Expert Opinion

Cellular delivery of siRNA and antisense oligonucleotides via receptor-mediated endocytosis

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Introduction: There is great potential for antisense and siRNA oligonucleotides to become mainstream therapeutic entities thanks to their high specificity and wide therapeutic target space compared with small molecules. Despite this potential, the pharmacological targets within the cells are less accessible to oligonucleotides that are hydrophilic and often charged. Oligonucleotides access their intracellular targets mainly by means of endocytosis, but only a fraction of them reach their targets, as delivery requires functional synergy of cellular uptake and intracellular trafficking.

Areas covered: This review provides an update on the progress of receptor-targeted delivery of oligonucleotides over the last 15 years and summarizes various targeting moieties for oligonucleotide delivery and coupling strategies. To inspire new strategies that can lead to oligonucleotides in the clinic, this review highlights how oligonucleotides successfully reach their intracellular targets by means of receptor-mediated endocytosis.

Expert opinion: Understanding the mechanisms of oligonucleotide internalization has led to greater cellular uptake and superior endosomal release through the rational design of receptor-targeted delivery systems. Further improvements will again depend on a better understanding of the intracellular trafficking of oligonucleotides.

Keywords: antisense, cellular delivery, endocytosis, oligonucleotides, siRNA, targeted delivery, trafficking

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1. Introduction

There is mounting interest in developing antisense and small interfering RNA (siRNA) oligonucleotides in therapeutic entities. Therapeutic oligonucleotides, recognizing their targets based on gene sequences, enjoy higher specificity than small molecules, which interact with their targets based on molecular conformation. Theoretically, therapeutic oligonucleotides can regulate the expression of any gene, and therefore 3000 – 10,000 disease-related genes make up the therapeutic target space for oligonucleotides, whereas that for small molecules has been estimated at 600 – 1500 genes [1]. Despite the enormous therapeutic potential, the pharmacological targets within the cells are less accessible to oligonucleotides that are hydrophilic and often charged macromolecules. Therefore, the size of the 'druggable' genome for therapeutic oligonucleotides largely depends on how effectively the oligonucleotides are delivered to their intracellular targets in disease sites.

Therapeutic oligonucleotides encounter their pharmacological targets within mammalian cells [2,3]. Classic antisense oligonucleotides regulate gene expression by RNaseH-mediated degradation of complementary pre-mRNA, which requires that oligonucleotides enter the nucleus of cells [3]. In the cytoplasm, antisense

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Article highlights.

- Cellular delivery is the key for clinical success of oligonucleotides.
- Cellular delivery requires functional synergy of cellular uptake and intracellular trafficking.
- Multifunctional targeting systems are a trend for oligonucleotide delivery.
- Targeting a specific trafficking pathway represents another promising strategy.

This box summarizes key points contained in the article.

oligonucleotides can function by translation inhibition and ribozyme-mediated cleavage [3]. Splice-switching oligonucleotides (SSO), chemically modified antisense oligonucleotides, alter alternative splicing of pre-mRNA in the nucleus and thereby can regulate gene expression for therapeutic purposes [4]. Generally, siRNAs are loaded onto the RNA-induced silencing complex (RISC) and then bind target mRNAs in the cytoplasm, triggering their degradation via Ago2-mediated cleavage. Initial evidence also indicates that siRNAs may trigger transcriptional gene silencing when they enter the nucleus and bind to promoter regions [2]. Regardless of the mechanisms, therapeutic oligonucleotides cannot access their pharmacological targets until they are present in the cytoplasm or nucleus in the cells.

Therapeutic oligonucleotides must cross numerous biological barriers to reach their intracellular targets following *in vivo* administration [5,6], and the ability to overcome these barriers determines their drug-like properties. After systemic administration, oligonucleotides 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 in order to approach the diseased cells in tissue. Finally, oligonucleotides have to cross the plasma membrane and the endosomal membrane to access their gene targets in the cytoplasm and/or nucleus of cells. Chemical modification of oligonucleotides has improved their drug-like properties such as nuclease stability and *in vivo* half-life [5]. However, precise delivery to the cells of interest and intracellular distribution remain key hurdles for the widespread use of therapeutic oligonucleotides [5,6]. Receptor-targeted delivery systems (**Figure 1**) allow not only preferential delivery of therapeutics to particular tissues and cell types of interest, but also favor distribution of the oligonucleotides to appropriate sites of action within cells. This review provides an update on the progress of receptor-targeted delivery of therapeutic oligonucleotides and highlights how the oligonucleotides reach their intracellular targets by means of receptor-mediated endocytosis. The objective of this review is to elucidate these cellular mechanisms and motivate more rational strategies leading to greater clinical successes of therapeutic oligonucleotides.

2. Receptor-mediated endocytosis

Like other biological macromolecules that are large, polar and sometimes charged, oligonucleotides reach their pharmacological targets from extracellular space by some form of endocytosis, which is subdivided into five major classes (**Figure 2**) [7]: i) the 'classic' clathrin-coated pit pathway (also known as clathrin-mediated endocytosis [CME]); ii) the caveolar pathway; iii) multiple noncaveolar and clathrin-independent pathways (CLIC pathways); iv) phagocytosis, which takes place mainly in 'professional phagocytes' such as macrophages and granulocytes; and v) macropinocytosis, in which the macromolecules are simply engulfed along with the ambient medium.

The first three classes often involve a cell surface receptor and are collectively termed receptor-mediated endocytosis. It is these pathways that are mainly utilized in cellular delivery of macromolecular drugs such as oligonucleotides. In general, receptor-mediated endocytosis of oligonucleotides includes three major steps:

- Receptor binding and internalization facilitate the initial entry of oligonucleotides into cells. Ligand–receptor recognition determines which target cells and tissues oligonucleotides are delivered to. After the ligand binds to the receptor, the ligand–receptor complex enters vesicles that bud from the cytosolic face of the plasma membrane and then pinch off, often with the assistance of the dynamin GTPase [8]. The vesicles are formed under assistance of clathrin or caveolin, which then selectively sort cargos to their destination. The endocytosis pathways are classified based on the proteins involved in the internalization step [7].
- Initial uptake is followed by sequential intracellular trafficking into a variety of low pH endomembrane compartments such as early endosomes and lysosomes. After internalization, many vesicles first traffic to early endosomes. In some instances, the receptors, which may be coupled with the cargos, are sorted into recycling endosomes and then head back to the cell surface, whereas in polarized cells they are transported across cells in a process known as 'transcytosis'. In other cases, the receptors enter late endosomes and further traffic to lysosomes, where they can be degraded. Vesicular fusion events are controlled by SNARE and SM proteins [9], whereas the complex flow of endomembrane traffic is guided by the Rab GTPases, which are localized in distinct membrane vesicles [10]. For example, transferrin receptor and epidermal growth factor receptors (EGFRs) converge in early endosomes after internalization; however, Rab11-containing vesicles deliver transferrin receptor to recycling endosomes and eventually back to the plasma membrane, whereas Rab7 guides the sorting of EGFRs to the lysosomes, where they are degraded.

