (yellow).

Several cations (K$^+$, Na$^+$ and TBA$^+$) diluted in different matrix (water, PB or PBS), with different concentrations (2, 4, 6 and 10 mg mL^{-1}) were tested as electrode inner solution. The TBACl solution with a concentration of 4 mg mL^{-1} dissolved in a PB matrix presented the most reproducible and controlled diffusion profile, due its high lipophilicity and size comparing to sodium and potassium ions. A steady-state was achieved 20 min after immersing the PSE in the PB solution (Fig. S1.2).
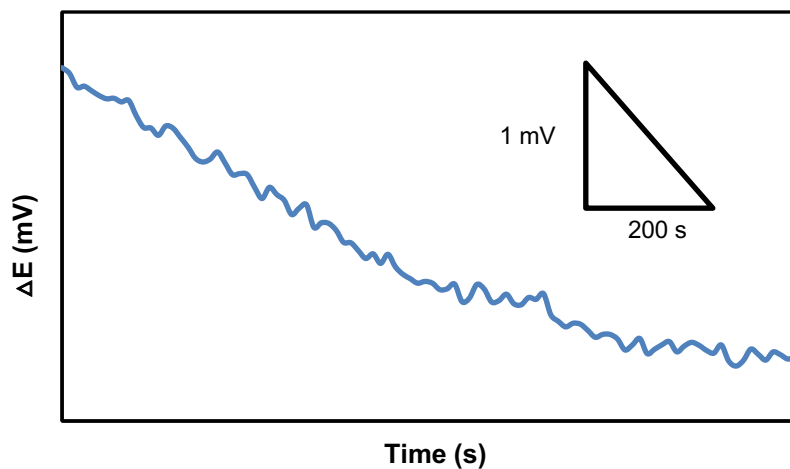
Fig.S1.2 – Passive diffusion profile of the bare PSE with 4 mg mL^{-1} TBACl diluted in a PB matrix as internal solution, obtained just after PSE immersion.

Moreover, stir effect studies confirm the benefit of the presence of TBA$^+$ gradient in the development of an immunosensor based on surface blocking principle (Fig. S1.3). Indeed, it was observed that TBA$^+$ flux amplifies the EMF signal obtained due the disruption of the confined surface layer caused by the stirring changes. Furthermore, the magnitude of the EMF step due to stir effects is related to the TBA$^+$ concentration in the PSE inner solution.
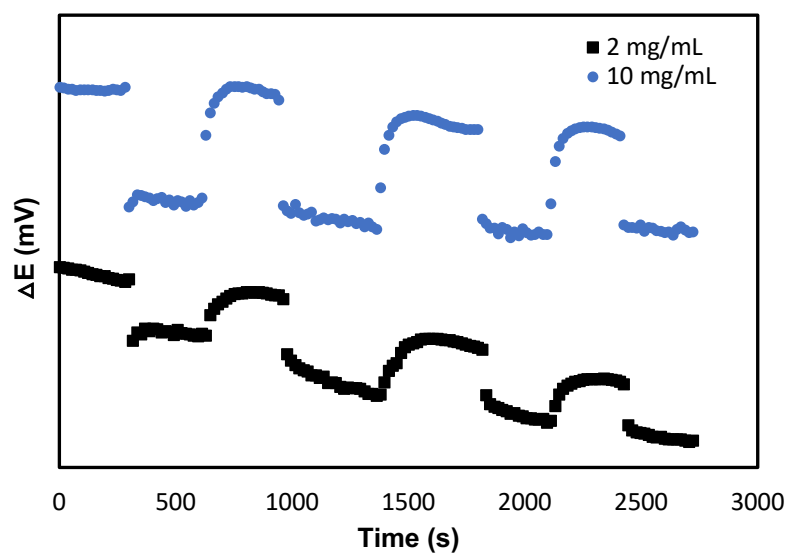
Fig.S1.3 − Stirring effect studies in bare PSE conditioned during the same time with an internal solution of TBACl in 0.01M PB pH 7 with different concentrations 2 mg mL $^{-1}$ (black) and 10 mg mL $^{-1}$ (blue);

S2. Immunosensing Interface Development

Table S2.1. Optimized parameters in immunosensing interface development.

Optimization	Immunosensing interface		Optimized parameter
	IMS 1	IMS2	
EDC/NHS concentration	x		
G4 PAMAM dendrimer concentration (0.5;2 and 5 %, IMS2)		x	0,5%
glutaraldehyde amine groups activation time (10 or 20 min. IMS2)		x	10 min.
activation method used (two steps or one step),		x	Two step activation protocol (GLU activation plus antibody incubation)
antibody incubation time (2h vs "overnight")	x	x	2h
BSA surface blocking effect (with or without blocking step)	x	x	With BSA blocking step (30min)
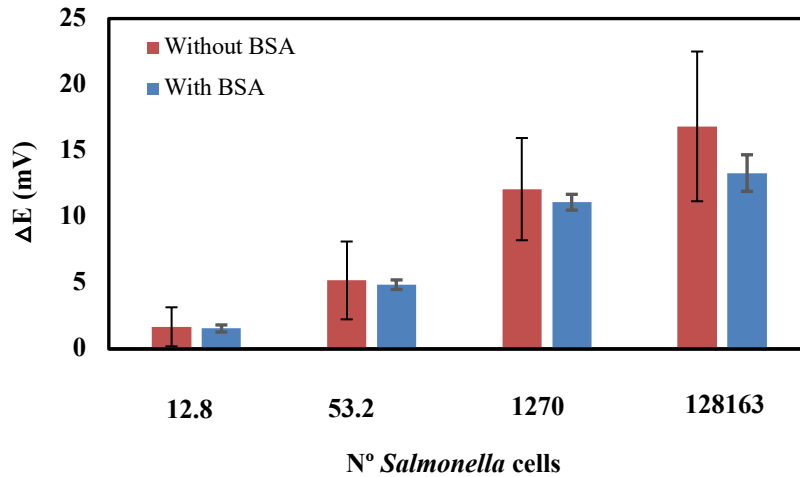
Fig.S2.1 – PSE modified with the IMS1, with (blue) and without (orange) the BSA modification step (n=3). The electrodes were internally conditioned with a TBACl 4mg/mL in 0.01M PB pH=7 solution during about 1.5 days.

S3. Immunosensing platform characterization: EIS

EIS experiments were performed with an Autolab Electrochemical Analyzer (PGSTAT128N, Metrohm). The readings were performed after each modification step at open potential circuit, using 5 mmol L^{-1} of Fe^{4-}/Fe^{3-} probe prepared in 0.1 mol L^{-1} KNO_3 added to a phosphate buffer (PBS) of 0.1 mol L^{-1} $Na_2HPO_4.H_2O$ and KH_2PO_4, pH 7.4, at single modulated AC potential of 411 mV, with an amplitude of 5 mVs^{-1} between 0.1–100 kHz.

All these experiments were made in a one-compartment three-electrode cell system comprising a bare or modified glassy carbon electrode (GCE) with 3 mm of diameter as working electrode, an Ag/AgCl (KCl 3 mol L^{-1}) electrode as reference and a platinum wire electrode as counter electrode. To obtain and process the EIS spectra the NOVA (version 1.7) software from Metrohm Autolab was used.

Typical Nyquist plots include a semicircle region at high frequency and a straight line at lower frequency range. As the goal was to evaluate the effectiveness of electrode modifications only the semi-circle part, controlled by the charge control processes in the interface between the GCE and the electrolyte was presented, excluding the Warburg impedance of the equivalent circuit. Additionally, a common double layer capacitance element was replaced by the constant phase element (CPE) to represent the presence of a non-ideal capacitor and the surface porosity and roughness, that was observed in SEM images [52].

As expected, the drop-casting of the carboxylated PVC sensing membrane on the bare GCE surface increased substantially the medium Rct value (2.41MΩ, ± 0.30 MΩ (n=3)) due to the electrostatic repulsion forces between the charged carboxylic acid groups (COO-) of the polymeric layer and the negatively charged redox probe, and also due to the insulating characteristics of the PVC matrix. Following, the activation of the PVC carboxyl groups by the EDC/NHS couple should originate a neutral ester intermediate, that eliminates the electrostatic repulsion from the previous step modification, facilitating the electron transfer, but originating also a high Rct value (1.05 MΩ; ± 0.10 MΩ (n=3), ΔRct= - 1.36 MΩ) due the presence of the same insulating PVC membrane backbone.

In the next modification steps, as the IMS thickness increased, the charge transfer to the GC electrode surface was successively more difficult due to the blocking effect caused consecutively by the antibodies, the BSA molecules and the antigen. The rise in Rct values confirms the assembling of antibody (1.72 MΩ; ± 0.19 MΩ (n=3), ΔRct= 0.689 MΩ) and the surface blocking by BSA (2.06 MΩ; ± 0.45 MΩ (n=3), ΔRct= 0.34 MΩ). The antibody-antigen complexation at two ST concentrations (2.89 MΩ (n=1), ΔRct = 0.828

MΩ for 10000 CFU and 2.96 MΩ (n=1), ΔRct= 0.070 MΩ for 20000 CFU) was also detected by an impedance increase. Figure 4-C shows a SEM image of three ST cells captured on the optimized immunosensing layer (IMS1) after the incubation with 10000 CFU of ST. The shortening in Rct differences between the several steps, can be related to the different concentration and size of the immobilized biomolecules, as the membrane permeability decreases until complete fouling that the several modifications can cause.

The first two modification steps (casting of the PVC-COOH membrane and EDC/NHS membrane esterification) are identical in the two immunosensing interfaces studied but in IMS2 a dendrimer layer was assembled on the EDC/NHS activated membrane. Upon introduction of this layer the electrode impedance decreases, despite the presence of a ticker barrier between the redox probe and GCE surface (0.19 MΩ ;± 0.08 MΩ, ΔRct=-0.85 MΩ). This observation can be explained by the shape and size of the dendrimer (hyper-branched structure with a high structural and chemical homogeneity, ± 4.5 nm), which enlarges substantially the area of the electrode and reduces the charge repulsion introducing 64 primary amines groups of the dendrimer instead of the carboxylic groups of the PVC [53, 54]. The pre-activation step with glutaraldehyde (0.32 MΩ; ± 0.69 MΩ, ΔRct=0.13 MΩ), the antibody immobilization (1.34 MΩ;± 0.71 MΩ, ΔRct=1.02 MΩ) and the BSA surface blocking step (3.11 MΩ; ±0. 27 MΩ, ΔRct=1.77 Ω) originate a successive increasing in the impedance of the modified electrode.

A

Legend:
- PVC_membrane
- PVC+EDC/NHS
- PVC+EDC/NHS+Ab (1/100)
- PVC+EDC/NHS+Ab(1/100)+BSA
- PVC+EDC/NHS+Ab(1/100)+BSA+ 10000 cells
- PVC+EDC/NHS+Ab(1/100)+BSA+ 20000 cells

B

Legend:
- PVC_membrane
- PVC+EDC/NHS
- PVC+Dendrimer_0,5%
- PVC+Dendrimer_0,5%+GLU_1%
- PVC+Dendrimer_0,5%+GLU_1%+Ab(1/100)
- PVC+Dendrimer_0,5%+GLU_1%+Ab(1/100)+BSA(1%)

C
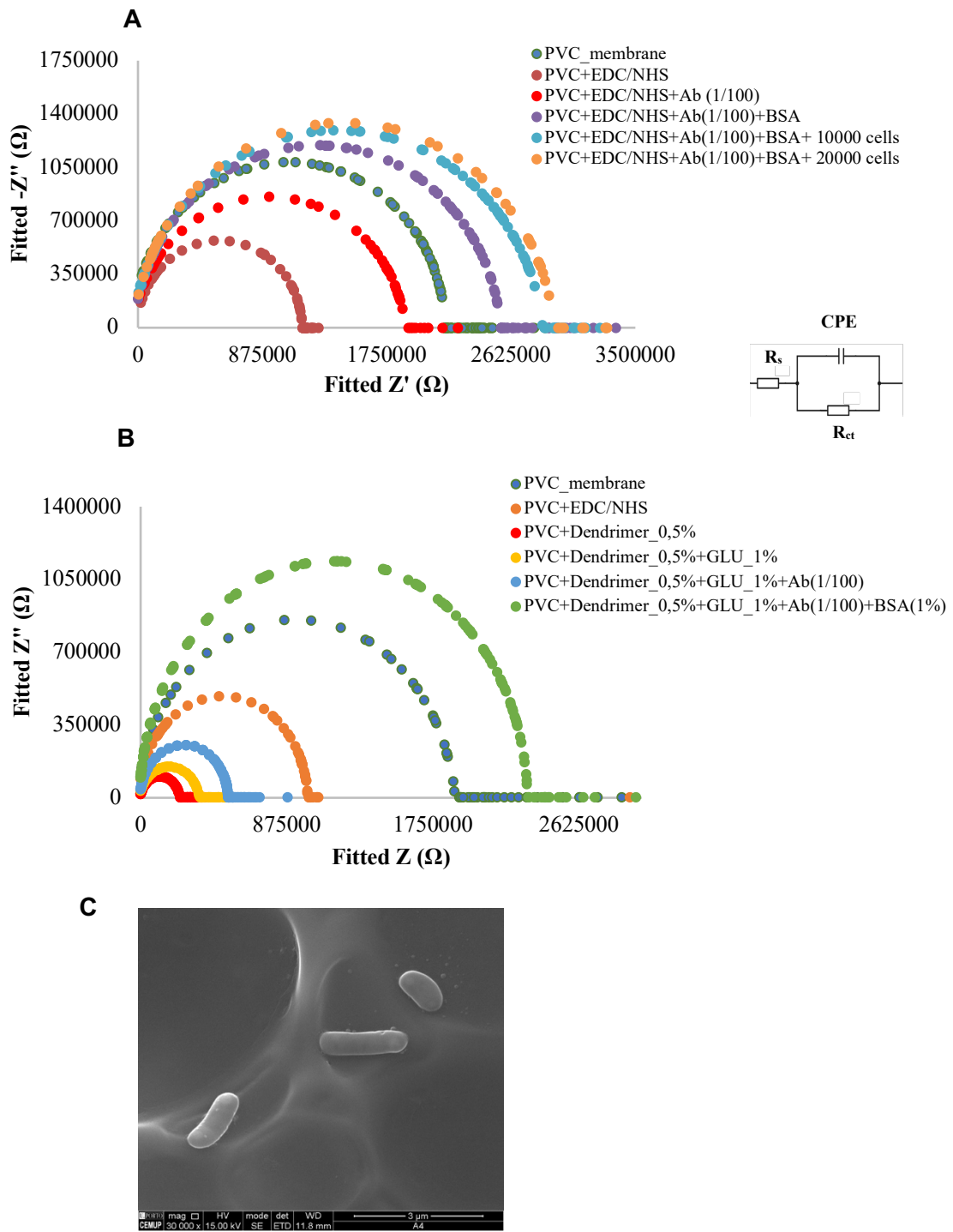
Fig.S3.1 – Nyquist plots of EIS at high frequencies showing the different electrode modification steps performed: (A) in IMS1 with Randle's circuit inset.; (B) and the IMS2 development; all in 0.1 mol L^{-1} KNO$_3$ containing 5 mmol L^{-1} of [Fe(CN)$_6$]$^{3-/4-}$ probe; (C) SEM image of a working area of a PSE modified with the IMS1, and incubated with 10000 cells mL^{-1} of ST.

S4. Immunosensor performance

S4.1. Stir-effect
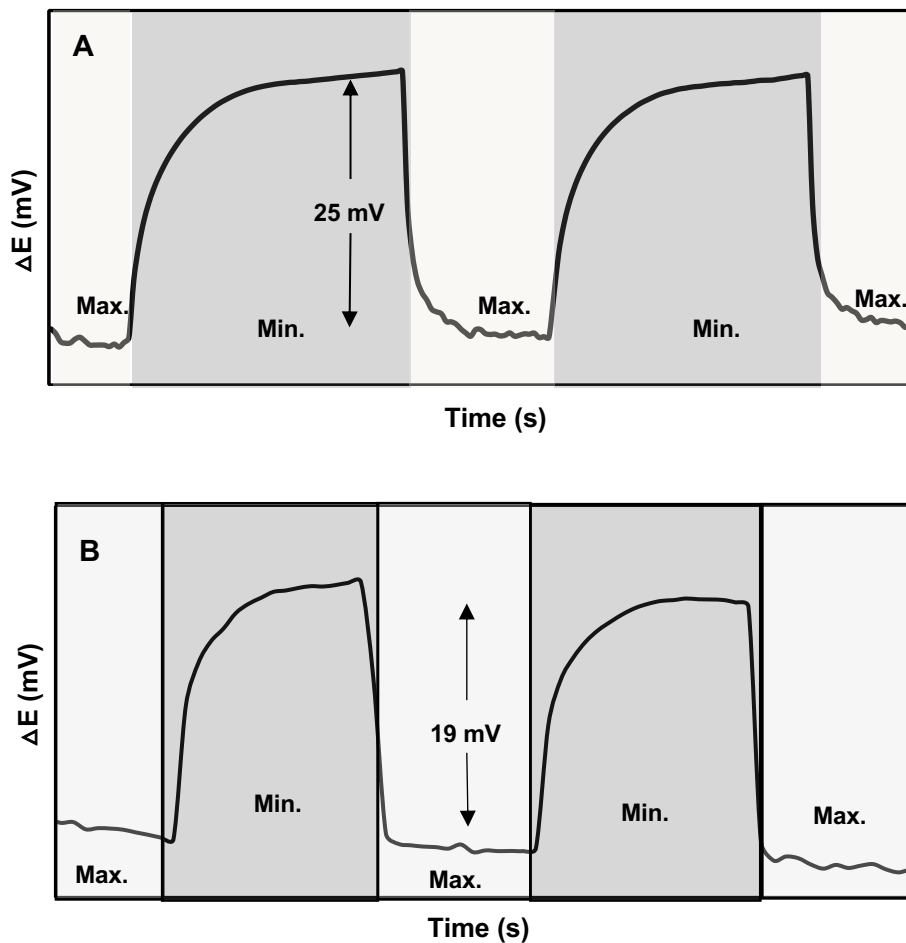
Fig. S4.1 – Stirring effect studies: (A) PSE modified with IMS1 and (C) IMS 2 both with TBACl (14.4 mM, 0.01M PB) during ≈ 24h.

S4.2. Matrix Effect
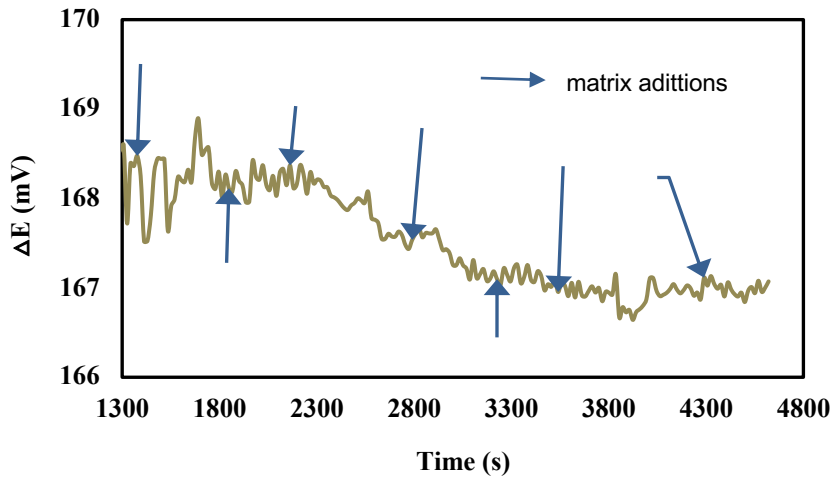
Fig.S4.2 – Matrix effect observed in optimized immunosensor modified with IMS1 and conditioned for 1.5 days with TBACl (4 mg mL $^{-1}$, 0.01M PB) as inner solution.

S4.3. Analytical parameters
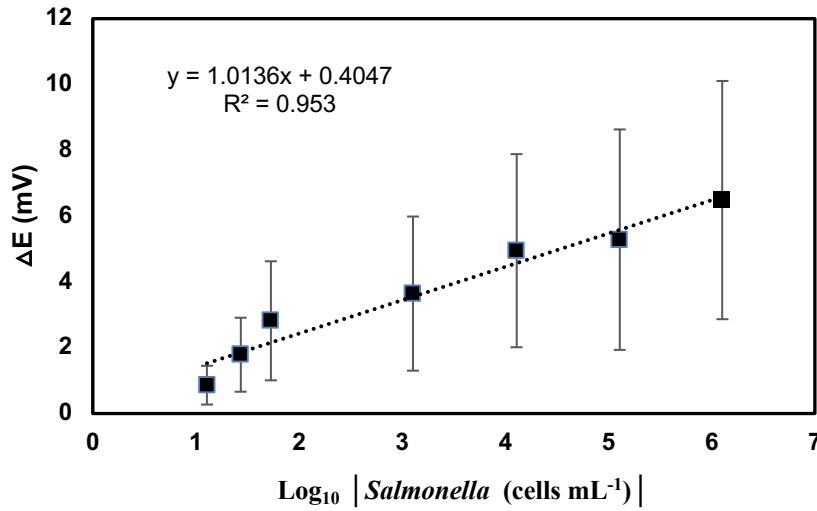
Fig. S4.3 – Calibration curve of PSE modified with IMS2 representing the average values ± the standard deviation values (STD) of 4 different assays (n=4).

