Nucleic acid aptamer-based methods for diagnosis of infections

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ABSTRACT

Infectious diseases are a serious global problem, which not only take an enormous human toll but also incur tremendous economic losses. In combating infectious diseases, rapid and accurate diagnostic tests are required for pathogen identification at the point of care (POC). In this review, investigations of diagnostic strategies for infectious diseases that are based on aptamers, especially nucleic acid aptamers, oligonucleotides that have high affinities and specificities toward their targets, are described. Owing to their unique features including low cost of production, easy chemical modification, high chemical stability, reproducibility, and low levels of immunogenicity and toxicity, aptamers have been widely utilized as bio-recognition elements (bio-receptors) for the development of infection diagnostic systems. We discuss nucleic acid aptamer-based methods that have been developed for diagnosis of infections using a format that organizes discussion according to the target pathogenic analytes including toxins or proteins, whole cells and nucleic acids. Also included is, a summary of recent advances made in the sensitive detection of pathogenic bacteria utilizing the isothermal nucleic acid amplification method. Lastly, a nucleic acid aptamer-based POC system is described and future directions of studies in this area are discussed.

1. Introduction

In today's highly mobile, interdependent and interconnected world, infectious diseases spread at a very fast pace and, consequently, they pose a serious global threat to public health (Morens and Fauci, 2013; Christian et al., 2013). The three major global threats of infectious diseases that exist today include emerging infections, antimicrobial resistance and healthcare-associated infections. Over the past decades, newly emerging pathogens, such as the severe acute respiratory syndrome (SARS) or middle east respiratory syndrome (MERS) coronavirus, avian influenza virus, henipavirus and ebola virus, have caused illness and death associated with significant economic burdens (Fauci et al., 2005; Morens et al., 2004; Fauci, 2001; Institute, 2003; Boucher et al., 2009). In addition, common microbes such as *Staphylococcus aureus* and *Mycobacterium tuberculosis* have continued to develop resistance to antibiotics (Fauci et al., 2005; Morens et al., 2004; Fauci, 2001; Institute, 2003; Boucher et al., 2009). As a result, more than two million people are now infected with antibiotic-resistant bacteria each year in the US alone, and at least 23,000 people ultimately die as a direct result of these infections (Solomon and Oliver, 2014; Fridkin et al., 2015; Frieri et al., 2017). Lastly, healthcare-associated infections (HAIs) also produce a significant socioeconomic burden ($150 billion per year in the US) arising from prolonged hospital stays, lasting disabilities and demand for new antibiotics (Magill et al., 2014). To combat the major issues surrounding infectious

diseases effectively, rapid and accurate diagnostic methods that can be utilized at the point of care (POC) need to be developed. The availability of these tests eliminates diagnostic delays and uncertainties, enabling timely initiation of proper antibiotic treatments and prevention of the spread of the diseases.

To date, bacterial culture and biochemical staining procedures are the clinical gold standard for bacterial pathogen identification even though these methods require long procedural times (up to several days) from specimen collection to final results and they have only limited use in identifying certain species. Thus, the landscape for infection diagnosis has begun to shift from traditional bacterial culture to POC test systems. For the development of POC diagnostic systems, biosensing technologies that consist of bio-receptors and transducers have been extensively investigated (Fig. 1) (Malik et al., 2013). Different recognition elements such as aptamers, antibodies and whole cells have been employed as bio-receptors for the identification of pathogens at the POC. Among these, considerable attention has been given to nucleic acid aptamers, oligonucleotides that have high affinities and specificities toward their targets and that are sometimes called "chemical antibodies" because of their similarities as the bio-receptors. These aptamers are assumed to form secondary/tertiary structures that provide binding pockets for targets (Patel et al., 1997). The high level of interest in these aptamers is also a result of their unique features compared to those of antibodies, which includes low cost of production that does not require living systems, easy chemical
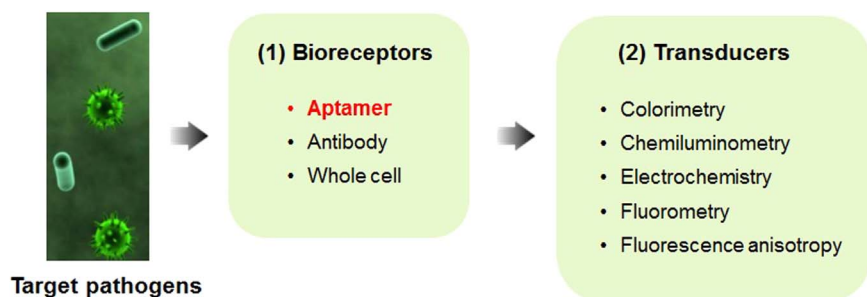
Fig. 1. A schematic diagram of infection diagnosis using a biosensor.

modification during the chemical DNA synthesis that leads to custom-tailored properties, low levels of immunogenicity and toxicity that do not interfere with the aptamer selection, and high chemical stability, binding affinity, reproducibility and reusability (Hong and Sooter, 2015).

Nucleic acid aptamers are screened using an in-vitro selection procedure called a Systematic Evolution of Ligands by EXponential enrichment (SELEX) discovered by Gold and Szostak in the 1990s (Ellington and Szostak, 1990; Tuerk and Gold, 1990). The SELEX process begins with combinatorial oligonucleotide libraries of up to 10^{13} to 10^{15} members, each consisting of an inner random region flanked by two constant regions for polymerase chain reaction (PCR) amplification (Zimbres et al., 2013; Hong and Sooter, 2015). The target molecule of interest is first incubated with the random oligonucleotide libraries (i: incubation) and the members of the libraries that bind to target molecules are separated from nonbinding aptamers (ii: separation). The retained libraries are then applied to PCR amplification (iii: amplification). In this manner, one round of SELEX, comprised of incubation, separation, and amplification is repeated until aptamers with the highest affinities are selected, which typically takes 8–15 rounds (positive selection). In addition, negative selection, in which the oligonucleotide libraries are incubated with negative targets and those that do not bind to negative targets are retained and subjected to further rounds of SELEX, is often executed to improve target specificity. Finally, the selected aptamers are cloned and sequenced to identify sequences and secondary/tertiary structures interacting with target molecules (see Supporting information for details).

