Comprehensive REVIEWS in Food Science and Food Safety

Emerging Biorecognition and Transduction Schemes for Rapid Detection of Pathogenic Bacteria in Food

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Abstract: The presence of unsafe levels of microorganisms in food constitutes a growing economic and public health problem that necessitates new technology for their rapid detection along the food continuum from production to consumption. While traditional techniques are reliable, there is a need for more sensitive, selective, rapid, and cost-effective approaches for food safety evaluation. Methods such as microbiological counts are sufficiently accurate and inexpensive, and are capable of determining presence and viability for most pathogens. However, these techniques are time consuming, involve destructive sampling, and require trained personnel and biosafety-certified facilities for analysis. Molecular techniques such as the polymerase chain reaction have greatly improved analytical capability over the last decade, achieving shorter analysis time with quantitative data and strain specificity, and in some cases the ability to discriminate cell viability. The emerging field of nanosensors/biosensors has demonstrated a variety of devices that hold promise to bridge the gap between traditional plate counting and molecular techniques. Many nanosensors/biosensors are rapid, portable, accurate devices that can be used as an additional screening tool for identifying unsafe levels of microorganisms in food products with no need for pre-enrichment. In this review, we provide a brief overview of available biorecognition–transduction techniques for detecting bacteria in food. We then discuss the advantages and disadvantages of each technique, and describe some recent biosensor or nanosensor technologies that are under development. We conclude by summarizing the opportunities and challenges in the field of pathogen monitoring in food systems and we focus the discussion on the strengths/weaknesses of the most popular biorecognition agents and transducer nanomaterials for biosensing.

Keywords: biosensor, nanotechnology, pathogen, rapid monitoring, spoilage

Introduction

Food spoilage and foodborne infections are 2 major problems that affect the global food industry. The rich nutrient matrix and high water activity of fresh and minimally processed foods create a favorable environment for the proliferation of specific spoilage organisms (SSOs) as well as foodborne pathogens (FPs). The economic implications of these problems are stunning: approximately one-third of the world's food production is lost annually, to a large extent due to food deterioration associated with microbial activity (FAO 2011). This waste not only impacts the food industry, but also has a direct effect on the water and energy sectors, as the energy embedded in wasted food in the United States is equal to approximately 2% of annual energy consumption, and the water associated with food waste is equal to about 24% of all the water consumed in agriculture (Cuéllar and Webber 2010; United Nations Environment Program 2013).

In the U.S., foodborne diseases annually cause over 3 million cases of illness, 10000 hospitalizations, and 200 deaths, a cost of $1.5 billion, and a loss of over 1000 quality-adjusted life years (Scallan and others 2011; Batz and others 2012). *Salmonella spp.*, *Listeria monocytogenes, Campylobacter* spp., *Staphylococcus aureus*, and the "big six" diarrheagenic *Escherichia coli* pathotypes (also known shiga-toxin-producing *E. coli*) are the most predominant FPs that persistently cause infections (Koluman and Dikici 2013). According to the reportable food registry released by the U.S. Food and Drug Administration (FDA 2016), *Salmonella* Typhimurium, *L. monocytogenes*, and *E. coli* represent 25%, 19%, and 1%, respectively, of the most frequently reported food safety hazards. The global impacts of this problem are even more alarming, as diarrheal and intestinal infectious diseases caused 38 million global cases of various diseases with 1.5 million deaths, leading to a loss of 84 million disability-adjusted life years in 2015 (World Health Organization 2015). The challenge of maintaining a safe supply of high-quality food products while minimizing losses is exacerbated by the growing consumer demand for more "natural" products with minimal processing and containing no "chemical" preservatives (Yue and Tong 2009; Banterle and others

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2012; Lê and others 2013). This has global impacts, as production and export of high-value commodities by developing nations must meet the strict quality and safety import requirements established in developed nations (labeling, certification, and traceability), in turn restricting the global trade of fresh produce (Unnevehr 2000; Quested and others 2010; Engler and others 2012). To meet the need of supplying fresh, safe food to a growing world population, rapid and sensitive monitoring techniques are needed that can determine the presence of SSOs and FPs.

In most countries, current mandatory standards for the direct monitoring of SSOs and FPs include microbiological techniques such as total viable counts (TVCs). Culturing microbes in selective or differential culture media generates colonies that can be easily and accurately distinguished based on their distinct colony phenotype by a trained microbiologist using a light microscope. Culture-counting methods are commonly performed in the presence of selective markers (such as antibiotics) to suppress growth of nontarget strains in the culture inoculum, significantly increasing the selectivity of the assay. Secondary validation is a critical aspect, as most positive results are regarded as "presumptive positive" until validated by standard culture-based methods. While microbiological counts provide valuable information, major drawbacks of these methods are: underestimation of bacterial populations due to dormancy state of viable but nonculturable (VBNC) cells (Dinu and others 2009), as well as the labor/time required to yield results (requiring 2 to 7 d for analysis). Long delays have significant economic/safety implications as product deterioration/loss and shipping complications can occur while the food product is being held.

Recent efforts in the field of whole pathogen food safety biosensors have focused on *in situ* rapid monitoring technologies after calls for stricter monitoring of transient pathogens (Isaacs and others 2005; FDA 2006; CDC 2009, 2011). Wang and Salazar (2016) recently reviewed the field of culture-independent pathogen detection methods, including toxins, and they provided a comprehensive review of the most common methods. Valderrama and others (2016) reviewed the state-of-the-art in commercially approved detection methods, and they noted that there is a need for approved devices that can rapidly detect viable pathogens without the need for pre-enrichment. Rapid detection technologies are typically organized into 3 categories: immunological methods, nucleic acid-based methods, and biosensors. Given the large number of emerging methods for rapid pathogen detection, it is important to discuss the technical differences between devices based on the molecular interactions of a biorecognition agent with the target, some of which fall distinctly within these 3 categories and others may satisfy more than 1 category (for example, immunoseparation with impedimetric detection and a secondary probe for confirmation/viability). Thus, in this review, we provide a brief overview of current and emerging detection techniques, and we then describe their advantages and disadvantages for food pathogen monitoring. We conclude by summarizing the opportunities and challenges in the field of pathogen monitoring in food systems and we focus the discussion on the strengths/weaknesses of the most popular biorecognition agents and transducer nanomaterials.

Sample preparation

While the primary focus of this review is biorecognition and transduction in sensing, sample preparation is an inseparable component of any sensor applied to the food matrix. Over the last 2 decades, much effort has focused on resolving this issue by development of sample pretreatment methods that can be integrated with biosensors. These approaches include microfluidic separation (Zuo and others 2013; Packard and others 2013; Clime and others 2015; Kim and others 2015a; Lee and others 2015b; Luka and others 2015; Gashti and others 2016), nanoparticle separation (Varshney and Li 2007; Kwon and others 2015; Wang and others 2015), magnetic relaxation switching (Chen and others 2015a), dielectrophoresis (Cheng and others 2009; Packard and others 2011; Wang and others 2012c; Yang 2012; Hamada and others 2013; Couniot and others 2015; Kim and others 2015b; Fernandez and others 2017), immunochromatography (Vyas and others 2015), acoustofluidic sorting (Li and others 2017), ferrofluidic manipulation (Kose and others 2009), or hydrodynamic focusing (Clime and others 2015). Although not reviewed in detail here, sample pretreatment is a major focus of biosensor research labs and the primary challenges are minimizing destructive sampling, need for pumps, energy, and use of exogenous chemicals. Methods are being developed by many research labs that are low cost and environmentally sustainable by using combinations of these technologies. In the next section, we focus on general principles of biorecognition and transduction.

Biorecognition and transduction

There are numerous effective biorecognition–transduction schemes for developing pathogen biosensors, from simple detectors (such as colorimetric assays, disposable sensor strips) to complex arrays (multiplexing biochips with microfluidics and dielectrophoresis). Regardless of the complexity of the device, the working scheme for a biosensor involves a 3-step process: biorecognition, transduction, and signal acquisition (referred to as RTA for short).

In the biorecognition step (Figure 1), a molecular interaction between the target and a macromolecular structure on the sensor results in highly specific binding. In the transduction step, selective binding of the target produces a change in energy state of the system. Transduction can be inherent (such as change in impedance due to antibody [Ab]–antigen binding in immunoassays), or engineered (cascade reactions involving Förster resonance energy transfer [FRET] as a function of nucleotide binding in polymerase chain reaction [PCR] or addition of exogenous reagents). Although not covered in this review, inclusion of nanomaterials such as nanocarbon (for example, nanotubes, nanosheets), nanometals (gold, platinum, metal oxides), and many other structures, has been shown to significantly improve transduction, and in some cases even biorecognition. The 3 most common classes of transduction are changes in mass, electrochemical, or optical properties, although other transduction processes are possible (such as magnetoelastic transduction). In the acquisition step, the change in energy state is measured and an output is correlated to analyte presence. Acquisition also includes *post hoc* analysis such as data filtering, statistics, machine-learning, and data visualization.

