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Functional nucleic acids for detecting bacteria

Abstract: Bacterial infection represents one of the leading causes of disease and death, and as such, bacterial detection is an important step in managing infectious diseases. The current protocol requires growing cell cultures, which can take several days. Fast detection of low copies of bacterial cells has thus posed an analytical challenge. Among the new strategies developed to achieve this goal, functional nucleic acids (FNAs) have emerged to be a promising platform. FNAs include DNAzymes, aptamers, and aptazymes, all of which can recognize analytes other than complementary nucleic acids. FNAs are obtained using a combinatorial biology technique called systematic evolution of ligands by exponential enrichment (SELEX). FNAs have been isolated against not only purified proteins and surface markers from bacterial cells but also whole cells. A diverse range of signaling mechanisms including fluorescence, color, and electrochemistry-based detection has been reported. Although the majority of current sensors cannot achieve single-cell sensitivity, with improved combinatorial selection techniques and the incorporation of nanomaterials to realize multivalent binding and signal amplification, FNAs represent a feasible solution for bacterial detection.

Keywords: aptamers; bacteria; biosensors; fluorescence.

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Introduction

It has been estimated that over a billion people develop bacterial infections each year (Deisingh and Thompson 2004). About 70% of these infections are related to foodborne pathogens and dirty water, whereas many others are hospital acquired (Kendall et al. 2006). Together with cancer and cardiovascular disease, infectious diseases are among the leading causes of death (Kochanek et al. 2010). With the rapid development of antibiotic resistance (Taubes 2008), the early detection of bacterial pathogens is critical for managing the spread of infection. To have a major commercial and societal impact, rapid detection is a key requirement because it can minimize the unnecessary recalls for the food industry and public panic (Benoit and Donahue 2003). Another area requiring rapid bacterial detection is clinical application, so that appropriate antibiotics can be administrated. Such analytical tools can also accelerate clinical research. For example, it has been suggested that the bacterial species in the oral environment can be used to predict the outcome of treatment (Rotstein and Simon 2004).

The traditional method for bacterial detection requires sample enrichment *via* cell culturing to form colonies for phenotypic or metabolic fingerprinting. Although it is still widely used today and serves as a benchmark for testing and validating other assays, this process is very time consuming, taking days to complete. Quick detection methods are required for the reasons outlined above. Each bacterial species can be identified based on certain nucleic acid sequences. For example, the ribosomal RNA (rRNA) is highly conserved throughout evolution and serves as a popular target for detection (Fuller et al. 2003, Riahi et al. 2011). The 16S rRNA is a commonly used target because it is very abundant (20,000 copies per cell) and contains both conserved and divergent regions (DeLong et al. 1989, Fuchs et al. 1998). However, rRNA is rich in secondary structures, retarding probe hybridization. In addition, in some cases, it is difficult to design specific probes because of its high sequence conservation (Liu et al. 2001). In light of this, some other genes have been suggested as potential targets. For many assays, polymerase chain reaction (PCR) is the key step to allow for ultrahigh sensitivity. Although, in theory, even a single copy of DNA can be indefinitely amplified, it is very difficult for practical assays to achieve such high sensitivity. The US Centers for Disease Control and Prevention (CDC) defines a measurable sensitivity limit of ten to a few hundred colony-forming units (CFU) depending on the type of pathogen. Practically, this is approaching the limit of PCR without enrichment culturing. In addition, many food components can inhibit DNA polymerases (Abolmaaty et al. 2007). Currently, the ultrafast PCR method can detect as low as 5 CFU in 20 min (Belgrader et al. 1999). Although this result is very encouraging,

PCR-based methods require cell lysis to extract the target DNA and lengthy sample pretreatment steps to suit the PCR conditions, which make the assay significantly more complicated than desirable. Furthermore, PCR reaction usually requires a laboratory environment, whereas desirable pathogen assays need to be performed in the field.

Immunoassays represent another major analytical method for detecting bacterial cells (Yeh et al. 2002, Gracias and McKillip 2004, Haggerty et al. 2005). For example, enzyme-linked immunosorbent assay (ELISA) has been used in clinical applications for a long time. However, immunoassays are quite time consuming because multiple steps of incubation and washing are required. It is often necessary to perform sample enrichment because most immunoassays require more than 10,000 cells/ml for detection. Most of these so-called rapid detection methods are used for initial screening, and validation still requires the traditional assays. Other rapid tests measure cell viability, growth, cellular metabolites such as adenosine triphosphate (ATP) (Siro et al. 1982, Sugiyama and Lurie 1994) and bacterial enzymes. Most of these methods mentioned above lack the required sensitivity, specificity, or generality. For example, although ATP can be detected with a diverse range of methods such as luciferase assays, a cell often needs to be lysed first, which complicates the operation and significantly dilutes the ATP concentration. Therefore, assays capable of on-site and fast detection with minimal use of analytical instruments are still needed (Zourob et al. 2008).

