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Bioconjugates for Targeted Delivery of Therapeutic Oligonucleotides

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Abstract

Bioconjugates have been used to deliver therapeutic oligonucleotides to their pharmacological targets in diseased cells. Molecular-scale conjugates can be prepared by directly linking targeting ligands with oligonucleotides and the resultant conjugates can selectively bind to cell surface receptors in target cells in diseased tissues. Besides targeted delivery, additional functionality can be incorporated in the conjugates by utilization of carrier molecules, and these larger conjugates are called carrier-associated conjugates. Both molecular and carrier-associated conjugates have achieved initial successes in clinical trials for treating liver diseases; therefore, currently the greater challenge is to deliver oligonucleotides to extrahepatic tissues such as tumors. This review will provide an update on the application of oligonucleotide conjugates for targeted delivery during the last decade. By identifying key elements for successful delivery, it is suggested that oligonucleotide conjugates with intermediate size, cell targeting ability, and endosomal release functionality are superior systems to advance oligonucleotides to achieve their full therapeutic potentials.

Keywords

antisense; siRNA; oligonucleotides; conjugates; targeted delivery

1. Introduction

Therapeutic oligonucleotides (ONs) provide opportunity for treating serious, life-threatening diseases with limited options using traditional small-molecule and antibody drugs. Those traditional medicines have been constrained by limited “druggable” targets. Small-molecule drugs can only target certain classes of proteins including G protein-coupled receptors, ion channels, enzymes, and nuclear hormone receptors [1], while the targets for antibody drugs are limited to cell-surface receptors and circulating proteins. In contrast, antisense and siRNA ONs can modulate the expression of any gene and thus can target any protein by

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inducing enzyme-dependent degradation of target mRNAs [2]. Further, steric-blocking ONs, including splice switching ONs (SSOs), and antagonomers of microRNAs and long non-coding RNAs, block the access of cellular machinery to pre-mRNA or mRNA without causing enzymatic degradation of the RNA [3, 4]. Through this mechanism, steric-blocking ONs can target non-coding RNAs that exert important regulatory controls on many biological processes [5]. Thus, the target space for ONs is even larger than the size of human proteome. Indeed, the positive outcomes from the clinical trials of microRNA antagonists [6] and SSOs [7, 8] have revealed the tremendous therapeutic potentials for ONs by targeting non-coding RNAs that are “undruggable” by small molecules and antibodies. Despite the enormous therapeutic potential, the development of ONs as therapeutic agents has been constrained by the inability of these membrane-impermeable molecules to reach their intracellular sites of action. Indeed, the FDA-approved antisense drug Mipomersen [9] and several other ONs in late-phase clinical trials [6, 10, 11] target the genes in the liver although ONs can also modulate extrahepatic targets. This limitation is largely due to the favorable physiology of the liver, being a well-perfused organ with a fenestrated endothelium, and endogenous ability of the hepatocytes to take up ONs and/or their delivery systems [12, 13]. Therefore, the success of ON delivery to intracellular targets in disease sites determines whether ONs can meet therapeutic demands that are unmet by traditional medicines.

Therapeutic ONs must overcome numerous biological barriers to reach their intracellular targets following systemic administration [12, 14] (Fig 1). After administration, ONs must avoid rapid degradation by nucleases in the bloodstream and fast clearance via the reticuloendothelial system and renal filtration. Then they need to cross the vascular endothelial barrier and diffuse through the extracellular matrix to approach the diseased cells in tissue. Chemical modification has improved nuclease stability of ONs and thereby their circulation half-life [12]. However, precise delivery to the cells of interest in disease tissues remains a key hurdle for the widespread use of therapeutic ONs [12, 14]. Size has a substantial impact on pharmacokinetics and biodistribution of ONs and their delivery systems. Materials with a size larger than 10 nm may have long circulation time in blood because of reduced renal filtration [12]. However, larger particles (>50 nm) may only access tissues where the vasculature is leaky, such as liver, spleen and some tumors [12, 15]. Thus, delivery systems with intermediate size (10-50nm) may have better chance to deliver ONs to diseased tissues. Even reaching diseased sites, ONs have to cross the plasma membrane and the endosomal membrane to access their target genes in the cytoplasm and/or nucleus of cells. Overcoming these cellular barriers is generally considered as the rate-limiting step for these membrane-impermeable molecules to reach their intracellular sites of action [12, 14].

