Chapter

Aptamers: Magic Bullet for Theranostic Applications

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Abstract

Aptamers are a short polymer of oligonucleotides (natural or modified) that can bind to its cognate target (small molecules to large macromolecules like proteins, cells, microorganisms etc.) with high affinity and selectivity. They can fold into unique secondary and tertiary conformation in solution (pH, ionic concentration) and bind to their targets in a specific manner (binding constants in sub-nano to pico molar range). They rival the monoclonal antibodies and other specific biological ligands with respect to affinity, stability, robustness, non-immunogenicity and facile to synthesis. Nucleic acid aptamers are selected from an oligonucleotide library by an iterative process called SELEX (**S**ystematic **E**volution of **L**igands by **Ex**ponential Enrichment Analysis). These aptamers are compatible to any kind of chemical modification, conjugation and functionalization. Briefly, this chapter discusses about the diagnostic and therapeutic application of aptamers.

Keywords: aptamers, SELEX, theranostics, chemical antibodies

1. Introduction

Aptamers (Latin word *aptus* means 'to fit' and Greek meros meaning 'part') are single stranded oligonucleotides, which act as synthetic ligands for its cognate target molecules. [1, 2] These molecules show high target specificity, selectivity and affinity, which resemble 'monoclonal antibody'. Similar to antibodies, aptamers also have immense potential to interact with their targets by structural recognition and thus they are termed as 'chemical antibodies'. [3] Different conformations allow aptamers to bind specifically with their target by 'lock and key' model. This hypothesis of binding mechanism is driven by the secondary and tertiary structure of aptamers in-bound state with their targets. Adopting various structures like hairpin loops, bulges, stem-loop, quartets, G-quadruplex, pseudo knots, aptamers can fit into the binding region of the target. [4] Intra and inter molecular interactions like hydrogen bonding, Vander Waals force, hydrophobic interaction, electrostatic forces play major crucial role in aptamer-target interaction. [5] However, the aptamers are primarily synthetic molecules and naturally occurring ribozymes are single stranded RNA molecules, which also have similar recognition domain acting in a similar manner. [6, 7] Aptamers are capable of forming various stable three-dimensional structures in physiological solution. The folding process in solution and the ligand-induced conformational switch is strongly dependent on the presence of divalent cations (magnesium, potassium etc.). [8] There are plethora of computer algorithms

enable sequence based modeling of secondary structure of the oligonucleotide aptamers which actually strengthen the predictability of strongest binders with lowest free energy. [9, 10] Aptamers fold into tertiary conformations and bind to their targets through shape complementarity at the aptamer-target interface. [11] An aptamer binds to a protein can modulate protein functions by interfering with protein interaction with natural partners. Similar to antibodies, aptamers can enter to specific target cells via receptor-mediated endocytosis upon binding to cell surface ligands. [12] Importantly, aptamers can penetrate into tumor cores much more efficiently than antibodies due to their ~20–25-fold smaller sizes compared with full sized monoclonal antibodies. [13, 14]

Monoclonal Antibody	Aptamer
Stability: Monoclonal antibodies require refrigeration to avoid denaturation. Limited shelf life. [15]	**Stability:** Aptamers do not require refrigeration. Indefinite shelf life. [16]
Immunogenicity: They can cause immunogenic response. [17]	**Immunogenicity:** Aptamers are non-immunogenic. [18]
Production: laborious, expensive, high batch-to-batch variation.	**Production:** simpler and controlled chemical reactions, little to no variation, automated, chemical synthesis, no contamination.
Size: Larger in size, they can resist filtration by the kidneys, long half-lives. However, their size prevents access to smaller areas. [19]	**Size:** Aptamers are small molecules. They are especially subject to kidney filtration, resulting in short half-lives. Compared to antibodies, aptamers can bind to smaller targets. [20]
Ability to modification: Antibodies cannot accommodate conjugates without negative consequences such as reduced activity.	**Ability to modification:** Easy to modify, modifications can also be incorporated during synthesis to prevent kidney filtration. [21]

Table 1.
Comparison between Monoclonal antibody vs. Aptamer:

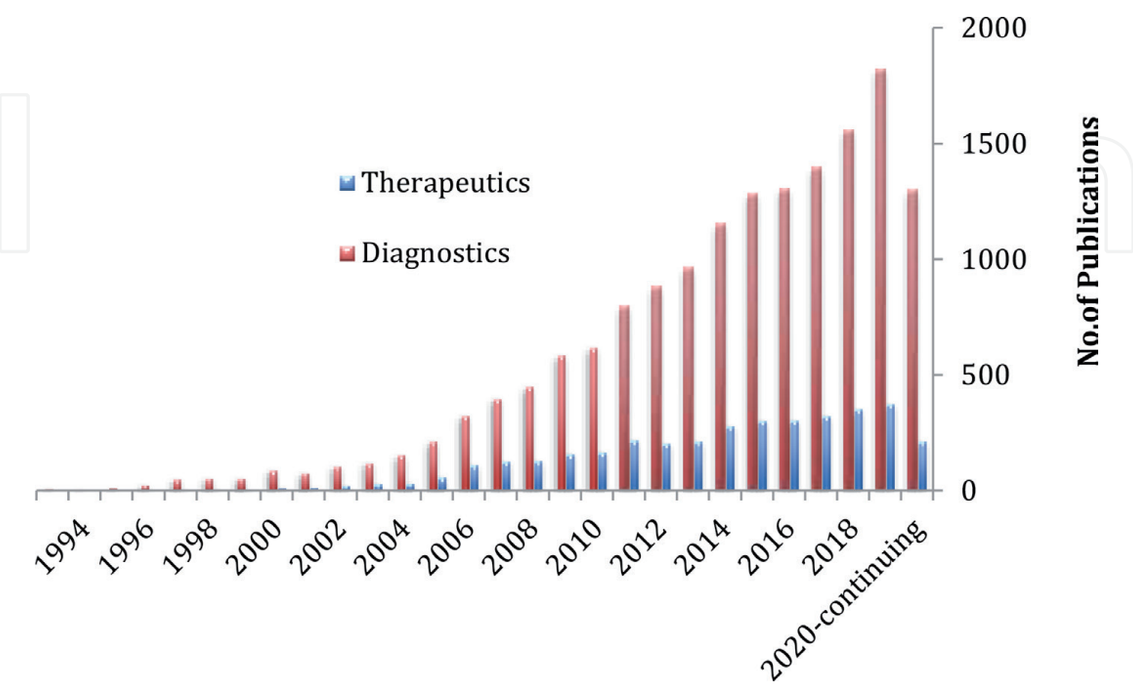

Figure 1.
Publication trend for Search strings: "Aptamers as diagnostics" and "Aptamers as therapeutics" (Source: Scopus).

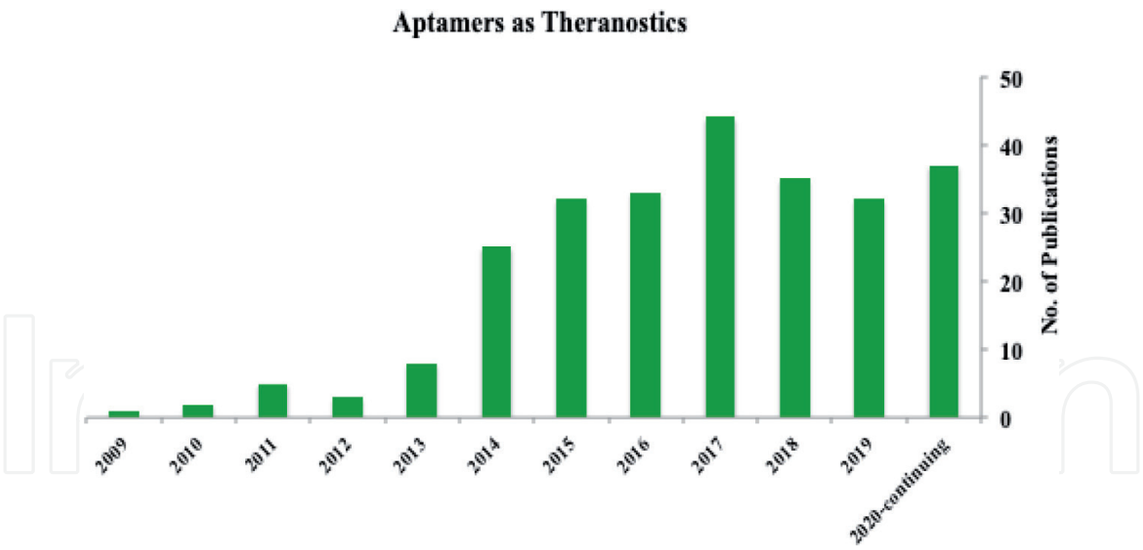

Figure 2.
Publication trend for Search strings: "Aptamers as theranostics" (Source: Scopus).