Functional nucleic acids (FNAs) are nucleic acids with chemical functions such as catalysis and molecular recognition (Li and Lu 2009). FNAs have experienced a rapid development in the past 22 years, and they represent a promising platform to develop biosensors (Katz and Willner 2004, Navani and Li 2006, Cho et al. 2009, Liu et al. 2009, Wang et al. 2009, Li et al. 2010, Lubin and Plaxco 2010, Zhou and Dong 2011). Many assays for microbial pathogens have also been reported (Liu et al. 2009, Hamula et al. 2011b, Wang et al. 2012). In this article, we present an overview on the analytical applications of FNAs and, in particular, its recent developments for detecting molecules related to bacteria.

Functional nucleic acids

FNAs describe a family of DNA and RNA molecules whose function goes beyond the recognition of complementary nucleic acids (Famulok et al. 2007). There are three major classes of FNAs used in analytical chemistry. The first class possesses catalytic activity. RNA-based catalysts (ribozymes) were discovered in the early 1980s (Cech et al. 1981, Guerrier-Takada et al. 1983). Ribozymes catalyze a diverse range of reactions such as RNA splicing (e.g., group I intron) and hydrolysis (e.g., the hammerhead ribozyme) (Symons 1992, Wilson and Szostak 1999, Joyce 2004). Many ribozymes have also been artificially created using *in vitro* selection. Although not found in nature, catalytic DNA (also known as deoxyribozyme and DNAzymes) was first isolated using *in vitro* selection in 1994 (Breaker and Joyce 1994). For biosensor development, DNA is a better candidate because it is >1 million-fold more stable than RNA (Li and Breaker 1999). In addition, it is much more cost effective to produce DNA and perform site-specific labeling. Figure 1A shows the secondary structure of a DNAzyme/substrate complex forming a bulged three-way junction. This particular DNAzyme (17E, the lower strand) cleaves its substrate at the single RNA linkage (rA, adenosine ribonucleotide) indicated by the arrow in the presence of Pb^{2+}, whereas other metal ions are much less effective for assisting this cleavage reaction. Therefore, this DNAzyme has been widely used to design sensors for Pb^{2+} detection (Li and Lu 2000, Liu and Lu 2003, Xiao et al. 2007). The DNAzyme (39E) in Figure 1B has a very similar structure but is active only in the presence of UO_2^{2+}, uranyl (Liu et al. 2007, Brown et al. 2009). With >1 million-fold selectivity for uranyl over other metal ions, this DNAzyme has been shown to be highly effective for uranyl detection.

The second class of FNAs is called aptamers, which are the nucleic acid equivalent of antibodies (Ellington and Szostak 1990, Tuerk and Gold 1990, Klussmann 2006). Most aptamers do not possess catalytic function but are capable of highly specific molecular recognition. Natural aptamers (i.e., riboswitches) have been found in many untranslated regions of bacterial mRNA, where they have the role of regulating gene expression (Winkler et al. 2002, Breaker 2004, Winkler and Breaker 2005). Aptamers had been isolated by a technique called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) 12 years before the discovery of riboswitches in 2002 (Winkler et al. 2002). To date, hundreds of aptamers have been obtained to bind to essentially any molecule of choice including metal ions, small organic molecules, peptides, proteins, whole cells, and various inorganic materials (Jayasena 1999, Wilson and Szostak 1999). Figure 1C shows a DNA aptamer that can selectively bind to adenosine and ATP (Huizenga and Szostak 1995). Figure 1D is a Hg^{2+}-binding DNA that forms thymine-Hg^{2+}-thymine base pairs (Ono and Togashi 2004).

A combination of aptamer and DNAzyme produces aptazymes, the third class of FNAs (Robertson and

