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Dr. Jaisree S
Assistant Professor, Central University Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Prakash Krishna B
UG Scholar, Final Year, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

Poojidha M
UG Scholar, Final Year, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

Corresponding Author:
Dr. Jaisree S
Assistant Professor, Central University Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Aptamers in diagnostic applications

Dr. Jaisree S, Prakash Krishna B and Poojidha M

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Abstract
Aptamers are single-stranded oligonucleotides capable of folding into specific structures and binding to targets such as proteins, exhibit greater affinity and selectivity due to their inherent shape-forming characteristics. In this review, we elucidate recent advancements in aptamer-based technologies, delve into the fundamental properties of aptamers, explore chemical modifications, examine various *in-vitro* selection processes, and assess different analytical techniques.

Keywords: Aptamers, chemical modification, SELEX, aptasensor, *in-vitro* selection, diagnostics

Introduction
Aptamers are single-stranded DNA or short RNA molecules typically comprising 10-80 nucleotides and weighing between 6-30 kDa (Sun *et al*., 2015) [1] (Hermann *et al*., 2000) [2]. The term “Aptamer” was coined by Andy Ellington and Jack Szostak in the year 1990 and it was derived from the Greek words "aptus," meaning 'to fit,' and "merus," meaning 'part' (Gold *et al*., 2021) [3]. Aptamers binds with their targets like antibodies and are also called as “chemical antibodies” (Baird *et al*., 2010) [4]. Aptamers are generated through a process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment), where a large random pool of oligonucleotides is subjected to iterative rounds of selection and amplification to isolate sequences that bind to the target molecule of interest. Through this process, aptamers with high affinity and specificity for their targets can be obtained. Aptamers have a wide range of potential applications in diagnostics, therapeutics, biosensors, and targeted drug delivery ‘/
Aptamers are easy to produce and scale up. The production time is relatively less compared to antibodies. These aptamers are chemically synthesized and has greater variability and less variation between batches. Aptamers can be designed to target the bacteria and can be used in the treatment of multi-drug resistant bacteria / emerging viruses. Aptamers can maintain their structure and function over a wide range of temperatures, making them thermally stable and eliminating the need for refrigeration during storage and transportation. It can be reused in various or multiple applications since they can be denatured by applying heat and renatured by cooling (Bruno *et al*., 2015) [6]. Aptamers offer several advantages over antibodies. They are easy to produce and scale up, with shorter production times. Chemically synthesized, they exhibit greater consistency and less batch-to-batch variation. Unlike antibodies, aptamers do not provoke an immune response, have a broader target range, and do not require animals for production. They possess superior renal clearance and penetrability, and their production can be upscaled rapidly (Baird *et al*., 2010) [4] (Darmostuk *et al*., 2015) [5] (Bruno *et al*., 2015) [6] (Doggrell *et al*., 2018) [7] (Röthlisberger *et al*., 2017) [8]. The detailed comparison between antibodies and aptamers is provided in Table.1.

Table 1: Comparison between aptamers and antibodies

	Aptamers	Antibodies
Target range	Wide and almost anything. We can develop aptamers even for non- immunogenic targets non- immunogenic targets and toxin	Targets must induce an immune response
Use of Animals	No	Yes
Reproducibility	Low batch to batch variation since it is chemically synthesized	Poor reproducibility with batch-to-batch variation
Cost effectiveness	Cost effective (One cent/ assay)	costlier
Storage	Withstand room temperature even without refrigeration	Freezing
Immunogenic in host	No	Yes (even for humanized mAb)
Molecular weight	6-30 KDa ; 1-2 nm in diameter	25-150 KDa; 10-15 nm in diameter
Penetrability	High	Low
Renal clearance	High	Low
Production time	Improvements in SELEX process takes 2-8 weeks or less	More than 6 months
Affinity and specificity	High affinity and greater specificity	High affinity and good specificity
Downstream applications	It can be used in different physical condition	Must be used near physiological condition

Structure of an Aptamer

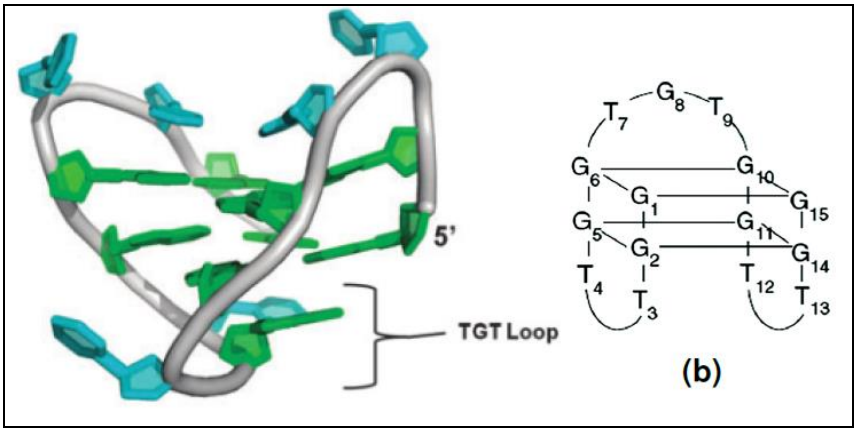

Fig 1: NMR image of an thrombin Aptamer (left) and Planar guanine tetrad of thrombin (right) formed via Hoogsteen base-pairing (*Source*: (Collie *et al*., 2011) [9].

Aptamers were discovered by Craig Tuerk and Larry Gold, as well as Andy Ellington and Jack Szostak, in 1990. The structure of the aptamer described in Figure 1 was the original work by Tuerk and Gold while investigating the translator switch of T4 bacteriophage 43 mRNA. This mRNA contains a hairpin and a Shine and Dalgarno domain at the 5' end of the start codon. Tuerk and Gold mutated the hairpin structure and identified eight nucleotides within it. Among the 65,000 combinations, they successfully isolated two aptamers, thereby established the SELEX process and obtained a patent for SELEX in 1995 (Baird *et al*., 2010) [4]. Despite over 5000 research articles following Tuerk and Gold's work, only one aptamer, Macugen, has been approved for therapeutic use. Macugen targets vascular endothelial growth factor (VEGF165) to treat age-related macular degeneration (Doggrell *et al*., 2018) [7].