A number of nucleic acid aptamers have been identified using this protocol. These include aptamers that are specific to pathogenic organisms such as gram-positive bacteria (*Staphylococcus aureus, Campylobacter jejuni, Listeria monocytogenes, Lactobacillus acidophilus, Streptococcus pyrogenes, Bifidobacterium bifidum*), gram-negative bacteria (*Escherichia coli, Vibrio parahemolyticus, Salmonella enterica*), *Mycobacterium tuberculosis*, viruses (Avian influenza virus, Human immunodeficiency virus, SARS coronavirus, Human papillomavirus, Hapatitis B virus, Hepatitis C virus, and Ebola virus), protozoa (*Plasmodium falciparum*), and fungi (Several species of *Penicillium, Aspergillus,* and *Fusarium*) (Table 1). In addition, aptamers that recognize enzymes such as thrombin and DNA polymerase have been utilized to detect pathogenic nucleic acids in a sequence-specific manner (Dang and Jayasena, 1996; Lin and Jayasena, 1997; Yakimovich et al., 2003; Friedrichs and Simmel, 2007; Kolpashchikov and Stojanovic, 2005; Ikebukuro et al., 2005; Bock et al., 1992; Macaya et al., 1993; Schultze et al., 1994).

In many types of biosensing platforms, nucleic acid aptamers have been employed as affinity receptors in combination with various signal transduction strategies including colorimetry, chemiluminometry, electrochemistry, fluorometry, and fluorescence anisotropy (Fig. 1) (Mok and Li, 2008; Li and Ho, 2008; Lautner et al., 2010; Qi et al., 2017; Beiranvand et al., 2017; Darbandi et al., 2017; Zhang et al., 2011). Most importantly, because aptamers change their structures upon binding to target analytes, they can be employed in homogenous assays that utilize a "mix and read" format that does not require

separation or washing steps (Deng et al., 2014; Sung et al., 2016; He et al., 2010). Furthermore, because nucleic acid aptamers are comprised of nucleic acids, they can be easily manipulated using isothermal nucleic acid amplification methods to enhance their sensitivities for detection of target pathogens (Magro et al., 2017; Maffert et al., 2017; Bi et al., 2017).

In this review, an overview of systems designed to diagnose infections that are based on nucleic acid aptamers is given using a format that organizes discussion according to their target analytes including pathogenic toxins or proteins, whole cells and nucleic acids. In addition, recent advances made in the development of techniques for sensitive identification of pathogens that rely on the isothermal nucleic acid amplification method are highlighted. Finally, an aptamer-based POC diagnostic system is discussed along with future directions of research in this area.

2. Nucleic acid aptamer-based infection diagnosis

In recent years, a large number of researches have utilized aptamers in the design of new biosensors (Hayat and Marty, 2014; Prieto-Simón and Samitier, 2014). The aptamer-based biosensors for infection diagnosis developed to date can be categorized into three classes according to their target pathogenic analytes, which include those that target pathogenic toxins or proteins, whole cells and nucleic acids. In addition, numerous isothermal nucleic acid amplification techniques have been utilized in aptamer-based infection diagnosis for the sensitive determination of pathogenic analytes.

2.1. Aptamer-based detection of pathogenic toxins or proteins

This section focuses on strategies employed to design systems for detecting pathogenic toxins or proteins, which take advantage of the unique structure switching property of nucleic acid aptamers upon interaction with these targets. The first example of an aptamer-based sensor of this type was created to detect ochratoxin A (OTA), a mycotoxin produced by several fungal species of the genera *Aspergillus* and *Penicillium*. Many food crops such as grapes and cereals have been infected by these fungi that produce carcinogenic and neurotoxic OTA, and this has posed serious problems for human and animal health (McKeague et al., 2014; Amaya-González et al., 2013). Thus, it is necessary to develop fast and reliable approaches for detection of OTA. Thus far, antibody-based immunoassays are the accepted standard for this purpose (Meulenberg, 2012), but generation of antibodies for OTA and other toxins is quite difficult because this substance is not tolerated by animals nor is it immunogenic. As a result, conjugation of toxins to carrier proteins is required to enable them to be immunogenic (Amaya-González et al., 2013).

In contrast, because aptamers that are produced using the chemical process termed SELEX have no limitation as to their target molecules, a great number of assays for OTA utilizing nucleic acid aptamers have been described (Rhouati et al., 2013; Park et al., 2014; Mun et al., 2014; Zhu et al., 2015). One example of this type of detection system utilizes a target-induced strand displacement protocol (Chen et al., 2012). In this

Table 1
Summary of nucleic acid aptamers for the detection of pathogens.

