

Nucleic Acid Aptamers: New Methods for Selection, Stabilization, and Application in Biomedical Science

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Abstract

The adoption of oligonucleotide aptamer is well on the rise, serving an ever increasing demand for versatility in biomedical field. Through the SELEX (Systematic Evolution of Ligands by EXponential enrichment), aptamer that can bind to specific target with high affinity and specificity can be obtained. Aptamers are single-stranded nucleic acid molecules that can fold into complex three-dimensional structures, forming binding pockets and clefts for the specific recognition and tight binding of any given molecular target. Recently, aptamers have attracted much attention because they not only have all of the advantages of antibodies, but also have unique merits such as thermal stability, ease of synthesis, reversibility, and little immunogenicity. The advent of novel technologies is revolutionizing aptamer applications. Aptamers can be easily modified by various chemical reactions to introduce functional groups and/or nucleotide extensions. They can also be conjugated to therapeutic molecules such as drugs, drug containing carriers, toxins, or photosensitizers. Here, we discuss new SELEX strategies and stabilization methods as well as applications in drug delivery and molecular imaging.

Key Words: Aptamer, Stabilization, Modification, SELEX, Imaging, Drug delivery system

INTRODUCTION

For diagnosis, therapy, or imaging of certain disease, specific targeting is an important issue. To this end, antibodies have been widely used to target molecules of interest and made a tremendous contribution to a wide range of applications based on molecular recognition (Jayasena, 1999). The IgG2a CD3-specific transplant rejection drug OKT3 (mumomab) was the first FDA-approved therapeutic antibody (Hooks *et al.*, 1991). Subsequently, diverse therapeutic antibodies have been applied to treat diseases (Knight *et al.*, 1993; Mazumdar and Greenwald, 2009; Pageau, 2009; Banaszynski and Kolesar, 2013). However, many limitations have been identified, including difficulties with production, immunogenicity, expensiveness, reoptimization with each new batch, sensitivity to temperature, and irreversible denaturation. Recently, chemical antibody called aptamer has emerged as a new targeting molecule.

Aptamers consist of nucleic acids that bind to diverse targets with high affinity and selectivity. They are screened through an *in vitro* process and usually have higher binding affinity than traditional antibody. Aptamers are produced chemically, and

no or little batch-to-batch variation is observed during aptamer production. Furthermore, aptamers can be easily modified to chemically conjugate with other molecules. Aptamer can also undergo reversible denaturation at high temperature, making it a very versatile tool for drug loading and antidote application. Moreover, aptamers elicit little or no immunogenicity in therapeutic applications (Eyeteck Study Group, 2002; Foy *et al.*, 2007; Zhu *et al.*, 2012a). The first aptamer-based drug called Macugen (pegaptanib) was FDA-approved in 2004 (Ruckman *et al.*, 1998), and many studies proceeded to test efficacy.

Here, the up-to-date methods for SELEX processes are introduced. Moreover, diverse strategies to increase aptamer stability in biological fluid are discussed. Also, its utility as a targeting molecule for imaging, diagnosis, therapy and targeted drug delivery are discussed.

WHAT IS AN APTAMER?

Aptamer is a single-stranded nucleic acid oligomer made of RNA or DNA which can bind to specific target molecule with high affinity and selectivity. Secondary or tertiary struc-

Open Access <http://dx.doi.org/10.4062/biomolther.2013.085>

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Received Oct 16, 2013 Revised Nov 5, 2013 Accepted Nov 5, 2013

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tures of aptamers allow target binding and determine target specificity and affinity (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Schneider *et al.*, 1995; Mosing *et al.*, 2005; Chen *et al.*, 2009). In the early 1990s, several research laboratories were independently experimenting with what Jack W. Szostak's group termed "*in vitro* selection" (Ellington and Szostak, 1990). To describe molecular recognition properties for what were nucleic acid-based ligands, they coined the term 'aptamer' using the Latin word "aptus", meaning "fitting" and the Greek word "meros", meaning "particulate". But naming aptamers was not nearly as interesting as discovering that their properties compete quite well with those of antibodies. Targets of aptamer may include, but are not limited to, metal ions (Kawakami *et al.*, 2000), small organic compounds (Mann *et al.*, 2005), biological cofactors (Lauhon and Szostak, 1995; Holland *et al.*, 2000), metabolites (Bruno *et al.*, 2008), proteins (Ruckman *et al.*, 1998; White *et al.*, 2001; Savla *et al.*, 2011) and whole organisms such as a virus (Tang *et al.*, 2009), bacteria (Hamula *et al.*, 2011), yeast (Kolesnikova *et al.*, 2010), or mammalian cell (Chen *et al.*, 2009). Aptamers can be easily modified by various chemical reactions to introduce functional groups and/or nucleotide extensions. Aptamers can be easily conjugated to therapeutic molecules such as drugs, drug containing carriers, toxins, or photosensitizers. Thus, aptamers are promising escort molecules for drug delivery systems.

NEW METHODS FOR IN VITRO SELECTION OF AP- TAMERS

SELEX is an *in vitro* selection method designed to identify aptamers that are selectively bound to target molecules with high affinity. Substantive studies on aptamers have progressed since the *in vitro* selection process called SELEX was first reported by Gold's and Szostak's groups (Ellington and Szostak, 1990; Tuerk and Gold, 1990). First, the nucleic acid library, which consists of 10^{14} - 10^{15} random oligonucleotide strands, is incubated with a target molecule. Then, the target-bound oligonucleotide strands are separated from the unbound strands. The target-bound DNA or RNA strands are eluted from the target molecule and amplified via polymerase chain reaction to seed a new pool of nucleic acids. This selection process is continued for 6-15 rounds with increasingly stringent conditions, which ensure that the nucleic acid obtained has the highest affinity to the target molecule (Fig. 1). SELEX method can be modified in a variety of ways to increase the specificity of aptamer and efficiency of SELEX.