There have been two broad approaches to the delivery of ONs besides administration of free ONs. One has been to incorporate ONs into various lipid or polymer nanocarriers [16-22]. A second approach has been to create molecular-scale or macromolecular conjugates where ONs are linked to ligands that can bind with high affinity to specific cell surface receptors thus promoting entry via receptor-mediated endocytosis [13, 23]. Chemical conjugation of targeting ligands and ONs produces well defined molecules that are physically stable and enjoy relatively broader tissue distribution than nanoparticles (NPs) [12]. Thus, ON conjugates may have better chance to realize potentials of ONs by accessing a broader

pharmacological target space. This review will provide an update on the progress of ON conjugates for delivery and highlight the mechanisms underlying functional delivery of the ON conjugates. The objective of this review is to motivate more rational strategies leading to greater clinical successes of therapeutic ONs.

2. Molecular conjugates

The simplest method to prepare ON conjugates is to directly link peptide, lipid, carbohydrate, or small molecule moieties at the 5' or 3' positions of ONs so as to provide selective binding to cell surface receptors in target cells. Conjugation of a ligand should not interfere with binding of the ON to its RNA target and the post-binding events. Single-strand ONs are more tolerable to modification as long as the ligands are relatively small [24-26]. In contrast, modification of the antisense strand of siRNA, especially its 5'-terminus, should be avoided because the 5'-terminus of the antisense strand must be phosphorylated to be recognized by the RNA-induced silencing complex [27]. A variety of linkages have been used to conjugate ligands and ONs including amide, thioether, thiol-maleimide, ester, and disulfide [23, 28, 29]. Some linkages are readily cleavable inside the cells, including disulfide, some peptides, and hydrazone, and they are needed to attain biological activity of ONs when large ligands such as antibody [30] or carrier proteins such as albumin [31] are linked the ONs.

Cell selective delivery through ligand conjugation depends on the binding affinity of the ligands to their receptors and the density of the receptors on the target cell surface [32]. Besides, the intrinsic uptake rate of the ONs also plays a role. "Free" phosphorothioate (PS) ONs can bind to membrane receptors and enter the cells via endocytosis [13], and their cellular uptake is more efficient than "free" siRNA ONs and neutral morpholino ONs. Therefore, integrin targeting RGD peptide modified siRNA showed about 20-folded higher uptake than "free" siRNA in cancer cells [33], while RGD modified PS ON only showed about 2-fold higher uptake than free SSO [26].

2.1 Carbohydrate-ON conjugates

The leading ON conjugate in clinical development is a carbohydrate-siRNA conjugate composed of chemically stabilized siRNA with a trivalent liver-targeting ligand [34]. Developed by Alnylam Pharmaceuticals, three siRNA conjugates targeting different liver-expressed genes are currently in Phase I/II clinical trials for the treatment of transthyretin amyloidosis, hypercholesterolemia and haemophilia, respectively (www.clinicaltrials.gov). In this conjugate, the 3' terminus of the siRNA sense strand is linked to three molecules of *N*-acetylgalactosamine (GalNAc), an asialoglycoprotein receptor ligand, by means of a triantennary spacer (Fig 2) [34]. Asialoglycoprotein receptor, mainly expressed in the hepatocytes, binds and then mediates endocytosis of the galactose-terminal glycoproteins, in order to remove them from circulation [35]. This receptor has been considered as one of the most promising membrane proteins for hepatic delivery since it exhibits high affinity and a rapid internalization rate via the clathrin-mediated pathway [35]. In addition, it is easy to couple the GalNAc ligands to ONs as the ligand conjugation step can be incorporated in the process of solid-phase synthesis of ONs [34]. Another advantage of GalNAc-siRNA conjugates is that subcutaneous administration of GalNAc-siRNA conjugates resulted in

robust gene silencing in liver with a median effective dose (ED₅₀) of 1 mg/kg following a single dose, while lipid nanoparticles (LNPs) of siRNA can only be given intravenously [34]. The effective delivery via subcutaneous administration highlights the superior diffusibility of the small-sized conjugates and less non-specific interaction in the injection sites for the hydrophilic GalNAc-siRNA conjugates. The GalNAc-siRNA conjugates are hepatotropic and long-acting and thus have the potential to treat a wide range of diseases involving liver-expressed genes [34]. Besides GalNAc, mannose 6-phosphate, another carbohydrate, has been linked to siRNA for targeted delivery to hepatic stellate cells that play a central role in liver fibrosis [36].