Table S4.3 − Electrochemical emerging biosensors for foodborne pathogens detection using common conductive transducers and paper-based platforms.

		Microorganism	Working electrode	Sensor format	Detection Technique	Working range	LOD	Detection Time	Sample	Refs.
Electrochemical sensors	**Common conductive transducers**	*Escherichia coli*	Gold Sensor Chip	Automated Enzymatic immunoassay	A	$10 - 3.97 \times 10^7$ CFU mL^{-1}	50 CFU mL^{-1}	8 min	water	[55]
		Staphylococcus aureus.	GCE	Label-free Aptasensor	P	$1 - 10^5$ CFU mL^{-1}	1 CFU	1 – 2 min	buffer saline	[56]
		Salmonella Typhimurium	GCE/CNT	Label-free Aptasensor	P	$0.20 - 10^3$ cells mL^{-1}	1 cell mL^{-1}	1 min	PBS	[57]
		Escherichia coli	GCE/CNT	Label-free Aptasensor	P	$4 - 2.40 \times 10^4$ CFU mL^{-1}	6 CFU in milk, 26 CFU in apple juice	Real-time to 20 min	PBS and apple juice	[58, 59]
		Staphylococcus aureus	GCE/CNT	Label-free Aptasensor	P	$1 - 10^8$ CFU mL^{-1}	$8 - 10^2$ CFU mL^{-1}	6 – 11 min	PBS	[59]
	Paper-based transducers	*Staphylococcus aureus*	carbon working paper-based electrode	Label-free immunosensor	A	$10 - 10^7$ CFU mL^{-1}	13 CFU mL^{-1}	30 min	spiked milk samples	[60]
		Escherichia coli O157:H7	nanocellulose-graphene-nanoplatinum material	Aptasensor	I	$4 - 10^5$ CFU mL^{-1}	≈ 4 CFU mL^{-1}	12 min	vegetable broth	[61]

	S.mutans, B. subtilis and *E. coli*	Paper/graphene PNIPAm/ID µE	electrically receptive Thermally responsive sensor	I	$10^1 - 10^5$ cells mL^{-1}	5 cells mL^{-1}	6 – 10 min	autoclave water, tap water, lake water and milk	[62]
	Salmonella Typhimurium	**PEDOT: PSS Paper-strip electrode**	**Label-free immunosensor**	**P**	**1 – 1.28 × 10^5 cells mL^{-1}**	**5 cells mL^{-1}**	**5 min detection <60 min assay**	**PB and apple juice**	**This work**
Optical/Colorimetric sensors	E. coli BL21 and *E.coli O157:H7*	sol–gel printed paper-strip	IMS paper-based lateral-flow sensor	Color intensity (analysis by ImageJ software)	$10^1 - 10^5$ CFU mL^{-1}	20 and 5 CFU mL^{-1} in PBS, for E. coli BL21 and for E. coli O157:H7 respectively	30 min – 60 min	PBS, beverage and food samples	[63]
	Salmonella typhimurium DNA	Paper-based microfluidic biosensor	DNA-sensor	Color change/color intensity	1 – 100 nM	1nM	5 min	saline sodium citrate buffer	[64]
	S. typhimurium	distance-based paper devices (chemometer)	Imunomagnetic separation coupled with colorimetric detection	color distance	$10^2 - 10^4$ mL^{-1}	10^2, 10^5 and 10^3 CFU mL^{-1}in culturing solution, bird fecal samples and whole milk	90 min	culturing solution, bird fecal samples and whole milk	[65, 66]
	E. coli O157:H7	Paper-based strip electrode	Peptide-magnetic separation coupled with	intensification of color (naked eye analysis or using	12 – 1.21 × 10^6 CFU mL^{-1}	12 CFU mL^{-1} in broth samples and 30 – 300 CFU mL^{-1}	30 s	ground beef, turkey sausage, lettuce and milk	[67]

			Colorimetric detection	ImageJ software)		in spiked complex food matrices			
	Staphylocous aureus	Paper/gold magnetic strip electrode	Peptide-magnetic separation coupled with colorimetric detection	intensification of color (naked eye analysis or using ImageJ software)	7.5 - 7.5 × 10^6 CFU mL^{-1}, in pure culture broth	7, 40 and 100 CFU mL^{-1} in pure culture, inoculated food produces and environmental samples respectively (upon visual observation)	Few minutes	pure culture, inoculated food produces and environmental samples	[68]

Amperometric (A); Carbon nanotubes (CNT); Colony forming units (CFU); Glassy carbon electrode (GCE); Impedimetric (I); Poly(3, 4-ethylenedioxythiophene)polystyrene sulfonate (PEDOT:PSS);

Poly(N-isopropylacrylamide) polymer/ Interdigitated micro-electrodes (PNIPAm/IDµE); Potentiometric (P);

S.4.4. Immunosensor Regeneration

As happens with other pH regeneration buffers, after the treatment with NaOH it was observed that the electrode needs very long time to recover and achieved to a new steady-state potential, affecting the baseline signal of the sensor [69]. In reverse pH condition (with Glycine - HCl solution) the sensitivity of the sensor also reduces, although not affect so drastically the immunosensor surface charge.   After the second regeneration cycle the response was only a third of the initial in both *Salmonella* concentration studied (10^4 and 10^6 CFU), perhaps by the partially protective action of the glycine when attached to the antibody.

Regeneration agent	Assay Number	*Salmonella* Concentration (CFU)	Initial response (%) [a]
UREA (8M)	1	10^4	100.00
		10^6	100.00
	2	10^4	96.34
		10^6	79.58
	3	10^4	46.00
		10^6	50.49
Glycine-HCl (0.2M pH 2.8)	1	10^4	100.00
		10^6	100.00
	2	10^4	55.62
		10^6	41.00
	3	10^4	32.78
		10^6	29.17

[a] Mean (n=3)

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2.4. A potentiometric magnetic immunoassay for rapid detection of *Salmonella typhimurium*

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Statement of contribution

The contribution of the candidate, Nádia F.D. Silva, in this work includes the literature review, investigation and writing of the original draft.

Analytical Methods

TECHNICAL NOTE

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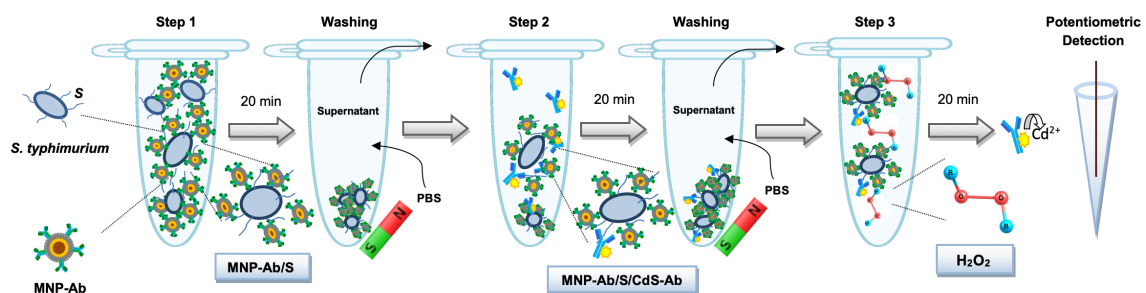
A potentiometric magnetic immunoassay for rapid detection of *Salmonella typhimurium*

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Potentiometric detection with homemade polymeric membrane microelectrodes was coupled to a magnetic sandwich immunoassay for *Salmonella typhimurium* determination. Cadmium and sodium ion selective electrodes were used respectively as indicator and pseudo-reference electrodes and were prepared in pipette tips to allow potentiometric measurements in microliter sample volumes. In the proposed method, the concentration of *S. typhimurium* was proportional to the amount of cadmium released upon dissolution of a CdS nanoparticle labeled to the secondary detection antibody. The limit of detection was 2 cells per 100 μL. The immunomagnetic assay with potentiometric detection is suitable for sensitive and rapid (average total time per assay of 75 minutes) detection of *S. typhimurium* in milk samples. The proposed method is easy to perform, safe, sensitive, and low cost and has potential for *in situ* analysis.

Introduction

Foodborne illnesses are a worldwide public health issue and *Salmonella* is one of the most frequently occurring pathogens in food. Analysis of *Salmonella* is usually based on culture and colony counting and the Polymerase Chain Reaction (PCR).[1–3] These methods have some disadvantages such as the need for intensive work by experts; it takes 2–3 days for the results to be known and up to 7–10 days for confirmation. Therefore, rapid and reliable monitoring methods are critical to ensure food safety. Immunoassays, exploring the selectivity arising from the use of immunochemical interactions, are the largest group of rapid methods used for this purpose.[3,4] Among them, the enzyme-linked immunosorbent assays (ELISA) are the most prevalent.

Immunoassays and immunosensors for pathogen analysis with electrochemical detection (EII), such as amperometry, electrochemical impedance spectroscopy and voltammetry, have attracted considerable interest due to the low cost and high sensitivity of these techniques.[4,5]

Potentiometric EII is scarce in the literature but this detection technique is an attractive tool as it shows an unrivalled simplicity of operation and instrumentation, the electrodes are easily miniaturized and it is possible to analyse turbid samples thus simplifying sample preparation.[6] On the other hand, over the last decade, research on ion selective electrodes (ISEs) has

led to drastic improvements of the limits of detection,[7] extending the applications to trace or femtomole analysis.[7,8]

The sandwich non-competitive heterogeneous immunoassay formats show some advantages for coupling to potentiometric detection because pathogen pre-concentration and separation from the sample matrix prior to detection is possible. This approach was explored in a previous study,[6] where the capture antibody was immobilized on microtiter plates but, to the best of our knowledge, coupling potentiometric detection with ion selective electrodes to labelled sandwich immunoassays using magnetic nanoparticles (MNPs) with immobilized capture antibodies for *S. typhimurium* detection has not been reported before.[4] Nevertheless, using receptors immobilized on MNPs opens new possibilities for further reduction of the volume used to perform the potentiometric measurements in immunoassays as it provides a simple and effective means for enhanced sample pre-concentration. This is a key feature because, in contrast with voltammetric techniques, the potentiometric signal is a direct function of the sample ion activity and is independent of the surface area of the sensing membrane. This characteristic of potentiometric detection has been explored for many years in intracellular analysis with ion selective microelectrodes.[8]

In this work, a cadmium selective polymeric membrane microelectrode (Cd-ISE) was developed and used as the sensor for detection in a labelled sandwich immunoassay. The homemade microelectrodes, the Cd-ISE and a sodium pseudo-reference electrode (Na-ISE), were prepared in pipette tips to allow potentiometric measurements in microliter sample volumes. The immunoassay was based on a sandwich format, where functionalized magnetic nanoparticles (MNPs) allowed us to capture *S. typhimurium* followed by a second binding to an

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antibody labelled with CdS nanoparticles.[9] The proposed method is rapid, safe, sensitive, low cost, and easy to perform and has potential for *in situ* analysis.

Experimental

Materials and apparatus

The potential difference (EMF) was measured with a 780 pH-meter from Metrohm and a potentiometric cell was placed in a Faraday cage. The micro-volume experiments were performed in 1.5 mL Eppendorf tubes. The Ag/AgCl inner electrodes were prepared by anodizing silver wires (AG005160 from Goodfellow) with 1 mm diameter using a power source ISO-TECH IPS-2303T (3 mol L^{-1} KCl; 1 mA cm^{-2}, for 60 s). A double junction commercial reference electrode (Orion 90-02-00) was used for the initial characterization of the pipette tip Cd- and Na-ISEs.

Deionized water (resistivity > 5 MΩ cm; Millipore Elix 3 Advantage) and reagents of pro-analysis quality or equivalent were used. For the preparation of the ISE membranes, 2-nitrophenyloctylether (NPOE), *N,N,N′,N′*-tetrabutyl-3,6-dioxaoctane-di(thioamide) (Cd-Ionophore-I), 4-*tert*-butylcalix[4]arene-tetraacetic acid tetraethyl ester (Na-Ionophore-X), potassium tetrakis(4-chlorophenyl)borate (TCPB), and polyvinylchloride (PVC) from Fluka (Selectophore) were used. A 0.1 mol L^{-1} phosphate-buffered saline (PBS) with 0.1 mol L^{-1} NaCl, with pH adjusted to 7.4, was prepared.

The *S. typhimurium* positive control, containing 5×10^9 cells per mL, was obtained from Kirkegaard & Perry Laboratories and the anti-*Salmonella* monoclonal antibodies (100 μg IgG in 1.0 mL of PBS) were purchased from Santa Cruz Biotechnology. The homemade iron/gold core/shell nanoparticles conjugated with anti-*Salmonella* monoclonal antibodies (Ab-MNP) and the secondary antibodies labelled with CdS nanocrystals (CdS-Ab, containing 1.25 μg mL^{-1} of Ab) used in the immunoassays were prepared and characterized in previous work.[9] Working solutions of the Ab-MNP, CdS-Ab and *S. typhimurium* were obtained by dilution with PBS and were stored at 4 °C until use.

ISE construction and evaluation

The Cd-ISE sensor cocktail was prepared by mixing 2.0 mg of Cd-Ionophore-I, 0.5 mg of the additive (TCPB) and the plasticizer (129 mg of NPOE) with 68 mg of PVC dissolved in tetrahydrofuran (THF). To prepare the Na-ISE sensor, 1.4 mg of sodium ionophore X, 0.4 mg of TCPB, 132.2 mg of NPOE and 66 mg of PVC dissolved in THF were used. The electrodes were constructed in 200 μL pipette tips, which were immersed in the respective sensor cocktail and filled by capillarity to a 7 mm height. The membranes were obtained after THF evaporation at room temperature and shielded from light for at least 12 hours. The Cd-ISEs were filled with a solution containing 10^{-7} mol L^{-1} $Cd(NO_3)_2$ and 10^{-5} mol L^{-1} $NaNO_3$; the conditioning solution was 10^{-2} mol L^{-1} $Cd(NO_3)_2$. For the Na-ISE, 10^{-3} mol L^{-1} $NaNO_3$ was used both as filling and conditioning solutions. The homemade Ag/AgCl electrodes were used as inner reference for both microelectrodes. They were evaluated in accordance with the IUPAC recommendations.[10]

The immunoassay

To start the immunoassay, it was necessary to optimize the dissolution with H_2O_2 of the CdS nanocrystal tags on the secondary antibody. First, to evaluate the time necessary to release Cd^{2+} from these nanoparticle tags, the microelectrodes were immersed in a mixture of 150 μL of 3% H_2O_2 and 5 μL of PBS until a stable EMF was attained.[6,11] Then, 5 μL of a CdS-Ab suspension was added and the potentiometric readings were recorded until a stable response was observed. Then, the characteristics of the potentiometric detection of CdS-Ab (1.25 to 125 ng mL^{-1}) were also accessed with 10 μL of 30% H_2O_2 and 10 μL of the CdS-Ab suspension. For each concentration three replicates were performed, using a reaction time of 20 minutes.

The procedure for *S. typhimurium* quantification is illustrated in Fig. 1. Briefly, 10 or 100 μL of pathogen solution was incubated consecutively with 10 μL of Ab-MNP and 10 μL of CdS-Ab suspension as optimised in a previous study.[9] For the potentiometric determination, the supernatant was removed, the MNP-bioconjugates were washed (with PBS) and the CdS tags were dissolved with 20 μL of 30% H_2O_2 for 20 minutes. Then, the EMF between the Cd-ISE and Na-ISE was measured and corrected for the EMF value in 20 μL of 30% H_2O_2. Blank assays, where the *S. typhimurium* solution was replaced by PBS, were also performed. Two replicates of standard and blank solutions were assayed. A calibration curve of the EMF against the logarithm of the *S. typhimurium* concentration (expressed in cells per mL) was calculated using the four-parameter (4P) model.[12] The limit of detection (LOD) was calculated based on the method described by Tijssen.[12]

This assay procedure was also applied to a commercial bovine milk sample, previously diluted with deionized water (1 : 5) and spiked with *S. typhimurium*. Blank assays using diluted non-contaminated milk were also performed.
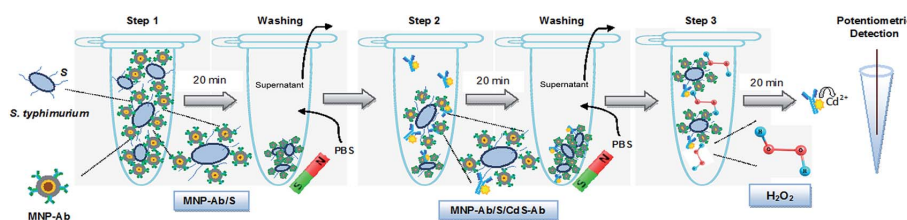

Fig. 1 Schematic representation of the potentiometric magnetic immunoassay for *S. typhimurium* quantification.

Analytical Methods

Results and discussion

Evaluation of the ISE

Firstly, the homemade Cd-ISEs were evaluated, using a commercial double junction Ag/AgCl electrode and 50 mL standard solution. Conditioning the Cd-ISE in 10^{-2}, 10^{-3} and 10^{-5} mol L^{-1} $Cd(NO_3)_2$ for 5, 24 and 48 hours showed that the best response was obtained after conditioning for 48 hours in the 10^{-2} mol L^{-1} solution. Three consecutive calibrations (1×10^{-5} to 1×10^{-2} mol L^{-1} Cd^{2+}) gave a slope of 25.7 ± 1.2 mV decade^{-1} (average $\pm$ standard deviation), with correlation coefficients (r) equal or superior to 0.998 and a limit of detection (LOD) of $3.0 \times 10^{-7} \pm 2 \times 10^{-8}$ mol L^{-1}. This LOD is close to the value reported by Thürer *et al.*[6] for pipette tip microelectrodes prepared with a different ionophore and is approximately ten-fold higher than the value reported by Wardak[13] for macro-electrodes (membrane with a 5 mm diameter) using the same ionophore but with sensing membranes constituted by multiple layers and containing ionic liquids. Repeatable response was obtained, with a coefficient of variation (CV) of 2.3% for fifteen consecutive measurements of 1.0×10^{-5} mol L^{-1} Cd^{2+} and the average stabilization response time was 40 s. The Cd-ISE maintained these response characteristics for at least three weeks. Similar performance was observed when the sample volume was reduced to 1.5 mL.

Homemade pseudo-reference microelectrodes (Na-ISEs) were used[6] to perform experiments using 20 μL of the sample. The Na-ISE showed a linear response ($r > 0.999$) towards sodium in the concentration range from 10^{-5} to 10^{-3} mol L^{-1} with a slope of 60 mV decade^{-1} and high stability (±0.2 mV min^{-1}). The operational lifetime of Na-ISE was around three weeks.

As we intended to explore pathogen pre-concentration from the samples it was decided to evaluate the performance of the ISE using 20 μL volumes of standard solutions. It was observed that the response of the Cd-ISE was linear ($r \geq 0.997$) between 10^{-5} and 10^{-3} mol L^{-1}, with a mean slope of 28.0 ± 0.9 mV decade^{-1} ($n = 4$) and a LOD of $5.8 \times 10^{-7} \pm 3.3 \times 10^{-7}$ mol L^{-1}. The results showed that an accentuated reduction of the sample volume rendered a slight increase of the LOD. However, the minimum amount of Cd^{2+} detected (*ca.* 1.1×10^{-12} mol)

is well below the value obtained for the 50 mL assays (*ca.* 2×10^{-8} mol).

Dissolution of CdS tags

To develop the immunoassay, it was necessary to optimize the dissolution of Cd^{2+} from the nanoparticle tags of the secondary antibody. The chosen procedure for the dissolution was oxidation with H_2O_2. This decision was supported by the favourable results reported by Numnuam *et al.*[11] for CdS tag dissolution in potentiometric detection of DNA hybridization. Thürer *et al.*[6] also used H_2O_2 to dissolve CdSe tags in a potentiometric immunoassay of mouse IgG. Typical results obtained for the dissolution of CdS-Ab are presented in Fig. 2 and show that after 12 minutes a stable potential reading was obtained. No interference was observed on the ISE response using H_2O_2 and the dissolution of the crystal was fast.[6]

Considering that a minimum volume of the solution to be quantified will conduce to a minimum amount of analyte detected, and that increasing concentrations of H_2O_2 accelerates the kinetics of dissolution,[6] it was decided to perform some experiments mixing 10 μL of the CdS-Ab suspensions with 10 μL of 30% H_2O_2. Twenty minutes was the time selected to ensure the dissolution of the CdS tags in 20 μL of H_2O_2. The good repeatability of the response obtained in these experiments is evidenced in Fig. 3 and showed good perspectives to achieve the potentiometric detection of *Salmonella*, lowering the minimum amount of Cd^{2+} detected.

