Chapter 16
Engineering Aptamers for Biomedical Applications: Part II

Laura Cerchia, Luciano Cellai and Vittorio de Franciscis

16.1 Introduction

The last decade has witnessed an enormous advance in the identification of disease-associated molecular targets for the development of new therapeutic and imaging agents. Indeed, the development of high-affinity reagents to selectively target affected tissues and organs has led to substantial advantages in combating important life-threatening diseases, including cancer. Major hurdles are represented by the lack of efficient, safe, and specific delivery systems able to selectively target the diseased tissue or organs. Until now, monoclonal antibodies are considered to be the molecules of choice predominantly used for cell-specific targeting. Nucleic acid aptamers have been recently shown to represent a valuable alternative to antibodies because may couple the advantages of their chemical nature to the high specific binding of antibodies to their proper targets. Indeed, aptamers have similar binding affinities as monoclonal antibodies, and like antibodies, they can be used for the recognition and sometimes the inhibition of disease-associated proteins [1, 2]. On the other hand, aptamers have a number of unique properties that make them particularly interesting for diverse areas, not just as alternatives to antibodies, but as main tools of medical and analytical strategies. They are produced chemically in a readily scalable process, thus avoiding the use of animal cells and assuring an easy, relatively inexpensive, and rapid production with high batch fidelity. At difference of protein-based reagents that undergo irreversible denaturation, functional aptamers, once denatured, can be regenerated

L. Cerchia (✉) · V. de Franciscis
Istituto per l'Endocrinologia e l'Oncologia Sperimentale del CNR "G. Salvatore",
via Pansini 5 80131 Naples, Italy
e-mail: cerchia@unina.it

V. de Franciscis
e-mail: defranci@unina.it

L. Cellai
Istituto di Cristallografia, CNR 00015 Monterotondo Stazione, Rome, Italy

W. Cai (ed.), *Engineering in Translational Medicine*,
DOI: 10.1007/978-1-4471-4372-7_16,

easily within minutes. Aptamers have shown no or low immunogenicity and are generally non-toxic [3, 4], which is an important advantage in comparison with antibodies for clinical applications requiring long period treatments.

Aptamers are short, structured single-stranded DNA or RNA able to bind, due to their specific three-dimensional shapes, with high affinity and specificity to a multitude of targets ranging from small chemical compounds to cells and tissues [1, 5–8]. They can be developed against almost any target protein through iterative cycles of in vitro screening of a combinatorial oligonucleotide library for target binding by a process named systematic evolution of ligands by exponential enrichment (SELEX) [9, 10]. Aptamers have a small size (ca. 15 kDa) which allows easy membrane penetration and short blood residence and can have been chemically modified to enhance their stability, bioavailability, and pharmacokinetics [11–13]. Furthermore, introduction of functional groups during the chemical synthesis of aptamers allows their subsequent derivatization with other molecules (nanocarriers, tracers for imaging, drugs). Aptamers can then perform therapeutic functions both acting as direct therapeutic agents and upon conjugation, as drug-targeting vectors, addressing drugs onto specific aptamer-sensitive targets [6, 7].

16.2 Aptamers Against Cell Surface Proteins as Delivery Systems: The Goal of Specificity

A great promise in developing specific molecular probes for disease biomarker discovery and for diagnostic and therapeutic applications is represented by the cell-SELEX strategy that allows us to select aptamers against even rare antigens if specifically expressed on living cells used as target for selection [6, 14–16]. This approach permits to select aptamers against cell surface transmembrane proteins, including receptor tyrosine kinase, in a physiological context, and can be carried out even when the purification of the target protein in native conformations can be difficult [17–22]. Furthermore, cell-SELEX can be developed without prior knowledge of the multiple proteins exposed on the surface of the target cells allowing the selection of aptamer ligands that specifically recognize a surface molecular signature specific of the cells.

As recently shown, when applied to cancer cell lines, cell-SELEX consents to select a set of aptamers that, by binding, distinguish with high accuracy even close tumor types in terms of malignancy, therapeutic response, metastatic potential, proliferation, and apoptotic rate, thus representing an ideal tool for supporting a more specific and selective antitumor therapy [6, 16, 18].

It has been reported that several aptamers by binding cell surface proteins are transported through the plasma membrane, thus acting as specific delivery agents for a variety of imaging and therapeutic agents including RNAs (siRNAs, miRNAs, antagomiRs) whose activity depends on their effective delivery to intracellular compartments [6, 23–26]. This approach combines the cell-type-specific

expression of proteins, as targets of therapeutic value, on the surface of cell populations, to the high affinity and specificity of aptamers versus such proteins. This means that aptamers may function as specific recognition ligands for targeted proteins exposed on the surface of the targeted cell type. Thus, aptamers can permit that conjugated reagents impart their therapeutic effect onto a specific subset of cells. Since non-targeted cells should not be exposed to the conjugated reagent, the risk of unwanted side effects is largely reduced.

The ability of aptamers to penetrate from outside the target cell to the cytosol, thus assisting the tissue-specific internalization of transported molecules, becomes crucial when an effective delivery strategy for those molecules is still lacking, as in the case of therapeutic antisense, siRNA or miRNA (ONs). Indeed, while the therapeutic value of sequence-specific gene silencing with small ncRNA is being evaluated in preclinical and clinical trials, the highly negatively charged RNA has the problem of requiring transport across cell membranes to the cytosol. Consequently, the development of vectors for specific ncRNA delivery is one of the key hurdles to overcome before RNA-based therapeutics can achieve widespread clinical use. A variety of natural and synthetic nanocarriers, including liposomes, micelles, exosomes, synthetic organic polymers, and inorganic materials, have been developed for the delivery of short ncRNAs, and some of them have entered clinical evaluation. However, these carriers are unable to specifically target a given cell or tissue unless driven by a recognition moiety as MoAbs or aptamers.

Several variants of the SELEX approach have been recently used by us and others to generate aptamers as specific ligands of membrane-bound receptors [19, 20, 27–31] or to select aptamers for cell-specific internalization [22]. These aptamers have been shown to be invaluable reagents for cell-specific targeting able to distinguish target cells both grown in vitro and in mouse xenograft in vivo. Some aptamers revealed as potent antagonist of the transmembrane receptor targets, and more recently, cell-specific aptamers have proved to mediate specific targeting of nanoparticles, anticancer drugs, toxins, enzymes, radionuclides, virus, and siRNAs (Fig. 16.1). The cargoes are attached to the aptamers either by direct conjugation or by using delivery carrier nanoparticles for loading [25].

16.3 The Conjugation of Aptamers

Biomolecules can be conjugated with other biomolecules, forming bioconjugates, and with chemical entities of a different nature, giving rise to assemblies endowed with new properties. In the field of medicinal chemistry, the goal of conjugation is, for example, the enhancement of drug potency, a more precise and effective targeting, an improvement in absorption, distribution, metabolism, elimination, and toxicity, the development of new therapeutic systems, the development of novel diagnostics and biosensors. Common components of conjugates are small molecules, such as drugs, biotin, fluorescent dyes, and larger molecules, such as proteins, oligosaccharides, nucleic acids, synthetic polymers, and also nanomaterials.

