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# The Potential of Modulating Small RNA Activity *In Vivo*

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**Abstract:** Small RNAs have shown to be ubiquitous, useful, post-transcriptional gene silencers in a diverse array of living organisms. As a result of homologous sequence interactions, these small RNAs repress gene expression. Through a process called RNA interference (RNAi), double strand RNA molecules are processed by an enzyme called Dicer, which cleaves RNA duplexes into 21-23 base pair oligomers. Depending on their end-point functions, these oligomers are named differently, the two most common being small interfering RNAs (siRNAs) and microRNAs (miRNAs). These small RNAs are the effector molecules for inducing RNAi, leading to post-transcriptional gene silencing by guiding the RNAi-induced silencing complex (RISC) to the target mRNA. By exploiting these small RNAs, it is possible to regulate the expression of genes related to human disease. The knockdown of such target genes can be achieved by transfecting cells with synthetically engineered small RNAs or small RNA expressing vectors. Within recent years, studies have also shown the important role of miRNAs in different diseases. By using several chemically engineered anti-miRNA oligonucleotides, disease related miRNAs can be specifically and effectively silenced. Since RNAi has developed into an everyday method for *in vitro* knockdown of any target gene of interest, the next step is to further explore its potential *in vivo* and the unique opportunities it holds for the development of novel therapeutic strategies. This review explores the various applications of small RNA technology in *in vivo* studies, and its potential for silencing genes associated with various human diseases. We describe the latest development in small RNA technology for both gene knockdown, and the inhibition of translational silencing in animal studies. A variety of small RNA formulations and modifications will be reviewed for their improvement on stability and half-life, their safety and off-target effects, and their efficiency and specificity of gene silencing.

**Key Words:** miRNA, RNAi, gene knockdown, *in vivo* therapy.

## INTRODUCTION

Many pathological conditions depend on abnormal gene expression levels. This could include the aberrant expression of endogenous or mutant genes, or the expression of foreign genes in an infected organism. With the introduction of nucleic acid-based inhibitors or antisense agents, a novel view on how to fight disease was established. In addition to strategies based on the inhibition of target proteins, the possibility of specific downregulation of pathologic genes emerged as an appealing strategy for treating human disease. Targeting the molecular level of disease by modifying gene expression with several types of antisense agents has advanced rapidly over the past 20 years, especially with the discovery of certain small RNA molecules with remarkable properties. The rapid advancement was primarily initiated by the sequencing of the human genome and the accompanied rapidly growing knowledge of the molecular causes of disease. After successful application *in vitro* and in small eukaryotic organisms like C. elegans, several of the antisense gene-silencers were prepared for *in vivo* studies in mammals.

The use of antisense agents started with antisense oligonucleotides (ASOs), short stretches of single-stranded RNA or DNA with sequence complementary to their target messenger RNA (mRNA). The idea that these ASOs could be used as specific inhibitors of gene expression was introduced in 1978 [1, 2]. The silencing mechanism of ASOs showed to vary depending upon the charged characteristics of the ASOs backbone [3]. Although much research was put into ASOs, interest eventually declined when the development of predicted therapeutic possibilities proved to be very time-consuming. Fortunately, gene-targeting strategies were given a boost with the discovery of RNA with catalytic activity, the so-called ribozymes (from *ribo*nucleic acid en*zyme*) in 1982 [4], which changed the perception of RNA as a simple bridge between DNA and protein. Since RNA can serve as a catalyst and as a carrier of genetic information, it holds both properties needed for life. This provided the basis for the "RNA world hypothesis", which proposes that our current DNA-, RNA- and protein-based world has evolved from an earlier exclusively RNA-based world, and started an exciting age of exploration of the functional RNA world. A unique property of the ribozyme is that it is able to break covalent bonds in RNA molecules with sequence specificity when guided by a unique substrate sequence [5] or when covalently joined to a specific antisense component [6]. This new knowledge further expanded the use of nucleic acid-based inhibitors of gene expression. Subsequently, both ASO and ribozyme strategies were further improved regarding stability, delivery, and efficiency of gene targeting, although issues still exist.