Compared to antibodies, aptamers can be produced using cell-free chemical synthesis and are therefore less expensive for large-scale manufacture. Aptamers exhibit extremely low variability between batches and have better controlled post-production modification, they are minimally immunogenic, and are small in size. (**Table 1**) The rapidly growing aptamer industry was predicted to reach US $244.93 million by 2020. [22] Presently more than 40 companies are actively engaged in diagnostics and therapeutics research to commercialize these "magic bullets" globally (EU countries, Asia, USA, UK etc.). [23] The largest company is "SomaLogic" (company based on SOMAmer- a patented "Slow Off-rate Modified Aptamer) founded by Prof. Larry Gold at Colorado, USA. Since the advent of aptamers scientists and researchers exploit different applications of aptamers that reflects the following trends in the publications. (**Figures 1** and **2**).

2. Basics of SELEX screening process

Back in 1990, two individual groups Prof. Larry Gold and Craig Tuerk from University of Boulder, USA and Prof. Jack Szostak and his student A.D. Ellington from Havard University, USA discovered the evolution process to obtain the oligonucleotide binders and they coined the term 'Aptamer' and the process as 'SELEX'. [24, 25] Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is a common screening process by which aptamers can be selected from an aptamer library which consist of $10^{24–25}$ number of various sequences. The method attempts to isolate an aptamer of interest from a pool of randomized library by an iterative cycle of incubation with the target, partitioning and amplification, until the pool of aptamers enriched enough to fit with the target. The SELEX procedure iterates over five basic steps- incubation of aptamer pools with the target, binding, partitioning and washing (to get rid of non-binders which are loosely bound with the target), then elution of positive target-bound aptamers and amplification of enriched pools. Traditionally, the positive pool eluted from last round is being analysed, and high-throughput sequencing is performed.

An array of different RNA and DNA aptamers were isolated against a vast array of targets: ions, [26] low molecular weight metabolites, [27, 28] proteins, [29–31] sugar moieties [32] lipids, [33] and even whole cells. [34, 35]

3. Library selection

To select highly selective, specific aptamers, design of the initial aptamer library is the first and foremost step. In case of determination of the length of the random region researchers should consider the sequence space and structural diversity. The complexity of the initial aptamer library depends on the length of the random window of the aptamer library (If the random window is 40 and if we consider DNA aptamer library, so the complexity of the library is: 4^40 that equals to $10^{24–25}$). [36]

Special libraries would consist of specifically designed flanking sequences directing the aptamers to form a specific secondary structure, or include modified nucleotides. In capture SELEX, there is unique docking sequence (12–14 nucleotides long) which enables the library in such a way, that highly sensitive aptamers can be fished out against small molecules. [37, 38] The extended genetic alphabets or combination of artificial xeno nucleic acids (XNA) greatly broaden the diversity of sequences and can influence the properties of the aptamers, such as their in vivo stability or nuclease resistance. [39–42] Modified nucleotides can be introduced either during the library synthesis or in the post-selection optimization.

In a review article, Maria *et al.* summarized all key features of designing nucleic acid libraries for SELEX like nature, composition of the library (RNA, DNA or modified nucleotides), the length of a randomized region and the presence of fixed sequences. Different randomization strategies and computer algorithms of library designs were also discussed. [43]

4. Various SELEX processes

Specific aptamers are screened by the iterative processs of SELEX from a highly diverse pool of oligonucleotides. [44–46] After the incubation of the random aptamer pool with the target followed by the removal of non- binding aptamers, the bound aptamer species are recovered. These recovered nucleic acid sequences are amplified with PCR (in the case of DNA aptamer) or RT-PCR (for RNA aptamers). In addition to selection against a purified target molecule, SELEX process can be performed against live bacterial cells and even in mammalian cell lines to isolate cancer cell specific aptamers and furthermore it can lead to the identification of novel biomarkers. [47, 48]

