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Modern Probe-Assisted Methods for the Specific Detection of Bacteria

Arghavan Shabani[1,2], Christophe A. Marquette[3], Rosemonde Mandeville[1], Marcus F. Lawrence[2*]

[1]Biophage Pharma, Montreal, Canada
[2]Department of Chemistry and Biochemistry, Concordia University, Montreal, Canada
[3]Laboratoire de Génie Enzymatique et Biomoléculaire, ICBMS UMR5246-Université Lyon 1, Villeurbanne, France
Email: *Marcus.Lawrence@concordia.ca

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Abstract

This review intends to present an overview of methods currently under development for the specific and sensitive detection of pathogenic bacteria that exist in a variety of human environments. Bacteria continue to be a major health threat in general, and much effort is being deployed to counteract this problem. In a first instance, current and efficient techniques in use for the detection of bacteria are described. In a second instance, this review serves to compare the more conventional techniques to emerging technologies for the direct (non-labelled) detection of bacteria (referred to as "biosensors"). These approaches are mainly optical, piezoelectric, and electrochemical in nature. They are cost-effective, quite sensitive, and potentially portable for rapid on-site/real-time detection, and rapid prevention. These devices are comprised of specific chemical/biochemical probes immobilized onto physical transducers. This work also presents comparisons between the efficiencies (assay time and sensitivity) of various techniques being employed.

Keywords

Bacteria Detection, Probes, Optical, Piezoelectric, and Electrochemical Biosensors

1. Introduction

Bacteria are microorganisms that can spread easily and rapidly in a moist environment at favorable temperatures.

*Corresponding author.

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They may affect animals and humans, causing a variety of infectious diseases. Many infectious diseases result from food and water contamination by pathogenic bacteria and they are the major cause of illness and death in many countries. Some types of bacteria are resistant to changes in environmental conditions and the disease they cause may result in a high fatality rate. It should be noted also that some of these pathogenic bacteria are considered to be potential biological warfare agents (see **Table 1**). *E. coli*, ever rampant, has become one of the most important organisms to cause disease. *E. coli* can easily spread and contaminate food such as ground beef, raw milk, chicken, and vegetables. *E. coli* O157:H7, first discovered in 1982, is the strain that is the most dangerous food-born pathogen. This bacteria strain produces a large amount of potent toxin, causing several types of damage such as hemolytic uremic syndrome and hemorrhagic colitis. Both cases cause watery diarrhea, followed by bloody diarrhea, kidney failure and in some cases death (especially in children) [1]. It has been determined that the *E. coli* O157:H7 strain is responsible for over 20,000 cases of diarrhea per year in the United States [2]. *Salmonella* is a Gram-negative bacteria and another food-born pathogen producing infectious diseases such as salmonellosis, typhoid fever, or other problems. Typhoid fever caused by *S. typhi* leads to 600,000 deaths annually, most cases occurring in South Asia, Africa, and South America [3]. Generally, infectious dosages by pathogenic bacteria such as *Salmonella* and *E. coli* are as low as 10 cells/mL [4], thus effective and sensitive methods for screening these microorganisms are essential.

2. Conventional Detection Methods

The widespread incidents of bacterial contamination of our environment, mainly related to our food and water resources, demand the development of effective testing and analysis techniques that specifically target these microorganisms. Effective testing requires methods of analysis that meet a number of challenging criteria such as short detection time and selectivity. In the following section, we consider many approaches for bacteria detection, from conventional methods to biosensor-based techniques.

The conventional approach to microbiological identification of bacteria involves plating and culturing methods, which allow a morphological evaluation of these microorganisms based on their ability to grow in various media. Almost all bacterial species can be detected using culture-based methods. One of the media used for culture purposes is MacConky's agar, which was first used to isolate *Enterobacteriaceae* from water, food, and urine in 1905 [2]. This is a nutrient medium that contains bile salts, lactose, and an indicator. By replacing lactose in the standard MacConkey's agar with sorbitol, the agar media was used to detect *E. coli* O157:H7 species by observing the formation colorless colonies. Lowenstein-Jensen (LJ) medium is another one that can be used to grow bacterial colonies. The LJ medium consists of glycerol, asparagine, some salts and egg, and was used to detect *Mycobacteria*. Blood agar and bismuth sulfate agar can also be used for the detection of *Bacillus* and *Salmonella* species, respectively. Although plating and culturing provide reliable results, they are time consuming. Completion of all the steps can take at least a few days, to several weeks, depending on the species isolated. For example, *tubercle bacilli* produce visible growth in LJ medium in about two weeks, and adding to this the time for clinical isolation, the process can extend to eight weeks [2].

General instrumental methods for bacteria detection include: microscopic methods, luminescence methods, flow cytometry, infra-red (IR) spectroscopy and mass spectrometry (MS). Among these methods, some have received less attention due to their limitations. For example, Rossi and Warner have reported on the identification of bacteria using IR spectroscopy in 1985 [6]. Using this approach, bacteria were introduced into the IR measurement cell and corresponding IR absorbance spectra were obtained. The main limitation of this technique is that the measurements of the chemical composition of the bacteria usually show similar (indistinguishable) results at the molecular level. Mass spectrometry has also been used to detect *B. anthracis*, but the method lacks sensitivity [7]-[11]. Another drawback is that mass spectrometers are expensive, they are not portable, and the experiment cannot operate under atmospheric conditions. The infectious dose for *B. anthracis* has been reported to be approximately 10^4 spores, and few rapid detection methods using mass spectrometry can detect spore counts below 10^5 cfu/mL [12]. In contrast to IR and MS methods, microscopy and flow cytometry techniques do not provide data based on the chemical components of the microorganisms at the molecular level, thus they are more accurate and more commonly used.

In microscopy methods, by labeling the cells with a specific dye, it is possible to visualize and identify a wide range of bacteria. For example, Huang *et al*. [13] have successfully used fluorescence microscopy for the identification of *S. tythimium* cells. The detection limit of the technique was reported to be 10^4 cfu/mL, with a total

Table 1. A list of pathogenic bacteria and related diseases [5].