2.2 Lipid-ON conjugates

Conjugation of siRNA or other ONs with a lipid tail has been shown to improve cellular uptake and *in vivo* pharmacokinetics of ONs [37-39]. This method, more specifically, conjugation of ApoB siRNA with cholesterol, led to the first example of gene silencing after systemic administration [38]. Cholesterol-siRNA conjugates can form particles with lipoproteins in blood circulation, which increases circulation time and promotes uptake into the hepatocytes via lipoprotein receptors [39]. The lipid-siRNA conjugates have been further optimized by using new lipids such as α -tocopherol [40] and by controlling the length of the linker between siRNA and lipid [41].

2.3 Small molecule-ON conjugates

Besides natural carbohydrate and lipids, small molecule drugs that demonstrate high binding affinity to their receptors have been used for ON delivery. Thus, mono- and multivalent conjugates of SSOs with anisamide, a small molecule ligand for the sigma receptor, have been synthesized and tested [42]. ONs have also been conjugated with folate [43] and with anandamide [44] that bind to the folate receptor and a cannabinoid receptor, respectively. All of these conjugates have shown to significantly enhance functional activity ONs in the absence of any transfection agent.

2.4 Peptide-ON conjugates

There are two types of peptides that have been used to ON delivery: cell-penetrating and cell-targeting peptides. Cell-penetrating peptides (CPPs) are the short basic amino acid-rich peptides, which can not only enter cells themselves but also facilitate the transport of molecular cargos across the plasma membrane. Many types of CPPs, including prototypical CPPs and those selected from structure-activity studies, have been directly linked to siRNAs, ASOs, and SSOs for *in vivo* delivery [45-47]. CPP conjugation has achieved more successes in delivery of SSOs, particularly uncharged morpholino or peptide nucleic acid oligomers. CPP-SSO conjugates have been used to correct defects involved in Duchenne muscular dystrophy and have shown good effects both in cell culture and in dystrophic mice [48-50]. CPP-based delivery of ONs is further discussed in a review by Boisguérin et al. in this theme issue.

Cell-targeting peptides are ligands for specific receptors that are overexpressed in diseased sites [32], and targeting peptide-ONs can provide additional disease selectivity compared to CPP-ON conjugates. siRNAs, ASOs or SSOs have been conjugated with peptides designed

to bind to specific receptors, for example, integrin $\alpha\beta3$ [26, 33, 51, 52], bombesin receptor [53], and insulin-like growth factor 1 receptors [54, 55]. Conjugates of ONs with integrin-targeting RGD peptide have been the most studied peptide-ON conjugate. In one study, siRNA was linked with mono-, bi-, tri-, and tetravalent cRGD peptides. The bi-, tri-, and tetravalent cRGD conjugates were taken up by integrin $\alpha\beta3$ positive M21⁺ human melanoma cells at approximately the same rate. However, only the tri- and tetravalent versions produced *RNAi* activity, while the bivalent version had little effect [33]. One cause of this discord may be the distinct internalization pathways that the peptide-ON conjugates undertake.

A recent development in peptide-ON conjugates was the use of chimeric peptides composed of multiple peptide domains with different functionalities. In one study, linking a muscle-specific peptide to a CPP peptide further improved the *in vivo* delivery of the CPP-SSO conjugate, producing functional correction of dystrophin and thereafter the improvement of the dystrophic phenotype [56]. In another study, peptides that include bombesin sequences for receptor targeting and a run of histidine residues for endosomal disruption were covalently linked to a SSO, and a trivalent version of this conjugate was prepared by means of a triantennary linkage. The trivalent conjugates that included both the targeting sequence and several histidine residues were substantially more effective than conjugates containing only the bombesin or histidine moieties, indicating the potential of creating molecular scale ON conjugates with both targeting and endosome escape capabilities [57].

