

High-Throughput Biosensors for Multiplexed Food-Borne Pathogen Detection

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Abstract

Incidental contamination of foods by pathogenic bacteria and/or their toxins is a serious threat to public health and the global economy. The presence of food-borne pathogens and toxins must be rapidly determined at various stages of food production, processing, and distribution. Producers, processors, regulators, retailers, and public health professionals need simple and cost-effective methods to detect different species or serotypes of bacteria and associated toxins in large numbers of food samples. This review addresses the desire to replace traditional microbiological plate culture with more timely and less cumbersome rapid, biosensor-based methods. Emphasis focuses on high-throughput, multiplexed techniques that allow for simultaneous testing of numerous samples, in rapid succession, for multiple food-borne analytes (primarily pathogenic bacteria and/or toxins).

TRF: time-resolved fluorescence

qPCR: quantitative polymerase chain reaction

1. INTRODUCTION

According to the U.S. Centers for Disease Control and Prevention, contaminated foods account for approximately 48 million illnesses; 128,000 hospitalizations; and 3,000 deaths per year in the United States alone (1). Traditional microbial culture methods can detect and identify a single specific bacterium, but they may require days or weeks to complete and often do not produce quantitative data. Detection of a few pathogenic bacteria in a complex food matrix requires methods of extraordinary sensitivity and specificity. The quest for faster, quantitative results has stimulated the development of rapid microbial detection methods, many of which are biosensor based, that exhibit assay times of minutes to hours (2–4). A biosensor is a device or process that produces a measurable signal when the target species binds to a biological molecule (e.g., an antibody, receptor, nucleic acid, or other biorecognition element). Biosensors generally exhibit a rapid response, high specificity, immunity from interference, and low detection limits. However, the high levels of nontarget microorganisms and other materials in food samples often interfere with biosensor operation, and the detection limits (often a single bacterial cell per 25 or 65 g of food) required for food safety are even lower than can be achieved with most biosensors. Therefore, the practice of mixed-culture enrichment (5, 6), which nonselectively concentrates sample bacteria via growth under appropriate conditions, and/or physical processes [e.g., filtration and/or employment of antibody-coated paramagnetic particles (7)], which separate or isolate targeted pathogens from sample matrices and/or other microorganisms and substantially increase their concentration, are typically essential prerequisites for effective biosensor assays of food-borne pathogens. Either of these concentration techniques must preserve the unique genetic and phenotypic features that allow for the detection and identification of the target organism. Underlying the processes of separation, isolation, concentration, and detection employed in the application of rapid methods is the biorecognition process: specific and high-affinity binding of the target species to a biomolecule.

The development of rapid methods initially emphasized speed, low detection limits, cost reduction, simplicity and automation, and robustness. More recently, biosecurity threats generated desire for rapid methods that have (*a*) multiplexing capability, that is, the ability to simultaneously detect multiple analytes, and (*b*) the ability to test more samples in order to reduce risk. Therefore, this review focuses on the development of integrated processes for high-throughput, sensitive, and cost-effective pathogen detection. An integrated process typically entails the capture and concentration of targeted pathogens, as well as biosensor reporting of captured signals. We begin by briefly characterizing the essentials of each step, then provide examples of applying integrated processes for the detection of multiple pathogens in various food matrices (ground meat, produce, milk, and liquid eggs) through the use of high-throughput platforms [time-resolved fluorescence (TRF), multiplex quantitative polymerase chain reaction (qPCR), DNA microarray, and protein (antibody) microarray]. We focus on approaches that apply external labels via “sandwich” and hybridization formats, which confer inherently greater target analyte specificity. However, external labeling is not always practical, particularly when one-step assays or detection of small analytes—which may be bound by only one capture or reporter molecule—are desired or required. For the latter applications, competitive and/or label-free systems (e.g., mass-sensitive biosensors, surface plasmon resonance, fluorescence microscopy, select one-step immunoassays, Fourier transform IR spectroscopy, surface-enhanced Raman spectroscopy, etc.) may be employed; these techniques have been reviewed elsewhere (4, 8–11).

2. BIORECOGNITION

Specific, high-affinity binding of a target species to a biological molecule is a key component of biosensor detection and of the separation and concentration processes described in this review.

Specificity is essential to prevent false-positive results (which may arise from binding of nontarget species, also known as nonspecific binding), and high affinity is required to achieve low detection limits and minimize false-negative results (which may arise from failure to bind the target). When the target species is a nucleic acid sequence, recognition can occur by the well-defined process of base-pairing of complementary sequences. A base-pairing biorecognition element can be rationally designed and readily synthesized for any known target sequence. DNA biosensors take advantage of the extraordinary discriminatory power imparted by specific nucleotide sequences for detection. This power can also be employed for separation and concentration through the use of surfaces (e.g., paramagnetic beads) coated with complementary sequences. A distinct feature of base-pairing biorecognition is the ability to modulate binding by controlling the temperature. This procedure allows fine-tuning of affinity, permits sensors to be reused, and is the basis of methods such as PCR for amplifying nucleic acid sequences. Base-pairing also enables detection modalities such as intercalating fluorophores and molecular beacons (12).

Biorecognition of other target species, such as whole bacterial cells, is based on less well defined mechanisms, exemplified by antigen-antibody and ligand-receptor interactions. These biorecognition elements cannot be rationally designed and synthesized; they must be isolated from an existing population of elements (i.e., a library) by a process that selects a single element or mixture of elements with the desired binding specificity and affinity. Antibodies, the most widely used biorecognition elements, are prepared by injecting the target species into an animal and recovering the mixture of (polyclonal) antibodies secreted by the immune system. Single (monoclonal) antibodies can be isolated after immunization through use of cell-culture techniques. Although such in vivo methods have yielded antibodies against a number of food-borne pathogenic bacteria and their toxins, they have failed to produce antibodies with sufficient affinity and/or specificity for several high-priority targets [e.g., *Listeria monocytogenes* (13)]. Furthermore, traditional methods for antibody production are time consuming, expensive, and highly empirical (14). The need for faster, cheaper, and more reliable production of biorecognition molecules has driven the development of more exotic in vitro techniques, namely phage display and aptamer approaches for generation and screening of biorecognition-element libraries based on antibodies and nucleic acids.

2.1. Phage-Displayed Single-Chain Variable Fragments

Antibodies are Y-shaped dimeric proteins composed of two heavy polypeptide chains and two light chains held together with disulfide bonds. Target binding occurs in the relatively small variable fragment (Fv) located at the ends of the Y. The two chains forming the Fv can be fused with a short linker peptide to produce a single-chain variable fragment (scFv) protein with binding properties similar to those of the native antibody. Phage display (15) is a powerful technique for producing scFv-biorecognition elements in vitro; this approach addresses the need for faster, cheaper, and more reliable production of biorecognition molecules. Phage display has been used to isolate antibody fragments that bind to bacterial pathogens (16) and bacterial protein toxins (17). Recently, phage display has been applied to generate specific antibody fragments for the detection of *L. monocytogenes* (18), a food-borne pathogen for which species-specific antibodies have been difficult to obtain by traditional methods (13, 19, 20).