Counter-SELEX

The counter-SELEX method was introduced to increase the efficiency of aptamer selection by traditional SELEX (Fig. 1) (Jenison *et al.*, 1994). Compared to traditional SELEX, counter-SELEX has a pre-clearing step using closely related structural analogs of the target to effectively discard non-spe-

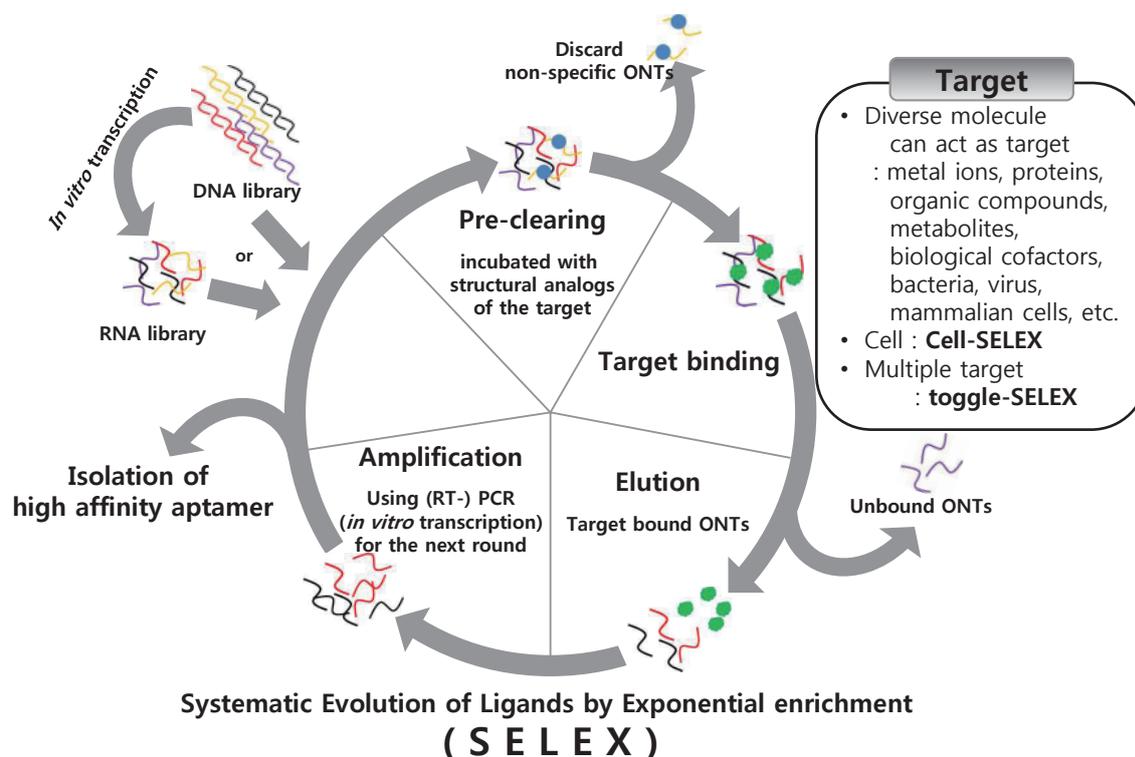


Fig. 1. Overview of SELEX scheme. Aptamers can be obtained through an iterative selection process known as SELEX (systematic evolution of ligands by exponential enrichment) by using single-stranded DNA or RNA. An initial pool of 10^{14} - 10^{15} random oligonucleotide (ONT) strands are subjected to binding with the target. Unbound ONTs are discarded and RT-PCR or PCR is performed to amplify the target-bound ONTs. This selection process is repeated 6-15 times using amplified ONTs as a new pool. This way, aptamers having high specificity and affinity are screened. Diverse molecules can be the target of the SELEX, including metal ion, protein, organic compound and cell. Toggle-SELEX performs SELEX with two different target molecules to obtain bispecific aptamers.

cific aptamers. This allows dramatic improvement in aptamer selection and can also be applied to other modified SELEX methods.

Cell-SELEX

The SELEX target is not limited to an individual molecule. The cell-SELEX strategy has been described that utilizes living cells as the SELEX target (Fig. 1). Although traditional SELEX is typically carried out using purified target molecules, whole live cells are also employed as selection targets. Not only normal/abnormal mammalian cells such as virus-infected and cancer cells but also live pathogenic organisms such as bacteria and viruses have been utilized as cell-SELEX targets (Tang *et al.*, 2009; Hamula *et al.*, 2011). The aptamers generated are functional with an original conformation of the target molecule on live cells. Compared to aptamers selected using a purified target, a cell-SELEX-derived aptamer has more possibility to be used directly for *in vivo* and clinical applications. A screened aptamer resulting from cell-SELEX using abnormal cells can be used to detect disease or cancer. Moreover, biomarkers can be used to identify the aptamer target for a specific abnormality (Blank *et al.*, 2001). Many strategies are available to select an anti-virus aptamer. First, SELEX can be performed using the virion directly (Wang *et al.*, 2000). Second, aptamers can be generated that specifically bind to virus-infected cells. Through virus infection, viral proteins exist on the host cell surface due to the viral gene or enveloped protein. SELEX provides screening of virus-specific aptamers that recognize virus infected cells. Furthermore, aptamers can be selected for host cell proteins if their expression levels are upregulated upon viral infection. Lastly, stable cell lines can be generated that express viral protein as a cell-SELEX target, and the aptamer obtained can be used to detect virus infected cells (Chen *et al.*, 2009). Furthermore, novel biomarkers can be discovered using the cell-SELEX technique. Because the target molecules of the aptamers generated by cell-SELEX may be previously unrecognized as cell-specific surface molecules, they could be novel biomarkers. Thus, cell-SELEX can be applied for *de novo* discovery of novel biomarkers for a desired cell by identifying the aptamer binding partner. The cell-SELEX concept can be extended for *in vivo* selection, which was first designed using a hepatic tumor xenograft mouse model (Mi *et al.*, 2010). Oligonucleotides were injected intravenously, liver tumors were harvested, and the injected RNA molecules were extracted and amplified. A tissue-specific aptamer can be more easily screened through this *in vivo* selection process. So, a screened aptamer may be a useful target for a tissue of interest without non-specific biodistribution in the *in vivo* application.

