

# Recent Progress in Aptamer Discoveries and Modifications for Therapeutic Applications

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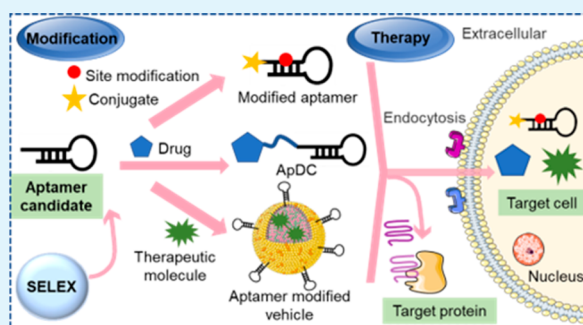
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**ABSTRACT:** Aptamers are oligonucleotide sequences with a length of about 25–80 bases which have abilities to bind to specific target molecules that rival those of monoclonal antibodies. They are attracting great attention in diverse clinical translations on account of their various advantages, including prolonged storage life, little batch-to-batch differences, very low immunogenicity, and feasibility of chemical modifications for enhancing stability, prolonging the half-life in serum, and targeted delivery. In this Review, we demonstrate the emerging aptamer discovery technologies in developing advanced techniques for producing aptamers with high performance consistently and efficiently as well as requiring less cost and resources but offering a great chance of success. Further, the diverse modifications of aptamers for therapeutic applications including therapeutic agents, aptamer–drug conjugates, and targeted delivery materials are comprehensively summarized.

**KEYWORDS:** aptamer, microfluidic SELEX, long-acting modification, aptamer–drug conjugate (ApDC), targeted delivery materials, nanoparticle, therapeutic application



## 1. INTRODUCTION

Aptamers are oligonucleotide sequences with a length of about 25–80 bases that mimic monoclonal antibodies. They generally fold into diverse three-dimensional structures that bind to specific targets. An aptamers selection process, named Systematic Evolution of Ligands by Exponential Enrichment (SELEX), was developed in 1990 by Tuerk and Gold,<sup>1</sup> and Ellington and Szostak.<sup>2</sup> A large number of aptamers targeting small metal ion, amino acids, organic molecules, proteins, viruses, bacteria, whole cells, and animals have been produced in recent years.<sup>3</sup> However, conditional aptamer discovery techniques are inefficient and laborious. Furthermore, these methods often fail to generate aptamers with enough binding ability compared to monoclonal antibodies.<sup>3</sup> In the past few years, much research has been carried out toward developing innovative techniques for producing aptamers with high performance consistently and efficiently, and at the same time with fewer resources and, above all, a greater chance of success.<sup>4</sup> The majority of efforts have focused on systematically altering every important step of the aptamer-generating process—selection, analysis, and sequencing—by applying many cutting-edge technologies in biology, physics, and chemistry, including microfluidic devices (M-SELEX)<sup>5</sup> and high-throughput sequencing (HTS).<sup>6</sup>

In practical applications, aptamers benefit from their convenience of generation, low manufacturing cost, little

batch-to-batch differences, reversible folding features, and very low immunogenicity compared to monoclonal antibodies (Table 1).<sup>7</sup> Further, the most important property of aptamers rests with the feasibility by which these oligonucleotide sequences can be easily modified and engineered into aptamer–drug conjugates (ApDCs)<sup>8</sup> and targeted delivery materials,<sup>9</sup> which facilitates their clinical translation into therapeutic applications. Most recently, a great many important applied paradigms have been demonstrated in the field of therapeutic research.<sup>3</sup> First, similar to monoclonal antibodies, aptamers act as inhibitors that can interfere with the normal function of a target protein and be applied as therapeutic agents directly after some chemical modifications in order to improve the ability to resist nuclease degradation and prolong the action time (as discussed in detail below).<sup>10</sup> Second, some aptamers are internalized after binding to receptors on the cell surface, which makes them valuable as targeting agents for microRNAs, small interfering RNAs (siRNAs), conventional small-molecule drugs

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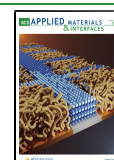
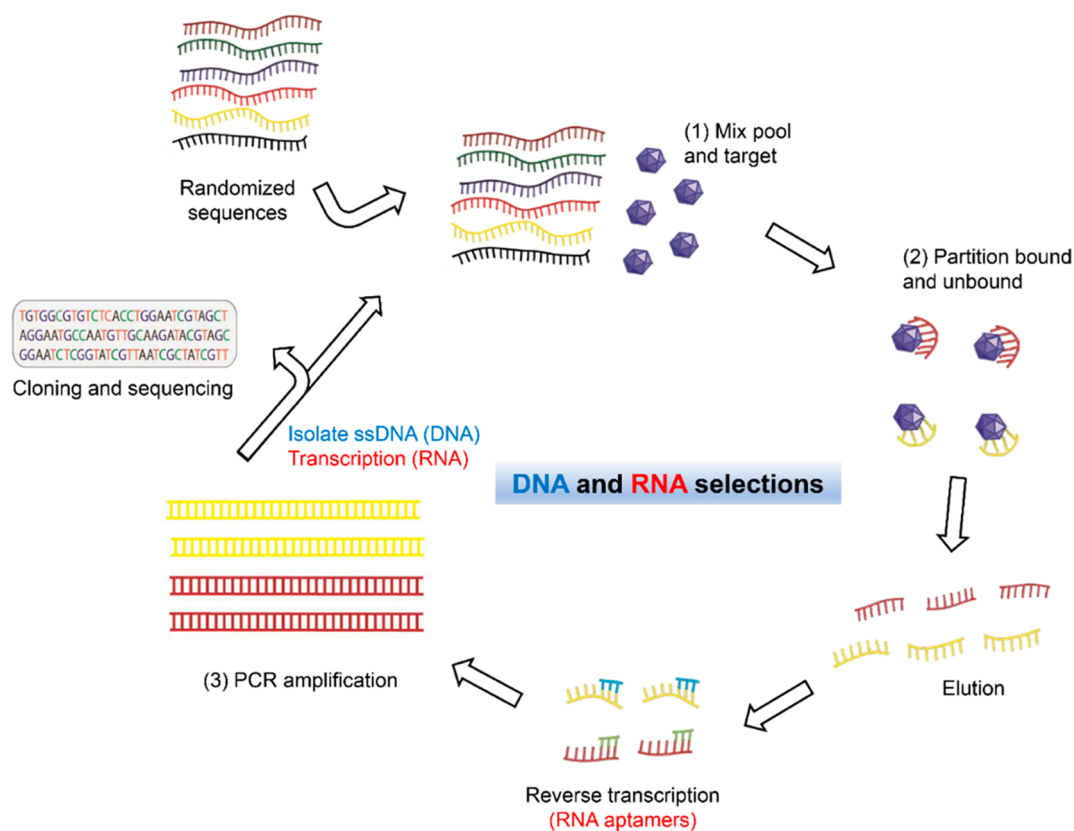
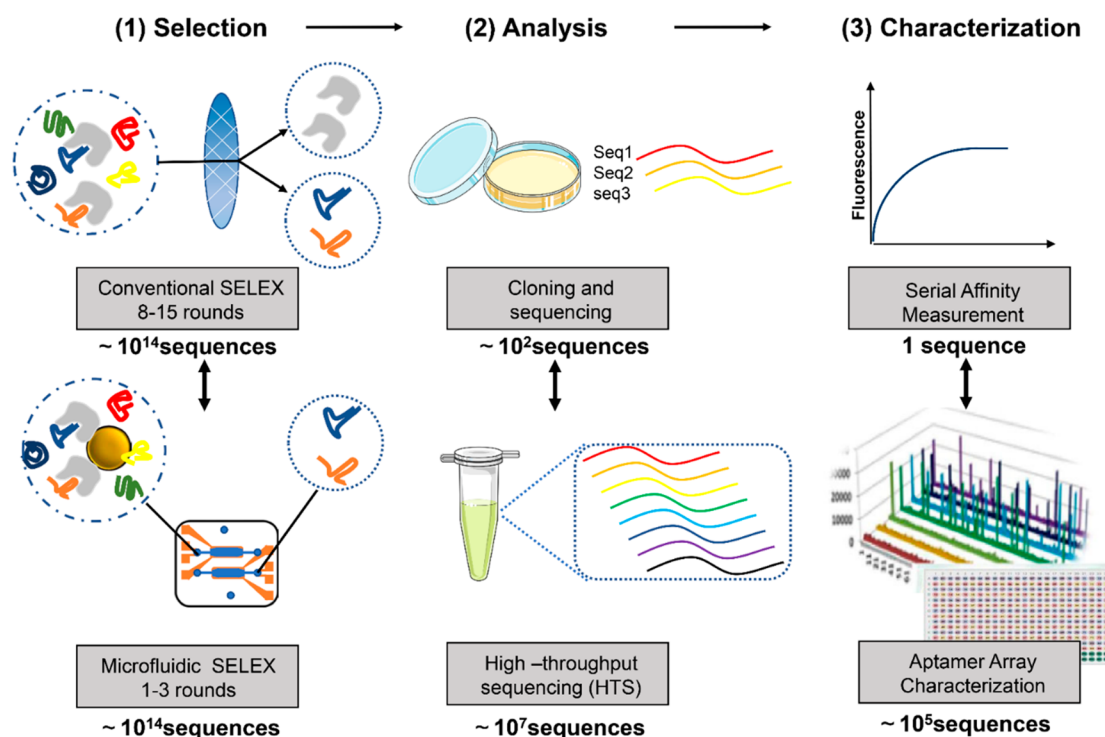


Table 1. Comparison between Nucleic Acid Aptamers and Monoclonal Antibodies

	nucleic acid aptamers	monoclonal antibodies
stability	withstand repeated rounds of denaturation/renaturation temperature resistant: stable at room temperature long shelf life (several years) can be lyophilized degradable by nucleases resistant to proteases	easily denatured temperature sensitive and require refrigeration to avoid denaturation limited shelf life must be refrigerated for storage and transport degradable by proteases resistant to nucleases
preparation	<i>in vitro</i> SELEX takes only 2–8 weeks no batch-to-batch variation cheap to synthesize	produced <i>in vivo</i> , more than 6 months batch-to-batch variations laborious and expensive
target potential	from ions and small molecules to whole cells and live animals	targets must cause a strong immune response for antibodies to be produced
size	small molecules	relatively large by comparison
modifiability	aptamers can be readily and easily modified without affinity loss	modifications often lead to reduced activity
affinity	high and increased in multivalent aptamers	dependent on the number of epitopes on the antigen
specificity	single-point mutations identifiable	different antibodies might bind the same antigen
tissue uptake/kidney filtration	fast	slow



**Figure 1.** Schematic of the selection process for DNA and RNA aptamer libraries. Starting with a randomized library incubated with the target (1), bound species are partitioned and stringently washed (2), followed by elution of the desired species. For RNA selections, recovered material must be reverse transcribed, followed by polymerase chain reaction (PCR) amplification (3) and transcription back into RNA to generate the library for the next round. DNA selections, however, are ready for PCR amplification after elution (3), but afterward must be separated from the complement strand before the resulting ssDNA pool can be used for the next round.



**Figure 2.** Schematic illustration of the conventional SELEX processes (upper) and microfluidic SELEX (lower). All three major steps of aptamer discovery (selection, analysis, and characterization) were optimized. The selection process with microfluidic devices allowed rapid and efficient partitioning of aptamer candidates from random nucleic acid libraries. Next, rather than identifying aptamers by individually choosing clones for sequencing, high-throughput sequencing (HTS) was utilized to analyze much larger pools of aptamers. Finally, the labor-intensive process of individually measuring aptamer affinity and specificity was conducted by using DNA microarrays and automated analysis.

(ApDCs), and beyond.<sup>11–13</sup> Lastly, attributed to their chemical synthesis properties, aptamers are easy to couple with liposomes and other carriers to form a smart delivery system, so that small molecules, peptides, nucleic acids, and even the CRISPR/Cas9 system can achieve targeted delivery.<sup>14</sup>

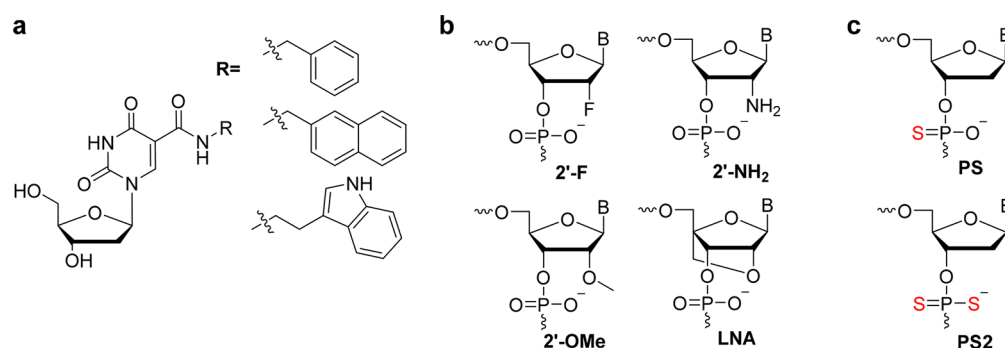
Although great improvements have been achieved, lags still exist in the clinically successful use of aptamer-based therapeutics compared to that of monoclonal antibody-based agents. Pegaptanib (Macugen; Pfizer/Eyetech)<sup>15</sup> is the only aptamer which has been approved by the U.S. FDA for clinical use. Currently, aptamers have two main weak points, i.e., susceptibility to the hydrolysis of nuclease and rapid clearance through glomerular filtration, which give aptamers a very short half-life *in vivo*.<sup>16</sup> These pose significant problems, which have hampered their clinical transformation. In order to increase the circulation half-life ( $t_{1/2}$ ), a number of methods have been attempted, focusing on either the chemical modification of the aptamer's nucleic acid chain or the conjugation of the aptamer with coupling agents to achieve long-lasting action.<sup>17</sup>

Herein, we address the innovative technologies in aptamer discovery, highlight the diverse modifications of aptamers for therapeutic applications, and summarize the promising delivery systems in aptamer-derived targeted materials. Despite the limitations, great progress has still been made in aptamer selection and modification, which may encourage researchers to continue pursuing aptamer-based therapeutics. This Review aims to provide a deeper understanding of the research and development regarding aptamer-based clinical therapeutic agents.

## 2. APTAMER DISCOVERY TECHNOLOGIES

SELEX is still a gold-standard strategy for the generation of nucleic acid aptamers. The selection cycle, whether for DNA or RNA sequences, on proteins, on cellular levels, or in living animals, requires three pivotal steps: (i) incubating a target with a library containing randomized sequences, (ii) partitioning bound sequences from non-bound sequences, and (iii) recovering and PCR amplifying the bound sequences.<sup>18</sup> The selection cycle is then repeated until the sequence is enriched with the desired affinity (Figure 1). Mostly, fundamental methods in the discovery of aptamers have not changed. However, several critical improvements have been meaningfully innovated in the way that aptamers can be characterized from the selection process, the target types that can be explored, and the modifications in which aptamers can be applied, expanding the practical applications of aptamers both *in vivo* and *in vitro*.<sup>19</sup> It should be noted that aptamers selected *in vitro* may not be functional *in vivo*.<sup>20,21</sup> SELEX based on living animals could be a promising solution.<sup>22</sup> In this approach, the oligonucleotide library is first administered to the animal through intravenous injection. Either a tissue or organ with specific research purpose is then harvested, and the bound oligonucleotide chains are extracted. The recovered sequences are then amplified by PCR to make a new library for further selection processes. This amazing methodology indicates the efficacy of SELEX based on living animals as a direct selection method for producing aptamers which are compatible with *in vivo* applications.