Following ASOs and ribozymes, a novel gene-targeting mechanism was discovered in 1998 in the nematode Caenor-

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habditis elegans [7]. This small RNA-based, naturally occurring, sequence-specific, posttranscriptional gene silencing phenomenon was termed RNA interference (RNAi). RNAi is triggered by the presence or introduction of double-stranded RNA molecules (dsRNA). Through an intracellular multistep process, specific small RNAs, called siRNAs, elicit powerful, targeted degradation of complementary RNA sequences [8]. It soon became clear that RNAi is evolutionary conserved as it also exists, although somewhat more complex, in vertebrates, including human. Because of its easy-to-use method *in vitro* and high specificity, RNAi showed to be a particularly powerful tool for targeted inhibition of gene expression of any selected target gene. As a result, our understanding of gene function improved rapidly and RNAi is now a well-established tool in biomedical research where it is being explored in high-throughput analysis, *in vitro* and *in vivo* functional studies, and for the development of gene-specific therapeutics. The success of RNAi was acknowledged by the Nobel Prize committee and the 1998 discovery of RNAi by Drs. Andrew Z. Fire and Craig C. Mello was awarded the 2006 Nobel Prize for physiology and medicine. Although being the most promising gene silencing tool so far, efficient delivery and side-effect issues have held back the *in vivo* applicability of this technique as well.

## BASICS OF RNAi

Prior to the discovery of RNAi in 1998, the phenomena of RNAi had been observed eight years earlier in transgenic plants where it was termed co-suppression [9]. This study demonstrated that, in an attempt to promote violet pigmentation in petunias, the introduction of dsRNAs for the pigmentation gene, resulted in complete and/or partly white flowers [9]. RNAi was demonstrated experimentally in C. elegans by Fire *et al*. [7], who showed that the injection of specific dsRNAs resulted in marked inhibition of gene expression, complementary to the dsRNA. Injection of dsRNA resulted in great efficiency of gene silencing, whereas sense or antisense RNA strands alone did not result in a significant reduction of targeted mRNA. A few years later, the mechanism of RNAi was experimentally demonstrated in a wide range of eukaryotic organisms including flies [10, 11], zebrafish [12], and finally in mammalian cells, including human [13]. The effector molecules of the RNAi mechanism were revealed by Zamore *et al*., who showed that the dsRNA was rapidly cleaved into small dsRNA strands with a length of 21 to 23 nucleotides (nt) called siRNAs [8]. The pivotal role of siRNAs in initiating RNAi was confirmed by the introduction of chemically synthesized siRNAs, which by themselves were sufficient for the induction of gene silencing [13]. Through biochemical analysis of the siRNAs, two distinctive features were found. The siRNA molecules possessed 2 to 3 nt overhangs at the 3' end and a monophosphate group on the 5'-terminal nucleotide, which indicated that siRNAs were the cleavage product of an endoribonuclease of the RNase III family [14]. This quickly led to the identification of Dicer as the enzyme required for cleaving dsRNA into siRNAs [15].

It is now clear that RNAi is an intracellular multistep process which initially begins with the cleavage of dsRNAs or short hairpin RNAs (shRNAs) [16] into siRNAs by Dicer (Fig. (**1**)). Dicer consists of two RNase III domains, a dsRNA binding domain, an N-terminal helicase domain and the RNA binding domain Piwi Argonaute Zwille (PAZ) [15, 17]. After cleavage, single stranded siRNAs are incorporated into the RNA induced silencing complex (RISC), constituted of at least Dicer, Transactivation Response Binding Protein (TRBP), and one Argonaute protein (Ago2 in human) [10]. The siRNAs are bound to Ago2, the catalytic protein component of the RISC, which is partially responsible for the selection of the siRNA guide strand on the basis of the 5' end stability in Drosophila [18, 19], and for the destruction of the siRNA passenger strand [20]. The RISC is activated upon ATP-dependent unwinding of the double-stranded siRNA into the single-stranded siRNA guide strand by RNA helicase activity [20]. Next, the activated RISC is brought in proximity to its target mRNA [21, 22], mediated through the hybridization of the antisense siRNA guide strand to its perfect complementary mRNA target site, which is then cleaved by the RISC nuclease Ago2 and further degraded as it has lost its protective ends [23, 24].

Anti-viral defence is one of the biological functions ascribed to RNAi, since RNAi has been shown to take part in a nucleic-acid-based immune system, protecting human cells from viral infection by degrading viral transcripts [25, 26]. Next to its important role as a regulator of gene expression through miRNAs, which will be discussed next, RNAi is also thought to be important in preventing transposon jumping [27]. Finally, RNAi is thought to contribute to genomic imprinting [28], to silencing of translationally aborted or overproduced mRNAs [29], or to tissue-specific gene expression by modulating DNA conformation [30], since RNAi is also capable of inducing heterochromatin formation [31] and DNA methylation [32].