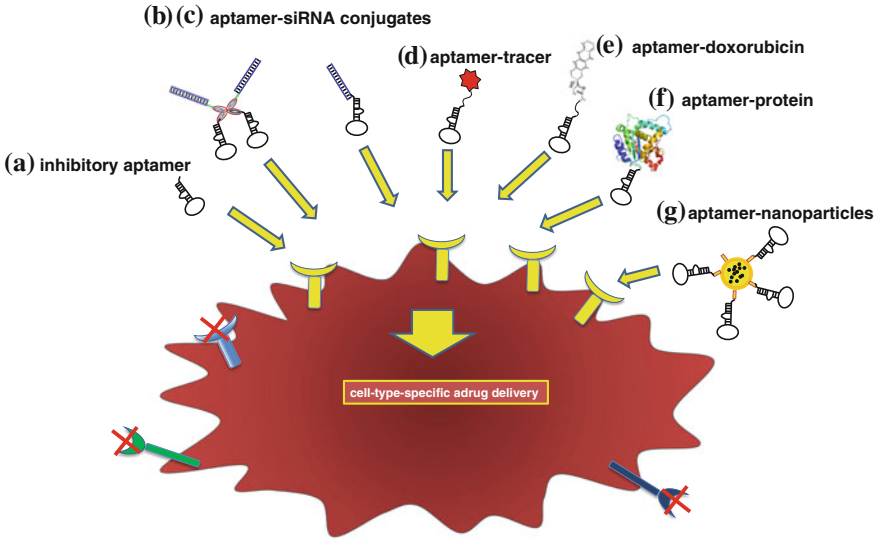

Fig. 16.1 Aptamers against cell surface proteins. Aptamers can act both as direct antagonist of the transmembrane receptor targets **a** and as delivery agents (**b**–**g**). **b** Aptamer–streptavidin–siRNA conjugates; **c** aptamer–siRNA chimeras; **d** aptamer–radionuclide/fluorescent agent conjugates; **e** aptamer–doxorubicin; **f** aptamer–protein conjugates; **g** aptamer–nanoparticles–drug conjugates

Aptamers are commonly synthesized by solid-phase chemical synthesis; therefore, chemical conjugation is possible at any position in the molecule, differently from proteins and peptides that can accept conjugation only on specific residues.

Common strategies for the post-synthesis, covalent bioconjugation of aptamers exploit the coupling of a terminal group like an amino group with a reactive succinimidyl ester or of a sulfhydryl group with a reactive maleimide.

Common strategies for the post-synthesis, non-covalent bioconjugation of aptamers are mainly based on interactions regulating nucleic acids and proteins structural assembly and association, involving the use of post-synthetic linkers, of sticky ends, or similar ways.

16.3.1 Conjugation of Aptamers with siRNA/miRNA

Because of their nucleic acid nature, the rational design of structured conjugates of aptamers with siRNA, with miRNAs, or even with larger therapeutic RNA-based molecules is greatly simplified. Recently, different groups have constructed distinct aptamer–siRNA conjugates for successful delivery of siRNA into target cells by employing diverse strategies to realize the chimeric molecules. Since aptamers

are commonly prepared by automated synthesis, they can be directly fused with their partner component in the chimeric molecule by the design and synthesis of single longer molecules. Additionally, particular labels, like many dyes or biotin, can be directly added as labeled phosphoramidite within the same process. In addition, aptamers and siRNA molecules can be assembled via a protein connector, or alternatively, completely RNA-based chimeric molecules can be generated by means of several approaches (see below).

16.3.1.1 Streptavidin–Aptamer–siRNA Conjugates

One of the first studies on the development of aptamer–siRNA conjugates involved the non-covalent conjugation of a 27-mer siRNA-targeting laminin A/C and GAPDH genes with the antiprostate-specific membrane antigen (PSMA) A9 RNA aptamer via a streptavidin bridge [32]. The siRNA aptamer and the RNA aptamer were chemically conjugated with biotin. Further, to enhance siRNA release in the cytoplasm, a reducible disulfide linker was designed between the sense strand of siRNA and the biotin group. In cell-based assays, the resulting conjugates were efficacious in silencing target genes at levels comparable with what observed with conventional lipid-based reagents.

16.3.1.2 Aptamer–siRNA/MiRNA Conjugates Consisting Only of RNA Components

Research has gone beyond with the aim to develop completely RNA-based delivery methods, thus reducing the various side effects associated with reagents such as proteins. The first report of a delivery system consisting only of RNA components was by McNamara et al. [33]. They described an approach in which the anti-PSMA A10 aptamer was covalently conjugated at the 3′ end to the 21-mer sequence complementary to the antisense strand of the siRNA and the chimeras were formed by annealing to the siRNA antisense strand. The resulting aptamer–siRNA molecules were shown to be selectively internalized into PSMA-positive cells and to effectively target the tumor survival genes polo-like kinase 1 and BCL-2. Further developments, including truncation of the aptamer portion, swapping of the sense and antisense strands of the siRNA portion, and addition to the chimera of a two-nucleotide 3′-overhang and of a PEG tail, produced analogous chimeric RNAs displaying enhanced silencing activity and specificity, and optimized in vivo kinetics [34]. By applying the same approach, PSMA aptamer–siRNA fusions were generated to target two key components of the nonsense-mediated mRNA decay (NMD) [35]. Tumor-targeted NMD inhibition forms the basis of a clinically feasible approach to enhance the antigenicity of disseminated tumors leading to their immune recognition and rejection.

Again by using the anti-PSMA A10 aptamer as a vector, Wullner et al. succeeded in generating bivalent aptamer–siRNA constructs in which the siRNA

against the eukaryotic elongation factor was used as a linker to join the two aptamers, or alternatively, the siRNAs were appended onto the 3′ ends of each aptamer [36].

Most work in the generation of aptamer–siRNA chimeric molecules has been performed by the group of Rossi JJ by using the 2′-F-modified RNA aptamer targeting the HIV-1 envelope glycoprotein gp120 as a vector. The gp-120 aptamer was covalently linked to siRNAs that target the HIV-1 *tat/rev* common exon, thus generating a novel anti-gp120 aptamer–siRNA chimera in which both components function as anti-HIV agents, thus showing a dual inhibitory function [37]. Further, a highly versatile approach was developed by the same group by using a G-C-rich dsRNA of 16 nucleotides "stick" as the scaffold to link the aptamer and the siRNAs. The two complementary sticky sequences were chemically conjugated at the 3′ end of the aptamer and of one of the two siRNA strands, thereby allowing the aptamer and siRNA portions to be non-covalently conjugated via Watson–Crick base-pairing by simple mixing. Both aptamer and siRNA were separated from each sticky strand by a flexible seven-unit three-carbon linker. The assembly proved efficacious both in vitro and in vivo [38, 39]. The functional assembly "aptamer–stick–siRNA" displayed a great versatility offering the possibility to easily combine different siRNAs to the vector aptamer, thus envisaging the possibility of using these chimeras for an antiretroviral siRNA combinatorial therapy [26, 40].

Recently, by using the delivery stick approach, we have generated completely RNA-based chimeric molecules containing internalizing aptamers coupled to therapeutic miRNAs that are down-regulated in human tumors and whose expression results in selective tumor growth inhibition. We have shown that when applied to cells expressing the specific aptamer target, the chimeric molecules are internalized and processed by Dicer, thus increasing miRNA cellular level and inhibiting miRNA target protein (our personal communication).

Two approaches have been reported to generate RNA-based chimeric molecules by directly fusing the aptamer with its partner component in a single longer molecule. In the first construct, Ni et al. [41] linked a short hairpin RNA against the DNA-activated protein kinase to a truncated A10 aptamer generating a single intact nuclease-stabilized molecule. The 3′-terminus of the A10 aptamer was conjugated to the passenger (sense) strand, followed by a 10-mer loop sequence and then by the guide or silencing (antisense) strand of the siRNA. In the second construct, chimeric molecules containing internalizing antimucin 1 (MUC1) aptamer fused to therapeutic miR-29b palindromic sequence were generated by direct synthesis. Applied to cells expressing the specific aptamer target, the chimeric molecules proved to be internalized and to increase miRNA cellular level [42].