One of the most important aspects in the detection of FPs is the type of biorecognition–transduction scheme used in the design. For qualitative or semiquantitative biosensing, the molecular binding between the target and the biorecognition agent is irreversible without addition of external reagents, or energy (for example, deoxyribonucleic acid [DNA] hybridization or antigen–Ab complex formation). These devices are referred to as detectors or assays throughout this review. Development of detectors and assays is a well-established field, and techniques such as the enzyme-linked immunosorbent assay (ELISA) have been widely used in the commercial field for decades, and thus serve as a gold standard for rapid pathogen detection (Valderrama and others 2016; Wang and

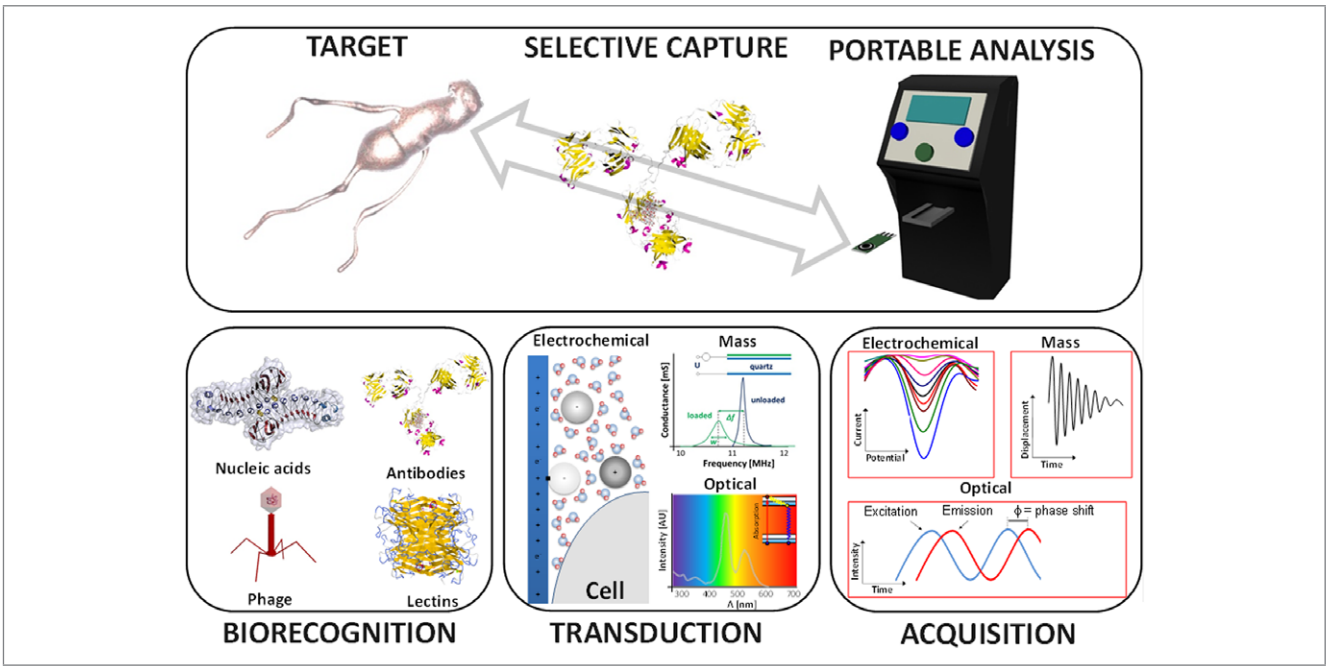

Figure 1–Biosensors are rapid handheld tools that can be used to measure the presence of target bacteria in food samples by selective capture. The general working scheme for a biosensor involves biorecognition of target analyte by capture probes such as nucleic acids, antibodies, phages, or lectins. This specific molecular interaction between the target and the macromolecular receptor probe leads to a change in energy state (transduction) that is measured using acquisition equipment. The 3 most common classes of transduction include electrochemical, mass/resonance, and optical.

Salazar 2016). For quantitative analysis, the biorecognition step must be reversible, with little to no hysteresis during repeated use. Conversely to detectors, sensors do not have inherent hysteresis and can produce quantitative results after multiple uses. The difference between these 2 (detector or sensor) is not merely semantic, molecular interactions between target and biorecognition element are the core principle of pathogen detection and dictate accuracy and precision. For example, disease risk models are limited by the quality and quantity of the data (Haas 2015), and thus uncertainties are inherently high when qualitative detectors are used to make quantitative predictions. Another matter complicating the usefulness of biosensors in food samples is the ability to detect cell viability, described briefly in the following section.

Determination of cell viability

While traditional microbiological culture-based methods discern viability, only a small percentage of microbes can be cultured in a Petri dish (Ramamurthy and others 2014) and the method thus underestimates microbial diversity. On the other hand, molecular methods such as PCR (Keer and Birch 2003) or metagenomic methods can capture viability of diverse microorganisms but require repetitive analysis over a long period of time from subsample populations, producing limited information on the viability of a specific sample (Cangelosi and others 2010). Further, use of traditional reverse transcriptase PCR (RT-PCR) or metagenomics analysis to differentiate between viable cells and free DNA fragments is extremely challenging. To avoid these problems, many techniques focus on cell membrane permeability as the marker of cell viability, which is based on the assumption that cell lysis is the dominant outcome along with cell death.

Relevant examples of cell permeability analytical techniques include cell live/dead labeling assays (Davey 2011), viability PCR (vPCR) (Nocker and others 2006), and molecular viability testing (MVT) (Weigel and others 2013; Do and others 2014). Cell labeling uses a combination of a membrane-impermeable stain such as propidium iodide and a membrane-permeable stain such as SYTO9 for analysis by fluorescence microscopy and/or flow cytometry. However, live/dead stains are known to have major issues with false positives (Stiefel and others 2015). The vPCR also uses the live/dead tagging concept, but the detection mechanism is based on inhibition of PCR amplification by a cell impermeant, photoactivated reagent (for example, propidium monoazide). MVT is also a PCR-based method but uses RT quantitative PCR (RT-qPCR) to detect production of a species-specific macromolecule in response to exogenous nutrients (Postollec and others 2011).

Other strategies for determining viability are based on the ability to maintain metabolism and homeostasis. As noted by Cangelosi and others (2010), these efforts have been marginally successful due to the stochastic variations that occur in the local microenvironment, particularly for single cells or small populations of cells (Kell and others 1998; Nyström 2001). The challenge of current technology for monitoring bacteria viability is the capability to detect a small number of viable cells in a complex matrix such as food.

Modern handheld (portable) sensors

Using schemes similar to those shown in Figure 1, a wide array of biosensors have been developed for food safety applications. Modern *in situ* sensors have high selectivity (specific to target bacteria), ultra-low detection limits, and fast response time (minutes), attributes that represent a major breakthrough over conventional laboratory analyses (discussed in detail below). Although the field of biosensing is well established (Turner 2013), there are only very few successful examples of pathogen biosensors in the food safety marketplace that demonstrate high specificity, quantitative sensitivity, and rapid response time in complex matrices (Valderrama and others 2016).

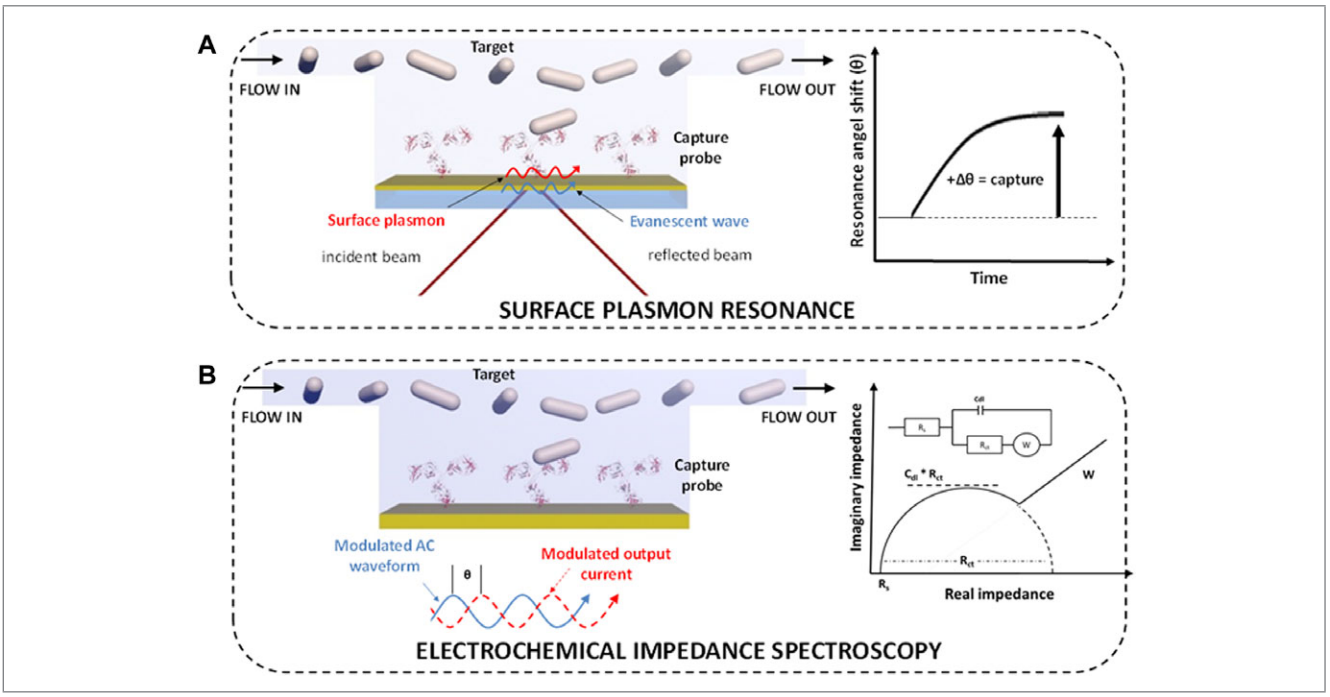

Figure 2–The 2 most common biosensor techniques for handheld biosensors are (a) surface plasmon resonance using an antibody as a capture probe, and (b) electrochemical impedance spectroscopy using an antibody as a capture probe. Flow-through SPR and EIS systems are shown here, although many formats are available (such as multiplexing, cross channel arrays, and others).

The 2 most common techniques for the development of handheld quantitative biosensors for pathogens are surface plasmon resonance (SPR) and electrochemical impedance spectroscopy (EIS). In SPR, shifts in measured evanescent waves are directly proportional to the binding of the target analyte and the biorecognition element (Figure 2A). In EIS, measured changes in impedance due to charge transfer resistance, double-layer capacitance, and/or Warburg impedance are directly proportional to target analyte binding (Figure 2B). See Supporting Information for a brief overview of SPR and EIS techniques (Figure S1 to S3). There are many other acquisition techniques available for the development of biosensors, but a detailed review of all available technologies is beyond the scope of this review. For recent seminal reviews on the general state of the art in biosensing not specific to food, see papers by Ronkainen and others (2010), Su and others (2011), and Turner (2013).

This review focuses on the most common biorecognition–transduction schemes used in pathogen detection. We then highlight some of the emerging techniques for direct monitoring of bacteria and then discuss the advantages/disadvantages of various biorecognition–transduction schemes. We conclude by discussing opportunities and challenges in the field of pathogen monitoring in food samples.

Pathogen Detection Schemes

Nucleotide-based detection

There are many types of nucleotide-based assays that are currently used to detect pathogens in food samples. The 4 types of probes used for specific gene targeting (such as nucleic acid binding) are based on DNA, ribonucleic acid (RNA), locked nucleic acid, or peptide nucleic acid; see Singh and others (2013) for a review. Although, there are too many variations of these techniques to cover in this review, the methods can be generally classified as either nucleotide hybridization or nucleotide amplification.