### MicroRNAs

Since RNAi could be induced by foreign dsRNA, and was therefore shown to be endogenous in several eukaryotic organisms, it was hypothesized that the mammalian cellular genome might encode some sort of RNAi inducing RNA. This was confirmed with the discovery of miRNAs, small RNA molecules that negatively regulate endogenous gene expression [33]. An important difference between siRNAs and miRNAs in mammalian cells is that the latter is endogenously present, whereas siRNAs are exogenously derived from e.g. viruses. The action of a miRNA had already been observed in 1993 [34], when the mechanism of RNAi was still unknown. MiRNAs are described as a class of short (~22 nucleotides), endogenously present, non-coding RNA molecules that negatively regulate gene expression by partially complementary base pairing to mRNA, inducing translational repression through mRNA destabilization and degradation [35-37]. In mammals, the cellular biochemical pathway is very similar to that of siRNA [29, 38]. Initially, a miRNA gene is transcribed by RNA polymerase II into variable length (100 to 1000's nt) primary transcripts called pri-miRNAs. These are then processed by the microprocessor complex, which in human consists of at least the RNase III protein Drosha and a dsRNA binding protein DGCR8 [39-41]. This complex binds the pri-miRNA and specifically cleaves at the base of the hairpin loops, releasing the 60 to 70 nt hairpin-shaped precursor miRNA (pre-miRNA) [39-41]. The pre-miRNAs are exported to the cytoplasm by exportin

5 [42, 43], and are further processed by Dicer into 22 nt long single stranded RNAs (mature miRNAs) and incorporated into the RISC, as described above (Fig. (**1**)). In contrast to siRNAs, which primary mode of action is target cleavage through perfect complementarity, miRNAs are partially complementary to their targets. MiRNAs bind predominantly to the 3'UTR of their target genes and only require a "seed" match of 7 to 8 base pairs between the 5' region of the miRNA and the 3'UTR of the target mRNA [36, 37]. Most miRNA targets are translationally repressed, however, mRNA cleavage can also occur [44]. Due to the partial complementarity, one miRNA could potentially regulate several distinct mRNA targets, thereby regulating a whole set of genes. Furthermore, target prediction algorithms have been generated, predicting that one specific gene could be targeted by numerous miRNAs.

So far, more than 550 miRNA genes have been identified in humans alone and many more have been predicted to exist [45-47]. The importance of miRNAs as biological regulators is recognized by predictions that miRNAs target over one third of all human genes and are often highly conserved across a wide range of species [30, 48, 49]. Moreover, many miRNAs are expressed in a tissue-specific manner which goes as far as organ-specificity or even expression restricted to single tissue layers within one organ [50, 51]. Because of their important roles in biological processes, abnormal expression or mutations in miRNAs or their target sites can affect cellular processes, even resulting in pathological changes, as shown for different forms of cancer [52]. Therefore, not only the silencing of coding genes is an appealing strategy for treating human disease, but also the silencing of disease-related miRNAs. Because miRNAs function through binding to their complementary mRNA sequences, two groups investigated whether oligonucleotides that were complementary to the miRNA would act as inhibitors of miRNA function [53, 54]. This allows miRNA loss-of-function studies *in vivo*, which lead to a better understanding of the precise molecular and biological functions of miRNAs, which are currently largely unknown for mammals. Understanding miRNA function will eventually lead to the development of new therapeutic applications.

## *IN VIVO* DIFFICULTIES FOR SMALL RNA MODULATION

As described above, small RNAs provide two ways of modulation, namely, knocking down gene expression, and the inhibition of translational silencing. Both can be exploited to study specific gene function *in vivo*, create loss-of-function animal models of human disease, or develop small RNA-based therapeutics for a variety of human diseases. For the successful application of small RNA therapeutics *in vivo*, it is essential to stably deliver these small RNAs to specific target tissues, with prolonged activity to inhibit gene function for a sufficient amount of time. However, small single stranded RNA molecules have a highly charged hydrophilic backbone, which makes them particularly vulnerable to enzyme degradation and complicates the diffusion through the cell membrane. In addition, efficient delivery is hampered by