2.5 Antibody-ON conjugates

Antibodies have been the primary choice as targeting moieties in targeted drug delivery and antibody-drug conjugates have achieved tremendous clinical success for cancer therapy [58]. This approach of direct conjugation has been extended to ON delivery; however, direct antibody-ONs conjugates haven't accomplished similar success as antibody-drug conjugates. In one study, a humanized monoclonal antibody was linked to siRNA via a reductive disulfide bond that is cleavable within cells [30]. Although antigen-specific binding and internalization of the antibody-ON conjugates were observed, single treatment of the conjugates failed to produce significant gene silencing. Co-treatment with chloroquine, an endosomal release reagent, caused *RNAi* activity, which indicated that functional delivery of the conjugates was limited by poor endosomal release [30]. Endosomal trapping of the antibody-ON conjugates was demonstrated by another study in which bispecific antibodies were used for binding to cell surface antigens with one binding domain and carrying antigen-modified siRNA with another domain [59]. Again, effective antigen-specific delivery of the antibody-siRNA conjugates did not lead to *RNAi* activity as the conjugates were not released from the endosomes. Formulating the conjugates into dynamic polyconjugates (DPCs) or into LNPs produced strong *RNAi* activity both *in vitro* and *in vivo* [59]. Although direct antibody-ON conjugates haven't achieved ideal therapeutic activity, those constructed via cationic carriers such as protamine have shown success both *in vitro* and *in vivo*. We will discuss this in Section 3.2.

2.6 Aptamer- and CpG-ON conjugates

Nucleic acid aptamers are single-stranded ONs with tertiary structures that bind to specific target molecules [60]. They are usually selected from pools of random-sequence ONs using an approach called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Aptamers not only bind to cell surface receptors to produce therapeutic activity by blocking the receptor, but also can enhance the endocytosis of cargo molecules that are linked to the aptamers, thereby acting as carrier molecules to deliver intracellular therapeutics, including drugs, toxins, and ONs [61].

Initial studies of aptamer-based ON delivery have targeted prostate-specific membrane antigen (PSMA), a protein that is expressed on the surface of some prostate cancer cells [62]. In one of the studies, biotin-labeled PSMA-specific aptamer and siRNA were linked via streptavidin and the conjugate produced *RNAi* activity specifically in PMSA positive cells [63]. Another study showed that two siRNAs, which were fused covalently to a PMSA aptamer, also targeted PMSA positive cells, and inhibited growth of prostate cancer xenografts following intratumoral injection [64]. The aptamer-siRNA chimeras have been optimized for extended plasma half-life by, for example, adding a terminal PEG, and the resultant conjugate showed potent antitumor activity after systemic injection into mice [65]. In addition, aptamer-siRNA chimeras of gp120-specific aptamer and a siRNA target HIV infected cells, and inhibit HIV replication through aptamer-mediated receptor blocking and *RNAi* activity [66, 67].

In another example of ONs as delivery agent, the siRNA targeting the immune suppressor gene Stat3 was linked to an immunostimulatory CpG ON to stimulate an anticancer immune response [68]. The binding of CpG-siRNA conjugate to the toll-like receptor 9 (TLR9) in immune cells not only caused various immune responses via endosomal TLR activation, but also triggered the internalization of the siRNA conjugate, leading to Stat3 silencing for greater antitumor immune response [68]. The CpG-siRNA conjugate was observed to first enter early endosomes, where CpG and siRNA parts of the conjugate are uncoupled by Dicer [69]. Diced siRNA molecules are then translocated to endoplasmic reticulum, where TLR9 facilitates release of the diced siRNA to interact with the *RNAi* machinery [69]. These findings suggest that the class of immunostimulatory siRNAs may benefit from activation of certain endosomal immune receptors, such as TLR9, by enhancing *RNAi* activity and synergistic therapeutics [69].

3. Carrier-associated conjugates

Besides cell selective delivery, additional functionality is often required for ON conjugates, including long circulation time and effective endosomal release. A carrier molecule can be used to achieve multiple functionalities and the resultant conjugates are significantly larger than free ONs. We call this type of ON conjugates as carrier-associated conjugates.