Aptamers adopt a stable G-quadruplex structure in guanine-cytosine-rich environments (GC), contrasting with the stable double helical structure of DNA and RNA. Their three-dimensional folding is crucial for target binding which is studied using techniques such as X-ray crystallography and NMR spectroscopy. Interactions between aptamers and their targets involve various forces, including Van der Waals forces, hydrogen bonding, electrostatic interactions, and shape complementarity (Zhou *et al*., 2006) [10]. The binding affinity of aptamers is quantified by the dissociation constant (Kd), which is derived from the ratio of dissociation rate constants to association rate constants (Koff/Kon). High-quality aptamers typically exhibit Kd values in the nanomolar (nM) to picomolar (pM) range. It is noteworthy that DNA aptamers are often more stable than RNA aptamers (Röthlisberger *et al*., 2017) [11].

Selex: (Systematic Evolution of Ligand by Exponential Enrichment) Process

SELEX is the process of selecting aptamers from an oligonucleotide pool of 10^{14} to 10^{15} random sequence. The conventional SELEX process involves the following steps (Figure.2)

Preparation of oligonucleotide pool: Generation and synthesis of random sequences (10^{14} to 10^{15}) of 30-50 oligonucleotides between two conserved binding sites.

Incubation: Incubation of aptamers with targets that are immobilized on solid surfaces will results in aptamer -target complex

Separation: This step includes separation of unbound sequences by membrane filtration or capillary electrophoresis or magnetic beads separation or through affinity columns

Amplification: It involves the amplification of bound aptamers by PCR or RT-PCR to generate sub-oligonucleotide pool Amplified aptamers are sequenced and subjected for selection. Usually the selection process is carried out for 8-20 rounds.

The duration of entire SELEX process ranges from weeks to months (Sun *et al*., 2015) [1] (Darmostuk *et al*., 2015) [5] (Blind *et al*., 2015) [12] (Mosing *et al*., 2005) [12].

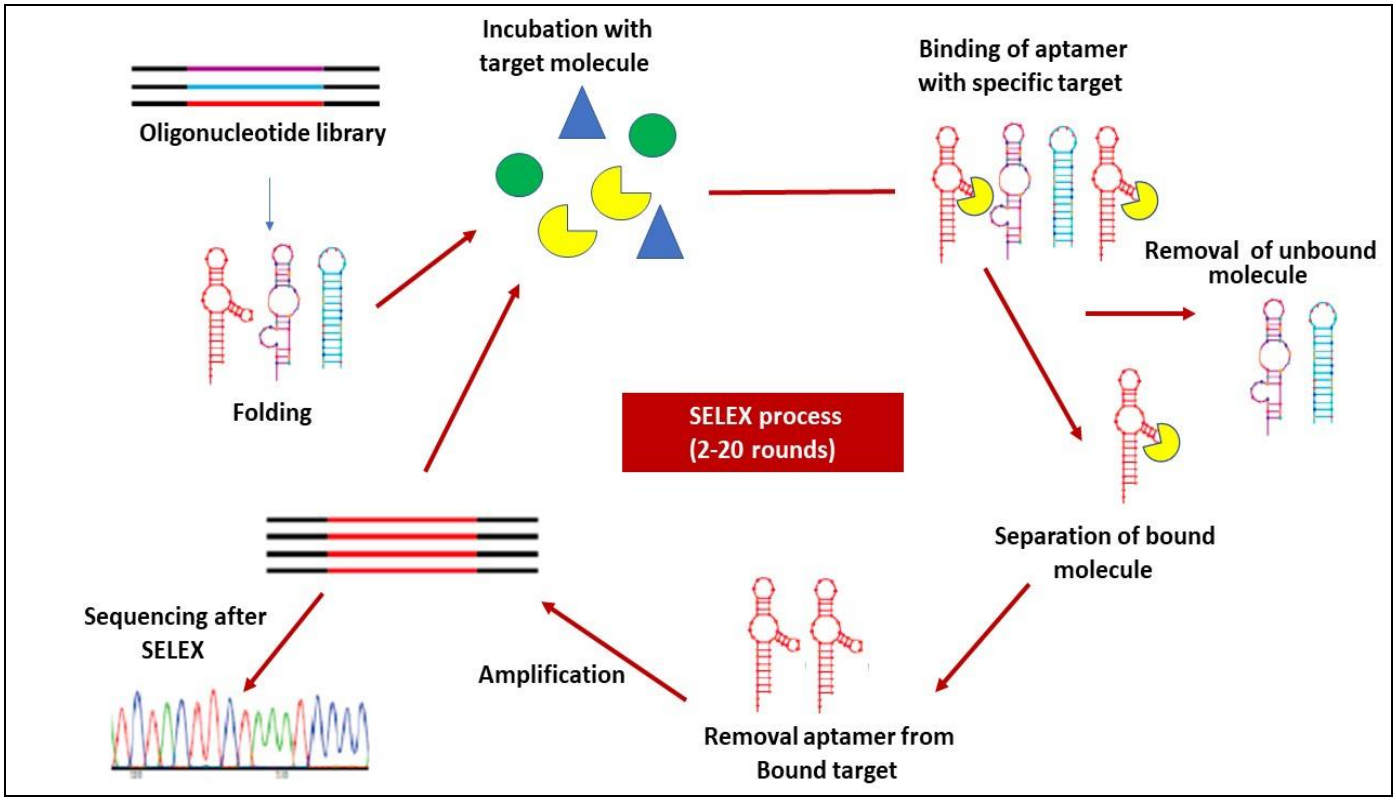

Fig 2: Schematic illustration of the SELEX process

Types of SELEX process

SELEX process has undergone several modifications since from the development in 1990. The different types of SELEX process are listed in Table 2.

