PROJECT NO: FP6-017969

BUGCHECK

A rapid hand-held analyser for control of microorganisms in the complete meat supply chain

Co-operative Research (Craft)

Horizontal Research Activities Involving SMEs

Publishable Final Activity Report

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Project Coordinator Name: Irene Larroy

Project Coordinator Organisation Name: Centre de Recerca i Investigació de Catalunya

Version 01 – Final

Table of Contents

PROJECT OBJECTIVES

Foodborne pathogens in processed ready-to-eat products pose a serious threat to consumers with compromised immune system. Sensitive, specific and rapid detection of such pathogens is thus essential at production level to prevent their entrance into the human food chain.

Conventional microbiological detection methods simply take too long (2 to 7 days) to detect and identify pathogens in food and no real time data is available. Other traditional testing methods, such as ELISA, are also relatively costly and time-consuming. Traditional methods require the taking of a product sample, its posterior culturing until sufficient microorganisms have been generated to enable ready detection on culture plates. While a number of methods such as PCR may provide faster detection (6-12 hours) they involve complex procedures and highly specialised trained personnel.

In today's modern food supply chain, products enter and leave the market within two to three days. Slow traditional analytical methods are clearly deficient as they enable contaminated meat products to reach the market, resulting in human disease and even mortality. Moreover, most analyses need to be carried out in large analytical laboratories as the required instrumentation is expensive and requires highly qualified staff. Only very large farms and slaughterhouses can thus afford to perform regular, on-site, microbiological checks.

During this project we will investigate, develop and validate a multi-analyte platform based on biosensor technology for the detection of the most common pathogens occurring in the meat industry (*Campylobacter*, *Salmonella* and *E. Coli*), including the most common strands of each. A biosensor-based approach presents a promising and sensitive alternative tool to detecting low numbers of cell within a few minutes (with no need for enrichment steps) as opposed to days.

The multi-analyte platform will be applicable at various stages along the food production chain, and for this reason it will need to be portable, cost effective, rapid and easy to use. The multi-analyte platform will use spectroscopic impedance methods to analyse the meat samples and will include a flow cell, into which the "plug & play" cartridges will be placed for measurement. Different cartridges will be developed for *Campylobacter*, *Salmonella* and *E. Coli* and will be removable after use. The developed platform will provide a sound basis for future implementation and analysis of other foodborne pathogens.

CO-ORDINATOR CONTACT DETAILS

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CONTRACTORS INVOLVED IN THE PROJECT

Partic. Role[1]	Partic. no.	Participant name	Participant short name	Country	Date enter project	Date exit project
CO	1	Centre de Recerca i Investigació de Catalunya, S.A.	CRIC	Spain	M1	M30
CR	2	Bio Sensor Technologie GmbH	BST	Germany	M1	M30
CR	3	BVT Technologies, a.s.	BVT	Czech Republic	M1	M30
CR	4	Palm Instruments BV	PALM	Netherlands	M1	M12
CR	4	Applied Research using OMIC Sciences, S.L.	AROMICS	Spain	M13	M30
CR	5	Investigaciones Bioquímicas S.L.	IBQ	Spain	M1	M12
CR	6	Atlangene Applications S.A.S	ATLAN	France	M1	M30
CR	7	Richard Woodall Ltd	WOOD	United Kingdom	M1	M30
CR	8	Nixon W & Sons LTD	NIXON	United Kingdom	M1	M12
CR	8	JCB Electromecánica, S. L.	JCB	Spain	M13	M30
CR	9	Selección Batallé	BAT	Spain	M1	M30
CR	10	Carton Poultry Group	CPG	Ireland	M1	M30
CO	11	Consejo Superior de Investigaciones Científicas - Centro Nacional de Microelectronica	CNM	Spain	M1	M30
CR	12	Feltalálói Es Kutató Központ Szolgáltató, Kft	FKK	Hungary	M1	M30
CR	13	Faculty of Veterinary Medicine, University College Dublin	UCD	Ireland	M1	M30

[1] CO = Coordinator / CR = Contractor

WORK PERFORMED AND RESULTS ACHIEVED

During first steps of the project, a market survey was carried out. The objective was to ensure that the technology developed corresponds to the needs of the market place, the price sensitivity, as well as the acceptance of the proposed equipment into the market. A questionnaire and a cover letter introducing the project and its objectives was draft and with the help of all partners were translated into different languages; English, German, Hungarian, French, Italian, Spanish and Czech. The questionnaire could be filled in on Internet web page: http://www.cric.es/questionnaire-bugcheck. The questionnaire was advertised on several on-line specialized magazines, a massive emailing was done, and a telephone survey was carried out to gain an improved and more in-depth overview. The most important conclusion gathered with the market survey was that the price of the analysis have to be around 10€ and the maximum result time around one day. Based on this market survey as well as the experience of the partners, the general input requirements were defined.

The next step in the project was to define the technical and biological requirements of the proposed system. Based on current epidemiological data available for incidence of foodborne disease in humans within the European Union, the pathogenic microorganisms to be detected will be *Salmonella*, *Campylobacter* and verocytoxigenic strains of *Escherichia coli*. Sampling procedures and criteria for sampling carcases will be carry on following the European Decision 471/2001 now incorporated in the Food Microbiological Criteria regulation (2073/2005).

Then, suitable antibodies in the three selected microorganisms were identified taking into account the specificity to recognise the corresponding pathogens, cross-reactivity towards other pathogens as well as the capability to be used for immobilisation techniques. First tests will be performed using non toxic strains of *Escherichia coli* for this reason the polyclonal antibody against this microorganism was purchased from AbCam plc. as well as the secondary antibody for the ELISA tests was also supplied by this copmpany.

Once the definition of the specifications was determined and the suitable antibodies purchased, the design and development of the four main parts of the BugCheck prototype started: the optimization of the immuno-functionalisation protocols, the interdigitated microelectrodes, the 'plug & play' cartridge and the Electrochemical Impedance Spectroscopy (EIS) instrumentation.

- Immuno-functionalisation protocols

  A number of gold immuno-functionalisation protocols have been optimised. Among the strategies based on Ab conjugation onto SAM-modified surfaces, using a Cysteamine SAM modified with carboxidextran appeared as the most efficient. This surface induced the lowest levels of protein non-specific adsorption detected and highest specific signals when an Ab-HRP model protein was being detected.

  Alternatively, several immunofunctionalisation protocols, based on biocomponent random deposition, have been developed. The best results were obtained for Ab direct adsorption, which is the simplest and shortest protocol assayed. Among the biotin-binding proteins evaluated, neutravidin produced the best results, generating surfaces more efficient and stable than other related proteins.

  There different optimised protocols on gold rods were developed during the project: SAM-Dextran immunofuntionalisation (only for protein detection), antibody direct absorption and neutravidin physisorption and affinity capture of biotinylated Ab.

- Interdigitated microelectrodes

  Interdigitated microelectrodes have been fabricated according to the proposal. These electrodes have been characterised by scanning electron microscopy, perfilommetry, conductivity measurements and also electrochemically by cyclic voltammetry. They have been found to how moderate electron transfer properties, but the effect of this in the performance of the BugCheck immunosensors was not significant, since the final measurements was not based in faradaic impedance but capacitance measurements.

  In general the detection limits of the immunosensing technique improved as interdigitated geometric features decreased. However, as the features were made smaller, the failure rate of the devices increased, probably arising from short circuiting between bands by debris present in the solution. Also, the impossibility to re-use the immunosensors rendered the chips useful for one measurement only.

- 'Plug & Play' cartridges

New disposable 'plug and play' cartridge based on a Printed Circuit Board of 75 mm long and 0.8 mm thick were designed with 6 mm in his narrow side to be housed on a 1.5 ml Eppendorf tube and with 14.5 mm in his large side to be connected to a standard SIM card reader connector.

- Electrochemical Impedance Spectroscopy (EIS) instrumentation

A microbial analyzer based on Electrochemical Impedance Spectroscopy were implemented. The system consist on an aluminium base in where the rotational thermostatised sample holder and a stainless steel prismatic rail are screwed. The sample holder has four wells in where 1.5ml Eppendorf tubes fits. The signal transduction circuitry is fixed to a linear sliding stage to allow the linear displacement thought the prismatic rail. The system are provided with a digital control to allow the data transfer to a PC. The characteristics of BugCheck instrumentation are listed in the following table:

Impedance Analyzer		
	DC-input potential range	0 to 2.5 V
	AC-input potential amplitude	5 mV to 100 mV
	Frequencies ranges	100 Hz to 10 kHz
	Current ranges	62.5 nA to 18.75 µA
	|Z| ranges	300 Ω to 80 kΩ
	Accuracy in magnitude of impedance	4.5 %
	Accuracy in phase	0.6 °
Thermostatic Sample Holder		
	Configurable temperature	30 °C to 80 °C
	Accuracy in temperature	0.3 °C
	Warm-up time	10 minutes (at 37 °C)
Other features		
	Weight	3.800 kgr
	Dimensions	26 cm x 21.7 cm x 40.5 cm
Estimated total price		**550 €**

At the end of the project, it was possible to detect *E. coli* and *Salmonella* in phosphate buffer solutions, using electrochemical impedance spectroscopy. The detection limit was strongly conditioned by the microelectrode geometry, reaching the best detection limits (around 10^4 CFU mL^{-1}) at the 10x10 interdigitated structures. On the other hand, when a more complex sample matrix, such as culture medium, was used, the detection was not possible at any concentration level. The reason is very likely to be found in the large amounts of organic matter that can readily adsorb on the chip surface, thus blinding the sensors. One possible way to over come these matrix effects may be the use of paramagnetic particles to extract the target pathogen from the sample and then transfer them to a cleaner environment, such as a phosphate buffer solution.

PUBLISHABLE RESULTS OF THE FINAL PLAN FOR USING AND DISSEMINATING THE KNOWLEDGE

Related with project results exploitation, the partners of the consortium agreed that the overall BugCheck system had very low commercial value and has no sense to commercialized it, however, there are some final results potentially exploitable:

A) Immobilization procedure

B) Biosensor software package

C) Plug and play cartridge system

D) Microelectrodes on silicon and glass

E) Validated method of the measurement

All SME partners were formally asked to express their interest in BugCheck project results and only three of them (BVT, BST and AROMICS) were interested in one or more of these exploitable results. An internal agreement was arranged between these SME partners.

Related with dissemination of the results, a website has been set up containing the public synopsis sheet, the objectives of the project and some general information about the partners. Regarding the communication among the partners, the same website contains a private password-protected area where all the documents of the project are available. The public website is at http://bugcheck.cric-projects.com

Other tasks related with dissemination matters have been done, for example, the project has been advertised in several publications on specialized magazines such us AgroMeat, EurooCarne and Azti tecanlia.

On the other hand, CRIC participated at ALIMENTARIA 2006, the International Food and Beverages, Exhibition, that was take place in Fira de Barcelona from 6 to 10 of March 2006. The space at ALIMENTARIA'06 was possible thanks to FITEC, a company dedicated to serve different industry activity sectors becoming the space R+D+I in the different scenarios of interrelationship and exchange between entreprenaurial world and research. ALIMENTARIA'06 allowed visitors to experience in a real way all innovations, to know the technological advances and to be well up on the last est the trends in the market. CRIC presented in AGROALIMENTARI'06 the BUGCHECK project in order to disseminate the project and his results beyond the consortium to a wider audience.

CRIC also participated in The Seventh Rothamsted International Biomarket, BIOPRODUCTS FOR FOOD AND HEALTH at Rothamsted Research, Harpenden, UK from 8 to 10 of November 2006. This event is an international conference and partnering event for those involved in research, knowledge transfer and product development leading to healthier, safer and better quality foods. During the BioMarket 2006 we had the oportunity to keep up to date participants about the BugCheck project.

Exploitation Board agreed give permission to CNM for their publication of results. Among their publications, they submitted a review article on pathogen detection published in Biosensors & Bioelectronics which was accepted and a second one on detection of E. Coli and Salmonella typhimurium using interdigitated microelectrode capacitive immunosensors to Analytical Chemistry which is pending. The full documents are provided below.

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Review

Pathogen detection: A perspective of traditional methods and biosensors

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Abstract

The detection of pathogenic bacteria is key to the prevention and identification of problems related to health and safety. Legislation is particularly tough in areas such as the food industry, where failure to detect an infection may have terrible consequences. In spite of the real need for obtaining analytical results in the shortest time possible, traditional and standard bacterial detection methods may take up to 7 or 8 days to yield an answer. This is clearly insufficient, and many researchers have recently geared their efforts towards the development of rapid methods. The advent of new technologies, namely biosensors, has brought in new and promising approaches. However, much research and development work is still needed before biosensors become a real and trustworthy alternative.This review not only offers an overview of trends in the area of pathogen detection but it also describes main techniques, traditional methods, and recent developments in the field of pathogen bacteria biosensors.

