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Cell-type-specific, Aptamer-functionalized Agents for Targeted Disease Therapy

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One hundred years ago, Dr. Paul Ehrlich popularized the "magic bullet" concept for cancer therapy in which an ideal therapeutic agent would only kill the specific tumor cells it targeted. Since then, "targeted therapy" that specifically targets the molecular defects responsible for a patient's condition has become a long-standing goal for treating human disease. However, safe and efficient drug delivery during the treatment of cancer and infectious disease remains a major challenge for clinical translation and the development of new therapies. The advent of SELEX technology has inspired many groundbreaking studies that successfully adapted cell-specific aptamers for targeted delivery of active drug substances in both *in vitro* and *in vivo* models. By covalently linking or physically functionalizing the cell-specific aptamers with therapeutic agents, such as siRNA, microRNA, chemotherapeutics or toxins, or delivery vehicles, such as organic or inorganic nanocarriers, the targeted cells and tissues can be specifically recognized and the therapeutic compounds internalized, thereby improving the local concentration of the drug and its therapeutic efficacy. Currently, many cell-type-specific aptamers have been developed that can target distinct diseases or tissues in a cell-type-specific manner. In this review, we discuss recent advances in the use of cell-specific aptamers for targeted disease therapy, as well as conjugation strategies and challenges.

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Introduction

Most therapeutic agents currently in clinical use, such as conventional chemotherapy,[1] radiotherapy,[2] immunotherapy[3] or gene therapy,[4] are, in general, not functionalized to selectively target the site of the disease. Thus, nonspecific biodistribution of the agent in the body significantly reduces the therapeutic index, and results in unwanted side effects associated with its distribution to nontargeted sites.[5] Achieving "targeted therapy" that can identify and attack diseased cells or organisms, while doing little or no damage to normal cells, is highly desirable.[6,7] Such targeting strategies largely rely on specific ligand-directed recognition events in which the targeting ligand exclusively identifies and binds to receptors it recognizes that are expressed on the diseased cells or tissues. In this way, the drug is localized and concentrated at the targeted site. Although some therapies may not need to be internalized by cells,[8,9] it might be beneficial for drugs that lack penetrability.[10] After ligand-receptor binding, the receptors are readily internalized by the cell and rapidly reexpressed on the cell surface to allow for repeated targeting and internalization. This cycle suggests that internalizing ligands may be more efficient and helpful than noninternalizing ligands.

With regard to internalized therapies, the advent of systematic evolution of ligand exponential enrichment (SELEX)[11–13] technology has inspired multiple groundbreaking studies that have successfully adapted internalizing aptamers that are cell-type-specific to target the delivery of active drug substances to diseased cells both *in vitro* and *in vivo*.[14] Nucleic acid aptamers are single-stranded DNA or RNA molecules, which can be selected from a combinatorial DNA or RNA library through SELEX technology. Similar to the antibody–antigen interaction, the unique three-dimensional recognition between aptamers and their target is exquisitely specific, and has a low nanomolar dissociation constant. However, large size of antibodies sometimes limits bioavailability and might prevent access to many biological compartments. The scale-up manufacture and chemical modification without affecting their activity also limit the application of antibodies. In this regard, nucleic acid aptamers have some advantages. Because of their additional superior characteristics,[15] including small size, high stability (dehydrated form), lack of immunogenicity, easy chemical synthesis, adaptable modification, and cell-free evolution, aptamers and aptamer-functionalized agents have been used extensively for targeted therapeutics, molecular diagnostics, *in vivo* imaging and tracking systems, biosensor systems, and biomarker discovery.[7,16–18] Moreover, the nature of nucleic acid provides another bonus that the function of aptamers can be modulated for desired therapy and drug delivery applications using the complementary oligo antidote strategy. The complementary base pairing can disrupt the interaction of aptamer and target, therefore, reversing the activity of the aptamers.[19] Through a rationally designed antidote, precise control and better timing of reversal aptamer activity *in vivo* may be possible.

Recently, advances in the development of DNA and RNA aptamers that specifically target cell surface receptors have motivated scientists to use cell-specific aptamers that are internalized as targeting ligands for targeted disease

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therapy. By functionalizing the cell-specific, internalizing aptamers with a drug or delivery vehicle, it is expected that the specific recognition and internalization of the therapeutic agents by the target tissue will be improved.[7] In addition, antidotes could be rationally designed to neutralize the interaction between aptamers and their targets[19], thereby providing a controllable targeted therapy system. Below, we review recent advances in using cell-specific aptamers for targeted drug delivery, and discuss the conjugation strategies and current challenges for developing aptamer-functionalized agents.

Selection and identification of cell-type-specific aptamers

Over the past 24 years, many refinements and modifications have been conducted to improve aptamer selection performance, including high efficiency, rapid speed, low cost, less labor, high stability, and specificity. To date, various aptamers have been evolved for thousands of targets. For example, through robust SELEX technology SomaLogic. (http://www.somalogic.com/Technology.aspx) has generated thousands of single-stranded DNA aptamers (as known as SOMAmer) for over 1,100 protein targets that cover a diverse set of molecular functions, including receptors, kinases, growth factors, and hormones. The Ellington laboratory generated a comprehensive online aptamer Database (http://aptamer.icmb.utexas.edu/), which collects and organizes all known information about aptamer selection. To select and identify cell-type-specific, nucleic acid aptamers to use as targeted drug delivery agents, two typical selection procedures have been used: (i) traditional purified membrane protein-based SELEX[20,21] and (ii) live cell-based SELEX.[22] A crossover strategy that combines these two procedures or *in vivo* selection procedure has also been used to identify cell or tumor tissue-specific aptamers, and many research and review articles describe the details of the selection. To access the full list of cell-type-specific aptamers, please refer to our previous review articles.[7,23]

Purified membrane protein as target for SELEX

In brief, a typical protein-based SELEX procedure contains four main steps: (i) mixing a nucleic acid library and the target purified membrane protein; (ii) partitioning target-bound sequences from unbound species; (iii) recovering the target-bound sequences; and (iv) reamplifying the recovered sequences. In addition to partitioning approaches that use traditional bead-, resin-, membrane-, or chip-based partition approaches, some novel isolation methods[24–27] (*e.g.*, capillary gel electrophoresis, flow cytometry, and microfluidic devices) have been adopted to accelerate the selection procedure, and dramatically reduce the number of selection cycles and time.

To date, most cell-specific aptamers have been successfully generated using the purified membrane protein-based SELEX procedure. For example, by immobilizing a purified fusion protein containing a modified extracellular form of prostate-specific membrane antigen (PSMA) on magnetic beads, Lupold *et al.*[28] successfully selected two 2′-fluoro-modified RNA aptamers (A9 and A10). In another example, CD4 RNA aptamers were isolated from Sepharose beads coated with soluble, recombinant CD4 antigen.[29] Recently, we used an isolation method based on traditional nitrocellulose membrane filters to generate a series of 2′-fluoro-modified RNA aptamers that specifically bound to the purified HIV-1 gp120 protein[30] or the B-cell-activating factor receptor (BAFF-R) protein[31] at a low nanomolar binding affinity. The selected aptamers specifically bound and were internalized by cells that expressed the target proteins. Recently, using a cellulose filter-based *in vitro* selection process, the Ellington group succeeded in identifying RNA aptamers that bind to the purified extracellular domain of human epidermal growth factor receptor (EGFR).[32] Despite of these examples, the number of membrane proteins that have been purified and can be used to select aptamers is limited. Because the purified protein-based selection procedure is also largely limited to targeting single pass receptor, it is challenging to develop aptamers for the many multiple transmembrane spanning receptors. Moreover, some membrane proteins of interest are insoluble or only actively functionalize when they are in their native conformation or in a multiprotein complex.[33] In some cases, aptamers that were evolved to bind to these target membrane proteins failed to recognize them when they were expressed on live cells or organisms.[34,35]

Live cell as target for SELEX

Live cell-based SELEX provides a promising alternative for generating aptamers that can recognize particular target membrane proteins under their native conditions.[22] In contrast to purified protein-based SELEX, live cell-based SELEX does not need information (*e.g.*, the native conformations, the biological functions, and the identities) about the proteins on the cell surface. The selection target is whole live cells, for which the cell-surface proteins keep their native conformation and active biological functions. The live cell-based SELEX strategy essentially relies on the differences between the target cell population (positive cells that express the target of interest) and the control cell population (negative cells that do not express the target protein).[36] Therefore, the procedure basically involves two selections: (i) a counter selection that removes nonspecific binding agents by incubating the nucleic acid library with the negative cells and (ii) a positive selection in which the nucleic acid library is incubated with positive cells and target-bound sequences are recovered. No matter which selection was performed first, researchers have succeeded in generating cell-specific aptamers. For example, Tan *et al.*[37,38] used the cell-based SELEX method to isolate a panel of cell-specific ssDNA aptamers that could specifically bind to CCRF-CEM cells (a T-cell acute lymphoblastic leukemia cell line), first by positively selecting and then by counter selecting. The selected aptamers specifically recognized cancer cells in patient samples and have been used for targeted drug delivery. Giangrande *et al.* used a modified "cell-internalization SELEX" approach to successfully enrich for 2′-fluoro RNA aptamers that could selectively bind and be internalized by breast cancer cells that expressed human EGFR 2 (HER2).[39] For each round of cell-internalization SELEX, the RNA libraries were first incubated with negative cells to remove nonspecific binding agents, and then supernatants containing the RNA sequences that did not bind

the nontarget cells were incubated with the target cells for positive selection.

Although successful in individual cases, one of the major challenges of cell-based SELEX is the nonspecific binding/uptake of nucleic acids to dead cells, thereby delaying target-specific sequence enrichment, even cause the failure of aptamer selection. Some selection procedures, such as intensive washing, detachment of adherent cells, high salt concentration, etc. inevitably result in damage or death to some fragile cells. The target receptor might be adversely affected under those conditions. The complexity of target cell surface markers or lower expression of the desired targets in cell-based SEELX generally increases the number of selection rounds (~14 cycles up to 25 cycles) in order to achieve a desired specific sequence. Consequently, it may often introduce the bias of enzymatic amplification reaction. Therefore, the careful recovery of health cell population expressing a higher level of active receptors before incubation with aptamer library is the key for successful selection.

In vivo selection of tissue-targeting aptamers

Additionally, live animal has also been used as target for SELEX.[40] In 2010, Mi *et al.*[41] described a unique *in vivo* selection approach for the selection of RNA-binding motifs using intrahepatic tumor-bearing mice. A random 2′-fluropyrimidine modified RNA library was intravenously injected into hepatic tumor-implanted mice. After 20-minute circulation, the live tumor tissues were harvested, and the injected RNA sequences were extracted, amplified, and used for the next selection injection. After 14 rounds of *in vivo* selection, they identified some RNA sequences that showed an enhanced affinity for aggregate CT26 tumor protein compared to normal colon. When administered systemically via tail vein injection, Cy3-labeled aptamer 14–16 localized exclusively to intrahepatic CT26 tumors, demonstrating successful isolation of tissue-specific, penetrating aptamers within living animals. The authors also identified an RNA helicase p68 was the target of the RNA aptamers. Most recently, a similar *in vivo* evolution strategy was applied in wide-type mice to identify 2′-fluoropyrimidine-modified RNA aptamers with enhanced penetration to brain.[42] After more than 15 rounds of *in vivo* selection, the aptamers have been identified which bound to brain capillary endothelia and penetrated into the parenchyma, probably providing a targeted delivery agent for the brain.