Nucleotide hybridization involves adsorption of oligonucleotide biorecognition probes, which are short sequences that are complementary to the target sequence in the pathogen strain. These probes are adsorbed to a substrate through covalent linkage. Common substrates include nanoparticles, fiber optics, metal electrodes, and nanocantilevers. If necessary, DNA in the sample is denatured (for example, by thermo/chemical cycling), and the sample is then exposed to the sensor surface that is coated with the complementary capture probe. When the target sequence(s) are present in the sample, hybridization occurs at the sensor surface and an output signal (such as fluorescence, mass change, or charge transfer) is correlated to the presence of target DNA. Singh and others (2013) have reviewed in detail the commercial systems and emerging devices based on DNA hybridization for developing rapid biosensors in pathogen detection. One of the most common types of nucleotide probes is the DNA microarray. A DNA microarray is a multiplexing system composed of numerous hybridization detectors in a biochip format (Ben-Yoav and others 2012). Thousands of oligonucleotides (typically 30 to 100 base pairs [bp] in length) can be qualitatively analyzed in a single microarray. In an array, target probes are labeled with a fluorescent dye (such as Cy5), and the biochip includes dye-conjugated reference sensors (such as cohybridized RNA) for cross-validation. With modern optical equipment, a single base match between an oligonucleotide probe on the microarray and the target gene sequences can be qualitatively detected in complex samples such as food.

The other major nucleotide technique involves DNA amplification via PCR. PCR-based methods are now conventional for the detection and identification of bacterial strains in food and there are many variations of the technique. For detailed reviews, see Saiki and others (1988), Settanni and Corsetti (2007), Ali and others (2014), and Law and others (2014). PCR amplifies a single copy of nucleotide in the presence of specific enzymes (such as DNA

polymerase). PCR primers are designed against target pathogen genes, and the final sequence is assessed to infer the presence of pathogens in the sample. PCR is a highly selective method that is capable of amplifying as few as 0.1 kilo base pairs (kbp) in a complex sample within as few as 12 h, which provides a qualitative assay of FPs (Wheeler and others 2011; McInerney and others 2014). qPCR is an advanced modality of PCR that monitors reaction kinetics in the early phases of PCR using fluorescence detection chemistries such as DNA intercalating-dyes (SYBR® Green) or hydrolysis probes (TaqMan chemistry) (Falentin and others 2011). For example, quantitative analysis of gene numbers can be conducted based on FRET between a donor dye and a quencher. qPCR technique provides distinct advantages over conventional PCR detection such as semiquantitative analysis, increased dynamic range of detection, enhanced precision, and faster analysis. RT-qPCR is a method that detects RNA expression via complementary DNA transcripts. RT-qPCR is still emerging, but is preferred by many labs since the estimation of quantitative transcript counts can be linked to strain-specific activity in the sample (Falentin and others 2011).

Other molecular methods include enzymatic techniques such as loop-mediated isothermal amplification (LAMP) (Nagamine and others 2001) and nucleic acid sequence-based amplification (NASBA) (Compton and others 1991) as well as nonenzymatic techniques such as split DNAzyme or self-assembly-based amplification (see review by Yan and others 2014 for details). LAMP is an enzymatic-based amplification method capable of 10^8 amplification in less than 1 h. Quantitative signal acquisition can be captured using turbidity, optical density, fluorescence, or visible color change (Notomi and others 2000). LAMP is a robust method that is not significantly affected by inhibitors, nontarget DNA, or alteration of physicochemical parameters. While LAMP requires fewer operation steps compared to qPCR, it requires a complicated primer design (commonly 6 primers), and has major limitations for multiplexing (Kokkinos and others 2014). Rather than competing directly with qPCR or RT-qPCR, the role of LAMP assays in many labs is for rapid "naked eye" qualitative confirmation (Yang and others 2010).

NASBA is an isothermal process that is capable of 10^7 amplification in less than 2 h. NASBA detects RNA sequences *in vivo* based on a 3 enzyme cascade (reverse transcriptase, RNaseH, and T7 RNA polymerase). This cascade serves to amplify sequences from an original single-stranded RNA template (Cook 2003). Amplicon detection is commonly based on enzyme-linked gel assays, bead-based enzymatic detection, electrochemiluminescence, molecular beacon technology, or fluorescent correlation spectroscopy (Sergentet and others 2008; Fakruddin and others 2013). Although not yet proven in food, Manojkuma and Mrudula (2006) note that in clinical use, NASBA has a higher analytical sensitivity than RT-PCR for pathogen detection. Although not covered here in detail, there are many other isothermal methods such as strand displacement amplification, invader assays, split DNAZyme amplification, and hybridization chain reactions (reviewed by Yan and others 2014).

Immunological methods

Ab-based assays (also known as immunoassays, immunodetectors, or immunosensors) are based on the selective interaction of an Ab with an antigen associated with a particular pathogen. The Ab-antigen complex forms a thermodynamically stable structure with a high level of specificity ($K_D = 1 \times 10^{-7}$ to 1×10^{-11} M) (Lequin 2005). Different types of biorecognition elements such as polyclonal Ab (common for cell capture), monoclonal Ab (single epitope recognition for high precision detection), recombinant Ab, Ab fragments, and single domain antibodies (also known as nanobodies) have been used extensively (Singh and others 2013). The most common qualitative test is ELISA. The basic approach for the development of an immunoassay involves 4 steps: (i) antigen identification on the target organism, (ii) synthesis of the Ab via phage display (recombinant method) or by using an animal host, (iii) engineering of a sensor platform, and (iv) design of the assay (immobilization of antibodies, sample preparation and analysis, and signal processing strategy). Typically, assays are based on optical and/or electrochemical transduction, although other approaches have also been developed. Even when not used directly as the analytical method, ELISA assays are generally used during Ab screening to identify Ab candidates with a high sensitivity, selectivity, and affinity for a constitutively expressed antigen of interest (epitope). The 2nd most common immunological-based method is a lateral flow assay, where samples flow by capillary force along an immunochromatographic line and generate detectable colors as an indication of absence/presence (Blažková and others 2009; Shan and others 2015).

The 3 most common types of immunoassay formats include: capture assays, sandwich assays, and subtraction assays (see Figure S4 and associated text for details). Sandwich assays are up to 5 times more sensitive than direct assays, although competitive adsorption between match pair Ab can induce error. To be successful, the target cell must contain at least 2 antigenic epitope sites that are accessible for Ab binding. Recent efforts have focused on the development of semiquantitative immunoassays using techniques such as SPR and EIS, among others (Mullett and others 2000). Some labs have recently enhanced performance by doping nanoporous membranes with Ab to reduce random orientation and conformational changes (Schibel and Ervin 2014). Among the transduction/acquisition approaches, plasmonic ELISA is emerging as an ultrasensitive method for recognition of pathogens and macromolecule markers of pathogen contamination in food (Bui and others 2015; Chen and others 2015b). Other common approaches include Ab immobilization in microfibers to enlarge binding area, or immunomagnetic separation (IMS) for specific concentration of bacteria from complex matrices (Sturbaum and others 2002).

Aptamer-based devices

In the last 2 decades, many devices have been developed based on immobilization of aptamers as the biorecognition element (reviewed by Kärkkäinen and others 2011). Aptamers are nucleic acid molecules engineered by using systematic evolution of ligands by exponential enrichment (SELEX), and they have been selected to bind 1 or more specific cell surface targets such as a particular antigen. Aptamers are stable for up to 2 y, relatively inexpensive, and have an affinity on the order of, or better than, monoclonal antibodies. There are fundamentally 3 types of aptamers, those based on DNA, RNA, or DNAzymes. As reviewed by Kim and Gu (2014), the 4 most prominent structural conformations used for DNA/RNA aptamers are the pseudo-knot (at least 2 stem–loop structures in which 1 stem is intercalated with the other stem), the G-quartet, the hairpin, and the stem/loop bulge (Figure 3). For example, Figure 3 (bottom panel) shows the double-hairpin 47-mer (DNA) developed by Ohk and others (2010) to target internalin-A, an invasion protein specific to *L. monocytogenes*. Internalins are specific to *Listeria spp.,* and are used by the bacteria to invade Ca^{2+} transmembrane proteins (known as cadherins) found along the

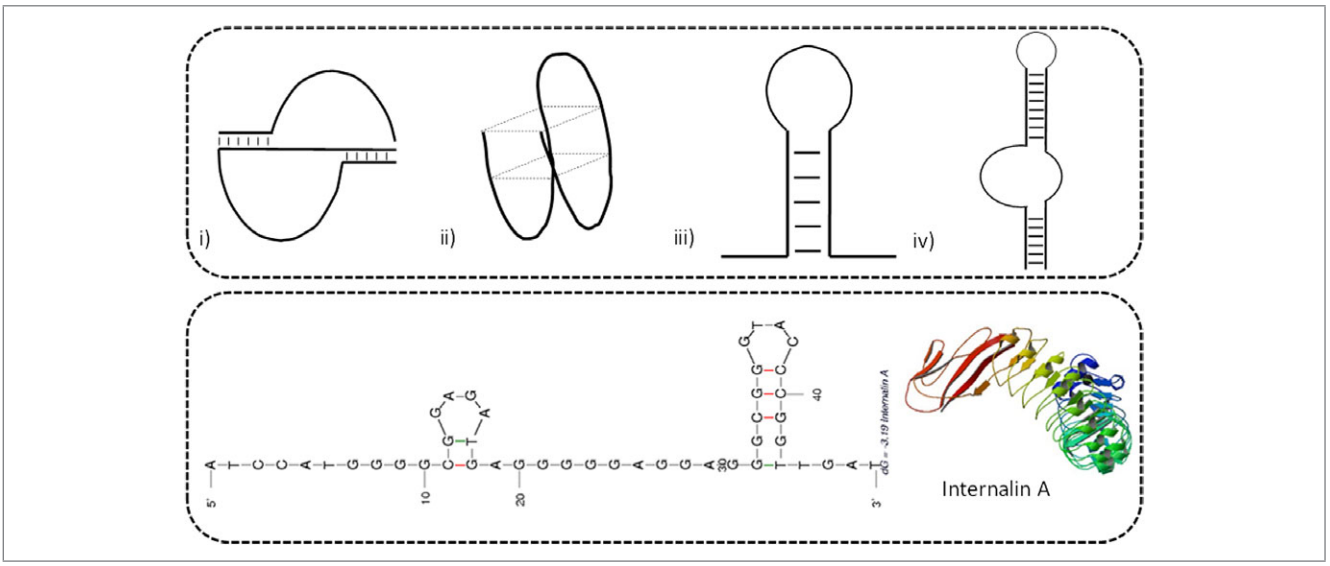

Figure 3–Aptamer specificity is a direct function of structural conformation. Top panel: Four common aptamer conformations include: (i) pseudo-knot, (ii) G-quartet, (iii) hairpin, and (iv) stem/loop bulge. Bottom panel: Stem–loop bulge DNA aptamer (47-mer) developed by Ohk and others (2010) for binding Internalin A on *Listeria monocytogenes* (Internalin A structure from Schubert and others 2002).

surface of mammalian cells. DNAzymes (not covered in this review) are primarily used for binding/reacting with small molecules in suspension and they have not been used for pathogen detection.

