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Mini Review

Nucleic Acid Aptamers as an Emerging Diagnostic Tool for Animal Pathogens

Tarun Kumar Sharma[1*], Ravi Shukla[2*]

[1]Center of Biodesign and DiagnosticsTranslational Health Science and Technology Institute, Gurgaon, Haryana, India–122016; [2]Centre for Advanced Materials and Industrial ChemistrySchool of Applied SciencesHealth Innovation Research Institute RMIT University GPO Box 2476, Melbourne 3001 VIC Australia
*Corresponding author: ravi.shukla@rmit.edu.au, tarun@thsti.res.in

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ABSTRACT

Various microbial and viral pathogens continuously posing threat to mankind and animals especially in agriculture economy based nations. In order to combat these challenges we need accurate diagnostic methods followed by proper treatment. Unfortunately contemporary detection methods are either time consuming or need sophisticated instruments so cannot be used for real field applications. Aptamer technology emerged as potential rival of antibody with several advantages over the later. Various aptamer have been generated against myriad of microbial and viral pathogens with the aim of detection and mitigation. In this review we tried to summarize the diagnostic application of aptamer technology for animal pathogens.

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INTRODUCTION

Once Watson and Crick demonstrated the structure of DNA in 1953, it was thought that this molecule of life carry genetic information from one generation to next. But beside this, nucleic acids (DNA and RNA) have been found to perform some other interesting functions. DNA and RNA are often considered as huge molecular hard disk for information storage, but they might also have great potential to be used as diagnostic tool and therapeutic agents (Daniela et.al., 2005; Tuerk et.al., 1990; Wilson et.al., 1999; Regina et.al., 2007). Due to the extensive structural and conformational topology single–stranded (ss) polynucleotides (ssDNA and RNA) can fold into diverse conformations and directly interact with cellular proteins and other ligands. Such studies have opened a new research field leading to the selection and design of functional nucleic acid molecules that are known as "aptamers". The name was derived from the Latin word "aptus", meaning "to fit", and these aptamers are agents to fit basically any given structure (Wilson et.al., 1999; Regina et.al., 2007). In 1990, Szostak and Gold independently pioneered an *in vitro* evolutionary process termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment) to identify nucleic acids (aptamers) specific to organic dyes and T4 DNA polymerase (Daniela et.al., 2005). Since then, a variety of aptamers have been generated against myriad of targets. Moreover several attempts has been made to improve and to develop variant of this process to generate aptamers for specific purpose (Daniela et.al., 2005; Wilson et.al., 1999;. Beauty of this process lies in the fact that aptamers can be generated against any target ranging from simple small molecule to complex protein to whole cell or organism (Cancer cells, Bacteria, Protozoan etc.). conceptually aptamers are quite different from antisense nucleic acid molecules, which are considered linear molecules and block transcription according to the Watson–Crick base–pairing model. Aptamers are molecules with complex 3–dimensional (3–D) structures which function through affinity–binding to target molecules, in a fashion similar to antibody . Aptamers can fold into 3–D structures as a result of intramolecular interaction and bind to targets through a small number of contact points which determine high specificity. They are more stable than antibodies and can undergo denaturation and renaturation (Daniela et.al., 2005; Tuerk et.al., 1990; Wilson et.al., 1999; Regina et.al., 2007). Aptamers (8–15 kDa) are smaller than antibodies (155 kDa) and therefore have higher permeability and can penetrate targets more easily. Reporter molecules, such as fluorophores, biotin, or nanoparticles, can be easily attached to aptamers by chemical synthesis. Aptamers are derived from an *in vitro* evolutionary process, so toxins or molecules that do not elicit immune responses can also be used as target to develop aptamers. The *in vitro* process can also be performed in non–physiological selection conditions. Moreover, SELEX confers aptamers with high specificity and is able to discriminate targets of slight structural difference, such as isoforms of protein enzymes. Aptamer have typical dissociation constants ranging from the low picomolar to low nanomolar or sub micromolar (Wilson et.al., 1999; Regina et.al., 2007). A head to head comparison of aptamer and antibody is given in table 1. SELEX is an effective technology for the *in vitro* selection of aptamers that have high specificity to and affinity for a particular target. Many aptamers not only evinced high specificity and affinity, but also interfere with biological functions of target molecules, thus presenting themselves as prospective therapeutic candidates. Prof. Ellington has created a comprehensive aptamer database which is an online resource of all selected aptamer sequences that may have diagnostic or therapeutic utility. This database is updated on monthly basis and is publicly available free of cost at http://aptamer.icmb.utexas.edu/ (Jennifer et.al., 2004).