DNA aptamer versus RNA aptamer

The main difference between DNA and RNA is the sugar present in the molecules. Deoxyribose sugar in DNA is less reactive because of C-H bonds, and its smaller grooves structures make it more stable and harder for enzymes to "attack" DNA. In contrast, ribose sugar in RNA is more reactive and not stable. However, modified bases are typically introduced in the starting RNA library used for a selection to increase nuclease resistance from the outset. The SELEX procedure initially starts from a single-stranded DNA oligonucleotide pool. After PCR amplification, the resultant double-stranded DNA is subsequently either *in vitro* transcribed into its corresponding RNA library for the isolation of RNA aptamers, or separated into its corresponding single-stranded DNA library for the selection of DNA aptamers via enzymatic digestion or streptavidin beads/resins when a biotinylating the 3′-primer is used in PCR reaction. In order to improve the RNA resistance to RNase degradation, the chemical modified nucleoside triphosphate derivatives (2′-aminopyridine, 2′-fluoropyrimide) can be routinely incorporated into RNA transcript using a mutant form of T7 RNA polymerase during the *in vitro* transcription.[16,43] Alternatively, after identification of individual aptamers, internal modifications can be chemically introduced to the aptamers, such as phosphor diester linkages (PS, phosphorothioate bond) or 2′ ribose position (2′-F, 2′-OMe, 2′-MOE, 2′-NH_2), L-form nucleotides (spiegelmer) or locked nucleic acids (LNAs).[44] However, post-SELEX modification might adversely influence molecular structure and binding affinity. Furthermore, in the case of DNA aptamer isolations, some modifications are intolerable in PCR amplification and can only be generated through solid-phase synthesis; they are not feasibly used during the SELEX procedure. Therefore, in the case of RNA aptamers isolations 2′-F or 2′-NH_2 modifications that are enzymatic tolerable provide an advantage for facilely selecting the nuclease-resistant aptamers without postmodification.

DNA and RNA aptamers are functionally similar.[17,45] One chemical modified RNA aptamer has so far been marketed for therapeutic application, and about ten aptamers are currently being evaluated in the clinic phase I and II, including DNA aptamer, RNA aptamer and Spiegelmer in which all of the sugars are the enantiomers of those found in biology nucleic acids.[46] The basis of the specific three-dimensional interaction is the structure of the aptamer, which can include stem, internal loop, hairpin, bulges, pseudoknot, kissing complex, or G-quadruplex motifs. Compared to DNA aptamer, RNA aptamer could form more diverse three-dimensional structures, probably providing strong binding affinity. Although DNA aptamers have better chemical stability, RNA aptamers have better conformational stability due to strong intrastrand RNA–RNA interactions.[47] Compared to RNA aptamer, a major benefit of DNA aptamers is that they can be generated in a large scale by solid-phase chemical synthesis with a cheaper price. Commercial solid-phase synthesis currently provides RNAs of less than 60 nt. But for DNA, up to about 400 nt could be synthesized with minimal side reactions. In particular, it is more difficult to incorporate various modifications into RNA during production with a high yield and purity, which will further increase the manufacturing costs.

Cell-specific aptamer-functionalized agents

The two main strategies for targeted delivery using cell-specific aptamers are: (i) aptamer-drug conjugates, in which aptamer is directly conjugated to the drug molecules *via* either a connector or a physical intercalation, and (ii) the aptamer-nanomaterials system, in which the aptamer is decorated on the surface of nanocarriers for targeted drug delivery. By covalently or physically functionalizing the cell-specific aptamers with drugs (*e.g.*, siRNA, microRNA, aptamer, chemotherapeutics, toxins, and enzyme) or delivery vehicles (*e.g.*, organic or inorganic nanocarriers), the diseased tissues will be specifically recognized and stimulated

to internalize the therapeutic compounds.[7,23,48–51] There are well-established methods for conjugating aptamers, and in this section, we highlight some of the representative agents that have been functionalized with aptamers and conjugation strategies that have been used.

Aptamer-therapeutic oligonucleotide conjugates

Since its discovery in 1998 (ref. 52), RNA interference (RNAi) has captured the attention of scientists, and has been investigated as a new class of therapeutics to treat human diseases, including wet age-related macular degeneration (AMD), asthma, pancreatic cancer, liver cancer, advanced solid tumor, respiratory syncytial virus, hepatitis B virus, and HIV-1.[53–55] The main classes of small regulatory RNAs that silence target messenger RNAs (mRNAs) in a sequence-specific manner include small interfering RNAs (siRNAs) and microRNAs (miRNAs).[56] To date, advances have been made in targeting RNAi delivery through the covalent conjugation or physical assembly of siRNA/miRNA with cell-specific internalizing aptamers.

Covalent RNA aptamer-siRNA/shRNA chimeras. Sullenger and coworkers were the first to describe a covalent RNA aptamer-siRNA chimera for cell-type-specific delivery of siRNA.[57] In this simple design (**Figure 1a**), a 2′-fluoro modified anti-PSMA aptamer (A10, 71 nt) was covalently joined to the sense strand of a 21-mer siRNA, and then annealed with an unmodified antisense strand. The resulting chimeric RNA molecule was selectively internalized into PSMA-expressing cells where it effectively knocked down expression of the targeted tumor survival genes polo-like kinase 1 (PLK1) and B-cell lymphoma 2 (BCL2), both in cultured cells and in animal models after intratumoral delivery. On the basis of their previous first-generation design, the Giangrande and colleagues further optimized a second-generation aptamer-siRNA chimera (**Figure 1b**),[58] in which a truncated version (39 nt) of the A10 aptamer and some rational structural changes of the siRNA were incorporated. The truncation of the aptamer did not affect the high binding affinity, but made the RNA amenable to large-scale chemical synthesis. Additionally, a 20-kDa polyethylene glycol (PEG) moiety was appended to the 5′ end of a PLK1 siRNA passenger strand by chemical synthesis, substantially increasing the circulating half-life from less than 35 minutes to greater than 30 hours, and improved the bioavailability of the aptamer-siRNA chimera. When the optimized version of the chimera was systemically administrated to a mouse model bearing human prostate cancer, the growth of PSMA-expressing tumors was dramatically repressed. Compared to the first generation chimera, the second-generation design allowed for a remarkably reduced therapeutic dose (1 nmol per injection at every other day for a total of ten injections versus 0.25 nmol per injection at every other day for a total of five injections), which minimized both the cost of treatment and the risk of harmful side effects. Recently, Gilboa *et al.*[59] used a PSMA aptamer-siRNA chimera to specifically deliver siRNAs that targeted nonsense-mediated messenger RNA decay factor (NMD) into prostate cancer cells. They found that systemic administration of PSMA-NMD siRNA chimeras significantly inhibited tumor growth in animal models of subcutaneous and metastatic tumors.

The same strategy has been applied for different aptamers and siRNA portions (**Figure 1a**). We developed a dual-functional, anti-gp120 aptamer-siRNA chimeric RNA,[30,60] in which both the aptamer and siRNA have potent antiHIV activities. A 2′-Fluoro-modified anti-HIV-1 gp120 aptamer was covalently fused to a 27-mer Dicer substrate siRNA (DsiRNA) that targeted the HIV-1 *tat/rev* common exon. When HIV-1-infected CEM-T cells and human peripheral blood mononuclear cells (PBMCs) were incubated with the aptamer-siRNA chimeras, the gp120 the chimera was internalized by the gp120 receptor, and we saw specific gene silencing. Moreover, the *in vivo* activity of the chimera was evaluated in an HIV-1-infected humanized Rag-Hu mouse model.[61] Systemic administration of the gp120 aptamer-siRNA chimera (0.25 nmol per injection

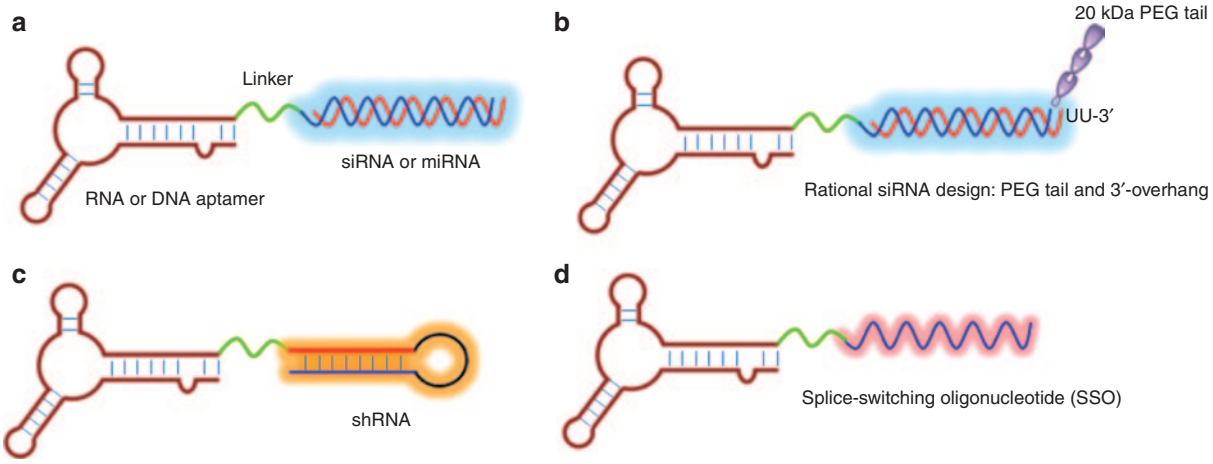

Figure 1 Covalent aptamer-therapeutic oligonucleotide chimeras. (**a**) Schematic of the first generation aptamer (RNA or DNA)-siRNA/miRNA chimera. The chimera was synthesized as two pieces followed by an annealing step to make the chimera. (**b**) Schematic of the optimized second-generation chimera. The aptamer portion was truncated from 71 to 39 nt. A two nucleotide (UU) overhang and polyethylene glycol (PEG) tail were added to the 3′-end of the guide strand (red) and the 5′-end of passenger strand (blue), respectively. (**c**) Schematic of the aptamer-shRNA chimera, which was synthesized as one piece. (**d**) Schematic of the AS1411 DNA aptamer-SSO chimera. SSO: splice-switching oligonucleotide.

at every week for a total of five injections) greatly inhibited HIV-1 replication and treated mice had a normal complement of $CD4^+$ T cells, which are usually depleted by viral infection. As expected, specific gene silencing and the siRNA were only detected in mice treated with gp120 aptamer-siRNA chimera, but not in mice treated with naked siRNA or a mutant aptamer-siRNA chimera. These results confirmed aptamer-mediated, cell-specific delivery of the siRNA and its RNAi activity. Most recently, we isolated several 2′-Fluoro-modified RNA aptamers that target B-cell-specific BAFF-R (B-cell–activating factor (BAFF)-receptor) and have nanomolar affinity.[31] Covalently generated chimeric RNA molecules that contain the BAFF-R aptamer and DsiRNA targeting signal transducer and activator of transcription 3 (STAT3) specifically delivered siRNA to BAFF-R expressing cells. These results demonstrate that the STAT3 DsiRNA was processed by Dicer and efficiently reduced the levels of target mRNAs for the proteins Jeko-1 and Z138 in human B-cell lines.