A phage-display library typically contains $\sim 10^{10}$ unique scFvs, from which virtually any target may be isolated. Selection of antibody fragments from phage-display libraries offers several advantages over traditional methods for antibody production (21). Antibody phage display involves isolating DNA sequences that encode the Fv light and heavy chains from a biological source such as mouse spleen. This DNA is cloned into the coat-protein gene of an engineered phage

scFv: single-chain variable fragment

(bacterial virus). When the phage genome is expressed, a chimeric protein consisting of an scFv fused to the native coat protein by a short peptide linker is “displayed” on the surface of the phage. Typically, the coat-protein gene is modified to include tag sequences (e.g., hexahistidine) for scFv purification, as well as promoters, restriction sites, and so on to facilitate cloning and modification of the scFv DNA. Millions of copies of the scFv can be produced by infecting a culture of host bacteria with the phage and allowing the phage to replicate. Generation of the library requires considerable time and effort that are comparable to those required to generate a monoclonal antibody. However, once the library is available, scFvs to virtually any target may be isolated from it through the use of an iterative multicycle process that can be either selective (positive panning) or subtractive [negative panning (22)].

2.2. Aptamers

Another strategy for the production of unique biorecognition elements uses aptamers, which are small nucleic acid molecules that exhibit high-affinity binding to target species. An aptamer library is simply a random sequence of 30 to 80 nucleotides, flanked by short fixed sequences encoding PCR primers for amplification. The flanking sequences may also include a transcriptase promoter sequence and restriction sites for cloning. A library containing more than 10^{12} unique members can be designed and prepared on a DNA synthesizer in one day at a cost of a few hundred U.S. dollars. Selection of binding aptamers is based on an iterative process termed SELEX (23), analogous to panning in phage display. Briefly, the target is mixed with the library; unbound aptamers are removed; and bound aptamers are recovered, purified, and amplified by PCR, yielding a population enriched in binding aptamers. Generally, the stringency of the selection is increased with each iteration, and negative selection is used to remove aptamers that bind nonspecifically. After several rounds of selection, the isolated aptamers are cloned into a bacterial host, individual clones are characterized, and final selection is performed. Aptamers have been isolated for more than 100 targets (24) including proteins, eukaryotic cells, spores (25), and bacterial lipopolysaccharides (26).

In some cases, aptamers exhibit binding constants that are higher than those of antibodies (27). Such biorecognition molecules have several potential advantages over antibodies and related proteins, and they may provide a unique biorecognition ability because pathogens that have evolved mechanisms to evade recognition by the immune system (e.g., by expressing host-like surface antigens) may be susceptible to aptamer recognition. Aptamers can be purified, analyzed, modified, and labeled using inexpensive, widely available molecular biology tools. Aptamers can be synthesized by the same processes used to prepare nucleic acid probes, allowing for the facile preparation of microarrays and other platforms targeting both nucleic acids and proteins. As simple nucleic acid polymers, aptamers provide detection avenues that are not available for proteins, such as molecular beacons (28, 29) and switches (30). Aptamer-target complexes can be reversibly dissociated by heat or chemical treatment, which allows reuse of the aptamer for capture or sensing (31). Individual and array biosensors based on aptamers exhibit rapid, reproducible detection of protein targets (32, 33).

3. SAMPLE PREPARATION: SEPARATION AND CONCENTRATION

The preferred detection limit for many pathogens is on the order of 0.1 cell ml^{-1} , whereas the typical rapid method can detect $\sim 10^3\text{--}10^6 \text{ cells ml}^{-1}$ (2). Pathogens must be separated and concentrated prior to biosensor detection to avoid false-negative results. This process may be achieved by conventional culture enrichment, physical separation and concentration methods, or a combination of the two (34). For air or water samples, nonselective physical methods such as filtration or

centrifugation are normally adequate for target separation and concentration. With food samples, however, such nonselective methods would overwhelm the biosensor with matrix constituents and background microorganisms. Selective separation and concentration methods using biorecognition are needed to avoid this problem. Biorecognition elements for separation or detection are generally immobilized on a support that ideally provides an inert, nonbinding surface. In practice, many sample constituents bind to the support surface, reducing the effective specificity of biorecognition and leading to false-positive results. This problem of nonspecific binding is ubiquitous and must be addressed at each stage of the assay to achieve reliable results. An important characteristic of separation and concentration processes is the concentration factor, namely the ratio of output to input target concentration. A concentration factor of 10^4 is required to match the detection limit of a typical biosensor (10^3 cells ml^{-1}) to the preferred raw sample–detection limit (0.1 cell ml^{-1}). If the physical method provides the required concentration factor, preassay cultural enrichment is needed to attain the preferred detection limit.

IMB:
immunomagnetic
beads

3.1. Immunomagnetic Beads

The introduction of immunomagnetic beads (IMB) has greatly improved the efficiency of bacterial cell separation (35, 36). These small paramagnetic particles (0.01–20 μm in diameter), coated with antibodies (some other biorecognition element that binds a target pathogen, cell fragment, or biomolecule would give rise to a term other than IMB), are generally mixed with the sample in a reaction tube so that the target binds to the bead surface. A magnet is then placed against the outer wall of the tube, pulling the beads (and the bound target) to the wall. The sample solution is poured off, leaving the concentrated bead-target complex behind. The method is rapid and efficient and can be used with food slurries containing large amounts of particulate matter. More recently, flow systems (e.g., Pathatrix[®]) have been introduced; such systems flow the sample over a bed of magnetically immobilized beads (37). This approach can potentially provide a much higher concentration factor than the tube method (~ 50 times higher). However, the availability of KingFisher[®], an automated and programmable magnet manipulator (38), offers similar convenience in a high-throughput sample-preparation format.

IMB were first used for the separation and concentration of various analytes more than 30 years ago (39, 40). A significant advance was the introduction of hydrophilic, monodisperse, uniformly dense, spherical beads 1–100 μm in diameter for use in chromatographic applications (e.g., 41–43). Eventually, monosized beads with homogeneously distributed colloidal magnetite were produced. Because small magnetite particles do not remain magnetized when removed from the external magnetic field [a property known as superparamagnetism (43, 44)], they can easily be removed from nonviscous fluids, washed, and resuspended for analysis. The IMB-based protocol was originally developed for the isolation of blood cells (45, 46).