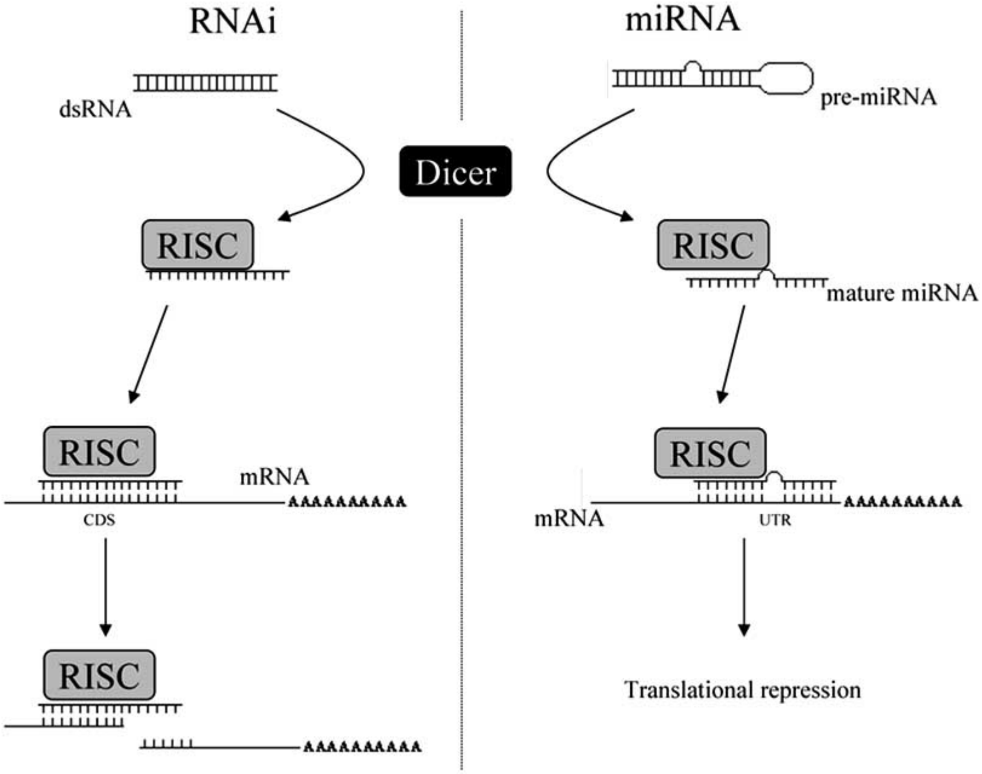


**Fig. (1).** Mechanism of RNAi and miRNA induced gene silencing. Both dsRNA and pre-miRNA molecules are cleaved into a single strand, loaded into the RISC complex, thereby leading to cleavage of the targeted mRNA via perfect binding (RNAi) or translational silencing of the gene via imperfect complementarity (miRNA).

(Dicer = endoribonuclease of the RNase III family, RISC = RNA-induced silencing complex, CDS = coding sequence, UTR = untranslated region, mRNA = messenger RNA).

non-specific uptake by cells and fast elimination by kidney filtration due to the small molecular mass. In general, the lifetime of small RNAs *in vivo* is insufficient for most human diseases. To achieve a therapeutic effect, sustained delivery is crucial. Vector based systems might provide a solution to this problem as they permit stable expression, but do require specialized delivery methods. Improving the efficient intracellular delivery of ASOs and siRNAs to target sites within the body is still a real challenge [55, 56]. Next to small RNA stability and delivery, the most important factor in gene-silencing experiments is the efficacy of the small RNA to target the mRNA or miRNA of choice. The targeting efficacy determines the time required to reduce protein or miRNA expression below the threshold level, critical for normal protein or miRNA function.

## MODULATING SMALL RNA ACTIVITY *IN VIVO:* GENE SILENCING THROUGH RNAI

To date, RNAi is the most promising strategy for the specific downregulation of pathologic genes. However, to achieve efficient gene-silencing, siRNAs need to be carefully designed. The efficacy and efficiency of gene-silencing can be strongly influenced by the composition and thermodynamic stability of siRNA duplexes [57]. Currently, several

**Table 1. Delivery Systems for *In Vivo* Delivery of Small RNAs and their Effects**

| Formulation | Effect | Example References |
|---|---|---|
| Liposomes | Complex formation or incorporation protects against nucleolytic degradation and renal clearance<br>Enhances cellular uptake through electrostatic interaction with the negatively charged cell membrane<br>Facilitates cytoplasmic delivery by destabilizing the endosomal membrane | [114]<br>[115]<br>[116]<br>[117]<br>[118]<br>[122] |
| PEGylation | Steric stabilization<br>Increases the hydrodynamic size which protects against renal clearance<br>Improves solubility which enhances biodistribution | [119] |
| SNALP | Full encapsulation protects against nucleolytic degradation and renal clearance | [107]<br>[123] |
| (Ga)lactosylated liposomes | Facilitates tissue/cell specific delivery in addition to liposomal effects | [124]<br>[125] |
| Atelocollagen, chitosan | Complex formation protects against nucleolytic degradation and renal clearance<br>Enhances cellular uptake through electrostatic interaction with the cell membrane<br>Prolonged release of small RNAs from complex | [130]<br>[131]<br>[132]<br>[133]<br>[134]<br>[135]<br>[136]<br>[137] |
| PEI | Complex formation protects against nucleolytic degradation and renal clearance<br>Enhances cellular uptake through electrostatic interaction with the cell membrane<br>Enhances cytosolic release | [139]<br>[140] |
| RGD-targeted PEG-PEI | Facilitates tissue/cell specific delivery in addition to PEI effects | [141] |
| Transferrin receptor-targeted cyclodextrin nanoparticles | Incorporation protects against nucleolytic degradation and renal clearance<br>Enhances cellular uptake<br>Tissue/cell specific delivery | [142]<br>[143] |
| Cholesterol | Binding to albumin and lipoprotein particles protects against renal clearance<br>Indirectly enhances tissue/cell specific uptake by binding lipoprotein receptors | [146] |
| Protamine-antibody | Complex formation protects against nucleolytic degradation and renal clearance<br>Tissue/cell specific delivery | [148] |
| Aptamer | Tissue/cell specific delivery | [149] |
| PLGA microspheres | Complex formation protects against nucleolytic degradation and renal clearance<br>Sustained release of small RNAs from complex | [150] |