Wheeler *et al.*[62] constructed CD4 aptamer-siRNA chimeras against the HIV-1 *gag* and *vif* or host CCRs gene to specifically knock down target genes in $CD4^+$ T cells, macrophages and cervicovaginal tissue explants. Vaginal administration of the chimeras significantly prevented vaginal transmission of HIV to cervicovaginal explants and to mice in a humanized mouse model. Using the cell-internalizing SELEX procedure, Giangrande *et al.*[39] selected and identified a series of RNA aptamers against HER2 that specifically recognized and were efficiently internalized by HER2-positive breast cancer cells. HER2 aptamer-Bcl-2-siRNA chimeras were selectively internalized into HER2-positive cells and silenced Bcl-2 gene expression. As a consequence of reduced Bcl-2 expression, the HER2-positive cells regained their sensitivity to low-dose chemotherapy (cisplatin).

Based on the original, fusion approach for covalent aptamer-siRNA described by Giangrande *et al.*[57] several different versions have been generated for targeted RNAi delivery. For example, Ni *et al.*[63] adapted this chimera design to deliver a radio-sensitizing siRNA to prostate cancer cells. An shRNA against the DNA-dependent protein kinase gene (DNAPK) was covalently fused with a truncated version of the PSMA RNA aptamer A10 (A10-3). The resulting aptamer-shRNA chimeras (**Figure 1c**) selectively reduced the expression of *DNAPK* in PSMA-positive prostate cancer cells, xenografts, and human prostate tissues. Importantly, this chimera dramatically and specifically improved the response of the PSMA-positive tumors to ionizing radiation, suggesting the RNAi-mediated by cell-specific aptamers could be used as a selective sensitizing agent to improve the treatment of high-risk localized prostate cancer. In addition to monovalent aptamer-siRNA designs, multivalent aptamer-siRNA chimeras have been generated to further increase the binding affinity and internalization efficiency of the chimeras. For example, Wullner *et al.*[64] rationally designed bivalent aptamer-siRNA transcripts to selectively silence the eukaryotic elongation factor 2 (EEF2) gene and consequently induce cell-specific apoptosis. In their designs, the siRNA itself served as a linker to connect the two PSMA aptamers or the siRNA was appended onto the 3′-ends of each PSMA aptamer to form bivalent aptamer-siRNA chimeras. Their results indicated that the bivalent design improved the cellular uptake of the chimeras, and led to the death of almost all PSMA-positive cells.

Covalent DNA aptamer-siRNA/miRNA/splice-switching oligonucleotides (SSO) chimeras. DNA aptamers have also been used to deliver siRNA, miRNA, or other therapeutics oligonucleotides to the target cells. For example, Zhu *et al.*[65] used a DNA aptamer that was converted from a reported CD4 RNA aptamer to deliver an siRNA for anti-HIV-1 protease (HIV-PR) into $CD4^+$ T cells. The CD4 DNA aptamer was covalently conjugated to the sense strand of the siRNA to form a DNA aptamer-siRNA chimera (DAS). An unmodified CD4 RNA aptamer-siRNA chimera (RAS) was also synthesized as a control. Both DAS and RAS were selectively taken up by $CD4^+$ T cells, but the uptake of RAS was much less than of DAS. DAS was much more stable than RAS in both 10% FBS medium and human serum. In the absence of transfection agents, DAS-suppressed HIV-PR mRNA levels in a dose-dependent manner in $CD4^+$ T cells, whereas no obvious gene silencing was seen in RAS-treated $CD4^+$ T cells. The authors attributed the differences to the poor stability of RAS (unmodified RNA aptamer-siRNA chimera version) in the cell culture medium.

Recently, a published anti-MUC1 (mucin1) DNA aptamer[66] was used to covalently construct a DNA aptamer-miRNA chimera.[67] Mucin1, a cell surface glycoprotein, is overexpressed and glycosylated on the cell surface of a majority of human adenocarcinomas.[68,69] Liu *et al.* first reported the MUC1 aptamer-mediated delivery of let-7i miRNA into OVACR-3 ovarian cancer cells. After cleavage by Dicer, the released let-7i significantly sensitized the cells to the effect of Paclitaxel (an anticancer drug), which inhibited cell proliferation. Dai *et al.*[70] also constructed a MUC1 DNA aptamer-miRNA-29b chimera to establish a targeted therapy for ovarian cancer. The miR-29b delivered as a MUC1 DNA aptamer, inhibited DNA methyltransferase (Dnmt1, Dnmt3a, and Dnmt3b) protein expression, subsequently inducing the reexpression of phosphate and tensin homolog mRNA and protein in OVCAR-3 cells. In addition, the aptamer-miR-29b chimera treatment significantly triggered cell apoptosis.

In 2012, Sullenger and coworkers[71] used G-quadruplex-forming AS1411 DNA aptamers to deliver SSOs into the nuclei of cancer cells (**Figure 1d**). As a form of antisense technology, single-stranded RNA oligonucleotides are designed to bind to a splice site or splicing enhancers, thereby blocking access to the endogenous splicing machinery and resulting in an alternate version of mature mRNA for translation. AS1411 (ref. 72), a 26-base guanine-rich DNA aptamer, binds to surface nucleolin,[73] a protein overexpressed in multiple cancer cells. Functionalizing AS1411 with therapeutic agents promoted its cellular internalization through its interaction with the nucleolin membrane protein. In a proof-of-concept study, the authors chemically synthesized an AS1411 DNA aptamer-single-stranded RNA (SSO) chimera to target cancer cells. This chimera altered splicing in the nuclei of treated cells to correct an aberrant stop codon within the luciferase reporter gene, and was more effective at lower doses as compared to nonaptamer-functionalized SSO.

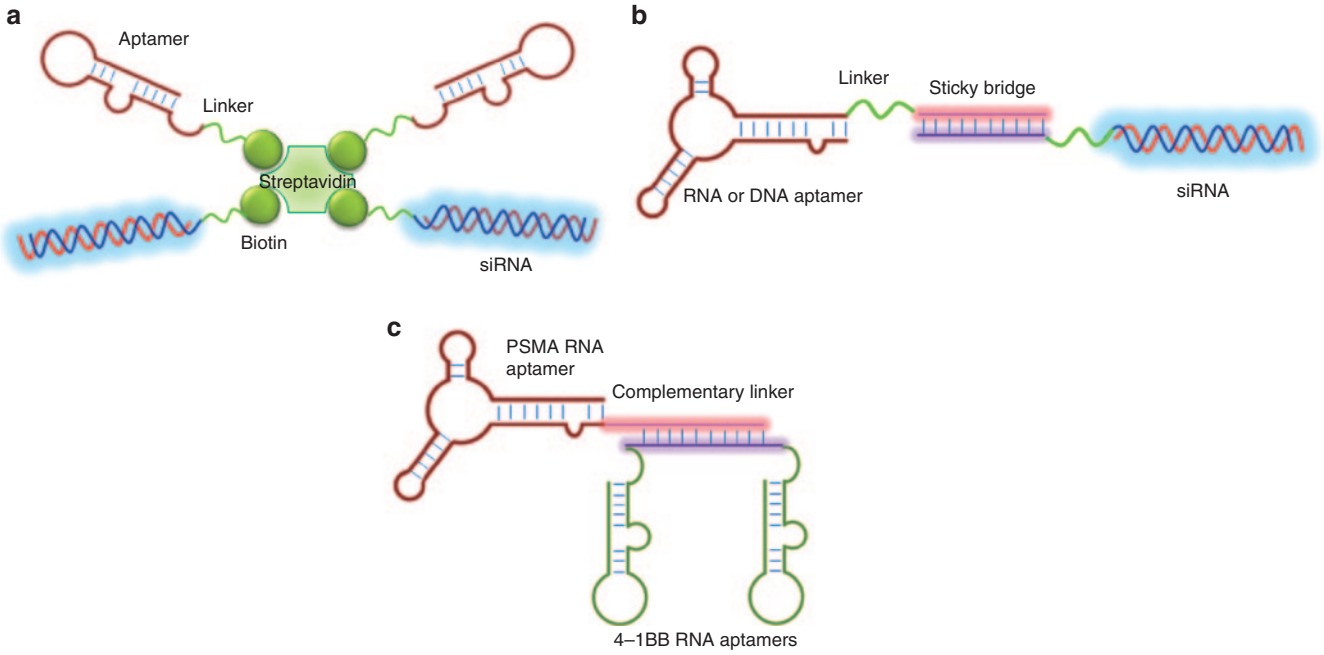

Figure 2 Noncovalent RNA aptamer- therapeutic oligonucleotide conjugates. (**a**) Schematic of the aptamer-streptavidin-siRNA conjugate. The siRNA and PSMA RNA aptamers were chemically conjugated with a biotin group. Then the two biotinylated siRNAs and two aptamers were non-covalently assembled via a streptavidin connector. (**b**) Schematic of the aptamer-sticky bridge-siRNA conjugate. The aptamer and siRNA were appended to complementary 17-nt 2′ OMe/2′ Fl GC-rich bridge sequences and were annealed by simple mixing that allowed Watson-Crick base pairing. (**c**) Schematic of a bispecific PSMA-4-1BB aptamer conjugate. The PSMA RNA aptamer and a bivalent 4-1BB RNA aptamer were tethered to complementary linker sequences and were hybridized together through Watson-Crick base pairing.

Noncovalent aptamer-siRNA or aptamer conjugation via a connector. Some connectors have used a noncovalently conjugated aptamer portion and siRNA portion (**Figure 2**), thereby providing a flexible method for assembling various aptamers and siRNAs. In 2006, Chu *et al.*[74] took the advantage of the specific interaction between biotin and streptavidin to connect a PSMA RNA aptamer (A9) with Lamin A/C or GAPDH siRNAs. In their design (**Figure 2a**), two aptamers and two siRNAs were modified with biotin and then were noncovalently, coassembled into a modular streptavidin connector. The resulting aptamer-streptavidin connector—siRNA—conjugates were internalized by PSMA-positive cells and specific knockdown of the target genes was seen in cultured PSMA-positive cells.

Recently, we designed a GC-rich "sticky sequence" as a connector to noncovalently connect RNA aptamers with various siRNAs.[30] In this design (**Figure 2b**), we chemically synthesized the RNA aptamer and siRNA with a 3′ carbon linker (C3), which in turn was attached to complementary 17-base 2′ OMe/2′ Fl GC-rich bridge sequences, thereby allowing the aptamer and siRNA to be annealed through Watson-Crick base pairing by simple mixing. The bridge facilitates the noncovalent binding and interchange of various siRNAs with the same aptamer. In a proof-of-concept study, we combined three different anti-HIV-1 siRNAs with a single gp120 aptamer. The resulting aptamer-sticky, bridge-cocktail siRNA conjugates were specifically internalized by HIV-1 cells that expressed gp120 and effectively suppressed the viral loads in HIV-1 infected cells (CEM-T cells and human PBMCs). In an HIV-1 infected humanized Rag-Hu animal model, systemic administration of this aptamer-bridge-cocktail siRNA construct knocked down the target mRNAs and potently inhibited HIV-1 replication.[75] When treatment with the aptamer-cocktail siRNA conjugate was suspended, HIV-1 levels rebounded in the majority of the treated humanized animals. Follow-up injections with the same conjugates were performed to determine if they could still inhibit viral levels. We found that retreating completely suppressed HIV-1 viral loads, and the effect lasted several weeks beyond the final injection. Similarly, this "sticky bridge" strategy was also used to conjugate the BAFF-R aptamer with STAT3 DsiRNA to target B-cells for RNAi treatment.[31] We demonstrated that the DsiRNA delivered by aptamers was processed by Dicer and incorporated in RISC (RNA-induced silencing complex) where it triggered potent knockdown of the STAT3 mRNA and protein in B-cell lines. Recently, Wang *et al.* used the "sticky bridge" method to conjugate an anti-CD8 DNA aptamer (CD8AP17s) with siRNA against Granulysin (GNLY).[76] The CD8 aptamer-GNLY siRNA conjugate was specifically internalized by human cytotoxic T lymphocytes (CTL), which significantly inhibited CTL-mediated drug hypersensitivity, and decreased cytotoxicity in *in vitro* models of Steven–Johnson syndrome/toxic epidermal necrolysis and graft-versus-host disease.