Bacterium	Site of infection/clinical syndrome
Bacillus anthracis	anthrax
Bacillus cereus	food poisoning/food-borne enteritis
Bacteroides sp.	abdominal sepsis, abscesses (including cerebral)
Bordetella pertussis	whooping cough
Campylobacter sp.	food-borne enteritis
Chlamydia pneumoniae	respiratory tract (atypical pneumonia)
Chlamydia trachomatis	genital tract, eye
Clostridium botulinum	botulism
Clostridium difficile	antibiotic-associated diarrhea (inc pseudomembranous colitis)
Clostridium perfringens	gas gangrene, abdominal sepsis, food poisoning
Clostridium tetani	tetanus
Corynebacterium diphtheriae	diphtheria
other ***Corynebacterium*** sp.	urinary tract, "line" colonisation/infection
Enterococcus spp. (formerly ***Streptococcus***)	urinary tract, "line" colonisation/infection, abdominal sepsis
Escherichia coli	urinary tract, abdominal sepsis, neonatal septicaemia/meningitis
Haemophilus influenzae	non-capsulate: respiratory tract (inc exacerbation COAD, middle ear)
Helicobacter pylori	atrophic gastritis, peptic ulcer disease
Klebsiella sp.	urinary tract, abdominal sepsis
Legionella pneumophila	Legionnaires disease (Pontiac fever, "atypical" pneumonia)
Listeria monocytogenes	septicaemia/meningitis (esp neonates & immunosuppressed)
Moraxella catarrhalis	respiratory tract (inc exacerbation COAD, middle ear)
Mycobacterium leprae	leprosy
Mycobacterium tuberculosis	tuberculosis
other ***Mycobacterium*** sp.	rarely tuberculosis, possibly other infections in immunosuppressed
Mycoplasma pneumoniae	respiratory tract ("atypical" pneumonia)
Neisseria gonorrhoeae	gonnorhoea
Neisseria meningitidis	septicaemia/meningitis
Proteus sp.	urinary tract, abdominal sepsis
Pseudomonas aeruginosa	urinary tract, abdominal sepsis, respiratory tract in cystic fibrosis patients
other *Pseudomonas* sp.	"line" colonisation/infection
Salmonella typhi/paratyphi	typhoid fever
other *Salmonella* sp.	food-borne enteritis
Shigella sp.	food-borne enteritis
Staphylococcus aureus	skin & soft tissue (eg abscess/cellulitis/fascitis), food poisoning & other toxin-mediated disease, endocarditis, osteomyelitis
Staphylococcus epidermidis	"line" colonisation/infection (& other prostheses)
Streptococcus agalactiae	neonatal septicaemia/meningitis
Streptococcus pneumoniae	respiratory tract (including lobar pneumonia, exacerbation COAD, middle ear), meningitis
Streptococcus pyogenes	skin & soft tissue (e.g. abscess/cellulitis/fascitis), pharyngitis (rheumatic fever, glomerulonephritis)
Streptococcus viridans	bacterial endocarditis
Vibrio cholerae	food-borne enteritis including cholera

analysis time of 4 hours. Luminescence-based systems have also been used for the detection of bacteria in the environment [14]. This detection approach is based on the oxidation of luciferin followed by light emission using ATP. The emission of light is proportional to the ATP concentration, and the process is catalyzed by luciferase. Another interesting instrumental method is flow cytometry. Using a flow cytometer, cells are stained with a dye and injected into a stream of sheath fluid, and are kept at the center of the stream. The cells in the sample are accelerated and pass, individually, through a laser beam (see **Figure 1**).

When a cell passes through the laser beam, it deflects the incident light. Light scattered from the interaction between the cell particle and the laser beam is collected by a lens and directed to the optical detectors. The detectors convert the light into an electrical signal. The light scattering gives information about cell size, shape and structure. Forward-scattered light (FSC) is proportional to the surface area or size of a cell and side-scattered light (SSC) is proportional to the granularity or internal complexity of a cell [15] (see **Figure 2**).

Flow cytometry is a practical technique for bacteria counting in clinical, environmental and industrial microbiology [16]. In the past, disadvantages associated to this method have been high cost of the instrumentation/analysis. However, the assays can be performed relatively rapidly and provide a solid ability to obtain quantitative results.

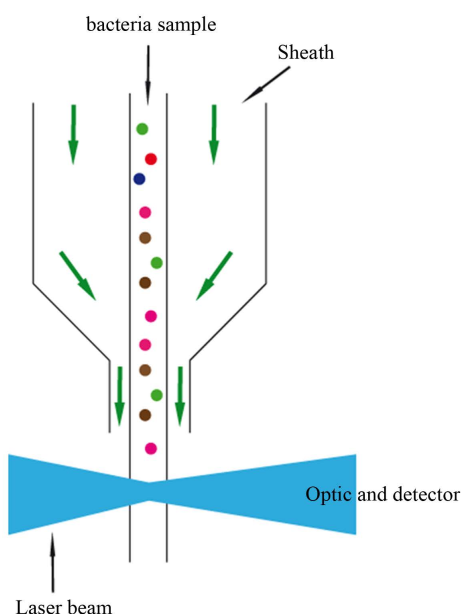

Figure 1. Schematic illustration of a flow cytometer.

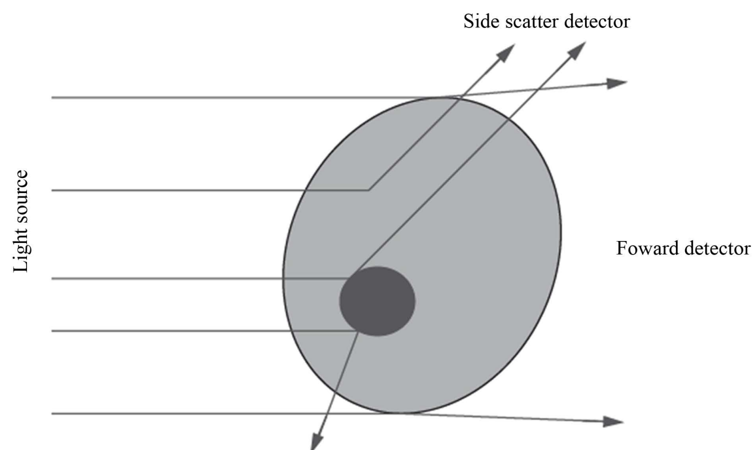

Figure 2. Light-scattering by a cell [15].

Wu *et al*. have demonstrated that flow cytometry used in conjunction with fluorescence microscopy, is a very efficient detection approach allowing to identify and quantify viable *E. coli* (using M13KE-TC labeled phages to recognize *E. coli ER*2738) [17]. Within 3 hours of measurement time, it was demonstrated that as few as 1% of viable *E. coli* cells could be quantified, in the presence of high concentrations (10^6 cells/mL) of non-viable (dead) and non-target bacteria.