Table 2: Types of SELEX

S. No	Modified SELEX	Description
1.	Counter SELEX	Used to select highly specific ligands that have the ability to discriminate between closely related closely related molecules by elimination of cross-reactive ligands to one or more ligands. E.g. Discrimination between theophylline and caffeine
2.	Capillary electrophoresis SELEX (Mosing *et al.*, 2005) [13]	It reduces the round of SELEX process from 20 to 4. It separates bound and unbound aptamers according to their electrophoretic mobility. Unbound aptamers migrate faster and pass through in waste. Bound aptamers move slow and are collected by applying pressure
	A. Micro free-flow electrophoresis (μFFE) SELEX (Jing *et al.*, 2011) [14]	SELEX process is carried out in micro FFE device. Partition efficiency is very high even in one round SELEX it provides aptamer with high affinity.
	B. Kinetic capillary Electrophoresis SELEX (NECEEM, ECEEM, Sweep CE)	An oligonucleotide library is incubated with a target molecule and allowed to reach equilibrium with free molecules, the mixture is injected into a capillary and separated by electric field. During the separation process, both unbound DNA and proteins migrate as a single electrophoretic zone, but DNA/protein complex dissociates slowly during migration creating nonequilibrium production of DNA and protein. Unlike NECEEM, in the ECEEM method the target molecule is present in the running buffer. ECEEM must be performed along with NECEEM.
3.	Cell SELEX (Morris *et al.*, 1998) [15]	Whole cell is used as target in the selection of ligands instead of a purified protein target.
	Cross over SELEX (Hicke *et al.*, 2001) [16]	Used to increase the specificity. Selection is carried out in two steps for each round. First round uses whole cell as a target and in second round purified protein is used as a target.
3.2	Differential cell SELEX (Catuogno *et al.*, 2017) [17]	Used to identify multiple ligands to know specific cell phenotype. Oligonucleotide pool is incubated with undesired cell phenotype and unbound ligands are separated and are allowed to bind with desired cells. Eg. To differentiate T cell lymphoma from B cell lymphoma
3.3	Fluorescence-Activated Cell Sorting (FACS)-SELEX (Mayer *et al.*, 2010) [18]	Fluorescent dye labelled aptamers is incubated with target cells and using cell sorting device the bound and unbound cells are separated. Aptamers from bound targets are further amplified and subjected to selection
3.4	Cell Internalization SELEX (Thiel *et al.*, 2012) [19]	For the target delivery of therapeutics into the cell the aptamers those are internalized into cells are selected by removing the surface bound aptamers with proteinase K and aptamer
3.5	Ligand guided SELEX (Catuogno *et al.*, 2017) [20]	In ligand guided SELEX primary aptamer and target complex is incubated with high affinity secondary ligand (mAb) which will displace aptamer. It is used to separate aptamer from the target and it is useful only in the case of known high affinity secondary ligands are available.
4	Magnetic bead based SELEX (Darmostuk *et al.*, 2015) [5].	Target is immobilized on magnet beads or Ligands are bound with magnetic beads for those targets which could not be immobilised
5	Microfluidic based SELEX	SELEX is carried on a microfluidic chips with automated system. Aptamers are mixed with

	(Qian *et al*., 2009) [21]	magnetic beads-coated target cells and the magnetic-bead complexes are recovered by applying a magnetic field. Bound ssDNAs are then mixed with magnetic beads-coated control cells. Control cells–aptamer complexes are collected and the supernatant are transported to the PCR chamber for amplification.
6	3D cell SELEX or *In vivo* SELEX (Souza *et al*., 2016) [22]	Aptamers are first subjected to negative selection in-vitro. Then applied for *in vivo* either in 3D cell culture or in animal models
7	In silico selection (Darmostuk *et al*., 2015) [5]	Selection and characterization of aptamers by docking using different bioinformatic tools like Rostta, FR3D, R3D align, Auto dock, DOVIS, Aptamotif, MPB bind and CLADE
8	SELEX with high throughput sequencing (Darmostuk *et al*., 2015) [5]	The genomic DNA library is used in Genomic SELEX in contrast to chemically synthesized library in classic SELEX. The genomic library is first transcribed into an RNA and then incubated with the target protein. Then the aptamers bound to the target are chromatographically separated, recovered from the target by protein denaturation, reversely transcribed into cDNA and amplified by PCR. A final pool of oligonucleotides obtained from nine selection rounds is characterized by HTS
9	Minimal primer or primer free SELEX (Darmostuk *et al*., 2015) [5]. (Catuogno *et al*., 2017) [17]	In genomic library primer binding sites with secondary structures self-anneal or anneal non-specifically. To overcome this primer binding sites are either removed or reduced before endonuclease treatment and allowed to bind with the target. The bounded molecules are re-annealed with primer and then amplification is carried out

Optimization of Aptamer

Aptamers have several advantages over antibodies and it is a promising alternative to antibody. But aptamers have limitations due to rapid renal clearance and inactivated by endonuclease. To overcome these limitations modifications of aptamers is practiced. The modifications that are commonly practiced are truncation, chemical modification, random mutation and bivalent and multivalent aptamers (Ni *et al*., 2017) [23] (Sharma *et al*., 2017) [24].

Truncation

Truncation of aptamer is the removal of primer binding domains from either side of the aptamer which increases affinity, specificity and reduces cost.

Chemical modifications

The chemical modification of aptamers (Fig. 3) include

1. Modification at 3’ end – Biotinylation at 3’ end prevents exonuclease digestion and reduces renal clearance
2. Modifications in the sugar ring - Substitution of 2’ position of sugar back bone of aptamers with hydroxyl or amino or fluoro or O -methyl offers increased resistance to exonuclease digestion. The other modification is Xeno -nucleic acid (XNA). XNAs are synthetic nucleic acids created by replacing the sugar-phosphate backbone or nucleobases with different chemical entities while preserving the base-pairing properties essential for genetic information storage and propagation. Xeno-nucleic acid contains modified sugars as in arabinonucleic acids (ANA). *Thermococcus gorgonarius* polymerase is required to amplify these types of aptamers in SELEX process. The common types of XNA are
   a. **Locked nucleic acid (LNA):** Ribonucleotides have methylene linkage between 2’ and 4’ of the ring. This leads to increased thermal stability and resistance
   b. **Unlocked nucleic acid (ULNA):** No Bond between c2 and c3 makes aptamer more flexible and thermostable but lacks a favourable quadraplex structure
   c. **F-ANA (2’ Deoxy- 2’ – fluoro D Arabinonucleic acid):** Substitution of fluoro group at 2’ position results in aptamer with increased 4 fold stable quadruplex structure and seven fold increased resistance to nucleases (Ruckman *et al*., 1998) [25] (Nsairat *et al*., 2020) [26].
3. Modification at 5’ end - Addition of PEG (40 KDa) or cholesterol at 5’ end enhances half-life of aptamers and reduces the clearance. When cholesterol was linked to the 5'-end of an aptamer via a phosphate spacer. This modified oligonucleotide, referred to as cholODN, which was then incubated with low-density lipoprotein (LDL), resulting in the formation of a complex termed cholODN-LDL aptamer. The cholODN-LDL aptamer has 10 times better half life than the original aptamer (De Smidt *et al*., 1991) [27].
4. Phosphate backbone modification - Replacement of phosphodiester with methylphosphonate or phosphorothioate or Triazole increased the stability of aptamers (Sharma *et al*., 2017) [24]
5. Base modification (SOMAmers)- Base modification increase the affinity of aptamer binding. At 5’ end of nucleotide hydrophilic and hydrophobic molecules having protein like chains or functional groups which are having inherent ability to bind targets are added. Commonly used hydrophobic modifications include the incorporation of 5-(N-benzylcarboxyamide)-2-deoxyuridine 5-BzdU), napthyl-dU (Nap-dU), Tryptamino - dU,(Trp-dU), Isobutyl-dU. These base modified aptamers are also called as SOMAmers (Slow Off-rate Modified Aptamers) (Ni *et al*., 2017) [23] (Sharma *et al*., 2017) [24].
6. Spiegelmers - The normal form of DNA is in D- form. The chiral transition from D- form to L-form of aptamers are not recognized by nucleases enzymes. L-form of aptamers are called as spigelmers. The disadvantage of Spiegelmer is the requirement of enantiomeric targets for SELEX process (Ni *et al*., 2017) [23] (Kong *et al*., 2013) [28] (Keefe *et al*., 2010) [29].