Capillary Electrophoresis-SELEX

The SELEX process has disadvantages in that it is time consuming to repeat the rounds. Some molecular biological methods have been introduced to SELEX to overcome these disadvantages. Capillary electrophoresis-SELEX (CE-SELEX) was designed for selecting aptamers to reduce repeating rounds with low dissociation constants (Mosing *et al.*, 2005). In this method, the nucleic acids that bind the target migrate with different mobilities than those of unbound sequences, allowing them to be collected as separate fractions. Although traditional SELEX requires 6-12 rounds, CE-SELEX significantly reduces the number of rounds and increases binding affinity

(Schneider *et al.*, 1995). CE-SELEX decreases the time, effort, and cost to screen a higher affinity aptamer compared to those of traditional SELEX. Based on CE technology, a non-SELEX method that selects an aptamer without amplification was demonstrated (Berezovski *et al.*, 2006). Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), a highly efficient affinity method, has been used to partition the DNA-target complex from the free DNA. An aptamer with nanomolar affinity for the protein farnesyltransferase was selected in a single round of partitioning using NECEEM (Berezovski *et al.*, 2005). The advantage of non-SELEX is its speed and simplicity. NECEEM-based non-SELEX selection took only 1 h in contrast to several days or several weeks required for a traditional SELEX procedure by conventional partitioning methods. This procedure can be automated using a single commercially available capillary electrophoresis instrument.

One step MonoLEX

The MonoLEX approach combines a single affinity chromatography step with subsequent physical segmentation of the affinity resin and one single final exponential amplification step of the bound aptamers (Nitsche *et al.*, 2007). Using the Biomek 2000, an automatic SELEX device (Cox *et al.*, 1998), Cox *et al.* demonstrated that 10 rounds of selection against eight targets in parallel can be performed in 4-5 days.

Microfluidic SELEX

Microfluidic SELEX (mSELEX) was designed that combines traditional SELEX with a microfluidic system. Protein immobilization is the most important aspect of mSELEX. A mSELEX prototype device was developed and biotinylated lysozymes were immobilized to silica microline previously coated with streptavidin (Hybarger *et al.*, 2006). In another study, a magnetic bead-based SELEX process with microfluidics technology and a continuous-flow magnetic activated chip-based separation device was developed (Lou *et al.*, 2009). Using this mSELEX method, an enriched aptamer pool was obtained that tightly bound to the light chain of recombinant *Botulinum* neurotoxin type A after a single round of selection

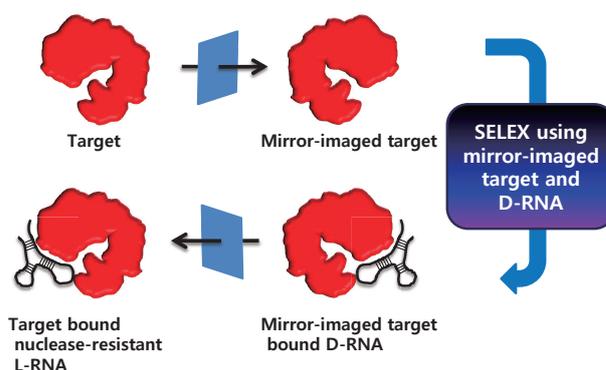
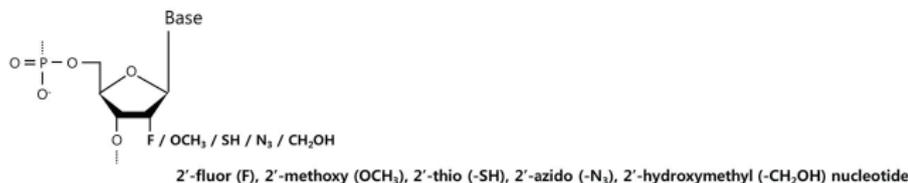
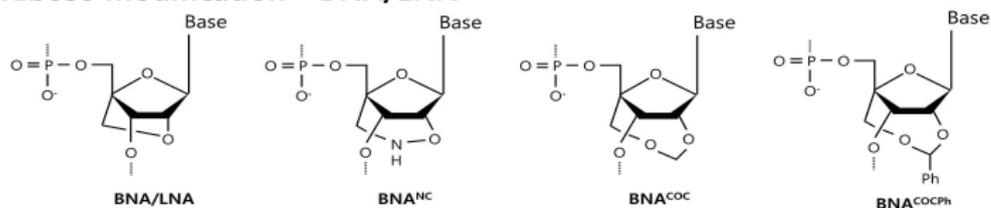


Fig. 2. Method for obtaining Spiegelmer. SELEX is performed with mirror-imaged target and D-form RNA. Based on the selected D-form RNA sequence, L-form RNA aptamer can be synthesized. Now L-RNA, the chiral form of the acquired D-form RNA, can bind to natural target. This L-form RNA is nuclease-resistant and suitable for *in vivo* application. The word 'Spiegelmer' is derived from German 'Spiegel' meaning 'mirror'.