3. Biosensor-Based Detection Methods

A biosensor is essentially an analytical device that integrates a biological/biochemical component with a physical transducer, for the detection of a specific analyte (or multiple analytes) present in a sample of interest [18]-[20]. The biological/biochemical species used for recognition purposes, usually referred to as probes, are wide ranging in nature (bacteriophages, cell receptors, enzymes, antibodies, nucleic acids, aptamers, etc.). The probe layer immobilized onto the transducer platform interacts/binds (recognizes) the taget analyte under study, and the interaction leads to a quantifiable signal. Biosensors are very versatile devices, their use has been extended to nanotechnological and nano-robotical applications [21] [22].

Depending on the method of signal transduction, biosensors can be classified into four major categories: optical, piezoelectric, electrochemical, and thermal sensors [23]. Each of these four sensor types can be adapted to perform either direct (label-free) or indirect (labelled) detection of target species. Efforts to commercialize biosensor technology are currently widespread, but progress in this area is still on-going due to problems that remain in achieving acceptable sensitivity and reproducibility. In the following section, the role of the most common biorecognition elements used in the construction of biosensors, and the improvements achieved by using optical, piezoelectric or electrochemical sensors for bacteria detection, are described. The most common biorecognition elements used in biosensor technology for the detection of bacteria are antibodies, nucleic acids, aptamers, and phages.

3.1. Antibodies for Bacteria Biosensors

Antibodies are used extensively as biorecognition elements. They can be used for pathogen detection or for detection of some of the pathogen components such as enzymes, toxins, pili, and spores. Based on the method of production, antibodies are categorized as polyclonal or monoclonal, monoclonal antibodies being the more specific and more expensive recognition elements. Antibodies can easily adsorb onto the transducer surface, or be immobilized by chemical conjugation or cross-linking onto the sensing surface through contact with functional groups such as amines and carboxylates.

Some publications have reported on the existence of peptides that are smaller than antibodies, that can specifically bind to pathogens [24]. The peptides can be produced from pathogen-binding proteins, they can also be synthesized *in vitro* following procedures similar to those used for aptamers. Fluorescent labelled peptides have been successfully used for the detection of bacterial toxin and spores [25]. Peptides have also been employed for the detection of different virus strains by ELISA and dot-plot assay [26].

3.2. Nucleic Acids for Bacteria Biosensors

Single-stranded nucleic acids can also be used as recognition elements to bind with complementary DNA or RNA sequences of a target microorganism. The oligonucleotides can be deposited onto the sensor surface by different means. For example, oligonucleotides modified with NH_2 terminal groups (which are readily available commercially) can be attached to suitable functional groups at the surface of a transducer through covalent bonding [27], or oligonucleotides modified with SH_2 terminal groups can be directly self-assembled onto gold surfaces [28].

3.3. Aptamers for Bacteria Biosensors

Aptamers, first reported on in 1990, are engineered nucleic acids that specifically bind to various targets of biological interest. The targets can be small molecules, peptides, proteins, nucleic acids or even whole cells. The advantage they offer over using antibodies or other alternative approaches is the simplicity associated with their production and isolation. They can be created completely *in vitro* by chemical synthesis, they are easy to store, and show non-immunogenicity in therapeutic applications [29]. Aptamers immobilized as probes are more resistive

than antibodies to freezing and drying reconstituting cycles, but they have a higher sensitivity to enzymatic degradation. To date, work on aptamers has mainly been limited to therapeutic applications, nevertheless the number of publications on their integration with sensing devices is on the rise.

3.4. Phages for Bacteria Biosensors

Phages are bacterial viruses that bind to target bacteria through specific receptors present at the surface of host cells. They inject their genetic material inside the cells and use the cell machinery to replicate. The fact that phages are capable of targeting specific bacteria makes them attractive candidates for use as probes in sensor devices. A few papers have reported on the use of phages as recognition elements to detect bacteria using fluorescence microscopy [30], using acoustic wave biosensors [31], or impedance spectroscopy [32]. We are describing here in more detail the biology and structure of phages, since they have emerged as elite probes for the specific detection of bacteria. Bacteriophages are small viruses that recognize specific receptors on the bacterial surface, to which they bind and then proceed to inject their genetic material. These viruses recognize target bacteria through receptors located on their tail. The target host of each phage is often narrowed to one species of bacteria, but several related species can sometimes be infected by the same phage. In effect, it is now well accepted that interactions between phages and bacteria are highly specific. They are very abundant organisms on earth. They can be found in large numbers wherever their host bacteria exist, such as in the soil, in sewage and feces, and in water [33].

As in the case of other viruses, phages are absolute parasites. They have no ability to generate energy and they have no ribosomes to produce proteins. They inject/transfer all the information for their production to an appropriate host. Each phage converts an infected bacterium into a phage-manufacturing system, which yields a large number of phage progeny. The degree to which different phages use part of the genetic machinery of the host varies. Some phages have fewer than 10 genes and they are totally dependent on bacteria cells, while some other phages have 30 to 100 genes, and they are dependent on proteins encoded by their own genetic material [34].

Different types of phages vary in size and shape. The size of most phages ranges from 25 to 200 nm, and the structure of the majority of phages is composed of a capsid (or head, which contains the genetic material), and a tail. The genetic material contains the nucleic acid genome (DNA or RNA) of the specific phage, covered by a protein coating which forms the capsid. The capsid is composed of many copies of different proteins which act to protect the genetic material from harmful substances. The tail is a hollow tube surrounded by a contractile sheath through which the nucleic acid passes and ends up being injected into the bacteria. At the end of the tail, there is a base plate and fibers, which enable phages to bind to the bacteria cells. It should be reiterated however that not all phages possess the tail and sheath [33]. The structure of phage T4 is shown (see **Figure 3**) as a model system.

Two categories of bacteriophages have been identified: lytic and temperate phages. Based on these categories, the phage follows either the lytic or lysogenic life cycle. In the lytic cycle, the phage converts a bacteria host cell into a phage factory to produce more phages, effectively destroying the bacteria (lysis), and releasing newly produced phages for the infection of other hosts. The lysogenic cycle on the other hand, is typically observed with phages containing double-stranded DNA. In this case, the phage DNA attaches itself to the host chromosome and leads to the formation of a new set of phage called a prophage. Virulent phages such as T4 usually lyse and destroy the host cells, while temperate phages, such as phage *lambda*, can adopt either the lytic or the lysogenic cycle.

The key factor dictating whether the lytic or the lysogenic pathway becomes operational is the relative expression rates of the phage repressor encoded by the *cII* gene (which promotes lysogeny) and the *cro* protein, to be able to switch off the expression of the repressor gene and initiate the lytic pathway [34]. For example, after infection of the host cells by phage *lambda*, a small proportion of these phages adopt the lytic pathway, while the majority of the phages enter the lysogenic cyle. These phages continue to replicate and produce clones of themselves containing phage DNA, and prevent further infection of bacteria.