With the increasing development of the conjugation strategies, the list of aptamers against surface epitopes that are being used as delivery agents for siRNA/miRNA is growing rapidly and now includes, in addition to PSMA and gp120 aptamers, those against CD4 [43], epidermal growth factor receptor 2 (HER2) [22], MUC 1 [30, 42], B cell-activating factor (BAFF) receptor (BAFF-R) [44], transferrin receptor [45], (see Table 16.1).

Table 16.1 Aptamers as delivery agents for siRNA/miRNA

Aptamer composition	Target	siRNA/miRNA delivery	References
RNA, 2′-F-Py	PSMA	siRNA	[32–36, 41]
RNA, 2′-F-Py	PSMA	miRNA	[51]
RNA, 2′-F-Py	gp120	siRNA	[37–40]
RNA, 2′-F-Py	CD4	siRNA	[43]
RNA, 2′-F-Py	HER2	siRNA	[22]
DNA	Mucin 1	miRNA	[30, 42]
RNA, 2′-F-Py	TfR	siRNA	[45]
RNA, 2′-F-Py	BAFF-R	siRNA	[44]

16.3.2 Covalent Conjugation of Aptamers with Drugs

Conjugation of aptamers with drugs can be used as a tool favoring specific cell targeting and internalization, in particular for drugs like antitumor drugs, which often lack cell specificity and produce life-threatening toxic side effects in patients.

Doxorubicin is the most utilized anticancer drug against neoplasms including acute lymphoblastic and myeloblastic leukemias, and malignant lymphomas, although producing heavy side effects among which cumulative cardiac damage. In order to improve its therapeutic potential, doxorubicin was covalently conjugated to DNA aptamer Sgc8c [46], selected for human T cell ALL CCRF-CEM cell lines, that can act as a drug carrier, targeting protein tyrosine kinase 7 (PTK7), a transmembrane receptor highly expressed in CCRF-CEM cells. To this aim, doxorubicin was reacted with N-(ε-maleimidocaproic acid) hydrazide to yield the C-13 (6-maleimidocaproyl) hydrazone derivative and then combined with the 5′-thiol-modified sgc8c DNA, prepared by automated synthesis. The linked sgc8c aptamer prevents the non-specific uptake of doxorubicin and decreases cellular toxicity to non-target cells.

However, despite the fact that anti-CEM/PTK7 aptamer has been reported to bind specifically to leukemia cell lines thus indicating PTK7 as a new biomarker specific for leukemia cells, Li et al. by examining how the aptamer performed with additional cell lines, which were not of hematopoietic origin, showed that it is possible that the aptamer instead of a specific binding to PTK7 may identify a propensity for adherence, thus needing further investigation [47].

16.3.3 Conjugation of Aptamers with Synthetic Polymers

Conjugation of biomolecules to synthetic polymers, such as polyethylene glycol (PEG), is used to increase the hydrodynamic volume to molecular weight ratio and thus improve their performance as therapeutics influencing their permanence in the body. Aptamers are relatively small, and charged molecules subject to rapid renal elimination and PEGylation of aptamers can overcome this potential limitation.

PEG shows low toxicity, non-immunogenicity, and high solubility in water and is commercially available in various configurations (linear, branched, or comb-shaped) and molecular weights and with a large variety of terminal functional groups suitable for conjugation.

PEGylated aptamers have been prepared by coupling the activated polymer to amino-modified aptamers [48]. In addition, comb polymers of PEG acrylate containing a pyridyl disulfide terminus have been reversibly conjugated to siRNAs [49].

As an example of more recent PEGylation chemistry, a 3′-thiol-modified (disulfide protected) 25-nucleotide DNA aptamer, selected against the protein core of MUC1 glycoprotein, was successfully conjugated via a maleimide–thiol reaction to a range of maleimide-activated PEGs. The affinity of the PEG–aptamer conjugate for the target resulted to vary according to the structure and conformation of the synthetic polymer [50].

PEG was also used as a spacer and linker for a second polymer, polyamidoamine (PAMAM), to construct a multicomponent, multifunction aptamer conjugate. PAMAM, due to the positively charged amino groups present on its surface, can bind, transport, and deliver therapeutic nucleic acids like siRNA and miRNA. The multicomponent conjugate was accomplished by combining first PEG with PAMAM and then adding aptamer A10-3.2, a shortened version (39 nucleotides) of PSMA-specific aptamer A10, more easily synthesized, more stable, and more efficient than A10 itself. In details, PAMAM dendrimer was reacted with α-maleimidyl-ω-N-hydroxysuccinimidyl polyethylene glycol to give PAMAM/PEG conjugate further reacted with sulfhydryl A10-3.2. The conjugate PAMAM/PEG/aptamer was used as a vehicle for the safe and effective target delivery of miR-15a and miR-16-1, identified as tumor suppressor genes in prostate cancer. These miRNA/PAMAM/PEG/aptamer conjugates proved ca. fivefold more active than miRNA/PAMAM/PEG conjugates, lacking the aptamer, in viability assays on PSMA-positive human prostate adenocarcinoma (LNCaP) cells [51].

16.3.4 Conjugation of Aptamers with Nanoparticles

The integration of cell-type-specific aptamers with nanocarriers like liposomes, micelles, synthetic polymer nanoparticles, carbon nanotubes, quantum dots (QDs), and other nanoparticles can produce new, versatile, and multifunctional specific delivery vehicles. Aptamer-functionalized nanoparticles have a size in the mid-nanometer range, allowing preferential accumulation in target tissues and organs through an enhanced permeability and retention effect, facilitating cellular entry by endocytosis. Conjugation with nanocarriers can also reduce renal clearance and improve circulation half-life and biodistribution in vivo [26].

16.3.4.1 Conjugation of Aptamers with Synthetic Polymer Nanoparticles

Aptamer–nanoparticles were prepared by first synthesizing a poly(lactic acid) (PLA)-block-PEG copolymer, with a terminal carboxylic acid functional group (PLAPEG- COOH), by ring-opening polymerization of the D,L-lactic acid dimeric lactide and OH-PEG3400-COOH. Nanoparticles were then prepared using the water-in-oil-in-water solvent evaporation procedure (double-emulsion method). These nanoparticles (Mn = 10,500) carry carboxylic acid groups available for covalent conjugation to amino-modified aptamers, forming a negative charge surface, which may minimize non-specific interactions with the negatively charged aptamers. The presence of PEG on particle surface enhances circulating half-life. The nanoparticles were conjugated with 3′-NH_2-modified A10 RNA aptamer-targeting PSMA and the resulting bioconjugates proved to efficiently target and take up by PSMA-expressing prostate LNCaP cells [52].

16.3.4.2 Conjugation of Aptamers to Drug Carrier Synthetic Polymer Nanoparticles

An aptamer–nanoparticles-based delivery system, targeting the transmembrane MUC1 protein, overexpressed in most malignant adenocarcinomas, has been realized for delivering the anticancer drug paclitaxel to MUC1-positive tumor cells. To this aim, the S2.2, 25-nucleotide MUC1 aptamer was synthesized as chimeric ON with a 3′-NH_2-modified 73-nucleotide spacer and covalently conjugated to a nanoparticle made of poly(lactic-co-glycolic-acid) (PLGA, 50:50, MW = 16,000). The paclitaxel–Apt–NP assembly was realized using the emulsion/evaporation method. The MUC1 Apt–NP system proved to enhance the delivery of paclitaxel to MUC1-positive MCF-7 cells in vitro [53].

16.3.4.3 Conjugation of Aptamers to Self-assembling Nanoparticles

Self-assembling nanoparticles, able to deliver siRNA and other therapeutics to targeted cells, can be obtained by fusion with the packaging phi29 motor RNA (pRNA). For example, a CD4 aptamer and a siRNA against survivine were covalently fused with pRNA and assembled into dimers that proved to specifically bind to CD4-expressing cells and to be internalized, knocking down the target transcripts [54].