Keywords: Biosensors; Pathogen detection; ELISA; PCR; SPR; QCM; Amperometry; Immunosensors; Salmonella; *E. coli*; Listeria; Legionella; Campylobacter

Contents

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1. Introduction and trends

This paper aims to give an overview of the field of pathogen bacteria detection. First, the main fields of application and bacteria are presented according to the academic literature over the past 20 years. Next, the main analytical methods shall be described. These descriptions will cover generic strengths and weaknesses from each method. Whenever possible, details such as time per analysis and detection limits will be given. Next, the role of biosensors in this important and challenging field will be addressed, and the main types will be covered. Recent breakthroughs, such as the applications of magnetic beads and microsystems, will be highlighted.

A comprehensive literature survey has been carried out for the present study. Because the literature related to pathogen bacteria is vast, our study focuses only on the analytical side: detection, identification and quantification, with an emphasis on biosensors. Pathogen detection methods are currently few but, due to the involvement of many different techniques (Pitcher and Fry, 2000; Stevens and Jaykus, 2004) between sample preparation (extraction and purification, enrichment, separation, . . .) and analysis, they are rich in complexity.

Conventional methods are used despite their long turnover times because of their high selectivity and sensitivity. Biosensors have the potential to shorten the time span between sample uptake and results, but their future lies in reaching selectivities and sensitivities comparable to established methods at a fraction of the cost. Although not so critical, issues such as ease of use, low maintenance and continuous operation also need to be considered.

1.1. Main areas requiring pathogen control: frequently found pathogenic bacteria

Pathogen detection is of the utmost importance primarily for health and safety reasons. Fig. 1 shows that three areas of application account for over two thirds of all research in the field of pathogen detection. These are the food industry (Leonard et al., 2003; Patel, 2002), water and environment quality control (Emde et al., 1992; Theron et al., 2000) and clinical diagnosis (Atlas, 1999). The remaining efforts go into fundamental studies (Gao et al., 2004; Herpers et al., 2003), method performance studies (Dominguez et al., 1997; Taylor et al., 2005) or development of new applied methods (Yoon et al., 2003; Ko and Grant, 2003).

Amongst the growing areas of interest, the use of rapid methods for defense applications stands out (Lim et al., 2005; Hindson et al., 2005). In fact, the number of publications dealing with these applications already account for over 1% of all publications in the field of rapid methods for pathogen detection since 1985.

The food industry is the main party concerned with the presence of pathogenic bacteria. The public health implications of failing to detect certain bacteria can be fatal, and the consequences easily make the news. Recently in Spain (July, 2005), a batch of contaminated pre-cooked chicken resulted in a salmonella outbreak causing 2500 sick people and at least one death by salmonellosis.

Although *Escherichia coli* is the most commonly and thoroughly studied model bacterium, Salmonellae account for the largest number of articles the number of articles reporting

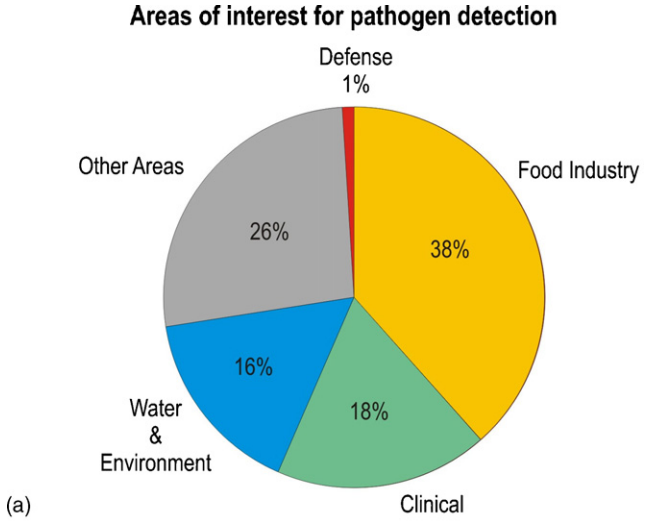

Fig. 1. (a) Distribution, by industry of application, of the relative number of works appeared in the literature on detection of pathogenic bacteria. (b) Distribution, by micro-organism, of the relative number of works appeared in the literature on detection of pathogenic bacteria.

rapid methods for its detection. Fig. 1b shows the distribution of scientific literature covering the detection of pathogenic bacteria.

In spite of our efforts to keep it down to a minimum, there may be some cases of overlap in our classification. Although in general it has been possible to correct possible overlaps, we cannot guarantee (nor do we pretend) that the categories in Fig. 1a and b are 100% mutually exclusive. In spite of this, we believe that they cast a good reflection of the existing literature.

The following sections describe the various approaches most commonly taken to detect and identify pathogenic bacteria. First, classic or traditional techniques are briefly summarised. Next, the uses of biosensors in their most important forms are described. Finally, a summary table is given where a comparison between methods can be made more easily.

1.2. Analytical methods in pathogen detection: trends

Fig. 2a compares the different methods used according to the number of publications where they are applied to the detection of any of the bacteria from Fig. 1. The most popular methods are, by far, those based on culture and colony counting methods (Leoni and Legnani, 2001) and the polymerase chain reaction, PCR (Bej et al., 1991). This can be explained on the grounds of selectivity and reliability of both techniques. Culture and colony counting methods are much more time consuming than PCR methods but both provide conclusive and unambiguous results. On the other hand, recent advances in PCR technology, namely real-time-PCR (Levi et al., 2003), now enable obtaining results in a few hours.

Biosensor technology comes with promises of equally reliable results in much shorter times, which is perhaps why they are currently drawing a lot of interest. However, there is still much work to do before biosensors become a real alternative. Fig. 2a and b suggest that biosensor technology may soon move ahead of *traditional* ELISA based methods, and their potential market (Alocilja and Radke, 2003) is very encouraging too.

Many biosensors rely on either specific antibodies or DNA probes to provide specificity. However, as Fig. 2 shows, the technology is very split when it comes to detection modes.

Fig. 2b points that biosensors' is the fastest growing pathogen detection technology.

The following sections will deal with each method in more detail.

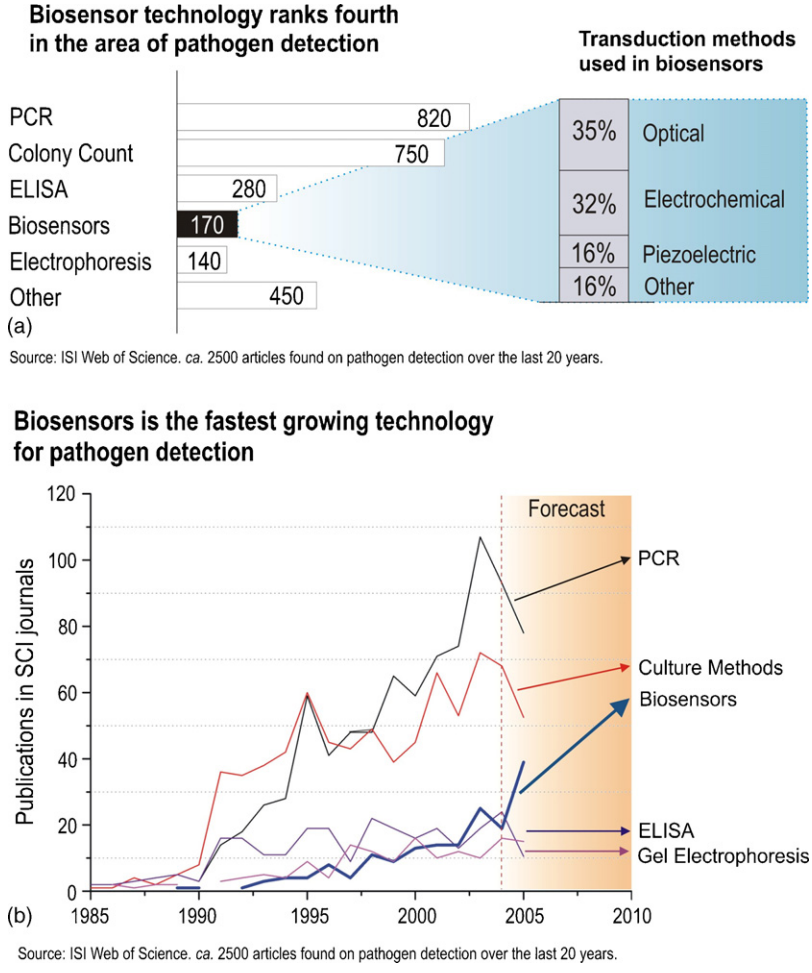

Fig. 2. (a) Approximate number of articles using different techniques to detect and/or identify pathogenic bacteria. Articles using more than one technique have been excluded in order to avoid overlap between categories. (b) Time series of the number of works published on detection of pathogen bacteria over the last 20 years. The fact that certain articles used more than one technique has been accounted for to make this graph.

2. Established methods in pathogen detection

Polymerase chain reaction (PCR), culture and colony counting methods as well as immunology-based methods are the most common tools used for pathogen detection. They involve DNA analysis, counting of bacteria and antigen–antibody interactions, respectively. In spite of disadvantages such as the time required for the analysis or the complexity of their use, they still represent a field where progress is possible. These methods are often combined together to yield more robust results.

2.1. Polymerase chain reaction

This is a nucleic acid amplification technology. It was developed in the mid 80s (Mullis et al., 1986) and it is very widely used in bacterial detection. It is based on the isolation, amplification and quantification of a short DNA sequence including the targeted bacteria's genetic material. Examples of different PCR methods developed for bacterial detection are: (i) real-time PCR (Rodríguez-Lázaro et al., 2005), (ii) multiplex PCR (Jofré et al., 2005) and (iii) reverse transcriptase PCR (RT-PCR) (Deisingh, 2004). There are also methods coupling PCR to other techniques such as, for example surface acoustic wave sensor (SAW) (Deisingh, 2004) or evanescent wave biosensors (Simpson and Lim, 2005).

The PCR is a lot less time-consuming than other techniques, like culturing and plating. It takes from 5 to 24 h to produce a detection result but this depends on the specific PCR variation used and this does not include any previous enrichment steps.

Fig. 3 illustrates the PCR method, consisting in different cycles of denaturation by heat of the extracted and purified DNA, followed by an extension phase using specific primers and a thermostable polymerization enzyme. Then each new double-stranded DNA acts as target for a new cycle and exponential amplification is thus obtained.

The presence of the amplified sequence is subsequently detected by gel electrophoresis.

Amongst the different PCR variants, multiplex PCR is very useful as it allows the simultaneous detection of several organisms by introducing different primers to amplify DNA regions coding for specific genes of each bacterial strain targeted (Touron et al., 2005). Real-time PCR permits to obtain quicker results without too much manipulation. This technique bases its detection in the fluorescent emission by a specific dye as it attaches itself to the targeted amplicon. Given that fluorescence intensity is proportional to the amount of amplified product (Cady et al., 2005), it is possible to follow the amplification in real time, thus eliminating laborious post-amplification processing steps such as gel electrophoresis. Different alternative probes, deriving from this principle, have been developed recently (TaqMan, fluorescence resonance energy transfer or molecular beacon probes) (Yang, 2004).

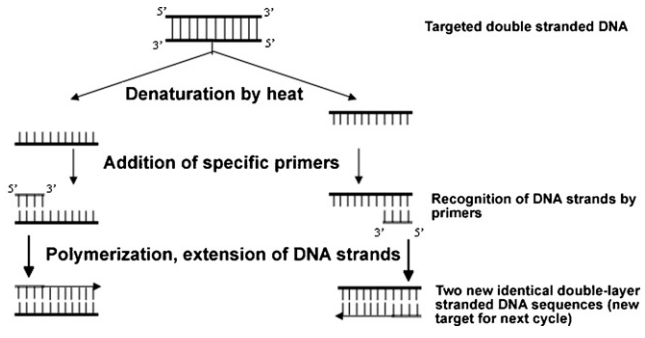

Fig. 3. Schematic representation of one PCR cycle taking place in thermocycler.

One of the limitations of PCR techniques lies in that the user cannot discriminate between viable and non-viable cells because DNA is always present whether the cell is dead or alive. Reverse transcriptase PCR (RT-PCR) was developed in order to detect viable cells only (Yaron, 2002). RT is an enzyme able to synthesize single-stranded DNA from RNA in the 5′–3′ direction. Several genes specifically present during the bacteria's growth phase can then be detected. This technique gives sensitive results without any time-consuming pre-enrichment step (Deisingh, 2004).

PCR may also be found coupled to other techniques. Examples are "the most probable number counting method" (MPN-PCR) (Blais et al., 2004), surface plasmon resonance and PCR-acoustic wave sensors (Deisingh, 2004), LightCycler real-time PCR (LC-PCR) and PCR-enzyme-linked immunosorbent assay (PCR-ELISA) (Perelle et al., 2004), the sandwich hybridization assays (SHAs) (Leskelä et al., 2005) or the FISH (fluorescence in situ hybridization) detection test (Lehtola et al., 2005).