guidelines on designing siRNA and shRNA have been published [57-61]. In addition, one can now make use of several online siRNA/shRNA design tools from both academic institutions and commercial companies. However, although predictions are improving, the gene-silencing efficiency of a number of selected candidate siRNAs still needs to be experimentally validated, because RNA-binding proteins and/ or intramolecular folding of the target mRNA may hinder antisense binding [62-65]. Therefore, selection of the target sequence is of great importance as well.

Yet, even the most carefully designed siRNA may still have significant sequence specificity problems [66, 67], since a match of only 7 nt is enough to induce miRNA-based gene-silencing [37]. In this way, siRNAs which are introduced into the cell, could exhibit miRNA function, inducing translational repression of one or more targeted genes. In addition, introducing siRNA or shRNA might disrupt the endogenous miRNA pathway through si/shRNA competition with pre-miRNA for exportin-5 or other parts of the processing machinery. This was shown in a study on the effect of high doses of shRNA in the livers of mice, where a significant number of mice died of dose-dependent liver injury, associated with the down-regulation of liver miRNAs [68]. Interestingly, it was shown that both siRNAs and shRNAs can compete against each other and with endogenous miRNAs for transport and for incorporation into the RISC *in vitro*, though the same siRNA sequences did not show competition when expressed from a miRNA backbone [69]. In contrast, a recent *in vivo* study showed effective target-gene silencing by systemic administration of synthetic siRNA without any demonstrable effect on miRNA levels or activity [70]. In general, when the goal is to silence a specific gene by means of siRNA, possible siRNA competition with the endogenous miRNA pathway should be taken into account.

Next to off-target effects due to sequence specificity, siRNAs are also able to provoke immune related side effects by inducing a type I interferon response through Protein Kinase R (PKR) [71, 72], and by activating the innate immune system *via* toll-like receptors (TLRs) [73-75], both RNA-sensing immunoreceptors. Fortunately, these immune responses can largely be avoided by delivering minimal amounts of siRNA, which are of appropriate length and depleted from certain TLR-associated RNA sequence motifs [76-78]. Altogether, the siRNA/target combination must function with great efficiency, so that only a minimal amount of siRNA is needed to effectively and specifically induce a translational block, minimizing non-specific and off-target effects which are often dose dependent. Unfortunately, most off-target and non-specific effects occurring *in vivo* haven't been documented in great detail. It is evident that, to fully exploit the *in vivo* potential of small RNAs, we need innovative delivery systems and optimal modes of administration, which minimize off-target and non-specific effects.

## Vector-Based Delivery

The *in vivo* delivery of siRNA molecules can be categorized into two general approaches: 1) the transient delivery of siRNA to the target tissue and 2) the inducible delivery of siRNA through shRNA-expressing vectors [79, 80]. Since mammalian cells lack the RNAi amplification mechanism that can occur in C. elegans, gene silencing is dependent on the effective number of siRNA copies delivered into the cells [81]. The use of shRNA-expressing vectors has the advantage that the RNAi effect can be more stable and sustained for a longer period of time [80]. In addition, inducible regulation has the advantage of keeping expression levels within physiological boundaries, whereas transient delivery of a single high dose or multiple doses of siRNAs might result in non-physiological responses. Additionally, vector-based RNAi allows the co-expression of reporter genes and the incorporation of regulatory elements to the promoter region of the expression vector. Successful shRNA delivery and gene silencing *in vivo* has been achieved by using adeno-associated viral (AAV) vectors [82-85] and lentiviral vectors [85-87]. Although the latter is associated with insertional mutagenesis and oncogenic transformation [88, 89]. Recombinant AAV vectors do not cause an inflammatory response, require a helper virus, and they integrate site specifically into the AAVS1 region of chromosome 19, which makes them more safe for *in vivo* use and gene therapy [90, 91]. Still, oncogenic mutagenesis cannot be excluded entirely, since approximately 10 percent of stably AAV transduced genomes have been reported to integrate into host chromosomes *in vivo* [92]. While the use of plasmid shRNA-expressing vectors provides a more safe approach, the successful application of this method is challenged by low transfection efficiencies and immunogenic side-effects [93]. Overall, strategies based on vector mediated small RNA delivery may possibly go together with serious side effects, which will hamper their *in vivo* use [94].