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Detection of microbial pathogens is really critical as accurate diagnosis is key to right treatment and cure. Detection, identification and quantification of microbial pathogens are crucial for public health protection. Areas where detection of microbial pathogens is critical include clinical diagnosis, water and environmental analysis, food safety, and biodefense. Microbial culture–based tests and molecular assays (immunological or nucleic acid technologies) are the most common methodologies currently used (Lazcka et.al., 2007; Edith et.al., 2009). These techniques are either time consuming or require sophisticated equipment and highly trained personnel, hence increasing the analysis cost. A robust and rapid detection technique should provide dependable, real time, on–site, easy to use, and inexpensive detection with improved or equivalent sensitivity, specificity and reproducibility of culture–based tests (Edith et.al., 2009). According to Lazcka et al., biosensor technology is the fastest growing area in rapid diagnosis of microbial pathogens (Lazcka et.al., 2007). The commonly used biological recognition elements in biosensor platforms are antibodies and nucleic acid probes.

Recently, aptamers based detection emerged as platform technologies for variety of diagnostic application and they are being used alone or in combinations of quantum dots, modified and unmodified gold nanoparticles, aptamers based dot blot assays and ELISA as an electrical biosensor and also in combination with quartz crystal microbalance and surface enhance raman scattering (Edith et.al., 2009). In this review, an attempt has been made to summarize aptamer based detection methods for microbial pathogens of animal importance.

Comparison of Aptamers with Antibodies

Aptamers are the rivals of Antibodies as they are stable, easy to develop and functionalized. More detail about aptamer advantages over antibody is given in Table 1.

Table 1: Comparison of Aptamers with Antibodies

S.N.	APTAMERS	ANTIBODIES
1	Binding affinity (kD) usually ranges from nanomolar to picomolar	Binding affinity (kD) in low nanomolar to picomolar range
2	Aptamer selection is a chemical process carried out in vitro and can therefore target any protein	Antibody generation requires a biological system, therefore difficult to raise antibodies to toxins (not tolerated by animal) or non–immunogenic targets
3	Can select for ligands under a variety of conditions for in vitro diagnostics	Limited to physiologic conditions for optimizing antibodies for diagnostics
4	Aptamer selection is a iterative process and usually requires 5–10 rounds and even more in some cases (varied from target to target)	Screening of monoclonal antibodies is a time consuming and expensive
5	Uniform activity regardless of batch synthesis	Activity of antibodies vary from batch to batch
6	Pharmacokinetic parameters can be changed on demand	Difficult to modify Pharmacokinetic parameters
7	Investigator determines target site of protein	Immune system determines target site of protein
8	Wide variety of chemical modifications to molecule for diverse functions (Flour and biotin labeling etc.)	Limited modifications of molecule
9	Return to original conformation after temperature insult	Temperature sensitive and undergo irreversible denaturation
10	Unlimited shelf–life	Limited shelf–life
11	No evidence of immunogenicity	Significant immunogenicity
12	Cross–reactive compounds can be isolated toggle SELEX strategy	No method for isolating cross–reactive Compound
13	Aptamer–specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse molecules