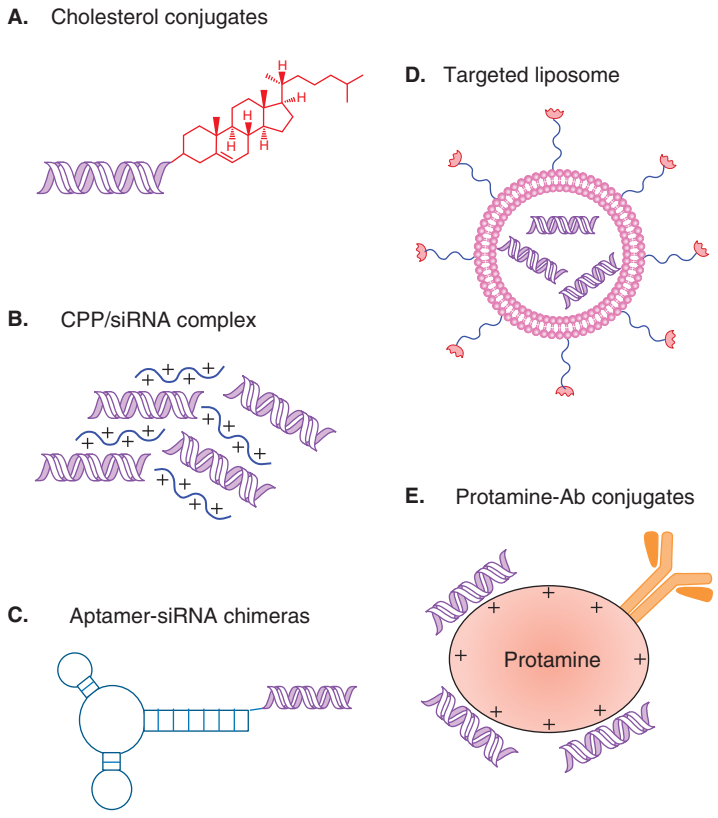

Figure 1. Delivery systems of therapeutic oligonucleotides. A variety of approaches have been used for oligonucleotide delivery, including: the direct conjugation of the oligonucleotides to targeting ligands, such as cholesterol (**A**) and aptamers (**C**); the association of the negatively charged oligonucleotides with a positively charged peptide (**B**) or protein linked to an antibody (**E**); or the encapsulation of the oligonucleotides into lipid or polymer nanoparticles (**D**).

- Ultimately, the oligonucleotides must exit from the endosomal vesicles to reach the site of action in the cytoplasm or nucleus. Endosomal release is a mandated step for delivery of oligonucleotide by means of receptor-mediated endocytosis [11]. Its importance has been highlighted by various examples in which the oligonucleotides are trapped in endosomal vesicles and treatment with endosomal disrupting agents improves the functional delivery of oligonucleotides without enhancing the overall cellular uptake [12,13]. After endosomal release, nuclear entry may not be the rate-limiting step for monomeric oligonucleotides in light of the observation that oligonucleotides rapidly accumulate in the nucleus within minutes of being microinjected into the cytoplasm of mammalian cells [14]. When oligonucleotides are bound to a nanocarrier, nuclear entry may become rate-limiting owing to the significant increase in size. In a typical delivery process, only some of the oligonucleotides reach their targets through the productive trafficking pathways; the others are sorted to non-productive processes, for example, degradation in the lysosomes and efflux from the cells. The distribution of the oligonucleotides between the productive and non-productive processes essentially determines their effectiveness. At present, therapeutic oligonucleotides are chemically modified to improve their nuclease stability, and they are stable in cellular environments. Therefore, export of oligonucleotides may represent an important cellular clearance mechanism. Cellular efflux of oligonucleotides has been observed in cell culture [15] and rat livers [16]; however, the exact mechanism is still unknown.

2.1 Research tools

Great efforts have been made to define the endocytosis pathways in cellular delivery of therapeutic oligonucleotides. Most of these studies involved two methods: colocalization of oligonucleotides to endocytosis markers; and inhibition of uptake and/or functional responses of oligonucleotides with pharmacological and genetic inhibitors of endocytosis.

In the colocalization experiments, the cells are treated with oligonucleotides, which are often labeled with fluorescent tag, and their possible overlap with specific endocytosis markers indicates the distribution of the oligonucleotides in the corresponding vesicle compartments. The endocytosis markers can be: ligands with known endocytosis pathways, such as