**2.1. Conventional SELEX.** Typically, a selection process begins with a nucleic acid library containing a random region with 20–60 nucleotides extended by fixed primer regions at both 5' and 3' ends.<sup>18</sup> Initially, a combinatorial oligonucleotide



**Figure 3.** SELEX with modified nucleotide bases, sugar rings, or phosphates. (a) Modifications (R) are employed for the selection of SOMAmers including benzyl, naphthyl, and indole (right). (b) Modifications (including F, NH<sub>2</sub>, OMe, LNA) on sugar rings can be incorporated into aptamers during selection. (c) Increased stability can also be garnered through phosphorothioate (PS) and phosphorodithioate (PS2) linkages.

pool consists of up to  $10^{15}$  unique oligonucleotide sequences theoretically, ensuring adequate structural diversity to obtain binders with high affinity. After repetitive selection cycles which consist of binding, partitioning, recovery, and PCR-amplification steps, aptamers with specific sequences are enriched and form the dominant library species (Figures 1 and 2). Recent progress in sequencing methodologies and computerized analyses has provided greater insight into the dynamics of aptamer generation during selections, enhancing the identification of effective sequences from earlier rounds of selection.<sup>23</sup> Nowadays, a series of new polymerases are available to harvest selection libraries with high stability to nuclease hydrolysis, diminishing the need for laborious post-selection modification. Stabilized derivatives with alterations on the sugar rings, including 2'-fluoro (2'-F) ribose, 2'-amino (2'-NH<sub>2</sub>) ribose, 2'-O-methyl (2'-OMe) ribose, or locked nucleic acids (LNAs, bridging the 2'- and 4'-ribose positions covalently) on the nucleotide residues, have been applied for incorporating unnatural nucleotides into oligonucleotides for several years utilizing a mutant of T7 RNA polymerase.<sup>19</sup> They are effective in extending the serum half-life for further therapeutic applications.<sup>24</sup>

**2.2. SELEX with Modified Nucleotide Bases, Sugar Rings, or Phosphates.** Interestingly, through modifications on the bases, aptamers with enhanced binding affinities are being produced, which endows them with protein-like properties.<sup>25</sup> In this field, SomaLogic has made considerable progress by using chemical modifications on bases to give aptamers more structural diversity and stronger target binding ability. Their “slow off-rate modified aptamers” (SOMAmers) demonstrate enhanced binding affinities and kinetics compared to conventional aptamers. Besides, the incorporation of these modifications on bases in their selection pool markedly increases the “hit rate”.<sup>26</sup> The advantage of this strategy arises from replacing the dT bases within oligonucleotide libraries with a dU base modified at the 5-position of the heterocyclic base.<sup>27</sup> With a number of hydrophobic replacements at the 5-position, including simple benzyl, a bit bigger naphthyl, and the more complicated indole, a great number of new aptamers have been discovered for targets that were unselectable previously (Figure 3a). Of additional note, SomaLogic has now identified great numbers of SOMAmers for more than 5000 diverse protein targets that are essential for either normal or disease conditions.<sup>28</sup> The company is exploring varieties of SOMAmers-based array techniques, including SOMAscan and SOMApanel, for disease-related proteomics and clinical applications.<sup>29</sup> Additionally, modifications on the sugar ring,

including 2'-F-ribose, 2'-NH<sub>2</sub>-ribose, 2'-OMe-ribose, or LNAs (bridging the 2' and 4'-ribose positions covalently) (Figure 3b), have been introduced by incorporating unnatural nucleotides into oligonucleotides using the mutational T7 RNA polymerase, enzymatically. All these techniques are effective in improving the stability to nucleases and prolonging serum half-life.<sup>30–33</sup>

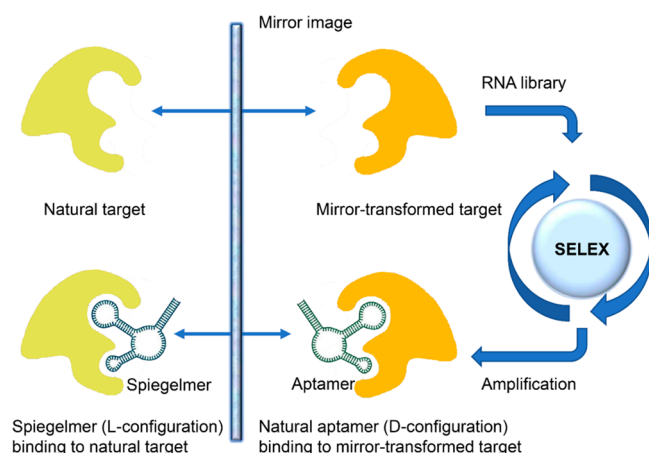
Phosphate linkage modifications can also be introduced into aptamers for stabilizing the chains of nucleic acids by replacing conventional phosphate (PO) backbones with sulfur-containing phosphate ester bonds<sup>34–36</sup>, including phosphorothioate (PS) bonds and more recently phosphorodithioate (PS2) bonds<sup>37,38</sup> (Figure 3c). It should be noted that the replacement of two non-bridging oxygen atoms in one phosphate ester unit with sulfur atoms (PS2) is achiral at phosphorus, which is similar to natural DNA. However, in the case of PS linkages, substitution is chiral (Sp or Rp configuration), which could adversely affect the biological function.

Additionally, Kimoto et al.<sup>39</sup> reported the incorporation of up to three unnatural nucleotides with the 7-(2-thienyl)imidazo-[4,5-*b*]pyridine (Ds base) nucleotides into an oligonucleotide library. The selection experiments were carried out against two protein targets, vascular endothelial cell growth factor-165 (VEGF-165) and interferon- $\gamma$  (IFN- $\gamma$ ). The resulting DNA aptamers had  $K_d$  values of 0.65 pM and 0.038 nM, respectively, with more than a 100-fold improvement in affinities compared to the aptamers containing natural bases. The group of Kimoto et al.<sup>40</sup> further developed a newer version of genetic alphabet expansion for SELEX (ExSELEX), applying a totally randomized sequences pool consisting of five components: one unnatural hydrophobic base (Ds base) and four natural bases. The hit rates of aptamers with high affinity were significantly improved by the increased diversity of the novel randomized library. With the improved library, a Ds base-containing aptamer targeting von Willebrand factor A1-domain (vWF) with significantly higher affinity was obtained. This new library strategy is expected to discover more high-affinity aptamers consistently.

Two new nucleotides,<sup>41</sup> 6-amino-5-nitro-3-(1'- $\beta$ -D-2'-deoxy-ribofuranosyl)-2(1H)pyridone and 2-amino-8-(1'- $\beta$ -D-2'-deoxyribofuranosyl)imidazo[1,2-*a*]-1,3,5-triazin-4(8H)-one (Z and P), were also added to the aptamer selection library used in a laboratory *in vitro* evolution experiment. Recently, Tan et al. introduced the artificial nucleotide to cell-based SELEX and generated aptamers for the regulation of protein activity.<sup>42</sup>

**2.3. Mirror Image Aptamers: Spiegelmers.** To bypass the degradation of nucleases in the body, the invention of mirror image aptamers (or “spiegelmers”, Figure 4) is a very different





**Figure 4.** Illustration showing the generation of an RNA-spiegelmer. As a first step, a mirror image of the natural target is synthesized. RNA oligonucleotides (aptamers) binding to the mirror-image selection target are then identified by *in vitro* selection from a synthetic oligonucleotide library in the natural D-configuration. The natural D-configuration is required because stereoselective enzymes are used for amplification, cloning, and sequencing of bound sequences. Identified sequences are finally synthesized using enantiomeric (L-) ribonucleotides. The resulting spiegelmer binds to the natural target.

and effective strategy.<sup>43</sup> Spiegelmers are oligonucleotide chains composed of L-nucleotide, as opposed to the natural sequences composed of D-nucleotides. They could form left-handed helices instead of the conventional right-handed helices.<sup>44</sup> They exhibit exceptional stability in serum, as they are recognized by ubiquitous nucleases. SELEX of Spiegelmers starts with natural sequences composed of D-nucleotides against mirror image targets such as D-peptides to obtain the aptamers with natural nucleotides.<sup>45</sup> The expected Spiegelmers (mirror versions) are then prepared in the inverted configuration as natural aptamers by chemical synthesis, which recognizes the natural target (L-peptide). The NOXXON company has filed a series of patents for the discovery, preparation, and application of spiegelmers. The company is taking advantage of the innovative discovery platform in developing spiegelmer-based therapeutics.<sup>46</sup> Currently, three spiegelmers have been developed for therapeutic applications: NOX-H94 (anti-hepcidin),<sup>47</sup> NOX-E36 (anti-CCL2),<sup>48</sup> and NOX-A12 (anti-CXCL12).<sup>49</sup> In phase 1 studies, all three candidates have exhibited good safety in healthy volunteers. Moreover, both NOX-H94 and NOX-A12 have also demonstrated exciting effectiveness in phase 2 studies. Extra clinical trials are currently being conducted in evaluations, with a number of other spiegelmers in the pipeline.

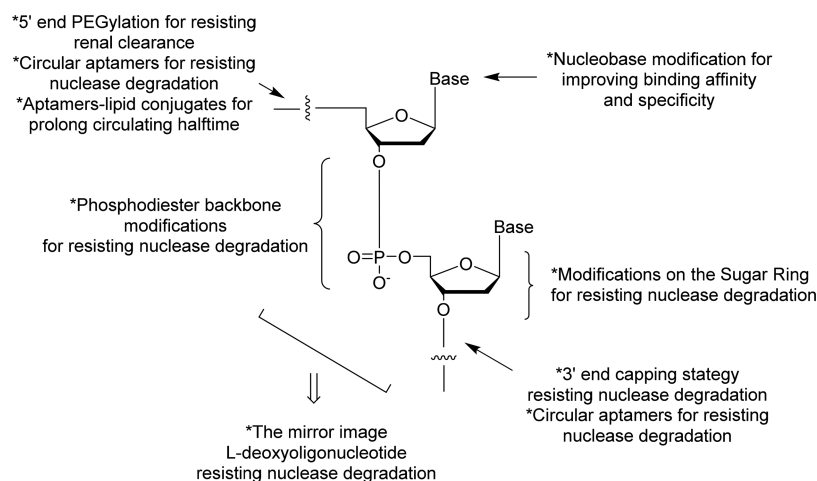
**2.4. Microfluidic SELEX with High-Throughput Sequencing.** Conventionally, SELEX begins with a randomized library of single-stranded nucleic acid sequences with diverse three-dimensional structures. The regions of randomized nucleotides in nucleic acid sequences supply the unique structure, supporting further biochemical properties. It employs the three main processes (described above) to generate aptamers that bind to a target specifically. Typically, a SELEX process concludes with the sequencing analysis of fewer than 100 sequences. However, general SELEX is restricted to the intrinsic inefficiencies in the separation steps. Irvine et al. demonstrated that, in one round of SELEX, the maximum enrichment that can be realized for a specified aptamer relative to another is equal to the ratio of their  $K_d$  values.<sup>50</sup> This indicates that several cycles of the selection process (10 or more in

common) are needed to achieve a manageable number of candidates that can be characterized experimentally when starting with  $\sim 1$  nmol ( $6 \times 10^{14}$ ) of molecules in a typical SELEX experiment. The requirement for multiple selection cycles becomes a problem, which could inevitably bring undesired biases, including the loss of rare binding sequences, biases in PCR process, and the unexpected amplification of non-target-specific sequences. As these biases continuously accumulate over multiple selection cycles, the chance of failed SELEX or obtaining aptamers with poor binding affinity will increase. To solve these problems and increase reproducibility, microfluidic devices are now designed to allow researchers to maintain strict experimental control over a small amount of target reproducibly.<sup>51</sup> Within the microfluidic device, there are a series of ferromagnetic items which are used to realize steady trapping of paramagnetic beads with the target coated on them. After the trapping of the bead, the washing buffer is passed through the device tubes directly, and subsequently the beads are treated with a highly stringent washing procedure. Benefiting from the automatic advantages of microfluidic devices, the majority of the unbound oligonucleotides are washed away within a few minutes. By stringently and precisely controlling the washing conditions, these studies exhibit that the use of automatic microfluidic devices can screen aptamers with higher affinity after fewer rounds of selection compared with general SELEX.<sup>52</sup> Typically, Sanger sequencing is applied when the candidate aptamers are screened to a relatively small number of oligonucleotides, and accordingly the most abundant oligonucleotides containing “consensus motifs” can be confirmed in general SELEX. However, the number of sequences that can be realistically obtained is relatively small, and these enriched oligonucleotides may not truly represent the best binders in a starting library. In order to overcome the defect of inherent low throughput of general sequencing and better analyze the enriched aptamer candidates, HTS is applied in the selection cycle together with microfluidic devices (Figure 2). HTS can discriminate the highest-fold enrichment in the early rounds of selection by monitoring the enrichment track of each selection cycle.<sup>53</sup> Many researchers have agreed that the aptamers with the best affinity are usually those that bind to the target most rapidly in the very early selection cycles. Accordingly, it is possible for microfluidic SELEX together with HTS to become an even more universal technique for aptamer discovery.<sup>52</sup>

### 3. LIMITATIONS OF UNMODIFIED APTAMERS FOR THERAPEUTIC APPLICATIONS

Although nucleic acid aptamers have many merits, their intrinsic physicochemical properties can lead to unsuitable pharmacokinetic curves caused by instability to nuclease degradation, rapid renal clearance, rapid distribution from the plasma to the specific tissues (the liver or spleen), polyanion effects, and non-specific immune response.<sup>3</sup> All these hinder their *in vivo* therapeutic potencies. To pave the way for the clinical application of aptamer-based therapeutics, extensive modifications on aptamer chains and conjugations with functional molecules are therefore needed.

**3.1. Sensitivity to Nucleases.** Due to the innate properties of oligonucleotides, aptamers suffer from rapid degradation mediated by ubiquitous nuclease.<sup>54</sup> The half-lives of unmodified nucleic acids are commonly about 5 min in serum and no more than 1 h in cells.<sup>21</sup> An anti-thrombin DNA aptamer was shown to have a half-life as short as 108 s *in vivo*,<sup>55</sup> while an RNA aptamer against keratinocyte growth factor was easily hydro-



**Figure 5.** Common strategies in the chemical modifications of nucleic acid aptamers and their purposes. Among modifications on the terminals of nucleic acids, modifications on the phosphodiester linkage, modifications on the sugar ring, and modifications on the bases, 3' end-capping with inverted thymidine and PEGylation has been the common strategy in the chemical modifications of nucleic acid aptamers for development of clinical therapeutics. Novel techniques including circular aptamers and lipid-conjugated oligonucleotides utilizing endogenous serum albumin are also reported.

lyzed within seconds in 90% human serum.<sup>56</sup> Accordingly, a series of techniques have been established to protect aptamers from nuclease degradation. Generally, the modification sites include (1) either end of the oligonucleotide, (2) the sugar ring, and (3) the phosphodiester backbone. In addition, the majority of the aptamers clinically applied are modified by capping the 3' end with inverted thymidine (pegaptanib, ARC1779, ARC1905, REG1, BAX499).<sup>57</sup> A number of modifications are possible on riboses, such as 2'-F, 2'-NH<sub>2</sub>, or 2'-OMe substitutions, isonucleotide substitutions, and replacement of ribonucleotide analogues with locked nucleic acid (LNA, linking 2'-O and 4'-C of the ribose with a methylene bond).<sup>58</sup> The application of spiegelmers to prevent aptamers from nucleases degradation is also an effective strategy.<sup>59</sup>

**3.2. Elimination by Renal Filtration.** In addition to the fast nuclease-mediated degradation, aptamers could also be eliminated through renal clearance rapidly.<sup>60</sup> The molecular weights of aptamers range from 6 to 30 kDa, and their average diameter is less than 5 nm.<sup>61</sup> Small aptamers are excreted rapidly through renal filtration when they are administered into the bloodstream in the non-formulated form, even adopting stabilizing modifications (described above). Accordingly, formulation with a bulky moiety is an effective way to prevent aptamers from being cleared by renal filtration and prolong the circulation half-lives. The main bulky moieties to enlarge the size of aptamers are polyethylene glycol (PEG),<sup>62</sup> proteins,<sup>63</sup> cholesterol,<sup>64</sup> liposomes,<sup>65</sup> and organic or inorganic nanomaterials.<sup>66</sup> Of all the moieties, PEG has been most widely applied in prolonging circulation half-life and improving the *in vivo* pharmacokinetic characterizations of aptamer candidates.<sup>67</sup> Aptamers formulated with high-molecular-mass PEG create covalent molecules with a molecular weight above 30–50 kDa (the cutoff value of the renal filtration).<sup>68</sup> Multimerizing single aptamers together is another method to increase the molecular weight of the aptamer. By coupling multiple aptamers into a complex, an increased retention time in circulation has been reported.<sup>69</sup>

**3.3. Toxicity of Aptamer-Based Therapeutic Agents.** The toxicity of aptamer-based therapeutic agents is also one of the influential factors for their clinical transformation. The use of

unnatural nucleotides may cause chemical toxicities or non-specific immune responses. It was reported that LNA-modified nucleic acids showed severe hepatotoxicity,<sup>70</sup> and 2'-F-modified RNAs affected the activation of pattern recognition receptors.<sup>71</sup> Consequently, modifications should be used with caution, and the actual application environment of the aptamers should be considered. The formulation of therapeutic aptamers is another origin of adverse responses that should be of concern. PEG moieties have been reported to cause serious immune reactions in the phase III study of aptamer-based therapy. Obviously, safety concerns are raised about the adoption of high-molecular-mass PEG moieties in aptamer modifications.<sup>72</sup> Accordingly, it is still desirable to seek a low-molecular-weight coupling agent without allergic responses to develop long-acting efficient therapeutic aptamers.