Unlike collisions between small molecules, interactions between IMB and targeted pathogens are not diffusion controlled. The Brownian motion of such large particles is extremely limited, and the metabolism-dependent motion of bacteria is quite slow (47). For IMB to physically contact and bind the bacterial target, either sedimentation of the particles through the sample (48) or flow of the sample over fixed IMB is required. The typical application of IMB for cell separation is based on the fact that the beads are slightly denser than the test fluid and therefore move under the influence of gravity (48, 49). Thus, in a gradually changing gravimetric field (i.e., in a shaking or rotating sample) the beads slowly pass through and sample the test fluid. Through the use of high-affinity antibodies, collisions between an IMB and the target cell generally result in binding. Cell capture is a first-order rate process in which the rate constant is a product of a mass transport term (γ , $\sim 3 \times 10^{-9}$ ml min^{-1} IMB^{-1}) and the IMB concentration. When the magnetic beads

greatly outnumber the target organism, the average number of cells captured per IMB complex is close to unity (48). As the cell-to-IMB ratio increases to greater than one, each bead can capture, on average, up to approximately 4 ± 1 cells (49). However, when the IMB capture more than one organism apiece, the efficiency of magnetic separation is somewhat lessened because the captured cells lower the density of the IMB-cell complex (50). Multiple target assays require simultaneous separation and concentration of all targets. A combination of IMB and various biosensor detection methods has been applied to assay various targeted pathogens in food matrices (51–54).

Combining IMB and biosensors involves pathogen capture using sedimentation-induced IMB motion and bead recovery using simple magnetic concentrators [e.g., Dynal® (Invitrogen™, Carlsbad, California, and Polysciences, Inc., Warrington, Pennsylvania)] as well as programmable devices [e.g., KingFisher (ThermoFisher Scientific, Waltham, Massachusetts)]. In conventional affinity-capture methods, the surface density of the antibody as well as the antibody orientation and mobility are important factors in capture efficiency. The dimensions of commonly used IMB (1–3 μm diameter) and targeted bacteria (approximately $1 \mu\text{m} \times 2 \mu\text{m}$) are comparable, and binding of a pathogen can cause a significant percentage of the antibodies on the IMB surface to become inaccessible to other targeted cells. Therefore, a high surface density of antibodies on IMB may not be necessary for the effective capture of pathogens. The sedimentation motion of IMB predicts that the capture of pathogens is directly proportional to the liquid volume swept (v_s) by the bead. For a spherical bead, this volume (V) may be estimated from Equation 1 (55):

$$V = \pi r_b^2 v_s = (\pi r_b^2)[m_b(1 - d_l/d_b)_g](6\pi \eta_l r_b), \quad (1)$$

where m_b , d_l , d_b , g , η_l , and r_b are the mass of the bead, the density of water, the density of the bead, gravitational acceleration, the viscosity of water (0.01 P at 20°C), and the hydrodynamic radius of the bead, respectively. Because m_b may be calculated from $(\frac{4}{3})\pi r_b^3 d_b$, V may be reduced to

$$V = 2gr_b^4(d_b - d_l)/9\eta_l. \quad (2)$$

For N beads applied, the total volume (V_T) traveled by the beads over the time of agitation, t , is

$$V_T = [2gr_b^4(d_b - d_l)/9\eta_l]Nt. \quad (3)$$

Equation 3 predicts that binding, and subsequent isolation, of targeted bacterial cells with antibody-coated beads will be more effective when beads of either increased density or hydrodynamic radius are employed. This prediction of variable bead/bacteria capture efficiency was empirically demonstrated through the use of IMB of differential sizes and densities for the binding and magnetic isolation of *Escherichia coli* O157:H7 cells, as monitored by light addressable potentiometric sensing (**Figure 1**) (55).

More recently, investigators studied the influence of the chemistry applied to conjugate antibodies to IMB (56). IMB with diameters of 2.8 μm and 1.0 μm were conjugated to antibodies against *E. coli* O157:H7 through different chemistry. The 2.8- μm beads (IMB-C, IMB-S, and IMB-T) exhibited better capture efficiency than the 1- μm beads (IMB-C1, IMB-S1, and IMB-T1) (**Figure 2**). Furthermore, IMB with antibodies conjugated through streptavidin-biotin interaction (IMB-S and IMB-S1) exhibited better capture efficiency than did the IMB with antibodies conjugated through either activated carboxyl functionality (IMB-C and IMB-C1) or tosylation chemistry (IMB-T and IMB-T1). Commercially available 2.8- μm IMB that used a proprietary conjugation process (IMB-D) were included for comparison. However, further experimental verification may be needed before these results may be generalized to other pathogens. Importantly, the methods used to link antibodies to magnetic beads affect the capture of targeted bacteria.

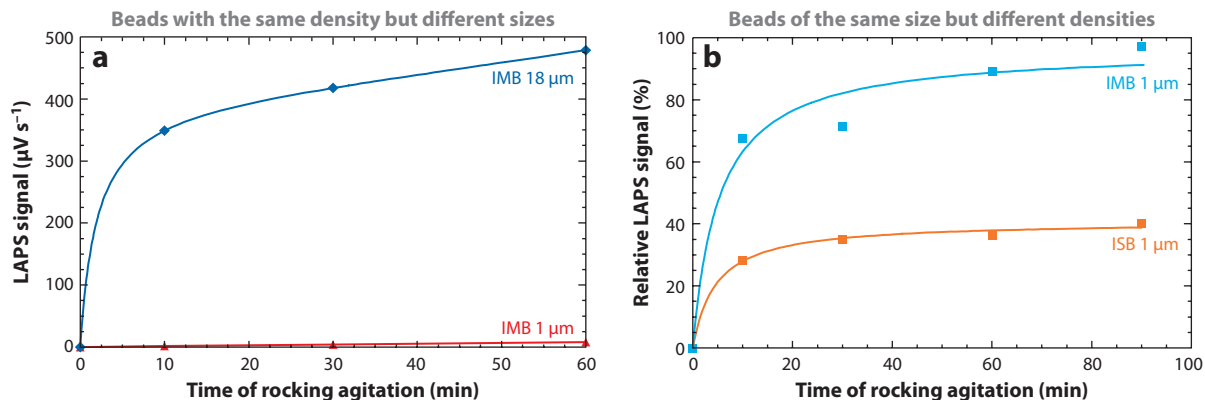


Figure 1

(a) Capture of *Escherichia coli* O157:H7 by immunomagnetic beads (IMB) of different sizes. Approximately 10^6 CFU of freshly cultured *E. coli* O157:H7 in 1 ml of phosphate-buffered saline (PBS) were mixed with 5,000 IMB (density, 2.50 g ml^{-1}), with diameters of 1 or 18 μm , coated with biotinylated anti-*E. coli* O157 antibodies. After the mixture was agitated for the indicated time, magnetism was used to separate free and bound bacteria. Light addressable potentiometric sensing (LAPS) was then used to detect bound *E. coli* O157:H7 (141). (b) Capture kinetics of *E. coli* O157:H7 by IMB (density, 2.50 g ml^{-1}) and immunopolystyrene beads (ISB) (density, 1.05 g ml^{-1}) of equal size (1 μm). Approximately 1.0×10^6 CFU of freshly cultured *E. coli* O157:H7 in 1 ml of PBS were mixed with 1.0×10^7 of IMB or ISB coated with anti-*E. coli* O157 antibodies. After the mixture was agitated for the indicated time, magnetic separation or sucrose density centrifugation was applied to separate free bacteria from IMB- or ISB-bound bacteria, respectively. The bound bacteria were then detected by LAPS.