4. Different Types of Biosensors

In order to detect bacteria efficiently, the analytical device needs to meet certain requirements that are outlined in **Table 2**. They should be able to specifically detect different types of bacteria, they should be able to distinguish between live and dead bacteria, their operation/manipulation should be simple and more importantly, they

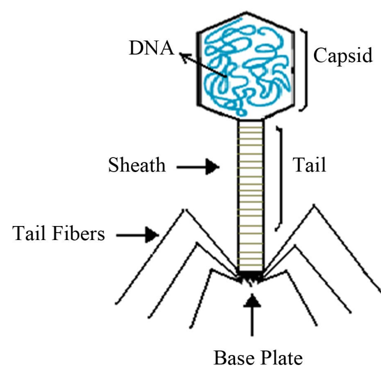

Figure 3. Structure of phage T4.

Table 2. Biosensor characteristics for bacteria detection [35].

Sensor Characteristic for Bacterial Detection	
Low detection limit	Ability to detect single bacteria
Assay time	5 - 10 min for a single test
Assay protocol	No reagent addition needed
Measurement	Direct, without pre-enrichment
Format	Highly automated format
Operator	Minimum skill required to use the assay
Viable cell count	Should discriminate between live and dead cells
Size	Compact, hand-held
Species selectivity	Ability to distinguish individual bacterial species

should be sensitive. As mentioned previously, the main categories of biosensors are based on optical, piezoelectric, electrochemical and thermal means of detection. In the following section, these techniques are described in relation to the detection of bacteria.

4.1. Optical Biosensors

Optical biosensors consist of a receptor immobilized onto a transducer, which enables the measurement of variations in light absorption/emission, refractive index, or thickness of a layer upon binding of the bacteria. Fluorimetry, surface plasmon resonance (SPR), functionalized optical fibers, and interferometry, are examples of techniques that have been adapted to bacteria detection.

An instrument based on fluorimetric detection, the AVL BDS-240, is a non-invasive automated system for the rapid detection of bacteria as well as some fungi. This instrument has an optical unit consisting of a filtered excitation source and a photodiode detection system. Since CO_2 is a product of bacteria metabolism, CO_2 optical sensors have been chosen to detect bacteria in human blood. During the bacteria's metabolic process, the CO_2 concentration increases, causing a change in fluorescence emission from a colorimetric pH indicator added to the sample [36].

An evanescent wave interferometer was also used to detect *Salmonella typhmurium* species [37]. In this case, a laser beam is directed into a wave guiding film and the light passes through the surface of the chip. The surface area functionalized with a specific receptor thus becomes the sensitive part of the chip. This system enabled the detection of 10^8 - 10^{10} cells/mL within 5 minutes. Although the system has a short detection time, it has a poor sensitivity.

More recently, surface plasmon resonance (SPR), is an optical technique that has been widely used for the detection of bacteria. A light beam is directed onto a metal film such as gold or silver at a fixed angle corresponding to what is called the resonance angle, oscillations of the free electrons at the metal surface (surface

plasmons) are induced, which generate a sensitive area on the surface called an evanescent field [38]. When the immobilized probe molecules bind to the targeted species, a variation in the surface plasmon oscillation frequency occurs, which is directly proportional to the change in the amount of bound, or adsorbed target (see **Figure 4**). The binding is detected by measuring the ensuing changes in the refractive index. A collimated polychromatic light beam is directed onto the prism at a specific angle and excites surface plasmon waves at the metal/dielectric interface. The binding of bacteria to the antibodies immobilized onto the gold film causes a change in the refractive index [39].

The first application of an SPR sensor for the detection of bacteria was reported by Fratamico *et al*. [40]. A sandwich assay was used to detect *E. coli* O157:H7 cells. A monoclonal antibody was immobilized onto the surface to capture the bacteria, and the captured bacteria were then further probed by a secondary antibody to increase the signal. No significant signal was observed using other (non-target) types of bacteria such as *S. typhmurium* or *Y. enterocolitica*. The sensor was able to detect 10^7 cells/mL, and the surface could be regenerated and reused for 50 measurements.

Numerous works based on using SPR for sensing purposes, and their corresponding limits of detection, have been reported. Obviously, many factors such as the efficiency of probe immobilization, or sample treatment methods, will have an effect on the detection limit of the system. In one study, Taylor *et al.* compared the observed detection limits for different sample treatment methods for the detection of *E. coli* O157:H7, and found the following: 10^7 cfu/mL for an untreated live sample, 10^6 cfu/mL for a heat-killed sample, 10^6 cfu/mL for a heat-killed and ethanol soaked sample, and 10^5 cfu/mL for a heat-killed and detergent lysed sample [39]. The difference in detection limits can be explained by the change in size and morphology of the cells. For heat-killed samples, *E. coli* O157:H7 cells can either change from their rod shape to become spherical, or be broken up into smaller pieces. Lysis also breaks up the cells, creating smaller pieces and increasing the concentration of detectable material. This facilitates mass transport, allowing material to more easily reach the sensor surface, therefore improving the overall sensitivity.

Usually, SPR sensors have been known to have high detection limits for the analysis of bacteria. This problem has been mainly attributed to the large dimensions of bacteria cells. Since detection with the SPR sensor depends on the ability of the analyte to reach the immobilized receptor at the surface, the large size of bacteria can make diffusion to the surface slow and limit the sensor response. However, in recent years major progress has been made in the development of SPR-based approach to detection of pathogenic bacteria. SPR has been demonstrated to be a very effective method to detect and quantify *E. coli* O157:H7 and methicillin-resistant *Staphylococcus aureaus* (MRSA) [41]-[43]. The detection is based on the specific detection of the penicillin binding protein, PBP2a. The measurements allow discrimination between the MRSA, and MSSA (methicillin-sensitive *Staphylococcus aureaus*) or BORSA (borderline oxacillin-resistant *Staphylococcus aureaus*), within 20 minutes, and at concentrations of 10 cfu/mL.