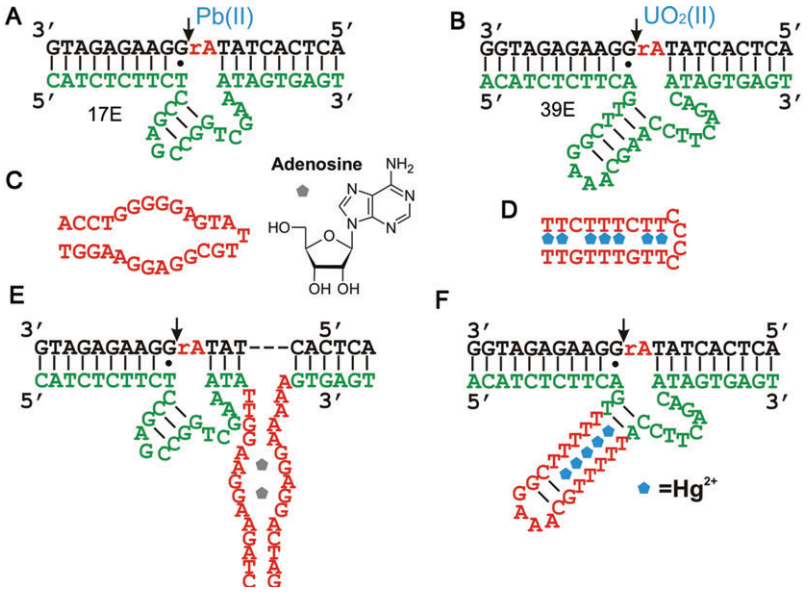

Figure 1 Examples of FNAs.
DNAzymes that need (A) Pb^{2+} or (B) UO_2^{2+} as cofactor to cleave RNA substrates. Aptamers that can bind to (C) adenosine or (D) Hg^{2+}. Aptazymes designed by the fusion of the (E) adenosine aptamer with the Pb^{2+}-specific DNAzyme or (F) Hg^{2+} binding DNA with the UO_2^{2+}-specific DNAzyme.

Ellington 1999, Burgstaller et al. 2002, Silverman 2003). Although DNAzymes are attractive for their catalytic turnovers, molecules other than metal ions cannot serve as effective cofactors. Fusion of an aptamer to DNAzymes can solve this problem. For example, by inserting the adenosine aptamer to the 17E DNAzyme (Figure 1E), the resulting aptazyme activity is controlled by adenosine binding (Wang et al. 2002). This system has been engineered into a colorimetric biosensor for adenosine detection (Liu and Lu 2004). In Figure 1F, the hairpin in the 39E enzyme was replaced by a thymine-rich DNA, and the active enzyme structure can only form in the presence of Hg^{2+}. A highly sensitive fluorescent sensor for Hg^{2+} was developed, and this sensor works only when both Hg^{2+} and uranyl are present (Liu and Lu 2007). Therefore, aptazymes combine the advantages of both DNAzyme and aptamers; they recognize a wide range of molecules and possess catalytic turnovers. Because most bacterial-related molecules are not metal ions, the direct use of DNAzyme for detecting bacteria has not been demonstrated. The other two classes, aptamers and aptazymes, have emerged to be promising molecules for rapid bacterial assays.

Advantages of FNAs

Because most reported FNA sensors for bacterial detection involve aptamers, a comparison between aptamers and antibodies for this analytical problem is given here. The biggest advantage is probably that aptamers are amenable to SELEX. Unlike the isolation of antibodies, aptamer selection can be carried out at designated conditions, and counter selections can be performed to remove cross activity, which is difficult to achieve in antibodies. For example, because SELEX does not involve live cells or animals, even toxic compounds and nonphysiological conditions can be used to obtain aptamers (Liu et al. 2003, Nelson et al. 2005). The binding affinity and specificity of aptamers can rival that of antibodies. SELEX can also be achieved in a relatively short period. The number of rounds in SELEX is determined by the selection efficiency. Traditional protocols that rely on target immobilization and heterogeneous separation limit the selection efficiency and the entire selection process may take a few weeks to months. With the new homogeneous techniques based on capillary electrophoresis, nonspecific interactions were minimized and aptamers can be obtained with just a few rounds of selection (Drabovich et al. 2005, 2006, Berezovski et al. 2006). This method allows a quicker response to an emergent new pathogenic species. Compared with antibodies, aptamers are usually smaller. Most aptamers are fewer than 60 nucleotides and can be immobilized with a much higher density. Because aptamers are chemically synthesized, there is very little batch-to-batch variation, and a wide range of chemical modifications can be incorporated for attachment chemistry and for fluorescence signal generation. For comparison, site-specific labeling

of antibodies is very difficult, and batch-to-batch variation can be quite large. In the past two decades, ample examples have shown the feasibility of using aptamers to achieve analytical applications (Cho et al. 2009, Liu et al. 2009), which can reach a similar performance and sometimes even outcompete their antibody counterparts. Finally, although aptamers can be denatured just like antibodies, aptamers are easily renatured while still retaining their function. DNAzymes and aptazymes share many of the same advantages, and for these reasons, FNAs have been a very popular choice for developing biosensors. The following contents of the review are focused on bacterial detection.