Target organism	Target	Type of aptamer	Length (nt)[a]	Affinity (K_d)	References
Staphylococcus aureus	Enterotoxin A protein	DNA aptamer	80	48.57 nM	(Huang et al., 2014)
	Enterotoxin B protein	DNA aptamer	78	N/A[b]	(DeGrasse, 2012)
	Whole bacteria	RNA aptamer	88	N/A[b]	(Han and Lee, 2014)
Campylobacter jenuni	Whole bacteria	DNA aptamer	80	292.8 nM	(Dwivedi et al., 2010)
Listeria monocytogenes	Internalin A protein	DNA aptamer	47	N/A[b]	(Ohk et al., 2010)
Lactobacillus acidophilus	Whole bacteria	DNA aptamer	78	13 nM	(Hamula et al., 2008)
Streptococcus pyrogenes	Whole bacteria	DNA aptamer	40	7 nM	(Hamula et al., 2016)
Bifidobacterium bifidum	Whole bacteria	DNA aptamer	80	11 nM	(Hu et al., 2017)
Escherichia coli	Release factor 1 protein	RNA aptamer	85	30 nM	(Sando et al., 2007)
	K88 fibrial protein	DNA aptamer	60	25 nM	(Li et al., 2011)
	Whole bacteria	RNA aptamer	64	110 nM	(Y.J. Lee et al., 2012)
Vibrio parahemolyticus	Whole bacteria	DNA aptamer	87	16.9 nM	(Duan et al., 2012b)
Salmonella enterica	Outer membrane protein C (ompC)	RNA aptamer	42	20 nM	(Han and Lee, 2013)
	Outer membrane protein (omp)	DNA aptamer	40	N/A[b]	(Joshi et al., 2009)
	Type IVB pili protein	RNA aptamer	71	7.2 nM	(Pan et al., 2005)
	Whole bacteria	RNA aptamer	76	123 nM	(Lavu et al., 2016)
	Whole bacteria	DNA aptamer	87	6.3 nM	(Duan et al., 2013)
Mycobacterium tuberculosis	MPT64 protein	DNA aptamer	34	N/A[b]	(Qin et al., 2009)
	Whole bacteria	DNA aptamer	97	31 nM	(Chen et al., 2007)
Avian influenza virus	Glycosylated hemagglutinin protein (Influenza A virus subtype H3N2)	RNA aptamer	94	N/A[b]	(Kwon et al., 2014)
	Hemagglutinin protein (Influenza A virus subtype H5N2)	RNA aptamer	90	N/A[b]	(Park et al., 2011)
	Hemagglutinin (Influenza A virus subtype H5N1)	DNA aptamer	115	4.65 nM	(Wang et al., 2013)
	PA endonuclease domain (Influenza A virus subtype H5N1)	DNA aptamer	64	137 nM	(Yuan et al., 2015)
	Hemagglutinin (Influenza A virus subtype H9N2)	DNA aptamer	68	N/A[b]	(Choi et al., 2011)
	Hemagglutinin (Influenza B virus)	RNA aptamer	114	720 pM	(Gopinath et al., 2006)
Human immunodeficiency virus	Reverse transcriptase	DNA aptamer	80	0.19 pM	(Mosing et al., 2005)
	SU glycoprotein	2′-F-RNA aptamer[c]	114	5 nM	(Khat et al., 2003)
SARS coronavirus	NTPase/Helicase (nsP10)	RNA aptamer	107	1.2 nM	(Jang et al., 2008)
Human papillomavirus	16E7 protein	RNA aptamer	75	1.9 μM	(Toscano-Garibay et al., 2011)
	Non-tumorigenic HF cell line	DNA aptamer	87	1.6 nM	(Graham and Zarbl, 2012)
Hepatitis B virus	Polymerase (P protein)	RNA aptamer	61	N/A[b]	(Feng et al., 2011)
Hepatitis C virus	Nonstructural protein 3	RNA aptamer	66	0.99 nM	(Urvil et al., 1997)
	Nonstructural protein 3	RNA aptamer	73	11.6 nM	(Fukuda et al., 2000)
	RNA polymerase	RNA aptamer	92	1.5 nM	(Biroccio et al., 2002)
Ebola virus	Inhibitory domain (eVP35)	RNA aptamer	70	3.7 nM	(Binning et al., 2013)
Plasmodium falciparum	Lactate dehydrogenase	DNA aptamer	36	38.7 nM	(S. Lee et al., 2012)
Several fungal species of the genera Penicillium and Aspergillus	Ochratoxin A	DNA aptamer	66	96 nM	(Barthelmebs et al., 2011)
Several fungal species of the genus Aspergillus	Aflatoxin B1	DNA aptamer	80	11.39 nM	(Ma et al., 2014)
Several fungal species of the genus Fusarium	Fumonisin B−1	DNA aptamer	96	100 nM	(McKeague et al., 2010)

[a] nt: nucleotides.
[b] N/A: not available.
[c] 2′-F-RNA aptamer: 2′-hydroxyl group in RNA aptamer is changed into 2′-fluorine to improve the stability.

method, a fluorescent label (FAM) linked to an aptamer fluoresces inefficiently because the aptamer is hybridized to a complementary blocker DNA containing a quencher (TAMRA). However, the aptamer changes its structure when it binds OTA, causing release of the blocker DNA and a consequent enhancement in the fluorescence intensity (Fig. 2A). In a similar way, novel nanomaterials, such as gold nanoparticles, single-walled carbon nanotubes and poly-vinyl pyrrolidone-protected graphene oxides, have been utilized as fluorescence quenchers in place of TAMRA in newly developed OTA sensing strategies (Duan et al., 2012a; Guo et al., 2011; Sheng et al., 2011). However, the assays described above require modification or immobilization of aptamers, which increase the overall cost and complexity of the assays.

To overcome this drawback, a new detection probe, consisting of a peroxidase mimicking DNAzyme, an OTA-specific aptamer, and a blocker DNA, was designed (Yang et al., 2012). In this method, the DNAzyme initially exists in a caged and inactivated state through its hybridization to the blocker DNA linked to the aptamer. In the presence of OTA, the blocker DNA dissociates from the DNAzyme as a result of

interaction of the aptamer with OTA. Consequently, the catalytically active free DNAzyme, produced in this way, promotes a process that leads to an optical response of a colorimetric substrate (Fig. 2B).

Label-free, fluorescent assays were also developed for the detection of pathogenic proteins. For example, Deng et al. designed a label-free, aptamer-based sensor for detection of H1N1 virus, which operates by targeting the hemagglutinin (HA) protein of avian influenza virus (Feng et al., 2015). This sensor consists of an aptamer for HA protein linked to an oligonucleotide sequence that forms a stem-loop structure. In the absence of HA protein, the hairpin-structured sequence is readily adsorbed on graphene oxide (GO), which prevents production of a new strand when treated with DNA polymerase and, as a result, a low fluorescence response occurs when the double-stranded DNA (dsDNA) specific fluorescent dye, SYBR green I, is added. However, when present, the HA protein binds to the aptamer causing the probe to disassociate from GO, an event that allows DNA polymerase to synthesize a new DNA strand to form a long dsDNA product. As a result, SYBR green I interacts with the dsDNA product to produce a high fluorescence response (Fig. 2C).
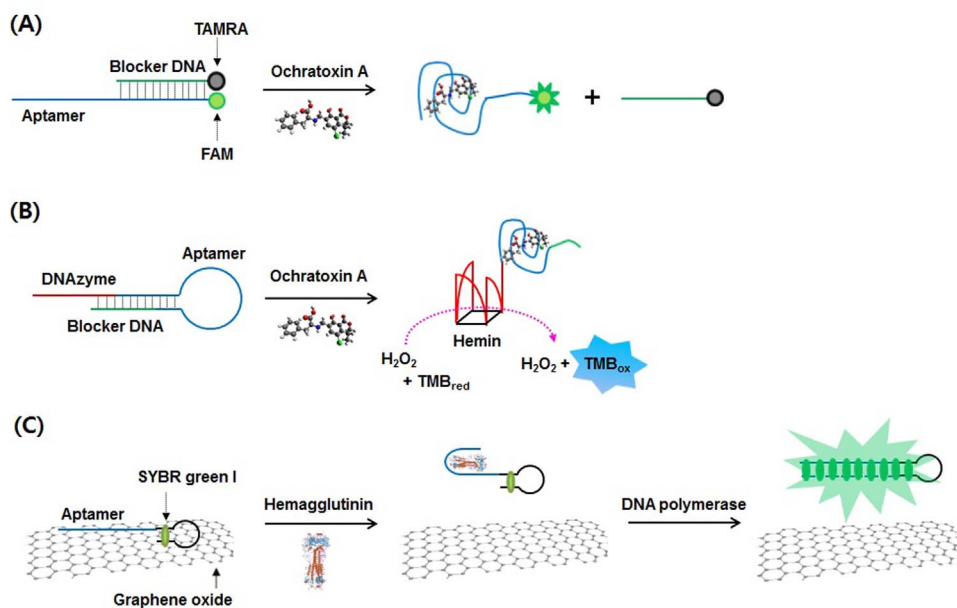
Fig. 2. Aptamer-based detection of pathogenic toxins or proteins. (A) An aptamer-based fluorescence assay for the detection of Ochratoxin A using an aptamer modified with a fluorescence label (FAM) and blocker DNA modified with a quencher (TAMRA) (Chen et al., 2012). (B) An aptamer-based label-free, colorimetric assay for the detection of Ochratoxin A using an aptamer linked to a peroxidase mimicking DNAzyme (Yang et al., 2012). (C) An aptamer-based label-free, fluorescence assay for the detection of hemagglutinin protein of H1N1 virus (Feng et al., 2015).