Optical fibres represent another interesting technology that has been adapted to the detection of bacteria. The structure of an optic fibre consists of polystyrene or silica glass with dopants such as Al_2O_3, B_2O_3, GeO_2. These dopants act to modify the optical properties of the fibres by raising their refractive index [44]. An optical fibre is composed of two main components: 1) the core with higher refractive index and 2) the cladding with lower

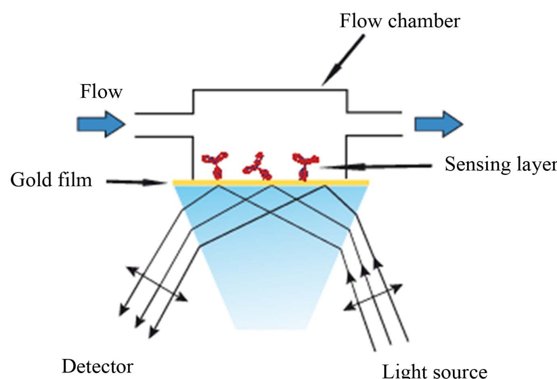

Figure 4. Simplified schematic of an SPR sensor.

refractive index. At a specific angle, incident light is transmitted through the optical fibre by total internal reflection, and a photo-detector can be used to capture the light either at the end of the fibre or at the cladding.

Ko *et al*. have used an optical fibre-based approach for the detection of *S. typhmurium* in ground beef [45]. Their fibres were functionalized using a silanization method, and labelled antibody-protein G complexes were then immobilized onto the cladding to form the evanescent wave-sensing region. The modified optic fibre was immersed into homogeneous ground beef containing *Salmonella* and the detection limit was determined to be 10^5 cfu/g, with measurements taking 5 minutes. Geng *et al*. have demonstrated the use of optical fibers for the detection of *Listeria monocytogen* cells in hot-dog or bologna [46]. In this sensor, polyclonal antibodies were immobilized onto polystyrene optic fibers through biotin-streptavidin chemistry. This immunosensor was tested with other (non-target) bacteria and showed good specificity toward the *Listeria monocytogen* species. The sensitivity of the sensor was 10^3 cfu/mL in pure culture grown at 37˚C. After enrichment steps, it took approximately 24 hours to detect bacteria cells in hot-dog samples, with a detection limit of 10 to 10^3 cfu/g. In another report a fiber optic chemiluminescence biosensor, coupled with a magnetic separation system, was used for the detection of *E. coli* O157:H7 in inoculated food samples including chicken, beef, and vegetables [47]. Briefly, a sandwich immunoassay consisting of *E. coli*-antibody coated magnetic beads, *E. coli* cells, and horseradish peroxidase (HRP)-labelled anti-*E. coli* coated magnetic beads, was formed. The bacteria cells were detected by collecting, through use of a fiber optic, the HRP-catalyzed chemiluminescence emanating from the surface of the beads. The advantage of this approach is its low detection limit (10^2 cfu/mL), without any need of an enrichment step, with a detection time of 1.5 hours.

4.2. Piezoelectric Biosensors

Piezoelectric biosensor devices are generally prepared by coating a piezoelectric transducer surface with a receptor, such as antibodies, followed by binding with bacteria. As the bacteria bind to the receptors, the mass at the surface of the piezo-sensor changes, and this is reflected by a variation of the piezoelectric crystal's resonance oscillation frequency. The most widely used piezoelectric material is quartz because it is easily available and has good thermal stability. Piezoelectric technology has been used for the detection of a variety of microorganisms, with a wide range of applications in the food industry, the environment, clinical diagnosis, and biotechnology in general [48] [49].

The detection of *S. enteritideis* is one example of the use of a piezoelectric device for the detection of bacteria [50]. In this sensor a piezoelectric crystal covered with layers of gold, silver, and palladium was then coated with polyethyleneimine to immobilize antibodies, allowing for recognition of the *S. enteritideis* species. Binding of the bacteria resulted in measurable changes in the crystal's resonance frequency parameter, giving a detection limit of 10^5 cfu/mL, with a detection time of 35 minutes. In other work, *S. typhmurium* was detected using a polyclonal antibody immobilized by the Langmuir-Blodgett method onto the surface of a quartz acoustic wave sensor [51]. The detection limit of this sensor was found to be a few hundred cells/mL, with a response time of less than 100 s over the range of 10^2 - 10^{10} cells/mL. The sensor's response was found to be linear with bacterial concentrations ranging from 10^2 to 10^7 cells/mL.

Another piezoelectric crystal sensor using antibodies has been developed for the detection of *Salmonella*, *E. coli*, *Shigella*, and *Yersiniapestis* [52]. In this case, crystals coated with antibodies were immersed in bacteria-containing solution for 45 minutes and, after washing and drying steps, the shift in resonance frequency was measured. A linear response was observed in the concentration range of 10^6 - 10^8 cfu/mL and the authors reported that the sensor could be reused at least 12 times. In another effort, a piezoelectric device was used to detect bacteria cells in drinking water [53]. For this purpose, anti-*E. coli* were immobilized onto crystals and used to detect *E. coli* K12. An identical crystal (not modified with anti-*E. coli*) was dipped in the same bacteria-containing solution to act as reference. The resonance frequency was measured as a function of *E. coli* concentration. The response range was found to be 10^6 - 10^9 cfu/mL. In this case it was not possible to regenerate the sensing surface. Attempts to remove the bound bacteria by washing the crystal with urea or glycine-HCl buffer resulted not only in removing the bacteria, but also the antibodies, therefore making reuse impossible. Other similar approaches were used for bacteria detection such as the quartz crystal microbalance (QCM) for the detection of *S. typhmurium* [54], or the detection of *Chlamydia trachomatis* in urine samples [55].

The main disadvantage with piezoelectric biosensors is the numerous washing and drying steps involved and the regeneration of the sensing surface layer. However, the problem of regeneration can be solved by manufac-

turing small crystals at low cost, therefore making the devices disposable, but these sensors may suffer from lack of sensitivity.

4.3. Electrochemical Biosensors (Amperometric, Potentiometric, and Impedimetric)

Electrochemical biosensors have some advantages over optical sensors. They are generally more sensitive, the equipment required for analysis is less complicated and less expensive. The electrochemical sensing electrodes are also well suited to miniaturization, making possible the development of small, portable, and potentially disposable sensors. Amperometry/conductometry, potentiometry and impedimetry are the main electrochemical methods used for biosensor applications to bacterial detection.