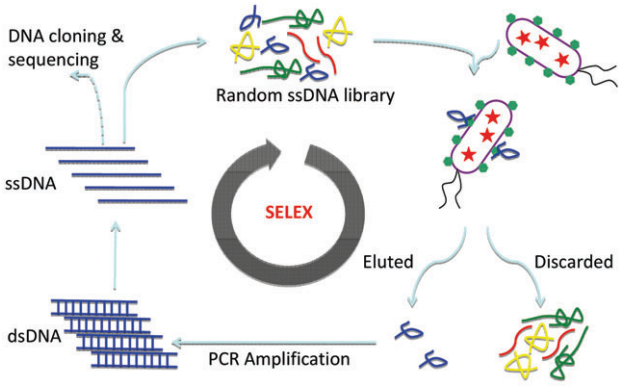

Figure 2 Schematic presentation of a typical aptamer selection process for whole bacterial cells.
The bacterial surface molecules are marked in green and the internal proteins or metabolites are represented by the red stars.

SELEX for bacterial targets

Whole-cell SELEX

To obtain aptamers specific for bacterial cells, a number of chemical species can be targeted. The most straightforward targets are the cell surface molecules, where even the whole cell can be used as the target for aptamer selection. Peptidoglycans are the major structural elements of bacterial cell walls, but to differentiate and identify specific cell types, molecular markers such as unique proteins need to be recognized. In addition, metabolites and proteins released by the cells have also been used to perform SELEX. In such a case, however, it can be difficult to identify the exact molecular target for the selected aptamers. Proteins and small molecules inside cells might also be used as targets for SELEX. In these cases, the FNA sensors need to cross the bacterial cell wall and cell membrane, which is a difficult task for DNA aptamers. Alternatively, cells have to be lysed, which is an undesirable operation and it dilutes the analyte concentration significantly.

The most direct way to target cell surface protein is to use whole cells as the target. The disadvantage here is that there is little control on the outcome of the selection and it is difficult to identify the molecular target of the aptamer. The selection is typically carried out by incubating bacterial cells with a DNA library with a size that can reach 10^{14}–10^{15} random DNA molecules (Figure 2). A small fraction of the DNA might fold into a conformation that selectively binds to bacterial surface molecules. The separation of binding and unbound DNA is usually achieved by a simple centrifugation step, and extensive washing can be carried out to remove nonspecifically bound DNA with a low binding affinity. The elution of aptamers is usually carried out under a denaturing condition, such as at high salt or high temperature. The selected binding DNAs are amplified by PCR, which generates double-stranded DNA. Because only one of the strands contains the aptamer sequence, the complementary strand needs to be removed, which is often achieved by introducing modifications to the PCR primers. For example, a biotin can be labeled on the primer for the complementary DNA, and the aptamer strand is then washed off after immobilizing the PCR product on a streptavidin column. Such selections are usually carried out for more than ten rounds until no more increase in binding activity is observed. At that point, the library is cloned into a vector and sequenced. To increase specificity and affinity, various modifications are often introduced. For example, counter selections with competing cells can be performed where sequences binding to competing cells are discarded. The incubation time can also be reduced to obtain sequences with higher binding affinity.

Cao et al. mixed a DNA library containing 45 random nucleotides with *Staphylococcus aureus*. The unbound DNAs were washed away while the cells associated with the binding DNAs were used as template for PCR amplification because they found that the cells alone did not produce specific amplicons (i.e., the aptamer elution step was eliminated). The library from round 3 was challenged with counter selection procedures against *Staphylococcus epidermidis* 26069, where only unbound strands were collected. The round 5 library was sequenced because it showed the highest binding activity. A total of five aptamers have been characterized, with dissociation constants (K_d) ranging from 61 to 210 nM. These aptamers were found to bind to different molecular targets on *Staph. aureus* by competition assays, leading to the possibility of using all

the five aptamers to increase the specificity against the target cell (Cao et al. 2009).

Group A streptococcus (GAS) is related to a diverse range of diseases such as streptococcal pharyngitis, scarlet fever, and endocarditis. Unlike other SELEX experiments performed on whole cells, where only single cell types were used, Hamula et al. (2011a) used a mixture of the ten most common GAS M-types in Canada because the goal was to obtain aptamers that can recognize all the major M-types. The separation of binding and unbound DNA was achieved by centrifugation, and 20 rounds of selection were performed. A number of different aptamer sequences have been obtained, and the best K_ds were lower than 10 nM. Binding was achieved for all the M-types in the selection experiment, and good selectivity was still shown against other bacteria. Because bacterial cells are much more robust and easier to culture compared to mammalian cells, a lot of SELEX work has been carried out by many different groups. Table 1 summarizes related work using whole cells as targets.