Determination of *S. typhimurium*

To evaluate the effect of the *Salmonella* sample volume on the performance of the immunoassay, calibration curves using 10 and 100 μL were studied (Fig. 4). The LOD using a sample volume of 10 μL was 1100 cells per mL. As expected, increasing the sample volume to 100 μL led to a more sensitive response towards the pathogen and the LOD was 20 cells per mL. These LODs are of the same order of magnitude as those reported recently in the literature coupling voltammetric detection to
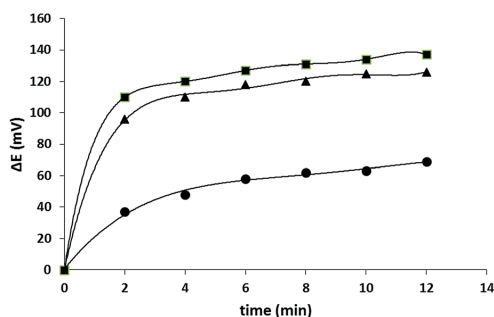
Fig. 2 Representative monitoring plots, with the Cd-ISE, of the dissolution of CdS-Ab suspensions (5 μL) ([CdS-Ab]: ● 6; ▲ 25; and ■ 125 ng mL^{-1}) in 150 μL of 3% H_2O_2 mixed with 5 μL of PBS.
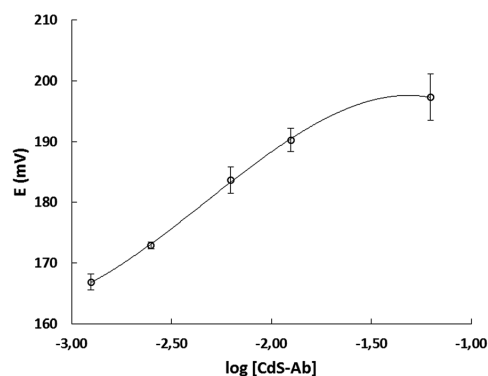
Fig. 3 Potentiometric response (average of three replicates and standard deviation) in 20 μL volume of CdS-Ab suspensions (μg mL^{-1}).
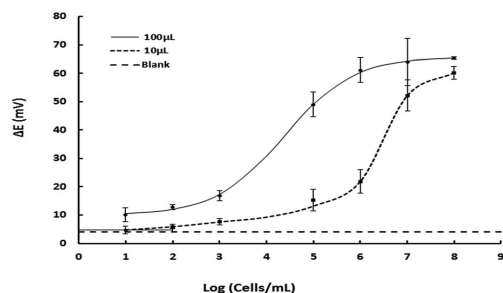
Fig. 4 Calibration plot potential difference (average of two replicates with standard deviation) *vs.* log[*Salmonella*] (10 to 1×10^8 cells per mL) for the sample volume indicated and blank assay.

immunoassays involving MNP and nanoparticle tags.[9] Nevertheless, in this work, potentiometry, characterized by its unrivalled simplicity of operation and instrumentation, was successfully used for detection.

The average recovery values obtained using 100 μL aliquots of contaminated commercial bovine milk were 84 and 104% respectively for samples with 1×10^2 and 1×10^3 cells per mL (CV < 5%). The results showed the applicability of the method to detect low *S. typhimurium* concentrations, in samples with a more complex matrix than PBS. The estimated average total time for a complete assay was 75 min.

Conclusions

Optimized potentiometric detection with miniaturized ISEs was coupled to a labelled sandwich immunomagnetic assay and a novel, rapid and highly sensitive method for *S. typhimurium* analysis was developed. A limit of detection of 2 cells per 100 μL of sample was attained, as a result of the unique performance of potentiometric detection in small sample volumes (in the order of 20 μL), the enhanced properties of magnetic nanoparticles for sample pre-concentration and the high specificity of antibody–antigen binding amplified by the introduction of nanocrystal tags. The results showed that potentiometric detection was suitable for sensitive and rapid (average total time per assay of 75 minutes) detection of *S. typhimurium* in milk samples. The potentiometric detection method described in this paper is simple and avoids using mercury(II) nitrate, a toxic reagent.[9] The developed potentiometric magnetic immunoassay could be applied to the control of food samples *in situ* owing to the simplicity of the procedure and the characteristics of the potentiometric instrumentation.

Acknowledgements

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CHAPTER 3   Electrochemical Biosensing of *Listeria monocytogenes*

3.1. Emerging electrochemical biosensing approaches for detection of *Listeria monocytogenes* in food samples: an overview

3.2. Electrochemical immunosensor towards invasion-associated protein p60: an effective strategy for *Listeria monocytogenes* screening in food

3.1. Emerging electrochemical biosensing approaches for detection of *Listeria monocytogenes* in food samples: an overview

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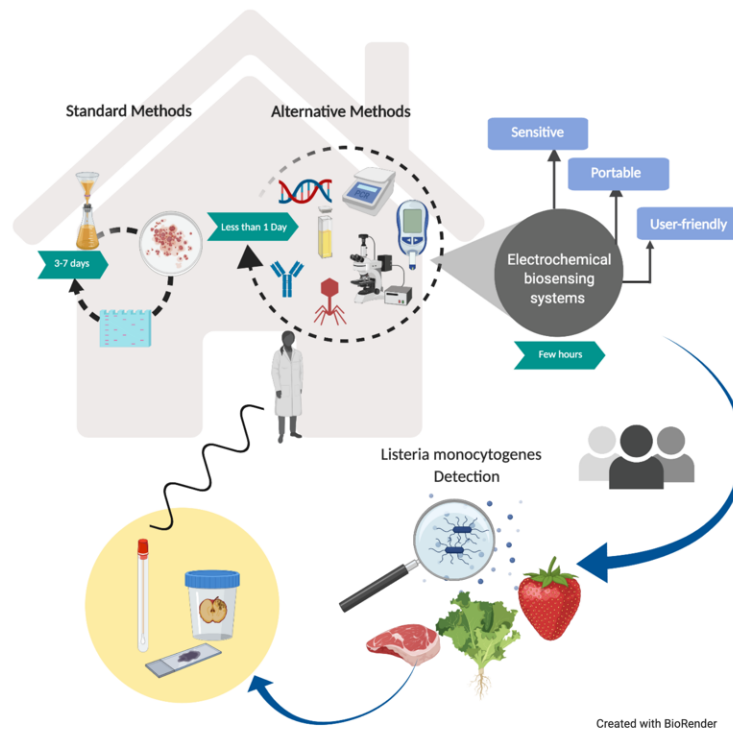
Statement of contribution

The contribution of the candidate, Nádia F.D. Silva, in this work includes the literature review, and writing of the original draft.

Emerging electrochemical biosensing approaches for detection of *Listeria monocytogenes* in food samples: an overview

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Abstract

Background:

Colony plate counting still remains the "gold standard" procedure for the identification of viable *Listeria monocytogenes* cells and to address microbiological quality assurance of foodstuff. However, this classical method lacks sensitivity and also requires an analysis time superior to the current demands in food industry. Hence, new time-enhanced methods based on improved cultural techniques, nucleic acid detection and immunological assays have raised as new validated alternatives. Nevertheless, those rapid methods still require specific and expensive equipment and imply a considerable workload.

Scope and approach:

In the last years, electrochemical biosensors and bioassays have been intensively investigated, emerging as excellent alternatives to surpass the disadvantages of the conventional and standard rapid methods Among others, they provide the desired fast, sensitive and selective response, towards a portable, cost-effective and user-friendly performance. Therefore, in this work, a comprehensive review about the foundations, current achievements and limitations described over the past fifteen years for electrochemical biosensing of *Listeria monocytogenes* in food products is presented.

Key findings and conclusions:

The latest innovations rely on the use of low-cost electrochemical transducers, integration of novel (nano)materials and incorporation of new bioreceptors in the sensing strategy. Single-cell detection and intelligent packaging are also growing trends. Regardless of the remaining challenges, that still need to be overcome, electrochemical biosensing seems to have a role as one of the promising strategies to lead the future of foodborne pathogen analysis.

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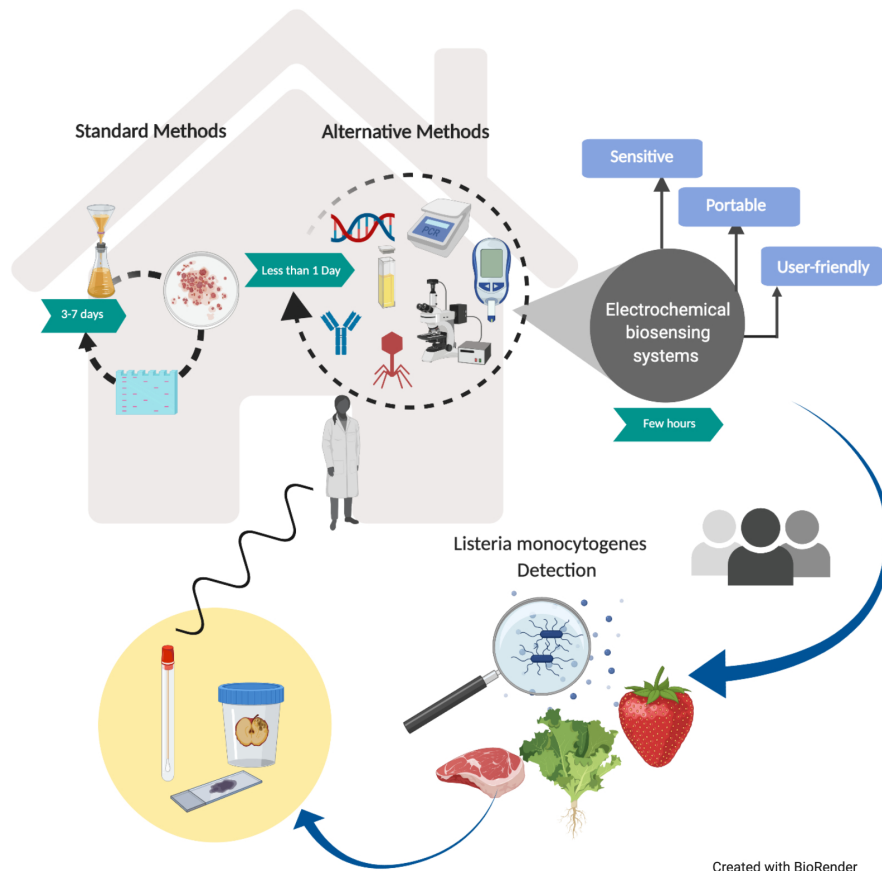
Keywords: Food safety; Foodborne pathogen; *Listeria monocytogenes*; Electrochemical biosensing; Rapid methods; Decentralized analysis

1. Introduction

Listeria monocytogenes

Listeria genus is a rod-shape Gram-positive bacillus that comprises seventeen different species (spp.) (Liu, et al., 2018). Among them, *Listeria monocytogenes* (LM) is responsible for causing listeriosis in humans (Hunt, Vacelet, & Jordan, 2017) and it was classified as an opportunistic, dangerous pathogen, especially for high-risk groups of population like pregnant women, children, elderly and immunosuppressed individuals. Listeriosis could lead to important illness scenarios such as meningitis, fetal anomalies, abortion, febrile gastroenteritis or even generalized infection (Liu, et al., 2018). Despite the low incidence that presents, when comparing to other common foodborne diseases (e.g. Salmonellosis or *E-coli spp*. infections), LM infection is associated to a greater number of hospitalization and a higher mortality rate (20−30%) (Cheng, et al., 2014; Liu, et al., 2018; Soni, Ahmad, & Dubey, 2018). This outcome can be explained by LM ability to grow in an extensive range of food (i.e., dairy products, raw and preserved animal proteins and vegetable products) (Välimaa, Tilsala-Timisjärvi, & Virtanen, 2015) and adverse environments (e.g., within a wide pH range, anaerobic conditions and at low temperatures) (Cheng, et al., 2014; Radhakrishnan & Poltronieri, 2017). Additionally, can also proliferate on food contact surfaces (e.g., stainless steel) (S. Silva, Teixeira, Oliveira, & Azeredo, 2008) while resisting to elevated concentrations of antimicrobial and antiseptic products (Välimaa, et al., 2015).

The contamination of the foodstuffs can occur at different points of the food chain, from production and processing to packaging and distribution, triggering a spoilage process that changes the biochemical properties of the initial product, making their consumption less desirable or even life-threating to consumers (Hameed, Xie, & Ying, 2018). To address this kind of biological hazards, quality preventive programs, such as hazard analysis and critical control points (HACCP) were implemented to evaluate and access the risk level at different stages of the food chain (Umesha & Manukumar, 2018). Moreover, since 2006 European Commission Regulation (EC) 2073/2005 addressed the objective of keeping the concentration of LM in food below the minimum infection dose reported to humans (100 CFU/g of food sample) (Commission (EC), 2005a). However, this limit was only accepted to foodstuffs unable to support the LM growth or right after being placed in the market, because a zero-tolerance policy relative to the presence of LM in ready-to-eat products was recently being implemented in several countries (Commission (EC), 2005b; Soni, et al., 2018). Nowadays, ready-to-eat products are

highly demanded due to its practical aspects that satisfy consumers' habits and lifestyle. Ready-to-eat products are characterized by having a long shelf-life, while stored at low but not freezing temperatures, and as its names indicate, are usually intended to be consumed directly without any previous washing or further cooking (Välimaa, et al., 2015). Due to those conditions, and despite the adequate packaging with a specific atmosphere (e.g., vacuum), these products are more easily contaminated and are therefore a niche in the food market that needs to be closely and strictly monitored respecting to LM presence.

Conventional methods for LM detection

The traditional colony counting plate remain the "gold standard" elected by a majority of food industries for LM identification (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010). In spite the high sensitivity that can achieve (1–5 CFU/25 g) and relatively low-cost (Välimaa, et al., 2015), they are negatively punctuated for being a laborious and time-consuming, as are only capable to provide quantitative results within a few days, sometimes just after one week period. Moreover, require trained experts to perform the analysis and involve an elevated workload, within a lengthy process of enrichment, isolation and detection, where automation difficulty would be an option. The elevated reagents consumption, analysis time and human resources involved also contribute to decreasing the cost-effectiveness of these traditional methods. Besides, official cultural methods established by different food safety regulation agencies (International Organization for Standardization (ISO), Food and Drug Administration (FDA), US Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS), and the Netherlands Government Food Inspection Service (NGFIS)) (Liu, et al., 2018; Zunabovic, Domig, & Kneifel, 2011) still require subsequent biochemical, molecular or serological tests to obtain confirmatory results out of presumptive colonies (Välimaa, et al., 2015; Z. Wang, et al., 2010). Moreover, the major difference between those standardized methods is the spectrum of food for which they are effective and validated. Indeed, only the ISO-11290 is regulated for all food matrices (Gnanou Besse, et al., 2019), comprising an overall detection limit of 5–100 CFU/25 g (Churchill, Lee, & Hall, 2006), still distant from the legislated limits of 1 CFU/25 g. According to ISO-11290 associated protocol, five days is the minimum time needed to perform the identification and enumeration of LM in food samples, demonstrating to be inappropriate to carry out

real-time detection in contaminated scenarios or to be applied in an industrial line or across food distribution supply chain for point-of-need high-throughput analysis (Rohde, et al., 2017).

Alternative methods for LM detection

LM high human pathogenicity added to zero-tolerance policies imposed by (EC) No. 2073/2005), has originated a hunt for a perfect tool to their sensitive and rapid screening at a lower cost. To this end, new alternative tests have been developed and established as official methods for the identification of LM in food samples according to the restricted validation procedures imposed by Association of Official Analytical Chemists (AOAC), Association Française de Normalisation (AFNOR), MicroVal ® or NordVal® (Rohde, et al., 2017). A detailed overview about those validated alternative methods, which is out of the scope of the present review, can be consulted in the works of Rohde *et. al.* (Rohde, et al., 2017) and Välimaa *et al.* (Välimaa, et al., 2015). Briefly, the majority of those new tests, generalized as "rapid" methods, consist of enhanced culture methods, acid nucleic acid analysis and immunological based techniques including some biosensors (Rohde, et al., 2017; Välimaa, et al., 2015).

Advanced plate counting methods based on improved culture strategies through the investigation of new media formulations, suppression of enrichment steps and reducing of incubation time are being implemented. For instance, Compass® *Listeria* by SOLABIA S.A.S. presents a "single plate" solution capable of reducing the whole time assay by at least 24 h (Rohde, et al., 2017). However, these methods still require high analysis time and present the possibility of false negatives results by the presence of other similar bacteria, like *Listeria innocua*, or by matrices interferents that can hide LM presence(Zunabovic, et al., 2011). Moreover, the use of highly selective media can jeopardize the method sensitivity(Liu, et al., 2018).

Immunological detection methods have been successfully used along the years for the detection of foodborne pathogens. They are based in an affinity reaction between an antibody and its specific antigen, through one or various epitopes, according to antibody clonality(Hameed, et al., 2018). Among others (e.g., enzyme-linked fluorescent assay, radioimmunoassay and immuno-electrophoretic or lateral flow immunoassay), enzyme-linked immunosorbent assay (ELISA) remains the most used test. Additionally, the conversion of ELISA methodology into commercial kits, also promote a large-scale utilization (Byrne, Stack, Gilmartin, & O'Kennedy, 2009). Regarding validated methods

for LM detection through nucleic acid analysis, traditional polymerase chain reaction (PCR) and real-time (quantitative) PCR are the most prevailing techniques. The widespread of PCR amplification techniques combined with the advances of equipment mechanization allowed to reduce not only the hands-on time but also the actual detection time to less than 24 h or 3–6 h for conventional or real-time PCR, respectively (Auvolat & Besse, 2016; Radhakrishnan & Poltronieri, 2017). Furthermore, other nucleic acid methods such as fluorescence in situ hybridization (FISH) (Fang, et al., 2018; Rocha, et al., 2019) and loop mediated isothermal amplification (LAMP)(Kim, Cho, Seo, Jeon, & Paek, 2012; D. Wang, et al., 2017) are also available (Välimaa, et al., 2015).

Notwithstanding the added value in terms of analysis time improvement, the new proposed non-cultural methods keep important restrictions. They are labor-intensive multistep techniques that generally requires high cost-equipment, as well as qualified staff to perform a non-decentralized analysis (Rohde, et al., 2017; Soni, et al., 2018). Furthermore, immunological based methods require a high amount of samples and overall reagents to perform the analysis, leading to the production of a considerable amount of waste (Soni, et al., 2018). Also, when they are applied in food analysis, often compels the use of filtration, separation/concentration or pre-enrichment steps to increase the sensitivity, which subsequently implies the increase of the total time of analysis to obtain a quantitative result. A pre-enrichment step of 16–24 h is usually required to attain detection limit ranges from 10^3 to 10^5 CFU mL^{-1} (D. Wang, et al., 2017). In terms of sensitivity, nucleic acid methods vanquish immunological-based assays, however, on the other hand, matrix interferences and false positives are also more pronounced in those amplification techniques (Farooq, Yang, Ullah, & Wang, 2018; Law, Ab Mutalib, Chan, & Lee, 2015; Liu, et al., 2018). Lastly, the fact that they are not able to distinguish between viable and non-viable cells is a limitation in comparison with conventional methods. To overcome this constraint, some alternatives using DNA staining permeable to dead cells and propidium monoazide pre-treatments are being associated with PCR (Radhakrishnan & Poltronieri, 2017), and LAMP (Fang, et al., 2018; Li, et al., 2017) respectively. The latter also presents an alternative to detect non-cultivable but viable cells, that only will be detected in standard methods that include an incubation period in a recovery media. However, further validation tests are necessary for their application in real scenarios.

According to the aforementioned, to date, and despite the scientific advances, the search for a perfect tool for the sensitive, cost-effective and rapid screening of LM remain of utmost importance. To this end, electrochemical biosensing techniques have been attracting considerable attention due to their simplicity, shorter detection times and

intuitive interfaces, allowing its manipulation by users with different levels of expertise. Thus, biosensors and bioassays with electrochemical detection have the potential to perform an important role as food screening analytical tools. Moreover, the compact format of their transducer surfaces and associated equipment increases the possibility for on-site real-time detection. The decentralization of the analysis would be an outstanding turning point in food industry routine (Radhakrishnan & Poltronieri, 2017).

In this work, it was intended to provide a critical overview of the emerging electrochemical biosensing systems developed for the detection of LM in foodstuff. The scope of this review was limited to the past 15 years of research and development.