3.1 Polymer as carriers

Polymers are often used to link to ONs to improve their therapeutic activity, for example, PEGylation is often used to increase the size of the ONs and thus increase the circulation half-life of the therapeutic ONs via reduced renal filtration [65]. In addition, amphipathic

polymers, which are capable of disrupting cell membrane, are often incorporated in the ON delivery systems for improving endosomal release of ONs.

The leading ON-polymer conjugate in clinical development is Dynamic Polyconjugates (DPCs) [70], which is currently being tested in a Phase II clinical trial for the treatment of Hepatitis B (www.clinicaltrials.gov). This delivery system is composed of several components, each designed to play a particular role in the siRNA delivery (Fig 3). The carrier molecule poly(butyl amino vinyl ether) (PBAVE) has side chains including alkyl groups interspersed with amines and thus this amphipathic polymer can disrupt cell membranes [70]. The membrane-disrupting activity of the polymer is masked by PEG via acid-cleavable carboxylated dimethyl maleic acid (CDM), and liver-targeting GalNAc ligands are also linked to the PBAVE polymer via this chemistry [70]. Further, the siRNA cargo is attached to the polymer by the bioconvertible disulfide bond. The PEG and the ligand are designed to be shed in the acidic environment inside the endosome, exposing the membrane-active polymer and triggering endosomal release; the disulfide linkage is cleaved in the reducing environment of the cytosol, releasing the siRNA from the polymer [70]. This polymer-based system with both cell targeting and endosomal releasing abilities was specifically delivered to hepatocytes after IV injection and produced effective gene knockdown and therapeutic activity in mice [70].

The original DPCs have been optimized by Arrowhead Research Corporation and other groups. Scientists at Merck & Co. Inc have focused on optimization of the carrier polymer in order to enhance the efficacy and safety of the delivery system [71-73]. The amphipathic PBAVE polymer in original DPCs may continue to interact with non-endosomal membranes after endosomal release and then cause side effects. Thus, in one study, disulfide bonds were incorporated in the backbone of the amphipathic polymers, and the resultant poly(amido amine disulfide) polymers provided degradability through reduction by glutathione in cytosol. Thus, the new DPCs of siRNAs produced excellent gene silencing *in vitro* and *in vivo* and did not cause significant toxicity [73]. In another study, by incorporating low *pKa* heterocycles, acid cleavable amino side chains, or carboxylic acid pH sensitive charge switches, the membrane activity of the polymers were triggered by the acidic environment inside the endosome and were then abolished after release into neutral environment in the cytosol. Thus, in animal studies, the new DPCs dramatically increased the therapeutic index to 10-15 from less than 3 for their original version [72].

A different strategy has been adapted to advance DPCs to clinical trials at Arrowhead Research Corporation. Instead of covalent attachment of siRNA to masked polymer in the original system, GalNAc-modified DPC polymer and cholesterol-conjugated siRNA are co-administered to target the same hepatocytes in the liver (Fig 2) [74]. The new delivery approach increased the efficacy over 500-fold over single use of cholesterol-siRNA and allowed over 90% reduction in target gene expression in mice and non-human primates [74]. A further optimization involves replacement of the synthetic polymer with a melittin-like peptide with similar endosome-specific lytic properties, which further simplified the preparation of the delivery system and simultaneously enhanced the siRNA delivery [75, 76]. The new system achieved excellent efficacy ($ED_{50} = 0.01$ mg/kg siRNA) in mice and nonhuman primates without causing significant toxicity after IV administration [75].

ARC-520, siRNA therapeutics that are delivered by this co-injection approach [76] are in a Phase II clinical trial for treating Hepatitis B (www.clinicaltrials.gov).