Apart from siRNA or SSO, therapeutic aptamers have been conjugated with a cell-type-specific aptamer for targeted therapy.[77] Gilboa and coworkers[78] described the development of bispecific ligands composed of nucleic acid aptamers to target costimularotry ligand to tumor cells *in vivo* (**Figure 2c**). In their previous study, a bivalent version of 4-1BB-binding aptamers has been shown to costimulate $CD8^+$ T cells and

promote tumor immunity in mice.[79] To achieve the tumor-targeted immune stimulatory ligand, which could mitigate the risk of autoimmunity, the bivalent 41-BB aptamer was conjugated to a PSMA RNA aptamer through complementary linker sequences. Their results showed that PSMA apatamer can specifically deliver costimulatory ligands (bivalent 4-1BB aptamers) to tumor cells *in situ* and enhance antitumor immunity. In pooly immunogenic subcutaneously implanted tumor and lung metastasis animal models, systemic administration of bispecific PSMA-4-1BB aptamer conjugates led to significant inhibition of tumor growth and long-tumor rejection, which was depended on homing to PSMA-expressing tumor cells and 4-1BB costimulation. In comparison with 4-1BB antibodies, this tumor-targeting costimulatory aptamer was more effective, suggesting a powerful approach to achieve protective antitumor immunity.

Aptamer-drug conjugates

Covalent conjugation of aptamers to a chemotherapeutic agent or protein via a chemical bond. Various functional groups, such as thiol, amino, or azide groups, are readily incorporated into nucleic acid aptamers through solid-phase synthesis, providing a means of attaching a single drug molecule, protein or nanomaterial to an aptamer. For example, an antitumor agent (Doxorubicin; Dox) has been covalently cross-linked with a thiol-modified, cell-internalizing DNA aptamer sgs8c through an acid–labile acylhydrazone linkage (**Figure 3a**), which facilitates cleavage of the chemical bond that releases the Dox molecule inside the acidic endosomal environment.[80] The DNA aptamer that was selected by the cell-SELEX method can specifically recognize and be efficiently internalized by CCRF-CEM cells that express its target protein tyrosine kinase 7 (PTK7).[37,38,81] The sgs8c aptamer-Dox conjugate not only showed antitumor activity similar to free Dox, but also preserved the high binding affinity of the parental DNA aptamer.

In another example, Boyaciogul *et al.*[82] identified a new DNA aptamer against PSMA and developed dimeric aptamer

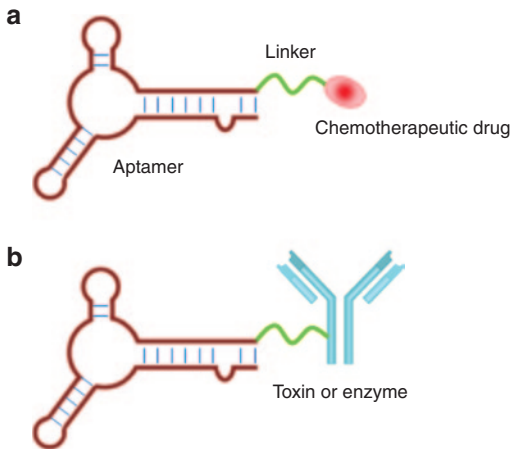

Figure 3 Covalent aptamer-chemotherapeutic agent or protein conjugation. (**a**) Schematic of an aptamer (DNA or RNA)-anticancer drug conjugate made using an acid-labile acylhydrazone linkage or formaldehyde linkage. (**b**) Schematic of an aptamer (DNA or RNA)-protein (rGel toxin or lysosomal enzyme) conjugate.

complexes (DACs) for the specific delivery of Dox to PSMA-positive cancer cells. Dox was covalently conjugated with DACs through a pH-sensitive linkage (formaldehyde), which promoted covalent attachment of Dox to genomic DNA after the Dox-DAC conjugate was internalized by the cell. The DAC selectively delivered Dox to C4-2 cells, where Dox was released in endosomes and then localized to the nucleus, displaying selective cytotoxicity to C4-2 cells.

In addition to small chemotherapeutic agents, proteins (enzymes or toxins) have been functionalized with cell-type-specific aptamers for targeted delivery (**Figure 3b**). The ribosomal toxin gelonin is a small N-glycosidase protein of approximately 28 kDa that causes cell death. Because neither gelonin nor its recombinant protein (rGel) can efficiently enter cells, Chu *et al.*[83] chemically generated RNA aptamer-gelonin conjugates to target and destroy cancer cells that overexpress PSMA. This conjugate had an IC_{50} of 27 nmol/l when used on PSMA-overexpressing cells, and when compared with PSMA-negative cells, the conjugate's toxicity was at least 600-fold higher. The PSMA aptamer was first modified with a cross-linker (SPDP agent, N-Succinimidyl 3-[2-pyridyldithio]-propionate), and the resulting intermediate product was subsequently conjugated with rGel (containing cysteine residues) through a disulfide linkage.

Chen *et al.*[84] conducted a parallel selection procedure to target the extracellular domain of the mouse transferrin receptor (TfR), which yielded an RNA aptamer (FB4) and a DNA aptamer (GS24). Both aptamers specifically bound to and were internalized by TfR-expressing murine cells, and colocalized with lysosomes. GS24 was chemically conjugated to a lysosomal enzyme, α-L-iduronidase. In the presence of periodate, the glycerol group at the 3′ end of GS24 was oxidized into aldehyde group, which then reacted with amino groups on the protein enzymes. When α-L-iduronidase-deficient mouse fibroblasts were treated with the aptamer-enzyme conjugates, they internalized it in a saturable manner, and consequently the defective glycosaminoglycan degradation in these cells was corrected.

Noncovalent conjugation of aptamers to chemotherapeutic agents through intercalation. With regard to some chemotherapeutic agents, aptamers themselves can act as both the carrier and the targeting ligand. Anthracycline drugs generally contain flat aromatic rings that can be intercalated into the G-C rich

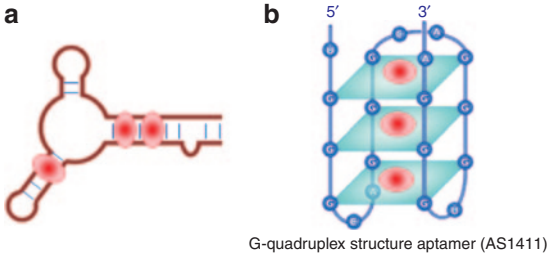

Figure 4 Noncovalent aptamer-chemotherapeutic agent conjugation. (**a**) Schematic of the physical conjugation between an aptamer (DNA or RNA) and anthracycline drug (Dox) through intercalation. (**b**) Schematic of the physical conjugation between a G-quadruplex structure aptamer (AS1411 DNA aptamer) and photodynamic agent (TMPyP4) interaction.

double-stranded region of aptamers. Because the intercalation is a noncovalent interaction and does not require any chemical modification of drugs or aptamers, such aptamer-drug conjugates simplify the formulation and protect the components in the drug delivery system from losing their bioactivity and binding affinity. For example, by using the ability of Dox to intercalate within the helical strand of nucleic acids (**Figure 4a**), Bagalkot *et al.*[85] physically conjugated Dox to the double-stranded region of the A10, a 2′-fluoropyrinidine-modified anti-PSMA RNA aptamer. Compared with free Dox, the resulting aptamer-Dox physical conjugate was selectively internalized by PSMA cells that expressed human prostate adenocarcinoma cell line (LNCaP). Flow cytometry analysis revealed that the fluorescence intensity of Dox when delivered by the aptamer to LNCaP cells was ~3.5-fold higher than that in PSMA-negative PC3 cells. Moreover, aptamer-Dox conjugates were more cytotoxic to LNCaP cells than to PC3 cells. Similarly, DNA aptamer-Dox complexes that have low nanomolar binding constants have also been formulated by intercalating Dox into the duplex structure of DNA aptamers (targeting MUC1, HER2, etc.).[86,87] Additionally, a tridentate version of the MUC1 DNA aptamer has been designed to enhance the ability of Dox to intercalate and increase its selective cytotoxicity.[88]

The G-quadruplex structure is a specialized G-rich DNA structure that is composed of four guanines through Hoogsteen hydrogen binding and is stabilized by monovalent cations. Because of its stability, the G-quadruplex structure is also used to carry a quadruplex ligand, such as 5, 10, 15, 20-tetrakis (1-methylpyridinium-4-yl) porphyrin (TMPyP4). As a quadruplex ligand, TMPyP4 contains aromatic rings with cationic charges, which let it bind to and stabilize the G-quadruplex formation. TMPyP4 is broadly used in photodynamic therapy (PDT) and provides a noninvasive method for treating solid tumors. However, TMPy4 lacks selectivity, and is usually cytotoxic to normal cells/tissue (**Figure 4a**). Shieh *et al.*[89] used G-quadruplex-forming AS1411 DNA