3.2. Filtration

Culture enrichment adds hours or days to the time required for microbial assays (delaying remediation or recall), is problematic when multiple pathogens must be detected, and prevents quantitative determination of the original pathogen concentration. Because of these and other well-known problems, physical separation methods such as immunoaffinity capture, centrifugation, and filtration are frequently suggested as potential alternatives to enrichment (e.g., References 34, 57, and

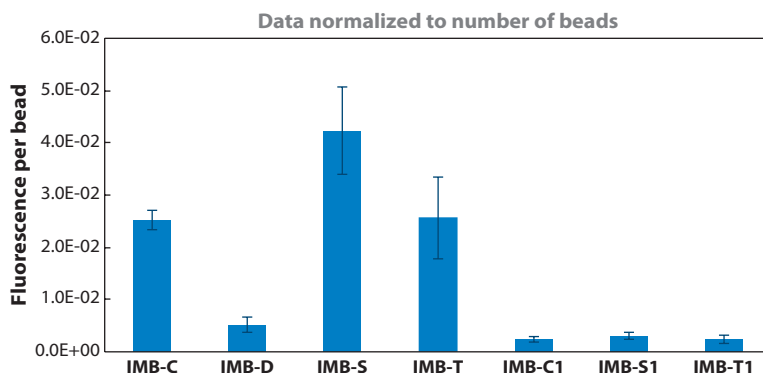


Figure 2

Capture of *Escherichia coli* O157:H7 strain B1409 from ground beef. Strain B1409 was inoculated into ground beef at a concentration of 1 CFU g^{-1} , enriched for 24 h in modified *E. coli* broth, and treated with different immunomagnetic beads (IMB). The captured *E. coli* O157:H7 were further linked to Eu-labeled anti-*E. coli* O157 antibodies and then detected by measuring the delayed fluorescence associated with Eu. The y axis values represent the average Eu fluorescence per individual bead.

CFU: colony-forming unit

LRF: leukocyte-removal filter

58). Through isolation and concentration of target organisms from a large sample, physical separation can allow quantitative, unbiased assay results for multiple targets; avoid problems with growth of nontarget microflora; remove interfering substances; and allow single-step processing of samples with minimal manipulation. Practical physical separation methods must process 100–1,000 ml of food homogenate (e.g., 10% homogenized ground beef in buffer) and concentrate a significant proportion of the target bacteria into a volume of 10 to 100 μl , at reasonable cost, in less than 1 h. When coupled to a rapid multiplexed detection system such as real-time PCR or qPCR, such methods could enable quantitative detection of multiple pathogens in a single food sample at 1 CFU g^{-1} within 2 h of sampling. This speed would represent an order-of-magnitude reduction in response time relative to enrichment-based assays, and it would allow the full potential of modern detection methods to be realized.

Although there exist a wide variety of physical separation techniques, including centrifugation, filtration, flotation, physico-chemical adsorption, biospecific adsorption, electrophoresis, dielectrophoresis, and liquid-liquid extraction (34), in practice such approaches are often limited to small volumes of relatively clean sample. Centrifugation, flotation, physico-chemical adsorption, electrophoresis, and dielectrophoresis fail when the sample contains high levels of particulates, proteins, or electrolytes. Gradient centrifugation and liquid-liquid extraction are impractical for use with large volumes, as are electrophoretic techniques. Currently, immunoadsorption on IMB is the most promising separation approach. This technique is typically conducted in batch mode on sample volumes of 1 ml, and IMB concentration is rapid, simple, readily automated, and capable of selectively concentrating the target pathogen from very complex matrices. However, relatively high concentrations (10^7 ml^{-1}) of expensive beads are needed for efficient recovery, which limits the technique's application to volumes of $\sim 1 \text{ ml}$ and concentration factors of ~ 100 (36, 48). Much larger ($\sim 500\text{-ml}$) sample volumes and concentration factors are practical when relatively few ($\sim 10^7$) beads are immobilized on a magnetic retaining device and the sample is pumped over the beads, but in such cases capture rate and efficiency are significantly lower, and many hours are required for recovery (59). The specificity of immunoadsorption also limits the technique to the few targets for which high-affinity antibodies are available. Larger sample volumes can be processed by flowing samples through immunoaffinity columns (60), but the samples must be free of large particles, and the same target limitations apply.

The small size of bacteria ($\sim 1 \mu\text{m}$) relative to that of eukaryotic cells ($\sim 10 \mu\text{m}$) and other food particulates suggests that filtration could be very effective for the rapid isolation and concentration of food-borne bacteria. Filtration has been effectively used to capture bacteria in milk and food homogenates (61), but the high solid content, broad particle-size distribution, and complex composition of typical food homogenates make filtration of more than a few milliliters extremely challenging. Until recently, large-volume filtration has been successfully performed only with dilute samples derived from rinsing or washing foods (62, 63). Recent research on filtration has led to the development of a novel filtration approach based on leukocyte-removal filters (LRFs) developed for blood transfusion. In this study, an LRF removed all particles larger than $10 \mu\text{m}$ from 100 ml of ground beef homogenate in 30 min, allowing capture of the bacteria on a $0.45\text{-}\mu\text{m}$ filter (with a $2\text{-}\mu\text{m}$ prefilter) and subsequent detection by direct plating with a detection limit of $\sim 0.25 \text{ CFU g}^{-1}$ (64). LRF filtration to remove most matrix constituents, followed by filtration, centrifugation, and/or immunoaffinity capture for concentration of the bacteria, appears to be a very promising alternative to enrichment.

4. BIOSENSORS

There has been a great deal of research on biosensors over the past two decades, and many advances in bacterial detection have been made (8, 65, 66). Researchers have drawn on a wide

array of techniques developed for the detection of proteins and other biomolecules, which has led to the development of rapid biosensor methods for bacterial detection that utilize cell components for signal transduction. These techniques include bioluminescence of ATP (67) and NAD(P)H (68). Examples of applications of various biosensors to bacterial detection through the integration of immunological recognition with numerous detection platforms include amperometry (69–71), antibody-directed epifluorescence microscopy (51, 72), electrochemiluminescence (73), fiber-optic biosensors (74), light addressable potentiometric sensing, (75, 76), luminescence (77, 78), quartz crystal microbalance (79, 80), surface plasmon resonance (81, 82), and TRF (54, 55). In addition, investigators have developed biosensor-based detection systems that do not employ antibody biorecognition; these systems include bacterial cell laser light scattering (83), bacterial colony laser light scattering (84), bacteriophage-induced changes in the target organism (85, 86), surface plasmon resonance (81), and PCR-based methods (87–90). The vast majority of these biosensors can detect relatively low levels of individual targeted organisms and provide many advantages over cultural methods (91, 92). In most current protocols, a food sample is tested for the presence of a single target. Multiple tests, often requiring different sample preparation and different assay conditions, are required to detect all potential pathogens. The time and resources needed for such single-sample, single-target assays constrain the number of samples or targets that can be tested and limit the ability to analyze putatively contaminated foods. High-throughput assays that can test for multiple targets simultaneously are needed to overcome this limitation.