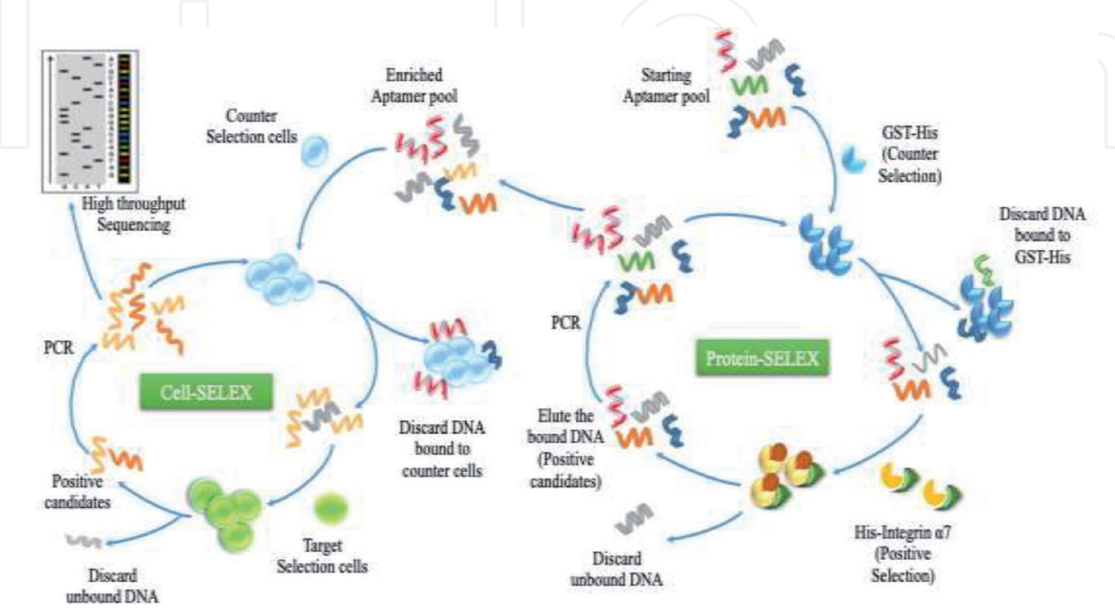

Figure 3.
Typical schema of Cross-over SELEX process.

A giant advancement of SELEX technology has been made since its discovery in 1990. Conventional SELEX is a well-established and effective method but due to its immense time- and labor-consumption, continuous improvement of alternative methods for aptamer selection has been inevitable.

High throughput SELEX (HT-SELEX), Functional screening (Microfludics or Flow cytometry based SELEX), Cross-over SELEX (where the target is alternatively changing from proteins and cells), (**Figure 3**) *in-vivo* SELEX, Spiegelmers selection, de-convolution SELEX are few examples of modern-era screening strategy of aptamers. [49] Cutting-edge functional screening process, the chemical modifications, Next-generation sequencing (NGS technology) enable SELEX more efficient, cost-effective and considerably less time-consuming.

5. Modifications of naturally occuring aptamers

DNA is the backbone of central dogma of our life cycle. Moreover, any form of nucleic acids play a crucial role in our genetic codon. DNA/RNA is an essential biomacromolecule consist of nucleotide bases such as adenine (A), thymine (T), uracil (U), guanine (G), cytosine (C).

There are various types of modifications (nucleotide base modifications, phosphate backbone modifications, peptide mimic oligonucleotides PNA etc.) available which can prevent aptamers from nuclease degradation. Locked nucleic acid (LNA) is one among them where 2′-oxygen has been linked to the 4′-carbon of the ribose sugar by a methylene bridge, thus completely locking the sugar into a 3′-endo conformation. LNAs increase the thermodynamic stability, binding affinity, and enable the oligonucleotides to prevent serum degradation. [50–52] These modifications enable the aptamers for biological applications.

Compared to LNAs, the unlocked nucleic acid (UNA) is an acyclic ribose derivative that has increased flexibility. UNAs do not consist the C2'-C3' bond, which confers the flexibility observed in this modified nucleotide. [53] LNAs increase the melting temperature of the nucleotide by 1–10°C per LNA insertion but UNAs reduce the melting temperature by 5–10°C retaining the nuclear resistance. In case of, Peptide nucleic acid (PNA) in which sugar-phosphate backbone is modified by short stretch of N-(2-aminoethyl)-glycine units connected by peptide bonds, enhances biostability of the modified candidates. [54]