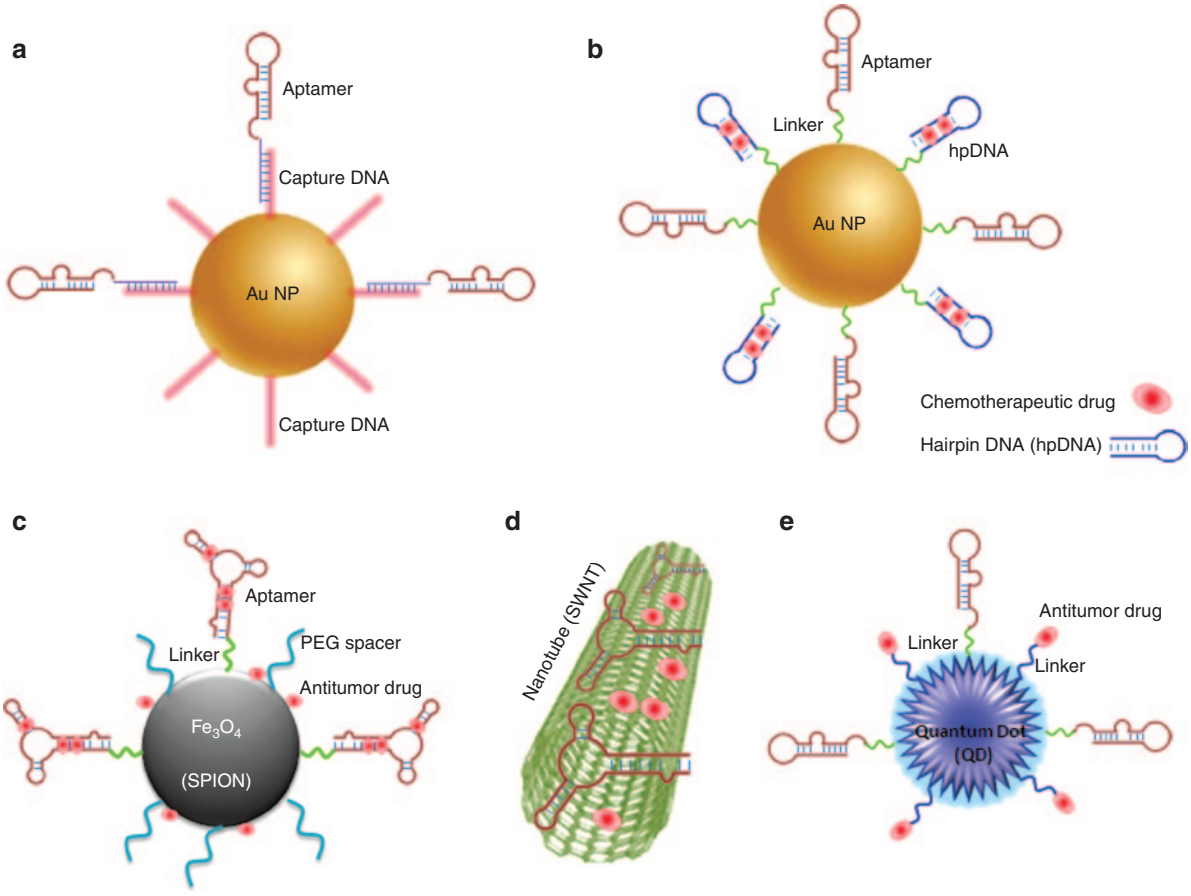

Figure 5 Aptamer-functionalized inorganic nanoparticles. (**a**) Schematic of the antiEGFR aptamer-AuNP conjugate created through a hybridization approach. AuNPs were first coated with short capture DNA sequences (pink) and were subsequently hybridized with corresponding complementary sequences that were appended to 5′-end of the antiEGFR RNA aptamer. (**b**) Schematic of sgs8c DNA aptamer/hpDNA-Dox/AuNPs. Using standard gold-thiol chemistry, the sgs8c DNA aptamer and hpDNA were assembled onto the surface of AuNPs. The double-stranded region within the hpDNA was used to load the chemotherapeutic drug Dox. (**c**) Schematic of aptamer-functionalized SPIONs. The PSMA RNA aptamer was covalently conjugated onto the surface of a SPION and then Dox was intercalated in the duplex region of the A10 aptamer and also complexed with the SPION through charge interactions. (**d**) Schematic of aptamer-functionalized single-walled carbon nanotubes (SWNTs). The sgc8c DNA aptamer and anthracycline agent (Dau) were loaded onto SWNTs through a π electron interaction. (**e**) Schematic of aptamer-functionalized QDs. The mucin 1 DNA aptamer was covalently linked to the surface of QDs through EDC/NHS chemistry. Dox was attached to QDs through a pH-sensitive hydrazone bond, which provided stability to the complex and facilitated the release of drugs in the endosome.

aptamers as carriers to deliver TMPyP4 into MCF7 breast cancer cells. In this system, six molecules of TMPyP4 were physically conjugated to the AS1411 aptamer by intercalation and the electrostatic attraction. The resulting apt-TMP complex was internalized, and MCF7 breast cancer cells accumulated much more TMPyP4 than did normal M10 epithelium cells. Consequently, use of light irradiation to activate the TMPyP4, induced specific photo-damage in MCF7 breast cancer cells.

Aptamer-nanomaterials as smart drug delivery systems

Nanomaterials have many advantages for targeted drug delivery. They possess a relatively large surface area, thereby providing a platform for conjugating multiple ligands (aptamers, antibodies, or peptides), various drugs (nucleic acid therapeutics, chemotherapeutic drugs, or antibody drugs), and signaling molecules (fluorescent dyes or radioactive agents). Currently, various nanomaterials, such as gold nanoparticles, iron oxide nanoparticles, carbon nanotubes, quantum dots, liposomes, micelles, dendrimers, polymers, or virus-based nanoparticles, have been combined with cell-specific aptamers to deliver therapeutic agents to specific cell targets or tissues (**Figures 5**–**8**).

Aptamer-functionalized gold nanoparticles. The many favorable characteristics of gold nanomaterials, which include inertness, high stability, lack of toxicity, good biocompatibility, and facile conjugation, make them highly attractive for a variety of uses in drug delivery.[90] For example, gold nanomaterials have functionalizable surfaces. Therefore, they can be functionalized by cell-type-specific aptamers for active targeting.

Ellington and coworkers[32] noncovalently linked the anti-EGFR RNA aptamer (J18, K_d of ~ 7 nmol/l) to the surface of gold nanoparticles (AuNPs) using a facile hybridization approach (**Figure 5a**). The AuNPs were first coated with short DNA capture sequences and then hybridized with the corresponding complementary sequences that were appended to the 5′-end of the anti-EGFR RNA aptamer. AuNPs that were linked to the antiEGFR aptamer were specifically internalized by EGFR-expressing cells through receptor-mediated endocytosis, and nonspecific absorption of AuNPs was reduced.

Through standard gold-thiol chemistry, Luo *et al.*[91] assembled the sgs8c DNA aptamer and hairpin DNA (hpDNA) onto the surface of AuNPs (**Figure 5b**). The repeated sequence d(CGATCG) within the hpDNA was used to load the anticancer agent Dox. Under optimal conditions, about 25 of sgc8c

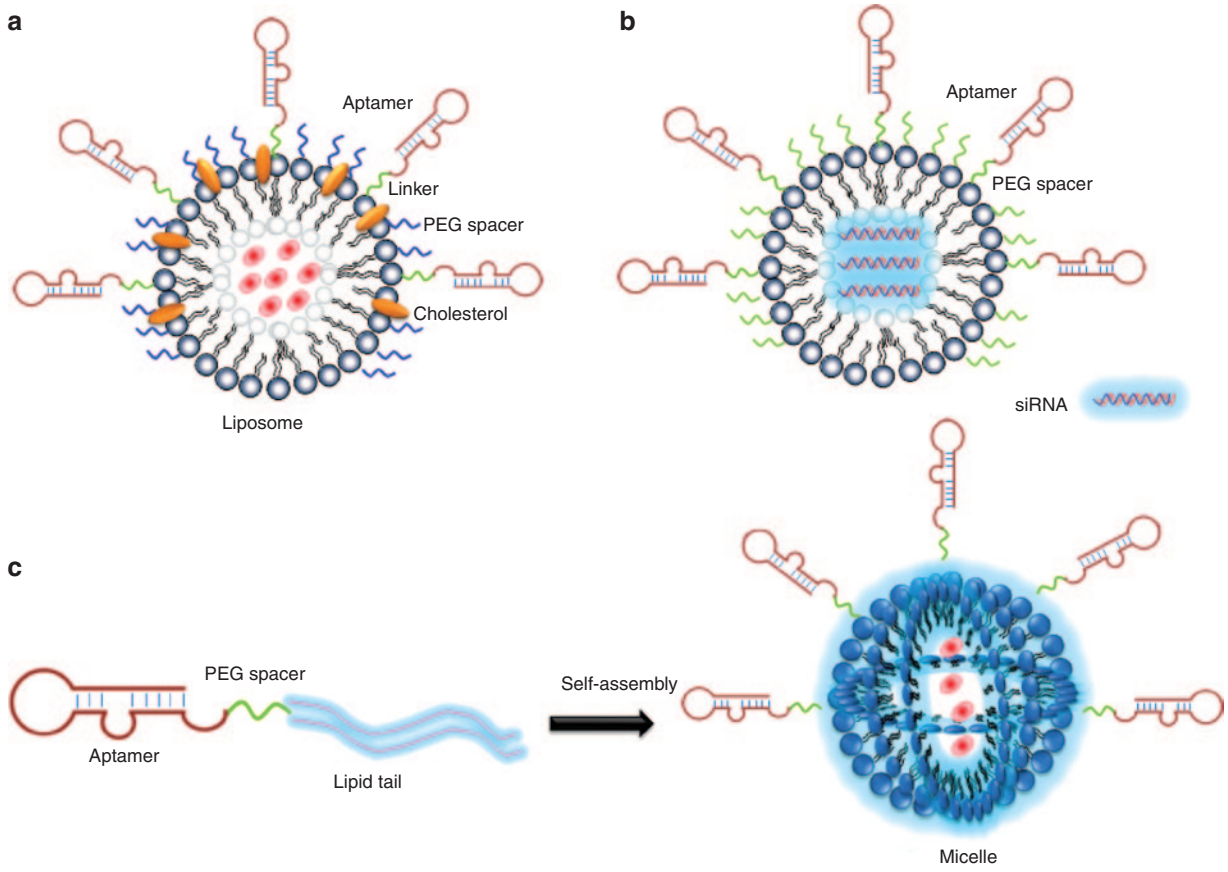

Figure 6 Aptamer-functionalized liposome and micelle nanoparticles. (**a**) Schematic of aptamer-functionalized liposome NPs for delivering antitumor agents. The sgc8 DNA aptamer was covalently conjugated to the surface of the liposome. A dextran molecule was then encapsulated into the core of the liposome. (**b**) Schematic of aptamer-functionalized liposome NPs for delivering siRNA. A TfR aptamer was used to functionalize stable, nucleic acid-lipid particles (SNALPs) containing siRNAs. (**c**) Schematic of aptamer-functionalized micelles for delivering drugs. A simple lipid tail phosphomidite with diacyl chains was attached to the end of a TDO5 DNA aptamer by using a PEG spacer. The amphiphilic aptamer-PEG-lipid tail molecule further assembled itself into a spherical micelle.

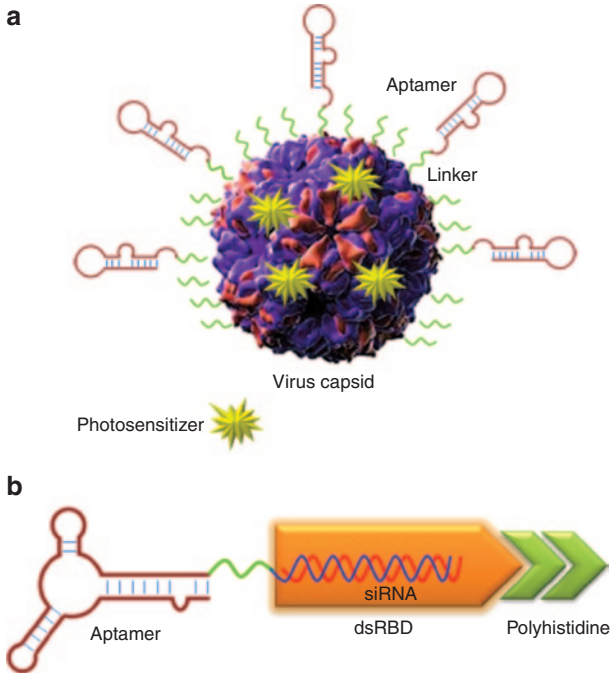

Figure 7 Aptamer-functionalized protein nanoparticles. (**a**) Schematic of aptamer-functionalized viral capsids. MS2 bacteriophage capsids were loaded with photosensitizer for targeted photodynamic therapy. Next, in the presence of the cross-linker reagent SPDP, the capsid exterior was covalently decorated with a DNA aptamer (AS1411) containing a 3′-thiol group. (**b**) Schematic of a protein complement for delivering aptamer-siRNA chimeras. The protein contains a dsRNA binding domain (dsRBD) for siRNA docking and a pH-dependent polyhistidine that will disrupt the endosomal membrane SPDP, succinimidyl 3-(2-pyridyldithio)propionate.

aptamer and 305 of Dox were successfully loaded on the surface of the AuNPs. The aptamer-functionalized AuNPs selectively targeted cancer cells and were more effective at killing targeted cancer cells compared to AuNPs without aptamer modifications. When illuminated with Plasmon-resonant light (532 nm), the conjugates released more Dox molecules, thereby enhancing the antitumor efficacy. In a similar manner, the Tan group described a gold nanorod (AuNR) functionalized with an sgc8 DNA aptamer developed for targeted PDT and photothermal therapy (PTT).[92] They designed an aptamer switch probe (ASP) that contained the 5′-end of the sgc8 leukemia aptamer conjugated to the surface of AuNRs by gold-thiol chemistry, and the 3′-end of the aptamer was linked chlorine e6 (Ce6), a photosensitizing molecule. Binding of the aptamer to the target cancer cells altered the conformation of ASP to drive Ce6 away from the gold surface, thereby producing singlet oxygens for PDT upon irradiation with light (λ = 812 nm). In addition, absorption of this radiation by AuNRs also destroyed the target cancer cells through a photothermal effect. Because of this synergistic effect, the therapeutic efficacy of aptamer-photo-sensitizer-AuNR conjugates was greater than that of PDT or PTT alone.