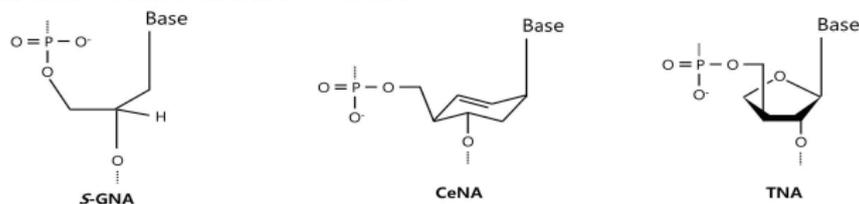
Ribose Modification – 2'-modification



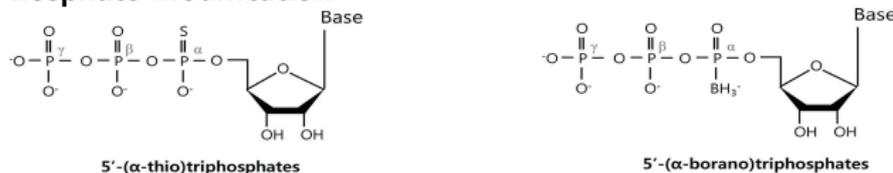
Ribose Modification – BNA/LNA



Ribose Modification – Others



Phosphate Modification



Base Modification

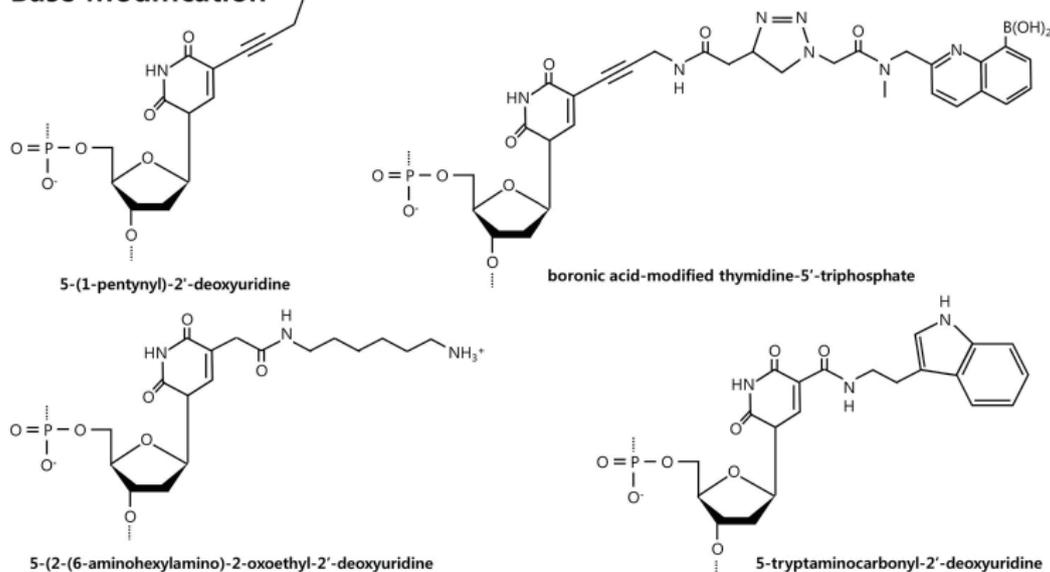


Fig. 3. Structure of chemically modified nucleotides (Modified form Kasahara and Kuwahara, 2012). The simplest ribose 2' modification is widely used to increase aptamer stability *in vivo*. Phosphate and base modification are also used for this purpose. Substitution of F, OCH₃, SH or CH₂OH for 2'-OH (H) is widely used. BNA/LNA was designed to structurally protect 2' site. In addition, thiol (S) or borane (BH₃) group is introduced to a phosphate to strengthen oligonucleotide backbone. Functional groups can also be introduced into the base.

with a 33 nM Kd value. Recently, a novel formulation of sol-gel protein microarray material was developed, which elicited physical properties suitable for protein immobilization, protein-protein interactions, and immunoassays (Kim *et al.*, 2006). Based on sol-gel, the authors developed a microfluidic device and method for binding nucleic acids to immobilized proteins and efficiently eluting and recovering the intact nucleic acids (Park *et al.*, 2009). The mSELEX system enhanced selection efficiency and reduced time and effort compared to those of traditional SELEX.

Toggle-SELEX

Furthermore, toggle-SELEX has been established for *in vitro* animal model assays using a single aptamer (Fig. 1). The “toggle” selection process was repeated during SELEX rounds using a target applied to human thrombin for even rounds and porcine thrombin for odd rounds to select a species cross-reactive aptamer (White *et al.*, 2001). A species cross-reactive aptamer from the toggle-SELEX strategy may contribute directly to animal models and clinical applications. Thus, toggle-SELEX makes the aptamer technique closer to a “bench-to-clinic” concept.

Based on these novel methods, high-throughput screening

of aptamers and improved efficiency of the SELEX process is possible. Moreover, consilience technology such as microfluidic technology and engineering could reduce effort, time, and cost of aptamer selection and analysis, while increasing its efficacy. Taken together, aptamers may play a key role in future theragnosis because of higher binding affinity, lower cost, and ease of synthesis compared to antibodies.

STABILIZATION OF THE OLIGONUCLEOTIDE APTAMER

One of the ultimate goals during aptamer selection is a clinical application or an escort molecule. However, unmodified nucleic acids *per se* are unstable in biological fluids due to enzymatic degradation or a short half-life. Various strategies have been established to increase serum stability and overcome the degradation of oligonucleotides by nuclease (Fig. 2, 3).

For example, the first FDA-approved aptamer drug Macugen, which specifically binds to VEGF₁₆₅, is a modified oligonucleotide prepared by SELEX processes (Ruckman *et al.*, 1998). The RNA aptamer was screened from a library of modified RNAs involving 2'-fluoropyrimidine nucleotides (U, C) and natural purine nucleotides (A, G) in which the 2'-posi-