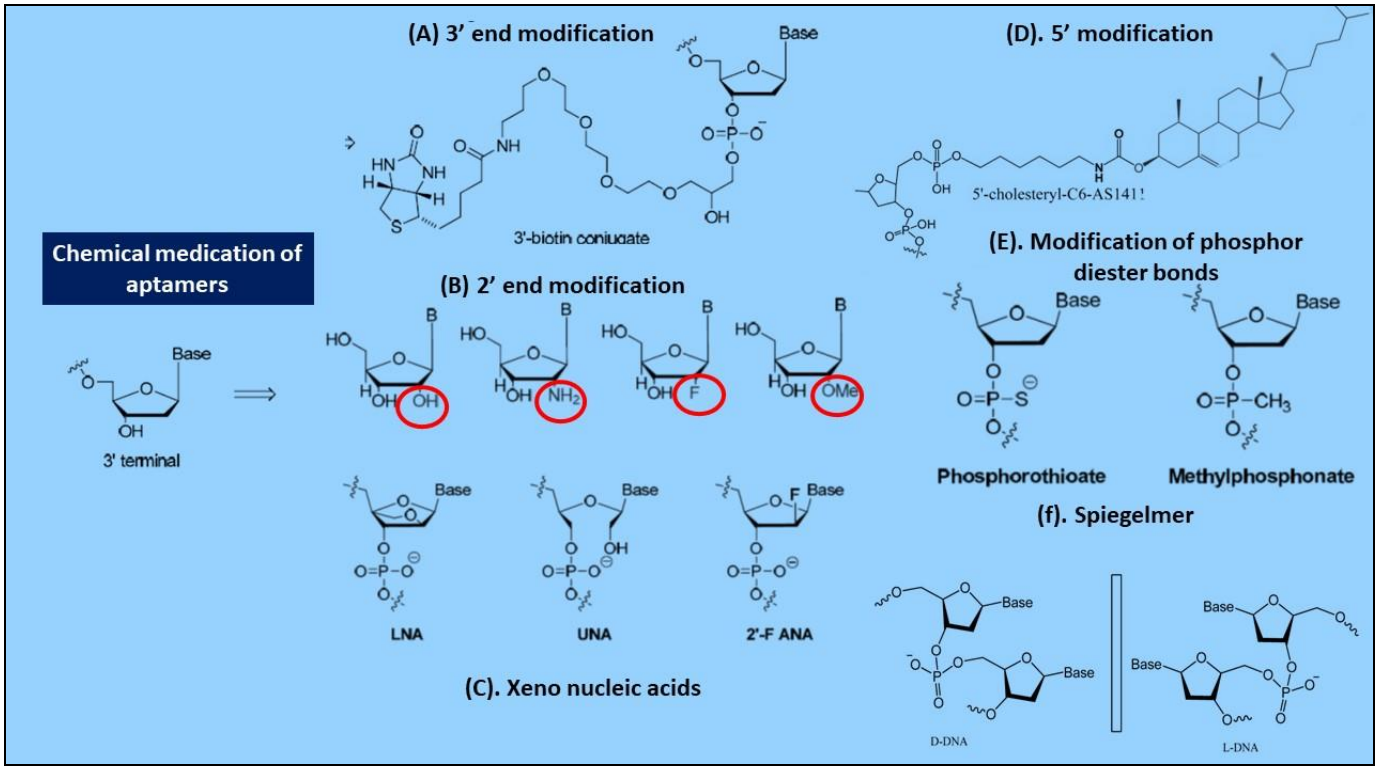

Fig 3: The common strategies in the chemical modifications of nucleic acid aptamers and their purposes. A. 3' end modification with biotin conjugation B. 2' Substitutions with OH/ NH2/ F/ O/ Me groups C. Structures of Xeno nucleic acids, locked nucleic acid (LNA), unlocked nucleic acid (UNA) and 2-deoxy-2-fluoro-D-arabinonucleic acid (2-F ANA) D. 5' end modification with cholesterol E. Modification of phospo diester bonds with methylphosphonate and phosphorothioate F. Spiegelmer – L form of aptamer

Construction of bivalent or multivalent aptamer

Bivalent or Multivalent aptamer of homologous or heterologous aptamer for the same target with 15 mer dA linker increases affinity, avidity and therapeutic efficacy of aptamers (Guo *et al*., 2016) [30]

Mutation

Affinity of aptamers can be increased by site directed mutagenesis or by non-homologous recombination (Sharma *et al*., 2017) [24] (Nonaka n *et al*., 2013) [31]

Characterization of Aptamer

Homology assessment by sequencing is the first step in characterization of aptamer. It helps to determine whether the aptamers share common structural motifs or they have distinct sequences. Various assays are used to characterize the binding ability and specificity of the target. Aptamers are evaluated based on colorimetric assays, fluorescence-based assays, gel-based assays, PCR based assays and in silico assays (Sharma *et al*., 2017) [24]

Colorimetric and fluorescence-based assays

These assays are used to evaluate the binding of aptamer with target based on colour change. Colorimetric assays include Aptamer-Linked Immunosorbent Assay (ALISA) or Enzyme-linked oligonucleotide assay (ELONA), also known as Enzyme linked Aptamer Assay (ELASA), dot blot, salt-induce Gold Nanoparticles (GNPs) aggregation assays and nanozyme (peroxidase like nanoparticles)-based assays, Dot-blot (biotin-streptavidin and gold nanoparticles based).