16.3.4.4 Conjugation of Aptamers to Form Micelles

An amphiphilic block copolymer made of a hydrophilic oligonucleotide and a hydrophobic polymer, in aqueous solution, can self-assemble into a spherical or a

nanorod-like micelle. An aptamer with a hydrophobic polymeric tail attached to its end can form a highly ordered micelle-like structure, in which the aptamer not only acts as the building block for the nanostructure, but also performs the recognition of its specific target. Furthermore, the dense packing of the aptamer in the micelle can greatly improve binding affinity to its specific targets. Micelles are also considered to be dynamic and soft materials, which may interact with the dynamic lipid bilayer of the cell membrane favoring drug delivery.

Aptamer TDO5, specific to Ramos cells (a B-cell lymphoma cell line), generated by automated synthesis, has been directly fused to a PEG linker carrying a diacyl lipid tail. This TDO5 amphiphilic conjugate self-assembled into a spherical micelle structure, as demonstrated by transmission electron microscopy (TEM). The average diameter of TDO5–micelles, estimated to contain 1,000 copies of conjugate unit, resulted 68 ± 13 nm, consistent with the hydrodynamic diameter of 67.22 nm measured by dynamic light scattering. The TDO5–micelles demonstrated extremely rapid recognition of the target cells, were found to enhance the binding capability of otherwise low-affinity TDO5 aptamer at physiological temperature, and helped cell internalization. Additionally, the aptamer–micelles displayed low k_{off} once on the cell membrane, high sensitivity, low critical micelle concentration values, great dynamic specificity in flow channel systems that mimic drug delivery in a flowing system, thereby appearing to function as an efficient detection/delivery vehicle in the biological living system [55].

16.3.4.5 Conjugation of Aptamers with Liposomes

Liposomes can be loaded with pharmaceuticals, in particular highly toxic or poorly soluble chemotherapeutics. A therapeutic aptamer–liposome (100 nm diameter) drug delivery system was molecularly engineered by assembling hydrogenated soy phosphatidylcholine, cholesterol, methoxy poly-(ethylene glycol)-distearoyl-phosphatidyl-ethanolamine and maleimide-terminated poly-(ethylene glycol)-distearoyl-phosphatidyl-ethanolamine, in a second step covalently linked to 3′-thiol-modified sgc8 aptamer, which has high binding affinity toward leukemia CEM-CCRF cells. The liposome system proved stabilized by PEG coating and able to bind target cells and deliver a model drug [56].

16.3.4.6 Conjugation of Aptamers with Gold Nanoparticles

Gold nanoparticles (AuNPs) hold great promise for biological and medicinal applications. AuNPs can be synthesized by reducing tetrachloroauric acid with trisodium citrate. AuNPs can be characterized by UV–vis spectroscopy and TEM. The concentration of the AuNPs can be calculated according to Beer's law using an extinction coefficient of $2.4 \times 108\ M^{-1}\ cm^{-1}$ at 520 nm for the 12 nm AuNPs [57].

They have unique colorimetric, conductivity, and nonlinear optical properties, easy surface conjugation with biological entities, high stability, biological compatibility, controllable morphology, and size dispersion. On the basis of their unique surface plasmon resonance (SPR) property, dispersed AuNPs appear red, whereas their aggregates appear purple allowing target-induced colorimetric assays generally based on their assembly or disassembly associated with target recognition [58].

Detection methods relying on the conjugation of ONs with AuNPs show more sensitivity than conventional assays based on ONs probes, so that AuNPs functionalized in particular with an aptamer (Apt) provide a powerful platform for targeted delivery, detection, and therapy. Some tests based on Apt-AuNPs have been commercialized [59].

AuNPs can be directly functionalized with thiolated aptamers and other thiolated ONs by chemisorption. With the introduction of the Apt-AuNPs platform, many kinds of analytes have been monitored on the basis of different mechanisms.

According to a bridging mechanism, AuNPs modified with aptamers for platelet-derived growth factors (PDGFs) produced a highly specific sensing system with detection limits of 3.2 nM [60]. Aggregation and redispersion of AuNPs connected to specific color changes can be achieved by inducing loss or screening of surface charges [61]. Assays based on the process of AuNPs disassembly can be performed with ODNs-AuNPs first cross-linked by an aptamer sequence to form aggregates that are forced to dissociate upon binding of a specific target [62].

Aptamers linked to the surface of solid AuNPs can result hindered in assuming the functional conformation with respect to free solution, and their target recognition ability can result impaired. To overcome this problem, AuNPs have been assembled with ONs complementary (cON) to a non-functional part of the aptamer sequence, allowing the subsequent hybridization of the aptamer, in this way leaving the functional part more exposed to solution and free to assume the appropriate conformation [63]. Taking advantage of the highly efficient fluorescence quenching properties of AuNPs for proximately fluorescent dyes through energy transfer processes (FRET) [64], this method of bioconjugation was applied to perform the multiplex detection of small molecules (adenosine, potassium, and cocaine) through the utilization of aptamer-based multicolor fluorescent AuNPs probes.

The strategy exploiting a 3′-FAM-Apt:cON-AuNPs probe has been applied to the monitoring of a protein, on the basis of the hybrid disruption and release of the aptamer from the Apt:cON-AuNPs assembly upon binding by its target protein. Upon hybridization, the fluorescence of the 3′-FAM-Apt is quenched through FRET by the AuNP, while in the presence of the target protein, the aptamer is induced to fold, fitting to its target and disrupting the hybrid, which causes 3′-FAM-Apt to be released and the generation of a fluorescence signal.

Recombinant human erythropoietin-α (rHuEPO-α) has been monitored by a simple signal transduction system made of 3′-FAM-modified aptamer (35 nt) hybridized to a partially complementary ON linked to AuNPs through a 5′-thiol linker. This simple method has shown high selectivity. It was observed that the

cON length and composition should be optimized; the average cON loading attached to each AuNPs can be estimated [65]; the Apt:cODN-AuNPs probe should be equilibrated in solution with, for example, BSA for preventing non-specific binding with any possible interfering proteins.

16.3.4.7 Conjugation of Aptamers to Quantum Dot Nanoparticles

Among aptamer–siRNA chimeric delivery systems, those based on inorganic nanoparticles are positively charged; consequently, negatively charged aptamer-siRNA may collapse onto their cationic carriers, thus affecting aptamer conformation and selective binding. To overcome this problem, aptamer–siRNA chimeras were linked onto quantum dot (QD) nanoparticles following a two-step approach. siRNA molecules (targeting eGFP), modified with a thiol reactive group, were first adsorbed onto polyethylene imine (PEI)-coated nanoparticles, and then, thiol-modified aptamers (targeting PSMA) were added to form aptamer–siRNA chimeras through the thiol-disulfide exchange reaction. The non-covalent adsorption facilitates the release of siRNA from the nanocarrier inside cells; additionally, neutralizing some of the positive charges on the nanoparticle surface, their interaction with aptamers is weakened, thus helping to retain aptamer properties [66]. Highly stable, water-soluble QDs were prepared by molecular self-assembly of hydrophobic QDs with amphiphilic copolymers poly (maleic anhydride-alt-1-tetradecene). Adding carbodiimide-activated QDs to polyethyleneimine yields single non-aggregated PEI-QDs. QDs excellent photoluminescent properties allows real-time monitoring.

Aptamer–siRNA chimeras directly adsorbed in one step onto nanoparticles elicited only ca. 8 % improvement in selective gene silencing with respect to non-targeted nanoparticle siRNA complexes, while the two-step approach produced 34 % more silenced cells.