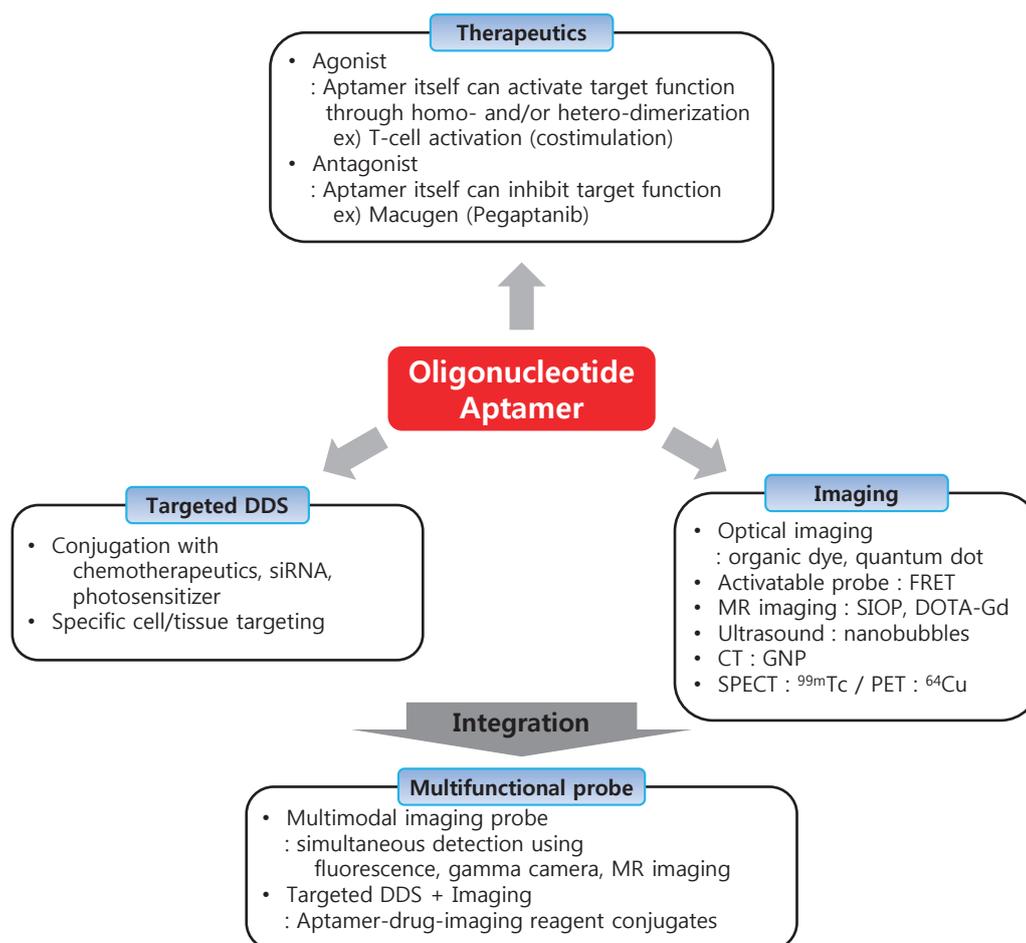


Fig. 4. Applications of aptamer. Aptamer can be used as therapeutics, targeted drug delivery system (DDS) and imaging. These three parts can be put together using aptamer and conjugation in multifunctional probe.

tion of ribose is a nuclease attack site. Then, the natural A and G are properly replaced with 2'-methoxy nucleotides to enhance nuclease resistance and avoid a decrease in affinity. Like Macugen, 2'-fluor or methoxy or amine substituted nucleotides was widely used to protect from nuclease (Zhou *et al.*, 2008, 2011a, 2011b, 2013) (Fig. 3). Additionally, 2'-thio (-SH), 2'-azido (-N₃), 2'-hydroxymethyl (-CH₂OH) modified nucleotides was developed for protection of oligonucleotide. Moreover, bridged/locked, S-glycerol, cyclohexenyl, and threose nucleic acid (BNA/LNA, S-GNA, CeNA, and TNA, respectively) were applied to aptamer building block (Horhota *et al.*, 2005; Ichida *et al.*, 2005; Kempeneers *et al.*, 2005; Tsai *et al.*, 2007; Kuwahara *et al.*, 2008; Barciszewski *et al.*, 2009; Kasahara *et al.*, 2013) (Fig. 3). For example, Schmidt group reported that LNA modification increased plasma stability more than 2'-OMe modification using TTA1 aptamer which bind to Tenascin-C (Schmidt *et al.*, 2004). Furthermore, for the substitute phosphodiester bond in backbone, 5'-(α -thio)triphosphates and 5'-(α -borano) triphosphates were developed (Andreola *et al.*, 2000; Lato *et al.*, 2002), with increased stability in the biological milieu (Yang *et al.*, 1999). Moreover, peptide nucleic acid (PNA) whose sugar-phosphate backbone is changed to repeating N-(2-aminoethyl)-glycine units linked by peptide bonds, increases biostability (Wittung *et al.*, 1994). In other aspect, base modification could increase aptamer stability in novel thrombin aptamer containing 5-(1-pentynyl)-2'-deoxyuridine (Latham *et al.*, 1994). In the thalidomide aptamer, a modified deoxyuridine bearing a cationic functional group via a hydrophobic methylene linker at the C5 position (Shoji *et al.*, 2007) could improve stability against nucleases and the bind-

ing affinity to target. Fibrinogen aptamer using boronic acid-modified thymidine-5'-triphosphate (Li *et al.*, 2008), TNFRSF9 aptamer using 5-tryptaminocarbonyl-dU (Vaught *et al.*, 2010) were also reported (Fig. 3). Certain thermophilic and mutant polymerases such as Pwo, Vent (exo-), Deep Vent (exo-), and KOD Dash have been actively developed for incorporation of modified nucleotides during polymerization (Gudima *et al.*, 1998; Kuwahara *et al.*, 2003; Leal *et al.*, 2006; Kuwahara *et al.*, 2009; Veedu *et al.*, 2009; Kuwahara *et al.*, 2010).

A chiral form of an aptamer is an alternative strategy to prevent enzymatic degradation in biological fluids by altering nuclease recognition. NOXXON Pharma established Spiegelmer (from German Spiegel "mirror", Fig. 2) to screen nuclease-resistant aptamers, which are RNA-like molecules built from L-ribose units. This Spiegelmer technology circumvents nuclease activity in biological fluids. In short, a chiral form of the target molecule and D-form oligonucleotides are used during the SELEX process. Subsequently, an L-form of the aptamer is synthesized that has the same sequence as the selected D-form aptamer. Thus, an enzymatic degradation-insensitive aptamer, which also binds to the original target molecule, can be obtained (Fig. 2) (Klussmann *et al.*, 1996; Nolte *et al.*, 1996; Williams *et al.*, 1997; Leva *et al.*, 2002).