2. Promising electrochemical biosensing approaches for LM detection

Biosensing techniques are translated as any procedure that uses a biorecognition element to detect a specific analyte (such as a biomolecule, microorganism, protein, etc.) combined with the transduction of said biological interaction into a quantifiable signal. More precisely, electrochemical transducers are able to convert the interactions between the biological or biomimetic recognition element - which can be immobilized on the transducer surface (biosensors) or not directly attached to it (bioassays) – and the target analyte, into a measurable electrical signal proportional to the analyte concentration in a simple and rapid way (Bettazzi, Marrazza, Minunni, Palchetti, & Scarano, 2017; Hammond, Formisano, Estrela, Carrara, & Tkac, 2016). In comparison with other transduction techniques, electrochemical biosensors have the advantages of not being restrained by the properties of the sample, such as colour or volume (Ricci, Adornetto, & Palleschi, 2012; Soni, et al., 2018). In fact, due to the continuous scale-down of electrochemical transducers and instrumentation, sample volumes in the range of micro- and nanolitres are being used. Therefore, electrochemical biosensing techniques have been raising as very appealing analytical tools for the development of new applications in a wide range of fields among which are food analysis and food safety (Felix & Angnes, 2018; Pedrero, Campuzano, & Pingarrón, 2009; Ricci, Volpe, Micheli, & Palleschi, 2007). Electrochemical biosensing devices can be categorized by the transducing techniques employed, which can be impedimetric, conductimetric, amperometric, voltammetric or potentiometric (Felix & Angnes, 2018), or according to their biorecognition element (Fig.1). The effectiveness of bioreceptors immobilization, binding availability, sensitivity and selectivity performance, as well as its availability, production-related costs and difficulties, are also key points in the development of highly sensitive electrochemical biosensing methods for foodborne pathogens detection. In recent years, different electrochemical-based strategies, based on traditional and novel bioreceptors for LM, have been reported for the sensitive, fast and accurate detection of this bacteria in food samples.
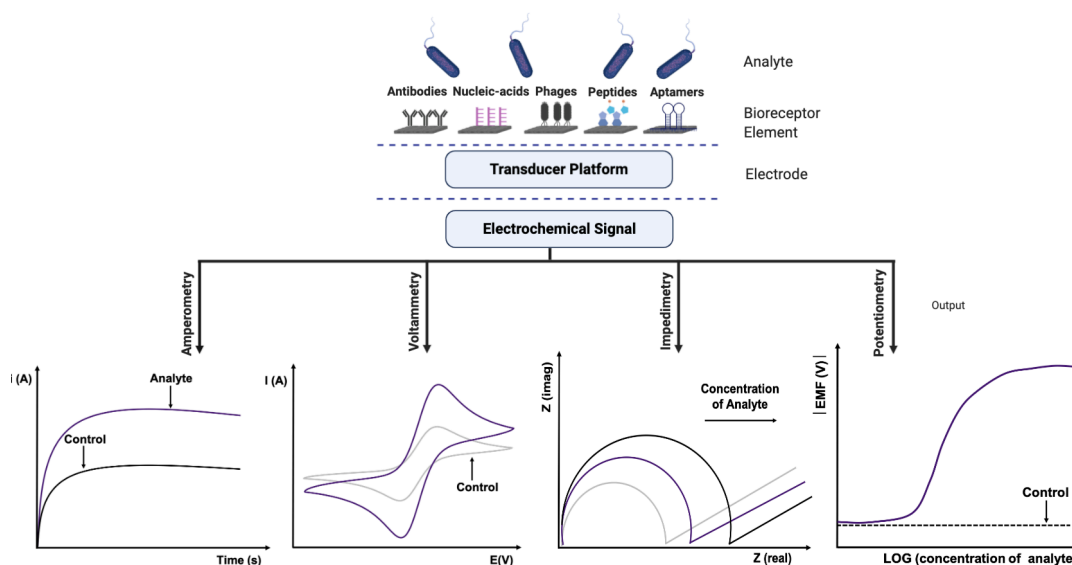
Fig.1 - Schematic representation of the key components of electrochemical biosensing strategies for *L. monocytogenes* detection. Created with BioRender.

Electrochemical antibody-based methods

Antibody-based methods have been successfully used along the years in the detection of foodborne pathogens resorting to electrochemical detection techniques. Table 1 presents a seriation of several electrochemical immunosensors and immunoassays. The majority of the presented immunosensors consist of label-free designs (Radhakrishnan, Jahne, Rogers, & Suni, 2013; Susmel, Guilbault, & O'Sullivan, 2003; Tully, Higson, & Kennedy, 2008; R. Wang, Ruan, Kanayeva, Lassiter, & Li, 2008), using electrochemical impedance spectroscopy (EIS) as transducing technique. By employing EIS in combination with a 'labeless' approach, Radhakrishnan, R., *et al.*, in 2013 (Radhakrishnan, et al., 2013), achieved the best limit of detection (LOD) founded in all the presented works. Using only a 11-mercaptoundecanoic acid self-assembled monolayer (SAM) gold electrode modification for the capture antibody anchorage, the authors achieved an excellent LOD, not only in buffer solution (5 CFU) but also in filtered tomato extract (4 CFU). LM exhibit a large size (0.5 µm×2.0 µm) and contains high amounts of antigen epitopes on the surface (Huang, et al., 2015), which favors their capture in a solid-liquid interface in comparison with smaller analytes. Additionally, the capture of few LM cells can block the charge transfer to the electrode surface, making EIS a plausible electrochemical technique to large bacteria detection (Yang & Bashir, 2008). However, a dramatic fouling effect could also be registered at higher concentrations (Campuzano, Pedrero, Yáñez-Sedeño, & Pingarrón, 2019). Herein, it's

important considering that the electron transfer blocking effect, can be caused directly, by the insulations layer formed by captured LM cells, as well as indirectly, by negative superficial charge that they present, which can repel the redox probe by itself (Susmel, et al., 2003).

The combination of conventional gold electrodes or gold microelectrodes with thiol SAM for covalent coupling of capture antibodies was reported as a promising approach to develop specific and sensitive immunosensors (Lazcka, Campo, & Muñoz, 2007; Ranjbar, Shahrokhian, & Nurmohammadi, 2018). Nonetheless, they are frequently associated with long-time electrode modifications and low stability of thiol-Au bounding, which may invalidate their potential use in real applications. In fact, none of the thiol self-assembled immunosensors for LM detection already published (Cheng, et al., 2014; Radhakrishnan, et al., 2013; Susmel, et al., 2003; R. Wang, et al., 2008), presented stability results. Notwithstanding this fact, the work by Cheng *et al*. (Cheng, et al., 2014) stood out, by developing a reusable device that allowed multi-measurements. More recently, carbon-based nanostructurated transducers demostrated their capabilities to improve the amperometric immunosensors stability and sensibility – even in real samples –, using more stable chemical bonds for antibody loading and resorting to shorter electrode modifications times (Davis, et al., 2013; Y. Lu, et al., 2016). However, despite the indubitable improving in shelf-life, their on-site and real-time application can be limited by the complex multi-step procedures and the need for sample pre-treatments. Three magnetic immunoassays, all from the same research group, with a similar sensing strategy (enzymatic) and transducing technique (EIS) were also reported for LM detection in a real sample proof application (lettuce) (Chen, et al., 2015; Chen, et al., 2016; D. Wang, et al., 2017). In those works, the use of immunomagnetic separation protocols, allowed to separate the LM cells directly from sample preparations simply resorting to a common magnet, and achieving high recoveries. The detection system of the described magnetic immunoassays was based on the products of urea enzymatic hydrolyzation, specifically the carbonate and ammonia ions that were formed in the reaction (Chen, et al., 2015). The increasing ionic strength of the media decreased the resistance at the electrode surface, in a manner that the concentration of LM could be correlated with the decreasing in charge transfer resistance value. Chen *et. al*. (Chen, et al., 2015), applied this detection mechanism using an interdigitated array microelectrode, reaching also a very good LOD of 300 CFU mL^{-1}. One year later, as this electrode presented a good reproducibility and stability (can be reused at least 50 times), its incorporation in an automatic fluidic system was described (Chen, et al., 2016). In addition to the technical improvement achieved due to the automation of the magnetic

assay, it also decreased the time required for the separation of the antigen from the sample and eliminate the tedious cleaning steps of the assay. Furthermore, this approach demonstrated to be more sensitive, with a LOD of 160 CFU mL $^{-1}$. The third related work (D. Wang, et al., 2017) (Fig. 2), did not present technical advances or particular assay optimizations compared to previous ones; still it is noteworthy the improvement in recovery rates and the low-cost of the electrode used. In conclusion, although all the three aforementioned works were still above the limits accepted by food safety agencies, they seemed to be good starting points to develop better and sensitive screening applications. In the future, more detailed studies to investigate stability and shelf life should be made.
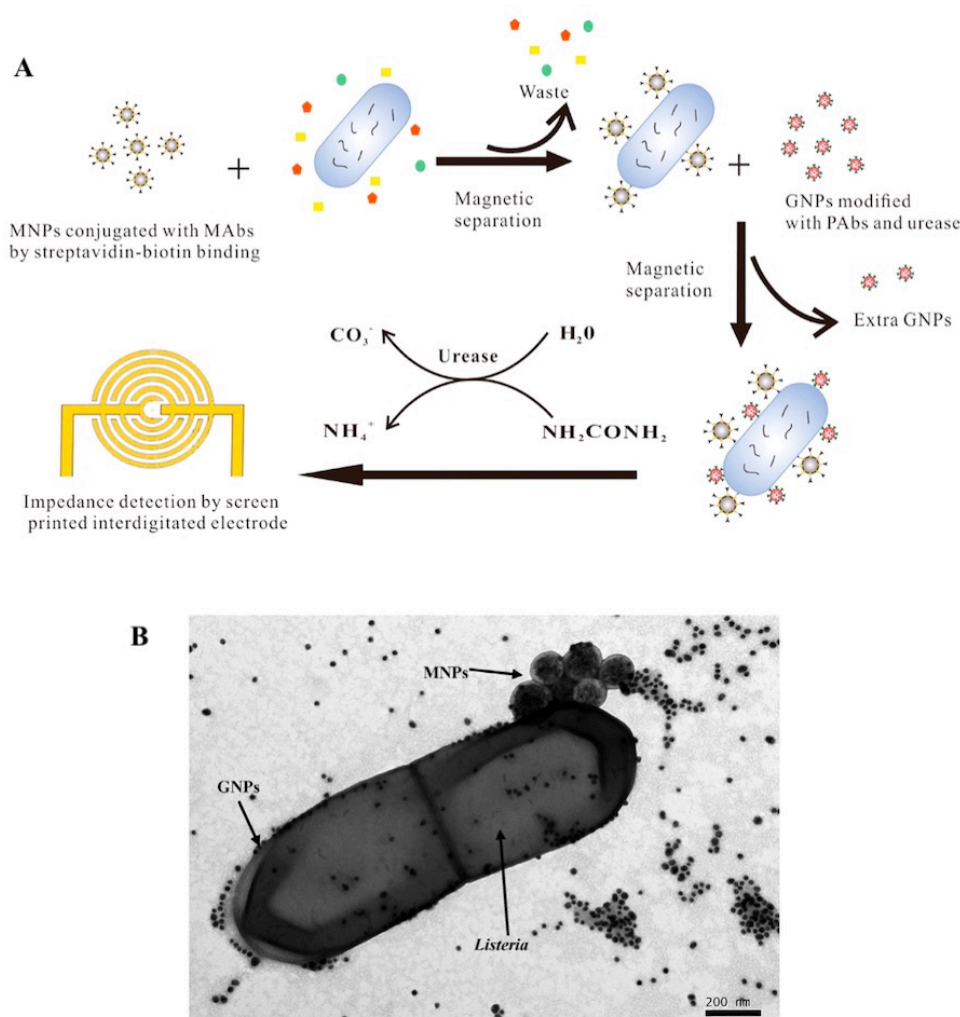
Fig. 2 - Illustration of an impedimetric bioassay with magnetic separation and enzymatic-based detection by using urease modified gold nanoparticles (A). TEM image of a *L. monocytogenes* cell representative of the sensing strategy developed. Reprinted with permission from (Wang, et al., 2017). Copyright (2017) Elsevier.

Regarding the use of antibodies in the development of bioassays it is important to stand out that the major limitation is related both with the production and purification steps,

which can be time-consuming, expensive and low profitable (Skottrup, Nicolaisen, & Justesen, 2008). Ensuring top quality of antibodies is crucial for a successful development that meets the sensing limits imposed by the legislation (K.-M. Lee, Runyon, Herrman, Phillips, & Hsieh, 2015; Nádia F. D. Silva, Magalhães, Freire, & Delerue-Matos, 2018). The existence of a few high-specific antibodies has been probably one of the main drawbacks in the development of antibody-based methods to the detection of LM. Accordingly, when compared with other foodborne pathogens, the number of works reported is reduced, despite its dangerousness to public health (Fig.3).
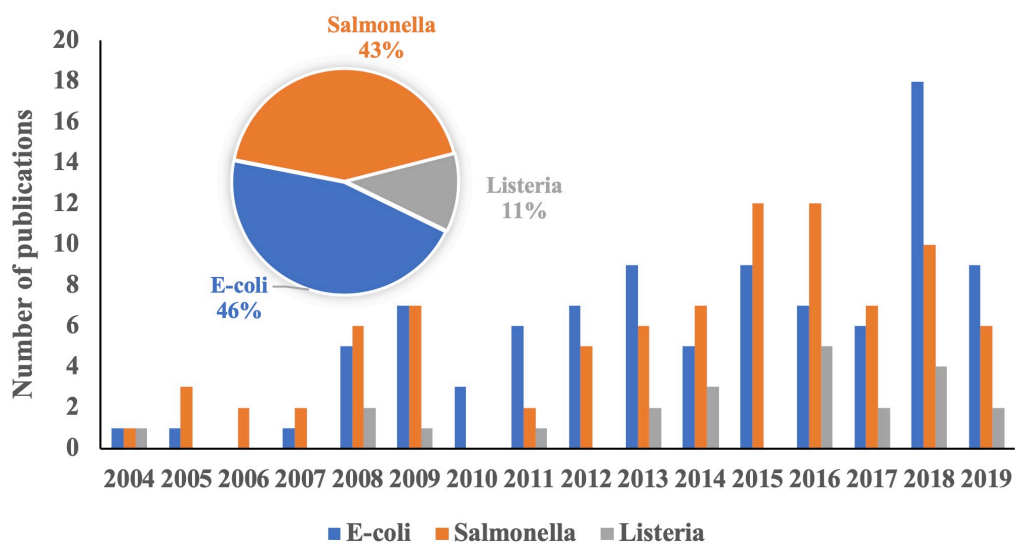
Fig. 3 - Distribution, by microorganism, of the state-of-the art for the detection of pathogenic bacteria: 205 articles were found in last 15 years using the keywords "*Salmonella*", "*E-coli*" or "*Listeria*", followed by "antibody", "detection" and "electrochemical". Source: ISI Web of Science (accessed on 09.09.2019).

Table 1. Electrochemical immunosensors and immunoassays for LM detection.

| Working electrode | Sensing phase[*] (preparation time) | Assay label | | Electrochemical technique | Assay time[**] | Linear range (CFU mL^{-1}) | LOD | Food application | Sample treatment | Reference |
		Type	Preparation time							
GE	13 h 40 min	HRP	n.a.	A	2 h 10 min	$10^2 - 10^6$	10^2 CFU mL^{-1} (in PBS) 10^3 CFU mL^{-1} (in milk)	Milk	-	(Cheng, et al., 2014)
IAME	4 h 30 min	Urease	2 h 30 min	EIS	1 h 50 min	$1.9 \times 10^3 - 1.9 \times 10^6$	1.6×10^3 CFU mL^{-1}	Lettuce	n.s.	(D. Wang, et al., 2017)
SPGE	3 h	Label-free	n.a.	EIS	2 h 30 min	-	≈ 1000 cells		-	(Susmel, et al., 2003)
SPCE electropolymerized with PANi	27 h	Label-free	n.a.	EIS	30 min	-	4.1 pg mL^{-1}	-	-	(Tully, et al., 2008)
TiO$_2$ nanowire bundle gold microelectrode	2 h 45 min	Label-free	n.a.	EIS	50 min	-	10^2 CFU mL^{-1}	-	-	(R. Wang, et al., 2008)
GE	2 h	Label-free	n.a.	EIS	n.s.	-	5 CFU mL^{-1} (in buffer) 4 CFU mL^{-1}	Tomato extract	Yes	(Radhakrishnan, et al., 2013)

							(in filtered tomato extract)			
SPCE	3 h	HRP	n.a.	A	30 min	2.25×10^1 – 2.25×10^5	2.25×10^2 CFU mL^{-1}	Wild blueberries	Yes	(Davis, et al., 2013)
IAME	3 h 40 min	Urease	2 h 30 min	EIS	2 h	3×10^1 – 3×10^4	3×10^2 CFU mL^{-1}	Lettuce	n.s.	(Chen, et al., 2015)
IAME	3 h 40 min	Urease	2 h 30 min	EIS	1 h 5 min	1.6×10^2 – 1.6×10^5	1.6×10^2 CFU mL^{-1}	Lettuce	n.s.	(Chen, et al., 2016)
MWCNT Fibers Electrode	5 h 30 min	HRP	n.a.	CV	30 min	10^2 – 10^5	1.07×10^2 CFU mL^{-1}	Milk	Yes	(Ying Lu, et al., 2016)

Amperometry (A); *Colony-forming unit (CFU)*; *Cyclic voltammetry (CV);* Electrochemical impedance spectroscopy (EIS); Gold electrode (GE); Horseradish peroxidase (HRP); Interdigitated array microelectrode (IAME); Limit of detection (LOD); Multi-walled carbon nanotubes (MWCNTs); Not applicable (n.a.); Not specified (n.s.); *Polyaniline (PANi);* Screen-printed carbon electrode (SPCE); Screen-printed gold electrode (SPGE).

[*] Sensing phase: recognition element immobilization (and preceding treatments related to the immobilization strategy) and blocking step (if applicable).

[**] Assay time comprises the major steps involved in the immunoassay development (i.e., immunoreaction events, labelling (if applicable), and reaction of the enzymatic label with the substrate (if applicable)); the (approximate) assay time was indicated according to the information provide in the consulted manuscripts.

[*/**] Overnight incubations were considered as a 12 h period for comparison purpose.

Electrochemical DNA-based methods

Similarly to antibody-based methods for LM determination, DNA-based ones have undergone tremendous evolution, especially in the time and in the number of steps needed to perform the analysis and in the sensitivity that it presents. In this literature review, the DNA-based detection methods research was limited to the electrochemical DNA-based sensors and assays (table 2). At this point, they are the ones closest to an on-site detection concept, offering the same specificity of PCR-based methods, in a more appealing and user-friendly format.

DNA-based sensors and assays may use a single strand of DNA as bioreceptor to detect a specific gene, or only a specific pathogenically associated sequence of it. Due to the high specificity of binding, it is expected that the phenomenon of hybridization occurs when the capture DNA enters into contact with its target (Kashish, Gupta, Dubey, & Prakash, 2015) and electrochemical transducing techniques are suitable for simple and rapid biosensing. To this end, EIS is often used to convert the hybridization, directly, into an impedimetric signal in label-free formats (Kashish, Gupta, et al., 2015; Kashish, Soni, Mishra, Prakash, & Dubey, 2015), whereas amperometric techniques are often employed into indirect measures, accomplished with the aid of redox mediators (Kavanagh & Leech, 2006; Niu, et al., 2017; Sun, et al., 2012) and enzymatic amplification methodologies (Brandão, Liébana, Campoy, et al., 2015; Kavanagh & Leech, 2006; Liébana, et al., 2016).

Kashish, *et al.* in 2005 (Kashish, Gupta, et al., 2015; Kashish, Soni, et al., 2015) developed two different label-free impedimetric genosensors directed to the hlyA gene that codes the listeriolysin O toxin, widely recognized as one of the major virulence factors of LM (Sharma & Mutharasan, 2013). The charge transfer resistance changes were related to the conformation of the capture DNA after and before its binding with increasing amounts of target. In order to amplify the obtained impedimetric signal, the authors opted for two distinguish methodologies to increase the area of the electrode and the biocompatibility of its surface: platinum nanoparticles dispersed in a biocompatible chitosan matrix and the use of a conducting polymer (poly-5-carboxy indole). While, in the later, poly-5-carboxy indole modified genosensor stood out by its high stability along various cycles of heating and cooling, which allowed carrying out a multi-analysis (Kashish, Soni, et al., 2015); platinum nanoparticle modified electrodes showed an exceptional fabrication reproducibility (relative standard deviation (RSD) < 10%) (Kashish, Gupta, et al., 2015). Despite these, both methods relied on pre-treatment techniques that use non-portable equipment making its on-site implementation

unfeasible. Moreover, Sun, W., *et al.* in 2012 (Sun, et al., 2012) and Niu, X., *et al.* in 2017 (Niu, et al., 2017) developed two similar methodologies to detect LM hly DNA sequence in PCR products from deteriorated fish meat, based on the monitoring of the hybridization reaction using methylene blue (MB) as electrochemical redox indicator and a carbon ionic liquid electrode (CILE) as transducer. MB interact with the phosphate groups present in DNA sequences electrostatically or by intercalation. Accordingly, was assumed that MB presents a greater affinity by a double DNA helix, resulting in higher signals in comparison with those obtained before the hybridization reaction. Additionally, the natural good features of CILE were exalted in both works by the use of derivatives of nanogold and graphene to modify the electrode surface, thus amplifying the electrochemical signal obtained. The work of Niu, X., *et al.* in 2017, (Niu, et al., 2017) reached a more attractive LOD (3.17×10^{-5} nM vs 2.9×10^{-4} nM), resorting to reduced time and fewer modification steps to the DNA biosensor fabrication. The major difference in the time was defined by the exclusion of the SAM construction used in the work of Sun, W., *et al.* for DNA probe immobilization. For that purpose, and by simply changing the order of the electrode modification protocol - gold electrodeposition before graphene partially electropolymerization -, the DNA probe was covalently attached to reduced graphene, which also resulted in a good reproducibility between electrodes (RSD = 3.7%) (Niu, et al., 2017).