The last example is label-free cantilever sensors for the detection of *Staphylococcus* enterotoxin B (SEB) and fumonisin B-1 (FB1)(Zhao et al., 2014; Chen et al., 2015). In these systems, binding of either SEB or FB1 to the corresponding aptamers immobilized on the surface of sensing cantilevers, promotes differential surface stress, which consequently creates a measurable deflection signal by the surface stress-based microcantilever. These sensors are able to detect SEB and FB1 over a wide range and with high sensitivities and selectivities.

2.2. Aptamer-based detection of pathogenic whole cells

In addition to toxins and proteins, whole pathogenic cells can be targeted by aptamers produced using the Cell-SELEX method (Morris et al., 1998; Chen et al., 2016). A number of aptamers have been uncovered that target cells of different pathogens including group A *Streptococcus, Salmonella* Typhimurium, *Lactobacillus acidophilus, Mycobacterium tuberculosis, Staphylococcus aureus, Vibrio parahemolyticus* and *Campylobacter jejuni* (Duan et al., 2013). These aptamers, when combined with different transduction methods (Fig. 1), serve as bioreceptors for specific detection of target pathogens. The first example of a system of this type is a fluorescent detector for *Salmonella* Typhimurium (*S.* Typhimurium), which causes devastating symptoms such as diarrhea, cramps, vomiting, and fever (Fig. 3A) (Duan et al., 2013). In this protocol, *S.* Typhimurium specific DNA aptamers either modified with a fluorescent label (FAM) or linked to magnetic nanoparticles are first incubated with a solution of *S.* Typhimurium, and then washed by using magnetic separation to remove unbound aptamers. Finally, the FAM labeled aptamers bound to *S.* Typhimurim are subjected to fluorescence based quantitative analysis. A similar, but more advanced, chemiluminescence system has been developed for the detection of *Escherichia coli* (*E. coli*). In this sensor, FAM labeled *E. coli* aptamers bound to the bacterial cells are magnetically separated from unbound aptamers by using graphene oxide (GO)/iron (Fe_3O_4) nanocomposites, which complex with the unbound aptamers through π–π stacking interactions (Fig. 3B) (Khang et al., 2016). Finally, the aptamers bound to *E. coli* are assayed using chemiluminescence resonance energy transfer arising from interaction of guanine in the *E. coli* aptamer with 3,4,5-trimethoxylphenylglyoxal hydrate (TMPG) in the presence of tetra-*n*-propylammonium hydroxide (TPA) and N,N-dimethylformamide (DMF). Although being simple and robust, the two new methods still require both fluorescent labeling and tedious washing steps.

To overcome these limitations, Qin et al. developed a label and washing free electrochemical protocol for the detection of *Listeria monocytogenes* (*L. monocytogenes*), which relies on the use of protamine, a group of arginine-rich polycationic proteins bearing positive surface charges (Fig. 3C). In this method, binding of aptamers specific to *L. monocytogenes* target cells effectively prevents them from interacting with protamine (Ding et al., 2014). Consequently, the presence of free protamine is detected by a potential change of a polycation-sensitive membrane electrode.

In addition, aptamer-based colorimetric strategies for the detection of pathogenic cells have also been developed (Wu et al., 2012; Bayraç et al., 2017; Bruno, 2014; Yuan et al., 2014a, 2014b; Hu et al., 2017; Lavu et al., 2016). A representative example is the approach devised for detection of *E. coli*, which utilizes polydiacetylene (PDA) vesicles. PDAs, formed by UV-light promoted polymerization of diacetylene (DA) monomers, have an initial blue color and undergo a blue to red color transition in response to external stimuli such as temperature, pH, surface pressure and molecular recognition (Fig. 3D)(Wu et al., 2012). Specific binding of aptamer-conjugated PDAs to target cells induces a blue-to-red color change of the PDA that is readily visible to naked eyes. This strategy was used to develop a colorimetric sensor that targets *E. coli* cells. In another effort, a unique sensor that utilizes a lateral flow test strip was developed for a simple, rapid and colorimetric detection of a pathogen. This sensor relies on an aptamer-based sandwich assay in combination with gold nanoparticles and quantum dots as the signal reporters. With the developed system, the target bacteria including *E. coli, L. monocytogenes* and *S. enterica* are successfully detected with high sensitivities and selectivities (Bruno, 2014).