Two basic approaches have been used for detecting multiple targets in a single sample: label multiplexing and spatial multiplexing. In label multiplexing, the sample is exposed to a mixture of probes (antibodies or nucleic acids), each of which carries a unique label. A multichannel detector is used to generate a separate signal from each probe. The number of targets is limited by the number of channels (labels) the detector can resolve. For example, with fluorescent lanthanide (93) or quantum dot labels (26), approximately five channels can be detected. Label multiplexing can provide very low detection limits and good quantitation, but it requires highly specific probes to achieve selectivity. The spatial multiplexing approach uses a large number of probes immobilized on separate sites with (at least) a single-channel detector. The probes are simultaneously exposed to the sample, and the bound target (protein, nucleic acid, cell, cell fragment, etc.) is detected either directly or through a common label. The number of detectable targets, which may be in the tens of thousands, is limited by the spatial resolution of the system. Examples of this approach include fiber-optic array biosensors (94), bead-based array sensors (95, 96), certain conceptions of mass-perturbance biosensors (97), and planar microarrays carrying protein (98–100) or DNA (101) probes. Planar microarrays are finding increasing use in drug-discovery and biomedical applications, and methodology and instrumentation have advanced rapidly in these areas. Laboratory spotters and readers/scanners are readily available; bedside diagnostic systems based on microarrays, as well as compact, inexpensive readers, are expected to become available within the next decade. Planar microarray biosensors for food-borne pathogens can leverage these advances and speed the development of practical assays. The ability to detect thousands of targets in a single assay has provided considerable impetus for the development of such biosensors. High selectivity can be achieved even with less-specific probes by requiring a response from multiple probes for a positive result.

4.1. Time-Resolved Fluorescence

Complexes (chelates) containing lanthanide cations such as Eu exhibit unusual fluorescence properties that make them excellent labels for sensitive, multiplexed target detection. The complexes can be excited over a broad range of wavelengths, and each lanthanide emits fluorescence in a

distinct, narrow peak that is widely separated from the excitation wavelength. These characteristics allow a mixture of lanthanides to be analyzed simultaneously. Lanthanide emission occurs over a much longer timescale than that of most other fluorescent materials. If a pulse of excitation light is used, the lanthanide fluorescence continues long after the fluorescence from interfering species decays away, which permits very sensitive detection of lanthanide labels (102). These unique fluorescent properties are exploited in a technique known as dissociation-enhanced lanthanide fluoroimmunoassay (DELFA). In most DELFA assays, a biorecognition molecule is labeled with a nonfluorescent lanthanide chelate by a proprietary method (Wallac Oy, Finland). After the biorecognition molecule binds to the target, fluorescence develops through the addition of an enhancement solution, which forms a new, highly fluorescent chelate inside a protective micelle. The fluorescence of the lanthanide is normally amplified 1–10 million times by the enhancement technique (103). DELFA has been applied through the use of TRF spectroscopic measurements to sensitively detect antibodies, microorganisms, drugs, and other therapeutic agents (104–107). A combination of IMB capture and concentration with TRF measurement of lanthanide-labeled antibody may be applied for pathogen detection in foods. Our results indicated that this combination can detect low levels (<1 CFU g^{-1}) of *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *S. enterica* serovar Enteritidis following enrichment at 37°C for 4.5 h in ground meats (52, 93) and in alfalfa sprouts (108). Also, this technology can detect Shiga-like toxins at levels of 5 to 50 pg ml^{-1} (109). The high sensitivity and specificity of the approach, together with the availability of a moderately priced, automated, high-throughput instrument, have led to the development of a rapid pathogen assay with a low probability of generating false-negative and false-positive results.

The combination of IMB capture and concentration and TRF detection (known as the IMB-TRF approach) has yielded demonstrable sensitivity and specificity for effective food pathogen detection (**Figure 3**) (93). Commercially available 96-well-format, high-throughput TRF readers can simultaneously measure the fluorescence associated with four different lanthanide cation labels (Eu, Sm, Tb, and Dy). Thus, when combined with suitable biorecognition reagents, many different pathogens and/or pathogenic indicators (including toxins) may be conveniently screened through the use of a single instrument. Combination of this approach with a commercially programmable 96-well-format paramagnetic bead concentrator may assure fast, economical, and high-throughput detection. This high-throughput IMB-TRF process was recently applied for the detection of outbreak strains of *S. enterica* serovar Enteritidis in shell egg contents (that is, in raw egg contained within an intact shell) (54).

4.2. Multiplex Quantitative Polymerase Chain Reaction

qPCR is an advanced PCR technique that has become invaluable for the detection of pathogens in food. Through the use of multiple TaqMan[®] probes labeled with spectrally distinct fluorescent reporters, a qPCR assay can be multiplexed to concurrently amplify and detect several different DNA sequences in the same reaction tube. Thus, several pathogenic species can be identified in a single assay by using multiple sets of target-specific primers and probes.

Using TaqMan-based PCR, investigators applied a recently developed multiplex qPCR assay to the simultaneous detection of *E. coli* O157, *Salmonella* spp., and *L. monocytogenes* in meat (110). The same principles were used for the development of a multiplex platform assay for the identification and differentiation of three *Campylobacter* spp. in chicken meat (89). Although qPCR is rapid, sensitive, and capable of detecting multiple DNA targets at the same time, extracting high quantities of quality genomic DNA directly from low numbers of target pathogens in food remains a challenge. Currently, the most successful PCR-based detection method requires selective