SELEX; an Evolution in the Test Tube

SELEX is an evolutionary technique to identify a molecular key of interest (aptamer), from a huge bunch of keys (random sequence nucleic acid library), that has high specificity and affinity for its molecular lock (targets of interest). In the past decade, variety of aptamers has been generated against myriad of targets ranging from recombinant protein to small molecule to bacteria and viruses. In addition numerous modifications and variations have been made to enhance the versatility, specificity and affinity of this technique. In general, the SELEX process includes the following steps: the first step is to design a random, single–stranded DNA library. This library is chemically synthesized with a centralized random sequence (35–50 nucleotides long) flanked by fixed sequences at either end which served as primer binding domain during PCR (Daniela et.al., 2005; Tuerk et.al., 1990; Wilson et.al., 1999; Regina et.al., 2007). The second step is to incubate the random library with the target. Usually, small molecules and proteins are immobilized on solid supports to generate affinity matrices while in case of microbial pathogens either pathogen specific protein or whole cell of a particular pathogen is used as a target for aptamer selection. The third step is to separate bound oligonucleotides from unbound this is usually achieved by number of stringent washes. The fourth step is to separate bound nucleic acid from the target followed by enrichment of binders using polymerase chain reaction (PCR). Since PCR product is double ssDNA, therefore, in order to begin next round of aptamer selection one need to generate ssDNA this is normally achieved through asymmetric PCR, denaturing PAGE or using biotin–streptavidin interactions. The fifth and final step is to elute the ssDNA and being next round of selection. The use of RNA aptamer requires have to go two additional steps; one is *in vitro* RNA transcription and other is reverse transcription polymerase chain reaction. In order to enrich the binder's population one has to go through these steps again and again. After reiterating these steps over several cycles (Figure 1), nucleic acids pool with high specificity to and affinity is cloned in appropriate cloning vector and subjected to sequencing.

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Then, binding affinity and specificity of nucleic acids with different motifs to target molecules can be tested and compared, and the aptamers with the highest affinity and specificity or other desired features is selected.

Modification of SELEX

Traditional SELEX protocol comprises iterative rounds of selection which is very time consuming and tedious job. In order to overcome this problem recently Andreas *et al.* introduced a single step MonoLEX process (Figure 2) of Aptamer selection (Nitsche et.al., 2007). This approach combined a single affinity chromatography step with subsequent physical segmentation of the affinity resin and one single final exponential amplification step of bound aptamers (Nitsche et.al., 2007). Therefore, this process improves the selection of high affinity aptamers by reducing the competition between the aptamers of different affinities during the PCR step.

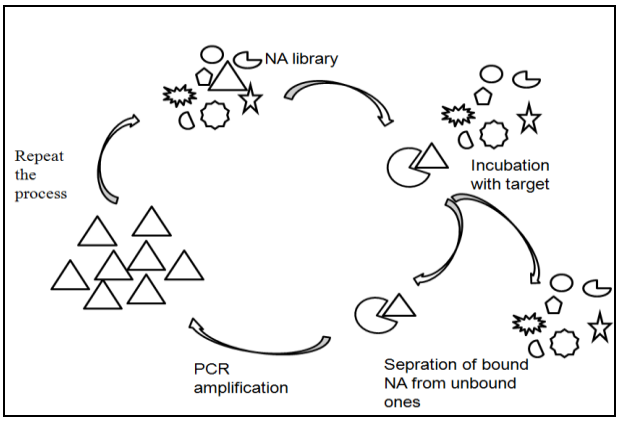

Figure 1: Schematic representation of SELEX (Systemic Evolution of Ligends by Exponential Enrichment); (NA: Nucleic Acid)