16.3.4.8 Conjugation of Aptamers to Magnetic Nanoparticles

Biocompatible aptamer-conjugated magnetic nanoparticles (ACMNPs) have been prepared by conjugating streptavidin-coated iron oxide nanoparticles with biotin-labeled aptamers and used for cancer cell detection and pattern recognition [67]. In fact, binding of the aptamer to target cells produces an aggregation of dispersed ACMNPs and this variation determines a decrease in spin–spin relaxation time (T_2) of adjacent water protons that can be measured by an NMR analyzer. The ACMNPs can detect as few as 10 cancer cells in a 250 μL sample; their specificity and sensitivity are maintained also in complex biological samples. Since cell lines more abundant in receptors produce comparatively larger T_2 variations, distinct recognition patterns can be associated with different levels of receptor expression in different cell lines. ACMNPs can be arranged in arrays able to recognize patterns of different types of cancer cells.

A nanosurgeon system was prepared by modifying the surface of commercially available carboxylated dextran-coated MNPs, with a diameter of 200 ± 50 nm, through conjugation, via the carbodiimide chemistry, with a 5′-NH_2-modified GB-10 aptamer, specifically targeting human glioblastoma cell lines. Aptamer-conjugated magnetic nanoparticles, controlled by an externally applied three-dimensional rotational magnetic field, proved able to perform selective surgical actions on target cells in in vitro studies [68].

16.3.4.9 Conjugation of Aptamers to Silica-Coated Magnetic Nanoparticles

ACNPs made of silica-coated magnetic (MNPs) and fluorophore-doped silica nanoparticles (FNPs) have been conjugated to highly selective aptamers to detect and extract targeted cells in a variety of matrices, according to dual nanoparticles-based assays. Following to an accurate process of optimization, the best performing ACNPs proved to be the 60 nm MNPs, with the most efficient capacity of extraction, and the tetramethylrhodamine-doped FNPs, showing the greatest signal-to-background ratio. The use of multiple aptamer sequences on the NPs increased sensitivity without impacting selectivity, allowing us to reach a theoretical limit of detection of 6.6 cells. This approach can be adapted for different types of cancer cells [69].

16.3.5 Conjugation of Aptamers with ^{99m}Tc for Single-Photon Emission Computed Tomography

Despite the fact that to date only a few aptamers have been developed as targeting agents in imaging modalities, very promising examples indicate their great potential in this field. Among them, the TTA1 aptamer directed against tenascin-C (TN-C) [70] has been extensively modified for performing single-photon emission computed tomography (SPECT), an imaging technique using gamma rays that allows for visualization of tumors at a spatial resolution in the submillimeter range. Indeed, to improve aptamer in vivo stability and guarantee a good blood persistence leading to sufficient signal-to-noise ratios for imaging, aptamer backbone (2′-F-Py containing RNA, 39 mer) has been further modified with the addition of 2′-OMe purine substitutions, a thymidine cap at the 3′ end and locked nucleic acids (LNA) in not binding critical stem [71]. The TTA1 derivative has been conjugated with mercapto-acetyl diglycine (MAG2) chelate via a hexyl-aminolinker at the 5′ end, labeled with ^{99m}Tc and administrated by intravenous injection in murine xenograft models of glioblastoma and breast cancer [48]. Data showed a rapid renal and hepatobiliary clearance, 0.2 and 1.5 %ID/g at 3 h, respectively, and a rapid tumor penetration (6 % injected dose at 60 min). Tumor retention was

durable (2.7 % injected dose at 60 min), and the tumor-to-blood signal was significantly high, thus enabling clear tumor imaging.

Also, DNA aptamers that target MUC1 have been labeled with ^{99m}Tc [72]. Four types of chelating agents have been coupled to the aptamer to generate novel complexes for diagnostic imaging: MetCyc, MAG3, DOTA, and porphyrin. Different from the monomeric Tc-MetCyc-Apt and Tc-MAG3-Apt, the tetrameric compounds, DOTA and porphyrin, conjugated with ^{99m}Tc were able to grab four aptamer molecules modified at 5′ by inserting amine groups to facilitate coupling with the ligands carboxylic terminations and at 3′ ends to protect against nuclease degradation. Despite the fact that the tetrameric complexes showed improved tumor retention and pharmacokinetic properties compared to the monomeric compounds, biodistribution studies have shown the presence of free Tc in the stomach and large intestine highlighting some kind of lack in the Tc–aptamer binding [73]. In another study by Pieve et al. [74], anti-MUC1 aptamers have been successfully conjugated to MAG2 chelator and labeled with ^{99m}Tc to analyze biodistribution of the aptamer in MCF7 xenograft-bearing nude mice. Even if the radiolabeled aptamers demonstrate good tumor uptake and clearance, they require further optimization before diagnostic use.

16.4 Modifications of Aptamers

RNA aptamers resulting from the SELEX process are single-stranded oligoribonucleotides composed in the average of ca. 80 bases. In general, they are not utilized as such but could be truncated to the minimal target-binding domain, usually down to 25–50 ribonucleotides, in order to reduce the risk of unwanted interactions. The reduction in the aptamer length, combined with different modifications (Fig. 16.2), may allow to first increase their stability in the biological media of application and second to render their chemical synthesis easier and more convenient in terms of final yield and accordingly also of economic cost.

It is well known that RNA aptamers, siRNAs, miRNAs, and other synthetic RNAs exposed to cells or tissues for performing therapeutic, diagnostic, or theranostic functions must be generally protected to overcome degradation by ribonucleases, with the consequent loss of their potential activity. In order to enhance resistance to nuclease attack, chemical modifications at position 2′ of ribonucleotides can be exploited with success, since ribonucleases utilize the 2′-OH group for the cleavage of the adjacent phosphodiester bond. In particular, substitution at the 2′ position of ribonucleotides with 2′-amino (2′-NH_2), 2′-fluoro (2′-F), or a variety of 2′-O-alkyl moieties has proven to lend resistance preventing degradation [75].

The most used chemical modification for the development of RNA aptamers stable in animal serum is the substitution of 2′-OH with 2′-F in pyrimidines. Such RNA can also be efficiently transcribed in vitro with a mutant viral RNA polymerase, thus facilitating its use in the SELEX process [9, 10, 75]. However, the

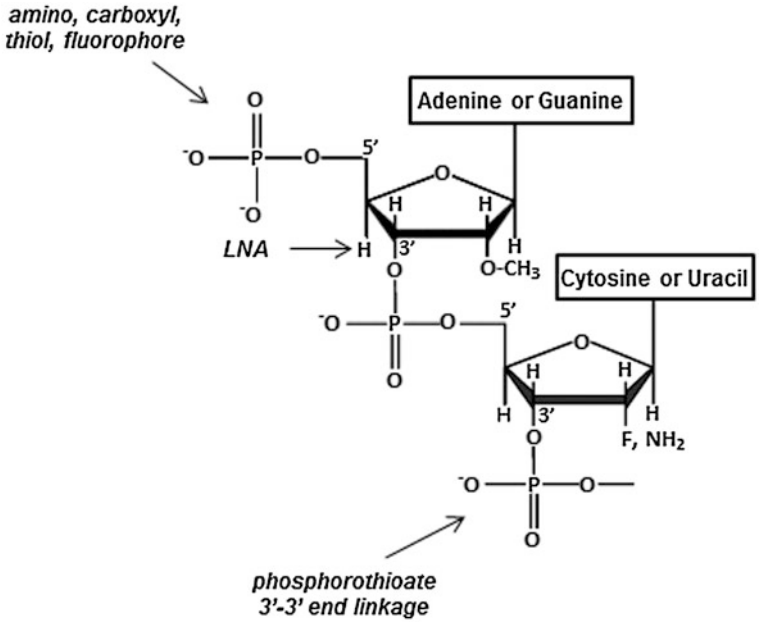

Fig. 16.2 Possible chemical modifications of aptamers

stability of RNA modified with 2′-fluoro-modified pyrimidines in the different contexts has not yet been well assessed.