5.1 Aptamers in Drug development pipeline

Aptamers have been incorporated in drug development pipeline as they have the capacity to block the downstream signalling (phosphorylation of kinases etc.) of different biomolecules. They can play an important role to regulate various cellular crosstalks. To screen therapeutic aptamers either DNA aptamers or 2′-fluoro modified RNA, a combination of 2′-fluoro pyrimidines and 2′-hydroxyl purines (fYrR) are of major interest. fYrR is the "nuclease stable RNA" and can be easily generated by Y639F modified T7 RNA polymerase. Fovista, an anti-platelet derived growth factor (PDGF) aptamer, was previously DNA aptamer but later modified to augment the stability with the addition of backbone modifications. [55] As with the 2′-fluoro modification, the 2'-OMe modifications adopt a C3'-endo conformation. US FDA approved the first aptamer (Macugen®, pegaptanib sodium) in 2004 against vascular endothelial growth factor for the treatment of age-related macular degeneration. [56] This aptamer was modified with 2′-fluoro-pyrimidines and 2'-O-methyl-purines. The stability of the small aptamer was a critical factor but later which can be circumvented with a 3′-cap and a polyethylene glycol

molecule, the half-life of Macugen® was extended to 131 hours at max. [57, 58] Anti-vascular endothelial growth factor (VEGF 165) aptamer Macugen, and an anti-Factor IXa aptamer REG1 were both selected from fYrR libraries, and subsequently 2′-O-methyl nucleosides have been incorporated in order to increase serum stability. [57]

There is a plethora of polymerase enzymes like KOD, Pwo, Phusion, Superscript III, vent (exo-), T7 polymerase have all been shown to be capable of incorporating modified triphosphates into DNA and RNA strands, which open up a new opportunities in aptamer selection strategies. [59] The use of Pfx DNA polymerase allows amplification of Ds-Px base pair in Ex-SELEX protocol where extended genetic alphabets were included in complexity of nucleic acid library. [60]

Several limitations of aptamers should be considered in the process of *in-vivo* applications of nucleic acid aptamers. Being polynucleotides, nucleic acid aptamers are naturally susceptible to enzymes degradation by exo and/or endo-nucleases, leading to a reduced *in vivo* circulatory half-life. This drawback can be alleviated by side chain chemical modifications to aptamers, incorporating unnatural nucleotide bases (locked and unlocked nucleic acids) and capping the aptamer ends, thus minimizing the susceptibility to endonuclease and exonuclease attack. [50, 51] Short blood residence time is another challenge with in vivo aptamer applications, which is due to fast removal of aptamer from the circulation by renal filtration as most aptamers have a size smaller than the renal filtration threshold of 40 kDa. [31] To achieve desired serum half-life, aptamers can be engineered by conjugation with a terminal polyethylene glycol (PEG), although this may compromise the extent of tumor penetration [61]. In some cases, post-SELEX modifications following the selection of aptamers may alter the 3-D structure of the aptamers, leading to the lost or altered binding affinity and specificity. Such problems can be prevented by using random aptamer pools containing modified nucleotides during the SELEX process. [62, 63] In addition, the ability of aptamers to interact with cells may decrease due to repulsion of nucleic acids by negatively-charged cell membranes. This can be refuted by increasing the binding affinity and specificity of aptamers toward their cell surface receptors to trigger receptor-mediated endocytosis.

In the field of oncology, two aptamers, namely, AS1411 and NOX-A12, have entered clinical trials. [45, 64] AS1411 (formerly ARGO100; Antisoma) is a guanine quadruplex aptamer obtained from a guanine-rich oligonucleotide library in the anti-proliferation screen, which is not a typical SELEX process. [65] The guanine quadruplex structure benefits AS1411 because it is resistant to nuclease degradation and enhances cell uptake. In *in-vitro* validations, AS1411 inhibits more than 80 types of cancer cell lines. In addition, the target of AS1411 has been verified to be nucleolin, and the relevant mechanism and specificity against cancer cells have also been described. In preclinical tests, AS1411 shows efficacy toward xenograft models, including non-small cell lung, renal, and breast cancers. It entered clinical trials in 2003 and passed phase II trials for acute myeloid leukemia. A subsequent phase II trial for renal cell carcinoma started in 2008 (clinical trial ID NCT00740441). [66] NOXA12 (Olaptesed pegol; Noxxon) is an L-form RNA aptamer known as Spiegelmer and is used for cancer therapy. NOX-A12 can bind to its target chemokine CXCL-12 and blocks its interaction with its receptor. [67] This disrupts the tissue gradient of CXCL-12 and reduces the cancer cell homing that might lead to tumor metastasis and drug resistance. [68] Currently, phase II clinical trials for NOX-A12 are underway for the treatment of chronic lymphocytic leukemia and refractory multiple myeloma (clinical trial IDs NCT01486797 and NCT01521533). [67] Aptamer based cancer therapeutics have immense potential for precise and less toxic treatment for cancer patients. [46]