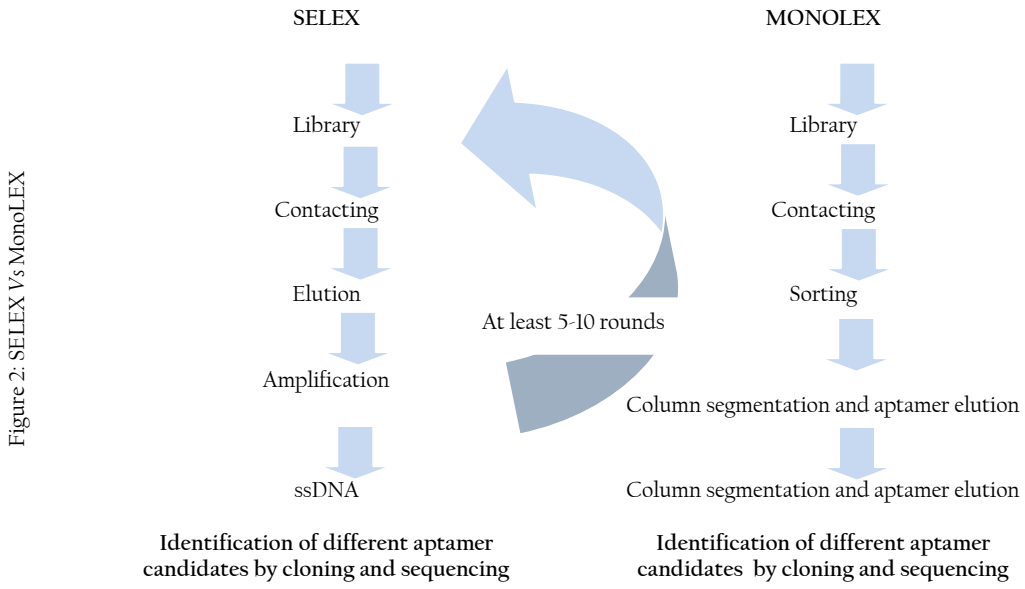

Figure 2: SELEX *Vs* MonoLEX

Aptamers against Bacterial Pathogens

Pathogenic diseases are among the most overwhelming burden across the world. They cause the suffering of hundreds of millions of people as well as an unknown figure of wild and domestic animals. Further the drugs present in the armory are very old and ineffective as pathogens have developed resistance against some of them. On the other hand process of developing new and improved anti–pathogenic drugs has been very slow. This is due to many factors, among them a significant lack of interest from the pharmaceutical industry as well as insufficient funding for drug discovery. Lastly, the drug discovery process involves screening of drug molecules from the huge library of synthetic compounds which is a slow and tedious process. Several attempts were made to generate aptamers against various bacterial pathogens which adversely affects animals and mankind.

DNA Aptamers Based Detection and Mitigation of Salmonellae

Salmonellae are one of the major causes of food borne sickness across the borders. It is responsible of about 1.4 million cases of salmonellosis including approximately 16,000 hospitalizations and 550 deaths only in United States (Joshi et.al., 2009). Salmonellae are also among bacterial pathogens in food animal species including cows (Wells et.al., 2001), pigs (Malorny et.al., 2005), chickens (Carli et.al., 2001), and turkeys (Nayak et.al., 2003). Amongst non–human cases, *S. typhimurium* is reported most frequently (Galanis et.al, 2006). Both classic zoonotic and food borne transmission of Salmonella spp. have been well documented for these animal species [Dejong et.al., 2005;

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Hendriksen et.al., 2004; Hsueh et.al. 2004; Schroeder et.al., 2005 Van et.al. 2004) . The organisms are shed in animal faeces periodically and at undetermined levels which makes its prevention and control a wild goose chase. Further detection of Salmonella and other bacterial pathogens in complex sample matrices such as feces, foods (Stevens et.al., 2004; Deisingh et.al., 2004), and environmental samples is really a challenging task because it needs sufficient enrichment in order to increase target copy number for downstream detection step. In addition, investigations at serovar level introduces complexity in diagnostic methodology. Though PCR can shorten the complexity but some time it fails or give false positive results when performed with complex biological samples (faces, food matrix etc.). In order to address such issues (Joshi et al., 2009) have demonstrated an aptamer based method for *S.typhimurium* diagnostics.

In this study, DNA aptamers were selected using subtractive SELEX strategy. *S. typhimurium* outer membrane proteins (OMPs) were used as target for aptamer selection while *Escherichia coli* OMPs and lipopolysaccharides (LPS) were used as for counter–selection. A total of 66 aptamer candidates were selected against *S. typhimurium.* Specificity of the selected aptamers was assessed by electromobility gel–shift analysis against *S. typhimurium* OMPs. On the basis of gel–shift assay five salmonella–specific aptamer candidates were selected for further characterization. Further, for capture assay two aptamer candidates (aptamers 33 and 45) were used to determined low–end detection limits which were 10–40 CFU. In order to determine the aptamer part which is specifically involved in binding to OMP of *S. typhimurium*, DNase protection assays were performed. Further, to identify the putative protein target of chosen aptamer candidates South–Western blot analysis coupled to mass spectrometry was performed. The results of mass spectrometry revealed ompA, ompD and ABC transporter as molecular targets of selected aptamer candidates. In addition Aptamer 33 was bound to magnetic beads and used for the capture of *S. typhimurium* seeded into whole carcass chicken rinse samples, followed by detection using quantitative real–time RT–PCR. In a pull–down assay format, detection limits were 10^1–10^2 CFU *S. typhimurium*/9 mL rinsate. Furthermore, the pull–down analysis using aptamer candidate 33 was corroborated on three naturally infected chicken litter samples proved their diagnostic utility for field application. This study exhibits the applicability of *Salmonella* specific aptamers for pre–analytical sample processing as applied to food and environmental sample matrices.