2.2. Culture and colony counting methods

The culturing and plating method is the oldest bacterial detection technique and remains the standard detection method. However, other techniques are necessary because culturing methods are excessively time-consuming. in the case of Campylobacter, 4–9 days are needed to obtain a negative result and between 14 and 16 days for confirmation of a positive result (Brooks et al., 2004). This is an obvious inconvenience in many industrial applications, particularly in the foods sector.

Different selective media are used to detect particular bacteria species. They can contain inhibitors (in order to stop or delay the growth of non-targeted strains) or particular substrates that only the targeted bacteria can degrade or that confers a particular colour to the growing colonies (rainbow agar from Salmonella detection (Fratamico, 2003)). Detection is then carried out using optical methods, mainly by ocular inspection.

2.3. Immunology-based methods

The field of immunology-based methods for bacteria detection provides very powerful analytical tools for a wide range of targets. For example, immunomagnetic separation (IMS) (Mine, 1997; Pérez et al., 1998), a pre-treatment and/or pre-concentration step, can be used to capture and extract the targeted pathogen from the bacterial suspension by introducing antibody coated magnetic beads in it (Gu et al., 2006). IMS can then be combined with almost any detection method, e.g., optical, magnetic force microscopy, magnetoresistance (Bead Array Counter) (Baselt et al., 1998) and hall effect (Besse et al., 2002),

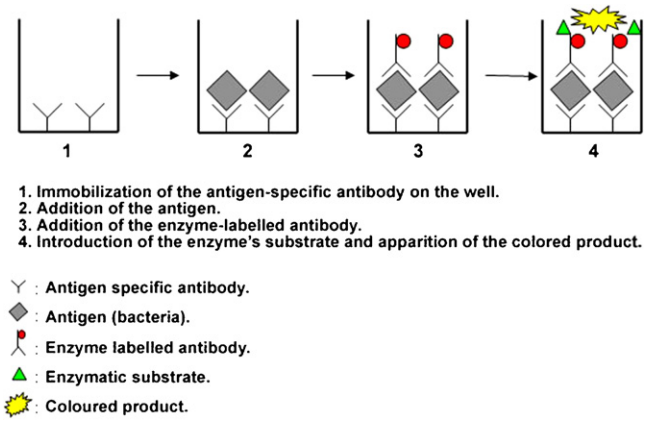

Fig. 4. Schematic representation of the sandwich-ELISA protocol.

amongst others. Custom derivatized magnetic beads are available from a number of companies, the most conspicuous of which is perhaps Dynal. Beads of widely ranging sizes (from a few nano-meters up to a few tens of microns) may be chosen depending on the application. Whilst large beads may be used for the measurement of intermolecular forces, smaller particles are best for the detection of small analytes where high sensitivity is critical. In the case of whole bacteria, the use of beads in the low micrometer range may provide the right balance between time and sensitivity.

Other detection methods are only based on immunological techniques; in this case the enzyme-linked immunosorbent assay (ELISA) (Crowther, 1995) test is the most established technique nowadays as well as the source of inspiration for many biosensor applications. ELISAs combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. Fig. 4 illustrates the principles of a typical "sandwich ELISA", which is the most common kind.

Next, an overview of recent works using biosensors in this field will be given. This overview aims to give a broad picture of the different existing technologies and working methodologies.

3. Biosensors in pathogen detection

Biosensors have recently been defined (http://www.biosensors-congress.elsevier.com/about.htm) as analytical devices incorporating a biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products, etc.), a biologically derived material (e.g., recombinant antibodies, engineered proteins, aptamers, etc.) or a biomimic (e.g., synthetic catalysts, combinatorial ligands and imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical.. The following sections classify biosensors according to their transduction methods.

3.1. Biological recognition elements and immobilisation strategies

There are three main classes of biological recognition elements which are used in biosensor applications. These are (i) enzymes, (ii) antibodies and (iii) nucleic acids. In the detection of pathogenic bacteria, however, enzymes tend to function as labels rather than actual bacterial recognition elements.

Enzymes can be used to label either antibodies (Ko and Grant, 2003) or DNA probes (Lucarelli et al., 2004) much in the same fashion as in an ELISA assay. In the case of amperometric (electrochemical) biosensors enzymatic labels are critical, as will be discussed below. More advanced techniques may operate without labelling the recognition element, such as the case of surface plasmon resonance (SPR), piezoelectric or impedimetric biosensors (Guan et al., 2004).

Because the use of antibodies in biosensors is currently more spread than that of DNA probes, the following sections deal mainly with antibody-based biosensors.

Antibodies may be polyclonal, monoclonal or recombinant, depending on their selective properties and the way they are synthesised. In any case, they are generally immobilised on a substrate, which can be the detector surface (Oh et al., 2005a), its vicinity (Radke and Alocilja, 2005) or a carrier (Ivnitski et al., 2000a).

This section addresses gold substrates only because of its importance in the area of immunosensors and DNA probes, which form the basis of most bacterial biosensors. Fig. 5 shows the three most frequent antibody immobilisation routes, which are:

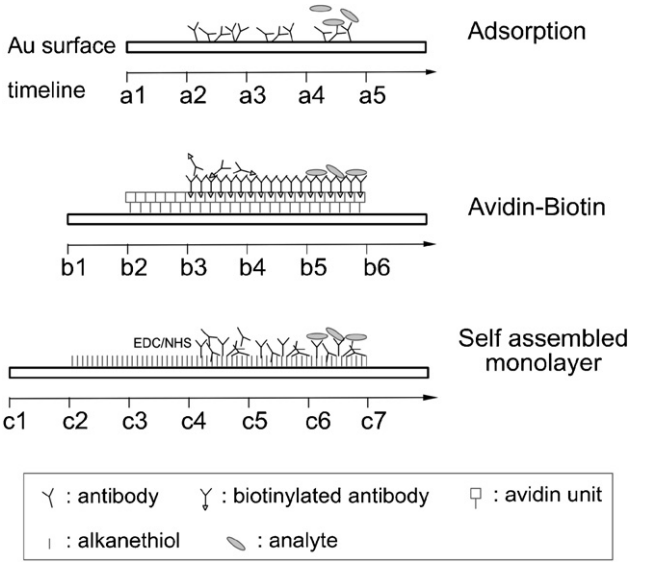

Fig. 5. Schematic representation of the main immobilization strategies and key steps involved. a1, Clean surface; a2, immersion in antibody solution; a3, wash step; a4, sample addition and a5, detection. b1, Clean surface; b2, avidin coating; b3, addition of biotinylated antibodies; b4, wash step; b5, sample addition and b6, detection. c1, Clean surface; c2, SAM formation; c3, activation in EDC/NHS; c4, antibody immobilization; c5, wash and blockage of unreacted active sites; c6, sample addition and c7, detection.

- Adsorption on gold.
- The Avidin–biotin system.
- Self-assembled monolayers (SAMs).

The bio-molecule immobilisation step is critical in the development of any sort of biosensor. It provides the core of the biosensor and gives it its identity. Moreover, the immobilised biomolecule needs to keep its original functionality as far as possible in order for the biosensor to work. This means that care must be taken so that the recognition sites are not sterically hindered. Another common reason for biosensor failure or underperformance is the chemical inactivation of the active/recognition sites during the immobilisation stages. There is no universal immobilisation method suitable for every application imaginable. When it comes to choosing the immobilisation method, there are other important factors that need careful consideration, e.g., the type of transduction used, the nature and composition of the sample and the possibility of multiple use of the biosensor. Brief descriptions of the three most common approaches follow.

3.1.1. Adsorption on gold

This is, undoubtedly the simplest, quickest and least reliable of the described methods. Since it consists in the random attachment of the antibodies on the substrate, the correct orientation of the binding sites cannot be controlled. The adsorption is non-specific and biosensor performance is seldom very good (Tombelli and Mascini, 2000). Karyakin et al. (2000) reported an approach using antibody adsorption whilst attaining a reasonable degree of performance. Fig. 5 outlines the principles of this method.

3.1.2. The Avidin–biotin system

This system is a simple and yet very effective way to anchor biomolecules to an avidin coated surface (Ouerghi et al., 2002). One of the most advantageous features of this system is that although the affinity constant between avidin and biotin is rather high (ca. 10^{-15} mol^{-1} L), the bonding is of non-covalent nature, which allows for multiple washing and re-use of the same sensing device (Tombelli and Mascini, 2000). An important drawback is the high cost of the reagents involved.

A glucose biosensor built on several avidin-biotinilated glucose oxidase layers is proposed by Anzai et al. (1998).

3.1.3. SAMs

Self-assembled monolayers are obtained by immersion of a gold plate in a solution containing a suitable surfactant in a high purity solvent (Bain et al., 1989). The most popular instances are those obtained by the immersion of gold in an ethanol solution containing disulphides or thiols (Su and Li, 2004). The packing and thickness of the formed monolayer is dictated by the radical attached to the sulphide atom(s) (Vaughan et al., 1999). An important group of compounds used in the formation of SAMs is that integrated by alkanethiols.

After formation of the monolayer, the bio-molecule of choice is linked to the other end of the thiol. Familiarity with the biomolecule is needed in order to achieve the optimum orientation and enhance biosensor performance. Depending on this, different forms of chemical modification and activation are required (Hermanson, 1996).

Due to the robustness of immunosensing devices based on SAMs, they can be found in a vast range of applications (Oh et al., 2003b; Vaughan et al., 2001; Mansfield, 2001).

Having covered the way in which antibodies and DNA may be immobilised on a transducer surface, we turn our attention towards the various measurement techniques available.

3.2. Optical biosensors

These are probably the most popular in bioanalysis, due to their selectivity and sensitivity. Optical biosensors have been developed for rapid detection of contaminants (Willardson et al., 1998; Tschmelak et al., 2004), toxins or drugs (Bae et al., 2004) and even pathogen bacteria (Baeumner et al., 2003). Recently, fluorescence and surface plasmon resonance, SPR, based methods have gained momentum because of their sensitivity.

3.2.1. Fluorescence detection

Fluorescence occurs when a valence electron is excited from its ground state to an excited singlet state. The excitation is produced by the absorption of light of sufficient energy. When the electron returns to its original ground state it emits a photon at lower energy. Another important feature of fluorescence is the little thermal loss and rapid (<10 ns) light emission taking place after absorption. The emitted light is at a longer wavelength than the absorbed light since some of the energy is lost due to vibrations, this energy gap is termed Stoke's shift, and it should be large enough to avoid cross talk between excitation and emission signals.

Antibodies may be conjugated to fluorescent compounds, the most common of which is fluorescein isothiocyanate (FITC) (Li et al., 2004). There are, however, other fluorescent markers. The use of lanthanides as sources of fluorescence in luminescent assays has very recently been reviewed (Selvin, 2002). Although lanthanides pose several important advantages (good stability, low background luminescence under normal light conditions and large Stoke's shift) compared to more traditional fluorophores, their use is very restricted due to safety reasons.

Fluorescence detection, in contrast to SPR, is also used in combination with established techniques such as PCR and ELISA. Such is the case of a hand-held real-time thermal cycler recently developed (Higgins et al., 2003). This analyser measures fluorescence at 490 and 525 nm, which enables the simultaneous detection of more than one microorganism. Although this work claims detection times of 30 min, it should be pointed that overnight culturing is required to achieve best results.

Fluorescence resonance energy transfer (FRET) biosensors (Ko and Grant, 2003) are based on the transfer of energy from a donor fluorophore to an acceptor fluorophore. Fig. 6 schematically shows how this kind of biosensor works. It is able to report whether a food sample contains salmonella down to a detection limit of 2 $\mu g\,mL^{-1}$.

3.2.2. Surface plasmon resonance

SPR biosensors (Cooper, 2003) measure changes in refractive index caused by structural alterations in the vicinity of a

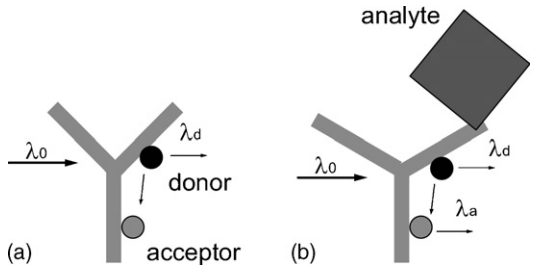

Fig. 6. Diagram of a FRET biosensor. The acceptor fluorophore responds to the excitation from the donor fluorophore only when the distance between them is short enough, e.g., when an antigen binds to the antibody.

thin film metal surface. Current instruments operate as follows. A glass plate covered by a gold thin film is irradiated from the backside by p-polarised light (from a laser) via a hemispherical prism, and the reflectivity is measured as a function of the angle of incidence, θ. The resulting plot is a curve showing a narrow dip. This peak is known as the SPR minimum. The angle position of this minimum is determined by the properties of the gold-solution interface. Hence, adsorption phenomena and even antigen–antibody reaction kinetics can be monitored using this sensitive technique (as a matter of fact, SPR is used to determine antigen–antibody affinity constants). The main drawbacks of this powerful technique lay in its complexity (specialised staff is required), high cost of equipment and large size of most currently available instruments (although portable SPR kits are also available commercially, as is the case of Texas Instruments' Spreeta system).