3.2 Protein as carriers

Human serum albumin (HSA) has a long history as a biodegradable, nontoxic carrier for small molecule and peptide drugs [77, 78]. With a diameter of ~ 7 nm that is close to the pore size of the glomerular filtration barrier, HSA exhibits minimal renal clearance and thus enjoys a long circulation half-life of 19 days [79]. Thus, albumin is an ideal carrier to increase the circulation half-life of drugs and ONs. Albumin has been used as carrier for targeted delivery of ONs [31, 80]. In one study, the SSOs are linked to albumin via reversible disulfide bonds. The albumin is also conjugated with PEG chains that terminate in an RGD ligand [80]. In a similar but simplified design, a morpholino ON was conjugated to a RGD peptide, and then, multiple RGD-ON conjugates were linked to a single molecule of HSA via disulfide bonds (Fig 4) [31]. Both methods produced small NPs with uniform and monodispersed size distribution at a diameter of 12-13 nm, which may be a favorable size for long circulation time [31, 80]. The intermediate sized HSA-ON conjugates could penetrate deeply and distribute throughout 3-D tumor spheroids (Fig 4), whereas the conventional NPs with sizes over 300 nm could only deliver to the cells on the surface of the tumor spheroids [31, 81, 82]. Utilization of HSA as carrier enabled displaying multiple (10-15) targeting ligands on the surface of the NPs, and this dramatically enhanced receptor-specific cellular delivery of ONs in integrin-expressing cancer cells (Fig 4). The targeted HSA-ON conjugates thus increased functional activity of the ON at low nanomolar concentrations without causing cytotoxicity [31, 80]. These delivery systems did not contain any endosomal release functionality yet. Thus, when the cells were further treated with Retro-1 that can cause endosomal release in the cells [83], functional delivery of ONs could be further enhanced by 5-fold [31].

Another protein carrier for ON delivery is a dsRNA binding domain. This protein is used for CPP-mediated delivery of siRNA. CPP conjugates with neutral ONs such PMOs and PNAs are more successful than those with negative charged ASN and siRNAs. The underlying cause may be the decrease of the net positive charge after conjugation of CPPs to polyanionic ONs. Thus, Tat CPP has been fused to a dsRNA binding domain, which binds siRNA tightly and masks their negative charges so as to allow CPPs to exert its membrane penetration ability [84]. This novel construct led to functional delivery of siRNA to tumors *in vivo* [84].

Protamine is a cationic protein that delivers siRNAs by binding them via noncovalent charge interaction. As one example, a single-chain antibody is fused with protamine, which binds siRNAs and forms a targeted delivery system [85, 86]. Antibodies targeting several membrane markers of tumors or lymphocytes have been utilized to deliver siRNAs to the tumors and HIV infected lymphocytes, which led to *RNAi* activity and effective therapeutics after systemic administration [85, 86]. Along the same line, an antibody has been fused with positively charged Oligo-9-arginine peptide, and thereby bound siRNAs targeting multiple viral proteins [87]. Systemic administration of the siRNA conjugates to HIV infected mice has achieved viral suppression [87]. As direct antibody-ON conjugates failed to produce

RNAi activity without using endosomal release reagents, it is believed that the cationic protamine and Oligo-9-arginine peptide may enhance endosomal release of the ONs [30].

3.3 DNA NPs as carriers

As we consider the range of carriers being investigated for the delivery of ON therapeutics, the ON itself is emerging as a viable option with uniquely suitable properties. Self-assembled nanostructures derived from nucleic acids (DNA and RNA) are increasingly being employed as agents for delivery of functional ONs and show significant promise for overcoming the current barriers to ON delivery [88-90]. In comparison to other methods, DNA nanotechnology is a highly efficient and controllable strategy for creating structures of defined shape and size through rational design and construction [91]. These DNA nanostructures with different sizes, shapes, and geometries have characteristic uniform size, precise addressability, excellent water solubility, and biocompatibility. Hence, they offer new opportunities for construction of nanostructures for biomedical applications.

DNA and RNA nanostructures are well suited for targeted drug delivery applications and offer the advantage of controlling the valence and geometry of attached targeting ligands. Nucleic acids based nanostructures can be functionalized or conjugated to a wide range of targeting ligands including small molecules, aptamers, peptides and antibodies. Several recent reports have described utility of 3-dimensional DNA and RNA caged structures for targeted delivery. Li *et al* reported a tetrahedral nanostructure appended with multivalent CpG ONs were effective in targeting TLR9 and producing an immunostimulatory response [92]. Guo and co-workers, working with modified multivalent pRNA nanostructures demonstrated targeted delivery of siRNA through folate receptor targeting [93, 94]. A recent report by Charoenphol and Bermudez described the successful use of DNA tetrahedron decorated with AS1411, an aptamer targeting the nucleolin receptor, and showed enhanced inhibition of tumor cell growth [95]. The Anderson group also utilized a DNA tetrahedron for siRNA delivery in an *in vivo* mouse model using tumor targeting folic acid [91]. In this study, DNA NPs allow precise control of the number and arrangement of these ligands, and interestingly the NPs carrying 3 folate ligands but at different positions had the same rate of cellular uptake, but produced diverse *RNAi* activity, leading to a rationale that their arrangement might affect the intracellular trafficking of the NPs.[91] The examples described here show the increasing use of nucleic acid based nanostructures for delivery of functional nanostructures.