Recently, Zhao *et al.*[93] modified a hollow gold nanosphere (HAuNS) with an RNA aptamer to carry an exceptionally high payload of Dox. Using standard gold-thiol chemistry, the surface of the HAuNS was chemically conjugated with a 39-mer RNA aptamer specific for CD30, a diagnostic biomarker for Hodgkin's lymphoma and anaplastic large cell lymphoma. The Apt-HAuNS-Dox specifically bound to target lymphoma tumor cells and was internalized. The Dox was then released inside the cell. Functional assays using cell mixtures indicated that Apt-HAuNS-Dox selectively killed the lymphoma tumor cells, whereas no effect was observed on off-target control cells grown in the same culture.

Aptamer-functionalized superparamagnetic iron oxide nanoparticles. Superparamagnetic iron oxide nanoparticles (SPIONs) are iron oxide particles of 1–100 nm in diameter. Because of their superparamagnetic properties and potential applications in catalysis, sensors, and MRI (magnetic resonance imaging), they have attracted extensive interest.[49] The hydroxyl and carbonyl groups on the surface of the SPION make them ideal for formulating targeted nanoparticle platforms. Farokhzad *et al.*[94] formulated PSMA aptamer-SPION bioconjugates for combined imaging and targeted drug delivery for prostate cancer (**Figure 5c**). Using a standard EDC/NHS (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride / N-hydroxysuccinimide) coupling chemistry, a 57-bp 2′-fluoro-modified PSMA aptamer (A10) that was modified with a C_{18}-amine at the 3′-end was covalently conjugated onto the surface of a SPION that was already coated with an antibiofouling polymer derived from carboxylic acid-PEG. Dox was intercalated in the duplex region of the A10 aptamer and also complexed with the SPION through charge interactions. The aptamer-functionalized SPION bioconjugates selectively delivered Dox to cancer cells expressing PSMA, without a significant loss of cytotoxicity. Moreover, when used as a targeted MRI contrast agent, the aptamer-functionalized SPION bioconjugates could detect PSMA-expressing prostate cancer cells with high sensitivity.

Aptamer-functionalized single-walled carbon nanotubes (SWNTs). Carbon nanotubes, discovered in 1991, are molecular-scale tubes of graphitic carbon. Because of their unique physical and chemical properties, such as photothermal, Raman properties, single-walled carbon nanotubes (SWNTs) have been used in the fields of electronics, biosensing, and drug delivery.[95] The sgc8c DNA aptamer that targets the leukemia biomarker protein PTK7 has been conjugated with Daunorubicin (Dau; a chemotherapeutic agent of the anthracycline family) and SWNTs to enhance the targeted delivery of Dau to Molt-4 cells, an acute lymphoblastic leukemia T-cell line.[96] Because SWNTs contain highly delocalized π electrons, the surface of SWNTs can be functionalized with agents that possess a π-electron-rich structure.[97] In this system (**Figure 5d**), the nucleotide bases of the DNA aptamer and the sidewalls of SWNTs form a stable noncovalent complex through a π-stacking interaction. In addition, Dau was efficiently loaded onto SWNTs through the same interaction. The resulting Dau-aptamer-SWNT tertiary complexes were taken up selectively by Mol-4 cells, and were cytotoxic to these leukemia cells. Dau was released from the tertiary complex in a pH-dependent manner, and a higher release rate was observed at pH 5.5 (similar to the endosomal environment). When an antisense sequence of the aptamer was added to Molt-4 cells, the specific cytotoxic effect mediated by the Dau-aptamer-SWNT tertiary complex

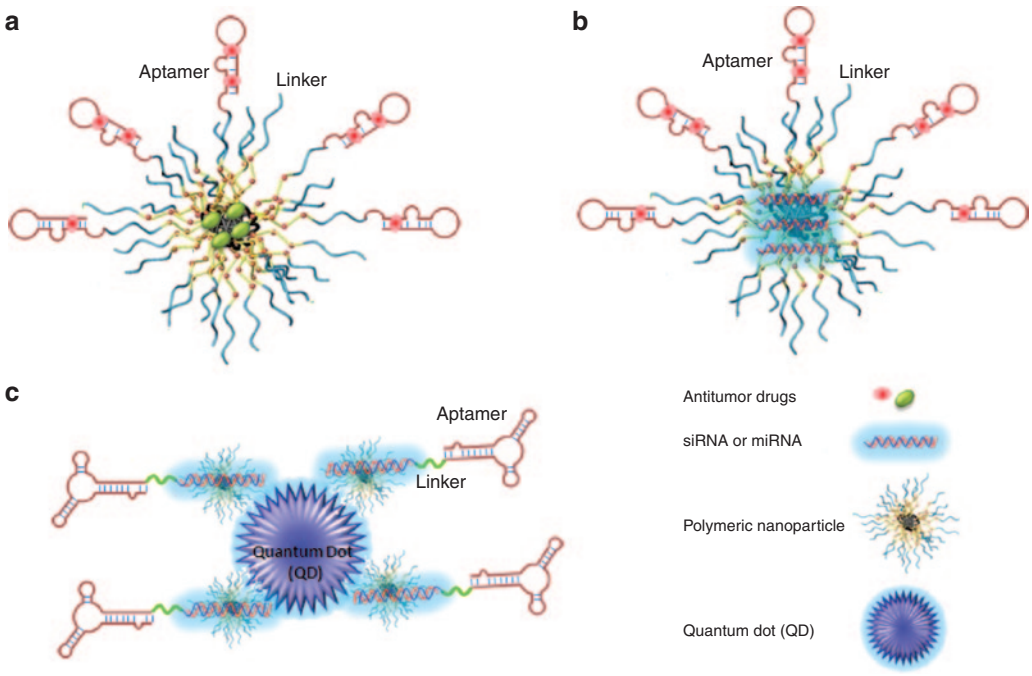

Figure 8 Aptamer-functionalized polymeric nanoparticles. (**a**) Schematic of aptamer-functionalized polymeric NPs for delivering antitumor drugs. The RNA or DNA aptamer was covalently functionalized onto the surface of the polymeric NPs. Antitumor drugs were either encapsulated into the core of the NPs or loaded onto the aptamers by intercalation. (**b**) Schematic of aptamer-functionalized polymeric NPs for delivering siRNA or miRNA. The siRNA or miRNA were complexed with cationic NPs through an electrostatic interaction. At the same time, antitumor drugs were intercalated into the aptamers for codelivery. (**c**) Schematic of a multifunctional PEI-coated QD system. First, thiol-modified siRNAs were adsorbed on PEI-coated QD nanoparticles through a noncovalent electrostatic interaction, which partially neutralized the nanoparticle surface's positive charge, thereby minimizing some of the nonspecific electrostatic interactions that occur between negatively charged aptamers and the nanoparticle surface. Next, the thiol-modified PSMA RNA aptamers were added to covalently conjugate to siRNAs through the thiol-disulfide exchange reaction to form aptamer-S-S-siRNA conjugates.

was antagonized, suggesting more control and better timing is possible using reverse aptamers.

Other aptamer-functionalized inorganic nanoparticles. Mesoporous silica nanoparticle (MSN), a form of silica, has many applications in medicine, biosensors, imaging, and drug delivery. The large surface area of their pores allows MSNs to be filled with drugs. Recently, Yang *et al.*[98] incorporated AuNRs into DNA-gated MSNs to achieve a drug-controlled release system, in which a quadruplex DNA aptamer (AS1411) served as both the targeting ligand and a gate keeper in response to a series of stimuli. In their system, a 12-mer sequence (DNA-1) was first covalently attached to the exterior of the MSNs through EDC/NHS chemistry. Next, the AS1411 DNA aptamer with a 12-base extension at the 3′-end, which was complementary to DNA-1, was hybridized with DNA-1. Thus, the resulting duplex DNA structure anchored on the MSNs surface could serve as a cap for trapping drug molecules (*e.g.*, Dox) within the pore channels. When the system was exposed to a laser beam that matched the absorption peak of the AuNR, photothermal heat converted from photoenergy dehybridized the duplex DNA structure, subsequently releasing the drugs from the pore.

Kim *et al.*[99] recently developed a multifunctional, cancer-targeting "theranostic" probe in a single system using a DNA aptamer (AS1411) and miRNA molecular beacon (MB) that was complementary to miR-221. The MB was conjugated to a magnetic fluorescent (MF) nanoparticle (MFAS miR-221 MB). The system was designed to simultaneously target cancer tissue, image intracellular miR-221 and treat carcinogenesis mediated by miR-221. MSNs were chemically synthesized and coated with a PEG-COOH spacer, to allow covalent conjugation with a 5′ amine-modified AS1411 aptamer or amine-modified miR-221 MB. The MB consisted of two parts: (i) a long oligonucleotide containing an amine-S-S-linker at the 5′-end, a Cy5 fluorescent reporter at its 3′-end, and the miR-221 binding sequence, which is a perfect reverse compliment to mature miR-221; and (ii) a short oligonucleotide containing a BHQ2 quencher at the 5′-end and a small annealing region that could hybridize with the longer oligonucleotide. This system (AS141l aptamer-conjugated MFAS miR-221 MB) displayed great selectively and delivery into various cancer cell lines. After internalization, the disulfide linkage between the miR-221 MB and the nanoparticle was cleaved in the reductive cytoplasmic environment, releasing the free miR-221 MB. If miRNA-221 was present in the cytoplasm of cancer cells, it would hybridize with miR-221, which would result in the short oligonucleotide containing the BHQ2 quencher, detaching from the long oligonucleotide containing the Cy5 reporter, thereby allowing fluorescence resonance energy transfer to generate a fluorescent signal. Furthermore, the hybridization of miR-221 with the miR-221 MB led to loss of miR-221 function, which reduced the expression of cellular oncogenes. This system shows great promise for

developing single probes that can be used both for diagnosis and treatment.