#### 4. APTAMER MODIFICATIONS FOR TRANSLATING INTO CLINICAL CANDIDATES

The applications of chemical modifications to protect nucleic acid aptamers from being degraded by endogenous, ubiquitous nucleases and from being cleared by resisting renal filtration have been valuable to get deeper into the development of already existing aptamers, or aptamers currently under clinical trials. Without the diversified modifications, the overall serum half-lives of DNA (2'-H) and RNA (2'-OH) are only mere minutes (described above), and therapeutic applications of aptamers would be at a far distant date (Figure 5, Table 2).

**4.1. Modifications for Protecting Aptamers from Being Degraded by Nuclease.** **4.1.1. Internucleotide Linkage with 3'-3' and 5'-5' Capping in the Terminus.** The 3'-3' and 5'-5' capping methods with an inverted nucleotide in the terminus were reported by Seliger to protect aptamers from being degraded by exonuclease.<sup>73</sup> Nowadays, it is common to modify aptamers with inverted thymidine in the 3'-terminus for aptamer-based therapy in either completed or ongoing clinical trials.<sup>27</sup> Through 3'-inverted thymidine, aptamers have a significantly increased stability and resistance to exonuclease in circulation (Figure 5).

**4.1.2. 2'-Substitutions and Phosphodiester Linkage Replacement.** RNA aptamers are commonly modified in 2'-OH

Table 2. Summary of Aptamer Modifications for Clinical Translation

drug name	target	modifications	circulation half-life (h)	phase	ref
Macugen (pegaptanib sodium)	VEGF	1. 27-nt RNA 2. 2'-fluoropyrimidines 3. 2'-O-methylpurines 4. 3'-inverted dT 5. 40 kDa PEG	18 (human plasma)	approved	15
ARC1905	C5	1. 38-nt RNA 2. 2'-fluoropyrimidines 3. 2'-O-methylpurines 4. 3'-inverted dT 5. 40 kDa PEG	>300 (rat serum)	2	156
E-10030	PDGF	1. 29-nt DNA 2. 2'-O-methylpurines 3. 3'-inverted dT 4. 40 kDa PEG	8 (rat serum)	3	157
REG1	coagulation factor IXa	<i>RB006 (drug):</i> 1. 37-nt RNA aptamer 2. 2'-ribose or 2'-fluoropyrimidine <i>RB007 (antidote):</i> 1. 17-nt 2. 2'-O-methyl 3. 40 kDa PEG	— —	2	158
ARC1779	A1 domain of von Willebrand factor	1. 39-nt DNA 2. 3'-inverted dT 3. 2'-O-methyl with a single phosphorothioate linkage 4. 20 kDa PEG	63 (human plasma)	2	92
NU172	thrombin	1. 26-nt DNA 2. unmodified	—	2	159
ARC19499 (BAX499)	TFPI	1. 32-nt RNA 2. 2'-3O-methylpurine 3. 3'-inverted dT 4. 40 kDa PEG	>72 (human serum)	1	160
AS1411 (AGRO001)	nucleolin	1. 26-nt DNA 2. G-rich DNA 3. PEGylated	—	2	161
NOX-A12	CXCL12	1. 26-nt DNA 2. G-rich DNA 3. PEGylated	>60 (human serum)	2	162
NOX-E36	CCL2	1. 40-nt RNA (Spiegelmer) 2. L-ribonucleic acid 3. PEGylated	>60 (human serum)	2	163
NOX-H94	hepcidin peptide hormone	1. 44-nt RNA (Spiegelmer) 2. L-ribonucleic acid 3. PEGylated	>60 (human serum)	2	164

positions with 2'-F, 2'-NH<sub>2</sub>, 2'-OMe, and LNAs.<sup>74</sup> Mostly, aptamers in clinical studies are chemically modified by fluorine or methoxy in the 2' position. LNA is a sugar ring modification linking the 2'-O and 4'-C of the ribose with a methylene bond. LNAs are promising candidate materials for nucleic acid drugs

due to their high nuclease resistance and excellent thermostability.<sup>33</sup> It was reported that a chimeric LNA/DNA aptamer targeting HIV-1 trans-activating response protein could remain intact for more than 20 h in serum.<sup>75</sup> Replacement of the phosphodiester bonds of aptamers with phosphorothioate or

methylphosphonate analogues is also a conventional strategy.<sup>76</sup> To enhance the stability of the phosphodiester backbone in the anti-thrombin aptamer, replacements with thiophosphoryl at different nucleotide sites were evaluated. An aptamer holding the sequence of d(GGSTSTSGGTGTGGSTSTSGG) was modified by thio substitutions at the loop regions, which resulted in a high stability against nucleases and a low hydrolysis rate in circulation while the anti-thrombin potency remained. Recently, Wang et al. introduced phosphorodithioate (PS2) substitutions to stabilize phosphodiester bonds.<sup>77</sup> PS2 modifications could also improve the binding between aptamers and their targets. PS2-modified aptamers possess a 1000-fold improved target binding affinity compared with an unmodified original aptamer (Figure 5).

**4.1.3. Spiegelmers.** Incorporating unnatural nucleotides into the oligonucleotide chain to improve aptamer stability is another feasible strategy.<sup>43</sup> Aptamers with the introduction of unnatural nucleotides are less susceptible to nuclease-mediated hydrolysis. Conventionally, the nucleases are chiral and accordingly recognize the substrate in a stereospecific way. Natural nucleic acids are made with D-nucleotides, while an oligonucleotide containing L-nucleotides avoids recognition and degradation by ubiquitous enzymes. These target-recognizing L-oligonucleotides (the mirror version of natural oligonucleotides) are called “spiegelmers”. D-Adenosine was the first target used to screen spiegelmers. The resulting L-RNA spiegelmer indeed exhibited considerable bio-stability. No detectable degradation in human serum was found, even after incubation at 37 °C for 60 h. NOX-1255 (targeting anti-gonadotropin releasing hormone, GnRH) is the first developed therapeutic spiegelmer which was generated with an increased affinity to the target (Figures 4 and 5).

**4.1.4. Modifications with D-/L-Isonucleoside.** Isonucleosides are nucleoside analogues in which the bases on the nucleosides are transferred to the 2' or 3' position (Figure 6). The

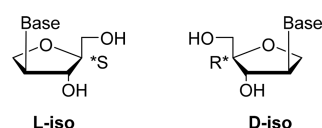


Figure 6. Chemical structure of D-/L-isonucleoside.

oligonucleotides assembled with isonucleosides possess an enhanced stability to nuclease-mediated hydrolysis.<sup>78</sup> Yang et

al.<sup>24,79,80</sup> reported the application of D-/L-isonucleoside in the modification of three aptamers (TBA, GBI-10, and AS1411). The replacement of some specific sites (like the loop areas) with D-/L-isonucleoside demonstrated a positive effect on stabilizing spatial conformation and improved the chemical stability. Additionally, it was also demonstrated that the modified aptamers had an enhanced resistance against biodegradation. Interestingly, the modifications with L-isonucleoside increased the biological activity of aptamers more significantly than modifications with D-isonucleoside.

**4.1.5. Nuclease-Resistant Circular Aptamers.** More recently, the emergence of circular aptamers was reported to overcome the key limitation of metabolic instability.<sup>81</sup> Cyclization of nucleic acids by linking 5'- and 3'-termini allows them to escape the primary degradation caused by exonucleases, serving as an easy and reproducible strategy to enhance the resistance of aptamers to nucleases.<sup>82</sup> King and colleagues<sup>83</sup> designed a kind of multivalent circular aptamer acting as an anti-coagulant. The cyclization of oligonucleotides can also increase their thermal stability, which ensures the conformational uniformity. Tan et al. reported that three aptamers, i.e., Sgc8,<sup>84</sup> TD05,<sup>85</sup> and XQ-2d<sup>86</sup> (targeting live cancer cells), were selected to establish bivalent circular aptamers. The cyclization method provides a feasible and efficient way to facilitate applications of the aptamer in diagnosis and therapy through improving their stability to nuclease and binding ability (Figure 7).<sup>87</sup>

**4.2. Long-Acting Modifications of Aptamers.** **4.2.1. 5'-End with Cholesterol.** Cholesterol has a strong affinity to low-density lipoprotein (LDL). It was introduced to the 5'-end of an aptamer chemically to form a cholODN and, further, the complex of cholODN-LDL compactly. This complex is highly resistant to nuclease hydrolysis in serum, resulting a 10-fold prolonged half-life compared to the unmodified version.<sup>88</sup> A RNA aptamer modified with 2'-F-pyrimidine was reported to be linked to cholesterol to form a chol-aptamer which can be absorbed into the cell and inhibit the replication of Hepatitis C virus RNAs.<sup>62</sup> No measurable toxicity of the chol-aptamer was observed *in vitro* or *in vivo*. Chol-aptamer also has no significant impact on the gene expression profile, especially the most common immune-related genes. In addition, no obvious abnormalities were detected in mice after *in vivo* administration of the chol-aptamer. Excitingly, compared to the unmodified aptamers, chol-aptamer

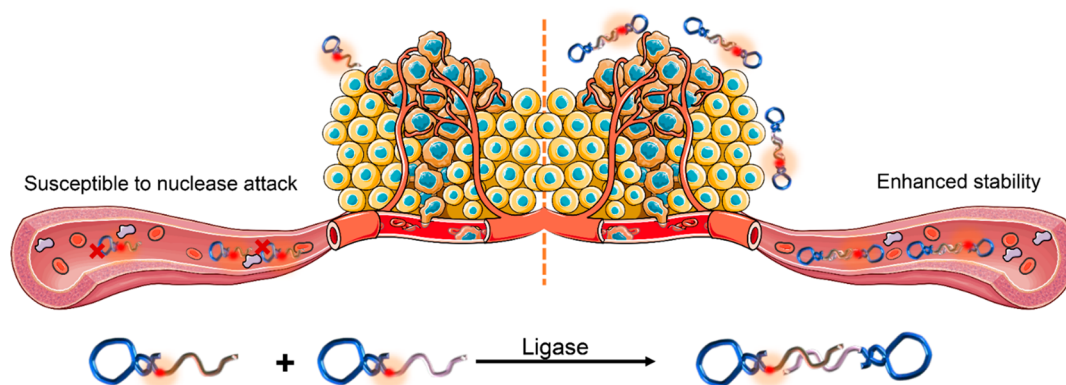


Figure 7. Schematic illustration of circular bivalent aptamers. The circular bivalent aptamers demonstrated improved *in vivo* stability and efficient accumulation and retention compared to “monoaptamers”.



had a much longer half-life and a 9 times lower plasma clearance rate (Figure 8).

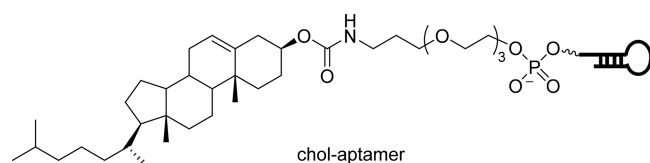


Figure 8. Structure of cholesterol–oligonucleotide conjugates.

**4.2.2. Dialkyl Lipid Modifications at the 5'-End.** Willis and co-workers<sup>65</sup> developed a diacylglycerol (DAG)<sup>89</sup>-conjugated aptamer targeting VEGF. The lipid tail of the DAG–aptamer conjugate was embedded in the lipid bilayer of a liposome, resulting in the improved activity. Compared to the unmodified aptamer, this DAG–aptamer–liposome complex possessed a much longer retention time in plasma (Figure 9).

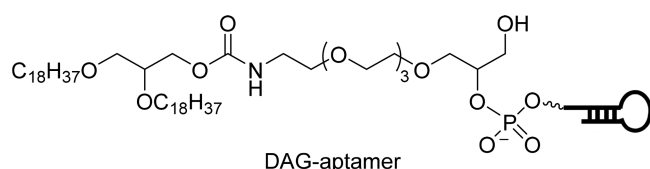


Figure 9. Structure of the dialkylglycerol<sup>89</sup>-modified VEGF aptamer.

**4.2.3. 5'-End PEGylation.** Pegaptanib (marketed as Macugen), with a PEG chain at the 5'-end, is the first therapeutic aptamer approved by the U.S. FDA.<sup>90</sup> It specifically binds to extracellular VEGF165 with a high affinity. By binding to the VEGF165, pegaptanib interrupts the interaction between VEGF165 and VEGF receptors and is used to treat age-related macular degeneration (AMD) by inhibiting VEGF-mediated biological functions on pathological vascular endothelial cells. The 5'-PEGylation modification contributes to the therapeutic activity and prolonged half-life of pegaptanib. Zimura, an aptamer against complement component 5 (anti-C5), is also modified with the same 5'-PEGylation as pegaptanib for further application. ARC1779 is another PEGylated aptamer targeting thrombin. It was developed to target the A1 domain of vWF (activated von Willebrand factor), exerting its anti-thrombotic activity.<sup>91,92</sup> Although PEG modification seems to have been the general strategy for long-acting aptamer modification, the high-molecular-mass PEG component possesses a very large molecular weight proportion, making it a serious limitation in increasing subcutaneous dosage to maximize therapeutic potential. Furthermore, it should be noted that serious or fatal immunoreactions to the PEG moieties have been reported in the clinical study, resulting in safety concerns with respect to the PEGylated agents in clinical application. Thus, it is still desirable to seek a new coupling agent without allergic responses to develop long-acting efficient therapeutic aptamers (Figure 10).

**4.2.4. N-Acetylgalactosamine (GalNAc) Modification.** GalNAc is a very good hepato-targeting group and has been

extensively applied in the study of targeted delivery of siRNAs and antisense oligonucleotides (ASOs).<sup>93–96</sup> Givosiran is the first GalNAc-conjugated nucleic acid drug approved for the treatment of acute hepatic porphyria.<sup>97</sup> In addition, many clinical nucleic acid therapies also use GalNAc modification strategies, like DCR-HBVS, DCR-PHXC, and IONIS APOC-III-LRx ([www.ClinicalTrials.gov](http://www.ClinicalTrials.gov)), which are still under clinical trials and have great prospects for listing.<sup>89</sup> GalNAc-ASO conjugates show 20- to 30-fold improved potency compared to unconjugated ASOs.<sup>98</sup> In addition, GalNAc modification can also reduce the dose to prolong the elimination half-life due to the decrease in the distribution of the off-target tissues of the drug and the increase in the concentration in the liver tissue.<sup>96</sup> Jayaprakash et al.<sup>99</sup> constructed two different siRNAs conjugated with GalNAc, and they found that the target gene MTTR was silenced for a considerably long period of time. Thus, GalNAc is also a promising candidate in the research on long-acting aptamer modification (Figure 11).

**4.2.5. Serum Albumin as a Carrier for the Long-Acting Modification of Aptamers.** Serum albumin is the most important carrier protein in serum, with an abundance of 40 mg/mL. It acts as a natural transporter of a series of active pharmaceutical molecules due to their attractive circulation half-time ( $t_{1/2}$ ) of about 20 days. Tan et al.<sup>100</sup> utilized a lipid with two octadecyl tails to delivery floxuridine homomeric oligonucleotide (LFU20), which “hitchhikes” the endogenous serum albumin. After being administered intravenously, LFU20 inserted into the hydrophobic cave of albumin to form an LFU20/albumin complex, which accumulated in the tumor by the enhanced permeability and retention (EPR) effect and internalized into the lysosomes of cancer cells. In brief, LFU20 non-covalently bonded with albumin, forming the LFU20/albumin complex which had a longer circulation time (Figure 12). Utilizing the specific binding between serum albumin and its corresponding ligands, Evans Blue (EB) should be noted as a valuable molecule. EB dye binds to serum albumin compactly and has a considerably prolonged half-life in blood. These characteristics make the EB molecule a promising carrier to extend the half-lives of aptamers by chemical conjugation.<sup>101–104</sup> Notably, it has been reported that EB–aptamer conjugates improved the delivery efficiency and retention time of aptamers to the tumor site by forming the long-acting aptamer/HSA complex.<sup>105</sup> As an endogenous substance, serum albumin exhibits considerable biological safety compared with most artificial carriers. In summary, adopting serum albumin as the carrier to prolong the circulation half-life is an alternative for improving the targeted performance of aptamers, which could facilitate their clinical translation.