2.3. Aptamer-based detection of pathogenic nucleic acids

Many attempts have been also made to detect nucleic acids of target pathogenic bacteria utilizing aptamers. An example of this type of detection system uses aptamers, linked to a target recognition sequence, that are covalently conjugated to their corresponding enzymes (Pavlov et al., 2005). In this system, thrombin, an important protease in the coagulation cascade, is initially inactivated by conjugated thrombin aptamers linked to the target recognition sequence. The presence of the target nucleic acids causes detachment of the aptamers from enzyme and, consequently, transformation of the inactivated enzyme to the active form, which promotes multiple catalytic reactions that result in the generation of highly amplified signals (Fig. 4A). Although this strategy, which incorporates enzyme-based signal amplification,
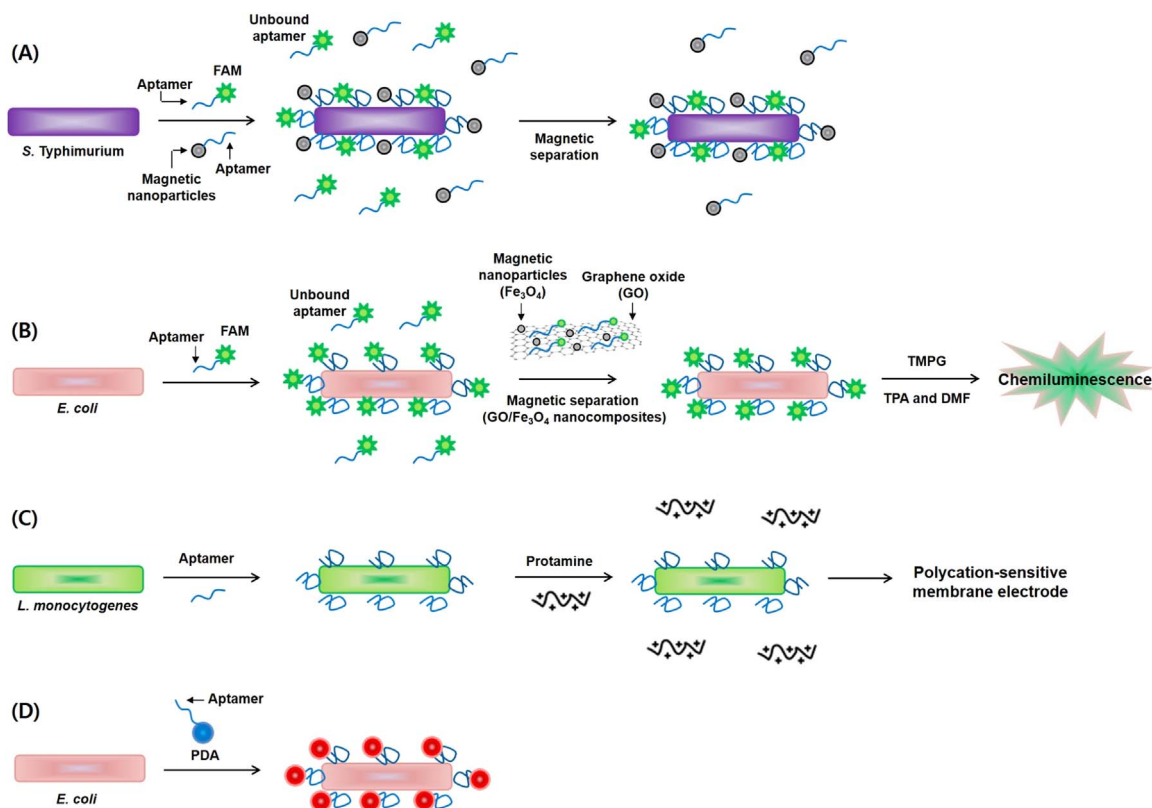
Fig. 3. Aptamer-based detection of pathogenic whole cells. (A) An aptamer-based fluorescence assay for the detection of *S*. Typhimurium using aptamers either modified with a fluorescent label (FAM) or linked to magnetic nanoparticles (Duan et al., 2013). (B) An aptamer-based chemiluminescence assay for the detection of *E. coli* using FAM-labeled aptamers and separation of unbound aptamers by graphene oxide (GO)/Fe₃O₄ nanocomposites (Khang et al., 2016). (C) An aptamer-based label-free, washing-free, electrochemical approach for the detection of *L. monocytogenes* using the electrostatic interaction of aptamers with protamine (Ding et al., 2014). (D) An aptamer-based colorimetric strategy for the detection of *E. coli* using polydiacetylene (PDA) vesicles modified with aptamers (Wu et al., 2012).

enables highly sensitive target DNA detection, it requires complicated and tedious modification processes, which could negatively impact the catalytic activity of enzymes (Park et al., 2015b). In addition, the method is difficult to apply universally to detect different target nucleic acids because the enzymes need to be modified with different DNA probes associated with each target.

To combat these disadvantages, Ikebukuro et al. designed an aptamer-based nucleic acid detection system that does not involve expensive modifications (Yoshida et al., 2006b, 2006a, 2013; Ikebukuro et al., 2008). In this method, thrombin aptamers are rationally engineered to contain a target recognition sequence. Thus, complementary target nucleic acids, through its hybridization to a target recognition sequence, regulate the aptamer's inhibition of the catalytic coagulation activity of thrombin (Fig. 4B). As a result, the target DNA can be sensitively detected without the requirement of expensive modifications.

Another interesting approach utilizes a DNA aptamer that possesses an inhibitory activity against DNA polymerase (Dang and Jayasena, 1996; Lin and Jayasena, 1997; Yakimovich et al., 2003; Friedrichs and Simmel, 2007; Kolpashchikov and Stojanovic, 2005). In this system, the DNA polymerase aptamer is rationally engineered to contain a target recognition sequence and, thus, DNA polymerase can be activated or inactivated by its hybridization to complementary target DNA (Fig. 4C) (Park et al., 2015b). The regulated DNA polymerase activity is simply measured by using a separated signal transduction module based on a primer extension reaction combined with TaqMan probes. Based on this working principle, Park et al. developed both signal-off and signal-on sensors for target nucleic acids that have high sensitivities and selectivities.

In addition, the practical applicability of this approach was recently demonstrated by its use in a POC diagnostic system, termed polarization anisotropy diagnostics (PAD), for rapid diagnosis of enteric fever and healthcare-associated infections (Fig. 4D) (Park et al., 2015a, 2016a). In this study, the entire assay protocol from bacterial RNA extraction to signal detection and portable devices that streamline the POC diagnosis were established. Most importantly, the developed PAD system has several advantageous features. First, it uses a dual-probe scheme wherein target DNA is recognized by a detection probe but the signal is generated by a separate, common reporter system. Thus, the probe can be applied to multiple targets with the minimum incremental cost. Second, because all reactions take place in a single tube without requiring any washing steps, assay procedure is highly streamlined. Third, the system that measures fluorescence anisotropy is inherently ratiometric, automatically canceling fluctuations in light intensity and common environmental noise (LiCata and Wowor, 2008). Owing to these novel features, the aptamer-based PAD platform is expected to be a powerful tool for rapid, culture-independent detection of infection and antimicrobial resistance.