## Unmodified Small RNA Delivery

Non-viral carrier systems allow a more safe delivery of catalytically active siRNAs. However when not using viral vectors, unmodified siRNAs are generally harder to deliver into the cell. Nevertheless, numerous *in vivo* studies have shown the systemic or local delivery of unmodified siRNAs. A major disadvantage of systemic delivery is the requirement of very high amounts of unmodified siRNA, which is accompanied by an increase in non-specific effects, like concentration-dependent immune responses. Moreover, the standard method used for systemic delivery of unmodified siRNA; hydrodynamic transfection (high-pressure high-volume injection) [79], has been shown to produce membrane defects and disturb the cell interior in mice [95]. Additionally, hydrodynamic delivery primarily targets highly vascularised organs, such as the liver, kidneys, and spleen. On top, the hydrodynamic transfection procedure is highly unsuitable for human clinical use. Local delivery of unmodified siRNA surmounts the use of very high doses since systemic (renal and hepatic) elimination and nonspecific delivery to other tissues is reduced. However, organ-wide gene-silencing through local administration is only successful in a very limited number of organs like liver, eye, lung and brain [80, 96-98], and subcutaneous tissue or tumours [99]. Local delivery in other tissues requires the use of more invasive methods. Overall, systemic delivery is the favourable route for administrating small RNAs, though, especially to become effective in human, this requires the protection of the small RNA against systemic degradation, and special agents for targeting and entering specific cells and tissues.

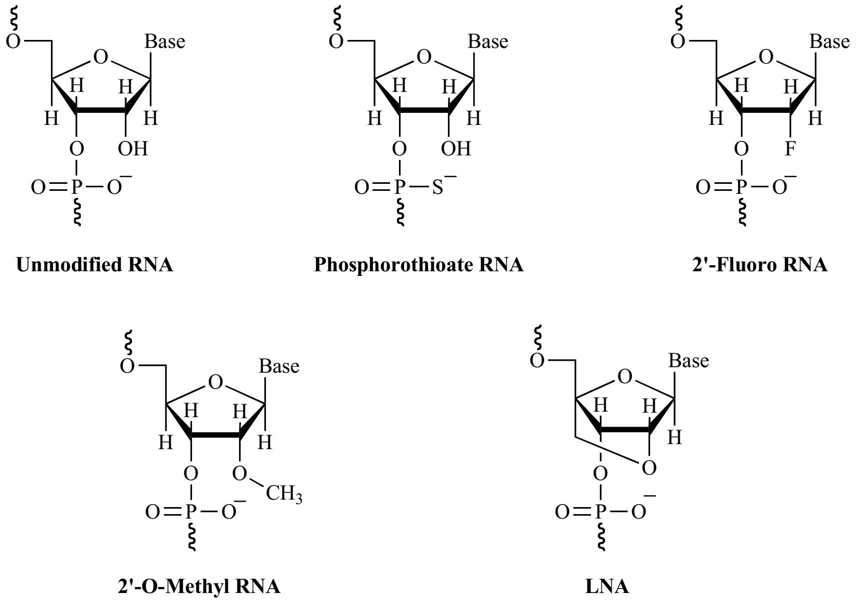
## Small RNA Modifications and Formulations for *In Vivo* Delivery

Alternative strategies for systemic delivery of small RNAs consist of backbone modifications, peptide-conjugations, pre-complexation with protecting and uptake-enhancing polymers and incorporation into lipids. All these siRNA modifications and formulations enhance systemic small RNA stability. In addition, polymer pre-complexation, lipid incorporation, and peptide-conjugations protect siRNAs against systemic elimination, enhance cellular uptake, and provide opportunities to target any specific organ, tissue or even cell type with smaller amounts of siRNA. Already many different small RNA modifications and formulations to improve stability and delivery have been employed by several groups.