It is noteworthy to mention that in culture media contaminated by mycoplasma, there are multiple mycoplasma-derived ribonucleases that can readily degrade also RNA modified with either 2′-F or 2′-O-methyl-modified pyrimidines [76].

Comparing the modification of aptamers with either 2′-NH_2 or 2′-F pyrimidines, it was concluded that (1) 2′-NH_2 modification decreases the thermal stability of model DNA/DNA, RNA/RNA, and RNA/DNA duplexes [77, 78] that, on the contrary, is increased by substitution with 2′-F [79–81]; (2) 2′- NH_2 groups increase in general the conformational flexibility of ODNs, and this may limit the binding affinity of 2′- NH_2-pyrimidine-modified aptamers, while 2′-F-pyrimidine-modified aptamers may adopt more rigid conformations and, thus, may exhibit higher binding affinities for their targets; (3) the chemical synthesis of 2′-F-pyrimidine-modified aptamers is more economical, i.e., the coupling efficiency of 2′-F-pyrimidine phosphoramidites during automated synthesis is greater than that of 2′-NH_2-pyrimidine phosphoramidites and the 2′-F groups do not require protection/deprotection steps [82].

Another way to make ONs resistant to nucleases is the transformation of the phosphodiester backbone into a phosphorothioate backbone, with the substitution of non-bonding oxygen of the bridging phosphate group with a sulfur atom, by the use of sulfurizing agents directly within the automated synthesis of the ONs. Analogously, resistance can be acquired by the synthesis of ONs as LNAs, by the use of the

appropriate phosphoramidites (Exiqon A/S), in which the ribose moiety is modified with an extra bridge connecting the 2′ oxygen and 4′ carbon [83]. The substitution of RNAs with LNAs leads to nuclease insensitivity and higher melting temperatures. For example, the introduction of LNA modifications in the TN-C aptamer significantly improved its plasma stability and enhanced its tumor uptake [71].

A challenging approach to enhance stability of aptamers is based on the selection of RNA aptamers binding to the mirror image of an intended target molecule (e.g., an unnatural D-amino acid peptide), followed by the chemical synthesis of the mirror images of the selected sequences, named Spiegelmers [84]. Since they are enantiomers of natural nucleic acids, they are not recognized by nucleases. Two Spiegelmers, NOX-A12 and NOX-E36, are in clinical trials for the treatment of type 2 diabetes and hematologic tumors, respectively [reviewed in 7, 8].

In order to render the chemical synthesis of RNA aptamers more efficient and less costly, systematic studies are usually carried out with the aim of defining the minimal length of the aptamer compatible with an acceptable binding affinity for the target. In fact, the chemistry at the basis of the oligoribonucleotide synthesis is such that the shorter is the sequence to synthesize, the higher is the final yield and the lower is the cost. A type of refinement consists in the screening of the single bases along the natural sequence that must/can be kept as unmodified ribonucleosides. A further refinement consists in protecting the 3′ end of the oligoribonucleotide from degradation by replacing a number of phosphodiester bonds with phosphorothioate bonds and to introduce an initial 3′-3′ oligodesoxynucleotidic capping junction.

With the support of computational programs, able to predict the conformational structure of the aptamer of interest, tracts can be identified along the sequence that can be substituted with non-nucleosidic bridges, like (CH_2CH_2O) units, thus improving stability and final yield, while reducing the cost.

16.5 Conclusions

In the last decade, researches in the field of aptamer have generated great interest because of their high potential as targeting agents. They discriminate between closely related targets and are characterized by high specificity and low toxicity, thus representing a valid alternative to antibodies for in vivo cell recognition. Further, due to their relatively simple chemistry, aptamers have several advantages that make this class of molecules as highly promising for their use in clinic. Development of new effective selection methodologies as, for example, whole-cell-SELEX, has recently offered the possibility to generate aptamers able to internalize into the cell in a receptor-mediated manner and thus molecules of choice for the cell-specific delivery of nucleic acid-based therapeutics. The increasing knowledge gathered in the last decade of the molecular mechanisms of RNA interference has boosted the interest in this class of molecules, including siRNAs, microRNAs, antisense oligonucleotides, and ribozymes as safe and

highly selective therapeutics. In this respect, because of their chemical nature, aptamers are more easier than antibodies to accept functional groups needed to create conjugates with nanoparticles or other macromolecules. Therefore, the combined advantages of cell-specific aptamers with those of nanoparticles and RNAi-based therapeutic agents provide now a very attractive and flexible new approach for selective delivery in the desired cells or tissue.

Acknowledgements This work was supported by funds from CNR, AICR No 11-0075 (L. Cerchia), MIUR grant, MERIT RBNE08YFN3_001 (VdF), AIRC No 13345 (VdF), AIRC No 11781 (L.Cerchia), by the Juvenile Diabetes Research Foundation International (JDRF, 5-2011-330), by the Compagnia di San Paolo, and from the Italian Ministry of Economy and Finance to the CNR for the Project FaReBio di Qualità.

References

1. Famulok M, Hartig JS, Mayer G (2007) Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. Chem Rev 107:3715–3743
2. Eaton BE, Gold L, Zichi DA (1995) Let's get specific: the relationship between specificity and affinity. Chem Biol 2:633–638
3. Foy JWD, Rittenhouse K, Modi M, Patel M (2007) Local tolerance and systemic safety of pegaptanib sodium in the dog and rabbit. J Ocul Pharmacol Ther 23:452–466
4. Yu D, Wang D, Zhu FG, Bhagat L, Dai M, Kandimalla ER, Agrawal S (2009) Modifications incorporated in CpG motifs of oligodeoxynucleotides lead to antagonist activity of toll-like receptors 7 and 9. J Med Chem 52:5108–5114
5. Mayer G (2009) The chemical biology of aptamers. Angew Chem Int Ed Engl 48:2672–2689
6. Cerchia L, de Franciscis V (2010) Targeting cell surface with nucleic acid-aptamers: the specific recognition of cancer cells. Trends Biotechnol 28(10):517–525
7. Keefe AD, Pai S, Ellington A (2010) Aptamers as therapeutics. Nat Rev Drug Discov 9:537–550
8. Esposito CL, Catuogno S, de Franciscis V, Cerchia L (2011) New insight into clinical development of nucleic acid aptamers. Discov Med 11:487–496
9. Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346(6287):818–822
10. Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505–510
11. Keefe AD, Cload ST (2008) SELEX with modified nucleotides. Curr Opin Chem Biol 12:448–456
12. Chelliserrykattil J, Ellington AD (2004) Evolution of a T7 RNA polymerase variant that transcribes 2′-O-methyl RNA. Nat Biotechnol 22:1155–1160
13. Burmeister PE, Lewis SD, Silva RF, Preiss JR, Horwitz LR, Pendergrast PS, Mccauley TG, Kurz JC, Epstein DM, Wilson C et al (2005) Direct in vitro selection of a 2-O-methyl aptamer to VEGF. Chem Biol 12:25–33
14. Morris KN, Jensen KB, Julin CM, Weil M, Gold L (1998) High affinity ligands from in vitro selection: complex targets. Proc Natl Acad Sci U S A 95:2902–2907
15. Shangguan D, Li Y, Tang Z, Cao ZC, Chen HW et al (2006) Aptamers evolved from live cells as effective molecular probes for cancer study. Proc Natl Acad Sci USA 103:11838–11843
16. Chen HW, Medley CD, Sefah K, Shangguan D, Tang Z et al (2008) Molecular recognition of small-cell lung cancer cells using aptamers. ChemMedChem 3(6):991–1001