Recently (Olga et. al., 2013) generated ssDNA aptamers against *S. enteritidis* and *S. typhimurium* using cell–SELEX method. They have also demonstrated inhibitory potential of aptamers for *Salmonella enteritidis* and *S. typhimurium*. Interestingly, this study revealed when a bunch of aptamers were used together they evinced strong bacteriostatic action which could be attributed to the decrease in their membrane potential.

DNA Aptamers Based Detection of Francisella Tularensis

Vivekananda et al. generated ssDNA aptamers that bind to *Francisella tularensis* subspecies (subsp) *japonica* bacterial antigen (Vivekananda et.al., 2003). *F. tularensis* is an intracellular, nonmotile, nonsporulating, Gram–negative bacterial pathogen that causes tularemia in man and animals. Their group successfully isolated a set of 25 unique aptamer candidates that can specifically bind to *F. tularensis* subspecies japonica. When these aptamers were tested in a sandwich Aptamer–Linked Immobilized Sorbent Assay (ALISA) and dot blot format, the aptamer cocktail exhibited unique specificity in its ability to bind only to tularemia bacterial antigen from subspecies japonica, holarctica (also known as palaearctica) and tularensis but not to *Bartonella henselae*. Moreover, no cross reactivity was observed either to pure chicken albumin or chicken lysozyme. Thus, it appears that this novel antitularemia aptamer cocktail could be used as prospective diagnostic tool for a potential biological warfare agent like *F. tularensis.*

Aptamer against Virulent Mycobacterium Tuberculosis:

Tuberculosis (TB) remains a serious threat to mankind in developing nations. It is responsible for more than five million deaths per year worldwide. One–third of the world's population is infected with *Mycobacterium tuberculosis* (MTB), the etiologic agent of TB. Global health problems attributed to TB need serious attention and in order to meet such challenges new and potent anti–TB drugs without cross–resistance with known antimycobacterial agents is urgently needed. (Chen et. al., 2007) generated first aptamers (ssDNA aptamer) NK2 using cell–SELEX against live MTB yielded aptamers that specifically recognize H37Rv over BCG. In addition, it modestly enhanced the survival rate of mice infected with the virulent strain H37Rv. Aptamer candidate (NK2) not only preferentially binds H37Rv but also increases the levels of interferon–γ in CD4+ T cells, and reduces bacterial counts in the spleens of infected mice (Chen et. al., 2007). Although neither the bacterial surface target nor the mechanism of protection has been identified, the authors proposed that NK2 most likely binds to and interferes with a self–protective H37Rv membrane protein, thus leading to more effective cell–mediated immunity. This strategy suggests aptamer NK2 can be used as diagnostic and therapeutic agent for MTB.

Chen et. al., 2009 have reported inhibitory action of aptamers NK2 for H37Rv by demonstrating that aptamer NK2 and 10^{th} pool potentially inhibits H37Rv invasion to macrophages. Further their results showed H37Rv treated with NK2 or 10^{th} pool significantly stimulated the secretion of interferon from macrophages as compared to control group.

Aptamer Based Detection of Foot and Mouth Disease Virus

Foot and Mouth Disease Virus (FMDV) is one of the serious threats to agriculture based economy of both developed and developing nations. It has caused several devastating outbreaks in the United States, United Kingdom and Asia including India. Due to such economic burden there is an urgent need to develop a rapid and reliable detection system for FMDV. Although several immunoassay and reverse transcription polymerase chain reaction (RT–PCR) and other nucleic acid amplification based methods are in place with wide range of FMD serotype specificity but unfortunately none of these methods is available in portable format or point of care test format.