### Chemical Modifications

Chemical modifications, including 2'-OH ribose residue substitutions, and phosphodiester backbone modifications have been shown to increase systemic siRNA stability. However, inside the cell, unmodified siRNAs show to be as resistant to degradation as modified siRNAs [100]. The RISC might be responsible for the protection of the siRNA guide strand from intracellular nucleases, which suggests that antimiR oligonucleotides, which will be discussed later, do not experience protection as they do not function through RNAi. Backbone modifications are primarily applied to the siRNAs passenger strand, because this strand plays no direct role in target silencing. Chemical modifications that block phosphorylation of the 5'-end of the guide strand impair RNAi, since the 5'-end phosphate of the siRNAs guide strand is required for Ago2 binding [101]. One major advantage of chemically modifying the siRNA passenger strand is that cells will not incorporate this strand into the RISC, preventing the non-target complementary strand to induce unwanted off-target effects. Partial substitution of the phosphodiester backbone with thioate linkages (Fig. (**2**)) at the end of one of the siRNA strands increases siRNA stability [102, 103] and biodistribution [104]. However, phosphorothioate backbones were shown to be cytotoxic and loss of silencing activity could occur [101-103, 105]. 2'-OH ribose modifications like 2'-fluoro (2'-F) (Fig. (**2**)) have shown diverging results; substitution of all pyrimidines with 2'-F increased plasma half-life to 1 day, compared to 1 minute for unmodified siRNAs, thereby retaining target silencing activity [100], whereas 2'-F substitutions for all the uridines decreased target silencing [106]. Interestingly, the increase in plasma stability did not lead to an *in vivo* extension or improvement of target gene silencing, indicating that *in vivo*, 2'-F modified siRNAs are no more potent than unmodified siRNAs [100]. Increased *in vivo* gene silencing has been achieved by chemically modifying all 2'-OH residues on both strands of the siRNA duplex, with 2'-F substitutions on all pyrimidine positions, deoxyribose and 2'-O-methyl (2'-O-Me) (Fig. (**2**)) substitutions in all purine positions on the sense and antisense, respectively [107, 108]. Additionally, in contrast to unmodified siRNAs, chemically modified siRNAs did not activate the immune response [108]. This was later confirmed by showing that immune activation by siRNAs can be completely abrogated by selective incorporation of 2'-O-Me, uridine or guanosine nucleosides into one strand of the siRNA duplex [109], by introduction of as little as three 2'-O-Me substitutions into the sense strand [110], or by 2'-O-Me modification of siRNA sense-strand uridine or uridine/adenosine residues [111].

One very promising backbone modification for siRNA is the so-called locked nucleic acid (LNA) (Fig. (**2**)). LNA nucleotides contain a methylene bridge between the 2′ and 4′ carbons of the ribose ring, which has been shown to greatly


**Unmodified RNA** **Phosphorothioate RNA** **2'-Fluoro RNA**

**2'-O-Methyl RNA** **LNA**

**Fig. (2).** Chemical structures of unmodified and chemically modified RNA used in the different studies. A phosphodiester backbone modification (Phosphorothioate), 2'-OH ribose residue substitutions (2'-Fluoro, 2'-O-Methyl) and a ribose moiety modification (LNA) are depicted. (LNA = locked nucleic acid).

improve bio- and thermal stability of siRNAs without adversely affecting their silencing efficiency [102, 112]. The LNA content and positioning are important for efficient gene inhibition, and reducing off-target effects. This was mediated by increased sequence specificity, lowering RISC incorporation of the siRNA passenger strand and by reducing the ability of improperly loaded passenger strands to cleave the target RNA [112]. Moreover, minimal 3' end LNA modification effectively stabilizes the siRNA and reduces off-target gene regulation compared with unmodified siRNA, *in vivo* [113].

In addition to backbone modifications, which primarily increase systemic small RNA stability, siRNA delivery formulations like lipid and polymer siRNA have shown to increase stability and enhance cellular uptake due to their positive charge. This facilitates complex formation with the small RNA, and allows electrostatic interaction with the negatively charged cell membrane. Moreover, complex formation and incorporation into liposomes prevents elimination by kidney filtration, allows the addition of surface molecules, and enables tissue specific targeting. The cellular uptake of these complexes occurs through vesicular mechanisms. For successful delivery, the release of the small RNAs from the endosome into the cytosol is essential. However, how these delivery systems facilitate endosomal release is not yet understood entirely.