3.4 Inorganic NPs as carriers

Inorganic NPs such as gold NPs have been used as nanocarriers for ON delivery. The so-called “spherical nucleic acids (SNAs)” are polyanionic structures comprised of densely packed and highly oriented ONs that are attached to the surface of gold NPs via metal-thiolative bonds. These NPs can effectively enter more than 50 different cell types without the aid of transfection agents and are able to produce antisense and *RNAi* activity [96]. In addition, topical application of SNAs carrying EGFR siRNA for 3 wks to mouse skin abolished EGFR expression and reduced epidermal thickness by 40% [96]. Mechanism studies have been followed to elucidate endocytosis pathways of SNAs [97, 98]. The latter study took advantage of great resolution of gold particles under transmission electron

microscopy and molecular tools that can manipulate endocytotic process. It demonstrated that the SNAs can bind strongly to class A scavenger receptors and undergo rapid cellular uptake via a lipid-raft-dependent, caveolae-mediated pathway. Evidence also showed that the SNAs enter early endosomes. However, it's still unclear on how they traffic after early endosomes and more importantly how they exit from the endosomal compartment. The original SNAs lack cellular selectivity, and thus a HER2 antibody was displayed on the surface of SNAs by conjugating the antibody with a complementary DNA sequence and binding to the SNAs. The resultant NPs produced cell-selective delivery and enhanced RNAi activity in HER2 positive cells [99].

Unique optical properties of some inorganic NPs via strong surface plasmon resonance absorption at visible and near-infrared wavelengths can produce photothermal effects to trigger endosomal release as well as release of ONs from the carriers [100]. In a study, hollow gold nanospheres with a size of 40nm were used for “photothermal transfection” of siRNAs toward cancer cells [101]. Both siRNA and folate ligands were linked to gold NPs via metal-thiol dative bonds. Targeted NPs exhibited significantly higher tumor uptake in tumor xenografts after I.V. administration. Cytoplasmic delivery of siRNA was triggered by light irradiation through photothermal effect, and thus *RNAi* activity was achieved only in tumors irradiated with light, but not in nonirradiated tumors, liver, spleen, kidney, and lung in the same mice, in spite of significant uptake of the siRNA NPs in these tissues. Thus, “photothermal transfection” of siRNAs via gold NPs may have advantage of low side effect [101].

4. Conclusion

Therapeutic ONs are delivered to their pharmacological targets in diseased tissues using free ONs, ON conjugates, or NPs. All three forms have achieved effective delivery to the liver and have led to FDA approval of the antisense drug Mipomersen [9] and several promising late-phase clinical trials. Thus, the greater challenge facing ON therapy is to deliver ONs to extrahepatic tissues including tumors, fibrotic tissues, heart, and muscle. Due to the lack of the favorable physiology and endogenous ON uptake ability, it is difficult to achieve effective intracellular ON concentration in those tissues by simply adapting liver delivery systems. Thus, the delivery systems for extrahepatic targets have to be further optimized and they must have three elements. First, they must have optimum size for long circulation and effective tissue penetration. Secondly, they must have cell targeting ability which enhances tissue accumulation and, more importantly, enables cell selective delivery. Thirdly, endosomal release functionality should be incorporated in the delivery systems.

Direct conjugates such as GalNAc-siRNA conjugates have achieved great success in liver delivery of ONs. Compared to larger NPs and carrier-associated conjugates, molecular conjugates are easy to prepare and may not have problems such as immunogenicity and biocompatibility that often hinder development of macromolecular drugs. Targeted ON conjugates can be easily changed to aim to extrahepatic tissues using other targeting ligands. However, single application of ligand-ON conjugates may not achieve functional activity in extrahepatic tissues in a similar level that was demonstrated in the liver. First, GalNAc-siRNA conjugates have similar size to free siRNA, indicating possible fast renal filtration.