The enzymatic amplification allied with DNA specificity is also a common strategy in label-based electrochemical biosensing. For instance, Kavanagh, P. and Leech, D. (Kavanagh & Leech, 2006) developed an enzyme sandwich assay through the non-covalent interaction of the avidin-conjugated glucose oxidase with the biotin modified target DNA sequence, once the hybridization took place. The mediation of glucose oxidation was made resorting to an osmium-based redox polymer. Besides the simplification of labelling process when compared with covalent tagging, and the benefit in terms of stability and reproducibility (CV<3.5%) of the electrode, the long preparation time (more than a day) remained as an important drawback. In any case, and despite its inadequacy to be used in point-of-care applications due to the short shelf-life of the transducer surface, it can be a good choice for multi-sample screening due to its regeneration capacity.

Other interesting alternative for LM DNA sensing was presented, for the first time, by Brandão, D., *et al.* in 2015 (Brandão, Liébana, Campoy, et al., 2015) and Liébana, S., *et al.* in 2016 (Liébana, et al., 2016). In those works, a magneto-genosensing assay using silica magnetic particles as a platform for DNA immobilization combined with PCR tagging amplification and electrochemical detection was proposed. That combination

allowed to reduce the overall assay time for each pathogen using a simple tagging strategy (Fig.4) (Liébana, et al., 2016). Moreover, a triple tagging-PCR multiplexed amplification (Brandão, Liébana, Campoy, et al., 2015) enabled to simultaneously detect and distinguish different bacteria while accomplishing a small LOD. Despite those great advances, more specific DNA sequences, allied with highly sensitive strategies, have to be employed with the aim of minimizing the need for amplification techniques and the inherent high-cost bulky instrumentation associated. Overall, the main drawbacks of most strategies presented are the need to use complex and time consuming (8−48 h) sample pre-treatments (pre-enrichment, DNA isolation, PCR or tagging PCR), before the electrochemical detection, as well as the requirement of high temperatures to denature the DNA during the assay, which also affects the simplicity, readiness and sustainability of the method.
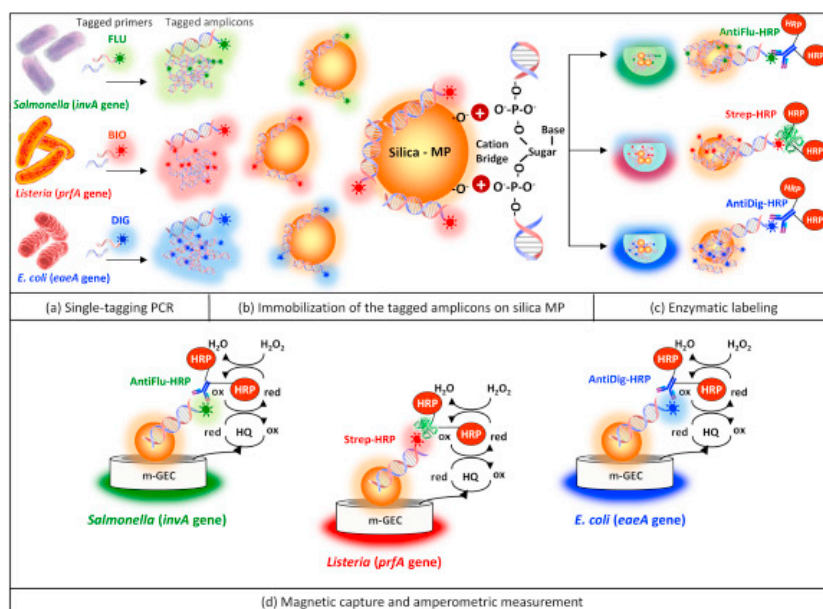
Fig. 4 - Schematic representation of simultaneous electrochemical magneto-genosensing of *S. enterica*, L. *monocytogenes* and *E. coli* by a multiplexed genoassay. Reprinted with permission from (Liébana, et al., 2016). Copyright (2016) Elsevier.

Table 2. Electrochemical DNA-based sensors and assays for LM detection.

Working electrode	Sensing phase[*] (preparation time)	Assay label	Electrochemical technique	Assay time[**]	Linear range	LOD	Food application	Sample treatment	Reference
GCE/PtNP	18 h	Label-free	EIS	20 min	$1.0 \times 10^{-12} - 1.0 \times 10^{-4}$ M	-	Milk	n.a.	(Kashish, Gupta, et al., 2015)
5C Pin-coated GE	5 h 30 min	Label-free	EIS	15 min	$1.0 \times 10^{-12} - 1.0 \times 10^{-4}$ M	2.34×10^{-13} M	-	-	(Kashish, Soni, et al., 2015)
GE	25 h	GOx	A	1 h	$1.0 \times 10^{-9} - 2.0 \times 10^{-6}$ M	0.2×10^{-9} M	-	-	(Kavanagh & Leech, 2006)
Au/GR/CILE	24 h 30 min	MB	DPV	n.s.[1]	$1.0 \times 10^{-12} - 1.0 \times 10^{-6}$ M	2.9×10^{-13} M	Fish	Yes[***]	(Sun, et al., 2012)
p-RGO/AuNPs/CILE	n.s[2]	MB	DPV	38 min	$1.0 \times 10^{-13} - 1.0 \times 10^{-6}$ M	3.17×10^{-14} M	Fish	Yes[***]	(Niu, et al., 2017)
m-GEC	n.a.	HRP	A	51 min[3]	-	12 pg µL⁻¹	-	-	(Brandão, Liébana, Campoy, et al., 2015)
m-GEC	n.a.	HRP	A	60 min[3]	-	13 pg µL⁻¹	-	-	(Liébana, et al., 2016)

Amperometry (A); Carbon ionic liquid electrode (CILE); Difference pulse Voltammetry (DPV); Electrochemical impedance spectroscopy (EIS); Glassy carbon electrode (GCE); Gold electrode (GE); Glucose oxidase (GOx); Horseradish peroxidase (HRP); Limit of detection (LOD); Magnetic graphite-epoxy composite electrodes (m-GEC); *Methylene blue* (MB); Not applicable (n.a.); Not specified (n.s.); Partial reduced graphene (p-RGO); Platinum nanoparticles (PtNP); Poly-5-carboxy indole (5 C Pin); Reduced graphene (RG).

[*] Sensing phase: recognition element immobilization (and preceding treatments related to the immobilization strategy) and blocking step (if applicable).

[**] Assay time comprises the major steps involved in the assay development (i.e., hybridization events, labelling (if applicable), and reaction of the enzymatic label with the substrate (if applicable)); the (approximate) assay time was indicated according to the information provide in the consulted manuscripts.

[*/**] Overnight incubations were considered as a 12 h period for comparison purposes.

[***] PCR amplification.

[1] 10 min of electrochemical detection but not specifies hybridization time.

[2] 30 min of preceding treatments related to the immobilization strategy but not specifies the probe immobilization time.

[3] Single-tagging PCR amplification time was not included in the Table. The authors indicate an overall analysis time of 3 h for electrochemical magneto-genosensing and PCR assay.

Novel bioreceptors: aptamers, bacteriophages and antimicrobial peptides

Due to the growing challenges in foodborne pathogens detection, the research towards the development of new biorecognition elements has been intensifying. To this end, in recent years, there has been an increase in the use of aptamers, antimicrobial peptides and bacteriophages as recognition elements to detect bacteria cells or related proteins in several electrochemical biosensing strategies (Ding, Lei, Ma, Gong, & Qin, 2014; Hills, Oliveira, Cavallaro, Gomes, & McLamore, 2018; C. X. Zhou, et al., 2016).

Aptamers are single-stranded DNA or RNA oligonucleotides purified *in vitro* with high specificity and selectivity for its specific target. They appear as an alternative to antibody-based methods, showing better resistance to stringent conditions, such as wider pH ranges or extreme temperatures, without losing its binding affinities (Zelada-Guillén, Blondeau, Rius, & Riu, 2013). Additionally, the use of aptamers as bioreceptors overcomes important issues associated with antibody production such as batch-to-batch consistency and related production costs. Hills *et al.*, in 2018 (Hills, et al., 2018) developed a label-free chitosan nanobrush border sensor trough the conjugation of DNA-aptamers or antibodies specific to LM on a reduced graphene oxide/nanoplatinum electrode surface. A pH stimulus was applied to the modified nanobrushes to boost the LM cells capture. With this approach was verified that aptamers have a high adsorption capacity when compared with antibodies, capable to detect through EIS LM concentrations in the range from 9 to 10^7 CFU mL^{-1} in 17 min including sample exposure and testing, in a homogeneous assay. Another label-free aptamer-based sensor, where the protamine-bacteria interactions and the potential change at an aptamer modified polycation-sensitive membrane electrode were used to detect LM, was present (Ding, et al., 2014). With this approach, a LOD of 10 CFU mL^{-1} was achieved just in 40 min, including LM incubation and analysis, resorting to a simple online filtration system as sample pre-treatment. Another impedimetric approach was developed by Zhou, C.X., *et al.*, in 2016 (C. X. Zhou, et al., 2016), taking into account the size and superficial charge of the *Listeria* cells, creating a surface blocking steric concept trough an aptamer modified nanoporous sensor and a redox probe. In that work, as *Listeria* cells were captured at the nanopore surface of the sensor, the ionic flux of the redox-probe through the pores decreased both by the physical blockage provoked by the large cells as well as by the repulsion of the redox-probe by the negative charge that the *Listeria* cells present at neutral pH. This electrochemical biosystem was able to detect 100 CFU mL^{-1} with high specificity, even in the presence of high levels of other similar pathogens like

E-coli. Besides the successful results presented in the aforementioned label-free aptasensors, it's also known that the immobilization of aptamers in solid surfaces can impair the ability of the aptamer to bind to its target, both due to conformational aspects of the DNA / RNA strand after immobilization, as well as due to steric hindrance issues (Ding, et al., 2014). Due to those reasons, it is important to carefully select the optimum conditions for immobilization and bioconjugation of the aptamer on the sensor surface to maximize the capture efficiency.

Bacteriophages, which are ubiquitous virus that can be found in abundance in natural biosystems, also appeared as important bioreceptors for LM and other foodborne pathogens. To replicate this virus infects its specific host cell, assaulting its metabolism. The bacteriophages only infects viable target bacteria, which are recognized by a specific membrane receptor present on its surface, similarly to some antibodies (Fischetti, 2011). After the infection, the metabolism of the host bacteria is compromised, while creating conditions for incessant phage replication within the bacteria cells. Beyond the excellent specificity and simple and low-cost production, phage have the extra advantage of discriminate between viable and non-viable cells (Rohde, et al., 2017). This fact, combined with high fast detection and elevated tolerance to severe conditions, such as organic solvents, extreme pH and temperature, makes bacteriophages a perfect candidate for biosensing (Ertürk & Lood, 2018). The application of bacteriophages in LM sensing is quite new and to date, only a few electrochemical phage-based methods for LM detection were reported. Nonetheless an intensive research towards the isolation of novel phage's for LM, such as the currently widely used P100, has been carried out (Chibeu, et al., 2013; Komora, et al., 2018; Y. Zhou & Ramasamy, 2019). Indeed, this phage was successfully implemented into commercial formulas like Listex[TM] P100, to reduce the probability of contamination in different food matrices (Chibeu, et al., 2013; Komora, et al., 2018). Recently Zhou, Y. and R.P. Ramasamy (Y. Zhou & Ramasamy, 2019) used this specific P100 bacteriophage conjugated with magnetic nanoparticles as capture element of a bacterial isolation/enrichment technique. The results presented higher capture efficiencies when compared with commercial antibody-modified magnetic particles (2.8 µm Dynabeads™), even in complex matrices like ground beef (Fig.5).

Along with bacteriophages, antimicrobial peptides (AMPs) have gained special attention by its high stability in harsh environments combined with being cost-effective bioreceptors (Etayash, Jiang, Thundat, & Kaur, 2014; Lv, Ding, & Qin, 2018). AMPs are peptides or portion of peptides able to recognize and kill its target host cells, by membrane surface elements presents at the cell surface. Herein, some biosensing strategies employing AMPs as bioreceptors for LM identification were emerging, namely

in optical/fluorescent (Hossein-Nejad-Ariani, Kim, & Kaur, 2018; Rocha, et al., 2019) and electrochemical systems (Etayash, et al., 2014; Lv, et al., 2018). However, only the labeled sandwich assay format were able to target LODs below the infection dose, perhaps due to the semi-selective affinity of some AMPs (Ding, et al., 2014). Accordingly, very recently (2018), Lv, E., *et al.*, developed a magnetic sandwich assay based on a short-peptide complementary pair specific for LM (Lv, et al., 2018). One of the peptides was used to modify a magnetic nanoparticle that was then used as capture, and the other was modified with an HRP enzyme and used as a secondary peptide to ensure an increased specificity to the assay. After the separation of the immunocomplex, the oxidation of 3,3',5,5'-tetramethylbenzidine was enzymatically induced, creating a change in ion selective membrane potential proportional with LM CFU. As a consequence of the good specificity of the peptide pair, the use of this simple sensing strategy allowed to reach an interesting LOD of 10 CFU mL^{-1}.
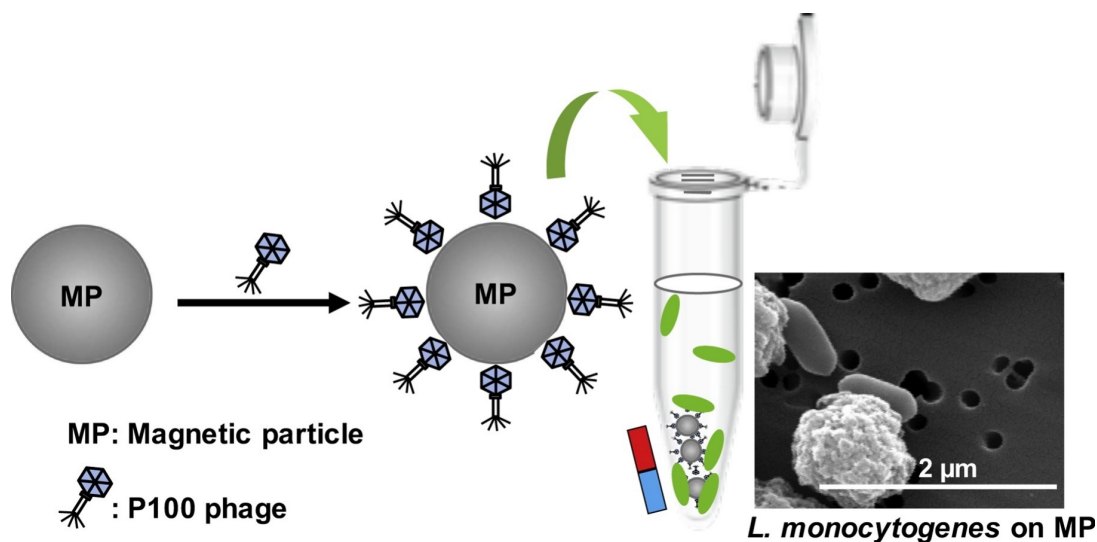
Fig. 5 - Isolation and separation of L. monocytogenes using bacteriophage P100-modified magnetic particles. Reprinted with permission from (Zhou & Ramasamy, 2019). Copyright (2019) Elsevier.

Nanomaterials in LM electrochemical biosensing

In comparison with other analytes, the detection of foodborne pathogens takes an extra analytical challenge due to the tightened limits of detection imposed by the current regulations (Commission (EC), 2005b; Soni, et al., 2018). This particularity allied to very complex matrices triggered the need for developing some strategies to increase the signal/background ratio (Välimaa, et al., 2015). Indeed, the integration of nanomaterials

revolutionized electrochemical biosensing by their intrinsic electrocatalytic properties. Additionally, it also contributed to improve miniaturization and automation (Krishna, et al., 2018; Lafleur, Jönsson, Senkbeil, & Kutter, 2016; Mukherji & Mondal, 2017).

Besides the specificity and availability of the bioreceptor, the electrochemical transducer has to be sensitive enough to detect the bioconjugation event. Nanostructured electrodes and the use of new electroactive labels seems to be the best approach to enhance the analytical performance of electrochemical biosensing methods achieving lower detection limits (Cho, et al., 2018). Different types of nanomaterials have been successfully employed in the development of electrochemical biosensing designs due to its intrinsic extraordinary properties (Dominguez, et al., 2017; Dridi, et al., 2017). Commonly, due to their large surface area and enhanced electron transfer features, they take part in biosensors transducer surface being used as a platform for bioreceptor anchoring, providing a stable and biocompatible immobilization(Y. Lu, et al., 2016; R. Wang, et al., 2008). Additionally, metallic-based nanoparticles, such as gold or silver, or quantum dots, can be used as electroactive labels. Nanomaterials can, therefore, performed important roles both for the nanostructuration of the transducer surface as well as electroactive labels, enhancing the analytical performance of electrochemical biosensing methods (Cho, et al., 2018; Kashish, Gupta, et al., 2015; Niu, et al., 2017; Sun, et al., 2012).

Regarding LM detection, the majority of antibody-based nanostructured sensors follows an enzymatic sandwich format, in which nanomaterials are inserted to serve as an enzymatic platform or as an electrochemical mediator of the reaction between the enzyme and the substrate, thus amplifying the signal obtained through the reaction. Additionally, the recycling and adaptation of well-known enzymatic amplification methodologies (like urease and HRP catalytic centers) increase the probabilities of obtaining a successful assay (D. Wang, et al., 2017), despite the impairment in cost-effectiveness. In label-free formats, nanostructured surfaces also prevent premature fouling (Zhao & Chen, 2019). In this sense, in general there has been a decrease in the use of SAMs to the detriment of the use of nanostructured electrode surfaces to the direct conjugation of the bioreceptor, due to their improved biocompatibility, high surface area, redox activity and catalytic efficiency (Zhao & Chen, 2019). The main drawback of these systems was the surface variability, which sometimes persists even after optimizing the synthesis of nanomaterials that are employed. Furthermore, the time-consuming steps that were needed to its biofunctionalization can also affect the reproducibility and simplicity of the method (Y. Wang, Shao, Matson, Li, & Lin, 2010; Yan, et al., 2016). Besides, more stability and shelf-life studies have to be made in an attempt to access its

practical usefulness. Additionally, would be necessary to explore the integration of these nanomaterials with the new sensing low-cost platforms (e.g. reusable sensors, paper-based, textile-based platforms).

Magnetic particles in LM electrochemical biosensing

In the absence of pre-enrichment steps, other strategies, such as centrifugation, filtration or magnetic separation, have emerged with the need of detection of low contamination levels with minimal sample processing *via* non-cultural method approaches (Krishna, et al., 2018). More specifically, the use of magnetic materials in biosensing of foodborne pathogens appear as an interesting, time-saving strategy to separate and concentrate the pathogen of interest. Magnetic and superparamagnetic particles can play a particularly interesting role in the development of biosensing strategies for LM detection, not only behave for separation processes but also as a solid surface were LM can be directly recognized by a specific bioreceptor, while non-specific targets are removed by a series of washing steps (Giri, Pandey, Neupane, & Ligler, 2016; Nadia F. D. Silva, Magalhaes, Oliva-Teles, & Delerue-Matos, 2015). Indeed, only the collected antibody-based methods for detection of LM that used immunomagnetic separation protocols presented real food sample applications with no other pre-treatment techniques and still ensuring that good LOD were achieved. Additionally, it can also be observed that both magnetic antibody (Chen, et al., 2015; Chen, et al., 2016; D. Wang, et al., 2017) and DNA-based assays (Brandão, Liébana, Campoy, et al., 2015; Brandão, Liébana, & Pividori, 2015; Liébana, et al., 2016) are the ones faster in their categories, comparing the times required to perform a full test from sample pretreatment till the final result. Some aptamer modified magnetic nanoparticles have also been developed for separation protocols but, to the best of our knowledge, it was only successfully applied to colorimetric/optical biosensors (Suh, et al., 2018; Zhang, et al., 2016). In the near future, the dissemination of the specific phages and peptides for LM could give a boost in this field, by their proven resistance to harsh media without loss of affinity (Malekzad, Jouyban, Hasanzadeh, Shadjou, & de la Guardia, 2017). So, it is expected better recuperation rates comparing with antibodies, even in complex matrices (Farooq, et al., 2018; Laube, Cortes, Llagostera, Alegret, & Pividori, 2014; Lv, et al., 2018; Y. Zhou & Ramasamy, 2019).