## 5. APTAMER-BASED TARGETED THERAPEUTICS

Aptamers are applied either as stand-alone therapeutics or as adjuvants for another therapeutic. Due to the ability to bind target molecules at an expected position, aptamers often serve as an excellent material for targeted delivery. They are superior components of targeted systems for drug delivery because of

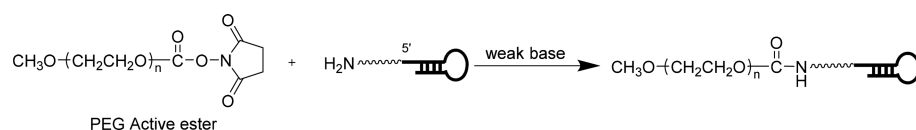
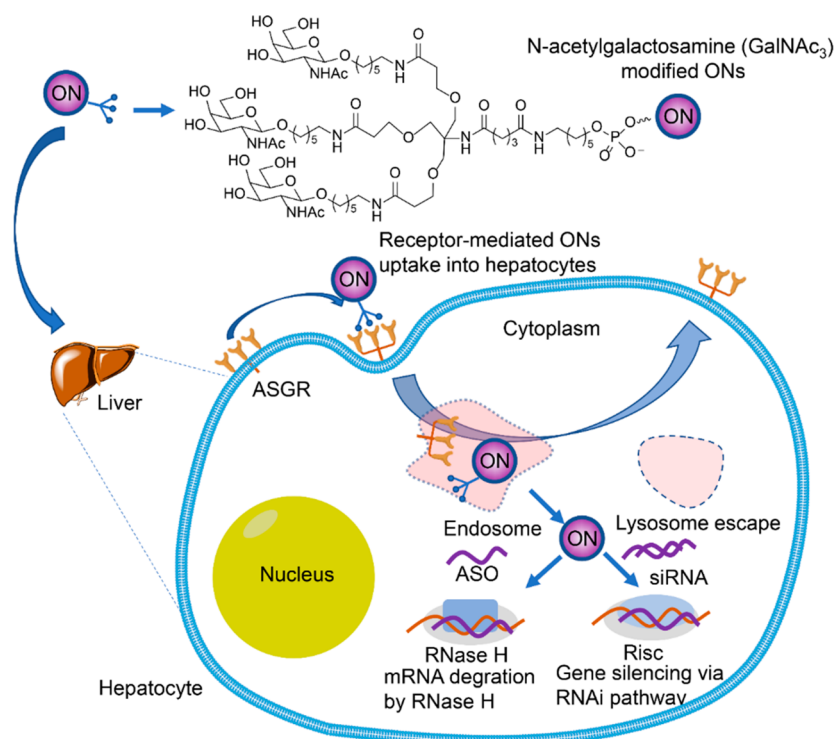
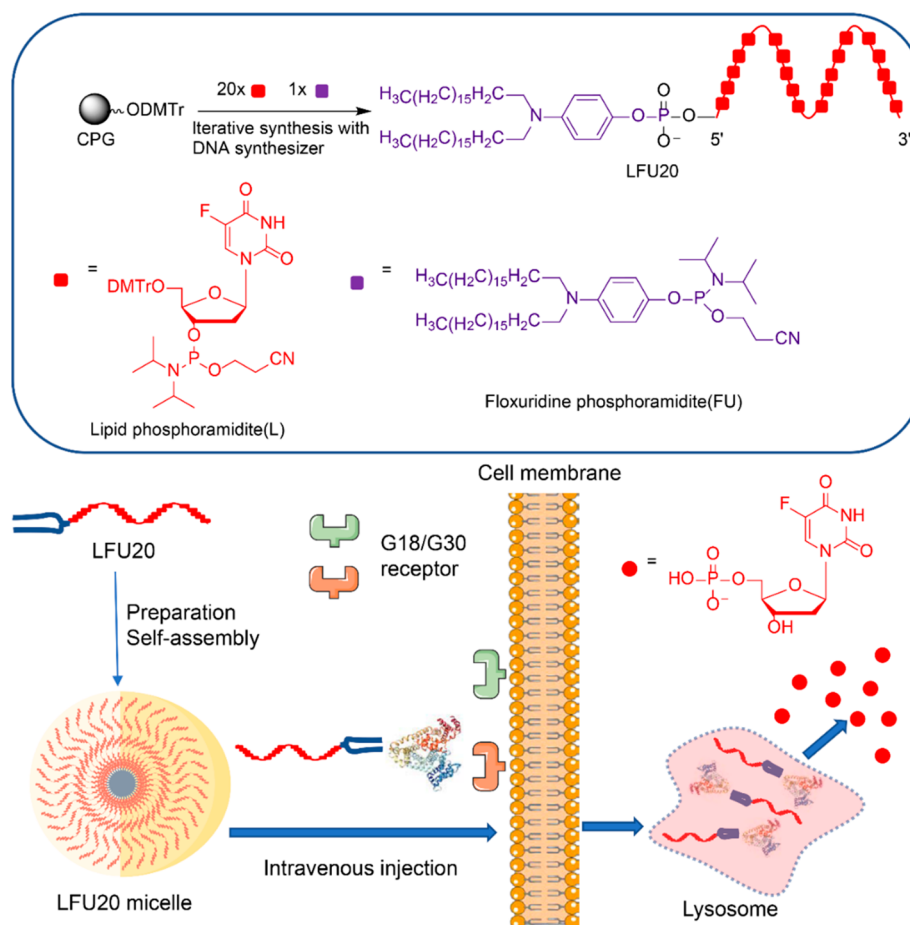


Figure 10. Reaction scheme of aptamer–polyethylene glycol (PEG) conjugate at the 5'-termini.



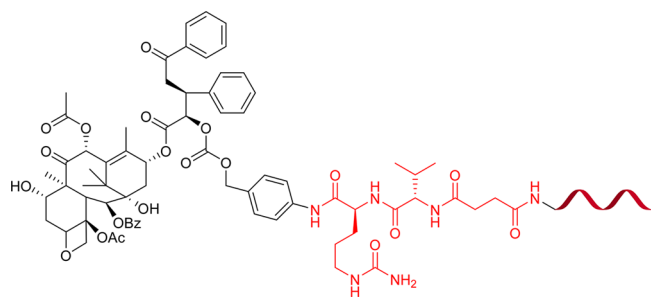
**Figure 11.** Schematic illustration of *N*-acetylgalactosamine (GalNAc)-mediated targeted delivery.



**Figure 12.** Illustration of the preparation of LFU20, self-assembly of LFU20 to a micellar nanostructure, non-covalent interaction of LFU20 with endogenous albumin after intravenous injection, and the subsequent cancer therapy of LFU20.

desirable characteristics including high affinity and specificity in target recognition, feasibility for chemical modifications, and low immunogenicity. According to the systems that have been modified, aptamer-guided drug delivery can be divided into three major categories: aptamer–drug conjugates (ApDC), aptamer–macromolecule conjugated systems, and aptamer–nanomaterial delivery systems. Nanomaterials possess several excellent characteristics, including a large surface-area-to-mass ratio, ultra-small size, and high reactivity. The relatively large surface area allows the materials to incorporate multiple targeting ligands or secondary therapeutic reagents, which makes them promising carriers for drug delivery. Nanoparticles, such as liposomes and hydrogels, also exhibit unique advantages, including high water solubility, enhanced accumulation at the tumor cells, prolonged circulation time in the blood, and inherent biocompatibility.<sup>106,107</sup>

**5.1. Aptamer–Drug Conjugates (ApDCs).** One of the limitations of traditional chemotherapeutic drugs is the lack of selectivity, which may lead to severe side effects. Daunorubicin and doxorubicin (Dox) are representative anthracycline antibiotics used in clinical for treating various kinds of cancers. However, severe side effects occur commonly because of the non-selective intercalation within the double-stranded nucleic acid of all cells. Nowadays, intensive advancements have been achieved in designing modified doxorubicin with targeted properties.<sup>108–110</sup> Bagalkot and colleagues<sup>111</sup> designed an aptamer–doxorubicin physical conjugate using a 2'-F RNA aptamer that binds to the prostate-specific membrane antigen (PSMA) with high affinity and specificity. It was reported that this conjugate could bind to the PSMA-expressing LNCaP cells specifically. Huang et al.<sup>112</sup> coupled doxorubicin with the sgc8c DNA aptamer using a covalent bond, which targeted T-cell acute lymphoblastic leukemia cells specifically. As a result, the non-target cells were not affected by sgc8c-Dox conjugates. In addition, Deng et al.<sup>109</sup> prepared a doxorubicin-functionalized aptamer complex (TLS11a-GC-Dox) which recognized HepG2 cells. Our group designed and synthesized a nucleolin aptamer–paclitaxel conjugate (NucA-PTX) that specifically delivered PTX to the tumor cells.<sup>113</sup> By coupling a tumor-targeting nucleolin aptamer (NucA) to the 2'-hydroxy of PTX via a cathepsin B sensitive linker, NucA-PTX remains intact in the bloodstream. Once tumor cells uptake the conjugates, the dipeptide bond linker of NucA-PTX is cleaved by cathepsin B, and then the free PTX is released for action. The conjugation of NucA facilitates the selective retention of the conjugated PTX in tumor tissue, further resulting in dramatically increased anti-tumor activity and decreased toxicity (Figure 13). Obviously, ApDCs demonstrate significant superiorities in many aspects compared with antibody–drug conjugates (ADCs). In the



**Figure 13.** Illustration of NucA-PTX conjugate. PTX and aptamer are coupled by a cleavable dipeptide linker.

generation of the targeting ligand, aptamers can be screened within several days via an efficient SELEX procedure instead of the complicated *in vivo* screening process involved in antibody discovery. Besides, aptamers can target a wide spectrum of molecules, including those with weak immunogenicity, which cannot be recognized by antibodies. Furthermore, in the process of preparations, aptamers can be chemically synthesized like common chemical entities under good manufacturing practice (GMP) conditions. The involvement of a series of cell colonies and living animals in the production of antibodies may cause an increase in contaminations and high batch-to-batch variation. In addition, in the process of application, ADCs could induce the body to produce antibodies against them, thus reducing the therapeutic effect. As low-molecular-weight molecules, ApDCs can barely induce antibodies, so they can be applied in the clinical over a much longer period. Currently, the preparation of ApDC mainly follows the methods developed for ADCs. As an alternative, automated modular synthesis of ApDCs had been proposed and developed, which may become a characteristic of ApDC.<sup>114</sup> Importantly, automated and modular technology may dramatically reduce the production difficulties and costs of ApDCs.

**5.2. Aptamer as an Escorting Material for the Targeted Delivery of Therapeutic RNAs.** The RNA interference (RNAi) strategy is a promising technique extensively applied for disease treatment. However, the individual RNA molecule may lack selectivity and specificity in systemic drug delivery, hindering the therapeutic translation of RNAi agents' broader application. Aptamer–RNA conjugated complexes have been widely researched for targeted delivery of bioactive RNA molecules, including small interfering RNA (siRNA), microRNA (miRNA), and small hairpin RNA (shRNA), to the target location.<sup>115,116</sup>

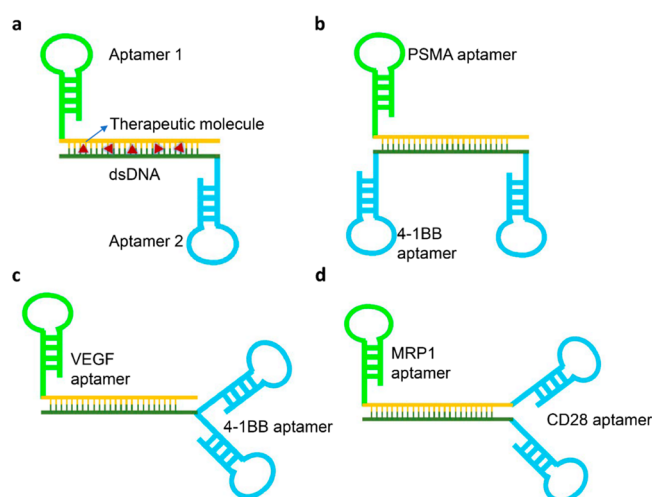
McNamara and colleagues developed the first aptamer–siRNA conjugates. The aptamer against human PSMA was covalently coupled to therapeutic siRNAs.<sup>117</sup> The conjugates targeted prostate cancer cells with PSMA expression and inhibited the target gene of siRNA, and consequently the tumor volume in a xenograft model of prostate cancer was diminished.<sup>118</sup> Our group reported aptamer-modified lipid nanoparticles (LNPs) containing siRNAs of osteogenic pleckstrin homology domain-containing family O member 1 (Plekho1) (CH6-LNPs-siRNA).<sup>119</sup> The introduction of an aptamer enhanced the knockdown efficiency of Plekho1 siRNA in *in vitro* determinations. In addition, CH6-LNP-siRNAs colocalized the siRNAs with cellular markers of osteoblasts and accordingly increased the bone anabolism in the test animal model. Nowadays, the aptamer–siRNA conjugating strategy for the targeting delivery of siRNA is being actively studied.<sup>120–122</sup>

Another representative RNA modified by aptamers for therapeutic applications is miRNA. It is mostly the deregulation of miRNA that is the cause of disease. The recovery of miRNA levels by efficient delivery is an attracting strategy. Esposito and colleagues reported an aptamer–miRNA conjugate which targeted the oncogenic receptor tyrosine kinase Axl and silenced the human *let-7g* miRNA in target cells.<sup>123</sup> Recently, a conjugated aptamer–miRNA-based targeted therapeutic was further reported to treat glioblastoma cells.<sup>124</sup> Anti-Axl aptamer and anti-PDGFR $\beta$  aptamer were designed as the carriers for anti-miR-10b and miR-137, respectively. These two aptamer carriers delivered therapeutic miR-137 and anti-miR-10b molecules to the glioma cell cluster, which inhibited the proliferation of glioblastoma cells.



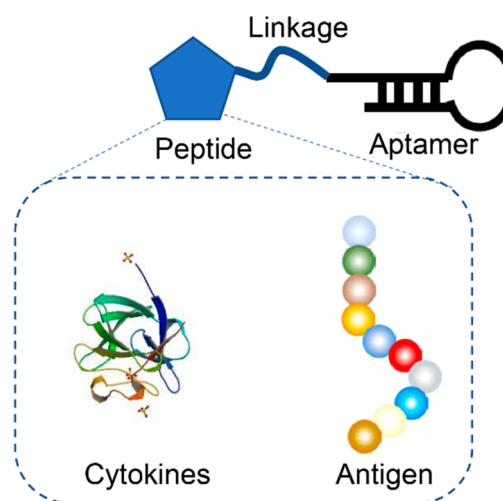
**5.3. Bi-specific Aptamer Systems.** Bi-specific aptamers are bivalent aptamers formed by the interaction or bridging of two aptamers. Due to their double-targeting advantages, bi-specific aptamers can stimulate or regulate the immune response between functional molecules and target cells.<sup>125</sup> Bi-specific aptamers can consist of two distinct aptamers which target two drug sites, or one therapeutic aptamer with the other one as a targeting moiety, or a bi-specific T-cell engager guiding cytotoxic T lymphocytes to malignant cells.<sup>126</sup> Gilboa and colleagues<sup>127</sup> reported a bivalent 4-1BB aptamer acting as a co-stimulant activated by T-cells in the fight against infection, and it was inactivated by the microenvironment after it entered into the tumor cell. PSMA is highly expressed in most prostate cancer cells, making it a significant biomarker for diagnosis and treatment. To avoid unwanted toxicities caused by non-targeting activation, a bi-specific aptamer containing anti-PSMA aptamer and anti-4-1BB aptamer was constructed to promote immune response to tumor tissues in model animals successfully. VEGF is widely expressed in most tumors and is the core protein in tumor angiogenesis. Schrand and colleagues<sup>128</sup> further reported a bi-specific aptamer covalently combining 4-1BB agonistic aptamer with VEGF targeting aptamer. This approach blocks VEGF expression in tumor cells and facilitates lymphocytes infiltrating tumor tissues through anti-4-1BB aptamer simultaneously. In another study, Soldevilla et al.<sup>129</sup> successfully suppressed the expression of Multidrug-Resistant-associated Protein 1 (MRP1) and simultaneously activated T-cells by stimulating CD28 receptor by constructing a MRP1-CD28 bivalent aptamer. This bi-specific aptamer promoted the suppressive effect in chemotherapy-resistant tumors. A significant proliferation of T lymphocytes in C57BL6 mice was observed, and meanwhile the aptamer showed little toxicity, thus prolonging the animals' survival (Figure 14).