4.3.1. Amperometric Sensors

Amperometric biosensors typically rely on an enzyme system that catalytically converts electrochemically non-active analytes into products that can be oxidized or reduced at a working electrode. The measurement is based on the variation in current as a function of applied potential. Neufeld *et al.* have reported on an amperometric method based on enzyme activity [56]. A bacteriophage is used to infect the bacteria species, causing the release of intracellular enzymes, and the activity of these enzymes is measured amperometrically. The product of the reaction between the enzyme and the substrate (*p*-aminophenyl-β-D-galactopyranoside) is *p*-aminophenol, which is then oxidized at a carbon electrode and the resulting current is monitored as a function of time. With this method *Staphylococcus* and *E. coli* cells were detected with a detection limit of 1 cell/100mL, within a period of 6 to 8 hours. Brooks *et al.* have developed an enzyme-linked amperometric method for the detection of *S. aureus* with a detection limit of 10^4 - 10^5 cfu/mL [57]. However, because of variations in the signals produced by the electrochemical detection step, this immunosensor suffered from lack of reproducibility.

Gehring *et al*. [58], employed an immunomagnetic separation system to electrochemically detect *Salmonella* species. In this method a sandwich immunoassay was formed using super-paramagnetic beads coated with anti-*Salmonella* and anti-*Salmonella* antibodies linked with alkaline phosphate. The complex immunoassay was performed by lowering the surface of graphite ink strip electrodes into the sample using a magnet, and voltammetry (current measured versus applied voltage) was used to detect the bacterial cells. This system was able to detect 10^3 cfu/mL within 80 min.

4.3.2. Potentiometric Sensors

Potentiometric biosensors are another type of electrochemical sensor, which measures the variation in potential that occurs when the analyte molecules interact with the probe-modified surface. In past years, an electrochemical approach using light addressable potentiometric sensors (LAPS), has been successfully used for the detection of pathogens. A LAPS is made up of a semiconductor chip (n-type silicon), covered with a silicon-dioxide insulating layer, placed in contact with the sample solution. The potential that results from the different charge distributions that exist at the insulating layer/solution interface and the semiconductor/insulator interface, is directly influenced by the binding interactions occurring at the probe-modified insulating layer surface, and the signal is enhanced by illumination with a modulated light beam [48].

Gehring *et al*. have also developed a LAPS system for the detection of *E. coli* O157:H7 cells in food samples using a polyclonal antibody as probe. Their system was able to detect 10^3 cells/mL [59]. The LAPS approach has also been used to detect *E. coli DH*5α in drinking water [60]. A glass cover slip was coated with primary anti-*E. coli* using a silanization method. *E. coli* in the drinking water was captured by the primary immobilized antibody, and then a secondary urease-*E. coli* antibody conjugate was used to link with the captured *E. coli*. The sample chamber was then washed with PBS, then urea was fluxed through the chamber and the reaction monitored. Urea is enzymatically converted to ammonia in proportion to the amount of bacteria cells initially present in the sample. The production of ammonia causes a change in the redox potential which is measured. The authors have reported that this system allows the detection of 10 cells/mL. Although the LAPS technique offers improvements over the more conventional methods of potentiometric detection of bacteria, there still remain issues in terms of reproducibility.

4.3.3. Impedimetric Sensors

In recent years, electrochemical transduction based on impedance techniques has received increasing attention

for applications in biological and biomedical detection. This is due to a number of factors such as: 1) impedance is one of the most important techniques for direct (label-free), real time detection; 2) the electrical properties of biological cells make them attractive analytes for detection using impedance-based methods; 3) impedance as an electronic detection system allows for the development/use of miniaturized biosensors (biochips), effectively providing access to smaller analytical devices rather than having to resort to using more cumbersome laboratory-based instruments [61].

The simplest impedance method for identification and quantification of bacteria is growth monitoring, which is based on the changes of impedance or conductance due to actual growth of bacteria, or a reaction resulting from the bacterial growth [62] [63]. Impedance microbiology is the basis for existing commercial impedimetric systems such as Bactometer® from Biomerieux, Bactrac® from Sy-Lab, and RABIT® from Don Whitley Scientific [64].

An important mechanism for detection based on growth makes use of the metabolic activity of the biological cells. The change in impedance is mainly caused by the release of ionic metabolites into the culture medium, as prescribed by the energetics of the live cell metabolism, which can be summarized as the consumption of oxygen and sugars by the bacteria and the generation of carbon dioxide and organic acids. For instance, a non-ionized glucose converted to two molecules of lactic acid lead to an increase in the conductivity of the medium. Furthermore, the metabolically driven combination of lactic acid with oxygen leads to the formation of carbonic acid, yielding more mobile carbonate ions and increased conductivity. Another contributing factor to changes in impedance is the possible ion exchange across the cell membrane. Ions such as K^+ and Na^+ are known to pass through ion channels in the cell membrane, which serves to adjust the osmotic difference between the interior and exterior of the cells [65]. Ion exchange causes changes in the ionic composition of the surrounding medium and therefore changes its electrical conductivity.

Even though growth-based impedance techniques are reliable and enable the detection of viable cells, they still suffer from high detection times and non-specificity. Usually, low cell numbers take a long time to grow (up to 24 hours) and the method cannot be used to identify specific strains of bacteria. Therefore, antibodies, nucleic acids, aptamers, peptides, and more currently phages, are being used consistently as probes for specific binding to target pathogens. Recent advances in the area of bacteria detection have been made by using electrochemical impedance spectroscopy (EIS). Most impedance biosensors for bacteria detection are based on using the electrically insulating properties of the cell membrane. When the cells attach to an electrode surface, the electrode surface area gradually gets covered with matter that reduces conductivity and therefore changes the impedance at the interface.

To obtain an impedance signal, the measurement can be performed in the presence or absence of redox couples such as $[Fe(CN)_6]^{3-/4-}$, which are referred to as faradaic or non-faradaic impedance measurement conditions, respectively [66]. Among the various recognition elements that were discussed previously, antibodies are the most commonly used bioreceptor for bacteria detection. Sensors based on the immobilization of antibodies require the attachment of a certain amount of bacteria cells to the electrode surface to produce a detectable signal. When antibodies are used as probes, two types of detection processes can be distinguished: 1) in the presence of a redox couple, the detection signal corresponds to changes in the faradaic impedance due to the biological events occurring on the surface, and 2) in the absence of a redox couple, the signal relates directly to the physical attachment of bacteria cells at the sensor surface, and the electrically insulating properties of the cell membrane [67]. Two important parameters to be considered when using EIS biosensors are the interfacial capacitance, also called the double-layer capacitance (C_{dl}), and charge-transfer resistance (R_{ct}) at the electrode surface. In certain applications, however, the key parameter of interest for detection purposes can be the conductivity of the medium (changes in solution resistance) [68].