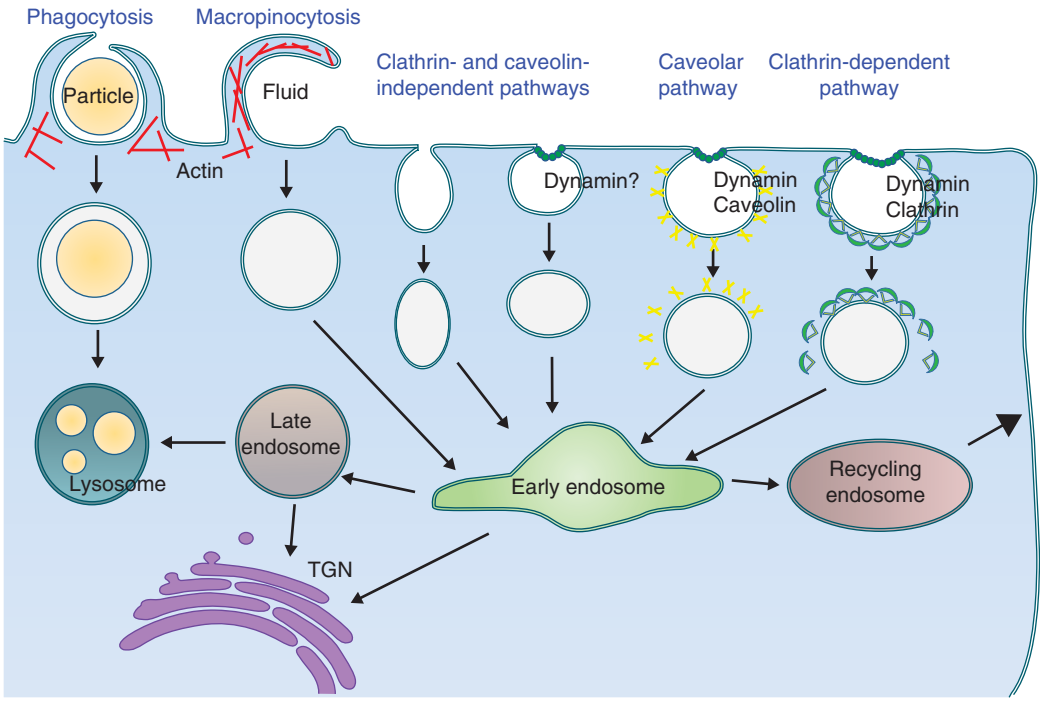

Figure 2. Endocytosis pathways. There are multiple pathways for cellular entry of macromolecules including oligonucleotides [110]. In all cases the initial step of endocytosis involves initial binding to the plasma membrane and engulfment of cargo into intracellular vesicles. The second step often involves sorting of the cargo through endosomes, which is followed by the final stage during which the cargo is delivered to its final destination or recycled to extracellular milieu.
TGN: *Trans*-Golgi network.

transferrin (clathrin-mediated endocytosis marker) and cholera toxin B (caveolar pathway marker); endogenous vesicle proteins, which are stained with their antibodies after the oligonucleotide treatment; and vesicle proteins fused with fluorescent proteins such as GFP, which are expressed from plasmids after being transfected into the cells before the treatment. The studies of colocalization should be performed with caution because artefacts have been associated with these methods. For example, the fixation step in immunostaining may cause relocation of the test molecules; this has generated artefacts in the study of endocytosis of cell-penetrating peptides (CPPs) [17,18]. On the other hand, the method using markers such as transferrin may not give specific localization of the oligonucleotides because the markers traffic through multiple endosomal compartments and may even use different trafficking pathways in different cell types.

Besides these technological problems, microscopic observations, along with cellular uptake study, do not distinguish the oligonucleotides finally reaching the gene targets from those that are sorted to non-productive compartments. This limits the strength of this method in resolving the oligonucleotides' trafficking patterns. Utilizing chemical and genetic inhibitors of endocytosis, coupled with functional assays, provides a useful complement to the colocalization experiments. Previous studies of oligonucleotide uptake have used several drugs extensively as endocytotic pathway blockers. However, such inhibitors often affect multiple uptake and trafficking pathways, and cause toxicity to many cell types. Genetic inhibitors of endocytotic pathways, such as dominant-negative Rab GTPases that selectively interfere with trafficking patterns, can provide extra mechanistic information. Moreover, Rabs knockout mice can provide valuable models to examine intracellular trafficking of oligonucleotides *in vivo*.

3. Endocytosis of free oligonucleotides

3.1 Antisense oligonucleotides

Antisense oligonucleotides can enter cells to some extent without delivery agents, and this ability has contributed to early clinical trials of these agents, administered as free oligonucleotides [3]. A great deal of effort has been made to resolve the unusual internalization mechanism for these charged macromolecules, and most of the studies have focused on phosphorothioate (PS) oligonucleotides owing to their wide clinical application. The various studies agree that the oligonucleotides enter cells by receptor-mediated endocytosis because: uptake of PS oligonucleotides is a saturable process [19,20]; and PS oligonucleotides have been found in intracellular vesicles [20]. However, the receptors that are responsible for oligonucleotides' cellular entry have not been identified. After

systemic administration PS oligonucleotides distribute mainly to liver and kidney, especially in Kupffer cells, hepatic endothelial cells and renal tubular cells [21]. Early studies using pharmacological inhibitors suggested that some scavenger receptors, which are highly expressed in these cells, are the receptors that mediate the preferential uptake of oligonucleotides [22-24]. However, knocking out class A scavenger receptor, which is highly expressed in Kupffer cells and hepatic endothelial cells, did not change uptake of PS oligonucleotides into these cells or the tissue distribution in mice [25], casting doubts on the scavenger receptor-mediated uptake mechanism. Other receptors have also been proposed as oligonucleotide receptors, such as Mac-1, a heparin-binding integrin that is mainly expressed in leukocytes [26]. However, none of them can explain the accumulation of PS oligonucleotides in the liver and the kidney after systemic administration. Cellular entry of PS oligonucleotides does not depend on actin and dynamin in cell culture [27], and they are sorted into vesicles other than clathrin-coated vesicles [19]. Evidence supports one of noncaveolar and clathrin-independent pathways for cellular uptake of PS oligonucleotides; however, the precise pathway is yet to be identified.

Interestingly, a recent study showed that locked nucleic acid (LNA) PS gap-mers can produce sequence-specific suppression of multiple targets at concentrations < 1 μM without using any delivery reagent [28]. Nuclear entry of the fluorescently labeled LNAs was not observed, but the free oligonucleotides were shown to traffic into P-bodies in the cytoplasm [28], where multiple proteins involving mRNA degradation are localized [29]. Further mechanistic studies are needed to determine whether the cytosolic localization of LNAs is associated with a new mechanism for antisense oligonucleotides in the cytoplasm.