**5.4. Aptamer–Peptide Conjugated Systems.** In recent years, the clinical progress of immunotherapy has developed rapidly. Up to now, there have been a variety of immune-checkpoint inhibitors, including anti-PD-1 molecules, anti-PD-L1 molecules, and anti-CTLA-4 molecules.<sup>130</sup> However, the



**Figure 14.** Schematic illustration of a bi-specific aptamer. (a) A bi-specific aptamer usually consists of two independent aptamers with a dsDNA linker; it can also serve as a small-molecule drug carrier in bi-specific drug delivery. (b) PSMA-dimeric 4-1BB aptamer conjugates. (c) VEGF-dimeric 4-1BB aptamer conjugates. (d) MRP1-dimeric CD28 aptamer conjugates.

existing immunotherapies have some problems in reaching the target tissue and penetrating the cell membrane of the target tumor. Aptamers provide an alternative to solve this problem, attributed to their biocompatibility and low immune toxicities.<sup>131</sup> Applications of aptamers for the targeted delivery of cytokine or peptide antigens have been widely studied.<sup>132–135</sup> The effectiveness of antigen presentation and the immunomodulation of vaccines play the core roles in the development of subunit vaccines. These studies have demonstrated efficient and specific ways for the delivery of peptide antigens toward antigen presenting cells (APCs).<sup>136</sup> Wengerter et al.<sup>134</sup> reported an aptamer-SIINFEKL chimera which achieved an upgraded level of T-cell proliferation and IFN- $\gamma$  and IL-2 production, and caused significant growth inhibition of the OVA-expressing B16F10 cells in mice. The aptamer was selected for targeting murine DEC205, a C-type lectin that is over-expressed on CD8 $\alpha$  dendritic cells (DCs), which is essential in antigen cross-presentation and activation of T-cell. The ovalbumin peptide SIINFEKL is an MHC-I-restricted antigen epitope targeting CD8 $^{+}$  DCs (Figure 15).

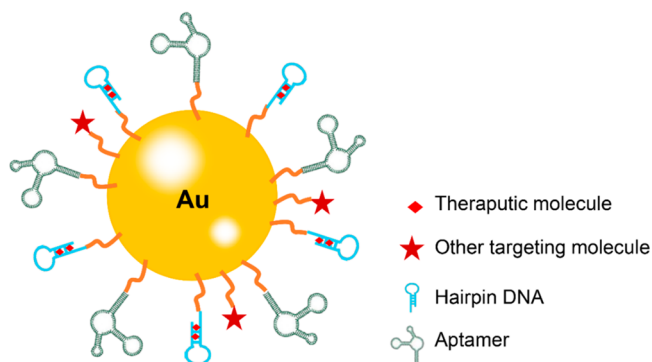


**Figure 15.** Schematic illustration of aptamer–peptide conjugated systems.

**5.5. Aptamer–Gold Nanoparticle Conjugated Systems.** There are a lot of advantages in gold nanoparticles (AuNPs), including biocompatibility, non-toxicity, easy modification, and high stability.<sup>137,138</sup> Luo et al.<sup>137</sup> designed a smart aptamer-based drug delivery system. The DNA aptamer sgc8c, serving as a targeting moiety against protein tyrosine kinase 7 (PTK7), was functionalized onto the surface of AuNPs. Additionally, hpDNA with a repeated CGATCG deoxyribonucleic acid unit was also assembled on the AuNP surface used as the delivery carrier for the chemotherapy drug doxorubicin. The system indicated a high loading ability and the ability to target CCRF-CEM (T-cell acute lymphoblastic leukemia) cells efficiently. Dam and colleagues<sup>139</sup> reported a nanoparticle composed of a gold nanostructured core and an anti-nucleolin aptamer (AS1411). No signs of significant toxicity were found at the highest tested dose (up to 48 mg/kg), and the tumor-specific accumulation was reported to be 5 times higher in invasive breast cancer tumors than in fibrosarcoma tumors (Figure 16).

**5.6. Aptamer–Liposome Delivery Systems.** Liposomes are broadly applied in the research focused on drug delivery. Their amphiphilic characteristics make them available for

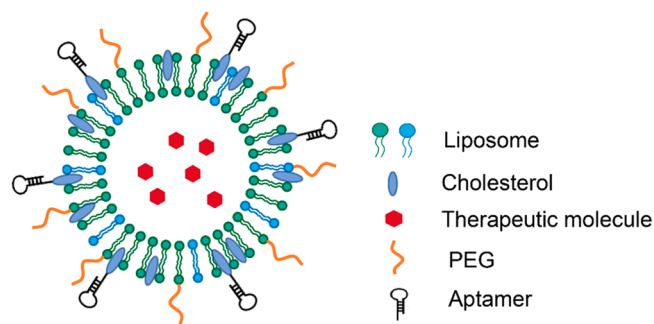




**Figure 16.** Schematic diagrams of nanoplateforms based on aptamer-conjugated gold nanoparticles for targeted drug delivery. The aptamer–AuNP complexes are usually composed of gold nanoparticles, aptamers targeting protein, and hpDNA serving as a drug cargo, while other targeting molecules are optional.

encapsulating a wide range of drug molecules.<sup>140,141</sup> Additionally, the surface of the liposome can be modified by different agents, such as high-molecular-weight PEG moieties, prolonging plasma half-lives and leading to efficient accumulation at the target positions.<sup>142</sup> Actually, there have been several liposome-based therapeutics approved by the U.S. FDA for clinical applications.<sup>143,144</sup> Currently, a number of studies have been reported to introduce aptamers for the application of liposomes. Cao and colleagues reported an aptamer-based liposome delivery system first in 2009.<sup>145</sup> They extended a 12-thymine sequence at the 3'-end of the nucleolin aptamer. Then a cholesterol tail for immobilization on the hydrophobic part of a PEGylated liposome was coupled to the 12-thymine linker, and the chemotherapeutic drug and the hydrophilic dye were encapsulated into the liposome. This combined system was demonstrated to deliver cisplatin to the target in a cell-specific manner. Alshaer et al.<sup>146</sup> incorporated an RNA aptamer with 2'-F-pyrimidine modification to the PEGylated liposomes which recognized CD44 receptor protein. This system was reported to demonstrate significantly enhanced ability to bind to cancer cells expressing CD44 compared with unmodified liposomes. Stuart et al.<sup>147</sup> developed an aptamer-modified liposome for delivering the zinc chelator specifically to prostate cancer cells. They reported that the zinc chelator was delivered to the prostate cancer cells specifically both *in vitro* and *in vivo* and inhibited tumor cell growth. Li et al.<sup>80</sup> developed an aptamer-modified liposome for the specific delivery of the anti-BRAF siRNA (siBraf) for treating malignant melanomas. The aptamer AS1411, bonded to nucleolin, was conjugated to PEG-DOPE as a targeting moiety. Increased siRNA accumulation was observed in melanomas with this strategy.

Our group discovered an aptamer (LC09) targeting osteosarcoma (OS) cells specifically and further prepared an LC09-modified PEG-PEI-cholesterol (PPC) lipopolymer. CRISPR/Cas9 plasmids encoding VEGFA gRNA and Cas9 were then encapsulated in the system.<sup>14</sup> The results indicated that aptamer LC09 facilitated the selective delivery of CRISPR/Cas9 in both orthotopic OS and lung metastasis, utilizing effective genome editing of VEGFA for tumor treatment without obvious toxicity. The combined system restrained both autocrine and paracrine signaling of VEGFA in tumor cells, facilitating the clinical translation of the CRISPR/Cas9 strategy into cancer therapies (Figure 17). Plourde et al.<sup>148</sup> demonstrated that aptamers were able to act as multifunctional



**Figure 17.** Schematic illustration of an aptamer-conjugated liposome.

excipients for liposomal formulations in addition to the targeting moieties. Ma et al.<sup>149</sup> designed a kind of neutral lipids that can interact with oligonucleotides via hydrogen bonding and  $\pi$ – $\pi$  stacking, which demonstrated superior properties over general commercial cationic transfection reagents. Nucleoside-based lipid headed with cytidine can deliver aptamer AS1411 with high transfection efficiency and low toxicity, which indicates that nucleoside-based lipids are promising carriers for transfecting therapeutic G4-aptamers and other kinds of oligonucleotides.

**5.7. Other Aptamer-Conjugated Systems.** To achieve the targeted delivery and efficient release of therapeutic molecules, other aptamer-conjugated systems have also been developed. Li et al.<sup>150</sup> constructed DNA nanohydrogels composed of three moieties, i.e., YMA (Y-shaped monomer A with three sticky ends), YMB (Y-shaped monomer B with one sticky end), and LK (DNA linker with two sticky ends). Hydrogels are cross-linked structures with hydrophilic materials that contain a large volume of water or biological liquids internally. Hydrogels with targeting functions were developed for a number of biomedical and pharmaceutical applications.<sup>151–154</sup> By constructing corresponding building blocks of different functional molecules, such as aptamers, sulfur-containing linkages, and therapeutic nucleic acids, the chemically prepared aptamer-functionalized nanohydrogels (Y-gel-Apt) were applied in targeted and stimuli-responsive therapy. The aptamer-functionalized nanohydrogels remarkably inhibited cell proliferation and migration in test A549 cells, while the control cells were unaffected. They are promising carriers for the targeted delivery of genes and drugs due to their superior advantages including simple and feasible self-assembly, efficient cellular uptake, and excellent biocompatibility. In addition, an oligonucleotide-based system containing ferrocene<sup>155</sup> was designed to solve the incompatibility in the enhanced permeability and retention effect (EPR). These nucleic acid assembly systems released highly reactive hydroxyl radicals into the microenvironment of a malignant tumor, realizing increased *in vivo* efficiency for cancer treatment.

## 6. CONCLUSIONS

Nucleic acid aptamers are chemically synthesized analogues (chemical antibodies) of conventional antibodies that exhibit a great many advantages in therapeutic applications. Aptamer technology demonstrates great potential in producing candidate bio-active agents and plays a dramatically important role in the subsequent therapeutic field. However, the discovery of aptamers with high performance is still a limit for aptamer-based research and industrial transformation. Fortunately, the introduction of new technologies including microfluidic devices and high-throughput sequencing has improved the hit rate of

SELEX. There have been great advances in material sciences and analytical methods in the past several years. Significant optimization has been made in the key processes of SELEX, including the design of a starting sequence pool (e.g., modifications on nucleic acid chains), automatic operation (e.g., microfluidic devices), and aptamer identifications (e.g., HTS). Presently, researchers have an ever-growing toolkit for aptamer discovery, and new technologies are being developed to increase selection hit rate and reduce the time and resources needed to generate new aptamers.

In addition, chemical modifications overcome the innate shortcomings of functional oligonucleotides, which is an attractive and efficient solution. As described in this Review, diverse strategies can be applied in aptamer modification to achieve a prolonged circulation half-life in the serum either directly in the SELEX process or by post-SELEX chemical preparation. Notably, hydrophobic fatty acid and amphiphilic PEG components have been widely used as long-lasting coupling agents in modifications of drug candidates. However, the high-molecular-mass PEG component possesses a very large molecular weight proportion, which is a serious limitation in subcutaneous dosage increase to maximize therapeutic potential. Furthermore, it should be noted that serious or fatal immune responses to PEG moieties have been reported in clinical studies. Accordingly, safety concerns are raised about the adoption of high-molecular-mass PEG moieties in aptamer modifications. Considering this, we expect that a small-molecule coupling agent utilizing serum albumin as the carrier to prolong the acting time could be an alternative to enhance the clinical translational value of nucleic acid aptamers.

As an attractive class of targeted moieties, aptamers can act as small molecules with superior flexibility and infiltrate target positions that may not be accessible to large-volume antibody molecules. Meanwhile, aptamers can also be applied in targeted therapy that cannot be realized by small-molecule drugs due to their dramatically high binding specificity, which is comparable to that of antibodies. These superior properties could give aptamers a niche in the therapeutic field between small-molecule drugs and high-molecular-mass antibodies. It is highly expected that innovative therapeutic applications will emerge through rational design and development to achieve a great pharmacokinetic profile and excellent clinical performance.

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Shuaijian Ni, Zhenjian Zhuo, and Yufei Pan wrote the manuscript. Yuanyuan Yu, Fangfei Li, Jin Liu, Luyao Wang, Xiaoqiu Wu, Dijie Li, and Youyang Wan contributed to the literature research. Lihe Zhang, Zhenjun Yang, Bao-Ting Zhang, Aiping Lu, and Ge Zhang revised and approved the manuscript.

## Notes

The authors declare no competing financial interest.

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## REFERENCES

- (1) Tuerk, C.; Gold, L. Systematic Evolution of Ligands by Exponential Enrichment: Rna Ligands to Bacteriophage T4 DNA Polymerase. *Science* **1990**, *249*, 505–510.
- (2) Ellington, A. D.; Szostak, J. W. In Vitro Selection of Rna Molecules That Bind Specific Ligands. *Nature* **1990**, *346*, 818–822.
- (3) Zhou, J.; Rossi, J. Aptamers as Targeted Therapeutics: Current Potential and Challenges. *Nat. Rev. Drug Discovery* **2017**, *16*, 181–202.
- (4) Kong, H. Y.; Byun, J. Nucleic Acid Aptamers: New Methods for Selection, Stabilization, and Application in Biomedical Science. *Biomol. Ther.* **2013**, *21*, 423–434.
- (5) Lou, X.; Qian, J.; Xiao, Y.; Viel, L.; Gerdon, A. E.; Lagally, E. T.; Atzberger, P.; Tarasow, T. M.; Heeger, A. J.; Soh, H. T. Micromagnetic Selection of Aptamers in Microfluidic Channels. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 2989–2994.
- (6) Reiss, D. J.; Mobley, H. L. Determination of Target Sequence Bound by Papx, Repressor of Bacterial Motility, in FlhD Promoter Using Systematic Evolution of Ligands by Exponential Enrichment (Selex) and High Throughput Sequencing. *J. Biol. Chem.* **2011**, *286*, 44726–44738.
- (7) Zhang, Y.; Lai, B. S.; Juhas, M. Recent Advances in Aptamer Discovery and Applications. *Molecules* **2019**, *24*, 941.
- (8) Zhu, G.; Niu, G.; Chen, X. Aptamer-Drug Conjugates. *Bioconjugate Chem.* **2015**, *26*, 2186–2197.
- (9) He, F.; Wen, N.; Xiao, D.; Yan, J.; Xiong, H.; Cai, S.; Liu, Z.; Liu, Y. Aptamer Based Targeted Drug Delivery Systems: Current Potential and Challenges. *Curr. Med. Chem.* **2020**, *27*, 2189.
- (10) Wei, L.; Cohen, A. Spotlight Commentary: Medicines Use During Pregnancy and Harmful Effects on Offspring. *Br. J. Clin. Pharmacol.* **2019**, *85*, 1393.
- (11) Wang, H.; Lam, C. H.; Li, X.; West, D. L.; Yang, X. Selection of Pd1/Pd-L1 X-Aptamers. *Biochimie* **2018**, *145*, 125–130.
- (12) Nozari, A.; Berezovski, M. V. Aptamers for Cd Antigens: From Cell Profiling to Activity Modulation. *Mol. Ther.–Nucleic Acids* **2017**, *6*, 29–44.
- (13) Tan, W.; Donovan, M. J.; Jiang, J. Aptamers from Cell-Based Selection for Bioanalytical Applications. *Chem. Rev.* **2013**, *113*, 2842–2862.
- (14) Liang, C.; Li, F.; Wang, L.; Zhang, Z. K.; Wang, C.; He, B.; Li, J.; Chen, Z.; Shaikh, A. B.; Liu, J.; Wu, X.; Peng, S.; Dang, L.; Guo, B.; He, X.; Au, D. W. T.; Lu, C.; Zhu, H.; Zhang, B. T.; Lu, A.; Zhang, G. Tumor Cell-Targeted Delivery of Crispr/Cas9 by Aptamer-Functionalized Lipopolymer for Therapeutic Genome Editing of Vegfa in Osteosarcoma. *Biomaterials* **2017**, *147*, 68–85.
- (15) Ng, E. W.; Shima, D. T.; Calias, P.; Cunningham, E. T., Jr.; Guyer, D. R.; Adamis, A. P. Pegaptanib, a Targeted Anti-Vegf Aptamer for Ocular Vascular Disease. *Nat. Rev. Drug Discovery* **2006**, *5*, 123–132.
- (16) Kovacevic, K. D.; Gilbert, J. C.; Jilma, B. Pharmacokinetics, Pharmacodynamics and Safety of Aptamers. *Adv. Drug Delivery Rev.* **2018**, *134*, 36–50.
- (17) Ni, S.; Yao, H.; Wang, L.; Lu, J.; Jiang, F.; Lu, A.; Zhang, G. Chemical Modifications of Nucleic Acid Aptamers for Therapeutic Purposes. *Int. J. Mol. Sci.* **2017**, *18*, 1683.
- (18) O'Connell, D.; Koenig, A.; Jennings, S.; Hicke, B.; Han, H. L.; Fitzwater, T.; Chang, Y. F.; Varki, N.; Parma, D.; Varki, A. Calcium-Dependent Oligonucleotide Antagonists Specific for L-Selectin. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 5883–5887.
- (19) Maier, K. E.; Levy, M. From Selection Hits to Clinical Leads: Progress in Aptamer Discovery. *Mol. Ther.–Methods Clin. Dev.* **2016**, *3*, 16014.
- (20) Keefe, A. D.; Pai, S.; Ellington, A. Aptamers as Therapeutics. *Nat. Rev. Drug Discovery* **2010**, *9*, 537–550.
- (21) Healy, J. M.; Lewis, S. D.; Kurz, M.; Boomer, R. M.; Thompson, K. M.; Wilson, C.; McCauley, T. G. Pharmacokinetics and Biodistribution of Novel Aptamer Compositions. *Pharm. Res.* **2004**, *21*, 2234–2246.
- (22) Mi, J.; Liu, Y.; Rabbani, Z. N.; Yang, Z.; Urban, J. H.; Sullenger, B. A.; Clary, B. M. In Vivo Selection of Tumor-Targeting Rna Motifs. *Nat. Chem. Biol.* **2010**, *6*, 22–24.