SPR has successfully been applied to the detection of pathogen bacteria by means of immunoreactions (Taylor et al., 2005; Oh et al., 2005a).

3.2.3. Piezoelectric biosensors

Piezoelectric sensors are based in the observation of resonance frequency changes on a quartz crystal microbalance (QCM) following mass changes on the probe/transducer surface (O'sullivan and Guilbault, 1999). The relation between mass and resonant frequency is given by the Sauerbrey equation:

$$\Delta F = \frac{-2.3 \times 10^6 F_0^2 \Delta m}{A}$$

where ΔF is the frequency change in Hertz, F_0 the resonant frequency of the crystal in MHz, Δm the deposited mass in grams and A is the coated area in cm^2.

As the literature shows (Pathirana et al., 2000; Wong et al., 2002; Vaughan et al., 2001), the use of QCM allows the detection of bacteria using probes modified with immobilised antibodies. Li et al. (2004) provide an example of how *E. coli* may be detected between 10^3 and 10^8 CFU mL^{-1} in 30–50 min. The antibody modified probe is immersed for an hour in a solution containing *E. coli*. It is then extracted, rinsed using PBS and dried under nitrogen (the Sauerbrey equation holds only for gas-phase measurements). The resonant frequency of the probe is finally measured and results are obtained within minutes after drying. The authors point in their conclusions that although the dip-and-dry method is more sensitive, reproducible and reliable than traditional flow-through methods, it is not as suitable for automation and therefore recommend that any further studies should be aimed to improving the flow-through method.

3.3. Electrochemical biosensors

These devices are mainly based on the observation of current or potential changes due to interactions occurring at the sensor-

Table 1
Detection of *E. coli*

Detection technique	Sample type	Time of analysis	Working range[a] (CFU mL^{-1})	Detection limit[a] (CFU mL^{-1})	Ref.
ELISA	Ground beef	Next day	10^3–10^4	1.2×10^3	Blais et al. (2004)
PCR-ELISA	Milk	5 h	10^0–10^4	100	Daly and Doyle (2002)
PCR-electrophoresis		2 h	10^1–10^4	1000	
Real-time PCR	Culture medium	5 h 20 min	5–5 × 10^4cells	5 cells	Fu and Kieft (2005)
	Ground beef	3 h 20 min		1.3×10^4 cells/g or 1.6×10^3 CFU mL^{-1}	
RT-PCR coupled to fluorescence	Drinking water	30 min	1–10^6	10^2	Higgins et al. (2003)
Fiber optic immunosensor	Culture	10 h	Tested up to 6.5×10^4	2.9×10^3	Tims and Lim (2003)
SPR biosensor	Culture	Not quoted	10^2–10^9	10^2	Oh et al. (2005b)
QCM Immunosensor	Culture/water	170 min	10^3–10^8	10^3	Brooks et al. (2004)
Amperometry	Culture	30 min	100–600		Abdel-Hamid et al. (1999)
Conductimetric biosensor	Mixed culture containing up to five different microorganisms	10 min	10–10^5	79	Muhhammad-Tahir and Alocilja (2003)
	Water				
	Vegetable wash water	6 min	10–10^6	81	Muhhammad-Tahir and Alocilja (2004)
Impedimetric immunosensors	Culture/water	10 min	10^4–10^7	10^4 in culture and 10^7 in water.	Radke and Alocilja (2005)

[a] Unless otherwise stated.

Table 2
Detection of *Legionella pneumophila*

Detection technique	Sample type	Time of analysis	Working range[a] (CFU mL^{-1})	Detection limit[a] (CFU mL^{-1})	Ref.
Colony count	Water	5–14 days	2.5–994	1	Villari et al. (1998)
PCR		1–2 h	0.015–150	1–10	
Sandwich hybridization assay (SHA)	Water	1–2 h		1.8×10^3 cells	Leskelä et al. (2005)
SPR	Culture	2 h 20 min	10^2–10^9	10^2	Oh et al. (2003a)

Table 3
Detection of *Campylobacter jejuni*

Detection technique	Sample type	Time of analysis	Working range[a] (CFU mL^{-1})	Detection limit[a] (CFU mL^{-1})	Ref.
ELISA	Bovine vaginal mucus and preputial washing	5 days	10^5–10^7	10^5–10^6	Brooks et al. (2004)
Real-time PCR-IMS	Chicken fecal suspension	4 h		100–150	Lund et al. (2004)
Total internal reflection fluorescente biosensor	Culture	Over 2 h		ca. 10^3	Sapsford et al. (2004)
Amperometric immunosensor	Culture and chicken carcass, wash water	2–3 h	10^3–10^7	2.1×10^4	Che et al. (2001)

Table 4
Detection of *Salmonellae*

Detection technique	Sample type	Time of analysis	Working range[a] (CFU mL^{-1})	Detection limit[a] (CFU mL^{-1})	Ref.
IMS-plating	Raw chicken	Next day		1–10	Mansfield (2001)
IMS-ELISA		Next day	10^6–10^9	10^6	
Electrochemical sandwich ELISA	Meat	Same day	Unknown	1–10 cells/25 g	Croci et al. (2001)
PCR-ELISA	Milk	Next day	1–10^8	10^3	Perelle et al. (2004)
QCM	Phosphate buffer	60 min	10^5–5×10^8	10^4	Wong et al. (2002)
Amperometric biosensor	Culture and water	1–2 h	Not specified	5×10^4	Brewster et al. (1996)

sample matrix interface. Techniques are generally classified according to the observed parameter: current (amperometric), potential (potentiometric) or impedance (impedimetric). Compared to optical methods, electrochemistry allows the analyst to work with turbid samples, and the capital cost of equipment is much lower. On the other hand, electrochemical methods present slightly more limited selectivity and sensitivity than their optical counterparts (see Tables 1–5 below). Fig. 7 compares the sizes of the various components of an electrochemical biosensor.

3.3.1. Amperometric methods

This is perhaps the most common electrochemical detection method used in biosensors. It works on the grounds of an existing linear relationship between analyte concentration and current. The sensor potential is set at a value where the analyte, directly or indirectly, produces a current at the electrode. In the case of biosensors, where direct electron exchange between the electrode and either the analyte or the biomolecule is not permitted, redox mediators are required (Eggins, 2002). Redox mediators are small size compounds able to reversibly exchange electrons between both the sensor and the enzyme of choice (e.g., ferricyanide, osmium or ruthenium complexes, dyes, etc.).

Many different combinations and strategies to build biosensors are possible. The actual choice depends on constraints imposed by sample matrix, analyte, or usability (Willner et al., 1997).

Bacterial biosensors do not differ much from more conventional biosensors (Ivnitski et al., 2000b). An interesting example

Table 5
Detection of *Listeria monocitogenes*

Detection technique	Sample type	Time of analysis	Working range[a] (CFU mL^{-1})	Detection limit[a] (CFU mL^{-1})	Ref.
PCR	Beef simple	Next day		1000 cfu/g	Liu et al. (2003)
Real-time PCR	Fresh product (salad)	Same day	100–1000	1000	Sapsford et al. (2004)
Magnetic DNA isolation-PCR	Milk	7 h	1–10^5	10	Wong et al. (2002)
QCM	Culture	30–60 min	10^7–10^8	10^7	Vaughan et al. (2001)
Amperometry	Phosphate buffer and milk	3–4 h	10^3–10^6	9×10^2	Crowley et al. (1999)
Amperometric immunosensor	Culture	Not less than 2 h	10^4–10^7		Susmel et al. (2003)

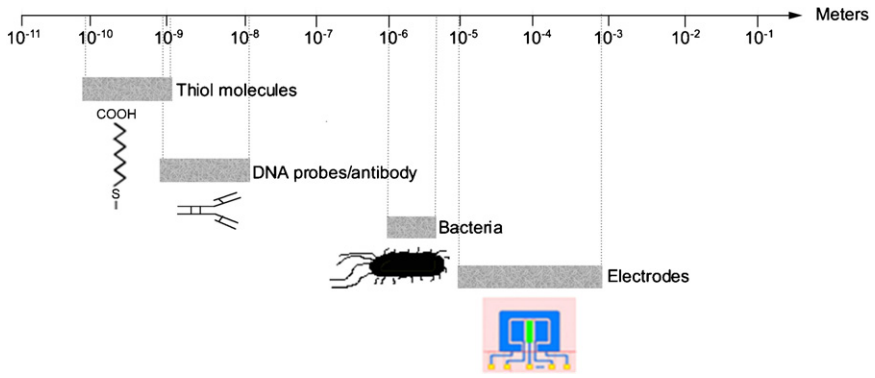

Fig. 7. Diagram representing the comparative sizes of the parts integrating a biosensor.

is found in (Abdel-Hamid et al., 1999). In this work, *E. coli* is detected in 30 min and between 100 and 600 cells mL^{-1} using a flow-through immunofiltration method coupled to amperometry. Fig. 8 shows how this disposable amperometric immunofiltration sensor works.

3.3.2. *Potentiometric methods*

These are the least common of all biosensors, but different strategies may be found nonetheless (Schoning and Poghossian, 2002). For example, they may consist of an ion selective membrane and some bioactive material, e.g., an enzyme. The enzyme catalysed reaction consumes or generates a substance which is detected by the ion-selective electrode. Since potentiometry yields a logarithmic concentration response, the technique enables the detection of extremely small concentration changes. Another approach involves the use of suitably modified ion selective field effect transistors (ISFETs) (Bergveld, 2003) which utilise the semiconductor field-effect to detect biological recognition events. ISFETs use an electric field to create regions of excess charge in a semiconductor substrate in order to enhance or decrease local conductivity. They consist of a p-type silicon substrate with two n-doped regions known as source and drain, separated by a short distance (gate) covered by a layer of insulator. The gate insulator is typically SiO_2 and it is covered by an ion selective membrane which is selectively permeable to a certain ion, e.g., K^+, Ca^{2+}, F^-, as described in (Munoz et al., 1997). More details on the functioning of ISFETs are reviewed in (Sandifer and Voycheck, 1999). The application of these devices in the area of biosensors is reasonably new (Schoning and Poghossian, 2002) and their use is not spreading as quickly

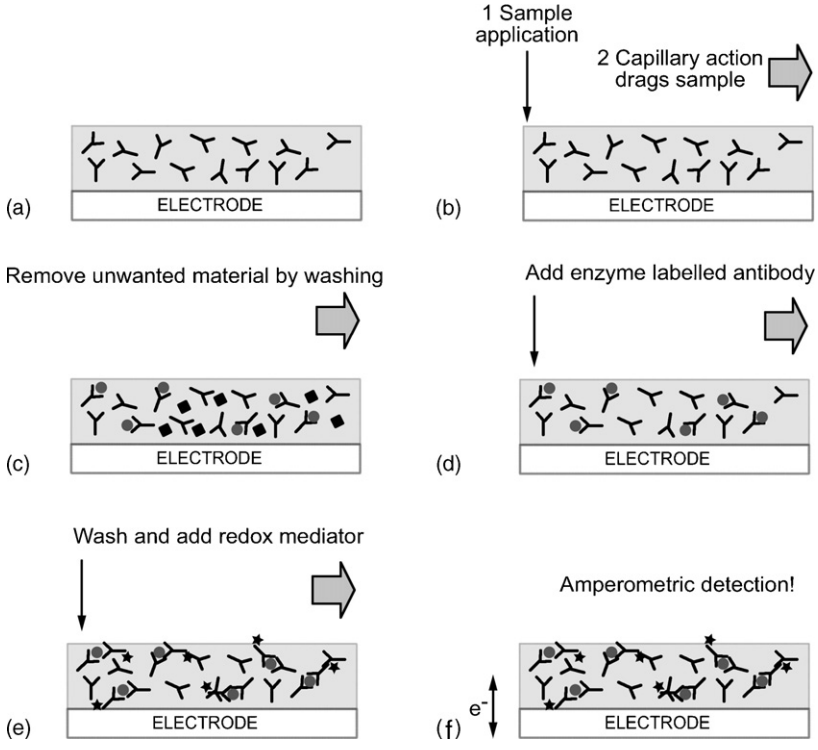

Fig. 8. Diagram of how an amperometric imunofiltration biosensor works.

as other electrochemical techniques due to, amongst others (i) problems related to production which include incompatibility of most biomolecule immobilization methods with the ISFET fabrication technology and difficult packaging and encapsulation at wafer level, (ii) poor detection limits, linear range and reproducibility and (iii) inadequate device stability.