Purified molecules as target

The selection outcome is more easily controlled if purified protein targets are used. This strategy, however, may require more steps to express and purify protein. *Salmonella enterica* serovar *Typhi* causes typhoid or enteric fever; it is an important pathogen that can be transmitted by food and water. The type IVB pili have been shown to be important for its pathogenicity. Pan et al. (2005) expressed and purified pre-PilS (structural protein of type IVB pili) as the target protein for selecting an RNA aptamer. The library contained a 30-nucleotide random region. After eight rounds of selection, the library was cloned and sequenced. One of the aptamers showed a K_d value of ~8 nM and has later been incorporated into an electrochemical sensor.

The *Escherichia coli* K88 stain has been identified as an important source of infection in both humans and animals. For example, the secreted K88 fimbriae are responsible for most newborn infections and post-weaning diarrhea (Frydendahl 2002). Li et al. (2011) pointed out that the survival and virulence of *E. coli* K88 depends on the ability of the fimbria protein anchoring to the intestines. Therefore, this protein was chosen as a target for detecting *E. coli* K88 (Li et al. 2011). The fimbria protein was adsorbed onto an ELISA plate to allow the nonbound DNAs to be washed away, and bound strands were released by heating at 95°C. A total of 11 rounds of selections were performed, and four aptamers were studied in detail, with K_ds ranging from 25 to 44 nM. The selected aptamers showed a higher affinity for the targeting K88 strain than the competing ones including *E. coli* K99, *E. coli* TOP10, and *S. aureus*.

Bannantine et al. (2007) used a lateral-flow chromatography technique to isolate aptamers against *Mycobacterium avium* subspecies *paratuberculosis*, which is related

Table 1 Literature examples of using whole bacterial cells as the target for SELEX.

Target	K_d	Reference
Vibrio parahemolyticus	16.88±1.92 nM	Duan et al. (2012a)
Group A *Streptococcus* (GAS)	9±1 nM	Hamula et al. (2011a)
Lactobacillus acidophilus	13±3 nM	Hamula et al. (2008)
Mycobacterium tuberculosis	130 nM	Chen et al. (2007)
Staphylococcus aureus	Not reported	Cao et al. (2009)
	8 CFU	Duan et al. (2012b)
Staphylococcus aureus enterotoxin B	Not reported	DeGrasse (2012)
Campylobacter jejuni	292.8±53.1 nM	Dwivedi et al. (2010)
Bacillus anthracis spores	Not reported	Bruno and Carrillo (2012)
		Fan et al. (2008)
		Bruno and Kiel (1999)
		Zhen et al. (2002)
Bacillus thurigensis spores	Not reported	Fan et al. (2008)
		Ikanovic et al. (2007)
Pseudomonas aeruginosa	17.27±5 nM	Wang et al. (2011)
Legionella pneumophila	Not reported	Rai et al. (2012)
Live African trypanosomes	60±17 nM	Homann and Goringer (1999)
	Not reported	Homann et al. (2006)
Trypanosoma cruzi	172±54 nM	Ulrich et al. (2002)

to Johne's disease. The recombinant target proteins from the MAP0105c gene were cloned into *E. coli*, expressed, purified, and deposited on a nitrocellulose membrane. The DNA library was then allowed to flow through the membrane, where only binding ones were retained and amplified (Takemura et al. 2006). Specific binding has been demonstrated for several selected aptamers, although no quantitative K_d values were reported.

Campylobacter jejuni is one of the four major foodborne pathogens defined by the US CDC. Bruno et al. (2009) extracted the surface proteins from live *C. jejuni* using 1.5 M $MgCl_2$. The extracted proteins were immobilized on magnetic microparticles with a tosyl modification. As a result, the bound and nonbound DNAs can be separated using a magnet. After five rounds of selection, six dominant aptamer sequences were obtained.

Lipopolysaccharides (LPS) are also known as endotoxins, which are an integral component of the outer membrane of Gram-negative bacterial cells. LPS is highly immunogenic and thus is an undesirable contaminant frequently found in various recombinant proteins and plasmid DNA of bacterial origin. An LPS contains three structural elements: O-antigen, core polysaccharide, and lipid A. Although LPS is not a protein, it is a general bacterial marker and has very important analytical applications. Using capillary electrophoresis to separate binding and unbound DNA, Lee and coworkers isolated DNA aptamers against LPS with K_d=11.9 nM (Wang 2008).