6. Aptamers as diagnostic agents

Aptamers can be used *in-vitro* and *in-vivo* as well. [69] In terms of *in vivo* diagnostics, 'escort' aptamers can be implied as vehicles for a detectable molecules, such as radionuclides, fluorophores etc. [70–72] The development of new agents like radio-pharmaceuticals is challenging. There are some important factors such as efficiency of the radiolabeling process, specific activity (radioactivity per moles e.g. Ci/μmol), chemical purity, radiochemical and chemical stability and shelf life of the final product. [73] Mostly, radiolabeling strategies for aptamers are similar as for proteins, or antibodies. Aptamers can be easily chemically modified at its 5′ or 3′ end with a desired functional group for radiolabeling (**Figure 4**).

Radiohalogens (fluorine-18, bromine-76, iodine-125 etc.) are the most commonly used for radiolabelling oloigonuclotides which are often accompanied with prosthetic groups. Recently, click-chemistry for radiofluorination was demonstrated on antisense oligonucleotides and siRNAs. [74, 75] Another report used photoconjugation as strategy for the radiofluorination of an aptamer. [76] Oligonucleotides have also been radiolabeled with the radiohalogens such as bromine-76 for PET imaging and iodine-123 for SPECT (Single photon emission computed tomography) imaging. In addition, iodine-125 has been used to radiolabel antisense oligonucleotides, aptamers and spiegelmers for theranostic applications. Due to the harsh and non-aqueous reaction conditions usually needed to radiolabel prosthetic groups, it is performed before the conjugation process to the oligonucleotide. [73]