Gel-based characterization

Polyacrylamide Gel Electrophoresis (PAGE) is used for the characterization of aptamer and it helps to study the structure of aptamer and target recognition site based on hydroxy radical footprinting and DNAse1 footprinting (Ditzler *et al*., 1998) [32]. Aptamers are polyanionic in nature and in the electric field unbound aptamer moves very fast and the aptamer-protein complex moves at a slower rate. Sometimes, the aptamer attains secondary and tertiary structure which reduces the migration of aptamer. This folded aptamer is misinterpreted as an aptamer-target complex. Hence, western blotting must be carried out to identify the protein aptamer complex (Joshi *et al*., 2009) [33]

Biophysical characterization

Aptamers are biophysically characterized based on Circular Dichroism(CD) test to predict secondary structures in aptamers. DNA has absorbance between 180-300 nm. B form of aptamers with stem-loop structure shows a positive peak between 260-280 nm. The AT rich DNA shows a negative peak from 245 to 250 nm. Aptamers in G4 form will show a positive peak at 260-265 nm and a negative peak at 240–245 nm. CD test is also used to differentiate wild aptamers from mutated aptamers and to assess the effect salts on aptamer's structure (Fujita *et al*., 2012) [34] (Kypr *et al*., 2009) [35]

Thermal shift assay

This test is used to measure the stability of aptamer. Aptamers are incubated with protein targets with a protein-dye SYPRO orange. When temperature is increased, the hydrophobic domains of proteins are exposed and release SYPRO orange thereby release increased fluorescence. Whereas, an aptamer-bound protein complex doesn't release SYPRO orange and there is decreased fluorescence. Melting curve analysis is used to evaluate the interactions between the aptamer and the target. Tm value is increased (thermal shift) when the aptamer binds with its targets. Usually, a thermal shift of 2-10˚C is observed when the aptamer bounds to its target (Rupesh *et al*., 2014) [36].

UV melting profile

Used to measure the stability of aptamer. It uses a UV spectrophotometer or CD instrument to measure absorbance at 260 nm. An increased Tm is observed when bound to its target and stable structure. An increase in Tm from 2-12 ˚C is observed when aptamer bounds to its target depending on the strength of interaction (Song *et al*., 2011) [37]

Computational tools

The following tools are used to predict aptamer structure (Sharma *et al*., 2017) [24] (Kinghorn *et al*., 2017) [38] (Gong *et al*., 2017) [39] (Caroli *et al*., 2016) [40]

a. Mfold – Predict RNA folding and secondary structure of aptamer
b. UNAfold – Predicts secondary structure and the propensity of G4 forming aptamers
c. QGRS mapper – Predicts composition and distribution of G4 forming G-rich segments
d. Autodock, ZDOCK, PatchDock, HADDOCK – Predicts interaction of aptamer with its target
e. APTANI – predicts target specific aptamers from HTS data
f. Rosetta, RNA composer – Gives 3D structure of aptamers

Aptamers in diagnostics

Aptamers have high affinity and specificity towards their target molecules. They are easy to synthesize and are not immunogenic. These unique properties made them more attaractive in the field of diagnostics. Aptamers are used in the pathogen detection, cancer detection, food safety assurance, environmental monitoring, water purification and in bio-imaging (Zhang *et al*., 2019) [41]. Several aptamers based diagnostics are in the pipeline and three companies, Neo Ventures Biotechnology Inc., SomaLogic, Inc (Boulder, CO, USA), Aptamer Sciences Inc. (AptSci, South Korea), and Base Pair (Houston, TX, USA) have commercialized their technology for the detection of Ochratoxin A (OTA sense®), Aflatoxin (AflaSense®) and for flowcytometry isolation of CD31, CD-31, EGFR, HGFR, ICAM-2, VEGFR-2 or HER-2 cells (AptoCyto®) (Kaur *et al*., 2018) [42].

Pathogen detection

Aptamers have been used successfully in the detection of bacterial, viral, parasitic and protozoan pathogen.

Bacterial pathogen

Aptamers have been used in the diagnosis of following Gram-positive and Gram- Negative bacterial pathogens viz. *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Salmonella spp*., *Shigella sonnei*, *Escherichia coli* O157, *Vibrio cholerae*, *V. parahaemolyticus*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *L. interrogans* (Zhang *et al*., 2019) [41] (Vishwakarma *et al*., 2019) [43].

Viral pathogen

Aptamers have been used in the diagnosis of several viral diseases viz. human pappilomavirus, herpes simplex virus, hepatitis C virus, Dengue virus, Zika virus, Japanese encephalitis virus, Tick borne encephalitis virus, Norovirus, SARS CoV, SARS CoV-2, Influenza virus, Rift valley fever virus, Dabie bandavirus, Ebola Virus, HIV and Hepatitis B virus (Choi *et al*., 2022) [44] (Chakraborty *et al*., 2022) [45].

Parasites

Aptamers have also been utilized to detect the parasitic pathogen *Plasmodium spp.*, *Leishmania infantum*, *Leishmania major*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Cryptosporidium parvum*, *Toxoplasma gondii*, *Schistosoma japonicum*, *Trichomonas vaginalis* (Brosseau *et al*., 2023) [46].

Aptamers in cancer detection

Various aptamers were developed in the diagnosis of cancer like breast cancer, colon cancer, T cell leukaemia, Burkitt lymphoma, hepatocellular carcinoma, malignant melanoma, prostate cancer. These aptamers were developed targeting cell surface markers, cancer specific proteins, Intracellular adhesion molecules and cell membrane associated antigens (Hu *et al*., 2022) [47] (Ruiz *et al*., 2018) [48].