Quantum dots (QD) are nanocrystals and important semiconductor materials. In modern biological analysis and medical research, scientists have taken the advantage of the extraordinary photostability of QDs for use in real-time tracking and imaging. Various targeting ligands have been used to modify QDs for targeted diagnosis and drug delivery. Savla *et al.*[100] reported the design and delivery of a tumor-targeted, pH-responsive quantum dot-MUC1 aptamer-Dox (QD-MUC1-DOX) conjugate for treating ovarian cancer (**Figure 5e**). The MUC1 DNA aptamer was covalently linked to the surface of the QDs through EDC/NHS chemistry. Dox was attached to QDs via a pH-sensitive hydrazone bond that provided stability to the complex and facilitated the release of drugs in the endosomes. The final QD-MUC1-DOX conjugates were much more cytotoxic to multidrug-resistant cancer cells than free DOX, and they preferentially accumulated in ovarian tumors.

Aptamer-functionalized liposomes. Liposomes are composite structures made of phospholipids. Since they were first described in 1964 by Alec Bangham, liposome technology has progressed quickly, and has been extensively applied to drug delivery.[101] Because of their specific structures, liposomes can both encapsulate hydrophilic therapeutic agents inside their aqueous core and load hydrophobic drugs within their lipid bilayer membrane. Currently, there are about 12 liposome-based drugs approved for clinical use and more are in various stages of clinical trials. For example, Doxil (Ben Venue Laboratories, Bedford, OH) was approved by FDA in 1995 for the treatment of chemotherapy refractory acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma. In order to selectively deliver the drug molecules to the sites of action, liposomes can also been decorated with various targeting ligands.

The Tan group successfully functionalized liposomes with the sgc8 DNA aptamer (**Figure 6a**).[102] Using maleimide chemistry, the sgc8 DNA aptamer was covalently conjugated to the surface of liposomes already coated with a PEG polymer to increase liposome stability. Dextran, was then encapsulated into the core of the liposome. The aptamer-conjugated-liposome drug delivery system targeted cancer cells with high specificity and efficacy. Cao *et al.*[103] have reported the controlled formation of aptamer-conjugated liposomes that encapsulate cisplatin. In this study, the authors used a nucleolin DNA aptamer (26-mer AS1411) that contained 12 extra thymines and cholesterol at the 3′-end. The cholesterol tag immobilized the aptamer on the liposome surface by inserting into the hydrophobic lipid membrane. The thymines served as a spacer to separate the aptamer from liposome surface, thereby preserving the aptamer's ability to bind to its target. Cancer cell-specific targeting and drug delivery was achieved in the breast cancer cell line MCF-7, which overexpresses nucleolin. Moreover, a DNA complementary to the aptamer was designed to disrupt the aptamer-mediated, targeted delivery, suggesting it could be developed into a controllable drug delivery system.

In addition to chemotherapeutic agents, aptamer-functionalized liposomes have been generated to selectively deliver siRNA (**Figure 6b**). To generate serum-stabilized aptamers that recognize the human TfR, Wilner *et al.*[104] used a cross-over selection procedure that combined traditional selection against the recombinant protein, followed by "functional" selection in live cells grown in media. A selected aptamer that was specific for human TfR was rapidly endocytosed by a variety of human cancer cells (*e.g.*, HeLa cervical cancer cells). A truncated version (c2.min, 42 nt, K_d of 102 nmol/l) was used to functionalize siRNA-containing stable, nucleic acid-lipid particles (SNALPs). The surface of PEG-2000 molecules was first replaced with a thiol-reactive PEG-2000-malemide. After the siRNA was encapsulated and dialyzed, a 5′ thiol-modified c2.min aptamer was covalently linked to the surface of the liposome. As expected, the TfR aptamer enhanced both liposome uptake and target gene knockdown (enhanced green fluorescent protein or Lamin A/C) in cultured cells compared to nonaptamer liposomes. Furthermore, SNALP-mediated delivery of siRNA did not induce nonspecific activation of the immune system.

Aptamer-functionalized micelles. Micelles are aggregates of surfactant molecules, such as fatty acids, soap, and phospholipids, which have a strong polar "head" and a nonpolar hydrocarbon chain "tail." In water, the nonpolar tails of the molecules clump into the center of a ball-like structure, the micelle. Polymeric micelles have been extensively researched in the field of drug delivery, and recently, a micelle was constructed as hybrid using hydrophilic oligonucleotides and hydrophobic polymers.[105] Thus, micelles are potential carriers for antisense oligonucleotides and drug molecules.

In a study reported by the Tan group (**Figure 6c**),[106] a self-assembled aptamer-micelle nanostructure was designed to achieve selective and enhanced binding of otherwise low-affinity aptamers under physiological conditions. The authors attached a simple lipid tail, phosphomidite, with diacyl chains linked to the end of a TDO5 DNA aptamer using a PEG spacer, which was selected to be specific to Ramos cells (a B-cell lymphoma cell line). The PEG spacer was inserted between the aptamer and lipid tail to minimize the adverse effect of the lipid tail on the aptamer. The amphiphilic aptamer-PEG-lipid tail molecule further assembled into a spherical micelle with an average diameter of ~68 nm. The final form of the TDO5-micelle not only enhanced binding selectivity (~80-fold) to the target cells at physiological temperatures, but also facilitated cellular internalization.

Using a different strategy, Mu *et al.*[66] developed aptamer-functionalized PEG-polylactic acid (PEG-PLA) micelles (APP) designed to target the TfR on brain endothelial cells. Flurbiprofen, a potential drug for therapeutic management of Alzheimer's disease, was encapsulated into APP micelles through a cosolvent evaporation method. Next, the flurbiprofen-loaded micelles were surface decorated with the TfR RNA aptamer (FB4) by covalent coupling using EDS/NHS chemistry. The FB4 RNA aptamer specifically binds to the extracellular domain of the mouse TfR. The resulting APP micelles significantly increased the surface association of micelles to the murine brain endothelial bEND5 cell line, which expresses TfR. In a similar study by Xu *et al.*[70], the PSMA RNA aptamer (A10) was covalently conjugated to APP micelles through a hyperbranched H40 polymer core

and EDS/NHS coupling. Dox was encapsulated into the hydrophobic core of the micelles. The targeted Dox-loaded micelles had a much higher level of uptake by PSMA-positive CWR22Rv1 prostate carcinoma cells compared to nontargeted micelles, leading to significantly higher cytotoxicity. Moreover, compared to systemically administered anticancer drugs, the aptamer-functionalized micelles were better distributed in *in vivo* PSMA-expressing prostate tumors. Through click chemistry,[107] an alkyne-modified PSMA A10 aptamer was also successfully conjugated to the surface of PEG-polycaprolactone (PCL) micelles to target the delivery of TGX-221, a potent cell membrane permeable inhibitor of the p110β catalytic subunit of PI3K (phosphatidylinositol 3-kinase).

Aptamer-functionalized protein-based nanoparticles. Because of well-defined geometry and homogeneity, viral capsids, which are rigid protein-based cages, have emerged as a nanostructural platform for modifying and conjugating active biomolecules or targeting ligands. Recently, Cohen *et al.*[108] reported using aptamer-decorated MS2 bacteriophage capsids, loaded with photosensitizer, for targeted photodynamic therapy *in vitro* (**Figure 7a**). Through an assembly packaging mechanism, about 250 porphyrin molecules were loaded into the MS2 capsids. Next, in the presence of the crosslinker reagent succinimidyl 3-(2-pyridyldithio)propionate (SPDP), the capsid exterior was covalently decorated with a DNA aptamer (AS1411) containing a 3′-thiol group. The resulting viral capsids induced specific cell death in MCF-7 breast cancer cells, but no phototoxicity was observed in control MCF-10A cells.

In addition, a protein nanoparticle delivery system that targets nucleoli has been developed.[76] Human serum albumin (HSA) is the most abundant protein in blood plasma and has more hydrophobic domains in its inner core and more hydrophilic domains on the surface. When the HSA disulfide bonds are reduced and hydrophobic domains are exposed, hydrophobic Paclitaxel (PTX) can be self-assembled into the core of HSA. In this example, PTX-loaded HSA nanoparticles were modified with the AS1411 DNA aptamer *via* EDC/NHS chemistry. The cytotoxicity of the resulting spherical Apt-HAS-PTX conjugates was enhanced in MCF-7 tumor cells, but there was almost no effects on control MCF-10A cells.

As previously mentioned, aptamer-siRNA chimeras have been successfully used for targeted RNAi delivery. However, some challenges still limit the efficiency of the RNAi, in particular, chimera endosomal escape. In comparison with the traditional transfection methods (using lipid-based transfection agents) in which 50 nmol/l siRNA could provide significant gene silencing, a large amount (200–1,000 nmol/l) of aptamer-siRNA chimeras is often required to achieve effective knockdown of target genes. Presumably, the vast majority of chimera is trapped in the endosome, thus reducing the access of siRNA by Dicer and RISC in the cytoplasm. Most recently, a universal small and simple protein was used as complements with endosome rupturing capabilities for delivery of siRNA-aptamer chimeras (**Figure 7b**).[109] The protein contains a dsRNA binding domain (dsRBD) for the siRNA docking and a pH-dependent polyhistidine that can disrupt the endosomal membrane. In contrast to common cationic nanocarriers that complex siRNAs via electrostatic injection, this protein does not rely on a high positive charge to interact with the siRNA molecules and can bind relatively long double-stranded RNA (>16 base pairs) through the dsRBD in a sequence-independent fashion. Because of these properties, dsRBD does not bind to the single-stranded region of aptamers and also avoids the adverse interference that arises from excessive positive charges. The enzymatic stability of siRNA was significantly enhanced when bound to dsRBD. Furthermore, in acidic compartments such as the endosome, histidine becomes protonated, which facilitates osmotic swelling and cargo release via the proton sponge effect. In this design, polyhistidine (His18) effectively destabilized the endosome, leading to significant gene silencing (>56% increase compared to the chimera alone at 100 nmol/l concentration).

Aptamer-functionalized polymeric nanoparticles. There are several examples of aptamer-functionalized natural or organic polymeric materials for targeted delivery of small drug molecules or RNAi therapies.[110] In 2004, the first example of an aptamer-modified polymeric nanoparticle for targeted drug delivery was described by Farokhzad *et al.*[111] (**Figure 8a**). Dextran was encapsulated within a synthesized PLA-block-PEG copolymer that had a terminal carboxylic acid functional group (PLA-PEG-COOH). The 5′ amine-modified PSMA RNA aptamer was covalently linked to the surface of polymer through EDC coupling chemistry. The specific internalization and drug delivery mediated by the PSMA aptamer was increased by up to 77-fold in tumor cells that expressed prostate-specific membrane antigen (PSMA). Since this study, many similar aptamer-functionalized polymeric nanoparticles have been reported for various drugs delivered into cultured cells or *in vivo* animal models.[112–115].For example, the Farokhzad *et al.*[112] formulated an A10 aptamer-functionalized poly (D, L-lactic-co-glycolic acid)-block-PEG (PLGA-b-PEG) copolymer designed to deliver doxetaxel (Dtxl). The Dtxl-NP-apt conjugates significantly enhanced *in vitro* cellular toxicity, and completely eradicated the tumors after a single intratumoral injection in a xenograft nude mouse model.