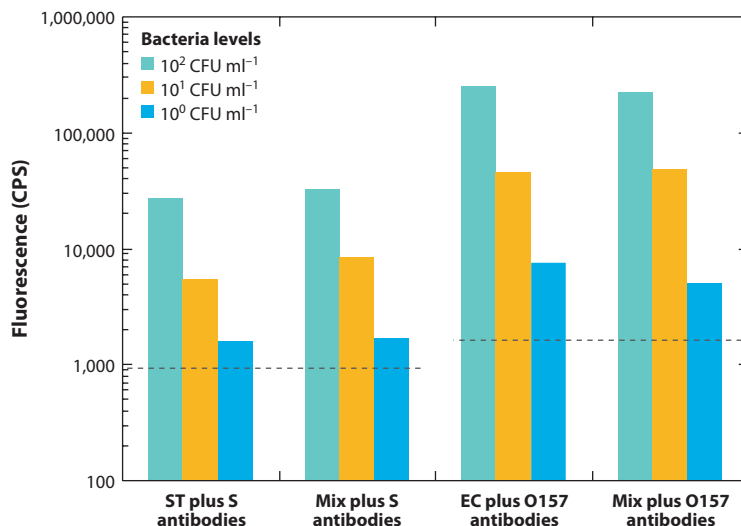


Figure 3

Simultaneous detection of spiked *Escherichia coli* O157:H7 and *Salmonella* in meats. Different levels of *E. coli* O157:H7 (EC) and *Salmonella enterica* serovar Typhimurium (ST), individually or in combination (mix), were spiked in amounts of 10^2 CFU ml $^{-1}$, 10^1 CFU ml $^{-1}$, and 10^0 CFU ml $^{-1}$ in hamburger, then enriched for 4.5 h (93). Beads coated with anti-*Salmonella* antibodies and anti-*E. coli* O157 antibodies were used to capture the pathogenic bacteria. Time-resolved fluorescence signals associated with Eu and Sm were used to measure the presence of *E. coli* and *Salmonella*, respectively. The averages of two independent measurements ($\pm 5\%$) are shown (gray dashed lines). The control (uninoculated hamburger) background was 1,179 counts per second.

culture enrichment, which increases the number of target pathogens in food samples to detectable levels. Food components may interfere with DNA amplification, resulting in PCR failure; thus, a successful PCR assay should be monitored via the addition of an internal, positive amplification control.

4.3. DNA Microarrays

DNA microarray technology is a very powerful tool for the simultaneous detection of large numbers (>104) of complementary DNA sequences in a sample. Therefore, this technology can be applied to genome-wide studies of gene expression, comparative genomic analysis, and single-nucleotide polymorphism detection in various biological systems (111, 112). DNA microarray entails printing of DNA probes, which are immobilized in discrete zones or spots measuring less than 1 mm in diameter, onto planar surfaces (or substrates), followed by exposure to samples containing DNA fragments. Complementary fragments in the sample hybridize to the probe spots, and unbound or weakly bound fragments are removed through washing. The amount of hybridized DNA on each spot is quantitated, typically by fluorescence. The numerous applications of microarrays include drug discovery (113), clinical diagnostics (114), and toxicogenomics (115). Although most microarray applications have focused on the analysis of gene expression, applications of DNA microarrays have expanded to include the detection and identification of bacteria and viruses (116–118). DNA microarrays have considerable potential to become rapid and sensitive methods for the detection of pathogenic microorganisms in foods for the following reasons: (a) They allow multiple microorganisms to be detected simultaneously; (b) general

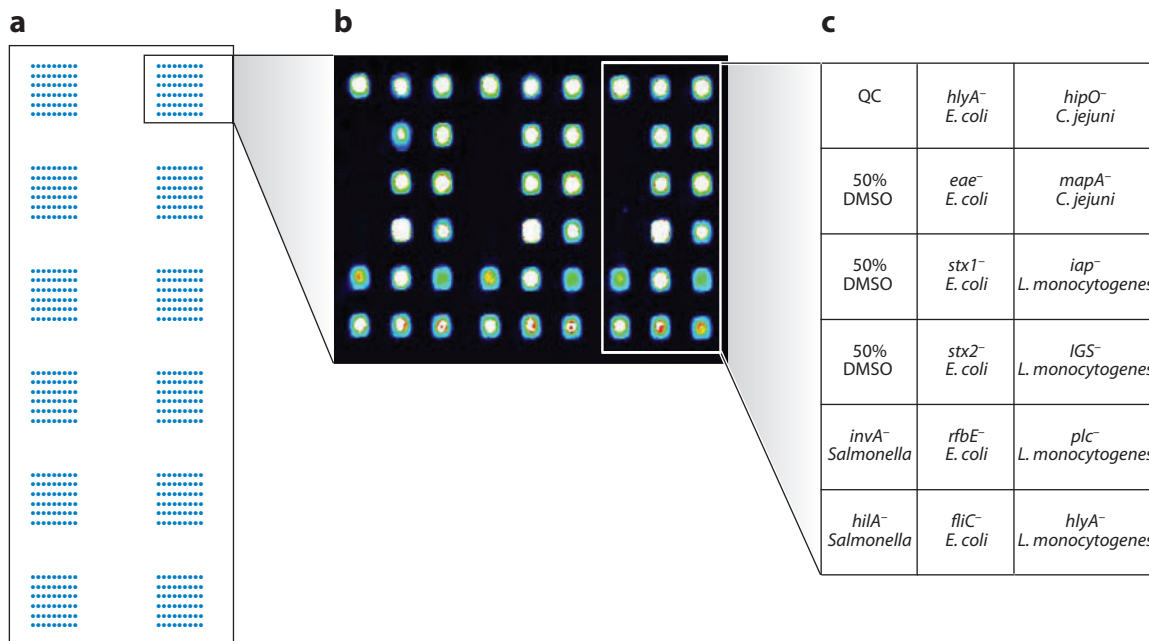


Figure 4

Layout of oligonucleotide probes on a pathogen detection microarray. (a) A microarray chip was spotted on 12 identical arrays. During hybridization, a chip was covered by a coverslip containing 12 attached frames (*partitioned sections*) for separating the subarrays. Individual subarrays were designed and used for analyzing an independent sample (b). A scanned image of a Cyto61-stained array shows the quality of microarray spots. Each subarray contains triplicate sets of probes, and the rectangle highlights a single set of probes. (c) Oligonucleotide positions in a single set of probes. QC represents a positive control probe. A solvent used to dissolve all of the probes, 50% dimethyl sulfoxide (DMSO), was employed as a negative control.

virulence factors as well as specific microorganisms can be detected; and (c) culture enrichment or growth of microorganisms is not a requirement for detection.

Recently, various types of microarrays have been developed and evaluated for microbial detection in food. Genetic markers used for microarray-based microbial detection include both “housekeeping” genes such as *16S*-23S recombinant DNA (119, 120), *gyrB* genes (121), and virulence genes (122, 123). Due to the specificity and cost of microarray assays, oligonucleotides (15–70 base pairs) are usually used (124). Microarray techniques have been used to distinguish not only different microorganisms (65, 125) but also different strains within the same species, such as *Listeria* spp. (126, 127) and *Salmonella* spp. (128). Although direct detection and discrimination of nucleic acids from bacteria are feasible, relatively few reports describe microarray detection following infectious food-borne outbreaks (129). The technology is well advanced, and a DNA microarray can be prepared for virtually any known set of uniquely distinct genetic sequences. Separation, purification, and concentration of unique nucleic acid sequences are expected to be a considerable challenge in the application of microarray techniques for utilization in practical food safety. Effective preparation of such unique nucleic acid samples will involve, at least, selective culture enrichment, filtration, and paramagnetic bead methodologies.