On the other hand, examples of ISFET based biosensors can be found using enzymes (EnFET), antibodies (ImmunoFET), DNA probes (GenFET) or even whole cells (CellFET). All of these kinds of BioISFETs share the problems mentioned above, each of them having its own merits and disadvantages. Similarly to the case of amperometric biosensors, EnFETs are by far the easiest to construct and operate. This is because the products of the catalytic reaction aided by the enzyme bring about local and measurable pH changes. ImmunoFETs and GenFETs are much harder to develop because translating the bio-recognition event into a measurable signal is a daunting practical problem. Last, CellFETs find application in the study of new drugs or environmental toxicity. They consist of an ISFET on which a cell, or a colony of cells is immobilised and which activity controls the recorded signal. Thus, the effect that toxins or any other chemicals have on living organisms can be directly assessed.

Evolving from BioISFETs, a recent technology combines potentiometry and optical detection. It is known as light addressable potentiometric sensor (LAPS) (Hafeman et al., 1988) and a commercial product, the Threshold Immunoassay System, is available and has successfully been applied to bacterial detection (Gehring et al., 1998).

LAPS is based on the coupling of a transient photocurrent to an insulated n- or p-doped silicon thin layer in contact with an electrolyte. This transient photocurrent is induced by the application of transient illumination using an intensity modulated light source such as light emitting diodes (LEDs). The magnitude of the induced photocurrent depends on the potential applied to the silicon plate. It is even possible to detect different physicochemical phenomena by using different light sources on different spatial regions. If these regions are structurally different then the control of several different parameters on a single device is possible. An area of demonstrated application of LAPS devices is in enzyme-linked type immunoassays (Piras et al., 1996).

3.3.3. Electrochemical impedance spectroscopy (EIS)

Impedance spectroscopy represents a powerful method for the study of conducting materials and interfaces (Barsoukov and Macdonald, 2005). In this technique, a cyclic function of small amplitude and variable frequency is applied to a transducer, and the resulting current is used to calculate the impedance (Barsoukov and Macdonald, 2005) at each of the frequencies probed. The amplitude of the current and potential signals and the resulting phase difference between voltage and current, which depends on the nature of the system under study, dictates the system impedance. That the impedance has a real and an imaginary component makes its mathematical treatment quite difficult and cumbersome. The imposed signal may involve a range of frequencies and amplitudes, and the results may be interpreted according to two routes. The most rigorous approach involves solving the system of partial differential equations governing the system (Gabrielli, 1990). The second way, which is often preferred because of its relative simplicity, consists in the interpretation of the data in terms of equivalent circuits (Gabrielli, 1990; Katz and Willner, 2003; Yang et al., 2004). The latter are made up of a combination of capacitors and resistors suitably arranged. Although this methodology is widely accepted because of ease of use, extreme care must be taken to ensure that the equivalent circuit obtained makes physical sense. In fact, the same impedance data may well be fit by several different circuits (Gabrielli, 1990; Barsoukov and Macdonald, 2005). Also, measuring the impedance at several frequencies may be useful when several parameters need to be determined.

EIS was initially used to quantify total biomass in a sample (Grimnes and Martinsen, 2000) and its application to DNA-probe or antibody modified electrodes has represented a breakthrough in selectivity (Mirsky et al., 1997). However, its detection limits are still poor compared to traditional methods (Radke and Alocilja, 2005). An advantage of EIS compared to amperometry or potentiometry is that labels are no longer necessary, thus simplifying sensor preparation.

Along these lines, Alocilja et al. reported a conductimetric method using polyclonal antibodies against *E. coli* (Muhhammad-Tahir and Alocilja, 2003). This is a single-use system consisting of four key parts, as shown in Fig. 9. The authors quoted a detection limit of 83 CFU mL^{-1} for this system and report that the signal decreases beyond 10^5 CFU mL^{-1}.

Last, impedance measurements also enable remote sensing, as described by Ong et al. (2001), where passive RLC sensors enclosed within the sample may be used to monitor temperature, permittivity, conductivity or pressure changes non-invasively. Because sensors may easily and cheaply be incorporated within the packaging, this approach would enable rapid and automated quality controls in the food industry.

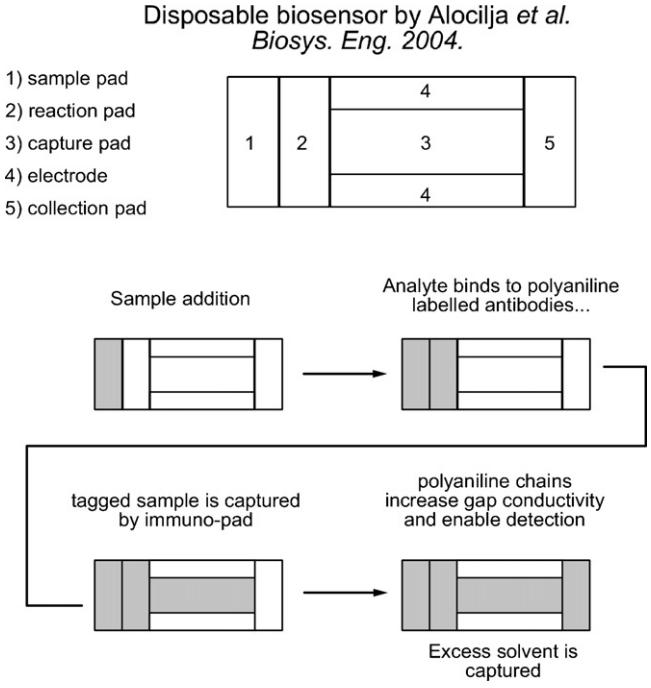

Fig. 9. Diagramatic representation of a disposable conductimetric biosensor.

Although impedimetric techniques are very promising, a lot of work is still needed in order to bring the technique up to a competitive level. Even the fundamental understanding of the phenomena involved in this type of immunosensors is largely to be developed. For instance, studies of the effect of electrode size and their separation distance has not been found in the recent literature, but it is not entirely unreasonable to believe that using the appropriate electrode configuration and sample pre-treatment steps, detection limits below 10^3 CFU mL^{-1} could be achieved.

4. New trends

More exotic approaches have been devised recently, such as the application of fractals theory to the analysis of biosensor data (Morris and Sadana, 2005). This kind of analysis not only enables the detection of pathogenic bacteria, but it also yields information about the binding and dissociation kinetics involved in the interaction of the pathogen with the biosensor surface. Although very powerful, this approach suffers from a very high degree of mathematical complexity.

The combined use of micro- and nano-fabrication techniques in the area of biosensors holds great promise and different applications are beginning to crop up (Carrascosa et al., 2006; Murphy, 2006).

Amongst the advantages of this smaller scale approach are: (a) the possibility of mass production and reduced unit costs, (b) it allows working with sample volumes in the range of nanolitres or less, which also implies that the cost of reagents is not too high, (c) micro-fluidics improve mixing rates and mass transport which is expected to result in much shorter analysis times, (d) the performance of multi-analyte analysis is enabled in the same device, which also shortens analysis time, and (e) because the volumes manipulated are so tiny, these devices provide more safety and they are more environmentally friendly. Power consumption is extremely low and contamination associated to waste material may be easier to contain due to the possibility to use tiny volumes and cartridge-like configurations.

To the best of the authors' knowledge, the first reports of bacterial detection at Microsystems dates back to the works of Bashir and co-workers (Gómez et al., 2001) in 2001. This work presents a microsystem capable of detecting listeria using impedance spectroscopy. Also in the same year, Woo and co-workers (Gau et al., 2001) reported the selective amperometric detection of *E. coli* (1000 cells; initial volume not quoted) in a very short time (40 min). It is interesting to note that both works rely on electrochemical and not optical detection.

Other examples combining pathogen detection and miniaturisation can be found in the literature (Busch et al., 2003; Gomez et al., 2002; Lagally et al., 2004). A very recent example of such a microdevice is given by Bashir et al. in this work (Gomez-Sjoberg et al., 2005), the authors describe a microelectromechanical system, MEMS, to monitor the metabolism of Listeria cells using impedance spectroscopy at a set of interdigitated electrodes. The detection follows a preconcentration step based on magnetic beads which the authors quote to attain concentration factors between 10^4 and 10^5. Once in the system, the sample undergoes dielectrophoretic separation of the cells, which are driven along a set of two electrodes towards the detector. Although the analysis time is shorter compared to traditional methods, it still requires at least 12 h, thus leaving room for some improvement. Along similar lines is the micro-fluidic lab-on-a-chip system developed by Baeumner et al. (Zaytseva et al., 2005), who use liposome amplified fluorescence detection of pathogenic bacteria or viruses based on a DNA/RNA hybridization reaction coupled to magnetic beads. The authors report analysis times of 15 min, including incubation steps, which is outstanding.

5. Summary and outlook

Traditional pathogen detection methods, although sensitive enough, are often too slow to be of any use. Therefore, new methods are needed that exceed their performance. Over the recent years, a lot of effort has gone into the study and development of biosensors of the most diverse nature, but their performance is irregular and still needs improvement. Tables 1–5 provide a summary of detection methods available against certain pathogens. The authors of this review believe that, in the near future, pathogen detection will undoubtedly benefit from the integration of biosensors into microdevices. Although, barring selectivity, performance will lie in a necessary compromise between time and sensitivity.

Optical techniques perhaps provide better sensitivity than electrochemical ones, but their cost and complexity makes them unattractive to most end users. Electrochemical techniques, on the other hand, are much easier to use but when it comes to detecting pathogens, their performance is still far from adequate. In order to become attractive, biosensors first need to show that they are capable of reaching at least the same detection levels as traditional techniques (between 10 and 100 CFU mL^{-1}). Next, they need to do so in a fraction of the time without overlooking cost.

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Detection of *E. coli* and *Salmonella typhimurium* using interdigitated microelectrode capacitive immunosensors.

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ABSTRACT This paper presents an immunosensing system to detect *E. coli* and *Salmonella* based on electrochemical impedance spectroscopy, EIS, at interdigitated electrode structures. Our results show the importance of good electrode design in the final detection limit. Four different structures have been fabricated and functionalised. Biotinylated polyclonal antibodies have been immobilised on neutravidin coated chips and BSA has been used to avoid non-specific adsorption. The immunosensor may be said to be capacitive since it is that part of the impedance which is used to monitor the presence of bacteria in phosphate buffer solution, PBS, samples. Detection limits around 10^4 – 10^5 cells mL^{-1} have been reached using chips featuring interdigitated structures of less than ten microns wide and 1.5 millimetres long. In both cases, the detection limits of the corresponding ELISA assays, using the same antibodies, was one order of magnitude higher (10^5 – 10^6 cells mL^{-1}). The analysis time, including sensor preparation was less than 5 hours.

KEYWORDS (Word Style “BG_Keywords”). Biosensor, immunosensor, Pathogen detection, *E. coli*, *Salmonella*, Electrochemistry, Impedance spectroscopy, EIS, Interdigitated electrodes, capacitive biosensor.

Introduction

The timely detection of pathogens is a subject of great importance. Particularly in the food industry, where goods can seldom be held during the several days required for standard methods to yield results. Conventional methods are highly selective and specific but, since they rely on a series of enrichment steps, they are too slow from the perspective of industrial needs. Rapid methods exist which are based on either DNA amplification or immunoassays. The problem with such methods is that they are not conclusive and, in case of a positive answer, the results always need to be confirmed using one of the standard methods. On top of this, only large production facilities may be able to afford the costs of a full-fledged laboratory, so that in many cases it is impossible to prevent the release of contaminated foods into the market.

This work presents a method for the detection of bacteria using *E. coli* and *Salmonella typhimurium* as model targets. The method can be described as an electrochemical impedance spectroscopy, EIS, based immunosensor. The use of biosensors for pathogen detection is increasingly gaining interest, and there are a number of different detection strategies and kinds of transducers. [1] Amongst these the most common are the optical and electrochemical methods. Other strategies such as piezoelectric or magnetic detection are also available, but they are somewhat less common. [2]

EIS encompasses a powerful set of electrochemical techniques. [3] EIS enables the characterisation of materials and the structure of interphases. It has been successfully used in biosensors to monitor processes of biological interest, such as the binding of proteins. [4-6] One of the features making EIS more attractive is that, unlike amperometric biosensors, it does not require the use of electroactive labels for detecting the biorecognition event. However, there are examples of impedance based biosensors that make use of enzyme catalysed processes. [7, 8] These examples monitor electron transfer rate [4] instead of changes in interfacial capacitance [9-12] or medium conductivity. [13] The system presented in this paper monitors the capacitive term of the impedance to determine the level of bacteria in a saline solution sample. The capture of bacteria by the antibodies on the electrode surface causes a decrease of the

relative permittivity of the interface and perhaps an increase in the distance over which the charge is distributed, [9] which brings about an overall decrease in the observed interfacial capacitance.