In addition to cell surface molecules, many secreted species have also been targeted. The protective antigen (PA) of *Bacillus anthracis* is an 83-kDa protein that plays a crucial role for the virulence of this bacterium. PA is secreted by the bacteria and binds to its surface receptors on mammalian cells. PA is then cleaved by proteases into two fragments. The larger 63-kDa fragment remains bound to the cell surface, and the smaller 20-kDa fragment is released. The 63-kDa fragment facilitates the endocytosis of other bacterial toxins and eventually leads to inhibited host immune response, killing of macrophages, and induction of cytokine overproduction (Collier and Young 2003). Choi et al. (2011) used the 63-kDa fragment as the target for SELEX, where the bound and nonbound DNAs were separated using a membrane filtration method. After eight rounds of selection, four aptamer sequences were carefully characterized and the best K_d was determined to be 1.3 nM.

Crude extracellular mixture as target

Li and coworkers considered that each bacterial cell might release certain specific molecules into the surrounding media and used crude extracellular mixture (CEM, essentially just the culture media) as the target for SELEX (Ali et al. 2011). They aimed to obtain signaling DNAzymes using CEM as a cofactor. The selection was performed by mixing a DNA library containing an RNA cleavage site, a fluorophore, and a quencher. The fluorophore was positioned right next to the quencher, separated by just the cleavage site, allowing the maximal signal change upon cleavage. The assumption was that certain molecules in the CEM might bind to the DNAzyme and assist in the cleavage of the substrate, leading to fluorescence increase. The resulting enzyme was fully capable of detecting the specific target (*E. coli* K12), and other bacterial cells did not produce any signal. Detection can occur with as low as 1 CFU, but cell growth still takes several hours.

Intracellular proteins as target

For analytical applications, intracellular protein targets are more difficult to reach because sensor probes have to cross the cell membrane, which is a challenging task for highly negatively charged DNA. Aptamer selection against intracellular targets has been motivated by therapeutic applications. For example, β-lactamases are responsible for most of the antibiotic-resistant activity in bacterial cells. Kim and coworkers have selected DNA aptamers against metallo-β-lactamases, for which no clinically useful inhibitors are available (Kim et al. 2009, Schlesinger et al. 2011). Quite surprisingly, the functional aptamer was determined to be just a 10-mer DNA hairpin with three base pairs in the stem region and a 4-nucleotide loop. This aptamer was reported to be a potent inhibitor of the target enzyme and was effective even at very low aptamer concentrations.

FNA sensors for bacterial detection

After obtaining FNAs with high binding affinity and specificity, the next step is to design a signaling mechanism so that a sensor can be produced. Because various fluorophores and other chemical groups can be attached to DNA at designated positions, the design of FNA-based biosensors is extremely versatile. In some cases, a signaling mechanism is incorporated into the SELEX process, and a good example is the selection using CEM as the target (see the Crude Extracellular Mixture as Target section). For most other selections, however, fluorophores are attached after the optimization of the aptamer sequence. In this

section, several literature-reported signaling methods for bacterial detection are reviewed.

Fluorescent biosensor

Fluorescence is a highly sensitive and versatile technique and has been used extensively in biosensor development. In addition to fluorescence intensity, many other properties such as fluorescence lifetime, polarization, energy transfer, and emission wavelength can be measured. The above signaling DNAzyme for the CEM is a good example of fluorescent biosensor (Figure 3) (Ali et al. 2011). Because the fluorophore and quencher are positioned right next to each other, the sensor background is extremely low, leading to a large signal enhancement in the presence of the target. Compared with simple binding, the DNAzyme catalytic reaction is likely to be more specific and thus less prone to false signals. Dwarakanath et al. (2004) reported that the emission of CdSe/ZnS core/shell quantum dots shifted from 520–600 to 440–460 nm upon binding to bacterial cells. It was hypothesized that the wavelength shift was due to the change in the chemical environment of the quantum dots at the cell surface.

Immobilized aptamers and ELISA

For aptamers targeting cell surface proteins as well as secreted proteins, ELISA-type assays are a popular choice, because they all need extensive washing to reduce nonspecific binding. Choi et al. (2011) adsorbed anthrax PA onto a polystyrene plate and added a biotinylated aptamer (Figure 4). After washing, a horseradish peroxidase-conjugated streptavidin was added, converting 3,3′,5,5′-tetramethyl benzidine to a yellow-colored product after acid treatment. Because the enzyme label has catalytic turnovers, a very high sensitivity can be achieved.

Using aptamer-functionalized magnetic beads and quantum dots (QD), Bruno et al. (2009) intended to develop a sandwich type of assay where the *Campylobacter jejuni* bacterium is bound between the magnetic bead and quantum dots. Interestingly, the authors found that the sandwich components adhered to the plastic cuvette even in food matrices, making a rapid homogenous assay possible. With a handheld fluorometer, detection in ~15 min was achieved. The detection limit was reported to be 2.5 CFU in buffer and 10–250 CFU in various food matrices. This magnetic and QD method should have a higher specificity because both labels are required to generate the signal. Although no enzyme was involved in this assay for signal generation, target immobilization was still a key component of this assay.

