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

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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

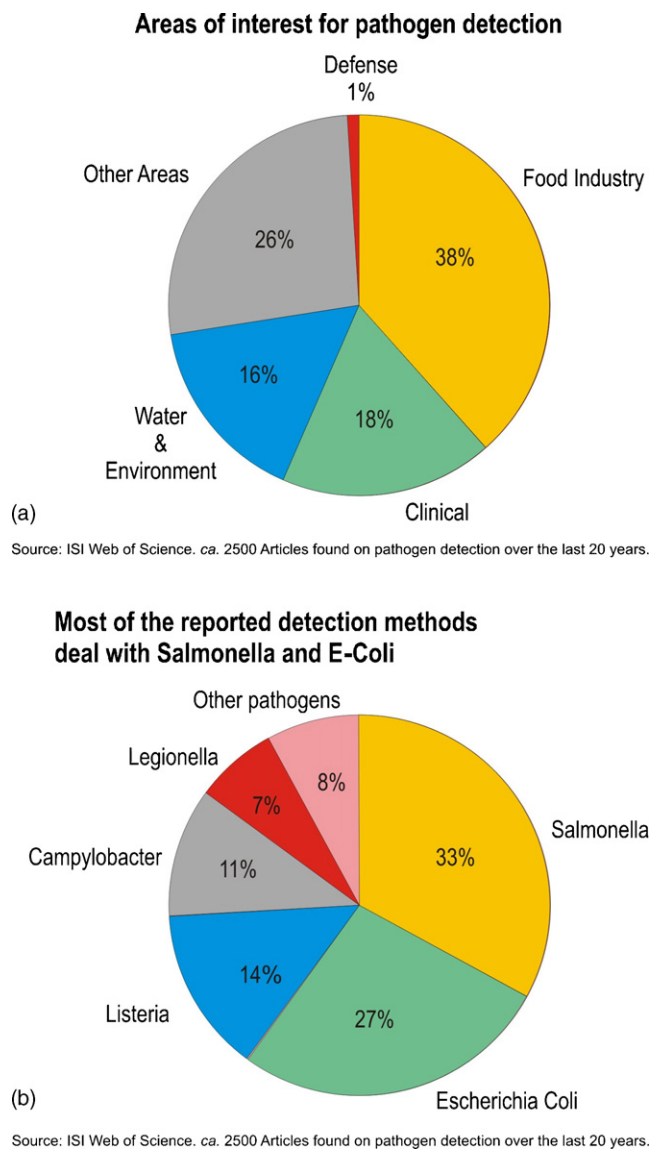

Fig. 1. (a) Distribution, by industry of application, of the relative number of works appeared in the literature on detection of pathogenic bacteria. (b) Distribution, by micro-organism, of the relative number of works appeared in the literature on detection of pathogenic bacteria.

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.

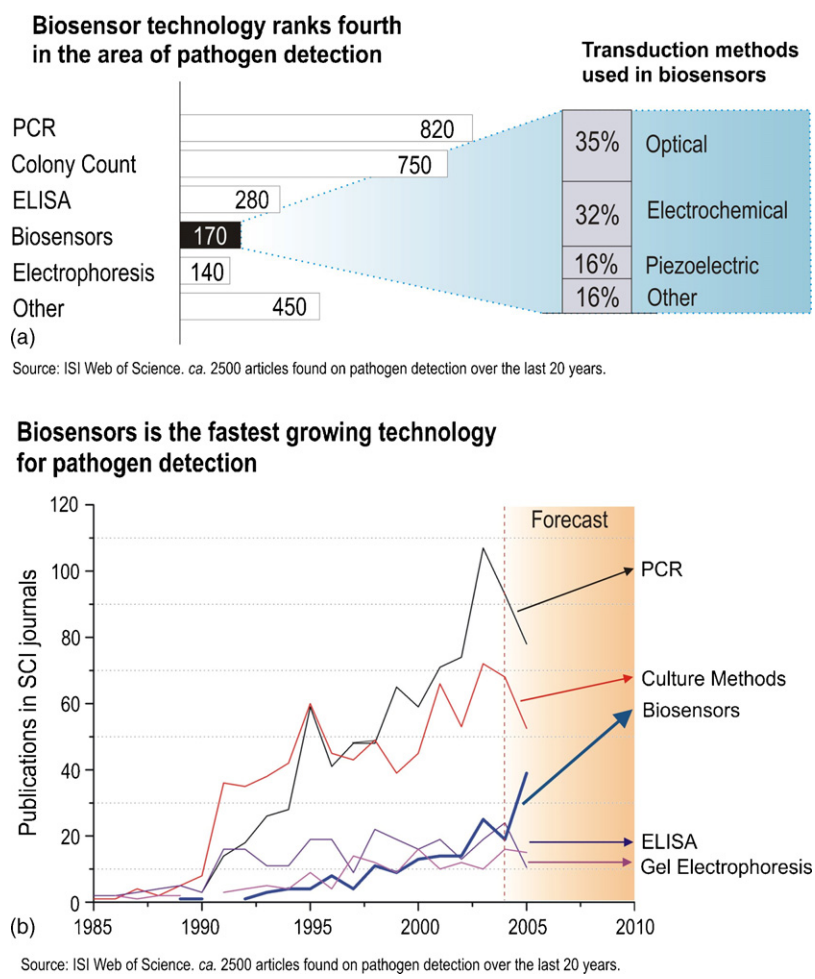

Fig. 2. (a) Approximate number of articles using different techniques to detect and/or identify pathogenic bacteria. Articles using more than one technique have been excluded in order to avoid overlap between categories. (b) Time series of the number of works published on detection of pathogen bacteria 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).