- (23) Cole, K. H.; Luptak, A. High-Throughput Methods in Aptamer Discovery and Analysis. *Methods Enzymol.* **2019**, *621*, 329–346.
- (24) Li, K.; Deng, J.; Jin, H.; Yang, X.; Fan, X.; Li, L.; Zhao, Y.; Guan, Z.; Wu, Y.; Zhang, L.; Yang, Z. Chemical Modification Improves the Stability of the DNA Aptamer Gbi-10 and Its Affinity Towards Tenascin-C. *Org. Biomol. Chem.* **2017**, *15*, 1174–1182.
- (25) Vaught, J. D.; Bock, C.; Carter, J.; Fitzwater, T.; Otis, M.; Schneider, D.; Rolando, J.; Waugh, S.; Wilcox, S. K.; Eaton, B. E. Expanding the Chemistry of DNA for in Vitro Selection. *J. Am. Chem. Soc.* **2010**, *132*, 4141–4151.
- (26) Strauss, S.; Nickels, P. C.; Strauss, M. T.; Jimenez Sabinina, V.; Ellenberg, J.; Carter, J. D.; Gupta, S.; Janjic, N.; Jungmann, R. Modified Aptamers Enable Quantitative Sub-10-Nm Cellular DNA-Paint Imaging. *Nat. Methods* **2018**, *15*, 685–688.
- (27) Gupta, S.; Hirota, M.; Waugh, S. M.; Murakami, I.; Suzuki, T.; Muraguchi, M.; Shibamori, M.; Ishikawa, Y.; Jarvis, T. C.; Carter, J. D.; Zhang, C.; Gawande, B.; Vrkljan, M.; Janjic, N.; Schneider, D. J. Chemically Modified DNA Aptamers Bind Interleukin-6 with High Affinity and Inhibit Signaling by Blocking Its Interaction with Interleukin-6 Receptor. *J. Biol. Chem.* **2014**, *289*, 8706–8719.
- (28) Joshi, A.; Mayr, M. In Aptamers They Trust: The Caveats of the Somascan Biomarker Discovery Platform from Somalogic. *Circulation* **2018**, *138*, 2482–2485.
- (29) Cotton, R. J.; Graumann, J. Readat: An R Package for Reading and Working with Somalogic Adat Files. *BMC Bioinf.* **2016**, *17*, 201.
- (30) Padilla, R.; Sousa, R. Efficient Synthesis of Nucleic Acids Heavily Modified with Non-Canonical Ribose 2'-Groups Using a Mutant T7 Rna Polymerase (Rnap). *Nucleic. Acids. Res.* **1999**, *27*, 1561–1563.
- (31) Ruckman, J.; Green, L. S.; Beeson, J.; Waugh, S.; Gillette, W. L.; Henninger, D. D.; Claesson-Welsh, L.; Janjic, N. 2'-Fluoropyrimidine Rna-Based Aptamers to the 165-Amino Acid Form of Vascular Endothelial Growth Factor (Vegf165). Inhibition of Receptor Binding and Vegf-Induced Vascular Permeability through Interactions Requiring the Exon 7-Encoded Domain. *J. Biol. Chem.* **1998**, *273*, 20556–20567.
- (32) Barciszewski, J.; Medgaard, M.; Koch, T.; Kurreck, J.; Erdmann, V. A. Locked Nucleic Acid Aptamers. *Methods Mol. Biol.* **2009**, *535*, 165–186.
- (33) Schmidt, K. S.; Borkowski, S.; Kurreck, J.; Stephens, A. W.; Bald, R.; Hecht, M.; Friebe, M.; Dinkelborg, L.; Erdmann, V. A. Application of Locked Nucleic Acids to Improve Aptamer in Vivo Stability and Targeting Function. *Nucleic. Acids. Res.* **2004**, *32*, 5757–5765.
- (34) Green, L. S.; Jellinek, D.; Bell, C.; Beebe, L. A.; Feistner, B. D.; Gill, S. C.; Jucker, F. M.; Janjic, N. Nuclease-Resistant Nucleic Acid Ligands to Vascular Permeability Factor/Vascular Endothelial Growth Factor. *Chem. Biol.* **1995**, *2*, 683–695.
- (35) Jhaveri, S.; Olwin, B.; Ellington, A. D. In Vitro Selection of Phosphorothiolated Aptamers. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2285–2290.
- (36) King, D. J.; Ventura, D. A.; Brasier, A. R.; Gorenstein, D. G. Novel Combinatorial Selection of Phosphorothioate Oligonucleotide Aptamers. *Biochemistry* **1998**, *37*, 16489–16493.
- (37) Yang, X.; Gorenstein, D. G. Progress in Thioaptamer Development. *Curr. Drug Targets* **2004**, *5*, 705–715.
- (38) Abeydeera, N. D.; Egli, M.; Cox, N.; Mercier, K.; Conde, J. N.; Pallan, P. S.; Mizurini, D. M.; Sierant, M.; Hibti, F. E.; Hassell, T.; Wang, T.; Liu, F. W.; Liu, H. M.; Martinez, C.; Sood, A. K.; Lybrand, T. P.; Frydman, C.; Monteiro, R. Q.; Gomer, R. H.; Nawrot, B.; Yang, X. Evoking Picomolar Binding in Rna by a Single Phosphorodithioate Linkage. *Nucleic Acids Res.* **2016**, *44*, 8052–8064.
- (39) Kimoto, M.; Yamashige, R.; Matsunaga, K.; Yokoyama, S.; Hirao, I. Generation of High-Affinity DNA Aptamers Using an Expanded Genetic Alphabet. *Nat. Biotechnol.* **2013**, *31*, 453–457.
- (40) Matsunaga, K. I.; Kimoto, M.; Hirao, I. High-Affinity DNA Aptamer Generation Targeting Von Willebrand Factor A1-Domain by Genetic Alphabet Expansion for Systematic Evolution of Ligands by Exponential Enrichment Using Two Types of Libraries Composed of Five Different Bases. *J. Am. Chem. Soc.* **2017**, *139*, 324–334.
- (41) Zhang, L.; Yang, Z.; Sefah, K.; Bradley, K. M.; Hoshika, S.; Kim, M. J.; Kim, H. J.; Zhu, G.; Jimenez, E.; Cansiz, S.; Teng, I. T.; Champanhac, C.; McLendon, C.; Liu, C.; Zhang, W.; Gerloff, D. L.; Huang, Z.; Tan, W.; Benner, S. A. Evolution of Functional Six-Nucleotide DNA. *J. Am. Chem. Soc.* **2015**, *137*, 6734–6737.
- (42) Tan, J.; Zhao, M.; Wang, J.; Li, Z.; Liang, L.; Zhang, L.; Yuan, Q.; Tan, W. Regulation of Protein Activity and Cellular Functions Mediated by Molecularly Evolved Nucleic Acids. *Angew. Chem., Int. Ed.* **2019**, *58*, 1621–1625.
- (43) Eulberg, D.; Klussmann, S. Spiegelmers: Biostable Aptamers. *ChemBioChem* **2003**, *4*, 979–983.
- (44) Hoehlig, K.; Maasch, C.; Shushakova, N.; Buchner, K.; Huber-Lang, M.; Purschke, W. G.; Vater, A.; Klussmann, S. A Novel C5a-Neutralizing Mirror-Image (L-)Aptamer Prevents Organ Failure and Improves Survival in Experimental Sepsis. *Mol. Ther.* **2013**, *21*, 2236–2246.
- (45) Klussmann, S.; Nolte, A.; Bald, R.; Erdmann, V. A.; Furste, J. P. Mirror-Image Rna That Binds D-Adenosine. *Nat. Biotechnol.* **1996**, *14*, 1112–1115.
- (46) Vater, A.; Klussmann, S. Turning Mirror-Image Oligonucleotides into Drugs: The Evolution of Spiegelmer((R)) Therapeutics. *Drug Discovery Today* **2015**, *20*, 147–155.
- (47) Boyce, M.; Warrington, S.; Cortez, B.; Zollner, S.; Vauleon, S.; Swinkels, D. W.; Summo, L.; Schwoebel, F.; Riecke, K. Safety, Pharmacokinetics and Pharmacodynamics of the Anti-Hepcidin Spiegelmer Lixapetide Pegol in Healthy Subjects. *Br. J. Pharmacol.* **2016**, *173*, 1580–1588.
- (48) Maasch, C.; Buchner, K.; Eulberg, D.; Vonhoff, S.; Klussmann, S. Physicochemical Stability of Nox-E36, a 40mer L-Rna (Spiegelmer) for Therapeutic Applications. *Nucleic. Acids. Symp. Ser. (Oxf.)* **2008**, *52*, 61–62.
- (49) Steurer, M.; Montillo, M.; Scarfo, L.; Mauro, F. R.; Andel, J.; Wildner, S.; Trentin, L.; Janssens, A.; Burgstaller, S.; Fromming, A.; Dummmler, T.; Riecke, K.; Baumann, M.; Beyer, D.; Vauleon, S.; Ghia, P.; Foa, R.; Caligaris-Cappio, F.; Gobbi, M. Olapted Pegol (Nox-A12) with Bendamustine and Rituximab: A Phase Iia Study in Patients with Relapsed/Refractory Chronic Lymphocytic Leukemia. *Haematologica* **2019**, *104*, 2053–2060.
- (50) Irvine, D.; Tuerk, C.; Gold, L. Selexion. Systematic Evolution of Ligands by Exponential Enrichment with Integrated Optimization by Non-Linear Analysis. *J. Mol. Biol.* **1991**, *222*, 739–761.
- (51) Sinha, A.; Gopinathan, P.; Chung, Y. D.; Lin, H. Y.; Li, K. H.; Ma, H. P.; Huang, P. C.; Shiesh, S. C.; Lee, G. B. An Integrated Microfluidic Platform to Perform Uninterrupted Selex Cycles to Screen Affinity Reagents Specific to Cardiovascular Biomarkers. *Biosens. Bioelectron.* **2018**, *122*, 104–112.
- (52) Gotrik, M. R.; Feagin, T. A.; Csordas, A. T.; Nakamoto, M. A.; Soh, H. T. Advancements in Aptamer Discovery Technologies. *Acc. Chem. Res.* **2016**, *49*, 1903–1910.
- (53) Pleiko, K.; Saulite, L.; Parfejevs, V.; Miculis, K.; Vjaters, E.; Riekstina, U. Differential Binding Cell-Selex Method to Identify Cell-Specific Aptamers Using High-Throughput Sequencing. *Sci. Rep.* **2019**, *9*, 8142.
- (54) Dougan, H.; Lyster, D. M.; Vo, C. V.; Stafford, A.; Weitz, J. I.; Hobbs, J. B. Extending the Lifetime of Anticoagulant Oligodeoxynucleotide Aptamers in Blood. *Nucl. Med. Biol.* **2000**, *27*, 289–297.
- (55) Musumeci, D.; Montesarchio, D. Polyvalent Nucleic Acid Aptamers and Modulation of Their Activity: A Focus on the Thrombin Binding Aptamer. *Pharmacol. Ther.* **2012**, *136*, 202–215.
- (56) Pagratis, N. C.; Bell, C.; Chang, Y. F.; Jennings, S.; Fitzwater, T.; Jellinek, D.; Dang, C. Potent 2'-Amino-, and 2'-Fluoro-2'-Deoxyribo-nucleotide Rna Inhibitors of Keratinocyte Growth Factor. *Nat. Biotechnol.* **1997**, *15*, 68–73.
- (57) Fine, S. L.; Martin, D. F.; Kirkpatrick, P. Pegaptanib Sodium. *Nat. Rev. Drug Discovery* **2005**, *4*, 187–188.
- (58) Veedu, R. N.; Wengel, J. Locked Nucleic Acid Nucleoside Triphosphates and Polymerases: On the Way Towards Evolution of Lna Aptamers. *Mol. BioSyst.* **2009**, *5*, 787–792.