17. Cerchia L, Duconge F, Pestourie C, Boulay J, Aissouni Y et al (2005) Neutralizing aptamers from whole-cell SELEX inhibit the RET receptor tyrosine kinase. PLoS Biol 3:e123
18. Cerchia L, Esposito CL, Jacobs AH, Tavitian B, de Franciscis V (2009) Differential SELEX in human glioma cell lines. PLoS ONE 4(11):e7971
19. Esposito CL, Passaro D, Longobardo I, Condorelli G, Marotta P, Affuso A, de Franciscis V, Cerchia L (2011) A neutralizing RNA aptamer against EGFR causes selective apoptotic cell death. PLoS ONE 6(9):e24071
20. Cerchia L, Esposito CL, Camorani S, Rienzo A, Stasio L, Insabato L, Affuso A, de Franciscis V (2012) Targeting Axl with an high-affinity inhibitory aptamer. Mol Ther 20(12):2291–2303
21. Xiao Z, Shangguan D, Cao Z, Fang X, Tan W (2008) Cell-specific internalization study of an aptamer from whole cell selection. Chemistry 14(6):1769–1775
22. Thiel KW, Hernandez LI, Dassie JP, Thiel WH, Liu X, Stockdale KR, Rothman AM, Hernandez FJ, McNamara JO 2nd, Giangrande PH (2012) Delivery of chemo-sensitizing siRNAs to HER2+-breast cancer cells using RNA aptamers. Nucleic Acids Res 40(13):6319–6337
23. Cerchia L, Esposito CL, Camorani S, Catuogno S, de Franciscis V (2011) Coupling aptamers to short interfering RNAs as therapeutics. Pharmaceuticals 4:1434–1449
24. Thiel KW, Giangrande PH (2010) Intracellular delivery of RNA-based therapeutics using aptamers. Thera Deliv 1(6):849–861
25. Zhou J, Rossi JJ (2011) Cell-specific aptamer-mediated targeted drug delivery. Oligonucleotides 1:1–10
26. Zhou J, Rossi JJ (2013) Aptamer-Mediated siRNA Targeting. In: Howard KA (ed) RNA Interference from biology to therapeutics. Advances in delivery science and technology
27. Li N, Nguyen HH, Byrom M, Ellington AD (2011) Inhibition of cell proliferation by an anti-EGFR aptamer. PLoS ONE 6(6):e20299
28. Lupold SE, Hicke BJ, Lin Y, Coffey DS (2002) Identification and characterization of nuclease-stabilized RNA molecules that bind human prostate cancer cells via the prostate-specific membrane antigen. Cancer Res 62:4029–4033
29. Li N, Larson T, Nguyen HH, Sokolov KV, Ellington AD (2010) Directed evolution of gold nanoparticle delivery to cells. Chem Commun (Camb) 46:392–394
30. Liu N, Zhou C, Zhao J, Chen Y (2012) Reversal of paclitaxel resistance in epithelial ovarian carcinoma cells by a MUC1 aptamer-let-7i chimera. Cancer Invest 8:577–582
31. Ye M, Hu J, Peng M, Liu J, Liu J, Liu H, Zhao X, Tan W (2012) Generating aptamers by cell-SELEX for applications in molecular medicine. Int J Mol Sci 13(3):3341–3353
32. Chu TC, Twu KY, Ellington AD, Levy M (2006) Aptamer mediated siRNA delivery. Nucleic Acids Res 34(10):e73
33. McNamara JO 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 24:1005–1115
34. Dassie JP, Liu XY, Thomas GS, Whitaker RM, Thiel KW, Stockdale KR, Meyerholz DK, McCaffrey AP, McNamara JO 2nd, Giangrande PH (2009) Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. Nat Biotechnol 27(9):839–849
35. Pastor F, Kolonias D, Giangrande PH, Gilboa E (2010) Induction of tumour immunity by targeted inhibition of nonsense-mediated mRNA decoy. Nature 465:227–230
36. Wullner U, Neef I, Eller A, Kleines M, Tur MK, Barth S (2008) Cell-specific induction of apoptosis by rationally designed bivalent aptamer-siRNA transcripts silencing eukaryotic elongation factor 2. Curr Cancer Drug Targets 8:554–565
37. Zhou J, Li H, Li S, Zaia J, Rossi JJ (2008) Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. Mol Ther 16:1481–1489
38. Zhou J, Swiderski P, Li H, Zhang J, Neff CP, Akkina R, Rossi JJ (2009) Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of dicer substrate siRNAs into HIV infected cells. Nucleic Acids Res 37:3094–3109

39. Neff CP, Zhou J, Remling L, Kuruvilla J, Zhang J, Li H, Smith DD, Swiderski P, Rossi JJ, Akkina R (2011) An aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4(+) T cell decline in humanized mice. Sci Transl Med 3(66):66ra6. doi: 10.1126/scitranslmed.3001581
40. Zhou J, Neff CP, Swiderski P, Li H, Smith DD, Aboellail T, Remling-Mulder L, Akkina R, Rossi JJ (2013) Functional in vivo delivery of multiplexed anti- HIV-1 siRNAs via a chemically synthesized aptamer with a sticky bridge. Mol Ther 21:192–200
41. Ni X, Zhang Y, Ribas J, Chowdhury WH, Castanares M, Zhang Z, Laiho M, DeWeese TL, Lupold SE (2011) Prostate-targeted radiosensitization via aptamer-shRNA chimeras in human tumor xenografts. J Clin Invest 121:2383–2390
42. Dai F, Zhang Y, Zhu X, Shan N, Chen Y (2012) Anticancer role of MUC1 aptamer-miR-29b chimera in epithelial ovarian carcinoma cells through regulation of PTEN methylation. Target Oncol 7:217–225
43. Wheeler LA, Trifonova R, Vrbanac V, Basar E, McKernan S, Xu Z, Seung E, Deruaz M, Dudek T, Einarsson JI, Yang L, Allen TM, Luster AD, Tager AM, Dykxhoorn DM, Lieberman J (2011) Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras. J Clin Invest 121(6):2401–2412
44. Zhou J, Tiemann K, Chomchan P, Alluin J, Swiderski P, Burnett J, Zhang X, Forman S, Chen R, Rossi J (2013) Dual functional BAFF receptor aptamers inhibit ligand-induced proliferation and deliver siRNAs to NHL cells. Nucleic Acids Res [Epub ahead of print]
45. Wilner SE, Wengerter B, Maier K, de Lourdes Borba Magalhães M, Del Amo DS, Pai S, Opazo F, Rizzoli SO, Yan A, Levy M (2012) An RNA alternative to human transferrin: a new tool for targeting human cells, Mol Ther Nucleic Acids 1:e21. doi: 10.1038/mtna.2012.14
46. Huang YF, Shangguan D, Liu H, Phillips JA, Zhang X, Chen Y, Tan W (2009) Molecular assembly of an aptamer–drug conjugate for targeted drug delivery to tumor cells. ChemBioChem 10:862–868
47. Li N, Ebright JN, Stovall GM, Chen X, Nguyen HH, Singh A, Syrett A, Ellington AD (2009) Technical and biological issues relevant to cell typing with aptamers. J Proteome Res 8(5):2438–2448
48. Hicke BJ, Stephens AW, Gould T, Chang Y-F, Lynott K, Heil J, Borkowski S, Hilger C-S, Cook G, Warren S, Schmidt P (2006) Tumor targeting by an aptamer. J Nucl Med 47:668–678
49. Heredia KL, Nguyen TH, Chang CW, Bulmus V, Davis TP, Maynard HD (2008) Reversible siRNA polymer conjugates by RAFT polymerization. Chem Commun 28:3245–3247
50. Da Pieve C, Williams P, Haddleton DM, Palmer RMJ, Missailidis S (2010) Modification of thiol functionalized aptamers by conjugation of synthetic polymers. Bioconjugate Chem 21:169–174
51. Wu X, Ding B, Gao J, Wang H, Fan W, Wang X, Zhang W, Wang X, Ye L, Zhang M, Ding X, Liu J, Zhu Q, Gao S (2011) Second-generation aptamer-conjugated PSMA-targeted delivery system for prostate cancer therapy. Int J Nanomed 6:1747–1756
52. Farokhzad OC, Jon S, Khademhosseini A, Tran T-NT, LaVan DA, Langer R (2004) Nanoparticle-aptamer bioconjugates a new approach for targeting prostate cancer cells. Cancer Res 64:7668–7672
53. Yu C, Hu Y, Duan J, Yuan W, Wang C, Xu H, Yang X-D (2011) Novel aptamer-nanoparticle bioconjugates enhances delivery of anticancer drug to MUC1-positive cancer cells in vitro. PLoS ONE 6:e24077
54. Shu Y, Cinier M, Shu D, Guo P (2011) Assembly of multifunctional phi29 pRNA nanoparticles for specific delivery of siRNA and other therapeutics to targeted cells. Methods 154:204–214
55. Wua Y, Sefaha K, Liua H, Wanga R, Tan W (2010) DNA aptamer-micelle as an efficient detection/delivery vehicle toward cancer cells. PNAS 107:5–10
56. Kang H, O'Donoghue MB, Liu H, Tan W (2011) A liposome-based nanostructure for aptamer directed delivery. Chem Commun 6:249–251