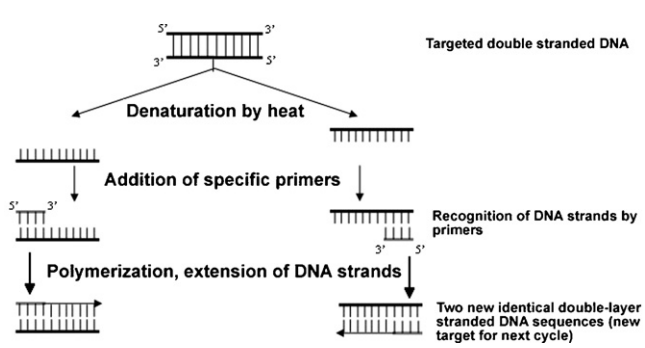

Fig. 3. Schematic representation of one PCR cycle taking place in thermocycler.

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),

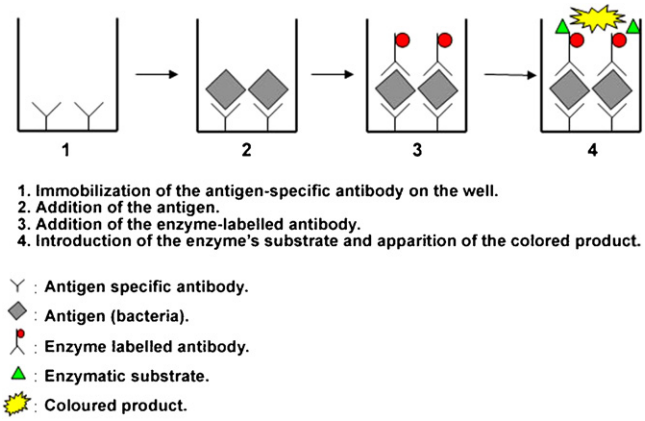

Fig. 4. Schematic representation of the sandwich-ELISA protocol.

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:

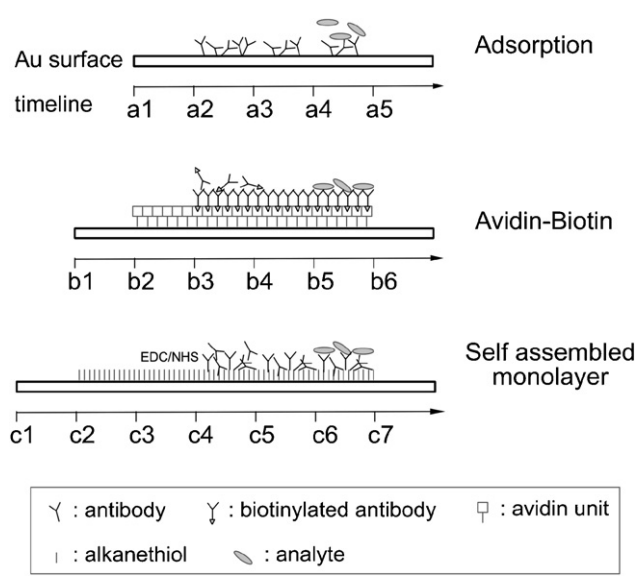

Fig. 5. Schematic representation of the main immobilization strategies and key steps involved. a1, Clean surface; a2, immersion in antibody solution; a3, wash step; a4, sample addition and a5, detection. b1, Clean surface; b2, avidin coating; b3, addition of biotinylated antibodies; b4, wash step; b5, 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

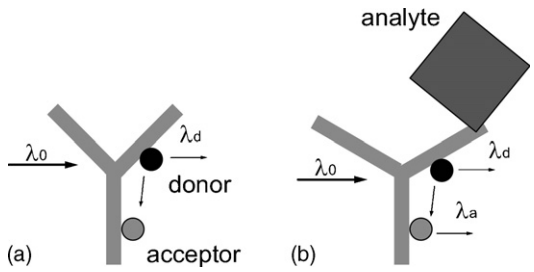

Fig. 6. Diagram of a FRET biosensor. The acceptor fluorophore responds to the excitation from the donor fluorophore only when the distance between them is short enough, e.g., when an antigen binds to the antibody.