- (59) Purschke, W. G.; Radtke, F.; Kleinjung, F.; Klusmann, S. A DNA Spiegelmer to Staphylococcal Enterotoxin B. *Nucleic Acids Res.* **2003**, *31*, 3027–3032.
- (60) Dass, C. R.; Saravolac, E. G.; Li, Y.; Sun, L. Q. Cellular Uptake, Distribution, and Stability of 10–23 Deoxyribozymes. *Antisense Nucleic Acid Drug Dev.* **2002**, *12*, 289–299.
- (61) Guo, P. The Emerging Field of Rna. *Nat. Nanotechnol.* **2010**, *5*, 833–842.
- (62) Lee, C. H.; Lee, S. H.; Kim, J. H.; Noh, Y. H.; Noh, G. J.; Lee, S. W. Pharmacokinetics of a Cholesterol-Conjugated Aptamer against the Hepatitis C Virus (Hcv) Ns5b Protein. *Mol. Ther.–Nucleic Acids* **2015**, *4*, e254.
- (63) Burmeister, P. E.; Lewis, S. D.; Silva, R. F.; Preiss, J. R.; Horwitz, L. R.; Pendergrast, P. S.; McCauley, T. G.; Kurz, J. C.; Epstein, D. M.; Wilson, C.; Keefe, A. D. Direct In Vitro Selection of a 2'-O-Methyl Aptamer to Vegf. *Chem. Biol.* **2005**, *12*, 25–33.
- (64) Heo, K.; Min, S. W.; Sung, H. J.; Kim, H. G.; Kim, H. J.; Kim, Y. H.; Choi, B. K.; Han, S.; Chung, S.; Lee, E. S.; Chung, J.; Kim, I. H. An Aptamer-Antibody Complex (Oligobody) as a Novel Delivery Platform for Targeted Cancer Therapies. *J. Controlled Release* **2016**, *229*, 1–9.
- (65) Willis, M. C.; Collins, B. D.; Zhang, T.; Green, L. S.; Sebesta, D. P.; Bell, C.; Kellogg, E.; Gill, S. C.; Magallanes, A.; Knauer, S.; Bende, R. A.; Gill, P. S.; Janjic, N. Liposome-Anchored Vascular Endothelial Growth Factor Aptamers. *Bioconjugate Chem.* **1998**, *9*, 573–582.
- (66) Zhou, J.; Soontornworajit, B.; Martin, J.; Sullenger, B. A.; Gilboa, E.; Wang, Y. A Hybrid DNA Aptamer-Dendrimer Nanomaterial for Targeted Cell Labeling. *Macromol. Biosci.* **2009**, *9*, 831–835.
- (67) Turecek, P. L.; Bossard, M. J.; Schoetens, F.; Ivens, I. A. Pegylation of Biopharmaceuticals: A Review of Chemistry and Nonclinical Safety Information of Approved Drugs. *J. Pharm. Sci.* **2016**, *105*, 460–475.
- (68) Moreno, A.; Pitoc, G. A.; Ganson, N. J.; Layzer, J. M.; Hershfield, M. S.; Tarantal, A. F.; Sullenger, B. A. Anti-Peg Antibodies Inhibit the Anticoagulant Activity of Pegylated Aptamers. *Cell. Chem. Biol.* **2019**, *26*, 634–644.
- (69) Borbas, K. E.; Ferreira, C. S.; Perkins, A.; Bruce, J. I.; Missailidis, S. Design and Synthesis of Mono- and Multimeric Targeted Radiopharmaceuticals Based on Novel Cyclen Ligands Coupled to Anti-Muc1 Aptamers for the Diagnostic Imaging and Targeted Radiotherapy of Cancer. *Bioconjugate Chem.* **2007**, *18*, 1205–1212.
- (70) Burdick, A. D.; Scialoja, S.; Mantena, S. R.; Hollingshead, B. D.; Stanton, R.; Warneke, J. A.; Zeng, M.; Martsen, E.; Medvedev, A.; Makarov, S. S.; Reed, L. A.; Davis, J. W., 2nd; Whiteley, L. O. Sequence Motifs Associated with Hepatotoxicity of Locked Nucleic Acid-Modified Antisense Oligonucleotides. *Nucleic Acids Res.* **2014**, *42*, 4882–4891.
- (71) Shen, W.; De Hoyos, C. L.; Sun, H.; Vickers, T. A.; Liang, X. H.; Crooke, S. T. Acute Hepatotoxicity of 2' Fluoro-Modified 5–10–5 Gapper Phosphorothioate Oligonucleotides in Mice Correlates with Intracellular Protein Binding and the Loss of Dbhs Proteins. *Nucleic Acids Res.* **2018**, *46*, 2204–2217.
- (72) Lincoff, A. M.; Mehran, R.; Povsic, T. J.; Zelenkofske, S. L.; Huang, Z.; Armstrong, P. W.; Steg, P. G.; Bode, C.; Cohen, M. G.; Buller, C.; Laanmets, P.; Valgimigli, M.; Marandi, T.; Fridrich, V.; Cantor, W. J.; Merkely, B.; Lopez-Sendon, J.; Cornel, J. H.; Kasprzak, J. D.; Aschermann, M.; Guetta, V.; Morais, J.; Sinnaeve, P. R.; Huber, K.; Stables, R.; Sellers, M. A.; Borgman, M.; Glenn, L.; Levinson, A. I.; Lopes, R. D.; Hasselblad, V.; Becker, R. C.; Alexander, J. H. Effect of the Reg1 Anticoagulation System Versus Bivalirudin on Outcomes after Percutaneous Coronary Intervention (Regulate-Pci): A Randomised Clinical Trial. *Lancet* **2016**, *387*, 349–356.
- (73) Ortigao, J. F.; Rosch, H.; Selter, H.; Frohlich, A.; Lorenz, A.; Montenarh, M.; Seliger, H. Antisense Effect of Oligodeoxynucleotides with Inverted Terminal Internucleotidic Linkages: A Minimal Modification Protecting against Nucleolytic Degradation. *Antisense Res. Dev.* **1992**, *2*, 129–146.
- (74) Ulrich, H. Rna Aptamers: From Basic Science Towards Therapy. *Handb. Exp. Pharmacol.* **2006**, *173*, 305–326.
- (75) Darfeuille, F.; Hansen, J. B.; Orum, H.; Di Primo, C.; Toulme, J. J. Lna/DNA Chimeric Oligomers Mimic Rna Aptamers Targeted to the Tar Rna Element of Hiv-1. *Nucleic Acids Res.* **2004**, *32*, 3101–3107.
- (76) Kibler-Herzog, L.; Zon, G.; Uznanski, B.; Whittier, G.; Wilson, W. D. Duplex Stabilities of Phosphorothioate, Methylphosphonate, and Rna Analogs of Two DNA 14-Mers. *Nucleic Acids Res.* **1991**, *19*, 2979–2986.
- (77) Wang, C.; Sun, Y.; Zhao, Q. A Sensitive Thrombin-Linked Sandwich Immunoassay for Protein Targets Using High Affinity Phosphorodithioate Modified Aptamer for Thrombin Labeling. *Talanta* **2020**, *207*, 120280.
- (78) Virgilio, A.; Varra, M.; Scuotto, M.; Capuozzo, A.; Irace, C.; Mayol, L.; Esposito, V.; Galeone, A. Expanding the Potential of G-Quadruplex Structures: Formation of a Heterochiral Tba Analogue. *ChemBioChem* **2014**, *15*, 652–655.
- (79) Cai, B.; Yang, X.; Sun, L.; Fan, X.; Li, L.; Jin, H.; Wu, Y.; Guan, Z.; Zhang, L.; Zhang, L.; Yang, Z. Stability and Bioactivity of Thrombin Binding Aptamers Modified with D-/L-Isothymidine in the Loop Regions. *Org. Biomol. Chem.* **2014**, *12*, 8866–8876.
- (80) Li, L.; Hou, J.; Liu, X.; Guo, Y.; Wu, Y.; Zhang, L.; Yang, Z. Nucleolin-Targeting Liposomes Guided by Aptamer As1411 for the Delivery of Sirna for the Treatment of Malignant Melanomas. *Biomaterials* **2014**, *35*, 3840–3850.
- (81) Liu, M.; Yin, Q.; Chang, Y.; Zhang, Q.; Brennan, J. D.; Li, Y. In Vitro Selection of Circular DNA Aptamers for Biosensing Applications. *Angew. Chem., Int. Ed.* **2019**, *58*, 8013–8017.
- (82) Di Giusto, D. A.; Wlassoff, W. A.; Gooding, J. J.; Messerle, B. A.; King, G. C. Proximity Extension of Circular DNA Aptamers with Real-Time Protein Detection. *Nucleic Acids Res.* **2005**, *33*, e64.
- (83) Di Giusto, D. A.; King, G. C. Construction, Stability, and Activity of Multivalent Circular Anticoagulant Aptamers. *J. Biol. Chem.* **2004**, *279*, 46483–46489.
- (84) Tan, J.; Yang, N.; Hu, Z.; Su, J.; Zhong, J.; Yang, Y.; Yu, Y.; Zhu, J.; Xue, D.; Huang, Y.; Lai, Z.; Huang, Y.; Lu, X.; Zhao, Y. Aptamer-Functionalized Fluorescent Silica Nanoparticles for Highly Sensitive Detection of Leukemia Cells. *Nanoscale Res. Lett.* **2016**, *11*, 298.
- (85) Mallikaratchy, P.; Tang, Z.; Kwame, S.; Meng, L.; Shangguan, D.; Tan, W. Aptamer Directly Evolved from Live Cells Recognizes Membrane Bound Immunoglobulin Heavy Mu Chain in Burkitt's Lymphoma Cells. *Mol. Cell. Proteomics* **2007**, *6*, 2230–2238.
- (86) Wu, X.; Zhao, Z.; Bai, H.; Fu, T.; Yang, C.; Hu, X.; Liu, Q.; Champanhac, C.; Teng, I. T.; Ye, M.; Tan, W. DNA Aptamer Selected against Pancreatic Ductal Adenocarcinoma for in Vivo Imaging and Clinical Tissue Recognition. *Theranostics* **2015**, *5*, 985–994.
- (87) Kuai, H.; Zhao, Z.; Mo, L.; Liu, H.; Hu, X.; Fu, T.; Zhang, X.; Tan, W. Circular Bivalent Aptamers Enable in Vivo Stability and Recognition. *J. Am. Chem. Soc.* **2017**, *139*, 9128–9131.
- (88) de Smidt, P. C.; Le Doan, T.; de Falco, S.; van Berkel, T. J. Association of Antisense Oligonucleotides with Lipoproteins Prolongs the Plasma Half-Life and Modifies the Tissue Distribution. *Nucleic Acids Res.* **1991**, *19*, 4695–4700.
- (89) Uludag, H.; Ubeda, A.; Ansari, A. At the Intersection of Biomaterials and Gene Therapy: Progress in Non-Viral Delivery of Nucleic Acids. *Front. Bioeng. Biotechnol.* **2019**, *7*, 131.
- (90) Gragoudas, E. S.; Adamis, A. P.; Cunningham, E. T., Jr; Feinsod, M.; Guyer, D. R. Pegaptanib for Neovascular Age-Related Macular Degeneration. *N. Engl. J. Med.* **2004**, *351*, 2805–2816.
- (91) Bae, O. N. Targeting Von Willebrand Factor as a Novel Anti-Platelet Therapy; Application of Arc1779, an Anti-Vwf Aptamer, against Thrombotic Risk. *Arch. Pharmacol. Res.* **2012**, *35*, 1693–1699.
- (92) Cosmi, B. Arc-1779, a Pegylated Aptamer Antagonist of Von Willebrand Factor for Potential Use as an Anticoagulant or Antithrombotic Agent. *Curr. Opin. Mol. Ther.* **2009**, *11*, 322–328.
- (93) Prakash, T. P.; Yu, J.; Migawa, M. T.; Kinberger, G. A.; Wan, W. B.; Ostergaard, M. E.; Carty, R. L.; Vasquez, G.; Low, A.; Chappell, A.; Schmidt, K.; Aghajan, M.; Crosby, J.; Murray, H. M.; Booten, S. L.; Hsiao, J.; Soriano, A.; Machemer, T.; Cauntay, P.; Burel, S. A.; Murray, S. F.; Gaus, H.; Graham, M. J.; Swayze, E. E.; Seth, P. P. Comprehensive Structure-Activity Relationship of Triantennary N-Acetylgalactosamine

Conjugated Antisense Oligonucleotides for Targeted Delivery to Hepatocytes. *J. Med. Chem.* **2016**, *59*, 2718–2733.

(94) Prakash, T. P.; Graham, M. J.; Yu, J.; Carty, R.; Low, A.; Chappell, A.; Schmidt, K.; Zhao, C.; Aghajani, M.; Murray, H. F.; Riney, S.; Booten, S. L.; Murray, S. F.; Gaus, H.; Crosby, J.; Lima, W. F.; Guo, S.; Monia, B. P.; Swayze, E. E.; Seth, P. P. Targeted Delivery of Antisense Oligonucleotides to Hepatocytes Using Triantennary N-Acetyl Galactosamine Improves Potency 10-Fold in Mice. *Nucleic Acids Res.* **2014**, *42*, 8796–8807.

(95) Kinberger, G. A.; Prakash, T. P.; Yu, J.; Vasquez, G.; Low, A.; Chappell, A.; Schmidt, K.; Murray, H. M.; Gaus, H.; Swayze, E. E.; Seth, P. P. Conjugation of Mono and Di-Galnac Sugars Enhances the Potency of Antisense Oligonucleotides Via Asgr Mediated Delivery to Hepatocytes. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 3690–3693.

(96) Crooke, S. T.; Baker, B. F.; Xia, S.; Yu, R. Z.; Viney, N. J.; Wang, Y.; Tsimikas, S.; Geary, R. S. Integrated Assessment of the Clinical Performance of Galnac3-Conjugated 2'-O-Methoxyethyl Chimeric Antisense Oligonucleotides: I. Human Volunteer Experience. *Nucleic Acid Ther.* **2019**, *29*, 16–32.

(97) Agarwal, S.; Simon, A. R.; Goel, V.; Habtemariam, B. A.; Clausen, V. A.; Kim, J. B.; Robbie, G. J. Pharmacokinetics and Pharmacodynamics of the Small Interfering Ribonucleic Acid (Sirna), Givosiran, in Patients with Acute Hepatic Porphyria. *Clin. Pharmacol. Ther.* **2020**, *108*, 63–72.

(98) Yu, R. Z.; Graham, M. J.; Post, N.; Riney, S.; Zanardi, T.; Hall, S.; Burkey, J.; Shemesh, C. S.; Prakash, T. P.; Seth, P. P.; et al. Disposition and Pharmacology of a Galnac3-Conjugated Aso Targeting Human Lipoprotein (a) in Mice. *Mol. Ther.–Nucleic Acids* **2016**, *5*, e317.

(99) Nair, J. K.; Willoughby, J. L.; Chan, A.; Charisse, K.; Alam, M. R.; Wang, Q.; Hoekstra, M.; Kandasamy, P.; Kel'in, A. V.; Milstein, S.; Taneja, N.; O'Shea, J.; Shaikh, S.; Zhang, L.; van der Sluis, R. J.; Jung, M. E.; Akinc, A.; Hutabarat, R.; Kuchimanchi, S.; Fitzgerald, K.; Zimmermann, T.; van Berkel, T. J.; Maier, M. A.; Rajeev, K. G.; Manoharan, M. Multivalent N-Acetylgalactosamine-Conjugated Sirna Localizes in Hepatocytes and Elicits Robust Rnai-Mediated Gene Silencing. *J. Am. Chem. Soc.* **2014**, *136*, 16958–16961.

(100) Jin, C.; Zhang, H.; Zou, J.; Liu, Y.; Zhang, L.; Li, F.; Wang, R.; Xuan, W.; Ye, M.; Tan, W. Floxuridine Homomeric Oligonucleotides "Hitchhike" with Albumin in Situ for Cancer Chemotherapy. *Angew. Chem., Int. Ed.* **2018**, *57*, 8994–8997.

(101) Chen, H.; Wang, G.; Lang, L.; Jacobson, O.; Kiesewetter, D. O.; Liu, Y.; Ma, Y.; Zhang, X.; Wu, H.; Zhu, L.; Niu, G.; Chen, X. Chemical Conjugation of Evans Blue Derivative: A Strategy to Develop Long-Acting Therapeutics through Albumin Binding. *Theranostics* **2016**, *6*, 243–253.

(102) Jacobson, O.; Kiesewetter, D. O.; Chen, X. Albumin-Binding Evans Blue Derivatives for Diagnostic Imaging and Production of Long-Acting Therapeutics. *Bioconjugate Chem.* **2016**, *27*, 2239–2247.

(103) Liu, Y.; Wang, G.; Zhang, H.; Ma, Y.; Lang, L.; Jacobson, O.; Kiesewetter, D. O.; Zhu, L.; Gao, S.; Ma, Q.; Chen, X. Stable Evans Blue Derived Exendin-4 Peptide for Type 2 Diabetes Treatment. *Bioconjugate Chem.* **2016**, *27*, 54–58.

(104) Ehlerding, E. B.; Lan, X.; Cai, W. "Albumin Hitchhiking" with an Evans Blue Analog for Cancer Theranostics. *Theranostics* **2018**, *8*, 812–814.

(105) Ding, D.; Yang, C.; Lv, C.; Li, J.; Tan, W. Improving Tumor Accumulation of Aptamers by Prolonged Blood Circulation. *Anal. Chem.* **2020**, *92*, 4108–4114.

(106) Li, Q.; Zhao, D.; Shao, X.; Lin, S.; Xie, X.; Liu, M.; Ma, W.; Shi, S.; Lin, Y. Aptamer-Modified Tetrahedral DNA Nanostructure for Tumor-Targeted Drug Delivery. *ACS Appl. Mater. Interfaces* **2017**, *9*, 36695–36701.

(107) Zhuo, Z.; Wan, Y.; Guan, D.; Ni, S.; Wang, L.; Zhang, Z.; Liu, J.; Liang, C.; Yu, Y.; Lu, A.; Zhang, G.; Zhang, B. A Loop Based and Ago Incorporated Virtual Screening Model Targeting Ago Mediated Mirna-Mrna Interactions for Drug Discovery to Rescue Bone Phenotype in Genetically Modified Mice. *Adv. Sci.* **2020**, 1903451.

(108) Meng, L.; Yang, L.; Zhao, X.; Zhang, L.; Zhu, H.; Liu, C.; Tan, W. Targeted Delivery of Chemotherapy Agents Using a Liver Cancer-Specific Aptamer. *PLoS One* **2012**, *7*, e33434.

(109) Deng, R.; Qu, H.; Liang, L.; Zhang, J.; Zhang, B.; Huang, D.; Xu, S.; Liang, C.; Xu, W. Tracing the Therapeutic Process of Targeted Aptamer/Drug Conjugate on Cancer Cells by Surface-Enhanced Raman Scattering Spectroscopy. *Anal. Chem.* **2017**, *89*, 2844–2851.

(110) Jing, P.; Cao, S.; Xiao, S.; Zhang, X.; Ke, S.; Ke, F.; Yu, X.; Wang, L.; Wang, S.; Luo, Y.; Zhong, Z. Enhanced Growth Inhibition of Prostate Cancer in Vitro and in Vivo by a Recombinant Adenovirus-Mediated Dual-Aptamer Modified Drug Delivery System. *Cancer Lett.* **2016**, *383*, 230–242.

(111) Bagalkot, V.; Farokhzad, O. C.; Langer, R.; Jon, S. An Aptamer-Doxorubicin Physical Conjugate as a Novel Targeted Drug-Delivery Platform. *Angew. Chem., Int. Ed.* **2006**, *45*, 8149–8152.

(112) Huang, Y. F.; Shangguan, D.; Liu, H.; Phillips, J. A.; Zhang, X.; Chen, Y.; Tan, W. Molecular Assembly of an Aptamer-Drug Conjugate for Targeted Drug Delivery to Tumor Cells. *ChemBioChem* **2009**, *10*, 862–868.

(113) Li, F.; Lu, J.; Liu, J.; Liang, C.; Wang, M.; Wang, L.; Li, D.; Yao, H.; Zhang, Q.; Wen, J.; Zhang, Z. K.; Li, J.; Lv, Q.; He, X.; Guo, B.; Guan, D.; Yu, Y.; Dang, L.; Wu, X.; Li, Y.; Chen, G.; Jiang, F.; Sun, S.; Zhang, B. T.; Lu, A.; Zhang, G. A Water-Soluble Nucleolin Aptamer-Paclitaxel Conjugate for Tumor-Specific Targeting in Ovarian Cancer. *Nat. Commun.* **2017**, *8*, 1390.