3.2 siRNA oligonucleotides

Cellular uptake of unmodified siRNAs is less efficient than that of PS oligonucleotides in melanoma cell line A375 (unpublished observation). This *in vitro* observation seems consistent with the fact that most siRNA clinical trials involve delivery agents such as liposomes [1]. However, an exceptional case is a Phase II clinical study in which naked siRNAs targeting p53 have been used to treat acute kidney injury [1]. More examples in animal models have supported that functional activity of free siRNAs in the renal proximal tubules is a general phenomenon [30-32]. Two-photon microscopy in live rats demonstrated that the fluorescently labeled siRNAs mainly accumulate in the renal proximal tubules after quick cellular entry from the apical membrane [30], supporting that there is a unique uptake mechanism for siRNAs to enter renal proximal tubular cells. Receptor-mediated endocytosis is generally accepted as the underlying mechanism, however, it is supported only by the observation that the internalized siRNAs stay in the vesicles in the apical domain of renal proximal tubular cells [30]. Besides uptake in the kidney, intranasal delivery of naked siRNAs to lung epithelium caused effective target gene suppression and prevented respiratory virus infection [33], indicating that free siRNAs can also be taken up by the cells in lung epithelia. A transmembrane protein, SID-1, has been identified as a receptor mediating siRNA cellular entry in *Caenorhabditis elegans* [34]. Transfection of the mammalian SID-1 homologue into PANC1 cells improved siRNA uptake and RNAi activity [35], whereas knocking it down or blocking it with an antibody caused decreased uptake of cholesterol-conjugated siRNAs in HepG2 cells [36]. Although SID-1 has been identified as a siRNA transporter, more studies are needed to examine whether it plays a role in RNAi activity *in vivo*, especially in the kidney and the lung, after free siRNA administration. In addition, mechanistic studies are needed to elucidate the mechanism for SID-1-mediated cellular entry.

The poor cellular uptake of naked siRNAs does not generate any functional activity in cell culture; however, moderate RNAi activity in cultured cells can be produced by naked siRNAs under stimulation of single-stranded PS oligonucleotides [37]. This unusual functional delivery of naked siRNAs depends on the cell type [38]. In the positive cell line ECV-304, the PS-stimulated siRNAs undergo a caveolar pathway and traffic to the rough endoplasmic reticulum (ER), whereas the siRNAs fail to reach it in the negative cell line SKRC-35, although similar total cellular uptake can be achieved in this cell line [38]. Most siRNAs are still trapped in vesicles after effective cellular entry by this delivery mode [13], and the RNAi activity caused by naked siRNAs under PS oligonucleotide stimulation can even be improved by improving the endosomal escape with a photochemical methodology [39] or conjugation of endosomal-releasing peptide [13]. Despite the interesting cellular mechanism in this strategy of siRNA delivery, nonspecific cellular uptake of siRNAs and the requirement of a larger amount of PS oligonucleotides can be hurdles for systemic therapy.

4. Delivery systems

4.1 CPPs as delivery vehicles

Cell-penetrating peptides are short basic amino acid-rich peptides that can not only enter cells themselves but also facilitate the transport of molecular cargos across the plasma membrane. Many types of CPP have been utilized in oligonucleotides delivery, as shown in **Table 1**. The first of them are prototypical CPPs, such as TAT and ANT, which are derived from natural transcription factors. In one of these studies, direct conjugation of both prototypical CPPs to antisense oligonucleotides led to effective suppression of P-glycoprotein, a difficult target protein, at concentrations < 1 μM [40], demonstrating initial success of this strategy. The CPPs of the next generation were engineered with extra properties through fusing with other peptides, and therefore are called chimeric CPPs. As an example, MPG consists of the fusion peptide domain of HIV-1 gp41 linked with the nuclear localization

Table 1. Cell-penetrating peptides as carriers.

Targeting moiety	Delivery system	Cargo	Purpose of study	Ref.
Ant CPP	Chemical conjugation	PNA	*In vitro* delivery to cultured neurons	[94]
Transportan and pAntp	Chemical conjugation	PNA	Intrathecal delivery	[95]
Tat and Ant CPPs	Chemical conjugation	PS ASO	*In vitro* functional delivery	[40]
Tat and Ant CPPs	Chemical conjugation	PS SSO	*In vitro* functional delivery	[96]
Synthetic $(R\text{-}Ahx\text{-}R)_4$	Chemical conjugation	PMO SSO	*In vitro* functional delivery	[42]
Synthetic $(R\text{-}Ahx\text{-}R)_4$	Chemical conjugation	PMO SSO	Systemic delivery to DMD mice model	[43]
Synthetic $(R\text{-}Ahx\text{-}R)_4$-muscle specific peptide	Chemical conjugation	PMO SSO	Systemic delivery to DMD mice model	[44]
R6-Penetratin	Chemical conjugation	PNA	*In vitro* functional delivery	[97]
Tat CPP	Chemical conjugation	siRNA	*In vitro* delivery and intracellular trafficking	[54]
Penetratin1	Chemical conjugation	siRNA	*In vitro* delivery to cultured neurons	[98]
Stearylated Transportan 10	Peptide/SSO complex	SSO	*In vitro* functional delivery	[99]
Penetratin EB1, an endosomolytic CPP	Peptide/siRNA complex	siRNA	*In vitro* functional delivery	[92]
MPG	Peptide/ASO complex	ASO	*In vitro* functional delivery	[100]
MPG variant	Peptide/siRNA complex	siRNA	*In vitro* functional delivery	[41]
MPG-8 and cholesterol-MPG-8	Peptide/siRNA complex	siRNA	*In vivo* delivery to tumors	[45]
HIV gp41-derived peptide	Peptide-PEI/siRNA conjugate	siRNA	*In vitro* functional delivery via improving endosomal release	[12]
KALA peptide	Peptide/siRNA-PEG complex	siRNA	*In vitro* functional delivery	[47]
Low-molecular-mass protamine	Peptide/siRNA complex	siRNA	Systemic delivery to tumors	[101]
Tat CPP	CPP-dsRNA binding domain/siRNA complex	siRNA	Systemic delivery to tumors	[46]

ASO: Antisense oligonucleotides; CPPs: Cell-penetrating peptides; DMD: Duchenne muscular dystrophy; dsRNA: Double-stranded RNA; PS: Phosphorothioate; PMO: Phosphorodiamidate morpholino; PNA: Peptide nucleic acids; siRNA: Small interfering RNA; SSO: Splice-switching oligonucleotides.

signal (NLS) of SV40 large T-antigen [41]. This amphipathic and cationic peptide not only forms stable complexes with anionic oligonucleotides but also enhances the endosomal release of the oligonucleotides [41]. Interestingly, a mutation that affects the nuclear localization of MPG prevented nuclear delivery of plasmid DNA, but promoted strong gene suppression caused by siRNAs, indicating the importance of subcellular distribution of oligonucleotides to their targets for their actions [41]. Other advanced CPPs have been designed from structure–activity studies (SAR) based on the older CPPs. For example, synthetic $(R\text{-}Ahx\text{-}R)_4$ was designed in extensive SAR studies in which the CPP-SSO conjugates were tested for reporter gene induction resulting from splicing correction [42]. This type of synthetic CPP-SSO conjugate has also been shown to restore dystrophin function in a Duchenne muscular dystrophy (DMD) mouse model [43]. Moreover, linking a muscle-specific peptide to the $(R\text{-}Ahx\text{-}R)_4$ peptide further improved the *in vivo* behavior of the CPP-SSO conjugate, even at lower doses, producing functional correction of dystrophin and thereafter improvement of the dystrophic phenotype [44].