2.4. Aptamer-based pathogen detection based on the isothermal nucleic acid amplification

Another strategy that has been used to design sensitive pathogen detection methods takes advantage of isothermal nucleic acid amplification. It is known that the amount of a target pathogenic marker required to cause a life-threatening illness is quite low. For example, in acutely infected patients, the bacteria level for enteric fever, a febrile illness caused by *Salmonella enterica* serovar Typhi and Paratyphi, is only 0.1–10 colony-forming units (CFU) per 1 mL of blood (Park et al., 2016b). Thus, devising new ultra-sensitive detection methods is highly important. Owing to the fact that aptamers are comprised of nucleic acids, they can be amplified to make aptamer-based pathogen detection
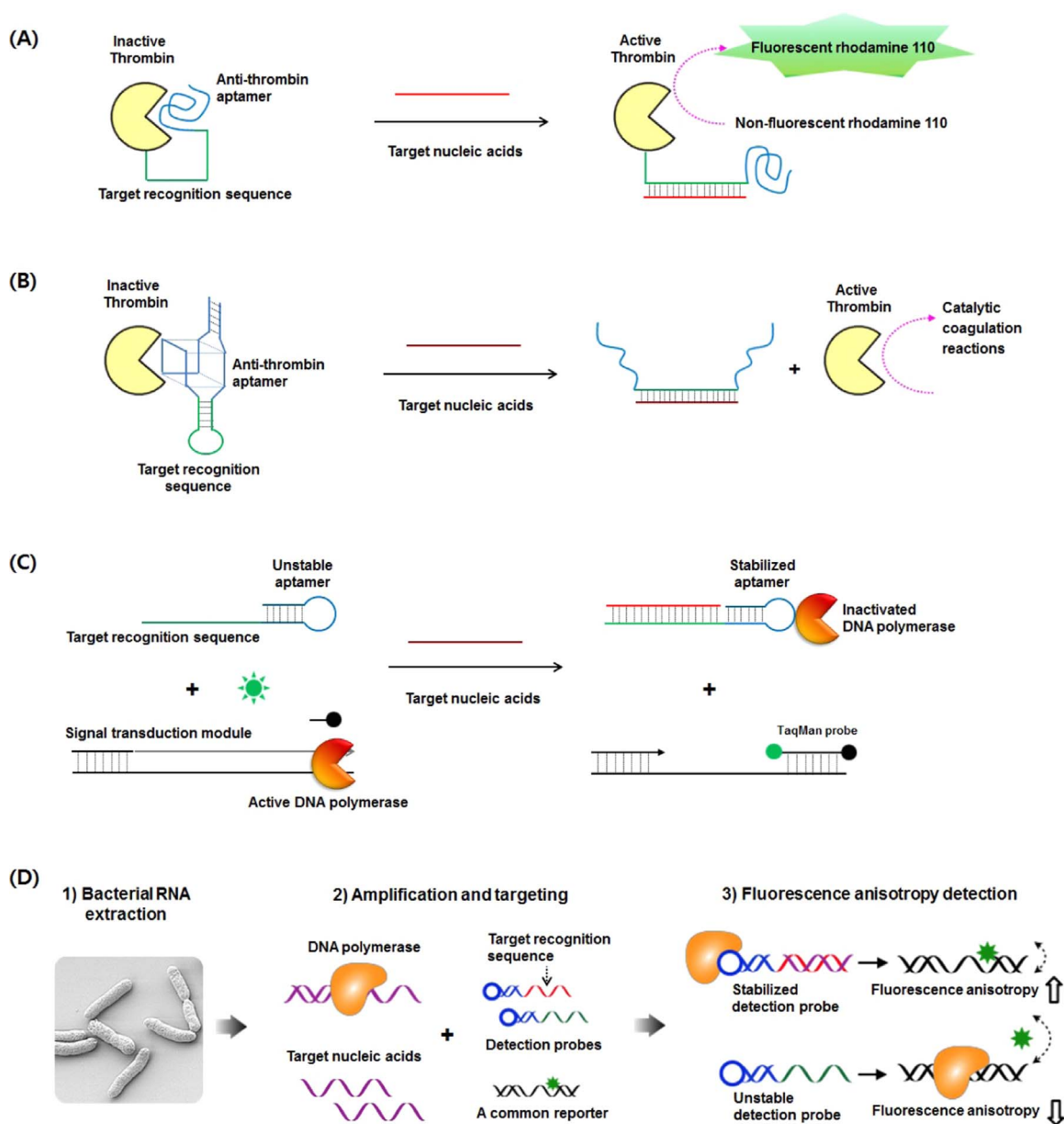
Fig. 4. Aptamer-based detection of pathogenic nucleic acids. (A) An aptamer-based detection of target nucleic acids using thrombin modified with thrombin aptamer linked to target recognition sequence (Pavlov et al., 2005). (B) An aptamer-based label-free assay for the detection of target nucleic acids using thrombin aptamer engineered to contain a target recognition sequence (Yoshida et al., 2006b; Ikebukuro et al., 2008). (C) An aptamer-based label-free assay for the detection of target nucleic acids using DNA polymerase aptamer engineered to contain a target recognition sequence (Park et al., 2015b). (D) An assay procedure for POC diagnostic system termed PAD using target-regulated DNA polymerase activity (Park et al., 2015a, 2016a).

systems more sensitive, a distinct advantage when compared to antibody-based detection approaches. In this regard, isothermal nucleic acid amplification techniques are quite promising because they do not require thermal cycling and, as a result, the assay protocol can be easily adapted to POC diagnostic systems. A variety of isothermal nucleic acid amplification techniques have been described and employed in aptamer-based assays. Readers are referred to recent reviews for a more comprehensive overview of isothermal nucleic acid amplification techniques (Bi et al., 2017; Deng et al., 2017; Giuffrida and Spoto, 2017; Zhao et al., 2015).

The first example of this type of detection system utilizes target recycling by exonuclease cleavage for ultrasensitive detection of ochratoxin A (OTA) (Fig. 5A). In this method, blocker DNA modified with an electroactive label (ferrocene) is immobilized on a gold electrode and hybridized with the aptamer. This causes ferrocene to be located far from the electrode. In its presence, OTA forms a complex

with the aptamer, which results in liberation of aptamer from the gold surface. This event produces the free blocker DNA, which forms a hairpin structure through its hybridization at each terminus that places ferrocene close to the electrode surface (Tong et al., 2011). More importantly, OTA is released from aptamer-OTA complex by the action of single-stranded specific exonuclease that digests the aptamer. As a result, an enhanced electrochemical signal is generated by recycling OTA.