For creating aptamer-based sensors (aptasensors), aptamers are immobilized on a surface using covalent linkage, encapsulation, or entrapment, typically by conjugating a chemical linker and a bioconjugate terminus (such as thiol group) to 1 end of the structure. Aptamers exposed to a contaminated sample selectively bind the target, and transduction (inherent or engineered) is measured (Kärkkäinen and others 2011; Wang and others 2012a). The most common detection strategies include SPR (Ashley and Li 2013), EIS (Queirós and others 2013), fluorescence (Maeng and others 2012), and mass-based acquisition techniques such as quartz crystal microbalance (QCM) (Shen and others 2007).

Lectin-based detection

Lectins are carbohydrate-binding glycoproteins expressed on cells within the immune system, most commonly for specific targeting of mono- and oligosaccharide components of polysaccharide structures on the bacterial cell surface (Cambi and others 2005). Lectins contain 1 or more carbohydrate recognition domains (CRDs), and the structure of the CRD determines the overall specificity. Lectins are typically either transmembrane proteins or secreted as soluble proteins. For sensing applications, the soluble proteins are preferred since transmembrane lectins are lipophilic and their poor stability deters applications in processing facilities or food transport due to excessive costs to maintain the protein in a stable membrane. Lectins are subdivided into several groups based on their structure and mode of action, including: (i) mannose-binding lectins (MBL), which bind cell surface glycans (Eddie and others 2009), (ii) Ca^{2+}-dependent (or C-type) lectins (Cambi and others 2005), (iii) N-acetyl-glucosamine-binding lectins, or N-type lectins (Ghazarian and others 2011), and (iv) fucose-binding lectins (Audfray and others 2012).

There are many carbohydrate-binding glycoproteins that have been characterized in the published literature, but a detailed review of all available lectins is outside the scope of this review. Rather, we provide examples of the basic structure for each lectin class and their respective target oligosaccharide in Figure 4. The 1st class of lectins, MBLs, binds a wide range of carbohydrate patterns on bacteria, viruses, protozoa, and fungi. Figure 4a shows the crystal structure of concanavalin A (ConA), a MBL derived from jack bean (*Canavalia ensiformis*) that is fairly nonspecific and interacts with a large number of surface proteins containing mannose carbohydrates (Cuatrecasas and Tell 1973; Reeke and others 1975). The 2nd type of lectin (C-type) is organized into 7 subgroups based on the order of the conserved protein domain (Drickamer 1993). Figure 4B shows the structure of a C-type lectin known as specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN-R1). SIGN-R1 is a principal receptor in the immune system for binding bacterial polysaccharide mannose residues, and is also involved in anti-inflammatory activity (Spinelli and others 2014). The cofactor (calcium) binding sites are denoted in the image. The 3rd type of lectin (N-type) binds to N-acetyl-D-glucosamine (NAG) on the outer membrane of bacteria. Some of the most widely studied N-type lectins include wheat germ agglutinin (WGA), *Maackia amurensis* lectin (MAL), and *Helix pomatia* agglutinin (HPA). The crystal structure of a WGA N-type lectin-binding NAG is shown in Figure 4C (Muraki and others 2002). The 4th type of lectin is a fucose (FUC) binding glycoprotein (or F type lectin). Figure 4D shows UEA, a F-type lectin from *Ulex europaeus* that has an affinity for fucose. Fucose plays an important role in bacterial adhesion and is common on the surface of many pathogenic bacteria, particularly those that form biofilms. Lectins are typically purified/isolated using a combination of standard protein precipitation and affinity chromatography techniques as reviewed by Nascimento and others (2012).

Based on the diverse molecular binding of lectins for various oligosaccharides, there is a great opportunity to engineer new classes of multiplexing biosensors that can detect pathogens (McKeague and others 2011; Wang and others 2012b). However, multiplexing arrays are necessary for determining target specificity based on binding affinity, since there is significant ligand cross-binding among various lectins. For example, the UEA lectin in Figure 4D will bind fucose, mannose, and NAG with varying affinities (with the highest affinity for fucose).

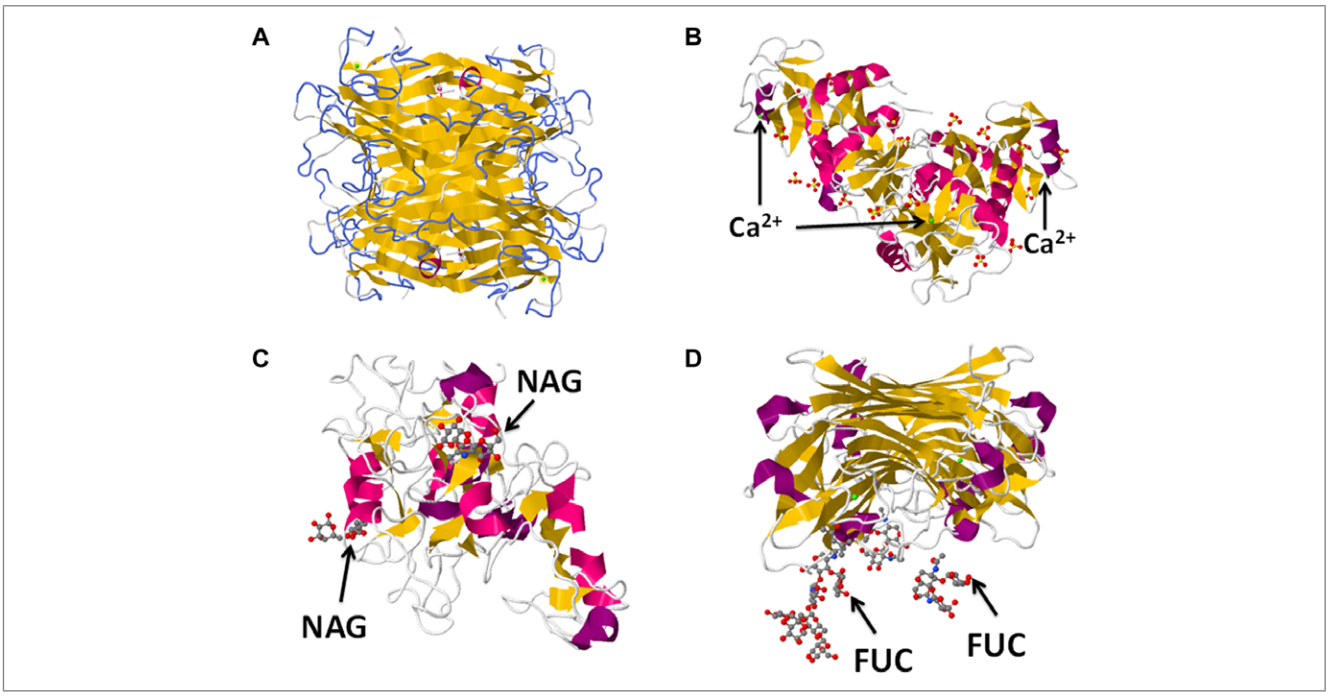

Figure 4–Basic structure of the 4 types of lectins: (a) crystal structure of concanavalin A (ConA), a MBL derived from the legume jack bean (Reeke and others 1975); (b) structure of a C-type lectin known as specific intercellular adhesion molecule-3-grabbing nonintegrin, or SIGN-R1; Ca^{2+} binding sites shown (Silva-Martín and others 2014); (c) structure of a wheat germ agglutinin N-type lectin binding to N-acetyl-D-glucosamine (NAG) (Muraki 2002); and (d) structure of F-type lectin from *Ulex europaeus* showing fucose (FUC) binding sites (Audette and others 2002).

Bacteriophage-based detection

Bacteriophages, or phages, are commonly used as biorecognition agents in pathogen sensing. Phages are viral parasites that infect a bacterial cell and induce virion multiplication through the host genetic machinery. Phage gene transfer takes place by the lytic pathway, temperate (or dormant) pathway, or horizontal gene transfer (see Rees and Loessner 2005). Most phages are strain-specific, and only a few show interspecies binding or specificity at the genus level (Chibli and others 2014). Singh and others (2013) provide an excellent detailed review of recent developments in bacteriophage-based sensors applied to pathogen detection. Lytic (wild type) phages have been used for biosensing, where changes in surface structure are measured by SPR or QCM. These devices have shown binding affinities that are comparable to monoclonal antibodies (Nanduri and others 2007). As discussed by Singh and others (2013), lytic phages have a number of major drawbacks for use in biosensing such as inherit biological activity. Thus, for the remainder of this section we focus on the following engineered phages: reporter phages, protein-expressing phages, and tailed phages.

The most common type of an engineered phage in biosensing is the reporter phage (Figure 5A). For engineering reporter phages, a gene (known as a reporter) is incorporated into the host chromosome, and it typically expresses a code for fluorescence or substrate-dependent colorimetry. The most common reporters are luciferase (*lux* or *luc*), β-galactosidase, ice nucleation genes, or green fluorescent proteins (*gfp*). Phage-based biosensors are rooted in the limitation that bacteriophages are incapable of expressing the reporter gene without infecting a host, thus the system is inherently selective and the "lights turn on" as a result of infection.

Another common phage-based technology is based on phage display. Phages are able to express peptides or proteins on outer surface structures, a process known as phage display. This technology uses genetically engineered recombinant phages wherein a bacterial gene encoding for a specific target peptide or protein is fused to the phage surface protein encoding genes (Smith and Petrenko 1997). Thus, a hybrid protein is expressed on the phage surface that displays the peptide or protein of interest (known collectively as a phage display peptide/protein [PDP]). As described by Løset and others (2011) in detail, peptides are typically displayed as fusions to the major coat protein (known as pVIII) or the minor coat protein (known as pIII) (Figure 5B). Phage display libraries are screened against targets of interest using a process called biopanning, which can be carried out using commercial kits to select custom affinity peptides for nearly any target (Fukunaga and Taki 2012). During biopanning, high-affinity phages are purified by isolating, and then sequencing after washing away unbound (or weak affinity) phages. Using this method, a number of novel peptides, cellular proteins, and Ab fragments have been developed for use as biorecognition agents (Singh and others 2013).