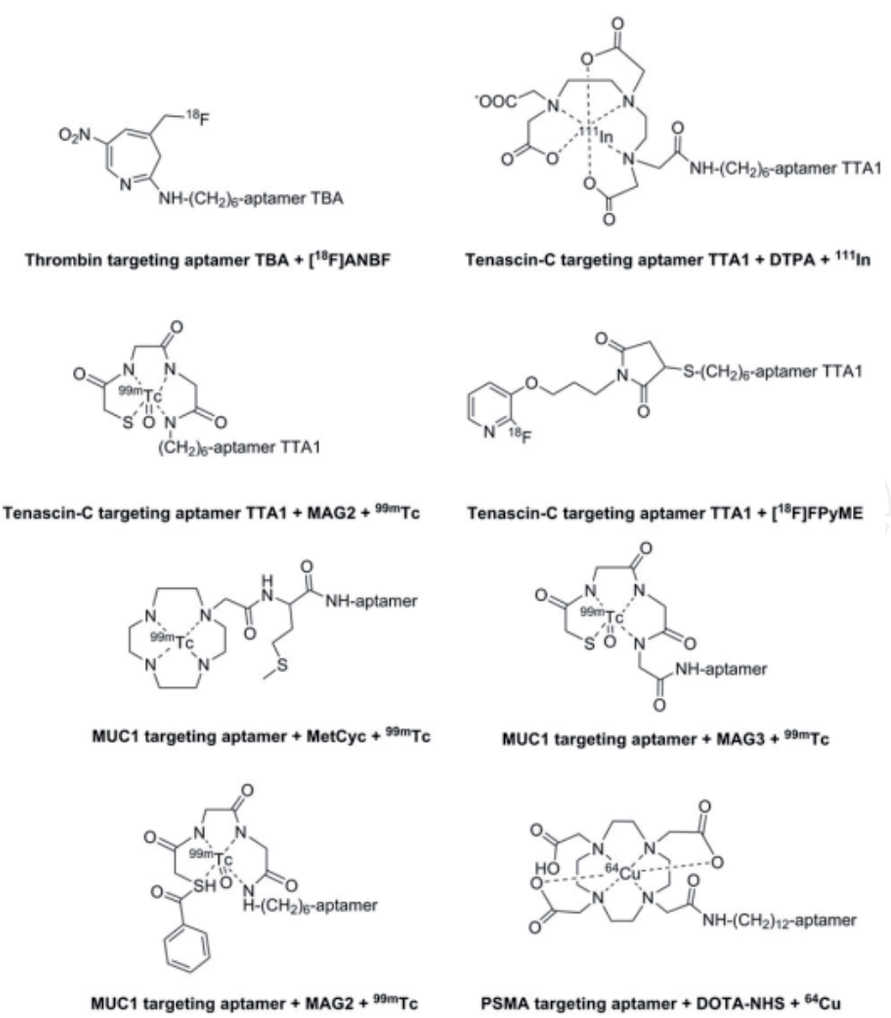

Figure 4.
Aptamers modified with radiolabelled molecules for disase diagnosis (Figure adapted from Gijs et al*) [73].*

Till date, a plethora of aptamers have been modified or labelled with radioactive molecules. Aptamers against several important biomarkers like PMSA, Tenascin C, thrombin, MUC1 were already exploited for radiolabelling. Aptamer-based radiopharmaceuticals were primarily developed for imaging and therapy of cancer diseases, metabolic disorders and others. The aptamers are mainly radiolabeled with technetium-99 m for SPECT (Single photon emission computed tomography), PET (Positron emission tomography) imaging. Very few aptamers were published related to PET imaging, and there is only one study of radiolabeled aptamers for therapy by Bandekar *et al.* [77] Other radiolabeled aptamers have only been tested for preclinical applications or in the course of preclinical assesement.

Molecular nuclear imaging technique is a diagnostic process of non-invasive visualization of any disease *in-vivo* at molecular level with high precision. For nuclear imaging, the probes used for radiolabelling has to be modified accordingly. Aptamers are the most promising candidates with versatile modification capibility, can be easily engineered for various imaging and other diagnostic purposes.

The first radiolabeled aptamer for nuclear imaging was discovered by Charlton et al. A DNA aptamer, NX21909, was selected against human neutrophil elastase, an enzyme which is secreted by neutrophils and macrophages during inflammation to kill pathogens. [78]

Aptamer TTA1, an RNA aptamer targeting the extracellular matrix protein tenascin C (TN-C), was the first radiolabeled aptamer which was used as molecular cancer imaging agent. Aptamer TTA1 was generated by a cross-over SELEX involving the purified recombinant TN-C protein and TN-C-positive U251 glioblastoma cells. [79, 80]

7. Lightup aptasensors for diagnostic applications

There are a unique group of aptamers (generally RNA aptamers) which can bind specifically with their cognate fluorogen molecules like DFHBI, thiazole orange, thioflavin T etc. [49, 81, 82] The non-fluorescent moelcules (native unbound state) become fluorescent (bound state) after binding to the aptamers and these "light-up" aptamers generate fluorescence signal. In the omni-presence of target molecules (small pre-miRNAs) and malachite green (fluorogen) light up aptasensors 'malaswitch' exhibit fluorescence enhancement. [83] We can engineer the small-molecule specific aptamers (like aptamers for some pesticides, toxins, small metaboltes) in such a way, that combined with light-up aptamers, they can generate a detectable signal. Light up aptasensors are promising alternative biosensor for label free sensitive detection of small molecules. [84]

8. Future perspectives

With more focus on *in vivo* studies for potential clinical applications, aptamers can be developed in combination with DNA nanostructures, nanomaterial, or microfluidic devices as diagnostic probes or therapeutic agents for cancers, infectious diseases, genetic, metabolic, neurological disorders, lifestyle diseases and several others. The use of aptamers as targeting agents in drug delivery can also be explored. Aptamers might be exploited to develop portable, low-cost and robust diagnostic kit using simple devices for real-time and on-site POC (point-of-care) detection and monitoring, instead of the laborious and time-consuming diagnostic tests currently available only in clinical labs. Regarding therapeutics approach, there is still untapped potential in the combination of the target recognition and strong

binding property of aptamers with exquisitely designed nanomaterials. It can be used as an effective alternative drug delivery platform. Variety of materials such as liposomes, polymer vesicles and silica nanoparticles, combined with DNA/RNA aptamers, has shown feasibility for use in *in vivo* targeted drug delivery. [85, 86] The integration of diagnostic capability with therapeutic interventions termed, as "Theranostics" is critical to address the challenges of disease heterogeneity and adaptation. Although aptamers have immense potential as theranostic agents, tailor-made modifications, validation of experiments need to be executed before aptamer-based drug delivery can reach clinical trials and eventually the patient management system.

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