The transducers used in this work are interdigitated structures of various sizes. One of the main contributions of this work is that we demonstrate the importance of transducer feature size on detection limit in electrochemical biosensors. In fact, better limits can be achieved when the features of the transducer are of the same order of magnitude as the target bacteria. Our best detection limit, slightly below 10^4 cells mL^{-1} for *E. coli*, is lower than that obtained by ELISA using the same antibodies (10^5 Cells mL^{-1}).[14]

Materials and Methods

Chemical reagents and instrumentation

All chemicals used were analytical grade and were used as received without any further purification. These were: potassium chloride (KCl; Fluka), potassium hexacyanoferrate ($K_4Fe(CN)_6$; Aldrich), hydrogen peroxide (H_2O_2; Aldrich, 30%), sulphuric acid (H_2SO_4, Aldrich, 60%+), Neutravidin (Invitrogen; Barcelona, Spain), Bovine serum albumin (BSA; Aldrich). Commercial PBS tablets (10 mM phosphate buffered saline, NaCl 13.8 mM; KCl 2.7 mM, pH 7.4) were dissolved as per provider (Invitrogen) instructions. The PBS-tween washing solution consisted in PBS containing 0.05% Tween 20x (Sigma). Rabbit polyclonal biotinylated antibodies, b-PAb, and HRP labelled Ab specific for *E. coli* and *Salmonella* were provided by AbCam (Cambridge, UK) and anti rabbit IgG goat PAb (Sigma; Barcelona, Spain) was used as negative control. Solutions were prepared using deionised water of resistivity not less than 18 MΩ cm^{-1}. Piranha solutions for cleaning were prepared by mixing one part (volume) of concentrated hydrogen peroxide (30%) and three parts of concentrated sulphuric acid. (*Caution: piranha solution is highly reactive and should be handled with proper protections*). The 0.5 mm diameter gold wire used for the ELISA control tests was provided by Sigma; Barcelona, Spain and cut into pieces 4 mm long.

The conductivity of the solutions used for the determination of cell constants was measured using a CRISON conductivity meter with temperature correction. Cyclic voltammetry measurements for the

characterisation of electrodes were performed using an Autolab PG12 potentiostat controlled by GPES 4 software running on a Windows XP based PC. Electrochemical impedance spectroscopy measurements were performed using the Autolab frequency response analysis FRA2 module, installed in the same PG12 potentiostat. The temperature of all solutions was controlled by means of a jacketed electrochemical cell connected to a thermostatic bath. ELISA tests were performed on a ThermoElectron Multiskan plate reader connected to a PC.

Electrode Fabrication

The fabrication of interdigitated electrodes using standard photolithographic techniques has been described elsewhere [15], but a short summary will be provided here for convenience. A four-inch diameter pyrex wafer was marked and thoroughly cleaned to improve the quality of subsequent stages. Next, a metal triple layer consisting of titanium (10 nm), nickel (10 nm) and gold (100 nm) was deposited by sputtering. A positive photoresin was then used to define the electrodes and contact pads after insolation through a clear field mask. The developed wafer was then etched in a series of chemical baths. Finally, the photoresin protecting the gold areas was removed in an acetone bath. A mixed silicon oxide (4000 Å) and silicon nitride (7000 Å) layer was deposited over the entire wafer. This passivating layer protects the contact pads and provides the final geometry to the metal parts on the chip. The wafer is then newly coated in photoresin which is insolated through a new mask and subsequently developed. The areas of exposed oxinitride containing the contact points and the electrode structures are attacked in a reactive ion etching process. Finally, the excess resin is removed and the wafer is exposed to oxygen plasma for three hours to clean the gold surfaces.

At this point, we need to divide the interdigitated structures in two groups depending on the way in which the electrodes are patterned. Using oxinitride for patterning improves the reproducibility of the fabricated structures. Unfortunately, we can not use oxinitride in all the devices. This is particularly so in the case of smaller structures, where feature size is comparable to the resolution of the photolithographic equipment, and even small misalignments could ruin the fabrication. As we found during the characterisation of our devices, those structures where oxinitride could not be used presented

certain variability between them and were in general smaller than the nominal sizes defined in the masks. The different structures and their actual dimensions will be presented in a later section of this paper.

Finally, the wafers are diced into individual chips with a footprint of 3x3 mm. These are then transferred and wire bonded to suitable print circuit boards. The encapsulating resin used to protect the connection pads of the PCB and wire bonds is an Epotek thermo-curable polymer.

Electrode Characterisation

Following their fabrication, the interdigitated structures were characterised by SEM microscopy, perfilommetry and conductivity measurements.

Figure 1 shows the SEM images and perfilometry data corresponding to the interdigitated structures used in this study. There are two different structures according to their manufacturing. The smallest structures are in effect protruding electrodes while the bigger ones are recessed at the bottom of oxinitride trenches. Oxinitride is used for electrode patterning because it improves the control over the final geometry, thus making the devices more reproducible. It also makes the devices more robust by clamping the electrode material to the chip substrate. On the other hand, when oxinitride is used the size of the devices is conditioned by the resolution of the photolithographic equipment and the tolerance of alignment between wafer and mask. The smaller structures used in this work, namely 10x10 devices, did not use oxinitride for patterning to avoid such problems. As summary table 1 shows, oxinitride-free structures suffered large deviations from their nominal sizes because they were over-attacked during the wet etch patterning step. The data displayed in table 1 was obtained from the SEM images.

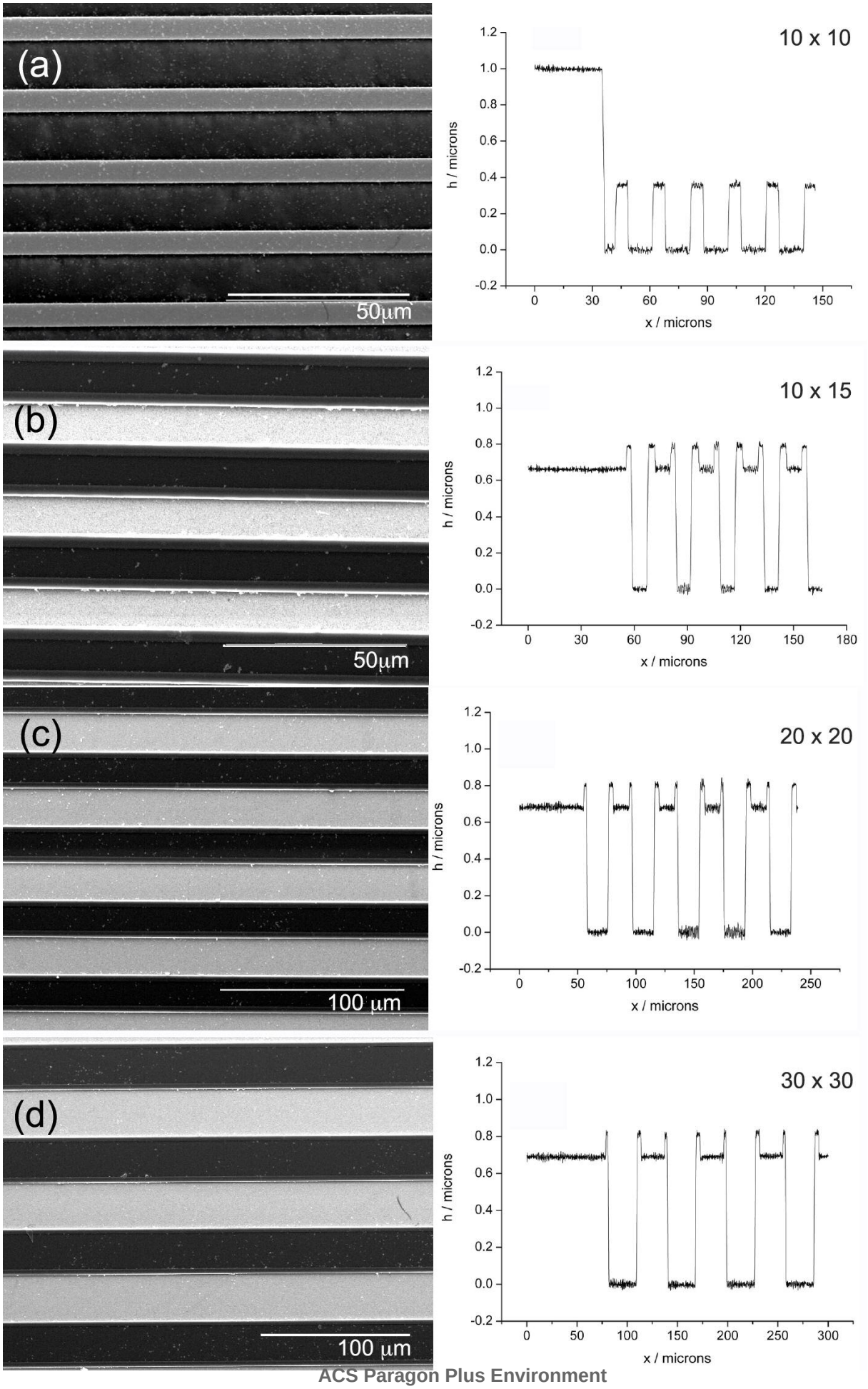
(a)
50μm
10 x 10
h / microns
x / microns
(b)
50μm
10 x 15
h / microns
x / microns
(c)
100 μm
20 x 20
h / microns
x / microns
(d)
100 μm
30 x 30
h / microns
x / microns

Figure 1.- SEM images and perfilometry data for the four interdigitated structures used in the current work. Structure (a) features protruding bands due to the lack of oxinitride while structures (b-c) are recessed in a 1 micron deep oxinitride layer.

Table 1.- Geometric features of the interdigitated structures used in the present work.

Device	Band / μm		Deviation in band size / %	Gap / μm		Deviation in gap size/ %
	Nominal	Actual		Nominal	Actual	
10x10	10	6.6	-34	10	12.5	25
10x15	10	10.7	7	15	15.2	1
20x20	20	19.8	-1	20	20.2	1
30x30	30	30.6	2	30	31.6	5

Another part of the characterisation involved the determination of the cell constant of each device in a series of conductivity standard solutions. The cell constant of a conductivity meter is the proportionality factor between the measured resistance and the conductivity of the solution. It is heavily dependent on the geometry of the cell used to measure the conductivity.

Its experimental determination only requires a set of solutions of known conductivity and a meter. In this work we used a Solartron frequency response analyser scanning between 50Hz and 100kHz between the two sets of digits in each chip. We used four chips of each geometry, and measurements were performed in triplicate. The electrodes were activated before the conductivity measurements by applying a potential around the hydrogen evolution zone for about 1 minute in 0.1M KCl. The degree of activation was then verified by cyclic voltammetry in ferrocyanide solutions of known concentration. The electrodes were considered active and suitable for impedance measurements when the cyclic

voltammograms of the two electrodes in each chip were equal. Only then the electrodes were used in further measurements.

The conductivity standards were prepared according to reference [16] and the temperature was controlled by means of a thermostatic bath. The results were compared to theoretical estimations according to the method described by Bergveld *et al.* in [17]. The dimensions used in the estimation of the theoretical cell constants were taken from SEM data. As shown in table 2, the experimental results are a bit on the low side but in quite good agreement with the theory. These deviations are attributed to the fact that the theory was developed for ideally flat, inlaid structures and it does not consider edge effects either. These conductivity measurements proved the correct functioning of the interdigitated electrode structures.

Table 2.- Summary of device characterisation and *E. coli* detection results for the four structures used.

Chip	10x10	10x15	20x20	30x30
N (x2)	54	42	22	18
Perimeter length / m	0.33	0.26	0.14	0.11
Electrode area / mm^2	1.13	1.28	1.35	1.62
k(Bergveld *et al.*) / cm^{-1}	0.160	0.180	0.300	0.390
k(experimental) / cm^{-1}	0.116	0.192	0.240	0.357
E. coli detection limit / Cells mL^{-1}	$1.50x10^3$	$1.30x10^4$	$2.70x10^4$	$2.50x10^6$

Electrode Functionalisation

The main drawback of EIS, as with most electrochemical techniques, is that it does not afford selectivity on its own. This antagonises with the concept of biosensors, where selectivity is a must. This is particularly important when dealing with complex samples where other processes may interfere with the measurement and become sources of error. In our case, selectivity was introduced by immobilising

either anti *E. coli* or anti *Salmonella* polyclonal antibodies. Figure 2 shows the surface structure of our immunosensor. It is based on the unspecific adsorption of neutravidin onto the gold interdigitated structures, followed by surface blocking with bovine serum albumin (BSA) and affinity capture of biotinylated polyclonal antibodies, b-PAb. Neutravidin is a variant of avidin from which carbohydrates have been removed. This lowers its isoelectric point and, reportedly, the level of unspecific binding characteristic of avidin while retaining its affinity for biotin. Neutravidin is known to be a suitable reagent in biosensor preparation. [18] In combination with BSA, the neutravidin-coated surface is expected both to promote orientation of the captured b-PAb and to avoid the unspecific adsorption of bacteria or other proteins/components potentially present in the sample under study. Although PAb may be less specific than MAb, they represent a cheaper alternative than the latter and allow detection of a wider range of microorganisms. This is of especial importance in our case, as a non-pathogenic *E. coli* strain had to be used against which no specific MAb could be found.