Although early efforts involved covalent conjugation of CPPs with oligonucleotides, recent studies have tended to utilize non-covalent, mainly electrostatic interactions between positively charged CPPs and negatively charged oligonucleotides to form stable complexes. This strategy has been further optimized by conjugating functional groups to CPPs [12,45,46] or oligonucleotides [47]. In one of these studies, MPG-8, an improved variant of the amphipathic CPPs, formed nanoparticles with siRNAs and generated efficient delivery of siRNAs into cultured cells and tumors on intratumoral injection [45]. Linking cholesterol to the CPPs further improved *in vivo* disposition of siRNA complexes, leading to reversal of tumor growth in mice after systemic administration [45]. In another example, Tat CPP was fused to a double-stranded RNA (dsRNA) binding domain, which binds siRNAs tightly and masks their negative charges in order to allow CPPs to display its membrane penetration ability [46]. This new construct led to functional delivery of siRNAs to tumors *in vivo* [46].

Despite rapid progress in the application of CPPs to oligonucleotide delivery, controversies still exist concerning, first, whether CPP internalization involves endocytosis and, if so, what the endocytic pathway is. Initial studies on prototypical CPPs such as TAT and ANT suggested a mechanism of receptor- and energy-independent translocation across the plasma membrane [48]; however, this observation was then considered as an artefact owing to the high membrane binding of CPPs [17,18]. By carefully removing the membrane-bound CPPs, the experimental data supported an active process of cellular entry of Tat CPP [49,50]. However, using similar tools of pharmacological inhibitors and endocytosis markers, various endocytosis pathways have been proposed for Tat CPP internalization, such as clathrin-mediated endocytosis [49], macropinocytosis [50,51] and caveolar endocytosis [52]. Knocking out dynamin [50], clathrin or caveolin [53] in the cultured cells, causing the molecular inhibition of

corresponding endocytosis pathways, did not prevent cellular entry of the Tat CPP, creating more uncertainties about the exact molecular mechanism of CPPs' endocytosis. The internalization mechanism of the CPPs coupled with oligonucleotides is more complicated and may be different from that of the CPPs themselves. In a study, whereas Tat CPP was localized in the nucleus in the cells, CPP-siRNA conjugates were localized to perinuclear sites, where siRNAs interact with RISC to cause RNAi [54]. It was suggested that siRNAs' interaction with RISC determines localization of the conjugate rather than the CPP peptide [54].

4.2 Small molecules as carriers

Conjugation of oligonucleotides such as siRNAs with a lipid tail has been shown to improve their cellular uptake, and thereby their ability to regulate gene expression [55]. This strategy, more specifically conjugation of apoB siRNA with cholesterol, led to the first example of RNAi activity *in vivo* after systemic administration [56]. A recent study indicated that cholesterol-siRNA conjugates preassembled with high-density lipoprotein (HDL) or low-density lipoprotein (LDL) further improved liver uptake and RNAi activity compared with the 'free' cholesterol-siRNA conjugate or that complexed with albumin. Knocking out the receptors for uptake of HDL or LDL reversed the improvement of tissue uptake of the conjugates complexed with the lipoproteins [36]. These data suggested that cholesterol-siRNA generates *in vivo* RNAi by forming lipoprotein particles that are internalized by receptor-mediated endocytosis [36]. Along the same lines, α-tocopherol, a vitamin E isoform, was conjugated to apoB siRNA in order to utilize lipoprotein particle-mediated hepatic uptake, and this conjugate achieved even greater RNAi activities compared with cholesterol-siRNA conjugates [57]. Nanoparticles have also been designed to mimic lipoprotein particles more closely. For example, siRNAs targeting hepatitis B virus (HBV) were encapsulated into DOTAP-cholesterol liposomes containing apolipoprotein A-I, a component of HDL [58]. These preassembled lipoprotein particles of siRNAs induced efficient and persistent antiviral effect in HBV-infected mice [58]. In addition, another lipophilic vitamin, vitamin A (retinol), has also been formulated into liposomes for siRNA delivery [59]. After systemic administration, the siRNAs targeting heat-shock protein were delivered to hepatic stellate cells by means of retinol-binding proteins, leading to the cure of liver fibrosis in rats [59].

Another widely used ligand for liver targeting is galactose. Asialoglycoprotein receptors, mainly expressed in the hepatocytes, bind and then mediate endocytosis of the galactose-terminal glycoproteins, in order to remove them from circulation [60]. Asialoglycoprotein receptor has been considered as one of the most promising membrane proteins for oligonucleotide delivery because it shows high affinity and a rapid internalization rate via the clathrin-mediated pathway [60] and it is easy to couple the ligands to oligonucleotides. Asialoglycoprotein receptor has been utilized in liver-specific oligonucleotide delivery after direct conjugation to oligonucleotides [61,62], polymer conjugation [63,64] and with galactose-containing liposomes [65]. More examples of oligonucleotide delivery systems using small molecules as carriers are given in **Table 2**.

4.3 Peptides and proteins as targeting moieties

As shown in **Table 3**, many peptides and proteins have been utilized to deliver oligonucleotides by targeting the receptors expressed in tumors, for example, integrins [27,66-68], bombesin receptors [69,70], insulin-like growth factor 1 receptors [71,72], transferrin receptors [73,74] and EGFR [75]. Although all these approaches have demonstrated functional delivery of oligonucleotides in cell culture, animal models, or even clinical trials, only a few of them aimed to elucidate the mechanisms of cellular delivery. Endocytosis of SSOs conjugated to Arg-Gly-Asp (RGD) [66] or bombesin [70] has been studied in detail. Both of the peptide-SSO conjugates follow the same pathways as their targeting ligands, that is, RGD-SSO utilizes the caveolar pathway [66], whereas bombesin-SSO follows the clathrin pathway [70]. Both of them traffic to deep endomembrane compartments such as late endosomes for bombesin-SSO and the *trans*-Golgi network (TGN) for both of them; however, how they are released into the cytosol is yet to be determined [66,70]. Besides direct conjugation, the SSOs and RGD peptides have been linked to albumin [67]. Interestingly, the resultant nanoparticle undergoes similar integrin-mediated endocytosis as the molecular conjugate, but demonstrates superior efficacy in a cellular model of aberrant splicing [67].