Several authors have inhibited *in vivo* clearance to increase half-life of aptamers. An anti-platelet derived growth factor aptamer was conjugated with 40 kDa polyethylene glycol (PEG) to slow down renal clearance (Floegel *et al.*, 1999). In another study, Healy *et al.* reported an aptamer conjugated with 20 and 40 kDa PEG to prolong aptamer half-life in circulation (Healy *et al.*, 2004). Furthermore, the DNA aptamer ARCC1779,

Table 1. Undergoing aptamer clinical trials.

| Name | Target | Condition | Phase |
|-----------------------------|--|---|-----------|
| Pegaptanib sodium (Macugen) | VEGF | Age-Related Macular Degeneration | Approved |
| EYE001 | VEGF | Macular Degeneration / Choroidal Neovascularization | Phase 2/3 |
| E10030 | PDGF | Age-Related Macular Degeneration | Phase 2 |
| Pegaptanib sodium (Macugen) | VEGF | Diabetic Macular Edema | Phase 2 |
| AS1411 | Nucleolin | Acute myeloid leukemia | Phase 2 |
| ARC1779 | A1 domain of von Willebrand Factor | von Willebrand Disease / Purpura / Thrombotic Thrombocytopenic | Phase 2 |
| REG1 (RB006 and RB007) | Coagulation factor IXa | Coronary Artery Disease | Phase 2 |
| NOX-E36 | CCL2 | Chronic Inflammatory Diseases / Type 2 Diabetes Mellitus / Systemic Lupus Erythematosus | Phase 1 |
| NOX-A12 | CXCL12 | Autologous or Hematopoietic Stem Cell Transplantation | Phase 1 |
| ARC19499 | tissue factor pathway inhibitor (TFPI) | Hemophilia | Phase 1 |
| ARC1905 | Complement Component 5 (C5) | Age-Related Macular Degeneration | Phase 1 |
| EYE001 | VEGF | von Hippel-Lindau Disease | Phase 1 |

which binds to the A1 domain of the von Willebrand factor, was also 5'-conjugated with a 20 kDa PEG to reduce renal filtration (Diener *et al.*, 2009). Many studies are underway to increase the biostability and half-life of aptamers in different settings. However, post-SELEX modification can influence target binding affinity or secondary and tertiary structure formation, which, in turn, can alter aptamer function.

APTAMERS AS AGONISTS/ANTAGONISTS

Individual aptamers can be equipped in a modular manner with additional functions and can be specifically tailored for many potential applications in biotechnology and molecular medicine (Fig. 4). In most cases, aptamers not only bind their cognate protein but also efficiently inhibit the function of target as an antagonist. Many aptamer therapeutic studies have been conducted to treat certain diseases by inhibiting therapeutic target activity and decreasing the partner binding property (Holahan *et al.*, 2011; Gissel *et al.*, 2012; Siller-Matula *et al.*, 2012; Vater *et al.*, 2013). Macugen, the first FDA-approved aptamer drug for age-related macular degeneration, is a typical antagonistic aptamer (Zhou and Wang, 2006). Macugen (pegaptanib) is a 28-base RNA oligonucleotide with two branched 20 kDa PEG moieties (Ruckman *et al.*, 1998). It selectively binds to the vascular endothelial growth factor (VEGF)₁₆₅ isoform when introduced intravitreally. Thus, the intracellular signal cascade is inhibited by the pegaptanib-VEGF₁₆₅ complex, which cannot bind to the VEGF receptor. Thus, pegaptanib has the potential to inhibit growth of blood vessels and vascular leakage by inhibiting the intracellular signal cascade induced by the pegaptanib-VEGF₁₆₅ complex. Diverse therapeutics based on antagonistic functions of aptamers are undergoing clinical trials (Table 1). They inhibit the signaling cascade by decreasing activity of the target protein or altering binding with the physiological binding partner.

The first report about aptamers as agonists came from the study that reported agonistic use of an aptamer with 4-1BB, which is a major costimulatory receptor (McNamara *et al.*, 2008). 4-1BB increased expansion and survival of activated T cells. Dimerization of the anti-4-1BB aptamer treated with the CD3 antibody not only increased T-cell activation and interferon- γ *in vitro* but also tumor regression *in vivo*. Moreover, Dollins *et al.* designed an aptamer-based agonist by dimerizing the OX4 RNA aptamer, which targets one of the tumor necrosis factor receptor families (Dollins *et al.*, 2008). Two 2'-fluoropyrimidine-modified RNA aptamers were selected that bind to CD28 (CD28Apt2 and CD28Apt7) (Pastor *et al.*, 2013). That study suggested that an agonistic dimerized anti-CD28 aptamer increases costimulatory efficacy and reduces therapeutic dose and toxicity compared to conventional agonistic anti-CD28 antibodies. Similar research on aptamers for use as costimulatory agonistic molecules is being conducted (Suntharalingam *et al.*, 2006; Römer *et al.*, 2011). Moreover, not only homodimerization of an aptamer for agonistic use but also heterodimerization of two individual aptamers that recognize different targets for dual purposes, specific targeting and stimulating, have been reported (Gilboa *et al.*, 2013). Two individual aptamers were heterodimerized including a 4-1BB binding aptamer to stimulate the immune response and a prostate-specific membrane antigen (PSMA) binding aptamer for prostate cancer targeting. This bispecific aptamer

was developed to regress prostate tumors through the host immune response. A bispecific aptamer is more effective than and synergizes with vaccination and exhibits a superior therapeutic index compared with nontargeted costimulation with 4-1BB antibodies or 4-1BB aptamers. Agonistic/antagonistic aptamers offer significant advantages over antibodies in terms of synthesis, cost, and immunogenicity as well as conjugation chemistry.