Recently, with the increasing availability of microbial genome-sequence data, microarray applications have rapidly advanced to the identification and characterization of numerous infectious agents, including microbial food-borne pathogens (130). Given that each microarray chip can accommodate many oligonucleotide probes, each at picomolar concentrations, the major

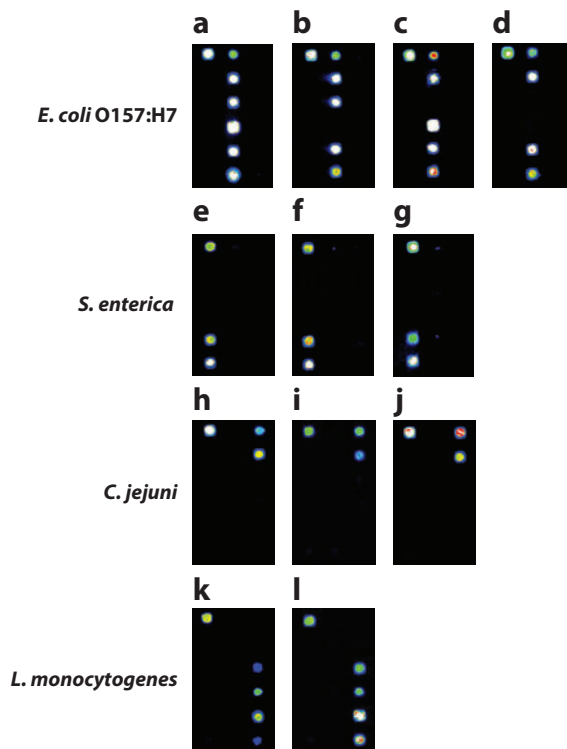


Figure 5

Specificity of a microarray for detection of food-borne pathogens. Genomic DNA from each of the following strains was preamplified in multiplex polymerase chain reaction and then analyzed by microarray. (a) *Escherichia coli* O157:H7 380–94 (*stxI*⁺, *stx2*⁺). (b) *E. coli* O157:H7 ATCC43890 (*stxI*⁺, *stx2*[−]). (c) *E. coli* O157:H7 B1409 ATCC43889 (*stxI*[−], *stx2*⁺). (d) *E. coli* O157:H7 B6–914 ATCC43889 (*stxI*[−], *stx2*[−]). (e) *Salmonella enterica* serovar Typhimurium ATCC14028. (f) *S. enterica* serovar Newport H1275. (g) *S. enterica* serovar Infantis F4319. (h) *Campylobacter jejuni* ATCC35918. (i) *C. jejuni* ATCC33560. (j) *C. jejuni* 81–176. (k) *Listeria monocytogenes* ATCC19111. (l) *L. monocytogenes* ATCC19115.

advantage of this technique is that detection, genotyping, and characterization of numerous microbial pathogens can be achieved in a single assay. It also has great potential for high-throughput screening of various food samples for multiple pathogens.

A whole-genome microarray approach, combined with bioinformatic data analysis, was used to study quorum sensing–regulated gene expression in *Campylobacter jejuni* (131). More recently, low-density oligonucleotide microarrays were designed and fabricated for the simultaneous detection of four major microbial pathogens in meat samples (Figures 4 and 5) (110). Other applications of DNA microarray for food-borne pathogen detection have been reported (132–134). However, for this approach to achieve routine diagnostic use, the specificity, sensitivity, speed, and cost of the assay must be improved.

4.4. Protein Microarrays

DNA microarrays are powerful, but they require the careful isolation and purification of DNA from the sample (e.g., microbial cell suspensions) prior to assay, and DNA detection is not suitable for analytes such as whole cells, cell fragments (lipopolysaccharides, cell wall components, flagella,

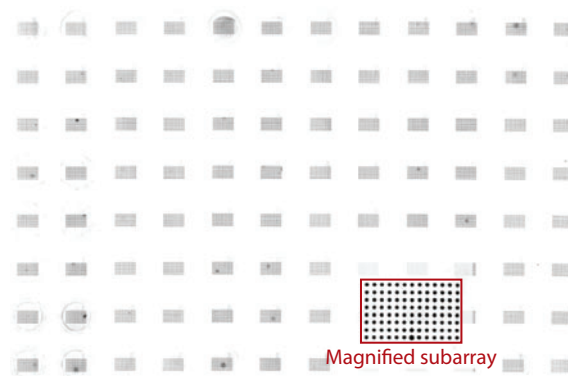


Figure 6

Sample 96-well microtiter plate-based microarray substrate containing $8 \times 13 \sim 100\text{-}\mu\text{m}$ subarrayed spots of fluorescent dye per well (generated with an Omnigrid Accent Pro microarray printer). The fluorescent scanned image is inverted.

blebs and vesicles, etc.), toxins, and metabolites. The technology for generating protein arrays and detecting binding is relatively less developed than for DNA microarrays, and considerable research is required to develop biorecognition elements (primarily antibodies), optimize immobilization of antibodies, and monitor/quantify binding of targeted analytes.

Since the inception of protein microarrays approximately 10 years ago, there has been a virtual explosion of research on the topic, ranging from analytes and applications to formats and detection systems (135). Similar to DNA microarrays, protein microarrays are composed of orthogonal arrays of biorecognition elements (antibodies, receptors, ligands, etc.) that are exposed to a sample, washed to separate unbound or unreacted material, and probed (typically with a fluorescently labeled reporter molecule such as an antibody conjugate) to detect binding of the targeted analyte. Probing is typically achieved via addition of a fluorescently labeled reporter molecule (often a fluorescent dye-conjugated antibody) in a fluorescent sandwich immunoassay format.

Pioneering research on protein-based antibody microarrays (98, 137, 136) has fostered the development of advanced formats for the simultaneous detection of bacteria and biomolecules in multiplexed, high-throughput platforms such as biochips (138), multichannel arrays (139), and 96-well microtiter plates (140), the last of which has the potential for high-throughput screening of samples (**Figure 6**). Although the microtiter-based antibody microarray exhibits poor detection limits of approximately 10^7 cells ml^{-1} for *S. enterica* serovar Typhimurium and approximately 10^6 cells ml^{-1} for *E. coli* O157:H7 in ground beef samples (**Figure 7**) (140), further refinement and expansion of a microtiter-based antibody microarray into automated, high-throughput platforms are expected to find application in future food safety methods, including adaptation to biosecurity applications. Regulatory efforts would benefit from the ability to rapidly (total assay time, <2 h) and simultaneously screen 96 samples for more than 100 analytes each. Recent efforts to optimize this assay have utilized centrifugation to enhance bacterial capture (bacterial capture at antibody-coated planar surface interfaces suffers from low efficiency) that has resulted in an improvement of approximately two orders of magnitude in bacterial detection limit, as well as a subsequent $\sim$ threefold reduction in total assay time (A.G. Gehring, unpublished observations). Antibody arrays with tangential flow systems have been demonstrated to achieve similarly improved capture efficiencies as well as favorable bacterial detection limits (139). Both of these microarray systems have detection levels for proteinaceous biomolecules (including bacterial toxins) that typically range from 0.1 to 100 ng ml^{-1} .