Besides studies to understand cellular mechanisms, the results from a clinical study have demonstrated that targeting transferrin receptor can deliver siRNAs to solid tumors and cause functional RNAi in humans [73]. In this study, siRNAs were formulated into cyclodextrin-based nanoparticles that were linked with transferrin. After systemic administration to humans, the targeted nanoparticles delivered siRNAs to the solid tumor, resulting in reduction in mRNA and protein levels of the target gene [73]. Another remarkable study has shown that coupling with a targeting peptide can overcome the blood–brain barrier and deliver siRNAs to the central nervous system [76]. In this study, a peptide derived from rabies virus glycoprotein (RVG) was fused with positively charged Oligo-9-arginine peptide, and was then complexed with siRNAs targeting Japanese encephalitis virus [76]. After systemic administration, the targeting peptide delivered siRNAs to neuronal cells, causing RNAi in the brain and ultimate strong protection against viral encephalitis in mice [76].

4.4 Antibody as carriers

Antibodies have been the primary choice as targeting moieties in targeted drug delivery, especially in tumor chemotherapy; this approach has been extended to oligonucleotide delivery and had similar successes, as shown in **Table 4**. In one of these

Table 2. Small molecules as carriers.

Targeting moiety	Delivery system	Cargo	Purpose of study	Ref.
Lipophilic phosphonium cation	Chemical conjugation	PNA	*In vitro* delivery to mitochondria	[102]
Derivatives of cholesterol and bile acids	Chemical conjugation	siRNA	*In vitro* functional delivery	[55]
Cholesterol	Chemical conjugation	siRNA	Systemic delivery to liver and jejunum	[56]
Cholesterol	Chemical conjugation	ASO for miRNAs	Systemic delivery to inhibit miRNAs	[103]
Cholesterol, bile acids and long-chain fatty acids	Chemical conjugation	siRNA	Systemic delivery and *in vitro* uptake mechanism	[36]
Cholesterol, apolipoprotein A-I	DOTAP-cholesterol liposomes complexed with apolipoprotein A-I	siRNA	Systemic delivery to liver to suppress HBV infection	[58]
Vitamin A	Liposome	siRNA	Systemic delivery to hepatic stellate cells	[59]
Vitamin E	Chemical conjugation	siRNA	Systemic delivery to liver	[57]
Galactose	Chemical conjugation	ASO	Systemic delivery to liver	[61]
Galactose	Chemical conjugation	PNA	Systemic delivery to liver	[62]
Galactose	Galactosylated cationic liposomes	siRNA	Systemic delivery to liver	[65]
Galactose	Ligand-polymer-siRNA conjugate	siRNA	Systemic delivery to liver	[63]
Galactose	Ligand-PEG-siRNA conjugate	siRNA	*In vitro* delivery to hepatic cells	[64]
Lactose	Ligand-PEG-siRNA conjugate	siRNA	*In vitro* functional delivery	[104]
Anisamide	Chemical conjugation	PS SSO	*In vitro* functional delivery	[105]
Anisamide	Liposome	siRNA	Systemic delivery to tumors	[106]
Dihydrotestosterone	Chemical conjugation	PNA	*In vitro* delivery to nucleus	[107]
Terpyridine	Chemical conjugation	PNA	*In vitro* uptake	[108]

ASO: Antisense oligonucleotides; DOTAP: *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methylsulfate; miRNA: MicroRNA; PEG: Polyethylene glycol; PNA: Peptide nucleic acids; PS: Phosphorothioate; siRNA: Small interfering RNA; SSO: Splice-switching oligonucleotides.

Table 3. Peptide and protein as carriers.

Targeting moiety	Delivery system	Cargo	Purpose of study	Ref.
Dimeric cyclic RGD	Chemical conjugation	PS SSO	*In vitro* functional delivery to A375 cells	[27,66]
Dimeric cyclic RGD	RGD-Albumin-SSO conjugate	PS SSO	*In vitro* functional delivery to A375 cells	[67]
RGD	PEI-PEG-RGD conjugate	siRNA	Systemic delivery to tumors	[68]
Bombesin	PEGylated liposome	siRNA	*In vitro* functional delivery	[69]
Bombesin	Chemical conjugation	PS SSO	*In vitro* functional delivery to PC3 cells	[70]
Insulin-like growth factor 1 peptide	Chemical conjugation	PNA	*In vitro* uptake	[71]
Insulin-like growth factor 1 peptide	Chemical conjugation	siRNA	*In vitro* functional delivery	[72]
Transferrin	PEGylated cyclodextrin nanoparticles	siRNA	Systemic delivery to solid tumors in clinical trial	[73]
Transferrin	OEI-HD Polymer-transferrin conjugate	siRNA	Systemic delivery to tumors	[74]
EGF	PEI-PEG-EGF conjugate	Synthetic dsRNA	Systemic delivery to tumors	[75]
LHRH	Micelles of siRNA-PEG-LHRH and PEI	siRNA	*In vitro* functional delivery	[109]
Rabies virus glycoprotein peptide	RVG-9R/siRNA complex	siRNA	Systemic delivery to brain	[76]
Bacterial toxin-derived peptide	Chemical conjugation	siRNA	*In vitro* functional delivery	[13]
KDEL motif, altering trafficking to ER	Chemical conjugation	ASO	*In vitro* functional delivery	[93]

ASO: Antisense oligonucleotides; CPPs: Cell-penetrating peptides; dsRNA: Double-stranded RNA; EGF: Epidermal growth factor; ER: Endoplasmic reticulum; LHRH: Luteinizing hormone-releasing hormone; PEG: Polyethylene glycol; PEI: Polyethyleneimine; PS: Phosphorothioate; PNA: Peptide nucleic acids; RGD: Arg-Gly-Asp; RVG: Rabies virus glycoprotein; siRNA: Small interfering RNA; SSO: Splice-switching oligonucleotides.

Table 4. Oligonucleotides as carrier.