A second example of this approach comes from studies by Tong et al. (2012) which focuses on the use of another isothermal nucleic acid amplification method, called rolling circle amplification (RCA), to amplify the output signal. In this method (Fig. 5B), the OTA aptamer preferentially hybridizes with a padlock DNA probe, which interferes with the circularization of padlock DNA. The presence of OTA results in formation of an OTA-aptamer complex, which causes release of the padlock DNA from the aptamer. As a result, padlock DNA probe becomes circularized by DNA ligase and is then effectively amplified by
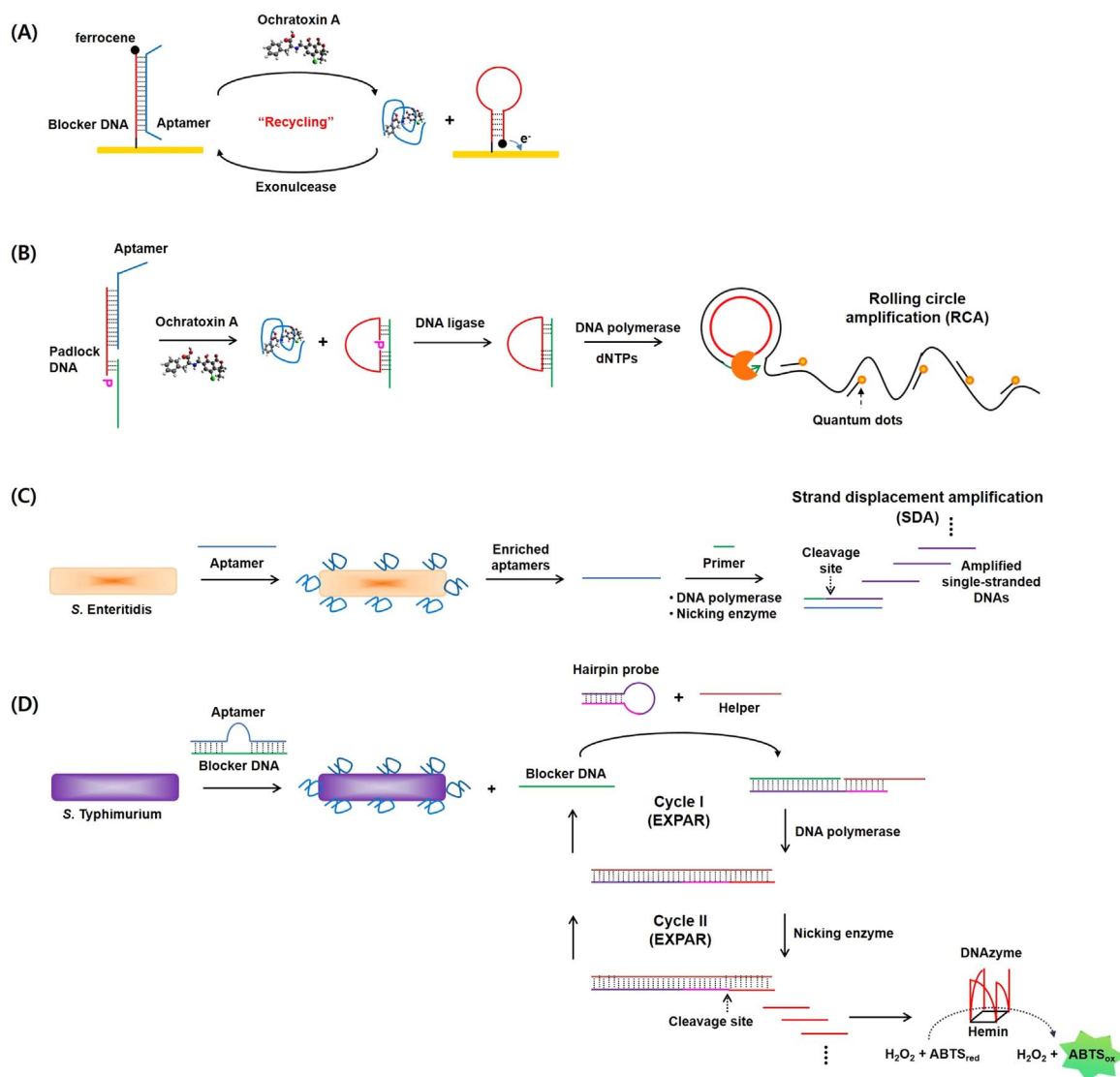
Fig. 5. Aptamer-based pathogen detection based on the isothermal nucleic acid amplification. (A) An aptamer-based electrochemical assay for the sensitive detection of Ochratoxin A using the target recycling by exonuclease cleavage (Tong et al., 2011). (B) An aptamer-based electrochemical assay for the sensitive detection of Ochratoxin A using target-promoted rolling circle amplification (RCA). P in the padlock DNA probe indicates the phosphate group at its 5′ terminus (Tong et al., 2012). (C) An aptamer-based sensitive detection of *S.* Enteritidis using the enriched aptamers as the substrate for strand displacement amplification (SDA) (Fang et al., 2014). (D) An aptamer-based colorimetric assay for the sensitive detection of *S.* Typhimurium using target-triggered exponential amplification reaction (EXPAR) (Qiu et al., 2016).

RCA to produce long single-stranded concatemeric products, which are detected by using fluorescence from quantum dots tagged on the RCA product.

In a similar way, isothermal nucleic acid amplification methods have been rationally integrated into aptamer-based sensing assays for the highly sensitive detection of pathogenic whole cells. One example of this application is found in studies by Fang et al., in which isothermal strand displacement amplification (SDA) was employed in a sensor for the detection of *Salmonella* Enteritidis (*S.* Enteritidis) (Fang et al., 2014). In this method (Fig. 5C), aptamers anchored on the outer membrane of *S.* Enteritidis serve as the template for subsequent SDA. Thus, aptamers enriched by the presence of target cells generate a large number of amplified ssDNA products through SDA that is mediated by the action of DNA polymerase and a nicking endonuclease. The final products are detected in a lateral flow biosensor system in which the capture DNA probes specific to the amplified ssDNA products are immobilized. Using this novel strategy, as low as 10 CFU of *S.* Enteritidis pathogens can be detected. The same group also demonstrated the universal applicability of this sensing strategy by employing it to detect *E. coli* (Wu et al., 2015).