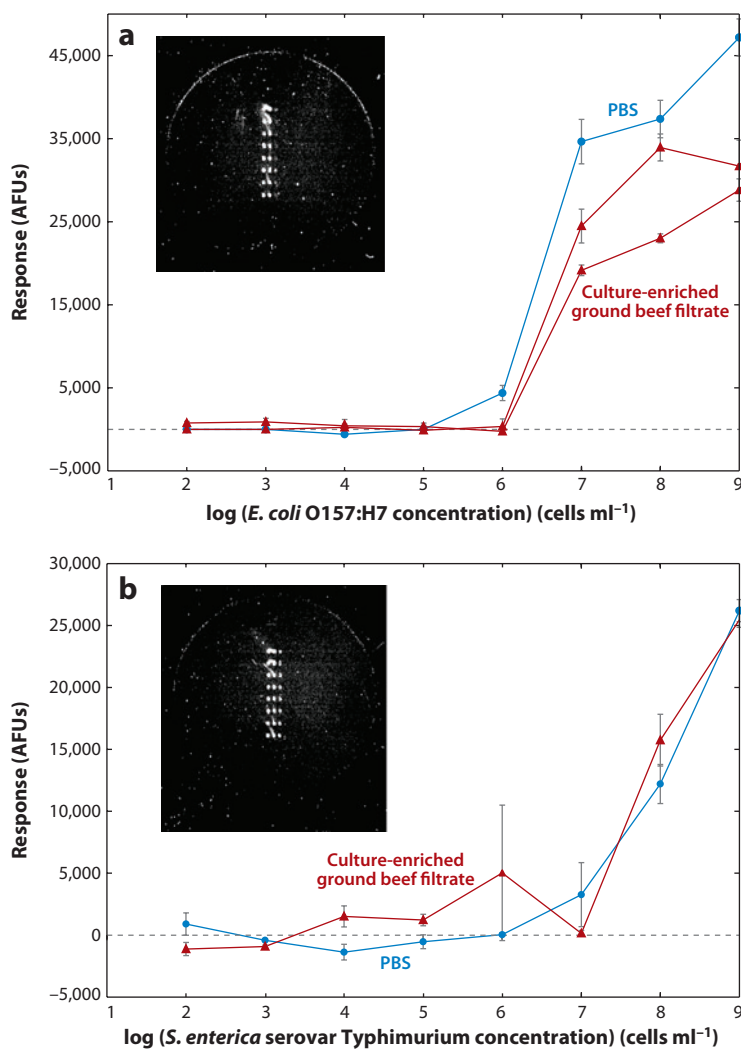


Figure 7

Multiplex detection of *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and chicken immunoglobulin G (IgG) via fluorescent sandwich immunoassay in microarray format. (a) Response versus concentration of *E. coli* O157:H7 (1×10^2 to 1×10^9 cells ml⁻¹) added to phosphate-buffered saline (PBS) or culture-enriched ground beef filtrate (results represent duplicate response curves) containing *S. enterica* serovar Typhimurium (1.0×10^8 cells ml⁻¹; eight spots in right column) and 100 μ g ml⁻¹ chicken IgG (eight spots in middle column). (Inset) Close-up of a microtiter plate well that contained 1×10^9 cells ml⁻¹ of *E. coli* O157:H7 (eight spots in left column). (b) Response versus concentration of *S. enterica* serovar Typhimurium (1×10^2 to 1×10^9 cells ml⁻¹) added to PBS or culture-enriched ground beef filtrate containing *E. coli* O157:H7 (1.0×10^8 cells ml⁻¹; eight spots in left column) and 100 μ g ml⁻¹ chicken IgG (eight spots in middle column). (Inset) Close-up of microtiter plate well containing 1×10^9 cells ml⁻¹ *S. enterica* serovar Typhimurium (eight spots in right column).

5. CONCLUSION

Food producers and regulators need rapid, reliable, and cost-effective detection approaches that can perform multisample, multianalyte detection of pathogens and toxins to ensure the safety of the food supply. Such detection methodologies will also provide data needed to carry out risk assessment, to develop and validate predictive microbial models, and to determine where intervention is most needed. Such information will assist the implementation of hazard analysis and critical control point programs by the U.S. Department of Agriculture Food Safety and Inspection Service, the U.S. Food and Drug Administration, and their regulated industries. With respect to food security, biosensor detection technologies will strengthen and expand laboratory preparedness and allow the development of rapid laboratory methods for the detection of select agents (microbial pathogens and toxins) in foods. Here we review several new and effective processes for sampling, isolating, and detecting pathogens from various food systems and matrices. Successful applications of these methods to the universal separation and concentration of the offending contaminant(s) will maximize their detection potential by biosensors. The advances described herein will become the basis for practical systems capable of multiple target assays in near-real time that have the potential for automation.

SUMMARY POINTS

1. Contaminated foods account for approximately 48 million illnesses; 128,000 hospitalizations; and 3,000 deaths per year in the United States alone. Therein lies a need for the development of rapid, biosensor-based methods as replacements for the more laborious and lengthy traditional bacterial culture methods.
2. Biorecognition elements, such as library-selected scFv antibodies and aptamers, are at the heart of biosensor-based rapid methods.
3. Microbial enrichment culture and physical processing (e.g., filtration and antibody-coated paramagnetic particle separation) are typically employed as prerequisites for enhancing the selectivity and sensitivity of rapid methods.
4. This review focuses on high-throughput biosensors that employ external labels and label multiplexing (TRF and qPCR) as well as external labels and spatial multiplexing (DNA- and protein-antibody microarrays).

FUTURE ISSUES

1. Further development of rapid methods is still needed and will require across-the-board improvements in speed, sensitivity, limit of detection, simplicity, cost, quantitation, specificity, portability, and robustness.
2. Because of the destructive nature of testing foods for microbial contaminants, it is not feasible to test entire product lots. In addition, there is the concern of infection by very small numbers of microorganisms, some of which (e.g., *E. coli* O157:H7) are classified as adulterants and have “zero tolerance” for detection. Therefore, a better understanding of sampling and/or further development of noninvasive testing is desired.
3. Epidemiological investigations undertaken to identify the source and spread of food-borne outbreaks will greatly benefit from improvements in both the rapidity and the accuracy of typing methods.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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