Tailed phages contain functional proteins at the tip of the tail-spike structure (or as part of the tail fiber) called receptor-binding proteins (RBPs). RBPs are highly selective and stable proteins that are responsible for triggering subsequent DNA translocation into the host after host-virion binding (Casjens 2011; Leiman and Shneider 2011; Parent and others 2012). Phages are capable of expressing several RBPs in a specific phage, and are currently being exploited for the development of RBP libraries that can be used to develop biosensors. Spinelli and others (2014) provide an excellent review of the structure and function of one of the most thoroughly characterized phages (*Lactococcal siphophages*). These phages are known to infect the Gram-positive bacterium *Lactococcus lactis,* a critical fermentative species in milk, cheese, buttermilk, and sour cream production. The structure of RBP from p2 and TP901-1 *L. siphophages* is shown in Figure 5C.

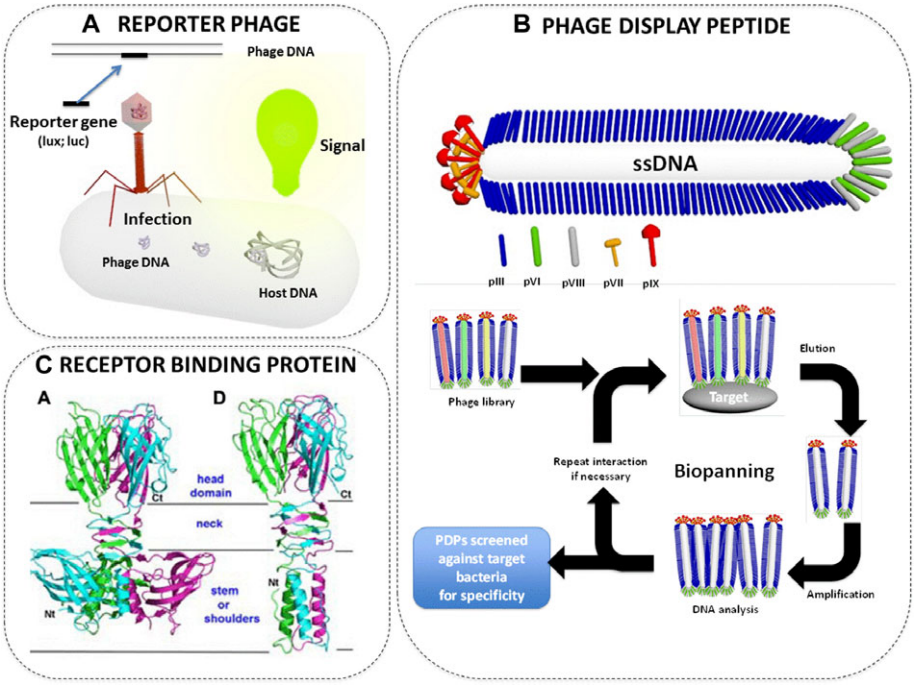

Figure 5—The 3 most common types of phage-based biosensors in recent literature sources are: (a) reporter phages, (b) phage-display peptides (PDPs); image based on Patwardhan and others (2007) and Løset and others (2011), and (c) phage receptor-binding proteins (RBPs) (Spinelli and others 2014).

Although we do not cover all biorecognition strategies in detail here, the sections covered above are the most common emerging methods to date. The following section highlights recent examples of sensors and assays for the direct monitoring of pathogenic bacteria in food based on the aforementioned recognition structures. Other emerging techniques such as surface-enhanced Raman spectroscopy (SERS) (Fan and others 2011), peptoid binding (Olivier and others 2013), and infrared spectroscopy (Wenning and others 2014) are also currently being used for detecting pathogens in food safety.

Examples of Devices for Pathogen Monitoring

Table 1 shows basic characteristics of recent devices for measuring pathogens in food. The discussion is separated by sections using the same format as the previous section, and does not cover all devices in the current literature, but highlights emerging trends.

Nucleotide-based assays

The lowest limit of detection (LOD) achieved for most nucleotide-based assays without preconcentration is 10^4 CFU/mL with 4- to 8-h response times (Singh and others 2013). Using preconcentration, LOD values as low as 1 to 10 CFU/mL have been attained in 8 h (Waage and others 1999; Lyon 2001). Bang and others (2013) developed a DNA microarray using random genomic DNA fragments, and reported a detection limit of 10^3 CFU/mL for *L. monocytogenes* in milk samples (16 strains of *Listeria* spp. were tested). In another study, a 1-pot isothermal microarray for detection of generic *Salmonella* spp. and *Salmonella* subspecies I (gene STM4057) was developed by Santiago-Felipe and others (2014). The array was capable of detecting 10^4 CFU/mL in powdered infant milk and chicken neck skin samples in less than 90 min. Mass-based devices such as the QCM by Xia and others (2008) have improved the detection limit to as low as 1.3×10^3 CFU/mL. Sharma and Mutharasan (2013) further improved on this detection limit (approximately 10^2 CFU/mL) by using piezoelectric nanocantilever hybridization sensors specific to the hemolysin gene (hlyA) of *L. monocytogenes*. This approach was also shown to be highly selective, with no significant loss of performance in the presence of 10^4 times excess nontarget genomic DNA extracted from *E. coli* JM 101 (an equivalent genomic DNA of 2.32 pg).

Work by Dzieciol and others (2013) demonstrated a qPCR assay for 1-step detection and identification of *Bacillus cereus* spores in artificially contaminated milk with a LOD of 10^3 CFU/mL using primers targeting *gyrB*. Bakthavathsalam and others (2013) reported a rapid method for *Salmonella* detection consisting of IMS followed by identification of target DNA *via* qPCR within 3 h. Silica-coated Fe_3O_4 nanoparticles were functionalized with *Salmonella* monoclonal antibodies and exposed to a suspension of heat-inactivated *Salmonella* cells. Advanced techniques such as multiplex-qPCR allow simultaneous analysis of multiple species in a sample. For example, Lee and others (2009) measured *S.* Typhimurium and *Salmonella* Enteritidis in beef and pork at levels as low as 10^3 CFU/mL in as few as 6 h. One of the most promising PCR developments involves combination of qPCR with a reverse transcriptase technique (or RT-qPCR), which has been used as a method for distinguishing between viable and nonviable cells (Zhou and others 2014). Remarkably, Zhou and others (2014) detected viable *Salmonella* at a detection limit of 10^1 CFU/mL in egg broth and milk using the *Salmonella*-specific sigDE operon as a viability indicator, together with IMS and solvent extraction.

Immunoassays

In general, immunoassays have LOD ranging from 10^1 to 10^3 CFU/mL and a response time of 2 to 6 h with a preconcentration step. Joung and others (2013) reported an impedimetric device

Table 1–Performance summary of some devices for direct measurement of pathogenic bacteria in food samples.

	Target (food sample or buffer)	Acquisition type	LOD (response time)	Reference
Nucleotide-based sensor	*L. monocytogenes* (milk samples)	DNA microarray	10^3 CFU/mL (24 h)	Bang and others (2013)
	Salmonella spp. (milk, chicken)	DNA microarray	10^4 CFU/mL (90 min)	Santiago-Felipe and others (2014)
	B. cereus (milk)	qPCR	10^3 CFU/mL (NR)	Dzieciol and others (2013)
	Salmonella spp. (milk, lemon juice)	qPCR	10^3 CFU/mL (3 h)	Bakthavathsalam and others (2013)
	S. epidermidis (buffer)	QCM	1.3×10^3 CFU/mL (5 h)	Xia and others (2008)
	Listeria monocytogenes (buffer w/ nontarget DNA)	Piezoelectric nanocantilever	Approximately 10^2 CFU/mL (60 min)	Sharma and Mutharasan (2013)
	S. Typhimurium *S.* Enteritidis (beef and pork)	Multiplex-qPCR	Approximately 10^2 CFU/mL (5 h)	Lee and others (2009)
	Salmonella spp. (egg broth)	rt-qPCR	10^1 CFU/mL (NR)	Zhou and others (2014)
Immunoassays	*E. coli* O157:H7 (whole milk)	EIS	84 CFU/mL (NR)	Joung and others (2013)
	Alicyclobacillus spp. (apple juice)	IMS + ELISA	10^3 CFU/mL (3 h)	Wang and others (2013a)
	E. coli spp. (buffer)	SPR	10 CFU/mL (2 h)	Maalouf and others (2007)
	Salmonella spp. (chicken)	ELISA	21 cells/mL (2 h)	Salam and Tothill, (2009)
	E. coli O157:H7 (spinach, milk, apple juice)	NP IMS + ELISA	10 CFU/mL (24 h)	Alocilja and others (2016)
Aptasensor	*Listeria* spp. (beef, chicken turkey)	Fiber optic	10^3 CFU/mL (2 h)	Ohk and others (2010)
	E. coli O157:H7 (buffer)	RT-qPCR/ELISA	10 CFU/mL (20 min)	Lee and others (2012)
	E. coli *S.* Typhimurium (milk, apple juice)	EIS	6 to 26 CFU/mL (approximately 75 s)	Zelada-Guillén and others (2010)
	E. coli O157:H7 (vegetable broth)	EIS	4 CFU/mL (12 min)	Burrs and others (2016)
Lectin-based sensor	*E. coli* (buffer)	EIS	10^3 CFU/mL (approximately 1 h)	Gamella and others (2009)
	Sulfate-reducing bacteria (buffer)	EIS	1.8 CFU/mL (approximately 2 h)	Wang and others (2012)
	E. coli (buffer)	QCM	10^2 CFU/mL (approximately 2 h)	Shen and others (2007)
	E. coli O157:H7 (cucumber, ground beef)	SPR	10^3 CFU/mL (NR)	Wang and others (2013a)
Phage-based sensor	*S.* Typhimurium (fat-free milk)	SPR	10^3 CFU/mL (20 min)	Lakshmanan and others (2007a)
	E. coli K12 (buffer)	SPR	10^2 CFU/mL (20 min)	Arya and others (2011)
	S. Typhimurium (buffer)	QCM	10^2 CFU/mL (3 min)	Olsen and others (2006)
	E. coli (buffer)	EIS	10^4 CFU/mL (4.5 h)	Gervais and others (2007)
	S. Typhimurium (buffer)	SPR	10^3 CFU/mL (30 min)	Singh and others 2010
	E. coli (milk)	EIS	10^3 CFU/mL (NR)	Shabani and others (2013)
	Campylobacter jejuni (buffer)	SPR	10^2 CFU/mL (NR)	Singh and others (2011)