57. Sun J, Guo A, Zhang Z, Guo L, Xie J (2011) A conjugated aptamer-gold nanoparticle fluorescent probe for highly sensitive detection of rHuEPO-α. Sensors 11:10490–10501
58. Yang L, Zhang X, Ye M, Jiang J, Yang R, Fu T, Chen Y, Wang K, Tan W (2011) Aptamer-conjugated nanomaterials and their applications. Adv Drug Deliv Rev 63:1361–1370
59. Giljohann DA, Seferos DS, Daniel WL, Massich MD, Patel PC, Mirkin CA (2010) In vitro selection of structure-switching signaling aptamers. Angew Chem Int Ed 49:3280–3294
60. Huang C, Huang Y, Cao Z, Tan W, Chang H (2005) Aptamer-modified gold nanoparticles for colorimetric determination of platelet-derived growth factors and their receptors. Anal Chem 77:5735–5741
61. Zhao W, Chiuman W, Lam JC, McManus SA, Chen W, Cui Y, Pelton R, Brook MA, Li Y (2008) DNA aptamer folding on gold nanoparticles: from colloid chemistry to biosensors. J Am Chem Soc 130(11):3610–3618
62. Liu J, Lu Y (2006) Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes. Nat Protoc 1:246–252
63. Zhang J, Wang L. Zhang H, Boey F, Song S, Fan C (2010) Aptamer-based multicolor fluorescent gold nanoprobes for multiplex detection in homogeneous solution. Small 6:201-204
64. Dubertret B, Calame M, Libchaber AJ (2001) Single-mismatch detection using gold-quenched fluorescent oligonucleotides. Nat Biotechnol 19:365–370
65. Zhang Z, Guo L, Guo AT, Xu H, Tang JJ, Guo XJ, Xie JW (2010) In vitro lectin-mediated selection and characterization of rHuEPO-α-binding ssDNA aptamers. Bioorg Med Chem 18:8016–8025
66. Bagalkot V, Gao X (2011) SiRNA-aptamer chimeras on nanoparticles: Preserving targeting functionality for effective gene silencing. ACSNano 5:8131–8139
67. Bamrungsap S, Chen T, Shukoor MI, Chen Z, Sefah K, Chen Y, Tan W (2012) Pattern recognition of cancer cells using Aptamer-conjugated magnetic nanoparticles. ACSNano.org 6:3974–3981
68. Nair BG, Nagaoka Y, Morimoto H, Yoshida Y, Maekawa T, Kumar DS (2010) Aptamer conjugated magnetic nanoparticles as nanosurgeons. Nanotechnology 21:455102. doi:10.1088/0957-4484/21/45/455102
69. Medley CD, Bamrungsap S, Tan W, Smith JE (2011) Aptamer-conjugated nanoparticles for cancer cell detection. Anal Chem 83(3):727–734
70. Hicke BJ, Marion C, Chang YF, Gould T, Lynott CK, Parma D, Schmidt PG, Warren S (2001) Tenascin-C aptamers are generated using tumor cells and purified protein. J Biol Chem 276(52):48644–48654
71. Schmidt KS, Borkowski S, Kurreck J, Stephens AW, Bald R, Hecht M, Friebe M, Dinkelborg L, Erdmann VA (2004) Application of locked nucleic acids to improve aptamer in vivo stability and targeting function. Nucleic Acids Res 32(19):5757–5765
72. Ferreira CS, Matthews CS, Missailidis S (2006) DNA aptamers that bind to MUC1 tumour marker: design and characterization of MUC1-binding single-stranded DNA aptamers. Tumour Biol 27(6):289–301
73. Borbas KE, Ferreira CS, Perkins A, Bruce JI, Missailidis S (2007) 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. Bioconjug Chem 18:1205–1212
74. Pieve CD, Perkins AC, Missailidis S (2009) Anti-MUC1 aptamers: radiolabelling with (99 m)Tc and biodistribution in MCF-7 tumour-bearing mice. Nucl Med Biol 36:703–710
75. Pieken WA, Olsen DB, Benseler F, Aurup H, Eckstein F (1991) Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. Science 253:314–317
76. Hernandez FJ, Stockdale KR, Huang L, Horswill AR, Behlke MA, McNamara JO (2012) Degradation of nuclease-stabilized RNA oligonucleotides in mycoplasma-contaminated cell culture media. Nucleic Acid Ther 22(1):58–68

77. Aurup H, Tuschl T, Benseler F, Ludwig J, Eckstein F (1994) Oligonucleotide duplexes containing 2′-amino-2′-deoxycytidines: thermal stability and chemical reactivity. Nucleic Acids Res 22:20–24
78. Lin Y, Qiu Q, Gill SC, Jayasena SD (1994) Modified RNA sequence pools for in vitro selection. Nucleic Acids Res 22(24):5229–5234
79. Cummins LL, Owens SR, Risen LM, Lesnik EA, Freler SM, McGee D, Guinosso CJ, Cook PD (1995) Characterization of fully 2′-modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease sensitivity. Nucleic Acids Res 23:2019–2024
80. Lesnik EA, Guinosso CJ, Kawasaki AM, Sasmor H, Zounes M, Cummins LL, Ecker DJ, Cook PD, Freier SM (1993) Oligodeoxynucleotides containing 2′-O-modified adenosine: synthesis and effects on stability of DNA:RNA duplexes. Biochemistry 32:7832–7838
81. Kawasaki AM, Casper MD, Freier SM, Lesnik EA, Zounes MC, Cummins LL, Gonzalez C, Cook PD (1993) Uniformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. J Med Chem 36(7):831–841
82. Ruckman J, Green LS, Beeson J, Waugh S, Gillette WL, Henninger DD, Claesson-Welsh L, Janjić N (1998) 2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). J Biol Chem 273(32):20556–205567
83. Petersen M, Wengel J (2003) LNA: a versatile tool for therapeutics and genomics. Trends Biotechnol 21(2):74–81
84. Eulberg D, Klussmann S (2003) Spiegelmers: biostable aptamers. ChemBioChem 4(10):979–983