Bruno et. al., 2008 developed a DNA aptamers against a 14 amino acid peptide from VP1 structural protein of FMDV this protein is highly conserved among 16 strains of 'O' serotype of FMDV. In this study they have utilized the high affinity and specificity of polyclonal aptamers to develop a novel FRET based assay that can confirm the presence of FMD within few minutes (Bruno et. al., 2008). This method could be adopted in portable format due to availability of hand held spectroflourimeter. They labelled VP1 structural peptide with Black Hole Quencher–2 (BHQ–2) dye and polyclonal aptamer population were labelled with Alexa Flour 546–14–dUTP by PCR and allowed to bind the BHQ–2 peptide conjugate. Following FRET aptamer–peptide complex, a light off or turn off kind response was observed within 10 minutes and this assay was able to detect as low as 25ng/ml VP1 peptide. Surprisingly, when individual aptamer candidate were used in similar kind of assay, they resulted in poor sensitivity and specificity which suggests that combinatorial action of aptamers leads to enhance sensitivity of assay. Further, unlike signalling aptamers (Jhaveri et.al., 2000) or aptamer beacons,

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(Hamaguchi et.al., 2001) these competitive FRET–aptamers attempt to place a known quencher target conjugate within the Förster distance of fluorophores in the aptamer–binding pocket and then compete it off with unlabeled analyte. In future, this approach could be used to develop point of care diagnostic assay for FMDV.

Selection of Aptamers against Live Trypanosomes

Among protozoan Trypnosomes are one of the serious threat to humans and cattle. It is the causative agent of sleeping sickness among cattle and humans. This disease is life threatening if remained undiagnosed and untreated. Moreover the available line of drugs causes serious adverse effects (side effects). In addition to that, development of drug resistance among trypanosomes is also a serious concern which has created a grave situation. Further, trypanosomes escape the immune response of invaded hosts through antigenic variation. This process is achieved through temporal expression of immunologically unrelated variants of surface glycoproteins (Blum et.al., 1993; Homann et.al., 1999). Fortunately, trypanosomes surface also contain some invariant proteins. In order to target such invariant surface protein Homann and Goringer designed SELEX experiment to generate RNA aptamers against invariant protein of live T. brucei with the aim to redirect the immune response to the surface of parasite using aptamers (Homann et.al., 1999). After 12 iterative cycles of selection and enrichment of binders, they were able to identify some pseudoknott aptamers that bind to blood stream stage trypanosomes and were not able to differentiate between two immunologically distinguishable T. brucei cell lines (MITat1.2 and MITat 1.4). Further they have also identified aptamer interaction partner be zero distance photo crosslinking and it was identified as 42kDa polypeptide. As expected this polypeptide was present on the surface of blood stream stage MITat1.2 and 1.4 but could not be identified on insect stage trypanosomes. Further experiments revealed that this 42kDa protein is a component of flageller pocket of the parasite. The same research group performed another study to determine fate of selected RNA aptamer (Homann et.al., 2001). They demonstrated when this aptamer binds to its target at flageller pocket it gets internalized and subsequently transported to lysosomes. This uptake pathway was visualized by flourphore labeled aptamer candidate. They further showed this intracellular internalization was sequence dependent as no such internalization was observed when truncated or random sequence was used. In addition to that, they have also reported that on internalization aptamer gets partially degraded to attain a stable 50 nucleotide structure. This work was sort of path breaking study which showed aptamer potential in mapping of surface associated protein to identify novel cell surface markers. Further, this work also suggests the application of aptamers as delivery vehicle to deliver aptamer–coupled compounds to lysosomal compartment.

CONCLUSION

Field of diagnostics always demand simple, rapid and reliable diagnostic methods that could also be adapted in point of care format for real field applications. Contemporary method of microbial pathogens relies on culture based, immunological and PCR based methods. All of these methods are tedious, time taking and require sophisticated instruments and skilled manpower. The field of aptamer technology opened a new vista in the field of diagnostics and therapeutics. Due to several advantages over antibody including negligible batch to batch variation these chemical rivals can easily be used to develop large scale point of care diagnostic assay. Further, ease in conjugation with gold nanoparticles (GNPs), quantum dots ribozymes and florophores makes them an attractive choice of diagnostic platform development. When these aptamer are conjugated with various signaling agents, they give visual readouts visible to naked eye. The enormous potential of aptamer remains to be exploited in the area of diagnostics.

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CONFLICT OF INTEREST

None

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