Circulation half-life of GalNAc-siRNA conjugates has not been reported; however, about half of the dose was accumulated in the liver [102], which is likely due to well-perfused liver and GalNAc targeting. In a less perfused organ, similar tissue accumulation of ONs is not expected; especially in that situation the liver is a non-target organ and may unfavorably trap ligand-ON conjugates. When low intracellular level is expected for extrahepatic application of ON conjugates, incorporation of endosomal release functionality becomes necessary. Indeed, fusion peptides of targeting domain and endosomal disruption domain have been reported [57]. Another strategy could be co-administration of endosomal reagents with ligand-ON conjugates, such as DPC polymers [75] and small-molecular ON action enhancers [83].

Carrier-associated conjugates provide several advantages over molecular conjugates and conventional NPs. Firstly, they are within the intermediate sizes (10-50nm) that are favorable to long plasma circulation and effective tissue penetration. The examples include polymer-ON conjugates (~10nm) [70], HSA-ON conjugates (12-13nm) [31, 80], DNA NPs (20nm) [103], and gold NPs (~40nm) [100]. Secondly, by using carrier molecules, multiple ligands can be displayed on the surface of the larger conjugates and thus superior targeting can be achieved. For example, HSA-ON conjugates that displayed 10 RGD ligands on the surface achieved significantly greater functional delivery of the model ONs than a direct RGD-ON conjugates only carrying a bivalent RGD peptide [26, 80]. Further, by precise positioning of targeting ligands using DNA NPs, favorable intracellular trafficking and superior functional activity can be achieved [91]. Thirdly, endosomal release functionality, including DPC polymer and photothermal materials, can be incorporated in the delivery systems for a precise co-delivery. Taken together, carrier-associated conjugates with intermediate size, cell targeting ability, and endosomal release functionality are superior systems to deliver ONs to extrahepatic targets.

Advance in ON-based therapeutics will continue to benefit from new delivery technologies and new materials. On the other hand, elucidating the underlying mechanisms leading to successful ON delivery will motivate rational design of new generation of delivery systems. Both will continue to advance therapeutic ONs to achieve the full therapeutic potentials.

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Abbreviations

ASO	antisense oligonucleotide
CDM	carboxylated dimethyl maleic acid
CPP	cell penetrating peptide
DPC	dynamic polyconjugate
ED₅₀	median effective dose
GalNAc	<i>N</i> -acetylgalactosamine
LNP	lipid nanoparticle

NP	nanoparticle
ON	oligonucleotide
PBAVE	poly(butyl amino vinyl ether)
PS	phosphorothioate
PSMA	prostate-specific membrane antigen
RGD	Arg-Gly-Asp
siRNA	small interfering RNA
SSO	splice-switching oligonucleotide
TLR9	toll-like receptor 9

Figure 1.

Biological barriers to systemic delivery of ON conjugates. After systemic administration, ON conjugates must avoid rapid degradation by nucleases in the bloodstream and fast clearance via the reticuloendothelial system and renal filtration. Then they need to cross the vascular endothelial barrier and diffuse through the extracellular matrix to approach the target cells in tissue. When arriving at the target site, ON conjugates have to cross the plasma membrane and the endosomal membrane to access their gene targets in the cytoplasm and/or nucleus of cells. (Partially adapted from Servier Medical Art, www.servier.com, with permission)

Figure 2. GalNAc-siRNA conjugates. Structure of the triantennary GalNAc-siRNA conjugate [34].

Figure 3.

DPCs. In the original DPC, siRNA is linked to DPC polymer by disulfide bond (left) [70]. In the new generation of DPCs, DPC polymer and targeted siRNA are co-injected to achieve liver delivery (right) [75].

Figure 4.

Targeted HSA-ON conjugates. A morpholino ON was conjugated to a RGD peptide, and then, multiple RGD-oligo conjugates were linked to a single molecule of HSA via a reductively responsive linkage and this led to 13nm-sized NPs (left). The targeted NPs showed receptor-specific delivery to cancer cell (right upper) and excellent penetration into 3-D tumor spheroids (right low).