NR, not reported; NP, nanoparticle; IMS, immunomagnetic separation; qPCR, quantitative polymerase chain reaction; rt-qPCR, reverse transcriptase quantitative polymerase chain reaction; EIS, electrochemical impedance spectroscopy; SPR, surface plasmon resonance; QCM, quartz crystal microbalance.

for label-free detection of *E. coli* O157:H7 in whole milk using a nanoporous alumina membrane modified with hyaluronic acid as the immobilization platform for the Ab. The immunodetector yielded detection limits as low as 84 CFU/mL (95% confidence) in milk samples after IMS. Wang and others (2013b) demonstrated an IMS-ELISA methodology for the detection of the SSO *Alicyclobacillus spp.* in apple juice, where magnetic beads decorated with polyclonal anti-*Alicyclobacillus* IgG were used to preconcentrate cells, and then the bacteria-rich buffer was tested using sandwich ELISA. The device had a response time of 3 h (not including incubation) with 96% specificity and 95% accuracy. Maalouf and others (2007) also developed an impedance device for detection of *E. coli* with a LOD of 10 CFU/mL and 10^3 CFU/mL for whole and lysed cells, respectively, in approximately 2 h. Salam and Tothill (2009) developed a sandwich ELISA device based on horseradish peroxidase with screen-printed gold electrodes; the device was used to detect *S.* Typhimurium at levels as low as 20 cells mL^{-1} in chicken meat samples.

Aptasensors

Aptasensors have a general LOD of 10^1 to 10^3 CFU/mL with a response time of 30 min to 2 h. Ohk and others (2010) developed a DNA-based aptasensor (Figure 3) for binding a cell surface protein known as internalin-A using a DNA 47-mer. The biosensor had a LOD of 10^3 CFU/mL, with a response time of 2 h. Aptasensors for detecting *E. coli* or *S.* Typhimurium have been used to detect bacteria at concentrations as low as 6 CFU/mL in complex matrices such as milk (Zelada-Guillén and others 2010). RNA aptasensors have also been used to measure pathogens. For example, a 64-mer for capturing *E. coli* O157:H7 based on binding to the number of O-antigens specific to O157:H7 lipopolysaccharides was used to develop an ELISA immunosensor with a LOD of 10 CFU/mL (no preconcentration) and a response time of 20 min (Lee and others 2012). Burrs and others (2016) extended this concept and used the same aptamer to develop a disposable (<$4/assay) paper-based electrochemical aptasensor for measuring *E. coli* O157:H7 in vegetable broth; the device had a LOD of 4 CFU/mL and a response time of 12 min with no preconcentration (10 min for incubation and 2 min for impedance measurement).

Lectin-based sensors

The LOD of lectin-based devices ranges from 10^1 to 10^3 CFU/mL with response times of at least 2 h or more. Lectins from *Triticum vulgaris* (WGA), *C. ensiformis* (ConA), *U. europaeus* (UEA), human fibronectin, *M. amurensis* (MAL), and *H. pomatia* (HPA) have been used to detect *E. coli* O157:H7, *S. aureus*, *S.* Enteritidis, and *L. monocytogenes* (Liu 2008; Wang and others 2013a). Gamella and others (2009) reported on lectin-modified screen-printed gold electrodes for the impedimetric label-free detection of *E. coli* with a detection limit of 5.0×10^3 CFU/mL. Wang and others (2013a) developed a SPR biosensor using lectin as the bioreceptor for the rapid detection of *E. coli* O157:H7 with a

Table 2–Summary of general parameters and challenges for emerging technologies used to detect pathogenic bacteria in food products.

Probe type	Response time	Specificity (%)	Limit of detection[a] (CFU/mL)	Ability to discriminate viability?	Challenges
Nucleotide-based sensors	2 to 24 h	>95	10^2 to 10^3	No[b]	• High cost, requires trained personnel. • High risk of false-positive detection. • Typically requires label.
Immuno-assays	8 to 24 h	>70	10 to 10^3	No	• Antibodies are often extracted from animals. • Typically requires label. • Qualitative results.
Aptasensors	1 to 3 h	>75	10 to 10^3	No	• Qualitative or semiquantitative analysis.
Lectin-based sensors	20 to 90 min	>60	10 to 10^3	Yes	• Relatively low affinity compared to other biological recognition elements.
Phage-based sensors	5 to 60 min	>90	10^2 to 10^4	Yes	• High phage purity is difficult to obtain with current extraction/purification methods. • Operating mechanisms are limited by the phage lysis time. • Stable immobilization of phages and phage RBPs on nonmetal surfaces is difficult.

[a] All LOD values represent data taken in food samples, including devices with/without IMS.
[b] Viability can be estimated by pretreating samples with reagents such as propidium monoazide (Li and others 2014) or monitoring the sigDE operon over time (Zhou and others 2014).

detection limit of 3×10^3 CFU/mL; a lectin from *T. vulgaris* was used as the binding molecule. Furthermore, the SPR biosensor was used to detect *E. coli* O157:H7 in contaminated cucumber and ground beef samples with a LOD of 3×10^4 and 3×10^5 CFU/mL, respectively.

Bacteriophage-based sensors

In general, the LOD of phage-based devices is 10^1 CFU/mL or greater with a response time of at least 1 h (including incubation) or longer. As reviewed by Singh and others (2013), phages have been used for qualitative identification of *E. coli*, *Mycobacterium*, *Salmonella*, *S. aureus*, and *L. monocytogenes*. Most phage reporters require approximately 24 h for analysis, and the LOD in real food samples is approximately 10 live bacteria per gram of food. Whole-bacteriophages have been used for direct detection of *S.* Typhimurium (LOD = 10^3 CFU/mL) using SPR (Lakshmanan and others 2007a), and also for detecting *Salmonella* with a magnetoelastic sensor in fat-free milk (LOD = 10^3 CFU/mL) (Lakshmanan and others 2007a). Arya and others (2011) developed a SPR phage biosensor for detecting *E. coli* K12 using T4 phage with a LOD of 7×10^2 CFU/mL. Olsen and others (2006) developed a QCM biosensor for detecting *S.* Typhimurium at levels as low as 10^2 CFU/mL using a filamentous phage. Gervais and others (2007) developed a PDP biosensor using T4 phages with the biotin-streptavidin system on gold electrodes; the LOD was 10^4 CFU/mL with a response time of 4.5 h. Singh and others (2010) developed a RBP sensor for targeting *S.* Typhimurium using a cysteine-tagged P22 phage with a LOD of 10^3 CFU/mL in buffer. The same group (Kropinski and others 2011; Singh and others 2011) also developed a SPR biosensor for *Campylobacter jejuni,* using RBPs expressed as a glutathione-S-transferase fusion protein, that were immobilized on glutathione-coated gold electrodes; the LOD was 10^2 CFU/mL in buffer.

Comparison of Current and Emerging Techniques for Monitoring Pathogens

A "systems level" analysis is needed for comparing biosensor technologies in order to provide necessary information for biosensors to fully penetrate the food safety market and to be used in industry (Valderrama and others 2016). Table 2 shows a summary of some important performance characteristics for sensors using the aforementioned biorecognition–transduction schemes, namely: response time, affinity (that is, specificity), LOD, and whether the test can determine cell viability. Some important discrepancies between biosensors and PCR that are not summarized in Table 2 are cost, ease of use, and device flexibility (these parameters vary too much to generalize in table format). Each of these is critical for adoption of a given technique by the food industry. The capital cost, training, and consumable/maintenance cost for PCR hardware is higher than that of most biosensors, even when considering that sample throughput is much higher. Response time is inherently important for food safety analysis, as products must wait for test results prior to shipping. Specificity over nontargets and LOD (with at 95% confidence interval or greater) are the most critical technical parameters when comparing emerging techniques with current standards (PCR, TVC).

There are many opportunities and challenges with nucleotide-based methods such as PCR and hybridization biochips. These methods are at least 12 h faster than culture-based methods, with specificity greater than 95%. There is no doubt that qPCR, RT-qPCR, LAMP, NASBA, and similar methods will continue to improve food safety monitoring for both industry and the consumer. Two persistent issues are (i) overestimation of bacterial numbers (false-positives) through quantification of naked nucleic acids, or (ii) matrix-induced inhibition of DNA polymerase (Dwivedi and Jaykus 2011). False-positive results induce unnecessary economic losses from wasted product that is still safe for consumption. In addition, qPCR is costly and requires trained personnel to conduct the analysis. Despite these disadvantages, one of the most exciting recent developments is the use of reverse transcriptase primers to distinguish between viable and nonviable cells. This advanced PCR technique is costly, but has one of the lowest LOD values in the literature for a culture-independent technique associated with viability testing. This method has great promise, and use of viability indicators such as sigDE operon are under investigation by a number of labs to refine the method (Barnett and others 2011; Shao and others 2011; Ruff and others 2012; Garrido and others 2013; Garrido-Maestu and others 2014; Li and others 2014; Vondrakova and others 2014; Zhou and others 2014; Meng and others 2015; Sebastião and others 2015). The drawback of this technique is the need for continuous monitoring of viability markers, complicating the assay, and adding analysis time and adding additional cost.

Immunoassays yield results within 8 to 24 h, and the LOD is comparable to other methods. Although detection of cells down to 10 CFU/mL is possible using nanoparticle-based IMS (Alocilja and others 2016), issues such as poor signal-to-noise ratio are a problem, and the unknown source of the noise is a primary focus of

research labs. Another deterrent to mainstream use of immunoassays is that selection of highly sensitive/selective monoclonal antibodies is time consuming and can be expensive (in addition to the need for a host organism, which has become unpopular for many labs). Without using IMS or secondary validation steps, the specificity can be low (around 70%), primarily due to matrix effects or cross-binding. Most bacterial strains share homologs of surface proteins, which is the source of the nonspecific binding (cross-binding). Inserting tags into Ab for immobilization significantly improves binding affinity, leading to improved specificity. Most immunoassays are limited to semiquantitative data due to hysteric (irreversible) binding between the Ab and target antigen. In other words, because of this hysteric interaction it is not possible to design an accurate sensor based on interactions between a single Ab type and the target antigen (direct capture), thus assays are developed with more than 1 binding event to produce semiquantitative data (see Supporting Information for description of sandwich assays and subtraction assays). Further, to date, no immunoassays are capable of determining cell viability. Thus, the issue of false-positive detection and the difficulty for obtaining precise quantitative data (such as number of microorganisms per unit of mass/volume of sample) limit the widespread applicability of Ab-based assays for the rapid monitoring in all conditions within the food industry.