3. Future directions in foodborne pathogen electrochemical biosensing

Nanoimpact electrochemistry: pursuing single entity identification

The presence of LM, even at low levels, is an indicator of unsanitary conditions and the food should be stated as unsuitable for consuming. Accordingly, the possibility of detection of living bacteria at a single-cell level would take the current analytical strategies to the next stage. One of the trends recently seen in the literature is the use of new nanoscale approaches to explore single(bio)entity sensing (Neves & Martín-Yerga, 2018). Namely, the possibilities of nanoimpact method through the electrochemical analysis of discrete collisions events is gathering considerable attention (Stevenson & Tschulik, 2017). New sensing strategies for individual foodborne pathogen cells were already reported. The detection of single *E. coli* cells decorated with silver nanoparticles, through the anodic striping charge resulting from the collision of the nanomaterials with a carbon electrode surface was described (Sepunaru, Tschulik, Batchelor-McAuley, Gavish, & Compton, 2015). Also, stochastic collisions of single *E.coli* and *B.subtilis* cells were accomplished by an electron transfer blocking strategy (Couto, Chen, Kuss, & Compton, 2018; Gao, Wang, Brocenschi, Zhi, & Mirkin, 2018; Ronspees & Thorgaard, 2018). These works demonstrated that the impact of the individual bacteria cells onto a microelectrode, where a redox mediator was being continuously monitored, could be related to the changes in the current that was being registered. The reported strategies, despite enabling a label-free detection of single bacteria, lacked selectivity as any species in the solution that adsorbs on the electrode surface could give place to an electrochemical response. Some strategies to improve specificity were proposed in the work of Dick *et al.* (Dick, Hilterbrand, Boika, Upton, & Bard, 2015), where a selective strategy for the electrochemical single detection of cytomegalovirus was studied. Until the moment, no similar approaches were published for LM, which is certainly because this is a very recent research line and the signs of progresses in this field are very incipient. In any case, nanoimpact electrochemistry is expected to be developed and extended to a broad spectrum of microorganisms, since the path in the direction of single unity detection is unquestionably opened.

Smart packaging: towards continuous monitoring

New analytical strategies to carry out a constant vigilance of the quality of ready-to-eat product while providing real-time information for the consumer is a hot topic in food industry (Yousefi, et al., 2019). The development of intelligent packaging technology is expected not only to improve food safety but also to help consumers making better decisions and reducing food waste. Accordingly, electrochemical (bio)sensing could give an important input in the development of those smart packaging strategies for continuous monitoring of food quality. To this extent, electrochemical sensing can be selected as the operating basis of important food quality indicators such as time−temperature (Mijanur Rahman, Kim, Jang, Yang, & Lee, 2018) and freshness indicators through the analysis of several metabolites related to bacterial growth (e.g., glucose, lactate, CO_2, O_2, biogenic amines, among others) (Fiddes, Chang, & Yan, 2014; Park, Kim, Lee, & Jang, 2015). Nonetheless, important obstacles still need to be overcome. The intelligent sensing devices have to comply with specific requirements of food safety. They have to be compatible with foodstuff, they should be recyclable and a multiplexed analysis of different freshness indicators for more assertive information should also be ideally accomplished. Regarding the transduction of electrochemical signal, they are expected to be self-powered devices that additionally ensure the real-time monitoring and data transfer for consumer smartphones or similar devices, with the aid of specific software applications, via Bluetooth, or even by the implementation of radio-frequency identification tags (Fiddes, et al., 2014; Ruiz-Garcia, Lunadei, Barreiro, & Robla, 2009). Despite the potential of electrochemical (bio)sensing for smart packaging, the development of a fully integrated and autonomous device for food spoilage monitoring is not yet a reality. Indeed, the development of a perfect stand-alone biosensor to be used for on-site analytical applications is still dependent on the concomitant advances in other parallel research fields, where an interdisciplinary and integrated network among material sciences, bio-interfaces, nano(electronics) and nanobiotechnology is required (Dincer, et al., 2019).

4. Final remarks

Currently, electrochemical biosensing methods appear as promising tools for the development of new sensing strategies for the rapid screening analysis of LM in food samples, especially during emergent contamination scenarios such as foodborne outbreaks. Therefore, rigorous studies on new biorecognition elements, novel transduction and labelling materials and original procedures are being carried out. Until the moment, the use of antibodies or nucleic acid as bioreceptor elements has prevailed within the reported works. However, the development of alternative synthetic bioreceptors with improved resistance and lower cost, such as aptamers, antimicrobial peptides and bacteriophages, has been emerging. Additionally, the use of nanomaterials as amplifiers of the electrochemical signal, electroactive labels or modifiers of the transducer surface, has also being successfully explored. In its turn, magnetic particles revolutionized the sample pre-treatment, by its simplicity, automation ability and capture efficiency rates. Due to its unique features, the use of magnetic particles allowed to combine purification, separation and biorecognition for the development of straightforward assays. In the future it would also be interesting to explore the selectivity of aptamers, peptides and phage's coupled with magnetic assays and combine it with the new electrochemical biosensing platforms, improving the actual recoveries rates in real food samples (Malekzad, et al., 2017). Novel tracing strategies for LM, such as nanoimpact electrochemistry and the implementation of intelligent packaging are still in its beginnings but, in the near future, could transform foodborne pathogen analysis. Therefore, electrochemistry assumes a remarkable role by its simplicity and portability, that allied with small-sized highly specific sensing platforms can raise a new generation of rapid devices for LM detection. However, even though the great achievements, important barriers in terms of knowledge transfer from the laboratory research to full validated commercial analytical instruments still remain (S. Wang, Chinnasamy, Lifson, Inci, & Demirci, 2016). Some of the major drawbacks are related to the limitations of the biological components of the sensing device such as instability, short-life and costs of production. The intensive research in artificial receptors can be a good option for surpassing the aforementioned restrictions. A higher degree of automation, which is also expected to decrease the workload, is also an important requirement (Pereira da Silva Neves, González-García, Hernández-Santos, & Fanjul-Bolado, 2018). Furthermore, a better harmonization among the different procedures for testing and validation should also be encouraged to facilitate a precise and objective comparison among the developed assays (Rohde, et al., 2017). Lastly, the cost associated to research,

development and implementation of new validated methods, complying with regulatory affairs, are also important constraints in the process of knowledge transfer (Pereira da Silva Neves, et al., 2018; Välimaa, et al., 2015).

In summary, the research in electrochemical biosensing devices for food safety has experienced a great evolution and new and exciting alternatives are being developed. Despite the continuous challenges and the technical limitations that persist, the scientific advances have been answering appropriately. Accordingly, in the next years, electrochemical sensing is expected to play an important role modelling food analysis towards a fast, sensitive, decentralized and continuous on-site monitoring.

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3.2. Electrochemical immunosensor towards invasion-associated protein p60: an alternative strategy for *Listeria monocytogenes* screening in food

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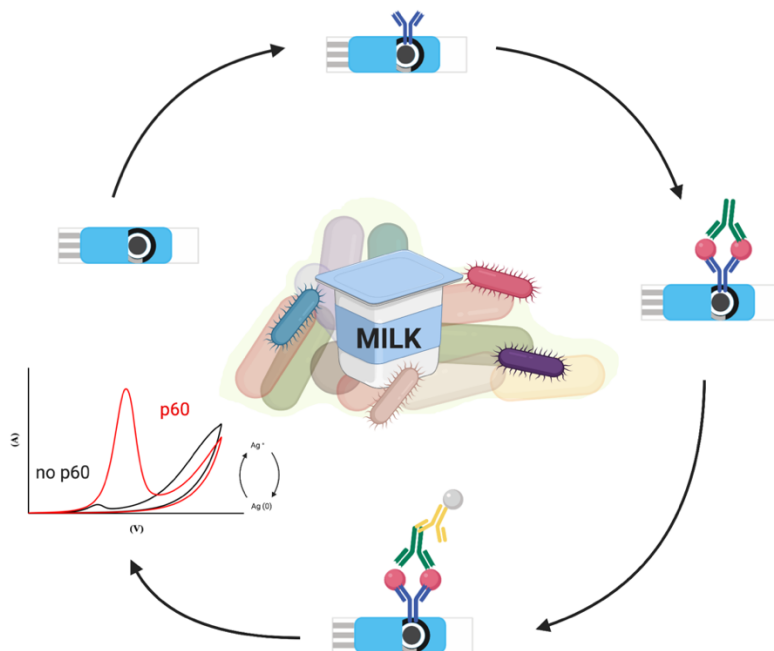
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Statement of contribution

The contribution of the candidate, Nádia F.D. Silva, in this work includes the literature review, investigation and writing of the original draft.

Electrochemical immunosensor towards invasion-associated protein p60: an alternative strategy for *Listeria monocytogenes* screening in food

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Abstract

This work reports the development of an electrochemical immunosensor for rapid, specific and decentralized detection of the invasion-associated protein p60 secreted by *Listeria monocytogenes*, a life-threatening foodborne pathogen. A disposable screen-printed electrode was used as transducer surface and a monoclonal antibody against a specific peptide sequence of *Listeria monocytogenes* p60 and a polyclonal antibody target-specific were used as the sandwich immuno-pair. The reaction was detected with the aid of an additional secondary antibody conjugated with the enzyme reporter (alkaline phosphatase) and a 3-indoxyl phosphate/silver ions as the mixture substrate. The analytical signal was acquired through the voltammetric stripping of the enzymatically deposited silver, which was directly correlated to p60 concentration in the sample. In optimized conditions, a limit of detection and quantification of 1.52 ng mL^{-1} and 5.06 ng mL^{-1} were achieved, respectively, in a useful time ($<$ 3 h). As proof-of-concept, the proposed immunosensor was successfully applied to spiked milk samples, demonstrating to be a suitable device for further use in real sample detection of *Listeria monocytogenes* in food products.

keywords: *Listeria monocytogenes;* p60 protein; electrochemical immunosensor; foodborne pathogens; food safety.

1. Introduction

Listeria monocytogenes (LM) is one of the most harmful foodborne pathogens [1, 2], being present in more than 95% of the cases of human listeriosis [3]. LM is an opportunistic bacteria that can quickly infect and spread into the host blood system [4], being particularly dangerous in high risk groups of population, like immune-depressed persons, elderly, children and pregnant women [2]. Listeriosis infection could lead to severe illness scenarios such as meningitis, sepsis or abortion and, even though presents a relative low incidence, this foodborne disease is associated with a high rate of hospitalization and death (20−30%) [2, 5, 6]. The consumption of contaminated food (dairy products, or raw animal proteins and vegetables) continues to be the main cause of infection, despite the several regulations that have been implemented along the years with the objective of keeping the concentration of LM in foodstuffs below the infection dose[7], as well as the zero-tolerance policies implemented in some countries for ready-to-eat products. This outcome results from the high pathogenicity associated to LM, combined with its capability of growing in adverse conditions such as low temperatures, wide pH's medias and anaerobic environments, while colonizing different surfaces [4, 8]. These characteristics difficult both the elimination or control of LM proliferation, even using aggressive disinfectants or advanced food preservation techniques. Additionally, it is known that some LM serotypes present resistance to antibiotics.

Traditional colony plate counting still remains the "gold standard" method for the identification of viable bacteria cells and to evaluate the microbiological quality of food products. However, the long analysis times which are characteristic of this classic method do not meet food industry current demanding for fast and high-throughput techniques. A usual waiting time of 5−7 days until obtaining a final result may originate non-controlled contaminations, since a food product with short shelf life, such as the ready-to-eat products, could be consumed before its microbiological quality is confirmed [9]. Hereupon, new methods based on enhanced biochemical techniques, nucleic-acid and immunological-based methods have emerged as rapid alternatives [3, 9, 10]. Immunological-based methods, such as ELISA commercial kits, are already able to offer a quantitative analysis in less than 24 h, although they involve high workload and its performance is limited to a laboratory facility [11]. Additionally, some of them need to resort to time-consuming pre-treatment techniques to isolate the LM from complex food matrix [4, 12]. The same setback is found in strategies based on nucleic acid-based methods, exacerbated by the need of expensive equipment's and expert technicians to perform the analysis. All of these constraints have hindered the implementation of rapid

methods for quality control routines of LM, both at production and consumer levels. Accordingly, the developing of faster, low-cost, portable and decentralized detection methods for LM detection are of upmost importance for food industry. Additionally, could be a starting point for implementing more effective control procedures throughout the whole food supply chain, which will undoubtedly lead to positive repercussions on public health [13].

The use of electrochemical techniques for the development of analytical devices have been attracting considerable attention due to their high sensitivity, simplicity, and user-friendly portable compact equipment [14]. More specifically, the combination of electrochemical disposable miniaturized transducers with the specificity of the immunoassays, appears as a good option for decentralized detection of LM. Indeed, electrochemical immunosensing strategies were already described for LM detection [6, 12, 15-18]. However, despite the improvement in the workflow and procedure's hands-on time, the developed strategies still lack in sensitivity and selectivity towards other non-pathogenic *Listeria spp*. or food components mixed in the sample. In order to accomplish a more specific detection, different genes (*hly*, *iap*) [19, 20] and their corresponding encoded proteins, listeriolysin O [21, 22] and p60 [4, 23], which are considered major virulence factors associated with bacteria pathogenicity [24], have been targeted for the detection of LM. Particularly, p60 protein, reveals an important role in host invasion, cell division and viability [24, 25] and besides being a surface protein is also secreted in large quantities into the growth media. These features make p60 an ideal diagnostic target for the development of immunological detection systems for *Listeria spp*. A few existent methodologies based on immunoassays with optical detection [3, 4, 13, 25, 26] were already reported for p60 determination however require long assays and/or time-consuming pre-enrichment steps for sample analysis. In order to overcome such limitations, the analytical features of electrochemical immunosensing presents a considerable potential.

Accordingly, in this work, the first electrochemical immunosensor for LM p60 protein determination is described. A pair of antibodies, generated against a peptide sequence of p60 only secreted by LM species, were successfully combined to design an electrochemical sandwich enzymatic immunoassay with improved performance. The developed method allowed a fast (total assay time $< 3h$; hands-on time < 15 min) and accurate determination of LM p60 protein. The proof-of-concept and applicability of the developed method for food safety has been successfully demonstrated by the analysis of spiked commercial milk samples.

2. Material and methods

2.1. Reagents and solutions

Mouse IgG p6007 monoclonal antibody (CAb), rabbit polyclonal antibody (DAb) produced against LM recombinant invasion associated protein p60, and recombinant P60 protein from LM (p60) expressed in *E. coli* were purchased from Adipogen, Life sciences. Anti-Rabbit IgG (Fc specific), highly cross adsorbed-alkaline phosphatase antibody (ALP-Ab) produced in goat, albumin from bovine serum (BSA), magnesium nitrate hexa-hydrate (99%), 3-indoxyl phosphate disodium salt (3-IP ≥ 98%) and Tris(hydroxymethyl)amino methane (Tris, ≥99.8%) were obtained from Sigma-Aldrich. Nitric acid (≥65%) was acquired from AppliChem Panreac and silver nitrate ($AgNO_3$) from Alfa-Aesar. All other reagents used throughout this work were pro-analysis quality (pa) or equivalent and were used without further purification. Milli-Q® water (18.2 MΩ cm^{-1} resistivity, at 25° C) obtained from a Simplicity 185 water purification system was used to prepare all the solutions used throughout this work. CAb, DAb, p60 and BSA solutions were prepared in 0.1 M Tris-HNO_3 buffer, pH 7. 2 (buffer 1), ALP-Ab was diluted in 0.1 M Tris-HNO_3 pH 7.2 buffer containing 2 mM Mg $(NO_3)_2$ (buffer 2) and working solutions of the substrate mixture (3-IP/Ag^+) were prepared using 0.1 M Tris-HNO_3 pH 9.8 buffer containing 20 mM Mg $(NO_3)_2$ (buffer 3) and stored protected from light until use. All of the working solutions were prepared daily and stored at 4−8ºC.

2.2. Apparatus and electrochemical measurements

Scanning Electron Microscopy (SEM) studies were carried out at CEMUP (Centro de Materiais da Universidade do Porto), Porto, Portugal, using Quanta 400 FEG scanning electron microscope, (SEM, FEI, Hillsboro, OR). Elemental analysis was performed using the same scanning electron microscope coupled with an EDAX Genesis X4M energy dispersive spectroscopy (EDS) operated at 15 KV with a detector type SUTW SAPHIRE analysis system of resolution 132.19. Linear sweep voltammetry (LSV) and cyclic voltammetry (CV) were performed using a potentiostat/galvanostat (PGSTAT101, Metrohm Autolab), controlled by NOVA 1.10 software.

Electrochemical impedance spectroscopy (EIS) experiments were performed with an Autolab Electrochemical Analyzer (PGSTAT128N, Metrohm) and the tests were conducted at an open circuit, recorded for 50 data points, at a single modulated AC potential of + 0.24 V with frequency

ranging between 10 mHz and 100 kHz. All these experiments were made using disposable screen-printed carbon electrodes (SPCE, Ref. DRP-110, Metrohm Dropsens) composed by a conventional three-electrode cell configuration: a working (WE, $\varnothing$ 4 mm) and an auxiliary electrode made of carbon ink and a pseudo-reference electrode made of silver. All measurements were performed, at least, in triplicate and were conducted at room temperature.

2.3. Optimized immunosensor assay for p60 detection

The establishment of the sensing phase was obtained by physical adsorption of the CAb in a non-modified SPCE surface. Accordingly, 10 µL of CAb solution (10 µg mL^{-1}) was dropped onto the WE and left to incubate overnight in a moist atmosphere at 4–8ºC. Then, the sensing phase (CAb/SPCE) was washed with buffer 1 to remove unbound antibodies and the free non-modified electrode spots were blocked with 40 µL of BSA solution (2 % w/v) during 30 min. Following a second washing step with buffer 1, the sensing phase was incubated during 60 min with 30 µL of a mixture containing different concentrations of p60 standard solutions (or milk spiked with p60), 2 µg mL^{-1} of DAb, and 0.5 % (w/v) of BSA. Afterwards, the electrode was washed with buffer 2 to eliminate the excess of analyte and DAb and 40 µL of ALP-Ab solution (1:25000, 40 µg mL^{-1}) was added and left to react for 60 min. After a final washing step with buffer 3, the enzymatic reaction was carried out over 20 min by placing 40 µL of 3-IP/Ag$^+$ (1 mM/0.4 mM) mixture covering all electrochemical cell. The enzymatic generation of metallic silver was reported elsewhere [28]. Briefly, ALP hydrolyzes 3-IP in an unstable indoxyl intermediate that will be oxidized along with silver ions reduction into metallic silver. Consequently, the amount Ag^0 formed at SPCE working electrode surface at the end of the enzymatic reaction will be proportional to the amount of the target analyte present in the sample. Thus, silver particles, co-deposited with indigo blue, were stripped anodically by applying a linear sweep voltammetry from + 0.0 V to + 0.4 V at a scan rate of 50 mV s^{-1}. Examples of typical cyclic voltammograms (CV) of silver re-oxidation were also presented.

2.4. Milk sample analysis

Skimmed milk samples purchased in a local supermarket, diluted (1:25) and spiked with different amounts of the p60 protein, were analyzed with the optimized immunosensor for p60 quantification without further pre-treatment techniques. Accordingly, four spiked samples with different final concentrations of p60 (10, 25, 50 and 100 ng mL^{-1}) and

control samples (non-spiked), were analyzed in triplicate, as proof-of-concept for the immunosensor's application in food.

3. Results and discussion

3.1. Development of an enzymatic sandwich immunosensor for p60

Fig. 1. illustrates the several steps necessary to the immunosensor development: construction of the immunosensing surface, optimization of immunoassay protocol and the electrochemical detection system applied. The proposed immunosensor development was composed of four essential steps: (i) immobilization of the anti-p60 CAb onto a SPCE WE surface (CAb/SPCE), (ii) BSA surface blocking step (BSA/CAb/SPCE), (iii) incubation of a solution containing different amounts of p60 and the detection antibody (DAb/p60/BSA/CAb/SPCE) and (iv) the sandwiched immunocomplex incubation with the ALP labelled secondary antibody. Once the ALP-Ab/DAb/p60/BSA/CAb/SPCE bioconjugate was formed, the biorecognition event was monitored by applying LSV.
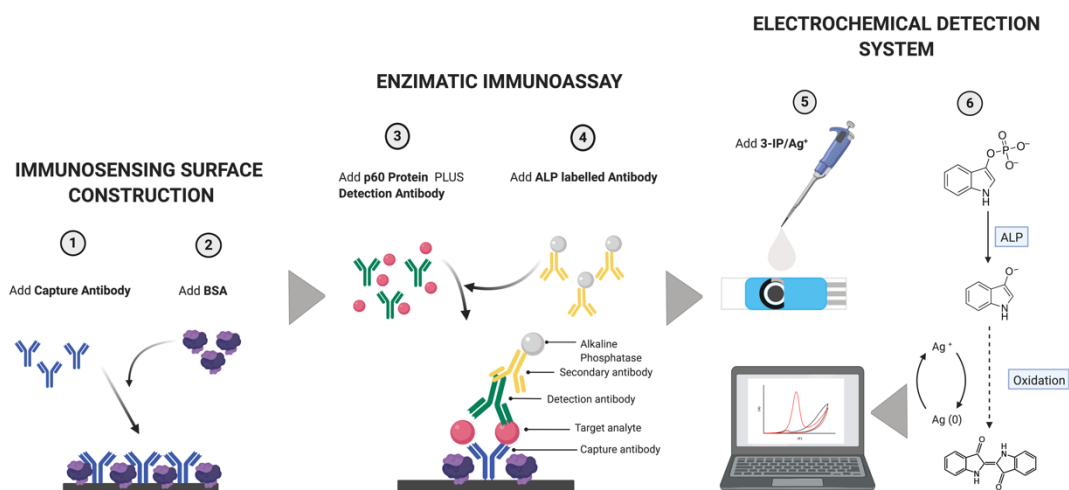

Fig. 1 - Schematic illustration of the several steps necessary for the immunosensor development. Created with BioRender.