(114) Wang, R.; Zhu, G.; Mei, L.; Xie, Y.; Ma, H.; Ye, M.; Qing, F. L.; Tan, W. Automated Modular Synthesis of Aptamer-Drug Conjugates for Targeted Drug Delivery. *J. Am. Chem. Soc.* **2014**, *136*, 2731–2734.

(115) Kruspe, S.; Giangrande, P. H. Aptamer-Sirna Chimeras: Discovery, Progress, and Future Prospects. *Biomedicines* **2017**, *5*, 45.

(116) Soldevilla, M. M.; Meraviglia-Crivelli de Caso, D.; Menon, A. P.; Pastor, F. Aptamer-Irns as Therapeutics for Cancer Treatment. *Pharmaceuticals* **2018**, *11*, 108.

(117) McNamara, J. O., 2nd; Andrechek, E. R.; Wang, Y.; Viles, K. D.; Rempel, R. E.; Gilboa, E.; Sullenger, B. A.; Giangrande, P. H. Cell Type-Specific Delivery of Sirnas with Aptamer-Sirna Chimeras. *Nat. Biotechnol.* **2006**, *24*, 1005–1015.

(118) Dassie, J. P.; Liu, X. Y.; Thomas, G. S.; Whitaker, R. M.; Thiel, K. W.; Stockdale, K. R.; Meyerholz, D. K.; McCaffrey, A. P.; McNamara, J. O., 2nd; Giangrande, P. H. Systemic Administration of Optimized Aptamer-Sirna Chimeras Promotes Regression of Psm-Expressing Tumors. *Nat. Biotechnol.* **2009**, *27*, 839–849.

(119) Liang, C.; Guo, B.; Wu, H.; Shao, N.; Li, D.; Liu, J.; Dang, L.; Wang, C.; Li, H.; Li, S.; Lau, W. K.; Cao, Y.; Yang, Z.; Lu, C.; He, X.; Au, D. W.; Pan, X.; Zhang, B. T.; Lu, C.; Zhang, H.; Yue, K.; Qian, A.; Shang, P.; Xu, J.; Xiao, L.; Bian, Z.; Tan, W.; Liang, Z.; He, F.; Zhang, L.; Lu, A.; Zhang, G. Aptamer-Functionalized Lipid Nanoparticles Targeting Osteoblasts as a Novel Rna Interference-Based Bone Anabolic Strategy. *Nat. Med.* **2015**, *21*, 288–294.

(120) Jeong, H.; Lee, S. H.; Hwang, Y.; Yoo, H.; Jung, H.; Kim, S. H.; Mok, H. Multivalent Aptamer-Rna Conjugates for Simple and Efficient Delivery of Doxorubicin/Sirna into Multidrug-Resistant Cells. *Macromol. Biosci.* **2017**, *17*, 1600343.

(121) Subramanian, N.; Kanwar, J. R.; Kanwar, R. K.; Krishnakumar, S. Targeting Cancer Cells Using Lna-Modified Aptamer-Sirna Chimeras. *Nucleic Acid Ther.* **2015**, *25*, 317–322.

(122) Herrmann, A.; Priceman, S. J.; Kujawski, M.; Xin, H.; Cherryholmes, G. A.; Zhang, W.; Zhang, C.; Lahtz, C.; Kowolik, C.; Forman, S. J.; Kortylewski, M.; Yu, H. Ctl4 Aptamer Delivers Stat3 Sirna to Tumor-Associated and Malignant T Cells. *J. Clin. Invest.* **2014**, *124*, 2977–2987.

(123) Esposito, C. L.; Cerchia, L.; Catuogno, S.; De Vita, G.; Dassie, J. P.; Santamaria, G.; Swiderski, P.; Condorelli, G.; Giangrande, P. H.; de Franciscis, V. Multifunctional Aptamer-Mirna Conjugates for Targeted Cancer Therapy. *Mol. Ther.* **2014**, *22*, 1151–1163.

(124) Esposito, C. L.; Nuzzo, S.; Kumar, S. A.; Rienzo, A.; Lawrence, C. L.; Pallini, R.; Shaw, L.; Alder, J. E.; Ricci-Vitiani, L.; Catuogno, S.; de Franciscis, V. A Combined Microrna-Based Targeted Therapeutic



Approach to Eradicate Glioblastoma Stem-Like Cells. *J. Controlled Release* **2016**, *238*, 43–57.

(125) Pastor, F. Tumor-Targeted Costimulation by Using Bi-Specific Aptamers. *Cancer Cell Microenviron.* **2016**, *3*, e1333.

(126) Boltz, A.; Piater, B.; Toleikis, L.; Guenther, R.; Kolmar, H.; Hock, B. Bi-Specific Aptamers Mediating Tumor Cell Lysis. *J. Biol. Chem.* **2011**, *286*, 21896–21905.

(127) Gilboa, E.; McNamara, J., 2nd; Pastor, F. Use of Oligonucleotide Aptamer Ligands to Modulate the Function of Immune Receptors. *Clin. Cancer Res.* **2013**, *19*, 1054–1062.

(128) Schrand, B.; Bereznoy, A.; Brennenman, R.; Williams, A.; Levay, A.; Kong, L. Y.; Rao, G.; Zhou, S.; Heimberger, A. B.; Gilboa, E. Targeting 4–1bb Costimulation to the Tumor Stroma with Bispecific Aptamer Conjugates Enhances the Therapeutic Index of Tumor Immunotherapy. *Cancer Immunol. Res.* **2014**, *2*, 867–877.

(129) Soldevilla, M. M.; Villanueva, H.; Casares, N.; Lasarte, J. J.; Bendandi, M.; Inoges, S.; Lopez-Diaz de Cerio, A.; Pastor, F. Mrp1-Cd28 Bi-Specific Oligonucleotide Aptamers: Target Costimulation to Drug-Resistant Melanoma Cancer Stem Cells. *Oncotarget* **2016**, *7*, 23182–23196.

(130) Sharma, P.; Allison, J. P. The Future of Immune Checkpoint Therapy. *Science* **2015**, *348*, 56–61.

(131) Hori, S. I.; Herrera, A.; Rossi, J. J.; Zhou, J. Current Advances in Aptamers for Cancer Diagnosis and Therapy. *Cancers* **2018**, *10*, 9.

(132) Dollins, C. M.; Nair, S.; Sullenger, B. A. Aptamers in Immunotherapy. *Hum. Gene Ther.* **2008**, *19*, 443–450.

(133) Zhu, G.; Chen, X. Aptamer-Based Targeted Therapy. *Adv. Drug Delivery Rev.* **2018**, *134*, 65–78.

(134) Wengerter, B. C.; Katakowski, J. A.; Rosenberg, J. M.; Park, C. G.; Almo, S. C.; Palliser, D.; Levy, M. Aptamer-Targeted Antigen Delivery. *Mol. Ther.* **2014**, *22*, 1375–1387.

(135) Liu, B. Y.; He, X. Y.; Xu, C.; Ren, X. H.; Zhuo, R. X.; Cheng, S. X. Peptide and Aptamer Decorated Delivery System for Targeting Delivery of Cas9/SgRNA Plasmid to Mediate Antitumor Genome Editing. *ACS Appl. Mater. Interfaces* **2019**, *11*, 23870–23879.

(136) Keler, T.; He, L.; Ramakrishna, V.; Champion, B. Antibody-Targeted Vaccines. *Oncogene* **2007**, *26*, 3758–3767.

(137) Luo, Y. L.; Shiao, Y. S.; Huang, Y. F. Release of Photoactivatable Drugs from Plasmonic Nanoparticles for Targeted Cancer Therapy. *ACS Nano* **2011**, *5*, 7796–7804.

(138) Alshaer, W.; Hillaireau, H.; Fattal, E. Aptamer-Guided Nanomedicines for Anticancer Drug Delivery. *Adv. Drug Delivery Rev.* **2018**, *134*, 122–137.

(139) Dam, D. H.; Culver, K. S.; Kandela, I.; Lee, R. C.; Chandra, K.; Lee, H.; Mantis, C.; Ugolkov, A.; Mazar, A. P.; Odom, T. W. Biodistribution and in Vivo Toxicity of Aptamer-Loaded Gold Nanostars. *Nanomedicine* **2015**, *11*, 671–679.

(140) Bamburowicz-Klimkowska, M.; Poplawska, M.; Grudzinski, I. P. Nanocomposites as Biomolecules Delivery Agents in Nanomedicine. *J. Nanobiotechnol.* **2019**, *17*, 48.

(141) Xing, H.; Tang, L.; Yang, X.; Hwang, K.; Wang, W.; Yin, Q.; Wong, N. Y.; Dobrucki, L. W.; Yasui, N.; Katzenellenbogen, J. A.; Helfferich, W. G.; Cheng, J.; Lu, Y. Selective Delivery of an Anticancer Drug with Aptamer-Functionalized Liposomes to Breast Cancer Cells in Vitro and in Vivo. *J. Mater. Chem. B* **2013**, *1*, 5288–5297.

(142) Ming, X.; Laing, B. Bioconjugates for Targeted Delivery of Therapeutic Oligonucleotides. *Adv. Drug Delivery Rev.* **2015**, *87*, 81–89.

(143) Chang, H. I.; Yeh, M.-K. Clinical Development of Liposome-Based Drugs: Formulation, Characterization, and Therapeutic Efficacy. *Int. J. Nanomed.* **2011**, *7*, 49–60.

(144) Zununi Vahed, S.; Salehi, R.; Davaran, S.; Sharifi, S. Liposome-Based Drug Co-Delivery Systems in Cancer Cells. *Mater. Sci. Eng., C* **2017**, *71*, 1327–1341.

(145) Cao, Z.; Tong, R.; Mishra, A.; Xu, W.; Wong, G. C.; Cheng, J.; Lu, Y. Reversible Cell-Specific Drug Delivery with Aptamer-Functionalized Liposomes. *Angew. Chem., Int. Ed.* **2009**, *48*, 6494–6498.

(146) Alshaer, W.; Hillaireau, H.; Vergnaud, J.; Ismail, S.; Fattal, E. Functionalizing Liposomes with Anti-Cd44 Aptamer for Selective Targeting of Cancer Cells. *Bioconjugate Chem.* **2015**, *26*, 1307–1313.

(147) Stuart, C. H.; Singh, R.; Smith, T. L.; D'Agostino, R., Jr.; Caudell, D.; Balaji, K. C.; Gmeiner, W. H. Prostate-specific membrane antigen-targeted liposomes specifically deliver the Zn(2+) chelator TPEN inducing oxidative stress in prostate cancer cells. *Nanomedicine* **2016**, *11*, 1207–1222.

(148) Plourde, K.; Derbali, R. M.; Desrosiers, A.; Dubath, C.; Vallee-Belisle, A.; Leblond, J. Aptamer-Based Liposomes Improve Specific Drug Loading and Release. *J. Controlled Release* **2017**, *251*, 82–91.

(149) Ma, Y.; Zhu, Y.; Wang, C.; Pan, D.; Liu, S.; Yang, M.; Xiao, Z.; Yang, X.; Zhao, W.; Zhou, X.; Li, Y.; Pan, Y.; Sun, J.; Wang, S.; Guan, Z.; Zhang, L.; Yang, Z. Annealing Novel Nucleobase-Lipids with Oligonucleotides or Plasmid DNA Based on H-Bonding or Pi-Pi Interaction: Assemblies and Transfections. *Biomaterials* **2018**, *178*, 147–157.

(150) Li, J.; Zheng, C.; Cansiz, S.; Wu, C.; Xu, J.; Cui, C.; Liu, Y.; Hou, W.; Wang, Y.; Zhang, L.; Teng, I. T.; Yang, H. H.; Tan, W. Self-Assembly of DNA Nanohydrogels with Controllable Size and Stimuli-Responsive Property for Targeted Gene Regulation Therapy. *J. Am. Chem. Soc.* **2015**, *137*, 1412–1415.

(151) Wei, X.; Tian, T.; Jia, S.; Zhu, Z.; Ma, Y.; Sun, J.; Lin, Z.; Yang, C. J. Target-Responsive DNA Hydrogel Mediated "Stop-Flow" Microfluidic Paper-Based Analytic Device for Rapid, Portable and Visual Detection of Multiple Targets. *Anal. Chem.* **2015**, *87*, 4275–4282.

(152) Yan, L.; Zhu, Z.; Zou, Y.; Huang, Y.; Liu, D.; Jia, S.; Xu, D.; Wu, M.; Zhou, Y.; Zhou, S.; Yang, C. J. Target-Responsive "Sweet" Hydrogel with Glucometer Readout for Portable and Quantitative Detection of Non-Glucose Targets. *J. Am. Chem. Soc.* **2013**, *135*, 3748–3751.

(153) Yang, H.; Liu, H.; Kang, H.; Tan, W. Engineering Target-Responsive Hydrogels Based on Aptamer-Target Interactions. *J. Am. Chem. Soc.* **2008**, *130*, 6320–6321.

(154) Zhu, Z.; Guan, Z.; Jia, S.; Lei, Z.; Lin, S.; Zhang, H.; Ma, Y.; Tian, Z. Q.; Yang, C. J. Au@Pt Nanoparticle Encapsulated Target-Responsive Hydrogel with Volumetric Bar-Chart Chip Readout for Quantitative Point-of-Care Testing. *Angew. Chem., Int. Ed.* **2014**, *53*, 12503–12507.

(155) Tan, J.; Li, H.; Hu, X.; Abdullah, R.; Xie, S.; Zhang, L.; Zhao, M.; Luo, Q.; Li, Y.; Sun, Z.; Yuan, Q.; Tan, W. Size-Tunable Assemblies Based on Ferrocene-Containing DNA Polymers for Spatially Uniform Penetration. *Chem* **2019**, *5*, 1775–1792.

(156) Biesecker, G.; Dihel, L.; Enney, K.; Bende, R. A. Derivation of Rna Aptamer Inhibitors of Human Complement C5. *Immunopharmacology* **1999**, *42*, 219–230.

(157) Jaffe, G. J.; Elliott, D.; Wells, J. A.; Prenner, J. L.; Papp, A.; Patel, S. A Phase 1 Study of Intravitreal E10030 in Combination with Ranibizumab in Neovascular Age-Related Macular Degeneration. *Ophthalmology* **2016**, *123*, 78–85.

(158) Vavalle, J. P.; Cohen, M. G. The Reg1 Anticoagulation System: A Novel Actively Controlled Factor IX Inhibitor Using Rna Aptamer Technology for Treatment of Acute Coronary Syndrome. *Future Cardiol.* **2012**, *8*, 371–382.

(159) Bunka, D. H.; Platonova, O.; Stockley, P. G. Development of Aptamer Therapeutics. *Curr. Opin. Pharmacol.* **2010**, *10*, 557–562.

(160) Waters, E. K.; Genga, R. M.; Schwartz, M. C.; Nelson, J. A.; Schaub, R. G.; Olson, K. A.; Kurz, J. C.; McGinness, K. E. Aptamer Arc19499 Mediates a Procoagulant Hemostatic Effect by Inhibiting Tissue Factor Pathway Inhibitor. *Blood* **2011**, *117*, 5514–5522.

(161) Mongelard, F.; Bouvet, P. As-1411, a Guanosine-Rich Oligonucleotide Aptamer Targeting Nucleolin for the Potential Treatment of Cancer, Including Acute Myeloid Leukemia. *Curr. Opin. Mol. Ther.* **2010**, *12*, 107–114.

(162) Hoellenriegel, J.; Zboralski, D.; Maasch, C.; Rosin, N. Y.; Wierda, W. G.; Keating, M. J.; Kruschinski, A.; Burger, J. A. The Spiegelmer Nox-A12, a Novel Cxcl12 Inhibitor, Interferes with Chronic

Lymphocytic Leukemia Cell Motility and Causes Chemosensitization. *Blood* **2014**, *123*, 1032–1039.

(163) Ninichuk, V.; Clauss, S.; Kulkarni, O.; Schmid, H.; Segerer, S.; Radomska, E.; Eulberg, D.; Buchner, K.; Selve, N.; Klussmann, S.; Anders, H. J. Late Onset of Ccl2 Blockade with the Spiegelmer Mnox-E36–3'peg Prevents Glomerulosclerosis and Improves Glomerular Filtration Rate in Db/Db Mice. *Am. J. Pathol.* **2008**, *172*, 628–637.

(164) Schwoebel, F.; van Eijk, L. T.; Zboralski, D.; Sell, S.; Buchner, K.; Maasch, C.; Purschke, W. G.; Humphrey, M.; Zollner, S.; Eulberg, D.; Morich, F.; Pickkers, P.; Klussmann, S. The Effects of the Anti-Hepcidin Spiegelmer Nox-H94 on Inflammation-Induced Anemia in Cynomolgus Monkeys. *Blood* **2013**, *121*, 2311–2315.