Aptasensors are extremely rapid (minutes to hours) and have detection limits in the 10 to 10^3 CFU/mL range with no preenrichment. As discussed previously, the affinity of aptamers for whole cell targets is comparable to and, in some instances higher than, monoclonal Ab (Que-Gewirth and Sullenger 2007; Dollins and others 2008; Song and others 2008). Aptamers are considerably smaller than antibodies, thus they allow higher densities of pathogen-sensing elements leading to higher sensitivity and lower nonspecific adsorption (Wang and others 2012c). Aptamers are nonimmunogenic and demonstrate high tissue penetration, similar to that of small molecules, facilitating possible future uses in food contact applications. Other major advantages of aptamers are the ability to amplify biorecognition probes without the need for host animals, and the wide range of antigen structures that can be targeted (Shao and others 2011; Ruff and others 2012). Aptamers have long shelf-lives (up to 2 y when stored in buffer at –20 °C), and thus can be synthesized to detect complex targets using basic knowledge of the target surface chemistry. Although aptamers are emerging as an alternative to immunological devices, they are plagued with issues of low signal-to-noise ratio in real food samples (in a similar fashion to Ab). The use of IMS and secondary validation steps improves this signal resolution problem, but to date there is debate as to whether Ab or aptamers are a "better" biorecognition element. The field of aptamer synthesis is relatively new, and thus there is limited knowledge on the specific interactions between the target structure and the aptamer. Recent efforts to develop computational tools aim at understanding aptamer–target interactions (Alfinito and others 2017), and there is great promise that these analytical platforms will provide much needed insight into pathogen binding so that devices can be engineered to optimize signal-to-noise ratio. To date, no aptasensors have been successfully demonstrated for determining cell viability in real food samples.

Lectin-based sensors are inexpensive, simple, and extremely rapid (minutes to hours), with LOD on the order of aptasensors. Lectins have been used in targeted capture using novel approaches such as the self-propelled microtubular nanoengines developed by Campuzano and others (2012), where microstructures conjugated to lectins autonomously "swim" in the solution to seek out and bind pathogens such as *E. coli* without the addition of exogenous labels. Although lectins have a low affinity relative to the other biorecognition agents discussed here, specificity can be significantly increased if multiplexing sensors are used to target pathogen-associated molecular patterns rather than an individual cell surface target based on a single interaction between target and lectin (Richards and others 2015). Furthermore, lectins can be easily regenerated for repeated measurements and thus can be used as a regenerable biosensor in processing lines, food packaging, and so on. To date no biosensors using lectins have been used for distinguishing viability, but there are a number of labs investigating use of multiplexing lectins based on the damage-associated molecular pattern (DAMP) system, which are lectins that are used to distinguish viable cells in the immune system based on DAMPs. DAMPs can facilitate cell viability detection due to the innate recognition of some lectins to bind dying cells based on changes in glycosylation patterns of cell surface glycoproteins (Mills 2011).

Phage-based biosensors have demonstrated excellent binding to pathogens. The binding strength of wild-type phages or RBPs to cell surface targets (such as β-galactosidase on *E. coli*) is typically equal to or significantly higher than monoclonal Ab (Singh and others 2013), and stability of RBPs to local changes in pH and temperature is typically higher than monoclonal Ab. Another advantage of phages is that binding affinity can be easily tailored: tags and linkers can be inserted without altering binding affinity, and multivalency can be engineered, particularly for RBP. One of the disadvantages of wild-type phages is contamination of bacterial protein, lipids, or carbohydrates during extraction of the phages from host cells (Singh and others 2013). As discussed by Singh and others (2013), there is a wide variety of methods that improve phage purity (including centrifugation, filtration, and size exclusion chromatography), but contamination persists as a major problem in the development of nonrecombinant phages. Another major disadvantage of wild-type phages is the inherent biological activity that can lead to lysis of target cells or enzymatic activity that potentially induces bias. Practical issues such as loss of binding affinity after drying (phage tail collapse) increase the cost of phage biosensors and the specificity decreases to that of aptamers and Ab. Phages are large relative to aptamers, which restricts use in some devices such as SPR, SERS, and QCM, where output signal is significantly reduced with increasing distance from the sensor surface. A major advantage of phage biosensors is the inherent ability to discriminate between viable and nonviable cells, due to the fact that phages cannot infect dead bacteria. However, phage reporters suffer from limitations such as phage multiplication inhibition due to DNA-restriction-modification systems, phage-inhibition genes, or bacteria antiviral immunity (see Singh and others 2013 for details). All of these are typically present in live pathogens, so the technique is therefore very restricted if a disinfection process is integrated into the management practices as a part of the pathogen monitoring program.

Although not reviewed in detail here, the BARDOT system (bacteria rapid detection using optical scattering technology) is a label-free, noncontact system used for rapid identification of colonies (Bayraktar and others 2006; Banada and others 2007, 2009). This system has multispecies capability and can provide rapid screening of large sample volumes. BARDOT is based on forward scatter phenotyping, and the accuracy depends on carefully controlled environmental conditions, sample transmittance, as well as large reference libraries for *post hoc* analysis by machine-learning (Rajwa and others 2010).

One major advantage of PCR for food safety is the flexibility of the technology. Multiple pathogens can be measured as well as a variety of food samples with an individual unit; the only requirement is that specific primers and reagents be purchased for a specific test. On the other hand, current biosensors applied in food safety tend to be specific to a food type and strain/serotype of bacteria, limiting the throughput. Despite the relatively low throughput, the cost of developing biosensors on disposable substrates (paper, biodegradable plastic, and so on) has decreased considerably in the last decade, which is facilitating development of devices that can potentially be used to augment/expand hygienic zoning strategies as food safety monitoring becomes more strict. For example, spot sampling food contact surfaces with low-cost, handheld biosensors that have response time on the order of 30 min and selectivity >90% can improve overall strategic safety plans beyond what is required by the Food Safety Modernization Act (FSMA) by providing high throughput screening capability and extending the spatial resolution in processing facilities. Due to these differences, a head-to-head comparison of these technologies is not fruitful; rather, each technology will play a specific role in the next generation of food safety monitoring strategies as the industry adjusts to higher food demand under the umbrella of stricter global regulations.

Challenges, future directions, and trends

The field of nanobiosensors has made exponential progress in the last decade, with advanced sensor modalities and hybrid nanomaterials facilitating enhanced LOD, response time, and sensitivity. However, there are challenges with the aforementioned technologies. Namely, the biggest challenges are selectivity over nontarget compounds, detection of viable cells, and lack of international standardization for technology research and development. With improvements in these areas, monitoring technology will likely significantly improve as social–technological components converge, as discussed below.

Selectivity. Fat and particulates can interfere with Ab–antigen interactions, and compounds including carbohydrates, polyphenolics, sodium chloride, sucrose, or lysine can alter DNA polymerase activity during PCR cycling (Dwivedi and Jaykus 2011). Matrix effects have been reported for many food products, including milk (Sharma and Mutharasan 2013), apple juice (Taylor and others 2006), broiler meat (Wei and others 2007), and cucumbers (Wang and others 2013b), among other examples. To resolve poor performance due to matrix effects, many methods require a preconcentration step such as centrifugation, filtration, IMS, magnetic nanoparticle separation, and microfluidic sorting, as discussed previously. Although rapid techniques are typically not as selective as molecular methods such as PCR (see Table 2), use of a preconcentration step resolves this problem and has secondary benefits that are similar to enrichment, including: (i) dilution of inhibitors from food sample, (ii) discrimination of cell viability, and (iii) revival of stressed cells that would otherwise not be detected as viable (Valderrama and others 2016). The recovery of preconcentration methods varies from as low as 10% to as high as 99% (Ben-Yoav and others 2012; Valderrama and others 2016; Alocilja and others 2016). The challenge with this approach is to maintain costs and throughput that are competitive with standard techniques without additional cost. For rapid techniques to have a clearly defined role in the mainstream market, the method should not require more than 1 h (total time) to produce a result, and ideally cost less than U.S. $5 per sample (with benefit exponentially increasing as analysis cost decreases). In addition to preconcentration, consideration of a secondary validation step is a critical aspect, as most positive results are regarded as "presumptive positive" until validated by standard culture-based methods. Although not covered in detail here, preconcentration, microfluidics, magnetic separation, and wireless sensor acquisition, are important areas of ongoing research that will lead to the incorporation of biosensors into large-scale and small-scale operations.

Detection of cell viability. Among culture-independent methods, one of the most important performance characteristics for next-generation methods is the ability to detect cell viability. While traditional culture-based methods discern viability, the method underestimates diversity as only a small percentage of microbes can be cultured in a Petri dish (Ramamurthy and others 2014). On the other hand, molecular methods such as PCR-based methods (Keer and Birch 2003) or metagenomic methods capture species diversity, but assessing viability is complex and requires repetitive analysis from subsample populations, producing limited information on the viability of a specific sample (in other words, there are problems with cross sectional viability determination) (Cangelosi and others 2010). Further, differentiating between viable cells and free DNA fragments with PCR or metagenomic analysis is challenging. Among the methodologies that avoid some of these inherent problems, most techniques focus on cell membrane permeability as the marker of cell viability. This type of assay assumes that lysis is the most dominant outcome following cell death. Examples of cell permeability analytical techniques include cell live/dead labeling assays (Davey 2011), vPCR (Nocker and others 2006; Nocker and Camper 2009), MVT (Cangelosi and others 2010; Weigel and others 2013; Do and others 2014).

Cell labeling uses a combination of membrane-impermeable stain (such as propidium iodide) and membrane-permeable stain (such as SYTO9) for analysis by fluorescence microscopy and/or flow cytometry. However, live/dead stains are known to have major issues with false positives (Stiefel and others 2015). The vPCR method also uses the live/dead tagging concept, but the detection mechanism is based on inhibition of PCR amplification by a cell impermeant, photoactivated reagent (such as propidium monoazide). MVT is also a PCR-based method but uses RT-qPCR to detect production of a species-specific macromolecule in response to exogenous nutrients. None of the biorecognition–transduction covered in this review provides direct information on cell viability. However, cell labeling can be easily combined with many of the devices to determine viability (Abu-Ali and others 2017).