In addition, an isothermal nucleic acid amplification method, which does not involve enzymes (e.g., DNA polymerase or nicking endonuclease), has been also utilized for the sensitive detection of pathogenic markers (Xie et al., 2016). One example is found in the detection of OTA that relies on the target-triggered hybridization chain reaction (HCR) (Wang et al., 2015). In this method, two types of metastable hairpin probes, one containing OTA-specific aptamer (hairpin 1) and the other containing a peroxidase mimicking DNAzyme in an inactivated state (hairpin 2), stably coexist, but the presence of OTA that interacts with OTA aptamer opens hairpin 1 and the exposed stem of hairpin 1 subsequently invades hairpin 2. In the same manner, hairpin 2 opens hairpin 1 and consequently multiple hairpins are assembled to generate a number of active DNAzymes, which effectively catalyze the color generation reaction, leading to an optical response of a colorimetric substrate.

The last example demonstrates how isothermal nucleic acid amplification serves as the foundation of a simple, sensitive and washing-free detection system for pathogens. The approach (Fig. 5D) utilizes the target-triggered exponential amplification reaction (EXPAR), an isothermal nucleic acid amplification process that takes place with a high

efficiency (Qiu et al., 2016). In this method, an aptamer specific to *S.* Typhimurium is partially caged by a blocker DNA, which is designed to open hairpin probe for the subsequent EXPAR (Cycle I). Thus, the presence of target bacteria liberates the blocker DNA, which unfolds the hairpin probe that enables hybridization of the helper. As a result, EXPAR aided by DNA polymerase and a nicking endonuclease occurs to recycle the blocker DNA (Cycle 1) and produce numerous DNAzymes (Cycle 2), that promote an intense colorimetric response through oxidation of 2,2′-azino-bis-(3-ethylbenzothiazoline)−6-sulfonic acid (ABTS) by the peroxidase mimicking activity of DNAzymes. Employing this novel method, target bacteria can be sensitively detected at levels as low as 80 CFU/mL without the requirement for tedious washing steps and expensive DNA modifications.

3. Summary and conclusions

A high demand exists for diagnostic systems for infectious diseases, which give clinicians the ability to determine the nature of the disease and most effective treatments rapidly. Testing systems of this type eliminate diagnostic uncertainties and, thus, reduce the empirical use of broad-spectrum antimicrobials and facilitate enrollment of new antibiotic treatments. The key requirement of an ideal diagnostic system is the presence of a bio-receptor that specifically identifies the target pathogen. Among many bio-receptors devised for this purpose, nucleic acid aptamers have received the greatest attention because they have unique features such as high stability, low production cost and inherent amplification abilities. Accordingly, various types of nucleic acid aptamer-based sensing strategies have been incorporated with different signal transducers and signal amplifying tools (e.g., isothermal nucleic acid amplification methods) to design systems for the detection of pathogenic toxins, proteins, cells, and nucleic acids.

4. Future perspectives

Despite being founded on well-established detection principles, the number of bio-sensors utilizing aptamers are limited because of the small number of aptamers that have been discovered thus far. It is believed that the scarcity of aptamers is a consequence of two factors. First, it has been reported that natural nucleic acids do not possess the full spectrum of chemical functional groups and conformational space needed to yield high-quality aptamers for many proteomic targets. Thus, it is expected that natural DNA aptamers for less than 30% of the human proteome have been identified (Wang et al., 2014). In this light, chemical methods for DNA synthesis have enabled the incorporation of special functional groups onto oligonucleotides and have led to a significant advancement in the number of diverse aptamers that are available (Kimoto et al., 2013). An application of this promising approach was recently reported by Gawande et al. (2017), in a study which not only demonstrated that chemical modification of oligonucleotides significantly increases their affinity, stability and inhibitory potency of aptamers, but also identified a new type of aptamer, called a slow off-rate modified aptamer (SOMAmer). Thus far, SOMAmers against 1300 different protein analytes have been discovered and made commercially available through SomaLogic (De Groote et al., 2017). Second, inefficiencies associated with the SELEX process contribute to the small number of aptamers that are currently available. To solve this problem, a number of methods (e.g., Hi-Fi SELEX (Ouellet et al., 2015), array-based strategies (Cho et al., 2015) and multiparameter particle display strategies (Wang et al., 2017)) have been developed, which have led to greater selection efficiencies (Zhang et al., 2016).

With these promising results as a foundation, more in-depth research is required in the future to expand aptamer target libraries that cover greater numbers of pathogens. Especially, aptamers that recognize antibiotic-resistance markers and antibiotics are in high demand because of the emerging antimicrobial resistance that increases morbidity, mortality and healthcare costs (Solomon and Oliver, 2014;

Fridkin et al., 2015; Frieri et al., 2017; H.M. Kwon et al., 2014; Y.S. Kwon et al., 2014b; Chen et al., 2017; Yan et al., 2016; Lan et al., 2017). In addition, the host response to the infection together with the pathogenic analytes such as toxins or proteins, whole cells and nucleic acids described in this review creates new attractive targets for the rapid diagnosis of infection especially in the areas where a low pathogenic burden causes a serious infection (Islam et al., 2016). The representative biomarkers in this type of assay are antibody secreting cells that are induced during infection (Jin et al., 2011, 2009; Saletti et al., 2013). In order to analyze infection-specific antibody secreting cells, a high demand exists for bioreceptors with high affinities and selectivities. Aptamers, which have many unique features as compared to those of antibodies, are expected to play a key role in the development of efficient analysis protocols for antibody secreting cells and, consequently, in devising rapid systems for diagnosis of infection.

Finally, robust and affordable POC platforms, which can be deployed in the developing countries where management of infectious disease is not effective, are also required. This goal will be achieved by collaborative efforts involving workers in diverse fields including nanotechnology, microfluidics, molecular biology and medicine (Nayak et al., 2016; Hsieh et al., 2014). Some of the promising results have already arisen from studies of systems for the detection of healthcare-associated infections and malaria infections (Park et al., 2016a; Dirkzwager et al., 2016). In the near future, these efforts should lead to development of the aptamer-based sensing platforms that will enable rapid and cost-effective detection of infectious diseases and, consequently, that will substantially reduce the human and economic burdens of diseases.

Acknowledgements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.11.028.

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