3.1.1. Immunoreagents selection

The use of highly specific antibodies to target the analyte is an essential requirement for the development of an efficient immunosensing method. Accordingly, a mouse monoclonal antibody specific for an epitope only present at LM p60 protein surface was selected to the immunocapture in pair with a matched polyclonal antibody obtained from the same supplier, that was able to recognize a different non-overlapping region of the whole protein. Thus, possible cross-reactions with p60-like proteins from other species are prevented [28, 29]. These differences in specificity allowed a non-competitive

binding of antibody pair to the target analyte [23, 29]. Subsequently, the secondary antibody labeled with the ALP used is specific for the Fc region of the detection antibody, ensuring no cross-reactions with the other IgG's used throughout the assay and against the paratopes binding region of the DAb. Notwithstanding these pre-selection study, the effectiveness of the immunosensing interface construction procedure (described in section 2.3) and the ability of the antibodies recognizing their ligands (affinity and specificity), such as possible blocking or inhibitory interaction between them were investigated and the results were presented in next sections (3.1.2 and 3.1.3).

3.1.2. Immunosensing surface characterization

Faradaic impedance spectroscopy, using the pair ferro-/ferricyanide as redox probe $[Fe(CN)_6]^{4-/3-}$ and CV experiments were conducted to evaluate the step-by-step construction efficiency along the immunosensing interface development. The differences in electron-transfer resistance observed in electrode/redox probe solution interface were used to access and compare the different assembling steps. Nyquist diagrams representations (real and imaginary impedances plotted against each other) are frequently used to characterization of impedance changes at transducer surface [30], namely the electron transfer at electrode surface which is represented at high frequencies (semi-circle portion) and a diffusion related electron transfer represented at lower frequencies (linear portion) [31]. Fig. S1(A), in supporting information (SI), shows a diffusion limited process with fast electron transfer kinetics related to bare SPCE. Moreover, a clear increase in Nyquist semi-circle diameter as well as an angle depression of linear plot at the lower frequencies, due to CAb and BSA incorporation can be observed. The CV data also corroborates the EIS information, by the successive decreasing of current values registered along the insulating characteristics of the CAb and BSA molecules used (Fig. S1(B)). The higher resistance of the subsequent surfaces to the diffusion and transference of $[Fe(CN)_6]^{3-/4-}$ electrons, confirm the successfully formation of the sensing phase, such as the effectiveness of BSA as blocking agent.

3.1.3. Evaluation of non-specific interactions

Possible non-specific adsorptions of the analyte, the antibody pair and ALP-Ab were assessed to confirm the suitability of the optimized immunoassay protocol for p60 protein detection. Moreover, the adequacy of the transducing electrochemical strategy used was also evaluated. For this purpose, solutions containing 0 ng mL^{-1} (I) and 100 ng mL^{-1} (II)

of p60 were analyzed resorting to a complete assay format and used as control. Moreover, the specificity of the CAb was also studied, using a negative control (without CAb). The effect of the absence of the DAb and Ab-ALP on the analytical signal were also studied. According with the results presented in Fig. 2, it was concluded that the antibody pair selectively recognized the analyte, according with the signal-to-blank (S/B) difference obtained for the control assays. Moreover, it was confirmed a correct performance of all immunoreagents, since no important cross-reaction interactions was observed. Regarding the electrochemical detection system, the enzymatically generation of metallic silver and its re-oxidation was evaluated, employing cyclic voltammetry, in the absence of 3-IP, Ag^+ or both (negative controls). The results were compared with an optimized assay (Fig. S2, SI). As was expected, no anodic signal for silver oxidation was obtained for the negative controls while for the optimized immunoassay a current signal for the metallic silver re-oxidation was recorded. In accordance, SEM images shown in Fig. S3 A (SI) indicated the presence of dense particles on the SPCE surface after the enzymatic reaction took place, which was further corroborated by EDS mapping analysis that effectively corresponded to silver deposits (Fig. S3 B, SI). Moreover, after silver re-oxidation, a second scan (Fig. S2) revealed the complete oxidation of silver in the first anodic sweep, thus confirming the effectiveness of LSV as the selected electrochemical technique.

3.2. Optimization of experimental conditions

An exhaustive optimization of the experimental parameters is fundamental to develop a successful immunoassay. Therefore, the variables of interest, such as reagents' concentration and incubation times, were selected according to their relevance for the assay. Those selected values were chosen according to a multi-parameter criteria based on the S/B ratio using the currents measured in the absence (B; 0 ng mL^{-1}) and in the presence (S; 100 ng mL^{-1}) of target protein, the anodic peak current differential (Δi_p) and the replica reproducibility attained in each experiment.
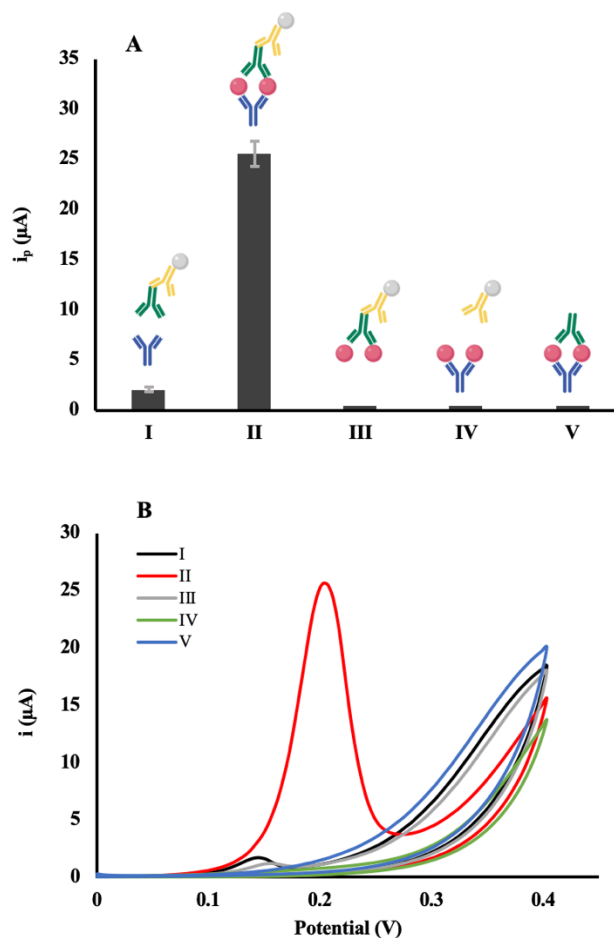
Fig. 2 - Evaluation of the non-specific interactions for an optimized immunoassay. (A) Peak current intensity obtained in the (I) absence (0 ng mL^{-1}) and in the (II) presence of p60 (100 ng mL^{-1}) for a complete immunoassay (control assays) and in the presence of p60 (100 ng mL^{-1}) but without (III) CAb, (IV) DAb and (V) Ab-ALP. (B) The corresponding cyclic voltammograms obtained. Experimental conditions: CAb (10 µg mL^{-1}); BSA (2%); DAb (2 µg mL^{-1}); ALP-Ab (1:25 000); 3-IP/Ag$^+$ (1.0 mM/ 0.4 mM). (A) Error bars as the standard deviation of three replicates. The illustrations were created with BioRender.

3.2.1. Optimization of the assay format

Following a time-saving optimization approach, four different designs composed by different number of incubation steps were compared: (i) four incubation steps (BSA, analyte, DAb, ALP-Ab), (ii) three incubation steps combining the target and detection antibody incubations in one step of 60 min (BSA, mixture of analyte and DAb, ALP-Ab); (iii) three incubation steps by joining the detection antibody and enzyme labelled antibody incubations in one step of 60 min (BSA, analyte, mixture of DAb and ALP-Ab) and; (iv) two step protocol (BSA, mixture of analyte, DAb, ALP-Ab). Fig. S4 correlates the analytical signal obtained in the presence and absence of p60 using different assay formats (i – iv). A similar S/B ratio was measured using format (i) and (ii), although the

second represents a 60 min total time saving comparing to standard format assay. In contrast, no signal was obtained, neither for blank or positive control solutions, for formats (iii) and (iv). This behavior could be possible attributed to steric hindrance impairment effects [32-34]. According to the data, format (ii) was chosen for further work.

3.2.2. Optimization of the capture, detection and reporter antibody

After the definition of the assay design, the influence of the capture, detection and the labelled secondary antibody concentrations, as well as the time of incubation of the reporter antibody in the immunosensor performance were studied. Different concentrations of CAb (2−10 μg mL^{-1}) adsorbed onto the immunosensing surface were evaluated and the data (Fig. 3 A) revealed that S/B ratio increased up to 5 μg mL^{-1}. For the subsequent tested concentration (10 μg mL^{-1}) the ratio between the i_p obtained for S and B seemed to stabilize. Nonetheless, the current measured in the presence of the analyte increased significantly. Indeed, the highest Δi_p and better reproducibility was accomplished with 10 μg mL^{-1} of CAb, suggesting a better orientation of the antibodies on SPCE surface, higher capture efficiency and less steric hindrance at this level of concentration [35, 36]. Bearing in mind that antibody orientation on the surface is an essential step in obtaining an immunosensor with high-performance, 10 μg mL^{-1} CAb was selected to continue the studies. According to the results obtained, additional concentrations of CAb were not considered due to the tendency of increasing the blank signal and also taking in account the sensor's cost. Afterwards, the effect of the DAb on the analytical signal was studied from 1–4 μg mL^{-1} concentration range. In Fig. 3 B can be seen that the obtained current increased with the loading of DAb up to 2 μg mL^{-1}, indicating that the lower concentration studied (i.e., 1 μg mL^{-1}) could be limiting the assay. However, the blank signal also increased with growing concentrations of DAb, which decreased S/B ratio. Hence, in order to minimize the influence of non-specific adsorptions, without affecting the immunosensor's sensitivity, the increment of the blocking agent concentration in 2 μg mL^{-1} DAb solution was evaluated with the aim of minimizing non-specific antibody binding. Accordingly, 2 μg mL^{-1} DAb solution (with BSA (0.5 %, w/v)) was selected to continue the studies and carrying out the subsequent enzymatic labelling. Bearing in mind the zero-tolerance policy imposed for LM in foodstuffs, for this specific parameter, it was opted to not prioritize the cost of the sensor in relation to a potential improvement in the sensitivity of the immunosensor. Lastly, the effect of the ALP-Ab dilution and the corresponding incubation time were also addressed. As shown in Fig. 3 C, with a dilution factor of 1:25 000 the highest S/B ratio, was achieved

and therefore selected. Regarding the effect of incubation time on anodic peak height (Fig. 3 D), the best compromise between S and B was observed for the longest time tested (i.e., 60 min). In view of the acceptable S/B achieved, longer assay times that would unnecessarily increase the total assay time were not considered. The concentration of enzymatic substrate (3-IP/Ag$^+$), enzymatic reaction time and buffers' pH were selected from previous works [34, 37, 38]. The results of the optimization studies were summarized in Table S1 (SI).
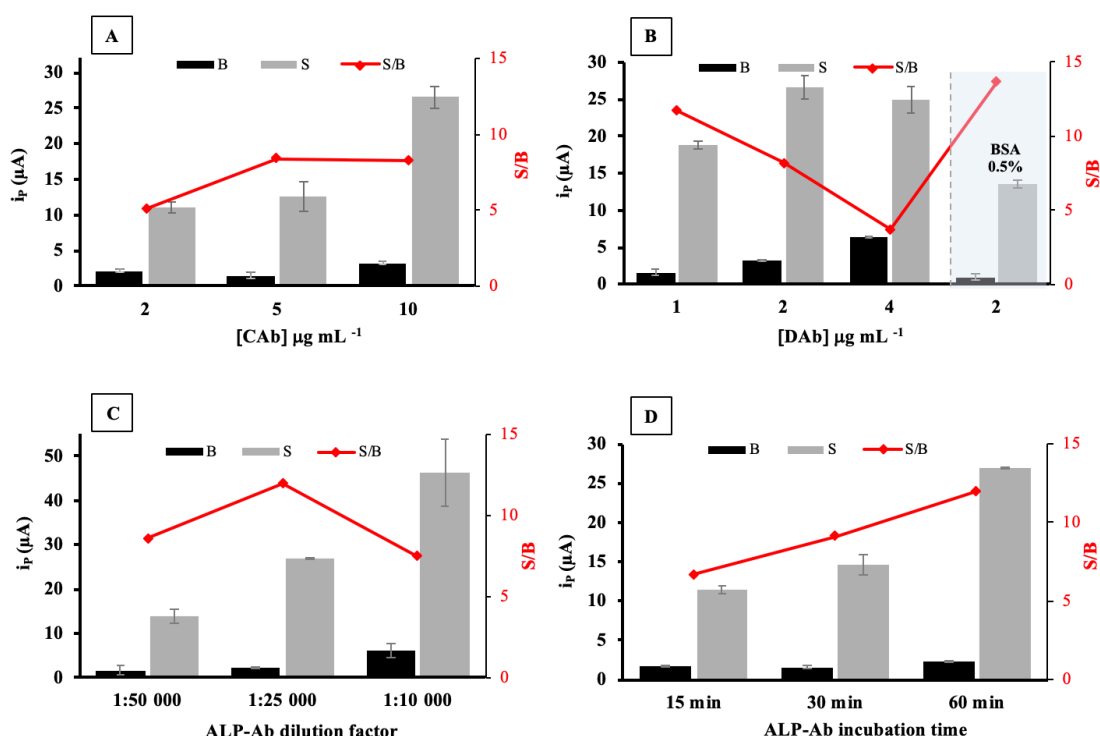

Fig. 3 - Dependence of the analytical signal obtained in the absence (B, 0 ng mL^{-1}) and in the presence (S, 100 ng mL^{-1}) of p60 standard solutions, as well as the corresponding signal/blank ratio (S/B) values, when evaluating the effect of following parameters in the immunosensing performance: CAb concentration (A); DAb concentration (B), ALP-Ab dilution (C), and incubation time of the ALP-Ab solution (D). Experimental conditions: CAb: 10 µg mL^{-1}, except in (A); BSA: 2% (A-D); DAb: 2 µg mL^{-1} in a 0.1% BSA solution (A, B white background) and 2 µg mL^{-1} in a 0.5% (B shadow background, C and D); ALP-Ab: 1:50 000, except (D); 3-IP/Ag$^+$: 1.0 mM/ 0.4 mM (A-D). Error bars estimated from the standard deviation of three replicates.

4. Analytical characteristics of the optimized immunosensor

4.1. Calibration plot

The dependence of the immunosensor response towards growing concentrations of p60, under the optimized conditions, in the range of 5–500 ng mL^{-1} was evaluated. A linear relationship was found between 5 and 150 ng mL^{-1} according to the following regression equation: i_p (μA) = 0.184 [p60] (ng mL^{-1}) + 6.356 (R^2 = 0.993). After the upper linear range value ($>$150 – 500 ng mL^{-1}) no significative differences in peak current intensity were verified, suggesting the saturation of the immunosensing response. The calculated limits of detection (LOD) and quantification (LOQ), set out by the equations LOD = 3s/m and LOQ = 10s/m, (where s is the standard deviation of the three blank measurements and m is the slope of the calibration line), were 1.52 and 5.06 ng mL^{-1}, respectively.
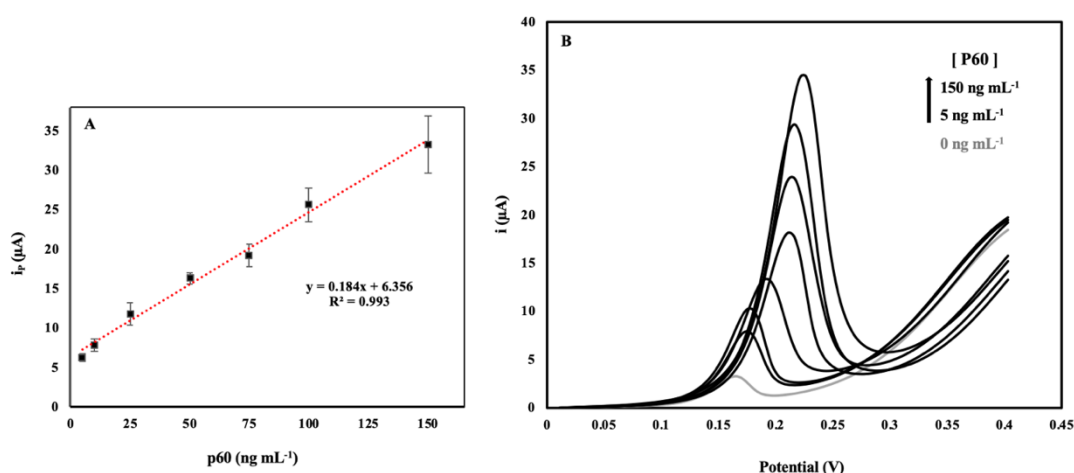

Fig. 4 - (A) Calibration plot obtained with optimized immunosensor for increasing concentrations of p60 standard solutions. (B) Examples of typical linear sweep voltammograms obtained within the linear range and the blank signal (0 ng mL^{-1}). Experimental conditions: CAb (10 µg mL^{-1}); BSA (2%); DAb (2 µg mL^{-1}); ALP-Ab (1:25 000); 3-IP/Ag$^+$ (1.0 mM/ 0.4 mM). (A) Error bars estimated the standard deviation of three replicates.

4.2. Precision

The intra-day and inter-day precision were evaluated. While the former was assessed by analyzing seven p60 concentrations within the linear range (3 replicas for each concentration), corresponding to a total of 21 different measurements in the same day; the inter-day precision was carried out by evaluating the reproducibility of three separate sensors in three different days for the same level of p60 concentration. An average

coefficient of variation (CV) of 8.58 % and 9.90% were achieved for intra-day and inter-day electrode precision, respectively.

4.3. Stability

To minimize the sensing phase construction time, its long-term stability and lifetime were assessed along a 30 days' period. For that purpose, the bioreceptor was immobilized on transducer surface according to the optimized protocol and the electrodes were stored at 2–8ºC. Firstly, the analytical signal obtained for a concentration of 25 ng mL^{-1} was measured at day 1 (control) and the acquired value was later compared with the responses obtained past 3, 7, 15 and 30 days (Fig. 5). The results revealed that the developed sensing phase is stable up to 7 days, without significant loss of the electrochemical signal. After 15 days of storage, the initial response decreased approximately 72%. Thus, the immunosensing surface, after being prepared and stored in adequate conditions, has a lifetime of one-week period.
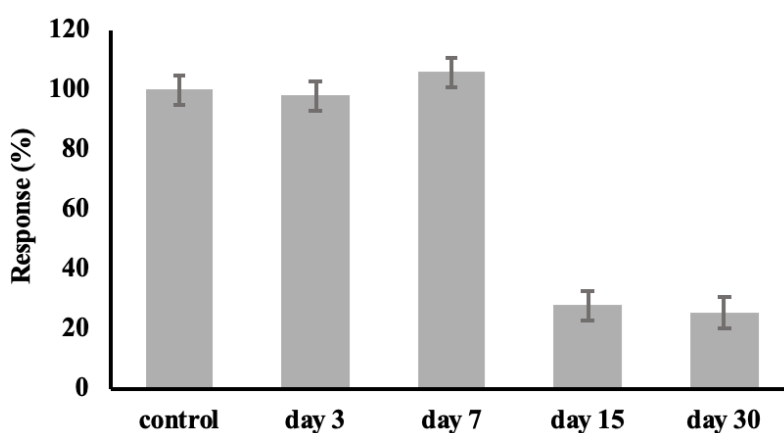

Fig. 5 - Study of the electrochemical signal obtained with the optimized immunosensor for a p60 concentration of 25 ng mL^{-1} after 3,7, 15 and 30 days of the immunosensing phase construction, relatively to day 1 (control). Experimental conditions: CAb (10 µg mL^{-1}); BSA (2%); DAb (2 µg mL^{-1}); ALP-Ab (1:50 000); 3-IP/Ag^{+} (1.0 mM/ 0.4 mM). Error bars estimated as the standard deviation of three replicates.

4.5. Milk sample analysis

The results obtained in the analysis of diluted milk samples spiked with 10, 25, 50 and 100 ng mL^{-1} of p60 were interpolated with the calibration curve performed in buffer solution. The respective recoveries (%) are shown in Table 1. A good recovery rate and

associated precision (average CV (%) <16) were achieved in all range of concentrations tested, with an average value of 97.92 %, proving the suitability of the developed immunosensor for screening proposes in a wide range of concentrations.