Aptamers in ensuring food safety

Several aptamers were developed to detect various food borne pathogens, adulterants, pesticides (Acetamiprid, malathion, atrazine, Edifenphos, Iprobenfos, Edifenphos, Phorate, Isocarbophos, phosalone, methamidophos, acephate, trichlorfon, dursban, Isocarbophos, profenofos, phorate, omethoate), drug residues (tetracycline, diclofenac, dopamine, ampicillin, neomycin B, enrofloxacin, penicillin G, florfenicol, sulfadimethoxine, lincomycin, diethylstilbesterol), food borne toxins (Aflatoxin, Zearalenone, Fumonisin, Anatoxin-a, Staphylococcus enterotoxins, okadoic acid), authentication and allergen (gluten) detection (Schmitz *et al*., 2020) [49] (Song *et al*., 2019) [50].

Aptamers in environmental monitoring

Aptamers have been used to detect aquatic toxins (microcystin, nodularin, malachite green; saxitoxin), pesticides, insecticides, antibiotics, industrial by-products (2,3′,5,5′-tetrachlorobiphenyl -PCB72, 2-hydroxyfluorene, BPA), pharmaceuticals (ibuprofen, estradiol) in water sources. For the soil monitoring aptamers have been developed to detect soil contaminants like lead and mycotoxins such as aflatoxin ; and ochratoxin. Aptamers have also been developed to monitor air quality by detecting toxic gases (radon, nitrogen dioxide, sulphur dioxide, and hydrogen sulphide), 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker for oxidative stress in urine due to air pollution (Zhang *et al*., 2019) [41] (McConnell *et al*., 2019) [51].

Aptasensors (Reich *et al*., 2019) [52] (Kim *et al*., 2016) [53] (Song *et al*., 2012) [54]

Biosensors are devices that transforms the recognition of targets into a physically detectable signal, such as optical, electronic mass or magnetic signal (Feng *et al*., 2014) [55]. Biosensors has two components: 1. Target recognition component 2. Signal transduction. Aptamer based biosensors are called as aptasensors. Aptasensors are constructed with different methodologies (Ruckman *et al*., 1998) [24] (Feng *et al*., 2014) [55] (Citartan *et al*., 2012) [56] viz. Colorimetric aptasensors, Fluorescence aptasensors, Chemiluminensce based aptasensors and Optical biosensors

Aptamer Based Diagnostic Platforms

Aptamer based biosensors using nanoparticles

Gold and magnetic nanoparticles are extensively used to design aptasensors because of their unique properties and functionalization. Gold Nanoparticles (AuNPs) - AuNPs have excellent biocompatibility, high surface area-to-volume ratio, and unique optical properties, such as surface plasmon resonance (SPR). AuNPs can be easily functionalized with

aptamers via thiol chemistry, where the aptamer is modified with a thiol group that binds strongly to the gold surface. The interaction between aptamers and their target molecules induces changes in the aggregation or dispersion of AuNPs, leading to detectable changes in their optical properties, which can be easily monitored. Magnetic Nanoparticles (MNPs) - MNPs, such as magnetite (Fe3O4) or maghemite (γ-Fe2O3), are commonly employed for their magnetic properties, allowing for easy manipulation and separation using an external magnetic field. In aptasensing applications, MNPs are often used for signal amplification or for separation purposes. For signal amplification, MNPs can be conjugated with detection molecules (e.g., enzymes, fluorescent tags) to increase the sensitivity of detection assays. In separation experiments, MNPs bound to aptamers can selectively capture target molecules from complex samples, enabling their isolation and subsequent analysis (Song *et al*., 2012) [54].

The AuNPs and MNPs in aptasensors when combined together offer synergistic advantages. MNPs can be used for target capture and concentration, while AuNPs can be utilized for signal transduction. This combination enhances the sensitivity and selectivity of the aptasensor (Zhao *et al*., 2020) [57]

Carbon nanomaterial based Aptasensors - Single walled carbon nanotubes' (SWCNTs), graphene and graphene oxide (GO) are the most promising nanomaterials for the applications in biosensors due to their unique electronic, optical and mechanical properties. They have large surface area for binding. Functionalized graphene can be used for label-free detection of target analytes, enabling simple and cost-effective sensor designs. biocompatibility and enhanced signal amplification capabilities (Wang *et al*., 2016) [58].

Aptamers in lateral flow assays

A reporter labelled antibody or aptamer is absorbed on the conjugate pad. A primary antibody or aptamer against the target analyte is immobilised on the test line and a secondary antibody or aptamer against the labelled bioconjugate is immobilised on the control line. Then the analyte is applied to the sample pad it migrates to the conjugate pad, where the target analyte binds to the labelled bioconjugate and this complex passes across the emmbrane via capillary movement to the test line where it is captured. The excess of labelled aptamer /antibody will then be captured at the control line. The appearance of both lines, test line and control line is indicative of a positive result, with a single line at the control line representing a negative result, whilst confirming correct assay function (figure 4). Different aptamer based lateral flow assays were develop for the diagnosis of different analyte viz. cocaine, aflatoxin, Arbovirus, ochratoxin, *S. enteritidis*, thrombin and β conglutinin (Jauset-Rubio *et al*., 2017) [59].