Flow cytometry

Flow cytometry is a commonly used analytical technique for cell counting, detection, and separation. In this technique, cells are individually passed through a small

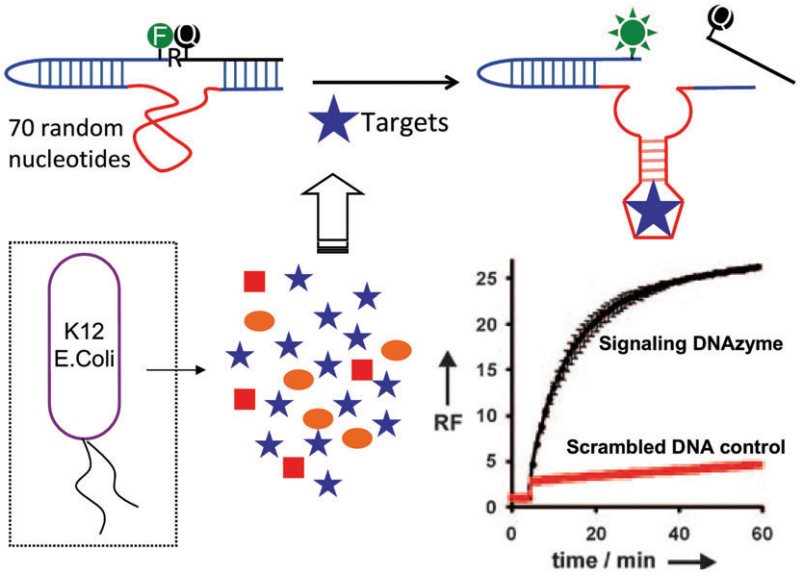

Figure 3 Schematics of signaling DNAzyme selection using extracellular mixture as the target.
The cell can release a large number of chemicals, and the selection was designed to allow chemicals specific to the cell to activate the DNAzyme. Reprinted with permission from Ali et al. (2011). Copyright 2011 John Wiley & Sons.

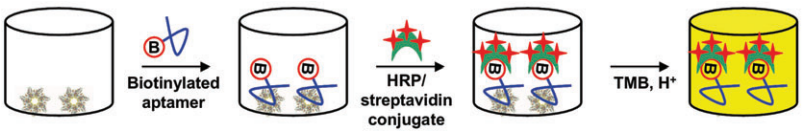

Figure 4 ELISA of anthrax PA using biotinylated aptamer and HRP conjugated with streptavidin.

capillary and are analyzed by spectroscopic techniques. Valuable information can be obtained based on the size and shape of cells using light scattering even without a fluorescent label. Specific fluorescent labeling allows a much higher sensitivity and specificity. To date, most fluorescent staining for flow cytometry has been made using labeled antibodies or small molecular probes. Fluorescently labeled aptamers can also be used. For example, most whole bacterial cell-based aptamer selections use flow cytometry to monitor the progress of aptamer selection (Chen et al. 2007, Hamula et al. 2008). Although most current flow cytometers are quite bulky, portable instruments are being developed for field applications.

Electrochemistry

Electrochemistry-based detection is advantageous in many aspects because it allows a fast response and can be readily miniaturized. However, a typical electrode-immobilized antibody-based binding to bacterial cells does not generate a detectable electrochemical signal. To overcome this problem, Zelada-Guillen and coworkers coated the electrode with carbon nanotubes (CNTs), which can serve as an efficient ion-to-electron transducer for potentiometric detection (Crespo et al. 2008). The authors used an RNA aptamer selected by Pan et al. (2005) that specifically binds to the type IVB pili of *Salmonella Typhi*. The aptamer was modified to contain a 3′ amine group to couple with carboxylated single-walled CNTs. The authors proposed that the aptamer/CNT conjugate was capable of both sensing and transducing the signal to electrode. One of the suggested mechanisms was the highly negatively charged aptamer binding and removal from the CNT surface, thus inducing the electric potential change. The sensor response time was <1 min, and even a single CFU can be detected. The selectivity was also tested against Gram-negative *E. coli* or Gram-positive *Lactobacillus casei*.

Lee and coworkers have fabricated single-walled CNT field effect transistors (FETs), where the conductance was modulated by the binding of *E. coli*. This was achieved by the immobilization of RNA aptamers selected against *E. coli* (So et al. 2008). This FET showed a >50% conductance drop upon the addition of *E. coli*, with no noticeable change produced even after exposure to high-density *Salmonella*, indicating excellent selectivity.