1) Faradaic impedimetric biosensors

There are now several reports on the impedimetric detection of pathogens in media containing a redox active species, typically the $[Fe(CN)_6]^{3-/4-}$ redox couple. The detection here is based on measuring variations of the charge-transfer resistance (R_{ct}) at the electrode surface, upon attachment of bacterial cells. Ruan *et al*. have reported on an electrochemical immunosensor for the detection of *E. coli* O157:H7, using $[Fe(CN)_6]^{3-/4-}$ as the electroactive redox couple in solution [69]. In this biosensor, anti-*E. coli* antibodies were immobilized onto a planner indium-tin oxide (ITO) electrode surface and used to detect *E. coli* cells. The sensor used secondary antibodies conjugated with horseradish peroxidase to generate insoluble products at the electrode surface. These insoluble products then act to prevent electron transfer at the surface and therefore cause a detectable variation

in impedance.

The overall biosensing system can be interpreted by an equivalent circuit as illustrated in **Figure 5(a)**. The proposed equivalent circuit includes the resistance of the electrolyte (R_s), the double-layer capacitance (C_{dl}), the charge (electron) transfer resistance (R_{ct}), and the Warburg impedance (Z_w). **Figure 5(b)** shows a typical Nyquist plot of the imaginary impedance (Z_{im}) versus the real impedance (Z_{re}), measured over a range of applied ac voltage frequency, which shows a combination of a semicircle and a straight line [66] (Bard and Faulkner, 2001). The semicircle relates to the faster electron-transfer processes occurring at the electrode surface (it appears in the high frequency domain of the Nyquist plot), while the straight line relates to the diffusion limited, mass transfer, processes that occur near the electrode on the solution side of the interface (it appears in the low frequency domain). The intercept of the semicircle with the Z_{re} axis at high frequency yields the value of R_s, and the diameter of the semicircle is equal to R_{ct}.

Among the various parameters present in the equivalent circuit, the electron transfer resistance, R_{ct}, was identified by Ruan *et al*. [69], as the main parameter being influenced by the binding of *E. coli*. After *E. coli* binding, the interfacial electron-transfer kinetics slow down and increase the electron transfer resistance. The increase in R_{ct} can be explained as bound cells inhibiting electron transfer between the electrode and he $[Fe(CN)_6]^{3-/4-}$ redox couple in solution. Ruan *et al*. showed that the value of R_{ct} increased with increasing bacteria concentration. This biosensor showed a linear response in the concentration range of 10^4 to 10^7 cfu/mL, with a detection limit of 10^2 cfu/mL.

Another example of a label-free electrochemical impedance sensor using a redox couple was reported by Yang and coworkers [70]. They developed an immunosensor for the detection of *E. coli* O157:H7, using interdigitated array microelectrodes (IDA). The sensing surface consists of indium-tin-oxide (ITO) modified with anti-*E. coli* antibodies. The antibodies were attached to the surface covalently through bonding between the carboxyl groups on the antibodies and the reactive hydroxyl groups on the ITO surface. The sample containing the target bacteria, in presence of the $[Fe(CN)_6]^{3-/4-}$ redox couple, was then deposited onto the functionalized microelectrode array. When the bacteria bind to the surface, they prevent the electron transfer between the interdigitated electrodes and increase the electron transfer resistance (**Figure 6**).

A linear dependence of the charge transfer resistance on the logarithm of bacterial concentration was observed in the range of 10^5 to 10^8 cfu/mL, with a detection limit of 10^6 cfu/mL. The detection limit for this approach is higher than that reported for the system used by Ruan *et al*. [69], or even for ELISA, but it is comparable to other immunosensor (antibody)-based methods using the quartz crystal microbalance (QCM) for the detection of *Salmonella* (with a detection limit of 10^6 cfu/mL) [71], and surface plasmon resonance (SPR) for the detection of *Salmonella enteritidis* and *L. monocytogens* (with a detection limit of 10^6 cfu/mL) [72], or the detection of *E. coli* O157:H7 (with a detection limit of 10^6 cfu/mL) [73].

2) Non-Faradaic impedimetric biosensors

Non-Faradaic impedance measurements mainly detect the attachment of bacterial cells onto the electrode surface, in the absence of a redox couple in the sample solution. Radke and Alocilja have developed one such impedance sensor for the detection of *E. coli* O157:H7 [74]. The sensor was made using a high-density gold interdigitated microelectrode array, with 1700 finger electrodes. Each electrode was 3 µm in width and their separation was 4 µm, therefore providing a large sensing area. The gold microelectrode array was functionalized using 3-mercaptomethyldimethyl-ethoxysilane (MDS) followed by *N-y*-maleimidobutryloxy-succinimide ester

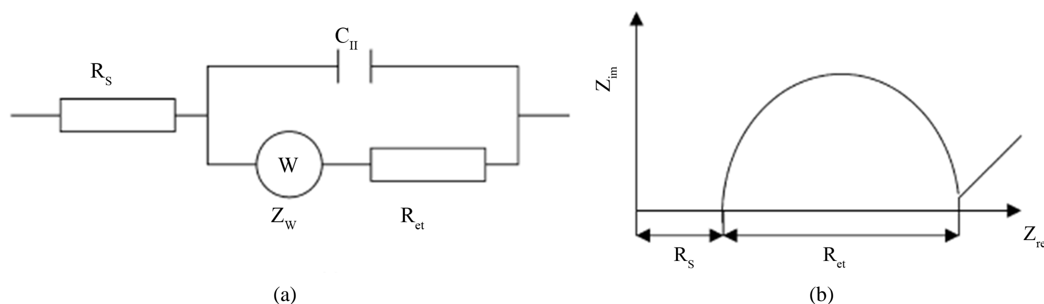

Figure 5. General equivalent circuit for an electrochemical cell in the presence of a redox couple (a). A typical Nyquist plot (Z_{im} vs Z_{re}) (b) used for the detection of *E. coli* O157:H7 cells.

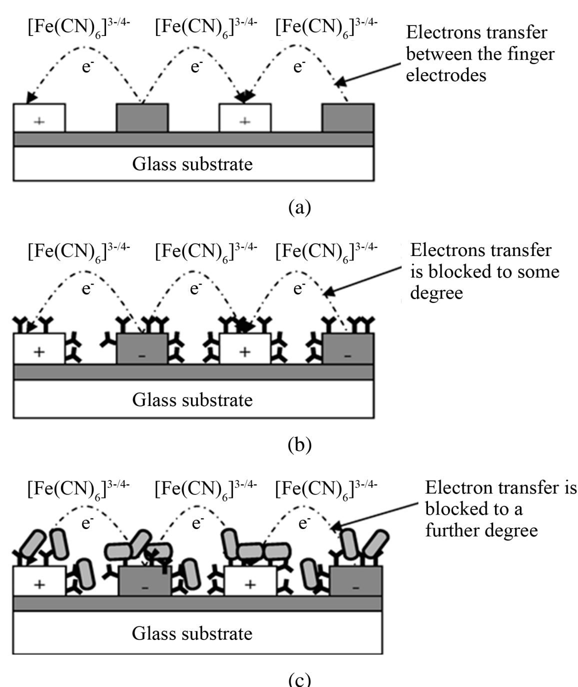

Figure 6. Direct impedimetric detection using interdigitated microelectrode arrays: (a) bare electrode; (b) with immobilized antibody; (c) with bound bacteria cells. The gray ovals are the *E. coli* O157:H7 cells; the Y shapes are the anti-*E. coli* antibody [70].