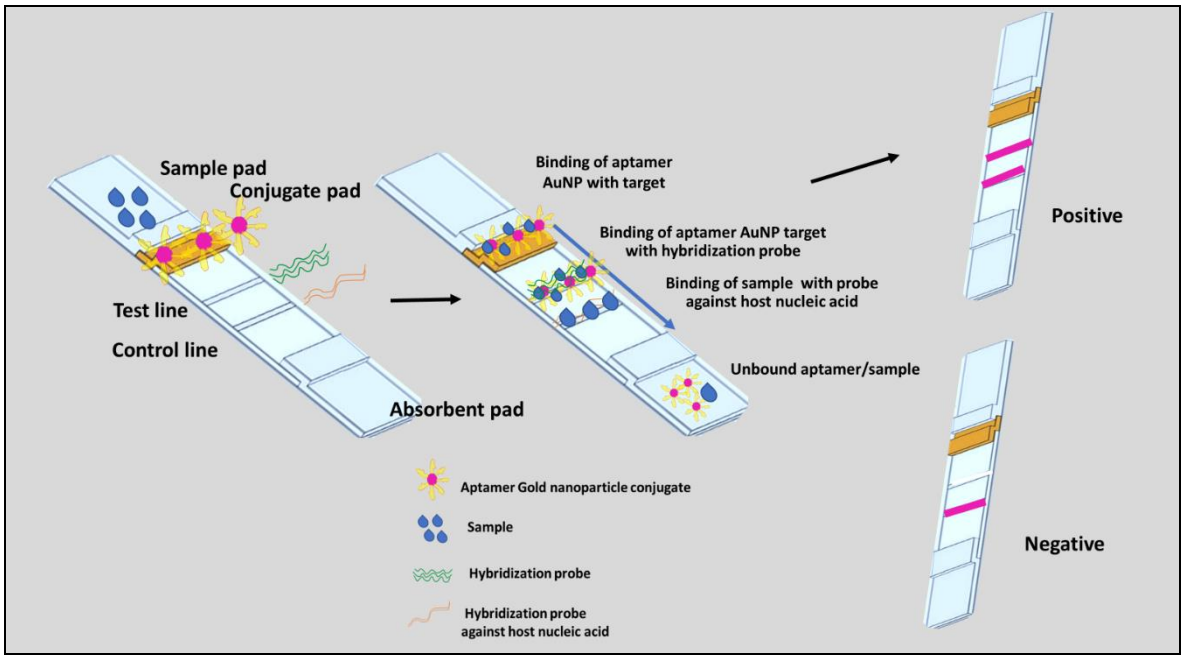

Fig 4: Aptamer based lateral flow assay

In the aptamer-based lateral flow assay, a target-specific aptamer conjugated with gold nanoparticles (AuNPs) is housed within the conjugate pad. The test line is impregnated with a hybridization probe specific to the target, while the control line contains a hybridization probe specific to host DNA. Upon loading the sample into the sample pad, it migrates into the conjugate pad. If the sample is specific to the aptamer, it binds with the AuNP-aptamer conjugate, causing this complex to move towards the test line. In the case of a positive sample, the AuNP conjugates with the hybridization probe, resulting in a pink coloration at the test line. Any remaining sample migrates towards the capture line, where it is captured by a host-specific probe, also producing a pink coloration. Consequently, the appearance of two pink lines indicates a positive result. If the sample is negative and fails to bind to the AuNP-aptamer, it will not be captured at the test line. However, the host DNA will be captured at the control line, leading to the appearance of only one pink line in negative samples.

Aptamer-Linked Immunosorbent Assay

Aptamer-Linked Immunosorbent Assay (ALISA), is a variation of the traditional ELISA (Enzyme-Linked Immunosorbent Assay) technique. In ALISA, instead of using antibodies as capture molecules, aptamers are employed. In ALISA format two different aptamers directed against the same target can be used. One aptamer with magnetic beads coated with magnetic beads as capture aptamers are immobilized onto the surface of a solid support, such as a microplate well. These aptamers selectively bind to the target

molecules present in the sample. After washing away unbound substances, the secondary aptamer conjugated with an enzyme or other bio molecules. This secondary aptamer binds to the target molecules captured by the immobilized aptamers, enabling the detection and quantification of the target analyte (Figure 5). ALISA has several advantages over traditional ELISA, including increased stability of aptamers, lower production costs, and the potential for high specificity and affinity to target molecules.

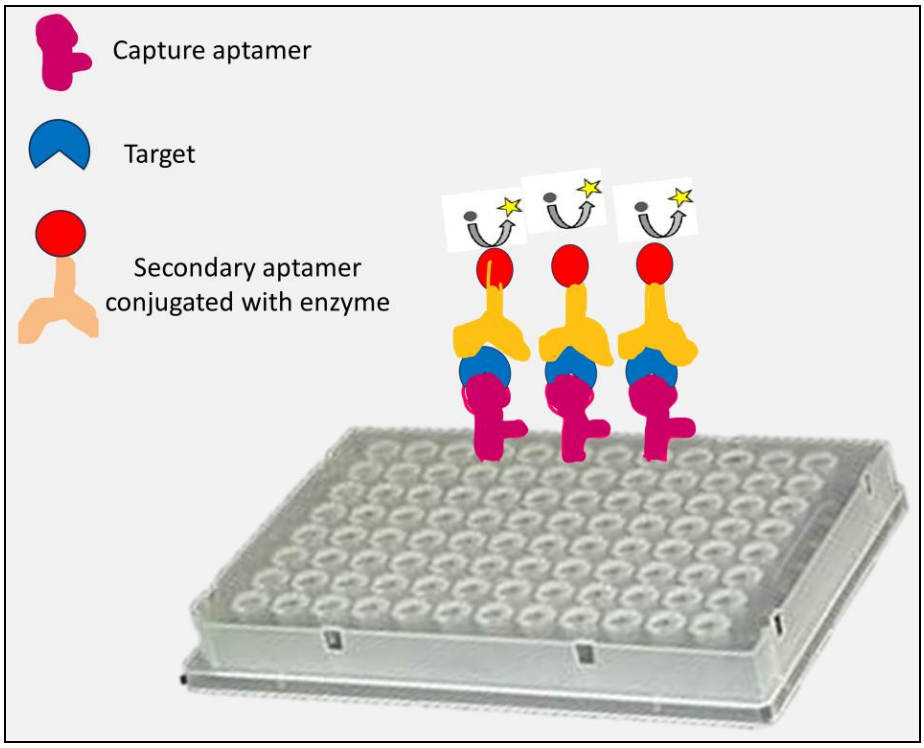

Fig 5: Schematic illustration of ALISA

Aptamers in western blotting

Conventional western blotting is a time-consuming process in which two types of antibodies are used. In aptamer based western blotting, RNA aptamer directed against His-tag and conjugated with quantum dots which are explained in figure 6 (Feng *et al*., 2014) [55].