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)

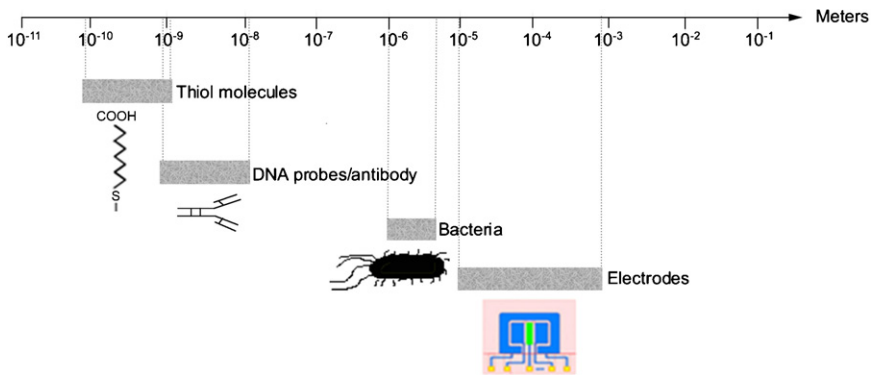

Fig. 7. Diagram representing the comparative sizes of the parts integrating a biosensor.

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

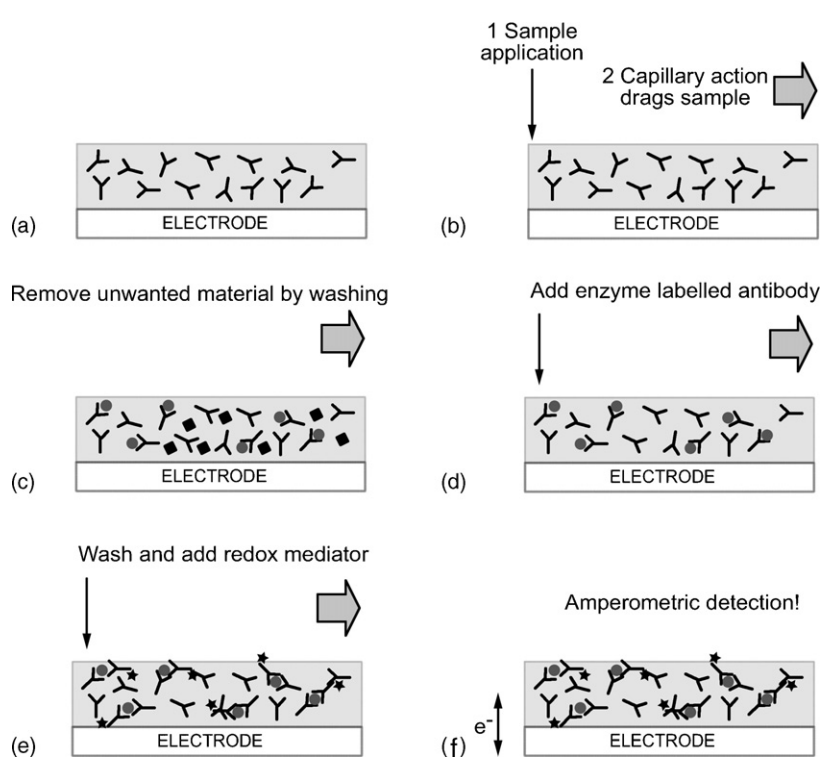

Fig. 8. Diagram of how an amperometric imunofiltration biosensor works.

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.

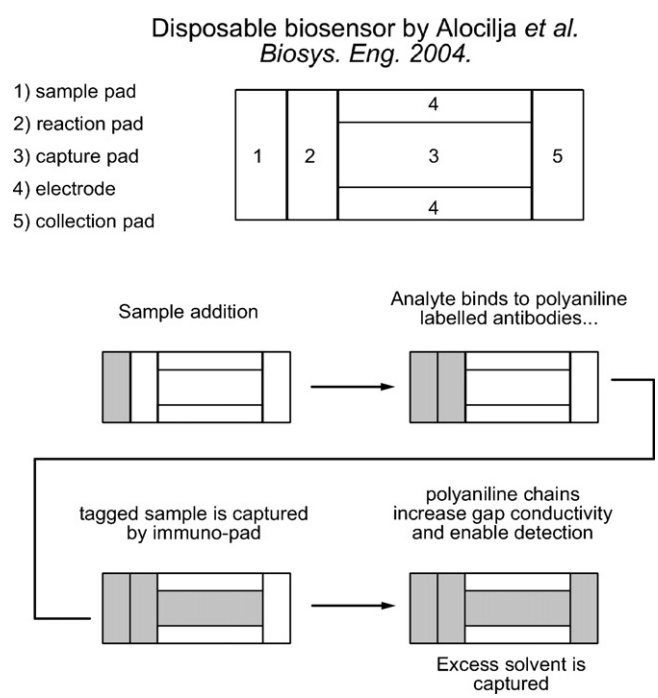

Fig. 9. Diagramatic representation of a disposable conductimetric biosensor.

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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