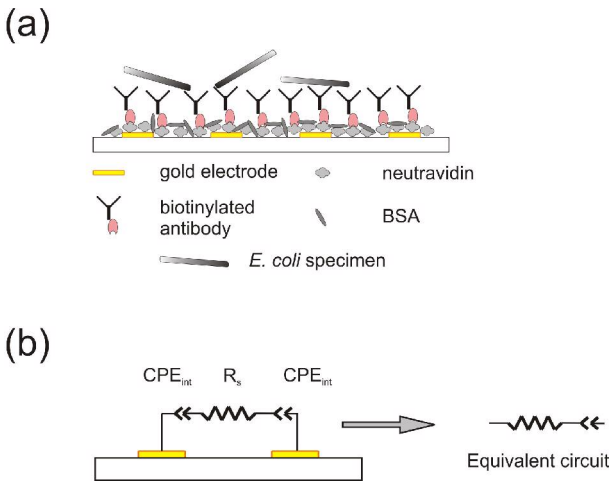

Figure 2.- (a) diagrammatic representation (not to scale) of the immunosensor surface. (b) equivalent circuit for the interdigitated structure in solution. Contact and substrate capacitances and resistances are negligible at the operating frequency range.

The surface functionalisation method was optimised by titration on small pieces of gold before transferring it to the electrodes. Once the electrodes are electrochemically activated and tested, they undergo extensive rinsing with ethanol and PBS. Next, they are incubated at 37 ºC for not less than one hour in a PBS solution containing 15µg/ml neutravidin, rinsed twice with PBS-Tween as to eliminate

weakly bound protein and once with PBS to remove the Tween. The biotinylated antibodies (15µg/ml in PBS) are then immobilised by affinity capture, incubating another hour at 37ºC, taking advantage of the strong interaction between biotin and neutravidin. Last, the surface is blocked for 2 hours at 37ºC with 2% (w/v) BSA in PBS. Thus, any bare gold spots are filled and non-specific binding of bacteria can be prevented. Weakly bound BSA is removed in a new rinse with PBS-Tween and PBS.

Other capture strategies have been assayed, including the use of streptavidin, anti-biotin antibodies and protein A. However, our results on both microtiter plates and gold surface indicated that the best performing method was, in all cases, neutravidin based in terms of low LOD, extended assay linear range and low level of nonspecific adsorption.

Handling and detection of bacteria

Escherichia coli K12 GCSC5073 (*E. coli*), *Salmonella typhimurium* ATCC 14028 wt, *Pseudomonas putida* KT2442, and *Staphylococcus aureus* were grown overnight at 37ºC until the exponential phase was reached. The count was done spectrophotometrically and by plating onto agar plates. The cultures were then aliquoted into eppendorf tubes (approximately $8x10^9$ cells per tube) and centrifuged for 10 minutes at 12000 r.p.m. The supernatants were discarded and the pellets were stored at -20ºC until needed.

Pellets containing the bacteria were transferred to a fridge at 4ºC for a period of ten minutes before reconstitution to spare the bacteria a too severe thermal shock that could gravely affect their viability. The re-suspension of the pellets is performed in two steps to avoid lumps and improve the homogeneity of the final suspension. 50 microliters of PBS are first pipetted into the eppendorf tube containing the pellet. After agitation and complete resuspension, the volume is completed with a further 0.95 mL of PBS. The tube is then vigorously shaken to complete the operation. A series of 1:10 dilutions are performed to produce bacterial suspensions containing from $8x10^9$ cells mL^{-1} down to 8 cells mL^{-1}. On completion of each experiment the concentration of the bacterial suspensions was verified by plate counting.

Two-electrode impedance measurements consisted in recording the impedance of freshly made immunosensor chips in a PBS solution at 37ºC. Each chip was then incubated for 30-40 minutes in a bacterial suspension of known concentration. Following this the chips were rinsed again and a second impedance measurement was obtained. The results were expressed as differences between the signals before and after exposure of the sensor to bacteria. As shown in figure 3, incubating the immunosensors in bacterial suspension for longer than 40 minutes increases the degree of unspecific adsorption. So this was also the incubation time used in impedance measurements.

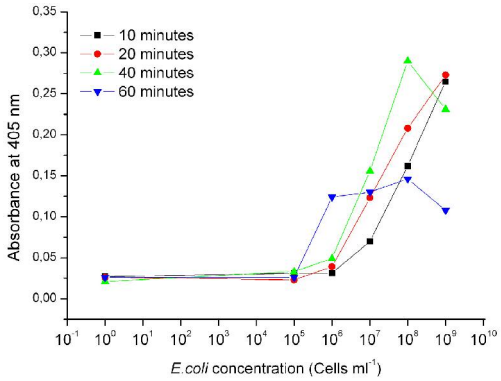

Figure 3.- Effect of incubation time on the bacterial capture effectiveness of the immunosensor surface. A time of 40 minutes was taken as best. The absorbance values given by the unspecific binding were subtracted from the specific signals.

The specificity of our functionalised surface has been assessed by comparison against surfaces coated with neutravidin and BSA but no Ab, and against surfaces coated with unrelated antibodies. Similarly, the anti-*E. coli* immunofunctionalised surfaces were assayed against unrelated bacteria. For these experiments, bare gold rods, 5mm long and 0.5mm in diameter, were used in addition to gold electrodes as to allow the simultaneous manipulation of higher numbers of samples. The Gold rods were immersed for 3 minutes in a freshly prepared piranha solution, sonicated for 3 minutes in isopropanol, rinsed in ethanol and thoroughly rinsed in PBS. The rods were at this point transferred to eppendorf tubes. A maximum of three rods per tube was used to prevent them from touching each other and scratching their surfaces, and thus facilitate surface coverage. Gold rods, functionalised with Ab, or covered in BSA

alone as a negative control for unspecific adsorption, were incubated with different concentrations of bacteria, washed 3 times with PBS and incubated with 0.5 µg/ml of HRP labelled Ab. Each gold rod was then separately transferred to individual microtitter plate wells and developed with 100 µL of enzyme substrate for 45 minutes at room temperature. The reaction was stopped with 50 µL of SDS 1% (w/v), and colour development recorded at A405 nm using a ThermoElectron Multiskan plate reader. For each condition, 3 rods were manipulated in parallel and the average and standard deviation of the registered values calculated.

Interpretation of impedance data.

The detection technique used in this study is based on Electrochemical Impedance Spectroscopy, EIS. This technique involves in our case the application of a sinusoidal function of potential through the working electrode. The current response of the system is measured and the impedance is calculated according to Ohm's general law:

$$Z=E/I= Z' + iZ''$$

where the impedance is generally a frequency dependent phasor composed by a real term (related to the resistive behaviour of the system) and an imaginary term (in the absence of inductive terms, such as our case, it is only related to the capacitive behaviour of the system). In order to interpret our data, we make use of so called equivalent circuits. An equivalent circuit is the expression of the physical system parameters in terms of electrical components; mainly resistors, capacitors or constant phase elements, to name but the most relevant to the present case. These elements are arranged in series or in parallel depending on how and when different events occur in the system under study. Although not the most rigorous approach, data interpretation via equivalent circuits is broadly accepted. In this context, however, it is important to avoid choosing circuits *a posteriori,* on the grounds of best fit to the data regardless of physical meaning. This is a completely wrong approach to the study of electrochemical systems using impedance spectroscopy.

In the present case, the electrochemical system under study consists of a pair of interdigitated electrode structures immersed in a saline solution and in the absence of any electroactive species. This

means that no faradaic contributions are expected in the observed impedance spectra. In all the experiments performed, the base potential was the system open circuit potential, the wave amplitude was 15 mV and the working frequency range was between 10Hz and 100 kHz. To reach an adequate equivalent circuit for our system, we initially envisaged the electrode-solution interface as a capacitor. The saline solution contained between a pair of electrodes behaves as a resistance in series with the interfacial capacitors, as shown in figure 2b. One could think of additional terms to add to the equivalent circuit, such as the geometric capacitance of the system, or the capacitances and resistances due to the connections between the frequency response analyser and the electrochemical cell. However, these terms are negligible at the working frequencies (not high enough for these elements to be visible) used in this study.

Despite the above discussion, the experimental response does not exactly match that for a series RC circuit. The reason is that the interface does not behave as a pure capacitor, but it deviates slightly presumably due to roughness or defects of the electrode surface and, perhaps also, due to edge effects. Therefore a constant phase element is used to represent the solution-electrode interface instead of a capacitor. A constant phase element [19] is a device used to account for non-idealities in a capacitor. [20] Its impedance is given by the formula:

$$Z'' = 1/(K\omega^n)$$

where K is a parameter related to capacitance although not a capacitance in itself, and n is related to the degree of deviation from $-\pi/2$, which is the phase angle for a pure capacitor. In the case of a constant phase element, the phase angle ϕ is:

$$\phi = -n\frac{\pi}{2}$$

This implies that as n approaches 1, the system behaviour approaches that of a true capacitance.

Finally, the overall impedance of our system can be simplified to:

$$Z = R_s - 2i/(K\omega^n)$$

The factor *2* arises from the fact that two sets of electrodes were identical and their capacitances are hence assumed to be equal. Impedance spectra were subsequently fitted to the above circuit using the ZView software (Scribner Associates, NC, USA). The results were then transferred to Excel for further analysis.

Results and Discussion

Electrode immuno-functionalisation and bacteria detection

All electrodes were cleaned and electrochemically activated prior to their functionalisation. Cleaning was done by gently rinsing in an ethanol stream, followed by a thorough rinse with deionised water. Next, the electrodes were electrochemically activated by applying a constant potential of -1.5V vs Ag/AgCl for about one minute or until a reversible and constant cyclic voltammogram was obtained in the presence of 1mM Ferrocyanide. Once activated, the electrodes were functionalised as described above and incubated in the presence of different bacterial concentrations. Electrodes lacking anti-*E. coli* or anti-*Salmonella* Ab, but having been functionalised with either an unrelated Ab or just Neutravidin/BSA, were used as negative controls to determine the degree of bacterial unspecific adsorption.

The presence of bacteria on the electrode surface can also be detected using impedance spectroscopy. Figure 4 displays two Bode plots from spectra recorded before and after incubation of an electrode in an *E. coli* suspension.

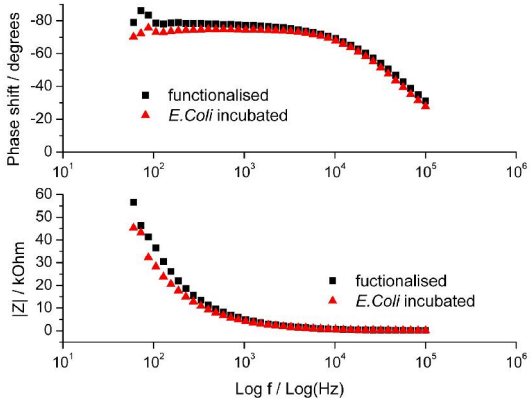

Figure 4.- Bode plots of two impedance spectra recorded before and after incubation of the immunosensor in a bacterial suspension. At the top, the change in phase shift versus logarithm of frequency and at the bottom the impedance module.

Bode plots are representations of impedance parameters versus frequency. Here we are using the impedance module, $|Z| = \sqrt{(Z')^2 + (Z'')^2}$ and the phase shift. The main differences are observed in the phase of the new spectra, which implies that interfacial changes have occurred. Under the present circumstances, such changes can only be due to the interaction of the targeted bacteria with the bound antibodies. The bacteria binding to the surface cause a decrease in the effective dielectric constant of the interface. This is observed as a net decrease of the interfacial capacitance or, in this case, in the capacitive term of the CPE. We have consistently observed this trend for all the electrode sets used, and it is consistent with data reported by other authors in the field. [11, 12, 21, 22] Figure 5 displays typical plots of the CPE capacitive term, *K,* versus the logarithm of bacterial concentration. The response is quite linear for bacterial concentrations above 10^3 cells mL^{-1} for *E. coli* and 10^5 cells mL^{-1} for *Salmonella*. This was also observed by Radke *et al.* in [23].The technique allows the approximate determination of the detection limit around $5x10^3$ cells mL^{-1}.