Standardization. While many pathogen biosensors in the published literature have merit (and likely a niche market), a systems level comparison (i.e., trade study) within the framework of food safety is nearly impossible due to a lack of standardization. For example, many industrial analyses use the term "sensitivity" to describe the probability of a test to detect a true positive, and the term "specificity" to describe the probability to detect a false negative (Valderrama and others 2016). Engineers working in technology development use the term "sensitivity" to denote the relationship between device input and output, where the term "LOD," used herein, denotes the true positive value. For example, response time should be calculated as the total time required for analysis from the moment a product is taken off the line to the time when the data are analyzed, or similar appropriate metric based on the application, including any time required for incubation, preconcentration, magnetic separation, filtering/centrifuging, or microfluidic sorting, as these add considerable analysis time. While culture-based techniques are often scrutinized as being "slow" (typically at

least 1 d), many biosensors and other techniques require hours or even days for preconcentration in addition to sample preparation that is typically not included in the reported "response time," resulting in a biased comparison. It is also an unfair comparison to directly compare a biosensor (which cannot distinguish viability) to a culture-based method that can report viable cell numbers. Within culture-based methods, there are specific technical issues that further convolute a direct comparison, such as a metabolic state known as VBNC, where pathogens are underestimated and food products may be released under a false negative test result (Li and others 2014). Thus, a direct comparison of "apples to oranges" does not advance the field, and perpetuates the problem rather than alleviating it. For a typical design, response time is reduced at the expense of higher LODs and lower sensitivity, which is an important trade-off to consider when thinking of safety monitoring at the systems level.

Response time is not the only performance characteristic that requires standardization. Calculation of LOD is not standardized, and some biosensor manuscripts report LOD values that have not been replicated, or results were conducted at a low confidence interval, both of which are not acceptable to meet industry standards. Selectivity is another major issue, and future manuscripts reporting new techniques should report validation studies that confirm strain/serotype specificity with a culture-based method, standardized/certified kit, or other approved standard methodology. For example, PCR (Marrazza and others 2001) or ELISA assays (Lee and others 2015a) can be used to validate biosensors together with replicates and controls that align with industry standards in lieu of culture-based techniques.

Most of the technologies discussed here are not yet commercially available and should be validated by organizations such as the Assoc. of Analytical Communities (AOAC-USA), the European Certification Organization for the Validation and Approval of Alternative Methods for the Microbiological Analysis of Food and Beverages (MicroVal), or the Assoc. Francaise de Normalisation (AFNOR-EU) as discussed by Valderrama and others (2016). In order for this to be a reality, the field of nanosensors/biosensors requires that a standard nomenclature (at the international scale) and performance characterization be used.

Once international standards are in place, trade studies can be easily conducted and nanobiosensors will progress in terms of technology readiness. Well-executed trade studies should be based on technical performance measures (TPMs) for various rapid methods, which are objective performance requirements that enable effective management. TPMs are used to derive measurable, quantifiable engineering requirements that have limited scope and are based on standardization. To be applicable, these standards must withstand the test of time and endure in the face of changes in regulations, policies, or consumer demands. If basic standards are established, research and trade studies for development of robust sampling techniques can be conducted to ensure accuracy/precision of a given method for product-specific pathogen detection. In addition to governing standards, increased data scrutiny regarding reproducibility/real sample analysis and comprehensive control studies are needed in the next generation of devices.

Emerging trends and the future of nanobiosensors in food safety. The grand challenge report released by the UN-FAO (FAO 2011) and also the FSMA report from 2011 each call specifically for new technologies to ensure the safe/secure supply of food in the face of a growing population and anticipated climate change effects. Development of socially relevant technologies in itself a major challenge (Scott and others 2016), and there are many groups around the world investigating this problem. As noted by Kagan (2016), nanoscale materials will play a major role in the development of new technologies at the nexus of food security and food safety. Nanoscale materials offer an opportunity to develop low-cost, low-power, lightweight, flexible, and nontoxic devices that are compatible with the safety, practices, and economy of the food industry. The promise of nanotechnology in future food chain systems is exciting, but the social acceptance by consumers, industry and regulators is a complex issue. As a starting point sensors based on new materials must be tested and validated under realistic operating conditions. However, a more pressing and complex challenge is the societal adoption and integration of new technologies. This social system includes consumers, but also government agencies, industry leaders, and even downstream wastewater treatment experts that process waste products related to the food industry.

Roco and others (2013) have developed a model paradigm for addressing this challenge using a process called convergence of knowledge and technology for the benefit of society (CKTS), which serves as a roadmap for researchers, citizens, industry, and policy makers. CKTS is an escalating process based on targeted interactions among transdisciplinary groups with a shared vision for integrating nanotechnology into society. CKTS, for short, has 4 integrated phases that constitute a convergence–divergence cycle. The 1st phase is a creative phase driven by synergism between multidisciplinary components. The 2nd phase involves integration and/or fusion of knowledge to develop a new system with a direct added value application. The 3rd phase is an innovation phase that results in new competencies, products and applications. The final phase is an outcome-based phase that consists of new applications and new spin-off technologies that initiate subsequent CKTS cycles based on societal need.

The field of nanotechnology and biosensors has a strong track record in the 1st 2 phases of CKTS and there is much published work in these 2 areas spanning engineering, science, technology, and social systems. For example, many current pathogen monitoring plans are focused on hygienic zoning, where high-care areas such as surfaces directly in contact with food are not tested rigorously due to the excessive cost and need to recall all upstream products if problems are identified (Shinbaum and others 2016). Therefore, most sampling plans focus on equipment in close proximity to food, food production areas, and areas outside of production lines. Individual facilities that choose to focus on monitoring food contact surfaces must hold products until test results are received, which is a major economic burden and also affects food quality. As discussed by Zwietering and others (2016), the frequency of sampling is related to the associated human health risk. Thus, a dynamic and flexible plan is developed using multidisciplinary knowledge (phase 1 convergence), and a monitoring system is established that has multiple technological options in the face of changing regulations and consumer demands (phase 2 convergence). Sensor networks are used to efficiently identify pathogens, serve as prompt indicators of contamination, and enable monitoring of the efficacy of good management practices, with the aim of reducing the time to respond so that microorganisms can be quickly reduced to acceptable levels. In this scenario, biosensors are used by some facilities to augment PCR-based monitoring and culture based techniques. Low-cost sensors can be used for sampling difficult-to-reach areas that are not frequently sampled, such as the underside of sinks or corners adjacent to processing equipment. PCR- and/or culture-based methods are the "workhorse" technology, where biosensors and other supporting technology are used to strategically monitor low-throughput zones as needed.

Another clear role for biosensors in this systems level monitoring plan is within food packaging and/or for monitoring shipping containers. There is a large collection of reviews, opinions, and theorems on these 1st 2 phases of CKTS in food safety technology, including this review.

The last 2 phases of CKTS are just beginning within the area of nanotechnology and biosensors for food safety, and this is an exciting new trend that will have immediate societal impact. Examples of phase 3 divergence include nanomaterials used as barriers, antimicrobial coatings, and spoilage indicators (reviewed by Duncan 2011). Recently developed materials have unique properties that will further extend these applications, including self-cleaning, self-healing, and ice-resistant materials (Hou and others 2015; Kreder and others 2016). These and other similar materials also have application in the medical and environmental industries (phase 4 divergence). The most important driver of the CKTS process is harmonious interactions between technology and social systems (Roco and Bainbridge 2003; Roco and others 2013). The convergence of knowledge, science, and technology achieves mutual compatibility, synergism, and integration, to create added value that is applied to ". . . unprecedented interconnected advancements in knowledge, technology, and social systems" (Roco and others 2013). Although nanotechnology offers the potential for direct interaction between social systems (consumers) and scientific discovery, to date this principle has not become commonplace. Indeed, safety regulations and legal ramifications are a factor in this lack of direct connectivity between food products and consumers, but future systems-level approaches will require feedback loops between society and food producers for successful implementation. Therefore, we anticipate that future efforts will adopt aspects of the CKTS methodology to redefine strategic monitoring plans and define best management practices in food safety nanotechnology, enabling new methodologies/technologies that will facilitate social convergence for the benefit of consumers and industry alike.

Conclusions

There are many choices available for developing RTA schemes in the development of pathogen monitoring technologies. Many *in situ* biosensors are providing rapid measurement of pathogens within a few hours, which is an exciting area of technology development. While there are many materials that can be used for specific binding of pathogens in food matrices, the choice of appropriate biorecognition agents and sensor designs is driven by cost, availability of materials, and sensor performance (including LOD, response time, specificity, and ability to detect viable cells). Comprehensive reviews are needed in order to collectively analyze trends in the field, but this is hampered by a lack of standardization among researchers and lack of scientific rigor when reporting sensor data. As research in nanomaterial synthesis progresses, there is great promise that biosensor technologies will augment culture-based techniques and molecular methods as the preferred *in situ* technology for food safety analysis. One of the ongoing areas of research that will lead to a game-changing performance demonstration for nanomaterial-mediated biosensors is the ability to combine preseparation/preconcentration, detection of pathogen presence, and viability all on a single low-cost sensor. Emerging biorecognition–transduction schemes provide an opportunity to develop small, rapid, wireless sensors that can be used to increase the spatiotemporal resolution of pathogen monitoring plans to ensure microbiological safety of food products, but these technologies must be beneficial for regulatory agencies, consumers, and industry. There are many different needs in the food chain related to food safety/food security. The technology community has risen to this challenge and developed a large library of molecular ensembles and different devices that can be used to make sensors, and now it is time to apply these divergent technologies for the benefit of society and the planet.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Basic operating principle for surface plasmon resonance (SPR).

Figure S2. An example of a typical sensorgram obtained for SPR for an antibody/antigen interaction.

Figure S3. (a) Schematic of electrical double layer formed at the surface of a charged metal electrode. The inner Hemholtz plane (IHP) and outer Hemholtz plane (OHP) are shown, as well as orientation of water molecules and ions. (b) EIS produces a Nyquist diagram that allows determination of the solution resistance (R_s), Warburg impedance (Z_w), double-layer capacitance (C_{dl}), and charge transfer resistance (R_{ct}). (c) A Randles-Ershler equivalent circuit model is fit to the empirical data based on minimization of chi-square for determining impedance values.

Figure S4. Schematic showing the 3 most common assay formats for immunosensors: (a) direct capture assay, (b) sandwich assay, and (c) subtraction assay.

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