(GMBS) acting as a cross-linker. Anti-*E. coli* were then immobilized onto the surface to detect bacteria cells. After immersing the sensor into the bacteria-containing solution, an increase in impedance was observed over the frequency range of 100 Hz - 10 MHz. The increase in impedance was attributed to the binding of the cells to the electrode surface (hence due to the electrically insulating property of their cell membrane). The sensor had a dose response to *E. coli* concentration from 10^4 cfu/mL to 10^7 cfu/mL.

Some researchers have developed impedimetric biosensors using microfluidic chips. An example of this type of sensor was reported by Boehm *et al.* for the detection of *E. coli* cells [75]. Antibodies were immobilized onto the glass surface of a microfluidic chamber, and the bacteria-containing solution was passed through the chamber causing the *E. coli* to be captured by antibodies, resulting in an increase of the measured impedance. The detection limit of this sensor was found to be 10^4 cfu/mL. In microfluidic-based sensors, injected bacteria cells tend to accumulate inside the chamber and enhance the signal, which is favorable to the detection of low bacteria concentrations.

In the last few years, work has been done on the development of electrochemical sensors using microelectrode arrays, potentially allowing multiplex detection of pathogenic bacteria. Previous studies performed with our group of collaborators has demonstrated the feasabilaty of disposable microelectrodes and chemistries electrografting of biomolecules for "biosensing" detection of pathogenic bacteria, based on the use of immobilized bacteriophages (acting as probes) on screen printed carbon . These phages have both a very high specificity for their bacterial targets have extreme stability in lyophilized or immobilized form, and there is no danger of contamination to humans.

For example, a novel method was presented for the specific and direct detection of bacteria using bacteriophages as recognition receptors immobilized covalently onto functionalized screen-printed carbon electrode (SPE) microarrays by Shabani *et al.* [32]. The SPE networks were functionalized through electrochemical oxidation in acidic media of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) by applying a potential of +2.2 V to the working electrode. Immobilization of T4 bacteriophage onto the SPEs was achieved via EDC by formation of amide bonds between the protein coating of the phage and the electrochemically generated carboxylic groups at the carbon surface. The surface functionalization with EDC, and the binding of phages, was verified by time-of-flight secondary ion mass spectrometry. The immobilized T4 phages were then used to specifi-

cally detect *E. coli* bacteria. The presence of surface bound bacteria was verified by scanning electron and fluorescence microscopies. Impedance measurements (Nyquist plots) show shifts of the order of 10^4 Ohms due to the binding of *E. coli* bacteria to the T4 phages. No significant change in impedance was observed for control experiments using immobilized T4 phage in the presence of *Salmonella*. Impedance variations as a function of incubation time show a maximum shift after 20 minutes, indicating onset of lysis, as also confirmed by fluorescence microscopy. Concentration-response curves yield a detection limit of 10^4 cfu/mL for 50 µL samples.

In another work [76], the carbon electrodes were initially functionalized through cyclic-voltammetric reduction of a nitro-aryl diazonium moiety, followed by further reduction of nitro groups to amino groups, and finally by treatment with glutaraldehyde. Functionalization of the carbon electrodes and the binding of *Gamma* phage were verified by X-ray photoelectron spectroscopy and time-of-flight secondary ion mass spectrometry, respectively. The *Gamma* phage-modified microarrays were then used to detect *B. anthracis Sterne* bacteria in aqueous electrolyte media. Faradaic impedimetric detection of bacteria in KCl solution containing the $[Fe(CN)_6]^{3-/4-}$ redox couple shows a gradual increase in Z_{re} values, taken from the extrapolation of the linear portion of Nyquist plots in the low frequency range, for sensors placed in contact with increasing concentrations of *B. anthracis*. ΔZ_{re} values vary approximately from 700 to 5300 Ohms for bacteria concentrations ranging from 10^2 to10^8 cfu/mL, respectively. These shifts in Z_{re} were attributed to a decrease in diffusion controlled charge transport to the electrode surface, which is related to the mass transfer-dependent component of the equivalent circuit (Z_w in **Figure 5(a)**), following capture of intact *B. anthracis*. No comparatively significant change in impedance was observed for control experiments using *E. coli* K12 as a non-specific target, even at a concentration of 10^8 cfu/mL. Concentration-response curves yield a detection limit of 10^3 cfu/mL for 40 µL samples. The possibility of reducing the detection time and detection limit of the system was demonstrated by integrating the impedance sensor with a magnetic bead manipulation system [77]. Also, the ability of the magnetic bead approach to effectively isolate/remove and detect specific bacteria in a more complex sample (milk), was demonstrated.

In general, impedimetric systems possess the versatility for the development and commercialization of biosensors for the direct (label-free), and simultaneous detection (multiplexed), of different bacteria present in a single sample. However, some characteristics that still deserve further attention are sensitivity and overall performance in more complex media.

5. Concluding Remarks

A range of methods that can be used for bacteria detection were reviewed, pointing out their limitations and advantages. Although conventional methods remain reliable, the development of novel biosensor technologies is expanding with the hope of providing more rapid, specific, and convenient/reliable methods for bacteria identification. Different types of biosensors based on the use of different transducers were described, with emphasis being placed on optical and electrochemical biosensors because of their growing use and simplicity of operation. Surface plasmon resonance and impedimetric sensors, have now emerged as excellent candidates to fulfill the current needs in the area of specific and rapid detection of bacteria, for preventative and therapeutic applications. Impedimetric sensors, in particular, carry the potential to meet and resolve additional technological concerns related to biosensor miniaturization and portability. It should be noted however, that single cell detection still remains a daunting challenge. Since the infectious dosage of pathogens such as *Salmonella* or *E. coli* O157:H7 is 10 cells/100mL, the biosensor should be able to detect as low as one bacteria, with a rapid analysis time and low cost.

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