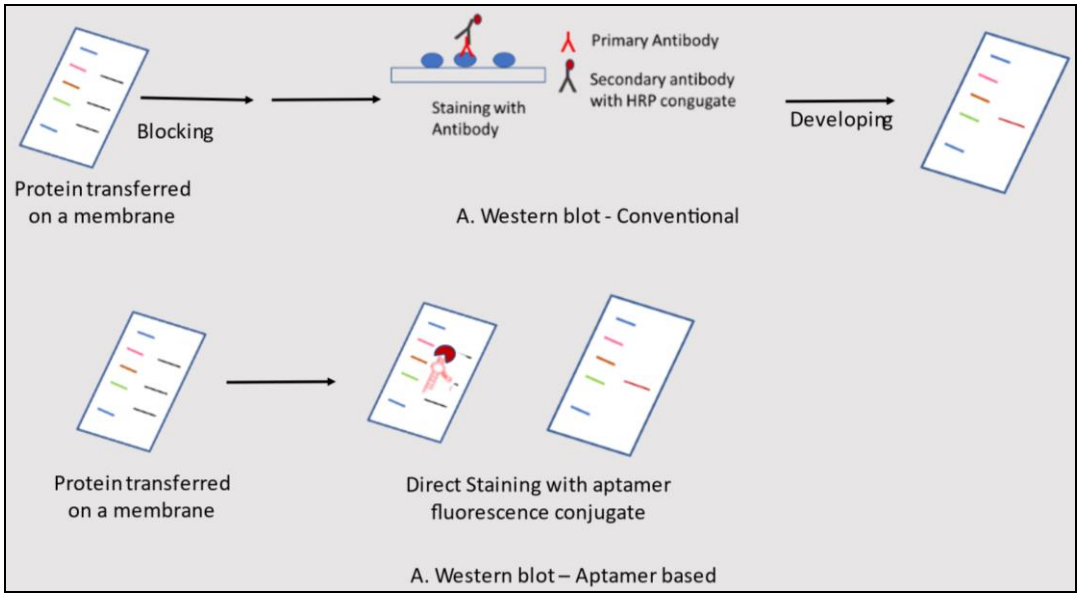

Fig 6: Comparison of conventional and aptamer based Western blotting (A) The conventional Western blot analysis which includes several steps like blocking, incubation with primary antibody, then with secondary antibody conjugate, developing with H_2O_2 (b) The aptamer-based Western blot analysis is very simple with

Aptamers in affinity purification

Aptamers are also used in affinity chromatography for purification of protein. Aptamers immobilized on polystyrene magnetic beads at 3'NH_2 terminal are used as functional affinity matrix. Once the target molecule is captured by the aptamers, it can be eluted from the solid support using specific conditions such as changes in pH, ionic strength, or competitive displacement by adding excess free aptamer or a molecule that competes with the target for binding (Schax *et al*., 2015) [60].

Aptamer based point- of- care (POC) test

Aptamer-based POC testing offers several advantages, including rapid results, high sensitivity and specificity, portability, ease of use, potential for multiplexing and on-site testing in dispensaries, hospitals and at home.

Aptamers enabled personal glucometer has been explored in POC testing of various analytes like dopamine and lead. (Hun *et al*., 2015) [61] (Gu *et al*., 2015) [62].

Smartphone enabled aptasensors have been developed for the detection of cadmium, mercury, ochratoxin, carcinoembryonic antigen (CEA), *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Salmonella Typhimurium* and visualization will be carried out with smartphone torch and camera (Song *et al*., 2022) [63] (Dirkzwager *et al*., 2016) [64].

3D printing of Aptamer assays

Functionalised aptamers are printed on 3mm Whatman filter paper using stereolithography – 3D printer. In this format it contains a spin column containing cotton wool packed syringe filter device followed by aptamer-functionalized Whatmann 3MM punched paper. All reagents including samples & buffers are applied by syringe. It is a colorimetric based test. Positive samples are detected by based on colour development (Dirkzwager *et al*., 2016) [65].

Aptamers in Bioimaging

Aptamers are used in bio-imaging study to understand the cell signalling pathways, molecular interactions, protein localization and to assess the dynamic changes in the cellular organelle (Le *et al*., 2017) [66].

Aptamers in confocal imaging

Aptamers labelled with Fluorophores serves as a probe in live cell imaging especially with 3D Laser confocal imaging.

Aptamers in Magnetic Resonance Imaging (MRI)

The MRI is a non-invasive imaging technique that provides detailed three-dimensional (3D) images of living systems with high resolution. In conventional MRI, contrast agents containing gadolinium ions (Gd3+) are often used to enhance image contrast. These contrast agents influence the relaxation times of surrounding water molecules, leading to brighter or darker regions in the MRI image, depending on their distribution. In aptamer tagged contrast agents, Gd3+ molecules are tagged with aptamer specific to the target to be imaged and used in MRI. Upon binding of aptamer with the target, it causes dissociation of GD3+ (contrast agent), results in magnetic relaxation and brightened image. Aptamer tagged with Gd3+ can be used for targeted imaging of cancer cells (Zhou *et al*., 2006) [10] (Huizenga *et al*., 1995) [67]. Aptamer based probes increased the target to background ratio by 4-fold due to rapid removal of unbound aptamers.

Aptamers in Positron emission tomography (PET) imaging

PET imaging is widely used in clinical oncology to diagnose cancer, neuro imaging, bio-distribution studies, and small animal imaging. In conventional PET imaging, radiopharmaceuticals (radioisotope usually $^{[18]}$ FDG or ^{15}O) is injected into live animals. When the radioisotope undergoes β decaying, releases positron which reacts with the inert electron in the tissue and releases two gamma rays that are detected by specialized gamma cameras to give 3D imaging. In aptamer assisted PET scanning instead of a drug aptamer targeting particular organelle is coupled with ^{18}F which will be injected into animal for capturing. The advantage of aptamer over antibodies in PET scanning is the unbound aptamers have good penetration, the unbound aptamers will be cleared rapidly from the system and it reduces background signal thereby improves image contrast (Wang *et al*., 2015) [68].

Conclusion

Aptamers are ssDNA or RNA, offers distinct advantages over monoclonal antibodies and has been exploited in all forms of diagnosis due to their unique properties. Aptamers serve as a versatile tool in the detection of various diseases, environmental pollutants, food adulterants and in assessing the air quality. They can easily be applied to complex biological samples such as blood, urine, and tissue extracts. A commercial market report from the USA predicted the aptamer market will attain tremendous growth in near future. The improved practices of SELEX and optimization facilitated the growth of aptamer-based diagnostics. However, thousands of studies have been carried out on aptamer-based diagnostics development only three were successfully commercialized so far. Hence, continued research and development in aptamer technology are required to further expand and develop highly sensitive, specific, and cost-effective diagnostic assays.

Competing Interests

Authors have declared that no competing interests exist.

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