For aptamers immobilized on an electrode surface, the binding of the target can increase the resistance of electron transfer to the electrode, providing a useful means for biosensor design. Lee and coworkers immobilized a DNA aptamer for LPS on a gold electrode (Figure 5) (Su et al. 2012). LPS is a component of the outer membrane of Gram-negative bacteria. Because LPS is highly immunogenic, detecting trace amounts of LPS is crucial for the medical, pharmaceutical, and food industries. By measuring the impedance of this electrode using $Fe(CN)_6^{3-/4-}$ as probe, the quantification of LPS from 0.001 to 1 ng/ml was made possible and the sensor can be regenerated.

Summary and future directions

In this review, we have summarized the development of using FNAs to develop biosensors for bacterial cell detection. In principle, FNAs can be selected to target any bacteria as long as certain molecular signatures are available to allow specific aptamer binding. A diverse range of signaling methods has been used, such as fluorescence, color, and electrochemistry-based detection.

Beyond the initial proof of concept, many challenges remain to be addressed. The food and pharmaceutical industries as well as many other health-care-related materials require “zero tolerance” to viable pathogens (Batt 2007). However, many studies have shown that it is a big challenge to discriminate between viable and nonviable

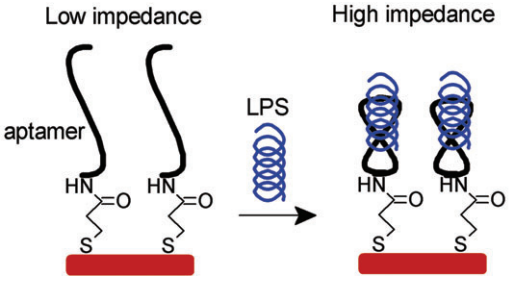

Figure 5 Electrochemical detection of LPS based on the impedance change upon target binding.

bacteria cells with current methods. It is particularly difficult to distinguish viable from viable but nonculturable bacteria. Taking advantage of the high DNA stability, some reports using nucleic acid as biomarkers with PCR amplification for detection showed promising results. These DNA assays are combined with other methods to obtain viability information. For example, viable cells might be floating and dead cells sink to the bottom of container. In addition, viable cells have a noncomprised membrane, and thus membrane permeability assays have also been used (Cenciarini-Borde et al. 2009). Compared with the nucleic acid sequence detection method, FNAs can be used to specifically target viable cells. Recently, Berezovski and coworkers obtained *Salmonella typhimurium* aptamers specifically for viable cells (K_d=25 nM) using Cell-SELEX and further developed an aptamer-based impedimetric sensor to achieve high sensitivity (600 CFU/ml) and selectivity toward live *Sal. typhimurium* bacteria (Labib et al. 2012). With more research efforts being devoted into the FNA field, better aptamers and DNAzymes will be isolated and characterized. Using whole cells as the target to perform SELEX appears to be the trend of research. Modified DNA bases can be used to enhance binding affinity. For example, the incorporation of boronic acid in DNA is likely to favor the binding of sugar moieties (Li et al. 2008), which are very abundant on bacterial cell surface. Another challenge is to obtain aptamers with appropriate selectivity. For example, in some applications, aptamers need to recognize a broad range of bacterial cells, whereas for other cases, a high specificity against a single cell species is critical. Fortunately, the outcome of aptamer selection can be directed by changing the selection conditions. Selected aptamers may in turn be used as a chemical biology tool for the study and manipulation of bacterial cells. The identification of surface markers, cell-specific binding, and inhibition of cell toxins are just a few examples of the possible applications.

Direct detection of ultralow copies of viable cells is still difficult. Single-cell sensitivity was possible using the DNAzyme to detect extracellular chemicals, but the cells need to grow overnight to produce such chemicals (Ali et al. 2011). Nanotechnology provides an excellent platform for increasing sensor sensitivity. For example, Tan and coworkers reported that using fluorescent-bioconjugated silica nanoparticles, single bacterial detection sensitivity was achieved, taking advantage of the fact that each silica nanoparticle can entrap a large number of organic dye molecules (Zhao et al. 2004). The electrochemistry-based detection methods reviewed above also have high sensitivity. Especially when conjugated with various nanomaterials, the surface area can be increased to further enhance sensitivity. Finally, many FNA-based assays are shown to be compatible with portable analytical devices such as handheld fluorometers, Raman spectrometers, portable flow cytometers, and PCR thermocyclers coupled with microfluidic devices, which will further push the field feasibility of using FNAs for bacterial detection (Shaikh et al. 2005, Bruno et al. 2012).

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