In addition, DNA aptamers have already been used for the same purpose. Gao *et al.*[82] isolated a DNA aptamer (GMT8) that could specifically bind to U87 human glioma cells. The DNA aptamer was covalently functionalized onto the surface of PEG-poly(ε-caprolactone) (PEG-PCL) nanoparticles for the targeted delivery of Dtxl. *In vitro* cell uptake assays showed that the GMT8 ApNPs significantly enhanced intracellular drug delivery and penetration of tumor spheroids. Moreover, Dtxl-loaded ApNPs were distributed to the tumor site, more than to normal brain tissue. At tumor sites, the ApNPs induced apoptosis and inhibited spheroid tumor growth. Similarly, Aravind *et al.*[116] functionalized PLGA-lecithin-PEG nanoparticles that were loaded with the anticancer drug Paclitaxel, using AS1411 DNA aptamers to specifically target tumor cells that overexpressed nucleolin receptors.

Aptamer-functionalized polymeric nanoparticles have been applied to nucleic acid-based therapeutics. Zhao *et al.*[117] noncovalently formulated a nanocomplex to target anaplastic large cell lymphoma (ALCL) cells that express CD30. CD30 is a cell membrane protein of the tumor necrosis factor receptor family that has been recognized as a unique biomarker on many lymphomas of diverse origins, and as an activation

molecule on B and T cells.[118] They directly mixed individual anaplastic lymphoma kinase (ALK) siRNA and antiCD30 RNA aptamers with poly (ethylenimine) (PEI)-citrate. The targeted nanocomplexes, which had an average hydrodynamic diameter of ~140 nm, were formed via electrostatic interactions. These nanocomplexes specifically silenced ALK gene expression, arrested growth, and induced apoptosis in ALCL cells. Using a different method (**Figure 8b**), Yang *et al.*[119] covalently conjugated the PSMA A10 RNA aptamer onto the surface of a cationic poly (DL-lactic-co-glycolic acid) (PLGA) polymer that encapsulated androgen receptor shRNAs. This A10-functionalized nanoparticle showed enhanced internalization, both by cells *in vitro* and by tumors in a xenograft model. Two injections of the nanoparticles efficiently silenced the androgen receptor gene, and rapid tumor regression was observed in PC-2/AR-derived prostate cancer xenografts in nude mice.

To confer the siRNA function with endosomal release, Gao *et al.*[120] engineered a multifunctional PEI-coated, QD system that was equipped with a proton sponge to enhance endosomal escape of the siRNA (**Figure 8c**). Unlike previous one-step, noncovalent incorporations of individual siRNAs and aptamers onto nanocarriers, which resulted in random orientations and conformations, they conducted a two-step process to graft the aptamer-siRNA chimeras onto the nanocarriers. First, the thiol-modified siRNAs were adsorbed on the PEI-coated QD nanoparticles through a noncovalent electrostatic interaction, which partially neutralized the positive charge on the surface of the nanoparticles, and thus minimized some nonspecific electrostatic interactions between negatively charged aptamers and nanoparticle surfaces. Next, the thiol-modified PSMA RNA aptamers were conjugated to the siRNAs via a thiol-disulfide exchange reaction to form aptamer-S-S-siRNA conjugates. The resulting PSMA aptamer-siRNA-nanoparticles selectively knocked down the target gene in target cells, and silenced it in 34% more of the total cell population than could nontargeted siRNA-nanoparticles.

Aptamer-functionalized dendrimers. Dendrimers are a class of highly branched, structurally well-defined chemical polymers. Their structure is characterized by a central inner core, surrounded by the layer of repeating units and an outermost layer of multivalent functional groups.[121] Dendrimers have been widely applied to catalytic chemistry, host-guest chemistry, and materials chemistry. Lee *et al.*[122] recently established a dendrimer-based nano-platform for effective chemoimmunotherapy. Dendrimers were the nano-platform, and were covalently linked to short, single-stranded DNA oligonucleotides and then hybridized with their corresponding complementary sequence that was appended to the 5′-end of a PSMA-specific RNA aptamer (A9). The resulting double-stranded hybrid and duplex region within the A9 aptamer allowed chemotherapeutic agents (*e.g.*, Dox) to be loaded via an intercalating interaction.

Through a covalent conjugation, a truncated PSMA RNA aptamer (A10-3.2) with a 3′-SH reactive group was chemically tethered to the surface of the poly(amido amine) (PAMAM) dendrimer.[123] In this design, negatively charged miR-15a and miR-16-1 were complexed with PAMAM dendrimers through an electrostatic interaction. PEG served as a spacer to separate the positively charged dendrimer from the aptamers, reducing the interference with the active folding of aptamers. The aptamer-PEG-dendrimer effectively delivered miR-15a and miR-16-1 to PSMA-overexpressing prostate cancer cells and had a tumoricidal effect. Aptamer-functionalized, miR-loaded PAMAM dendrimers showed a ~5.7-fold lower IC_{50} value than did nontargeting miR-loaded PAMAM controls.

Aptamer-functionalized nanomaterials for combination therapy. Two or more therapeutic drugs can be administered simultaneously using one delivery system. Combination therapies have been applied to chemotherapy, radiation therapy, photodynamic therapy, and gene therapy, thereby providing synergistic or additive efficacy, especially when targeting tumors and HIV-1. For example, Zhang *et al.*[124] developed an aptamer-targeted codelivery system for both hydrophobic and hydrophilic drugs (**Figure 8a**). Through a nanoprecipitation method, Dtxl was loaded into a PLGA-b-PEG block copolymer, and then a PSMA aptamer was used to covalently functionalize the surface of the polymer through EDC/NHS chemistry. Finally, they took advantage of the ability of anthracycline drugs to intercalate Dox within the duplex region of the aptamer, resulting in a vehicle to selectively codeliver Dtxl and Dox to PSMA-expressing cancer cells. Similarly, the Farokhzad group codelivered cisplatin and Dtxl to prostate cancer cells. Dtxl was encapsulated in the nanoparticles by nanoprecipitation[113] and the Pt(IV) hydrophilic prodrug was covalently conjugated to A10 aptamer-functionalized PLGA-PEG nanoparticles via an esterification reaction.

In the study by Kim *et al.*[125] (**Figure 8b**), a 5′-amine-modified PSMA RNA aptamer was covalently conjugated to the surface of a polymer composed of branched PEI that was grafted with PEG (PEI-PEG) to codeliver Bcl-xL shRNA and Dox. The shRNA was complexed with the aptamer-functionalized PEI-PEG nanoparticle through an electrostatic interaction, and the Dox molecule was loaded into PSMA aptamer by intercalation. The addition of Dox did not affect aptamer binding or uptake by cells. The combinatorial formulation synergistically induced selective cell death of prostate cancer cells, compared to a treatment where the therapeutics were simply mixed.

Conclusion and challenges

Since the first SELEX experiment was reported by Tuerk and Gold in 1990 (ref. 12), aptamers have spawned a productive academic and commercial industry. It only took 15 years for the first therapeutic aptamer, Pegaptanib (Macugen), to be approved by FDA for the treatment of neo-vascular AMD.[126] Moreover, many novel aptamer-based therapeutic agents are currently being evaluated in clinical trials.[17] There is a large, compelling body of literature that reveals that these short single-stranded nucleic acids can bind to almost anything, from small metal molecules to whole organisms and even tissues in live animals. The relatively small size of aptamers might enable us to access epitopes that are unavailable to antibodies, thereby making cell-type specific aptamers well suited to specifically target cells or tissues for drug delivery.

Several major challenges still remain that hinder the rapid transition of aptamer-mediated technology from the research laboratory to the clinic. The pharmacokinetics of a therapeutic agent is determined by their chemical and physical properties including the stability (nuclease/protease-resistance), the uptake and biodistribution in tissue and the circulate half-time in the blood organisms. Antibodies are not susceptible to nuclease degradation. Moreover, due to their large size, antibodies possess better pharmacokinetic properties, preventing renal filtration and extending circulating half-life. As single-stranded nucleic acid molecules, aptamers have a relatively smaller size and molecular weight (30–45 nt, 10–15 kDa) than antibodies, aptamers are subject to nuclease-mediated degradation, rapid renal filtration, and rapid biodistribution from the plasma compartment into the tissues. Proper modifications of aptamers can help to improve their pharmacokinetics for clinical application. However, it is necessary to precisely incorporate and carefully choose modifications to achieve effective functions. After chemical modification, aptamers should still preserve their active folding properties and biological activity. To date, aptamers have been conjugated to PEG or cholesterol to increase their bioavailability and circulating half-life *in vivo*. The pharmacokinetics study of Macugen that is 40 kDa PEGylated vascular endothelial growth factor aptamer showed its half-life was 9.3 hours and 12 hours in plasma after intravenous injection and subcutaneous injection, respectively, and 94 hours in vitreous humor.[127,128] Incorporating protective groups, such as thiol-phosphate, 2′-Fluoro, 2′-amino, 2′-OMe, etc., in the phosphate backbone or 2′-position of the ribose sugar, enhances their nuclease resistance.[43]

With the growing clinical development and market, there is a demand for efficient, cost-effective manufacturing of oligonucleotides. Additional challenges are large scale production, in particular, of RNA therapeutics that have special modifications. Commercial solid-phase synthesis using phosphoramidite generally provides RNAs of less than 60 nt. It is more difficult to chemically synthesize longer RNA molecules (>80 nt) with high purity and yield. Incorporating various modifications during production will further increase the manufacturing costs. Advances in chemical synthesis will hopefully solve these problems in the near future. Currently, *in vitro* transcription mediated by mutant enzymes has been used to produce 2′-fluoro or 2′-OMe-modified longer RNAs, cheaply and efficiently. In addition, experiment- or computer-guided approaches have been used to rationally truncate the aptamers.

Despite limited toxicological information about aptamers, potential toxicities due to nonspecific immune activation, polyanionic effects, and tissue accumulation of oligonucleotide materials may occur in response to long-term, repeated administration. For example, high concentrations of nucleic acids may cause nonspecific, off-target protein interactions, and trigger the polyanionic effect.

For the aptamer-mediated RNAi delivery, endosomal escape issue of aptamer-siRNA chimeras still contains a major concern. This limitation may explain why higher concentrations of chimeras would be required in those pioneer studies. Several groups had a tough time achieving effective gene silencing using atpamer-siRNA chimeras without using very large amounts of materials even in cell culture, even though large amounts of aptamers are observed to be entering cells. These observations have raised the challenges about the clinical viability of the aptamer-siRNA approach, and encouraged the researchers to further optimize the design or seek other alternatives for small RNA delivery. An obvious solution to this problem is to combined aptamer-mediated RNAi delivery system with nanocarriers (*e.g.*, liposomes, polymers or proteins) with endosome rupturing capabilities. Additionally, multiple aptamers and therapeutic agents (*e.g.*, siRNA, chemotherapy agents, etc.) could be equipped to one single nanocarrier system, which may further increase binding affinity of aptamer and loading capacity of drugs.

When combining aptamers with nanomaterials for targeted therapy, the biological incompatibility, toxicity, nondegradable nature, and passive entrapment of the nanomaterials by the liver, lungs or kidney should be taken into account. Despite of various organic and inorganic nanomaterials reviewed in this article, continued efforts are still required to further refine the formulation and conjugate strategy for their clinical translation. For example, the formulation of aptamer chimeras with a cationic nanocarrier, we should rationally design the conjugation procedure. Nonspecific electrostatic condensation generally compromises even destroys the targeting capability of aptamers. Ideally, the resultant aptamer-siRNA nanocarrier system should maintain the correct structure and make the binding domain of aptamers exposed for the target receptors. Taken together, the above-mentioned development and challenges promise to bring more novel ideas and increase progress in the field of aptamer-mediated targeted therapy.

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