Targeting moiety	Delivery system	Cargo	Purpose of study	Ref.
HIV-1 envelope antibody and ErbB2 antibody	Antibody-Protamine/siRNA complex	siRNA	Systemic delivery to HIV-infected cells and tumors	[77]
LFA-1 antibody	Antibody-Protamine/siRNA complex	siRNA	Systemic delivery to activated leukocytes	[78]
CD7 antibody	scFv-9R/siRNA complex	siRNA	Systemic delivery to suppress HIV infection	[79]
β_7 Integrin antibody	Immunoliposome	siRNA	Systemic delivery to leukocytes	[80]
Anti-transferrin receptor antibody	Immunoliposome	siRNA	Systemic delivery to tumors	[81]
LFA-1 antibody	Immunoliposome	siRNA	Systemic delivery to HIV-infected leukocytes	[82]

LFA-1: Lymphocyte function-associated antigen 1; scFv: Single-chain variable fragment; siRNA: Small interfering RNA.

examples, a single-chain antibody has been fused with positively charged protamine, which binds anionic siRNAs and forms a stable complex [77,78]. Antibodies targeting several membrane markers of tumors or lymphocytes have been utilized to target siRNAs to the tumors and HIV-infected lymphocytes, which led to successful outcomes of RNAi after systemic administration [77,78]. Along the same lines, anti-CD7 antibody has been fused with positively charged Oligo-9-arginine peptide, and was thereby complexed with siRNAs targeting multiple viral proteins [79]. Systemic administration of the siRNA complexes to HIV-infected mice has achieved viral suppression [79]. Another way of coupling antibodies with oligonucleotides is traditional immnoliposome strategy, in which siRNAs are encapsulated in the liposomes that are coated with targeting antibodies. This strategy also achieved promising results in suppressing tumor growth or HIV infection in animal models [80-82]. In spite of the encouraging results from preclinical studies, the internalization mechanism for oligonucleotides coupled with antibodies remains unclear.

4.5 Oligonucleotides as carriers

Nucleic acid aptamers are single-stranded oligonucleotides with tertiary structures that bind to specific target molecules. They are usually selected from pools of random-sequence oligonucleotides using a SELEX (systematic evolution of ligands by exponential enrichment)-type approach. Aptamers not only bind to cell surface receptors to cause effects by themselves, but also can enhance the endocytosis of bound cargo molecules, thereby acting as carrier molecules to deliver intracellular therapeutics, such as drugs, toxins and oligonucleotides. Examples using aptamers as carriers in oligonucleotide delivery are given in **Table 5**.

Several aptamer-based delivery strategies have targeted prostate-specific membrane antigen (PSMA), a protein that is widely expressed but only observed on the surface of some prostate cancer cells [83]. Initially, biotin-labeled PSMA-specific aptamers and siRNAs were linked via streptavidin and the conjugate caused RNAi specifically in PMSA positive cells [84]. A recent 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 [85]. The aptamer-siRNA chimeras have been optimized in aptamer size, nuclease stability and plasma half-life by, for example, adding a terminal PEG, and the resultant conjugate showed potent antitumor activity after systemic injection into mice [86]. Aptamer-siRNA chimeras have also been generated by fusing the HIV gp120-specific aptamer and a tat/rev-specific siRNA, and the chimeras target cells infected with HIV-1 and can inhibit HIV replication through aptamer-mediated receptor blocking and RNAi activity [87,88].

In another example of oligonucleotides as delivery agent, the siRNA targeting the immune suppressor gene *Stat3* was linked to an immunostimulatory CpG oligonucleotide in order to stimulate an immune response to kill tumor cells [89]. The binding of CpG-siRNA conjugate to the toll-like receptor 9 (TLR9) in immune cells not only caused various immune responses by means of endosomal TLR activation, but also triggered internalization of the siRNA conjugate, leading to silencing of the immune suppressor gene for greater antitumor immune response [89]. The CpG-siRNA conjugate was observed to colocalize with TLR9 within perinuclear endocytic vesicles, and then transiently interact with Dicer, which processes functional siRNAs [89]. However, the molecular mechanism of the endocytosis is yet to be elucidated.

5. Conclusion

Both antisense and siRNA oligonucleotides can enter cells and produce moderate pharmacological responses without delivery agents. However, this effectiveness of naked oligonucleotides is limited to the liver and the kidney, where the oligonucleotides largely accumulate after systemic administration, and is further restricted to special cell types. Resolving the underling mechanism for oligonucleotide uptake in these cell types will allow rational redesign of the oligonucleotide chemistry, leading to broader distribution and thereby wider application of therapeutic oligonucleotides. Furthermore, utilization of targeted delivery systems not only improves the uptake and thereafter the effectiveness of oligonucleotides, but also makes oligonucleotides available to more tissues and cell types.

Table 5. Antibody as carriers.

Targeting moiety	Delivery system	Cargo	Purpose of study	Ref.
Anti-PSMA aptamer	Chemical conjugation	siRNA	Systemic delivery to tumors	[85,86]
Anti-gp120 aptamer	Chemical conjugation	siRNA	*In vitro* functional delivery	[87,88]
CpG oligonucleotide	Chemical conjugation	siRNA	Systemic and local delivery to tumor-associated dendritic cells	[89]

PSMA: Prostate-specific membrane antigen; siRNA: Small interfering RNA.

CPPs were one of the first delivery vehicles utilized for oligonucleotide delivery and at least one of them is close to entering clinical study [43]. Although CPPs enhance the uptake and effectiveness of oligonucleotides, the improvement is universal to almost all the cell types, which may cause toxicity and off-target effects. Coupling CPPs with targeting ligands is a useful way to optimize CPP-based oligonucleotide delivery systems further. The advantages have been demonstrated in a study in which a muscle-specific peptide was coupled to a CPP peptide so that functional correction of dystrophin could be achieved at lower doses of the therapeutic oligonucleotides conjugated with this chimeric targeting peptide [44].

Various chemical entities, including small molecules, peptides, antibodies and aptamers, have been used as targeting ligands for oligonucleotide delivery. However, most of the delivery systems target the receptors that are expressed in tumors and the liver, which may be a limiting factor for broader application of therapeutic oligonucleotides. It is necessary to identify membrane receptors that are expressed in tissues other than tumors and the liver and that undergo efficient endocytosis. G-protein coupled receptors (GPCRs) may be a superior choice for this purpose. The GPCRs comprise the largest receptor family in the human genome with ~ 850 members [90]. Individual members of this family often display differential expression in various tissues [91]. Therefore, an oligonucleotide delivery system that binds a specific GPCR member can potentially lead to targeted delivery to a particular tissue. Bombesin peptide, a GPCR ligand, has been successfully utilized in oligonucleotide delivery [69,70], suggesting that targeting GPCRs is suitable for efficient delivery of oligonucleotides. Another way to generate more new targeting ligands is to use combinatorial library approaches, such SELEX for aptamers, phage display for antibodies, and RNA display for peptide domains. Aptamers and antibodies have been applied successfully in oligonucleotide delivery for tumor therapeutics, and it is expected that more of them will be selected for oligonucleotide-based therapeutics for diseases other than tumors.