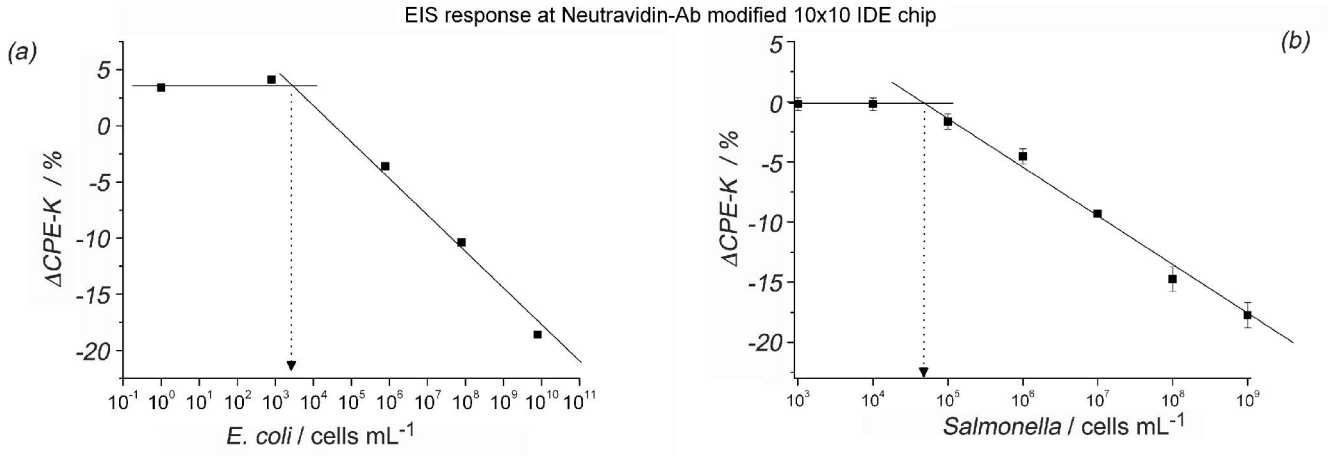

Figure 5.- Immunosensor response to suspensions of different concentration in (a) *E. coli* and (b) *S. Typhimurium*. The figure displays the change in the capacitive term of the interfacial CPE after fitting

the data to the equivalent circuit displayed in figure 2b. The detection limit is below 10^4 and 10^5 Cells mL^{-1} respectively.

The controls on the specificity of the antibodies conducted on the gold rods through the ELISA testing are represented in figure 6. These results show that the signal detected are truly specifically due to the attachment of the targeted bacteria and also shows us that, as for *E. coli*, the impedance methods allows to low down the detection limit for *Salmonella* compared to the ELISA testing (from 10^6 to 10^5 cells mL^{-1}).

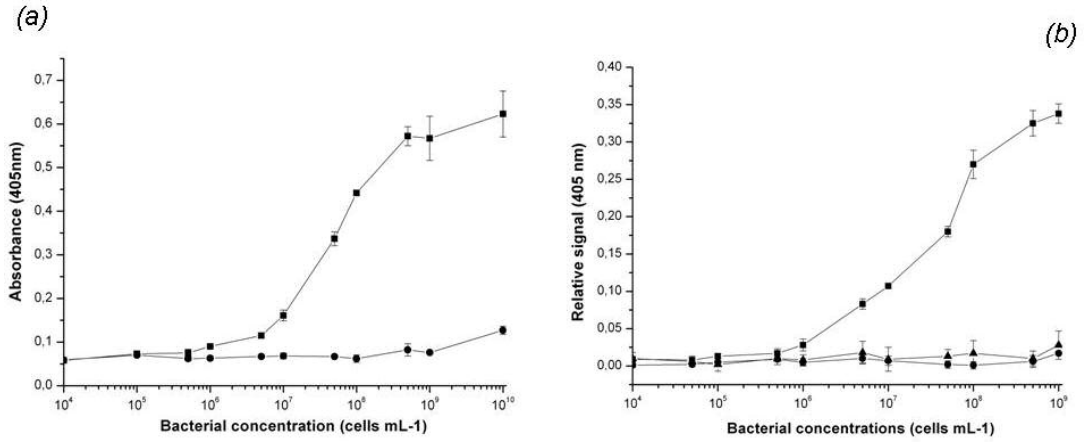

Figure 6.- Results obtained for the ELISA tests performed with the gold rods showing (a.) the specific recognition of *Salmonella Typhimurium* by the anti-*Salmonella* PAb (■) as the lack of unspecific binding on the BSA coated rods (●) and (b.) the specific recognition of *E.coli* by the anti-*E.coli* Pab (■). *Pseudomonas putida* (▲) and *Staphylococcus aureus* (●) were not detected at these functionalised surfaces. The relative signal were calculated for each bacterial concentration and assay conditions by subtracting signals registered on the negative control gold surfaces (treated with BSA but not Ab) from signals registered on Ab-functionalised gold surfaces.

Effect of electrode feature size and geometry

During these experiments the captured bacteria appeared either isolated or forming small clusters but always scattered over the functionalised surface. Thus, the level of coverage was low in most cases and more so for the lowest concentrations tested. This was the reason to study whether using electrodes of

different sizes would contribute differently to assay sensitivity. Interdigitated electrodes which features are given in table 1 were used to detect *E. coli*. Our results suggest that electrode size and geometry have a strong impact on sensitivity, to the presence of *E. coli*. Figure 7 shows that the best detection limits were achieved at the 10x10 electrode sets. These electrode sets contained the narrowest bands (*ca.* 7 μm) separated by very narrow gaps (*ca.* 13 μm). Since the chip size was the same for all the electrode sets, the smaller the features, the larger the number of microbands per chip. Under these conditions, the electrode perimeter increased in the following order: 30x30<20x20<10x15<10x10 while the gold surface area decreased in the in the opposite direction. These two parameters could be correlated to the detection limit found at each electrode for *E. coli,* and in both cases suggest that reducing electrode size improves sensitivity. This is because although the footprint of the four devices is the same, the actual transducer area decreases. Therefore, the perturbation produced in the interphase by the binding of bacteria becomes more significant as feature size decreases. [23] As it was described above, bacteria are randomly captured all over the electrode surface. The impedance measured does not reflect local changes but it gives information on the overall system impedance. Thus, as electrode features become smaller, it is easier for the system to pick up on small interfacial impedance changes. The CPE quality factor, *n*, was much more difficult to correlate with bacterial concentration.

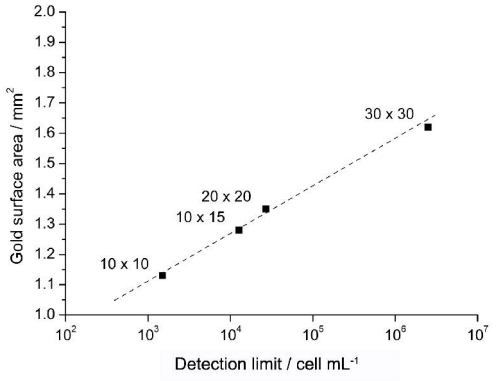

Figure 7.- Correlation between the detection limit for *E. coli* and the gold surface area of the four different transducers employed.

When it came to solution resistance, *Rs*, the 10x10 device was different from the rest in that a correlation could be observed between this parameter and cell concentration. This was attributed to the particular feature of the 10x10 electrode set, which lacked oxinitride between the gold bands (figure 1). Because there are antibodies over the entire surface of the transducer and not just the gold, bacteria are also trapped in the gaps, thus disrupting the current paths between neighbouring bands. As a result, the observed solution resistance increases. The data suggest that this field disruption is more marked for protruding than it is for recessed electrodes. Figure 8 shows *Rs* versus bacterial concentration for the 10x10 structure and for the other devices. This figure shows the different effect that *E. coli* binding has on the solution resistance measured at devices using or lacking oxinitride. Although this may not be conclusive evidence of the presence of *E. coli*, it may help in cases where the capacitance measurements are dubious.

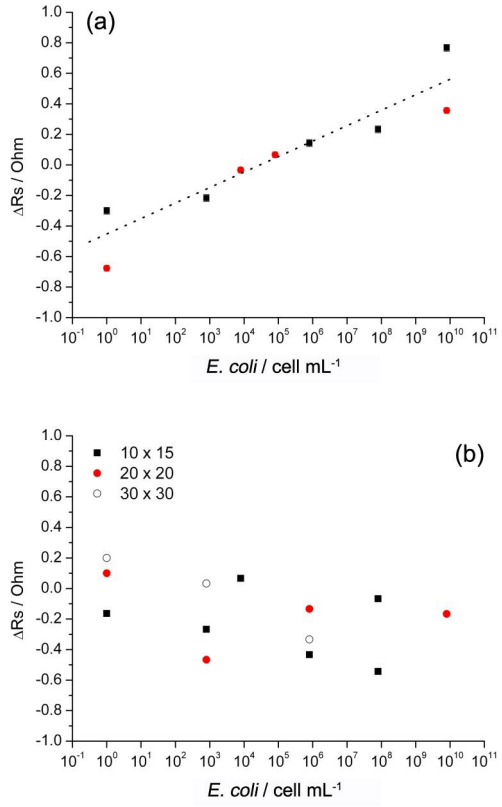

Figure 8.- Effect of *E. coli* on the measured solution resistance. There appears to be a correlation with bacterial concentration for the oxinitride free structure but not for the others.

Other authors have reported that the detection limit of immunosensors may be improved by using (a) the most suitable surface functionalisation strategy, (b) sample enrichment steps and (c) signal amplification strategies. Our results presented here show that the design of the transducers plays an equally important role in the detection. Using interdigitated electrodes which features are comparable in size to the target analyte has enabled us to reach detection limits well below ELISA in a very short time. In all cases the detection was performed after incubating the functionalised transducers in samples containing *E. coli* for 30 minutes only. The measurement itself takes about 10 minutes. The longest part is the electrode preparation and functionalisation, which is typically done in about four hours.

Sensor specificity and re-usability

The selectivity of our immunosensors has been assessed by determining the degree of non-specific adsorption of bacteria. We found that while the degree of unspecific adsorption is almost negligible on gold surfaces coated with neutravidin and BSA but lacking antibodies, the capture of unrelated bacteria depends on the specificity of the Ab used. In a first approach, we functionalised a series of 10x10 electrodes using anti *E. coli* antibodies and exposed them to suspensions of *Staphylococcus aureus* of increasing concentration. In order to observe significant changes the concentration of staphylococcus needs to be above 10^7 cells mL^{-1}. Although *Staphylococcus* is different from *E. coli* in shape and size, these results suggested that our immunosensor are specific towards *E. coli*. We expect that the future incorporation of MAb to the sensing surface may improve the specificity of the sensor further.

One important limitation of the present immunosensors is that they can only be used once. Work is currently in progress to develop an antibody recovery protocol which may enable breaking the bond between antibodies and *E. coli* specimens attached to them. There are many reports in the literature on the use of piranha solution to remove biomolecules and bacterial debris from the gold surface. Allegedly, this strategy allows for successive cycles of functionalisation and detection with the same devices. We have attempted this route without success. In our experiments, piranha solutions were so aggressive that even exposure to it for a few seconds caused irreversible damage to the electrodes in most cases. This was particularly bad in the 10x10 devices where titanium and nickel, two easily

oxidisable metals, are directly exposed to the solution at the electrode edges. Electrochemical reactivation is also out of the question because while it enables the removal of most of the neutravidin and BSA from the surface, the thin gold layer becomes damaged randomly. The net result is that subsequent impedance spectra differ from the original ones and the simple equivalent circuit given above no longer fits the data. It is likely that we are observing the corrosion of the newly exposed nickel spots as a Warburg-type element.

Conclusions

We have reported the development and fabrication of a capacitive biosensor for the fast and reliable detection of *E. coli*. A series of different interdigitated gold structures were produced photolithographically. These were then functionalised following a protocol optimised for polyclonal anti-*E. coli* antibodies.

The results confirm that the design of the transducers plays an important role in the detection, and the best results were obtained using electrodes displaying interdigitated features comparable in size to the target analyte. The chips were functionalised by anchoring biotinylated PAb to a neutravidin coated surface. Non specific adsorption of unwanted bacteria was avoided with a BSA blocking step. Nos significant cross reactivity has been found against other antibodies or against *Staphylococcus* either.

The method presented here enables the detection after exposing the immunosensors to bacterial samples for 40 minutes. The detection limits are around an order of magnitude better than those reported for ELISA ($1x10^4$ *vs.* About 10^5 cells mL^{-1}). However, it is important to bear in mind that the above results were obtained in PBS solutions, which, compared to real samples and even culture media, provide a very favourable environment for the detection. Work is in progress to measure in such complex media, but preliminary results in culture medium showed that in spite of the use of BSA as anti-blocking agent, fouling of electrodes occurs which hinders the detection.

It is likely that real samples will present similar problems related to the presence of other proteins and organic matter and that the detection limit will be affected. [24] Thus, we advocate the incorporation of sample pre-treatment steps that eliminate or reduce matrix effects. One attractive alternative is the use of

functionalised magnetic particles. The latter can be used to trap *bacteria* from the original sample matrix, be extracted using a magnet and concentrated in a small volume of clean solution. Then they can be put in contact with the immunosensor and continue with the detection as usual. Based on our observations presented here, it is expected that magnetic particles bring further advantages. In addition to ridding the detection of matrix effects, they may also amplify the detection signal because of the bigger size of the particle-bacteria conglomerate.

Another important issue to resolve is the regeneration of the electrodes. At present it is not possible to use the same transducer more than once. This is a serious problem because it makes the detection method rather expensive. There are three possible routes to make the electrodes suitable for multiple uses. The first involves the removal of the protein layers from the electrodes without damaging the gold. The second route involves regenerating the antibodies using a non-destructive method such as the glycine solutions reported elsewhere [25]. The last approach is about devising a system that does not require the functionalisation of the electrodes.

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