TARGETED DRUG DELIVERY

Targeted drug delivery is one of the most important areas in therapeutic research. Aptamers exhibit many desirable properties for targeted drug delivery such as ease of selection and synthesis, high binding affinity and specificity, low immunogenicity, and versatile synthetic accessibility. The therapeutic agents that have been delivered using aptamers as the targeting ligands can be categorized into three major classes of drugs, toxins, and small interfering RNAs (siRNAs) (Fig. 4) (Zhang *et al.*, 2011).

Doxorubicin (Dox) used in chemotherapy for hematological malignancies, carcinomas, and soft tissue sarcomas can intercalate within the double-stranded CG sequences of DNA and RNA. Based on the intercalation property of Dox to the aptamer, targeted Dox delivery for targeting cancer and Dox-mediated cancer therapy has been actively studied (Liu *et al.*, 2012; Meng *et al.*, 2012; Subramanian *et al.*, 2012). A Dox intercalated aptamer that binds to PSMA, which is abundantly expressed on the cell surface of a human prostatic adenocarcinoma (LNCaP) metastatic lesion has been studied. PSMA expressing LNCaP-specific cell death was observed by treatment of Dox-intercalated PSMA-specific 2'-fluoropyrimidine aptamer, but not in PSMA-negative PC3 cells (Bagalkot *et al.*, 2006). Covalent conjugation of aptamer harboring the drug is relatively stable such that drug release during transport is prevented before the drug localizes at its cellular target. Drugs can be chemically modified to form stable ester, amide, and disulfide bonds that serve to conjugate the drug to the aptamer. The sgc8c DNA aptamer which specifically binds to protein tyrosine kinase 7 (PTK7) was covalently conjugated with Dox using hydrazone (Huang *et al.*, 2009). As a result, PTK7 expressing CCRF-CEM cell-specific Dox delivery and cell death were observed. Other chemotherapy drugs such as daunorubicin, thalidomide, and dactinomycin can also intercalate to double-stranded oligonucleotides. They are expected to show a good therapeutic effect in specific cancers, and their application in various aptamers is actively sought.

The small size and lipid bilayer structure of liposomes make them an excellent drug delivery carrier. Aptamer-tagged liposomes were established that contained the anticancer drug paclitaxel and a fluorescent dye (Nile Red) for tumor-specific drug delivery and imaging (Aravind *et al.*, 2012). In another study, Cy3-labeled, carboxylated, thiolated oligonucleotide aptamer (thioaptamer) against E-selectin (ESTA, E-Selectin ThioAptamer) was designed. This modified ESTA was coupled to an amino PEGylated stealth liposome to make the vasculature targeting ESTA-conjugated liposome (Mann *et al.*, 2011), which achieved tumor vasculature-specific drug delivery. Similarly, this aptamer can be covalently coupled via NHS chemistry to toxic proteins. The targeting properties of the anti-PSMA aptamer can also be combined with the toxic characteristics

of gelonin, a ribosomal toxin (Chu *et al.*, 2006a). Moreover, a photosensitizer-aptamer conjugate was developed for cancer-specific delivery to be used in photodynamic cancer therapy (Yang *et al.*, 2011; Han *et al.*, 2013).

siRNA delivery to specific cells using an aptamer has been actively investigated. In these studies, receptor-mediated endocytosis of the aptamer is necessary. In general, non-covalent conjugation through a connector and covalent linkage through formation of an aptamer-siRNA chimera are two major strategies for aptamer and siRNA conjugation. In 2006, two biotinylated anti-PSMA aptamers were linked to two biotinylated anti-lamin A/C siRNAs using streptavidin as the connector (Chu *et al.*, 2006b). As a result, PSMA-positive prostate cancer cell-specific siRNA was internalized and siRNA-specific gene expression was inhibited *in vitro*. However, the siRNA release mechanism and an *in vivo* experiment have not been demonstrated. The “sticky bridge” concept was used to non-covalently conjugate the anti-gp120 aptamer with siRNAs to suppress HIV replication *in vitro* (Horhota *et al.*, 2005). An aptamer-siRNA chimera was developed using an aptamer consisting of single-stranded oligonucleotides. An aptamer was covalently linked to the passenger strands of siRNAs followed by annealing of the siRNA guide strands to the passenger strands to create a functional siRNA duplex (McNamara *et al.*, 2006). That study found that aptamer-siRNA chimera-mediated gene silencing was dependent on Dicer and occurred via the RNAi pathway. Moreover, anti-PSMA aptamer-Plk1 did not trigger interferon responses *in vitro*, and promoted tumor regression in a xenograft model of prostate cancer. Structural modifications were introduced that increased the valency of the chimera aptamer to enhance therapeutic efficacy of the aptamer-siRNA targeting PSMA-expressing system (Wullner *et al.*, 2008). In their design, the siRNA sequence served as a linker to join the two aptamers, or to join the 3' end of one of the aptamer dimers as a shRNA. Such modifications did not affect the critical sequence of the targeting regions and facilitated formation of the active conformation. Targeted siRNA delivery using an aptamer-siRNA chimera is being actively studied (Neff *et al.*, 2011; Zhu *et al.*, 2012b; Hussain *et al.*, 2013).