The coupling of targeting ligands with oligonucleotides includes chemical conjugation, non-covalent coupling and encapsulation in nanoparticles. Chemical conjugation of targeting ligands and oligonucleotides produce well-defined monomeric molecules that are physically stable and enjoy relatively broader tissue distribution compared with nanoparticles [5]. Recently, simple complexes of CPPs with siRNAs have shown positive results in cell culture and animal models. In more advanced constructs, targeting peptides are fused with oligonucleotide-binding peptides, such as Oligo-9-arginine [76,79], protamine [77,78] and dsRNA binding domain [46]. These non-covalent coupling strategies avoid the sometimes complicated conjugation chemistry and minimize the possible interruption between the targeting ligands and the oligonucleotides. Furthermore, the oligonucleotides may dissociate earlier from the targeting groups in intracellular trafficking owing to the loose interaction with the targeting ligands. Whether this leads to quicker endosomal release is yet to be tested. In spite of the promise, the delivery constructs need more characterization; for example, *in vitro* and *in vivo* stability, the sizes, and electrostatic properties of the complexes. The last coupling strategy is to formulate the oligonucleotides into nanoparticles containing targeting ligands, and the resulting constructs have demonstrated numerous successes in cell culture, animal models and a clinical study [73]. In particular, this type of strategy may benefit tumor therapeutics via active targeting and passive targeting through enhanced permeability and retention (EPR) effects. Recently, cyclodextrin-based nanoparticles coated with transferrin have been shown to deliver siRNAs to solid tumors in humans and cause gene specific RNAi activity [73], which was the first siRNA clinical trial to use a targeted nanoparticle as delivery system.

6. Expert opinion

Cellular delivery of oligonucleotides requires functional synergy of cellular uptake, vesicular trafficking and endosomal release. These steps govern the magnitude and duration of oligonucleotide exposure to the targets, and ultimately determine its effectiveness. Depending on their relative efficiency in different delivery modes, each step may become rate-limiting and control the cellular response of oligonucleotides. In that case, identifying the rate-limiting step by mechanistic studies can help to design a specific strategy to overcome this barrier. By rational design and selection of the targeting ligands, substantial successes have been achieved in improving total cellular uptake of oligonucleotides. When significant amounts of oligonucleotides enter the cells by means of

endocytosis, endosomal release has become the rate-limiting step and therefore studies have recently emerged exploring endosomal release of oligonucleotides into the cytoplasm. For example, even under stimulation of PS oligonucleotides, uptake of a surprisingly large amount of naked siRNAs generates only moderate RNAi activity in cultured cells [37]. Attributing limited efficacy to intracellular sequestration of siRNAs, an endosomal release signal peptide from bacterial protein toxins has been conjugated to siRNAs [13]. Although the conjugation did not enhance the cellular uptake of siRNA it improved specific RNAi activity dramatically [13], highlighting that endosomal release becomes the rate-limiting step when significant amount of siRNAs can enter the cells under stimulation. In another example, various CPPs were tested in functional delivery of siRNAs by forming complexes with siRNAs [92]. Although all of them significantly improved the cellular uptake of siRNAs, only penetratin analogue EB1, with endosomolytic properties, generated effective RNAi activity in cell culture [92]. In the last example, a multifunctional delivery system was constructed by linking *N*-acetylgalactosamine and siRNAs to an amphipathic polymer PBAVE, which is an endosomolytic agent [63]. This so-called dynamic polyconjugate system with both cell targeting and endosomal releasing abilities was delivered to the liver and caused local RNAi activity [63], signifying a future trend of more sophisticated and multifunctional systems for oligonucleotide delivery.

Besides engineering delivery systems with endosomal escape ability, another strategy is to target specific intracellular trafficking pathways, which can lead to quicker and more efficient endosomal release. This indirect strategy for endosomal release was first demonstrated in a study in which antisense oligonucleotide had been conjugated to tetrapeptide KDEL, a motif mediating the retrieval of ER luminal proteins from the Golgi apparatus [93]. Although the peptide conjugate was internalized less effectively than the free oligonucleotides, it was fivefold more efficient at suppressing target gene expression [93]. A further localization experiment showed that only the peptide conjugates were transported to the ER, where the oligonucleotides can be quickly released to the cytoplasm [93]. Similar lessons were learned from a study on intracellular delivery of SSOs [66], in which a bivalent RGD peptide was conjugated to an SSO that corrects splicing of a firefly luciferase gene mutant. The RGD-SSO accumulated in cells twofold higher than free oligonucleotide; however, it achieved a sevenfold greater effect on luciferase induction [66]. The endocytosis pathway of the conjugate involved an initial caveolar-mediated uptake followed by trafficking to the TGN [66], whereas the free oligonucleotide undergoes an unconventional endocytosis pathway that does not depend on the functions of actin and dynamin [27], indicating that the pharmacological effect of an antisense oligonucleotide depends not only on total cell uptake but also on intracellular trafficking.

In summary, understanding the mechanisms of oligonucleotide internalization has motivated some rational strategies of targeted delivery of oligonucleotides, leading to greater cellular uptake and more efficient endosomal release, and thereafter superior efficacy of the therapeutic oligonucleotides. However, these strategies often utilize more sophisticated and multifunctional systems. This increases the optimization steps in the drug development and may cause more potential problems in clinical study, such as immunogenicity and biocompatibility of the complicated carriers. Targeting more efficient intracellular trafficking pathways may lead to simpler delivery systems and avoid the problems with the complicated systems. For this purpose, many mechanistic studies need to be carried out in order to understand the subcellular trafficking of oligonucleotides. In addition, whether the trafficking mechanisms that are discovered with cellular models also act in animals is still unknown, and studying the complex endocytosis of oligonucleotides *in vivo* persists as another big challenge. Nevertheless, the further improvement of oligonucleotide delivery in light of the endocytosis mechanisms will continue to move therapeutic oligonucleotides closer to being a clinical reality.

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Declaration of interest

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