Table 1. Recovery results for the determination of p60 in spiked milk samples.

p60 added (ng mL^{-1})*	p60 found (ng mL^{-1})	Recovery (%)
0	< LOD	
10	9.18 ± 2.3	91.84
25	26.24 ± 6.4	104.96
50	48.51 ± 2.5	97.01
100	97.86 ± 8.2	97.86

*A dilution factor of 1:25 was applied to all the milk samples used in the recovery studies.

Results are given as average ± standard deviation of three replicates.

5. Conclusions

This work reports the first electrochemical immunosensor for quantification of p60 invasion associated protein from LM. The proposed immunosensor, resorts to the recognized specificity of an immunoassay-based strategy and the simple, low-cost and user-friendly electrochemical transducing, to perform a decentralized analysis of LM in food. In optimized conditions the immunosensor exhibits an excellent sensitivity through the detection of a recombinant p60 protein (LOD of 1.52 ng mL $^{-1}$) in a rapid assay (less than 3h). Moreover, this method represents an overall improvement in step procedures and in reducing hands-on time ($<$ 15 min), comparing with standard ELISA methodologies, along with miniaturized and portable instrumentation. In addition, through the direct and specific detection of this well-established major virulence factor, the proposed immunosensing approaches could settle the foundations to better address pathogenicity, virulence and viability in food contamination scenarios, as an interesting alternative to cells directed electrochemical immunosensors. Due to the abundance of the p60 in LM growth media and cells surface and the excellent analytical performance exhibited by the developed electrochemical immunosensor - even in food samples, the developed electrochemical strategy could be an asset in the routine of food quality control at the different levels of the food chain.

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Supplementary Information

Electrochemical immunosensor towards invasion-associated protein p60: an alternative strategy for *Listeria monocytogenes* screening in food

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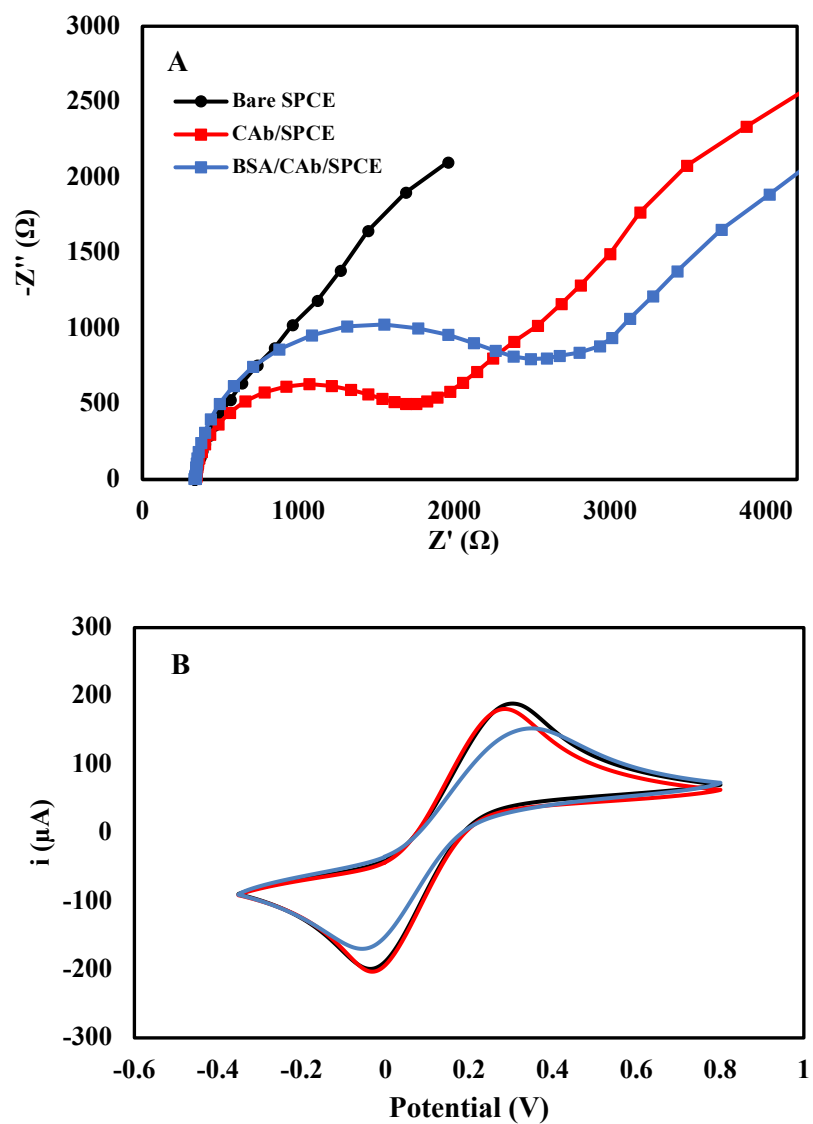[**]cmm@isep.ipp.pt

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Fig. S1 - Study of the sensing phase modification. (A) Nyquist plots of electrochemical impedance spectroscopy measurements for a bare SPCE (black line) and after the SPCE modification with the optimized CAb concentration (10 $\mu g\ mL^{-1}$; red line), and the subsequent blocking step with BSA (2% (w/v); blue line); (B) The corresponding cyclic voltammograms; all in 0.01 mol L^{-1} $NaNO_3$ containing 10 mmol L^{-1} of $[Fe(CN)_6]^{3-/4-}$ probe, acquired from - 0.4 V to + 0.8 V at a scan rate of 50 mV s^{-1}
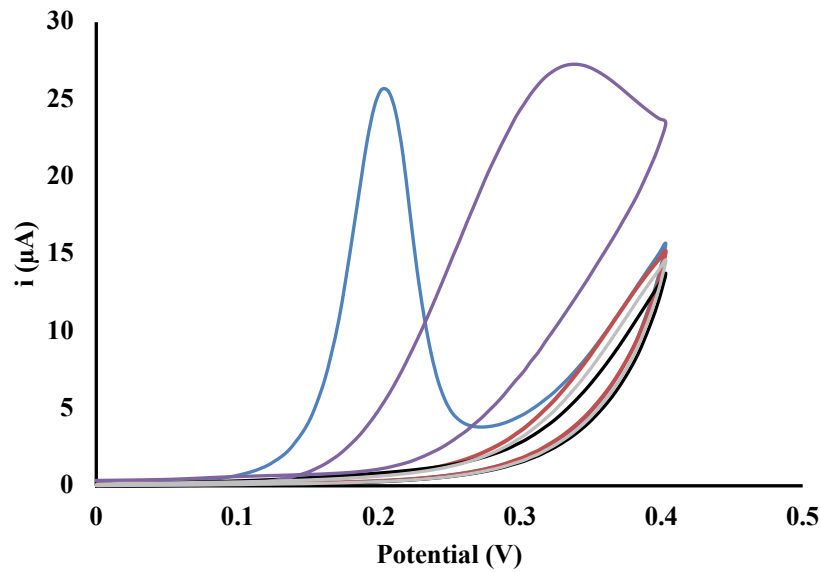
Fig. S2. Evaluation of the adequacy of the electrochemical detection mechanism based on 3-IP/Ag$^+$ substrate: Cyclic voltammograms obtained in the presence of p60 (100 ng mL^{-1}) with a complete immunoassay (control, blue line) and followed by a second scan (orange line). Cyclic voltammograms obtained in the presence of p60 (100 ng mL^{-1}) but without 3-IP (grey line); Ag$^+$ (yellow); or both 3-IP/Ag$^+$ (grey solid line) in the substrate mixture. Experimental conditions: CAb (10 µg mL^{-1}); BSA (2% (w/v)); DAb (2 µg mL^{-1}); ALP-Ab (1:25 000); 3-IP/Ag$^+$ (1.0 mM/ 0.4 mM), except mentioned otherwise.
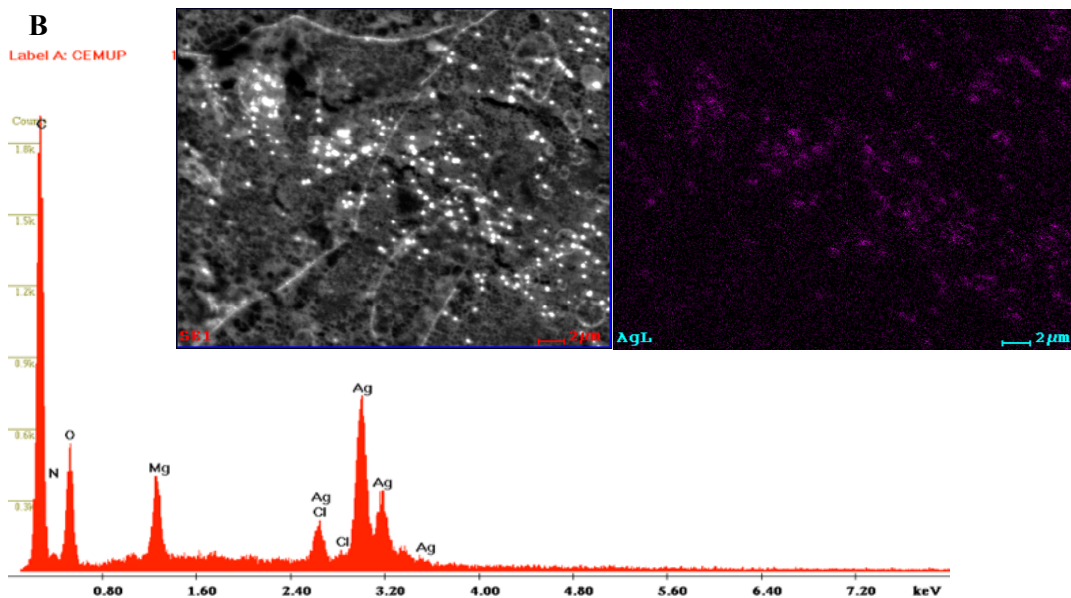
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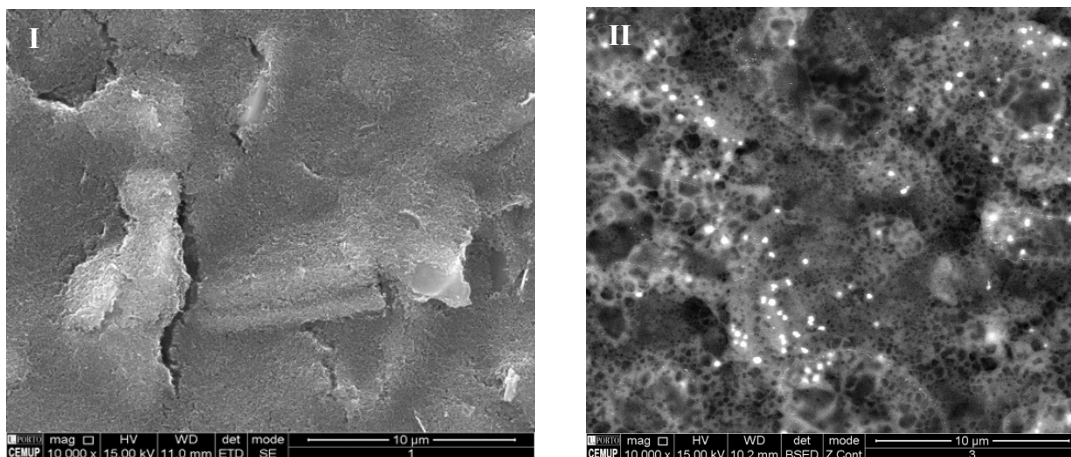
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Fig. S3 - SEM images (A) of a bare SPCE working electrode surface (I) and an optimized 3IP-Ag$^+$/Ab-ALP/DAb/p60/CAb modified SPCE (II). (B) EDS mapping of silver deposition.
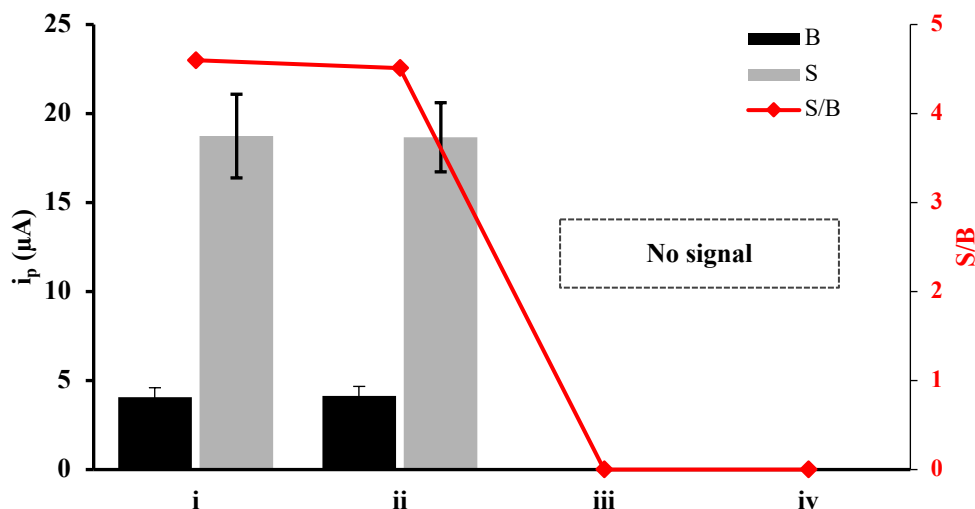
Fig. S4. Voltammetric responses obtained in the absence (B, 0 ng mL^{-1}) and in the presence (S, 50 ng mL^{-1}) of p60 standard solutions, as well as the corresponding signal/blank ratio values (S/B, red line), according with the assay format studied. Format (i): incubation of p60, DAb and ALP-Ab in separated steps of 60 min each; Format (ii): p60 and DAb incubation in one step of 60 min followed by 60 min ALP-Ab incubation; Format (iii): 60 min of p60 incubation followed by DAb and ALP-Ab incubation in one step; Format (iv): 60 min of p60, DAb and ALP-Ab incubation in one step. Experimental conditions: CAb (5 µg mL^{-1}); BSA (2%); Dab (2 µg mL^{-1} in a 0.01% BSA solution); ALP-Ab (1:50 000 in a 0.5% BSA solution); 3-IP/Ag$^+$ (1.0 mM/ 0.4 mM). Error bars were estimated from the standard deviation of three replicates.

Table S1 - Optimization of the different experimental variables involved in the preparation of the electrochemical immunosensor for p60 detection.

Variable	Checked range	Selected value
nº of incubation steps	2, 3 or 4 steps	3[*]
[CAb], µg mL^{-1}	2– 10	10
[DAb], µg mL^{-1}	1 – 4	2
ALP-Ab, dilution factor	1:10 000 – 1:50 000	1:25 000
ALP-Ab incubation time, min	15 – 60	60

[*]obtained by mixture of analyte and DAb.

CHAPTER 4 Conclusions and Future Prospects

This last chapter includes the main conclusions obtained from this thesis and presents some future prospective.

Conclusions and Future Prospects

Foodborne illnesses are caused by ingestion of water or food contaminated by pathogenic microorganisms, like bacteria and virus, pesticides residues or other toxins. Among other food contaminants, bacterial infections still stand today a worldwide public health issue, causing millions of deaths and hospitalizations every year, despite the several control and identification measures imposed by food control regulations and the innumerous alternative methods developed in past years. Accordingly, the main aim of this work was to develop innovative analytical methods, which simultaneously incorporate features like simplicity, rapidness, low-cost and minimal sample preparation. Moreover, also sought to achieve further improvements in autonomy and portability comparing to the present validated methods, in order to fulfil the present need of a decentralized method for out-of-lab foodborne detection.

There is a multiplicity of food-borne microorganisms able to infect humans with different impact on public health. This work only relies on two of the most frequently occurring and life-threatening pathogens in food, namely *Salmonella* and *Listeria spp.,* with special focus to *Salmonella typhimurium* and the *Listeria monocytogenes* serotypes. Among electroanalytical techniques, potentiometry with ion selective electrodes has recognized merits in the detection of small ionic analytes at low cost and using simple and portable instrumentation. Accordingly, this electrochemical technique played a prominent role in the immunosensing systems created for *Salmonella typhimurium*, since a zero-tolerance policy was imposed by the food quality control standards for this specific microorganism. Two different label-free immunosensing approaches were developed resorting both to an ion selective electrode as transducer element, potentiometry as transducing technique and to the surface blocking principle and a zero current passive ion flux as detection mechanism, but using different immunosensing platforms, electrode substrates and construction designs. In the first work, gold nanoparticles formed in-situ on a polymer inclusion membrane has been successfully used as a biocompatible sensing platform for bioreceptor conjugation. Surprisingly, this construction shown that besides influence the correct bioreceptor anchorage, the nanostructured surface also promotes the amplification of the measured potentiometric signal, when a surface confined immunoreaction was accessed concomitantly. Moreover, it was verified that the ratio between the AuNPs size and bioreceptor has an important role in the development of this type of label-free potentiometric immunosensors, according to its target binding capacity, which was assumed here being related with capture antibody physical and conformational changes experienced in different immunosensing interface construction

protocols. Accordingly, since the signal amplification was obtained through the developed immunosensing interface, without resorting to redox labels or enzymatic amplification, this reliable method can be easily applied to a different bacteria-antibody couple, simply changing the specific antibody and optimizing the particle size distribution or the membrane surface coverage on the polymer inclusion membrane.

The second label-free potentiometric immunosensor was developed on a paper-based platform. The strategy presented in this work is a simple and accessible methodology to control ionic flow through the polymer membrane. The developed paper-strip electrode prototype also shows potential to be applied in other media or other microorganisms simply by using different ion selective polymer sensors, marker ions and different specific antibodies. Both proposed potentiometric immunosensors shows potential for on-site food control owing to the easiness of the experimental procedure and the simplicity and portability of the potentiometric instrumentation. Additionally, the use of a label-free format allowed to increase the methods cost-efficiency, with apparently no loss of accuracy, comparing with labelled-approaches presents in the literature. Low limits of detection of 5–6 cells mL^{-1} were achieved in less than 1-hour assay. Notwithstanding the add-value of the developed immunosensors, the possibility of improving the recovery rates obtained without increasing the total time of analysis was equated. Therefore, in the third work miniaturized ion-selective electrodes were coupled to a labelled sandwich immunomagnetic assay, in which the enhanced properties of magnetic nanoparticles for sample pre-concentration were explored, by capturing the *Salmonella typhimurium* cells in milk samples both to the high specificity of antibody–antigen binding amplified by the introduction of nanocrystal tags. The home-made cadmium ion selective polymer electrodes developed in this work achieved to a high analytical performance even in small sample volumes. Moreover, the developed immunoassay proved to be highly effective in the accurate selection of the target bacteria and in its quantification by indirect correlation of the cadmium nanocrystals used as labels in the final step of the assay. A limit of detection of 2 cells per 100 mL of sample was attained in an average total time per assay of 75 minutes.

Listeria monocytogenes presents a high human pathogenicity, persistency and resistance to adverse environments. By these features, the development of accurate analytical devices for detection of this foodborne pathogen was also considered in the development of this thesis. Thus, the analytical method developed for *Listeria monocytogenes* aimed to address the main flaws of conventional methods in food quality control, according to a realistic contamination scenario. Among others, ready-to-eat products are one of the food categories most are more easily contaminated and likely to

generate uncontrolled widespread contamination, since their shelf-life don't fit with the standard methods analysis time. Accordingly, the shortening of assay times relatively to the standard methods paired with the high sensitivity imposed by food control regulation were the parameters which were given the highest emphasis. Therefore, the last experimental work presented reports the first electrochemical immunosensor for rapid, specific and decentralized quantification of p60 invasion associated protein from *Listeria monocytogenes*. The target analyte and the overall immunoreagents selected show a high affinity for their conjugates, which resulted in the development of a high specificity sandwich assay. Moreover, the enzymatic amplification coupled with voltammetric stripping analysis proven to be a good methodology to address to low concentration levels of analyte (LOD of 1.52 ng mL^{-1}) even in complex food samples (LOD of 5 ng mL^{-1}, in milk). Moreover, this method presents important hands-on time improvements ($<$ 15 min), comparing with standard ELISA methodologies, along with miniaturized and portable instrumentation. Due to the abundance of the p60 in LM growth media and cells surface and the excellent analytical performance exhibited by the developed electrochemical immunosensor, the developed electrochemical biosensing system could be an asset in the routine of food quality control, especially for ready-to-eat market niche, which still has no adequate food control solution.

The research in electrochemical biosensing devices for food safety has experienced a great evolution and new and exciting alternatives are being developed. In this context, the developed analytical systems presented here achieved to a prominent place in terms of overall sensitivity and analysis time improvements, but above all asserts as low-cost food control tools, which can predict a closer path to the decentralized implementation food safety methodologies at all levels of the product chain. Notwithstanding, further research is needed to increase the robustness of manually constructed electrodes, and clarify the real advantages associated with different nanostructured electrochemical platforms. Additionally, the activity potential and the benefits of magnetic separation in simplification of pre-treatment food sample protocols will have to be further studied to fully understand the analytical potential of the developed prototypes. In this field, it is envisaged that the use of innovative bioreceptors, more resistant to different pH's, ionic concentrations and different temperature conditions, such as phage's, peptides or artificial biorecognition elements, leverage better recovery rates in food samples.