APTAMER-BASED IMAGING

Another application of aptamers is diagnosis via *in vivo* and *in vitro* imaging, using an aptamer that is conjugated to a fluorophore, quantum dots, or other material such as gadolinium, which is useful for magnetic resonance imaging (MRI) (Fig. 4). Optical imaging is a cost-effective imaging method that typically uses fluorescent or bioluminescent molecules. Aptamer-based optical imaging can be divided into direct targeting and activatable probes (Hong *et al.*, 2011). Direct conjugation with an aptamer and a fluorescent molecule is widely studied (Shi *et al.*, 2010; Cui *et al.*, 2011; Talbot *et al.*, 2011; Zhang *et al.*, 2012; Song *et al.*, 2013; Zhang *et al.*, 2013). It is the simplest way to image via a visualized aptamer by fluorescence. Activatable probes are based on conformational changes in aptamer. It is designed such that fluorescence will “turn-on” when the aptamer binds to its target. Typically, a fluorescently labeled substrate is designed to be maximally quenched by a quencher in close proximity because of FRET (McIntyre and Matrisian *et al.*, 2003; Tsien, 2005). For example, Zhang *et al.*, designed a bifunctional fluorescent oligonucleotide probe for

the detection of adenosine triphosphate (ATP) and thrombin (Tmb). The molecular beacon contains two hairpin loops that serve as the sensing elements, and a fluorophore at one end and a quencher at the opposite end, as the reporter (Li *et al.*, 2013). “Turn-on” of fluorescence mechanism by quenching is widely used in aptamer-based biosensor. Comparing to a conventional “always-on” probe, the activatable probe could substantially minimize the background signal originating from non-target tissues, thereby giving significantly enhanced image contrast (Hong *et al.*, 2011). Not only optical imaging, but superparamagnetic iron oxide nanoparticle-aptamer conjugate was used to MR imaging (Yigit *et al.*, 2007). T1-weighted MR imaging was designed that uses aptamer-gadolinium-tetraazacyclododecanetetraacetic acid (DOTA-Gd) (Xu and Lu, 2011). It consists of anti-adenosine aptamer and its complementary sequence which conjugated with DOTA-Gd. Formation of adenosine-aptamer complex leads to release of DOTA-Gd-complementary sequence, which allows MR imaging.

Aptamer can also be applied for targeted ultrasound imaging using covalent conjugation of nanobubbles (Wang *et al.*, 2011). These aptamer-conjugated nanobubbles can be harnessed to release certain amount of drugs upon targeted ultrasound-trigger (Maul *et al.*, 2010). Another application of aptamer is radioisotope labeling and its use in SPECT and PET. The TTA1 aptamer that can bind to tenascin-C, which is overexpressed in many solid tumors, was labeled with ^{99m}Tc for SPECT imaging (Hicke *et al.*, 2006). ^{99m}Tc-labeled TTA1 aptamer exhibited rapid blood clearance with a circulation half-life of <2 min but tumor penetration in several xenograft models. The durable tumor retention in combination with fast blood clearance yielded an excellent tumor-to-blood ratio, and various tumors were clearly visualized by planar scintigraphy. Furthermore, PET imaging application was studied using ⁶⁴Cu-labeled RNA aptamer (Rockey *et al.*, 2011). In this study, the choice of chelators and radiolabeling parameters such as pH and temperature were investigated for the development of ⁶⁴Cu-labeled RNA aptamers for potential PET imaging. In other aspect, aptamer can be coupled to iodinated nanoparticles (NPs) or polymer-coated Bi₂S₃ NPs which can be used as computed tomography (CT) contrast agents (Cai and Chen, 2007). Because each imaging method has its own advantages and disadvantages, a multimodality imaging probe based on AS1411 aptamer was evaluated (Hwang *et al.*, 2010). For this purpose, a cobalt-ferrite NP coated with rhodamine was modified with the AS1411 aptamer, which was then conjugated to a chelating agent and further labeled with ⁶⁷Ga. This multimodal imaging probe could be simultaneously used for fluorescence, gamma camera and MR imaging *in vivo*.

There are also attempts to not only image but simultaneously treat the disease. Diagnosis and therapy or theragnosis can be much simpler and easier when the disadvantages of each part are complemented through conjugation with aptamers. Recently, drug-loaded aptamer-gold nanoparticle (GNP) conjugate was designed for combined CT imaging and chemotherapy (Kim *et al.*, 2010). The PSMA aptamer was conjugated with GNP for CT imaging, and Dox was loaded as a chemotherapeutic drug for prostate cancer targeting. This conjugate has been successfully used for targeting, imaging, and therapy of PMSA-expressing prostate cancer cells. Similarly, anti-mucin1 aptamer-QD-Dox conjugate was designed for optical imaging and chemotherapy of ovarian cancer (Salva *et al.*, 2011). The key to effective and successful imaging

would be the development of delivery platforms having high efficiency and ultrasensitive molecular probes for specific targets of interest.

SUMMARY AND CONCLUSIONS

Aptamers have many advantages compared to traditional targeting molecules and antibodies including high affinity, low cost and immunogenicity, thermal stability, ease of production and high reproducibility. Diverse molecules such as chemotherapy drugs, toxins, siRNAs, imaging probes, and nanomaterials can be conjugated to aptamers, which serves to meet the increasing demand for versatile applications. Many new SELEX methods have been developed to easily and rapidly select for an aptamer. Compared to the traditional SELEX method, microfluidic, CE-SELEX saves time and effort in addition to providing better affinity and selectivity. In the near future, high-throughput selection of aptamers will allow various rapid aptamer selections and this will help to replace the role of antibodies in many applications from research to the clinic. Aptamers help to overcome the problem of immunogenicity when using antibodies in clinical applications and have provided a safe therapy and/or diagnosis without an immune response. Furthermore, aptamers are easily obtained by an *in vitro* chemical reaction and can overcome ethical issues of antibodies obtained from animals at a high cost. Natural oligonucleotides are unstable in biological fluids because of endogenous nuclease. This problem can be addressed by using modified oligonucleotides during SELEX or by a post-SELEX modification. Although a modified aptamer is obtained, careful consideration will be needed for post-SELEX modifications because the modification can affect properties of the aptamer such as binding affinity and/or function. Aptamers can be easily conjugated with other molecules in a chemical reaction. Aptamer-imaging probe conjugates can be used in various imaging modalities including optical imaging, MRI, PET, SPECT, CT, and ultrasound. Moreover, aptamers can also be conjugated with anti-cancer drugs. An aptamer-based multimodal imaging probe loaded with drug(s) will allow more precise real-time monitoring and therapy. The theragnostic potential of aptamer is gaining new momentum and will see a most auspicious development.

ACKNOWLEDGMENTS

The present research was conducted by the research fund of Dankook University in 2011.

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