## Lipid Carriers

Since their widespread use in *in vitro* studies, cationic liposomes (Fig. (**3a**)) have been one of the first adopted methods for the *in vivo* delivery of small RNAs. They can be seen as nonviral envelopes that mediate cellular uptake, and protect the small RNAs against nuclease degradation and renal excretion. Several groups have used Roche's cationic lipid DOTAP for successful delivery of siRNAs *in vivo* [114], resulting in a 70% and 37% reduction of functional expression of TNF-α and vasopressin receptor V2, respectively [115, 116]. Several other types of cationic liposomal/siRNA formulations have been successfully used for systemic delivery, including LIC-101 liposomes/siRNAs [117], NeoPhectin-AT cardiolipin/siRNAs [118], and AtuFECT01 cationic liposomes/siRNAs [119]. This last study also demonstrated the advantage of using poly ethylene glycol (PEG)-ylated liposomes (Fig. (**3a**)). PEGylation sterically stabilizes the nanoparticle, and can reduce immunogenicity and nonspecific interactions. However, multiple administrations of PEGylated liposomes have been shown to induce an anti-PEG immune response [120, 121]. The neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) has been used successfully, though at very high concentrations, making it very expensive for human clinical use. Injections of 150 mg/kg body weight of neutral DOPC liposome/siRNAs targeting the oncoprotein EphA2 twice a week (for 4 weeks) resulted in a 10-fold and 30-fold higher tumour accumulation than that of DOTAP/siRNAs and naked siRNAs, respectively [122]. Stable nucleic acid lipid particles (SNALPs) (Fig. (**3a**)) have also mediated effective siRNA gene targeting; SNALPs increased systemic half-life from 2 minutes to approximately 6.5 hours [108], and a small single dose of 2.5 mg/kg body weight reduced target gene expression by more than 90% in non-human primates [123].

Lipid carriers can be modified with cell type-specific ligands for tissue or cell specific delivery of small RNAs, thereby minimizing off-target effects. Lactosylated and galactosylated cationic liposomes have been used for hepatic parenchymal cell specific delivery of siRNAs, with significant gene knockdown and no toxicity [124, 125]. However, even with cell-specific delivery, certain problems still remain for liposomal systems, since cationic liposomes can significantly induce the immune response [126-128]. Consequently, modifications of naturally occurring lipids like cardiolipin, a component of the inner mitochondrial membrane, are being developed to minimize liposomal toxicity. Another point that should be addressed, is that cationic lipids alone were shown to alter gene expression of treated cells when analyzed by microarray-based gene expression profiling [129].

## Nanoparticles/Cationic Polymers

*In vivo* studies have shown some success in polymer and nanoparticle delivery of siRNAs. Positively charged macromolecules used for *in vivo* delivery of small RNAs include atelocollagen, chitosan, and polyethylenimine (PEI). Atelocollagen is a highly purified pepsin-treated type I collagen which increases cellular uptake, is resistant to nucleases, has prolonged release of oligonucleotides, and displays low immunogenicity and toxicity *in vivo* [130]. *In vivo* delivery of enhancer of zeste homolog 2, phosphoinositide 3'-hydroxykinase p110-alpha-subunit and fibroblast growth factor siRNAs complexed to atelocollagen (Fig. (**3b**)) have shown efficient inhibition of tumour growth [131, 132]. Moreover, the complexes remained intact for at least 3 days and did not activate the immune response [132]. Other non-cancer related *in vivo* studies showed that siRNA/atelocollagen complexes were effectively delivered into the brain [133], and detectable in graft vein wall after at least 7 days [134].

Chitosan is a linear polysaccharide, produced by deacetylation of chitin, and has been used in a number of studies to coat nanoparticles for *in vivo* siRNA delivery (Fig. (**3c**)). The use of siRNA/chitosan at very small amounts of 0.15 and 1.5 mg/kg body weight administered intravenously every 3 days in mice resulted in tumour growth inhibition of over 90% and no toxicity [135]. Effective *in vivo* RNAi was also achieved through nasal [136], and intratumoural [137] administration of siRNA/chitosan formulations. It is however crucial to mention that next to its anti-bacterial activity, chitosan can cause anti-tumour activity *via* activation of the immune system [138].

Several *in vivo* studies have used polyethylenimines (PEIs) as polymeric delivery systems for small RNAs (Fig. (**3d**)). PEIs are synthetic polymers of various shapes and sizes, which allow noncovalent complexation with nucleic acids. Next to protection against nucleolytic degradation, PEI increases cellular uptake through endocytosis, and enhances cytosolic release. Intraperitoneal injections of low molecular weight PEI-complexed, but not of naked siRNAs targeting the HER-2 receptor led to significant reduction in tumour growth in a mouse tumour model [139]. Intraperitoneal and subcutaneous injections of PEI-complexed siRNAs targeting BCR/ABL1 leukemia fusion protein also led to significant inhibition of tumour growth, without a measurable induction

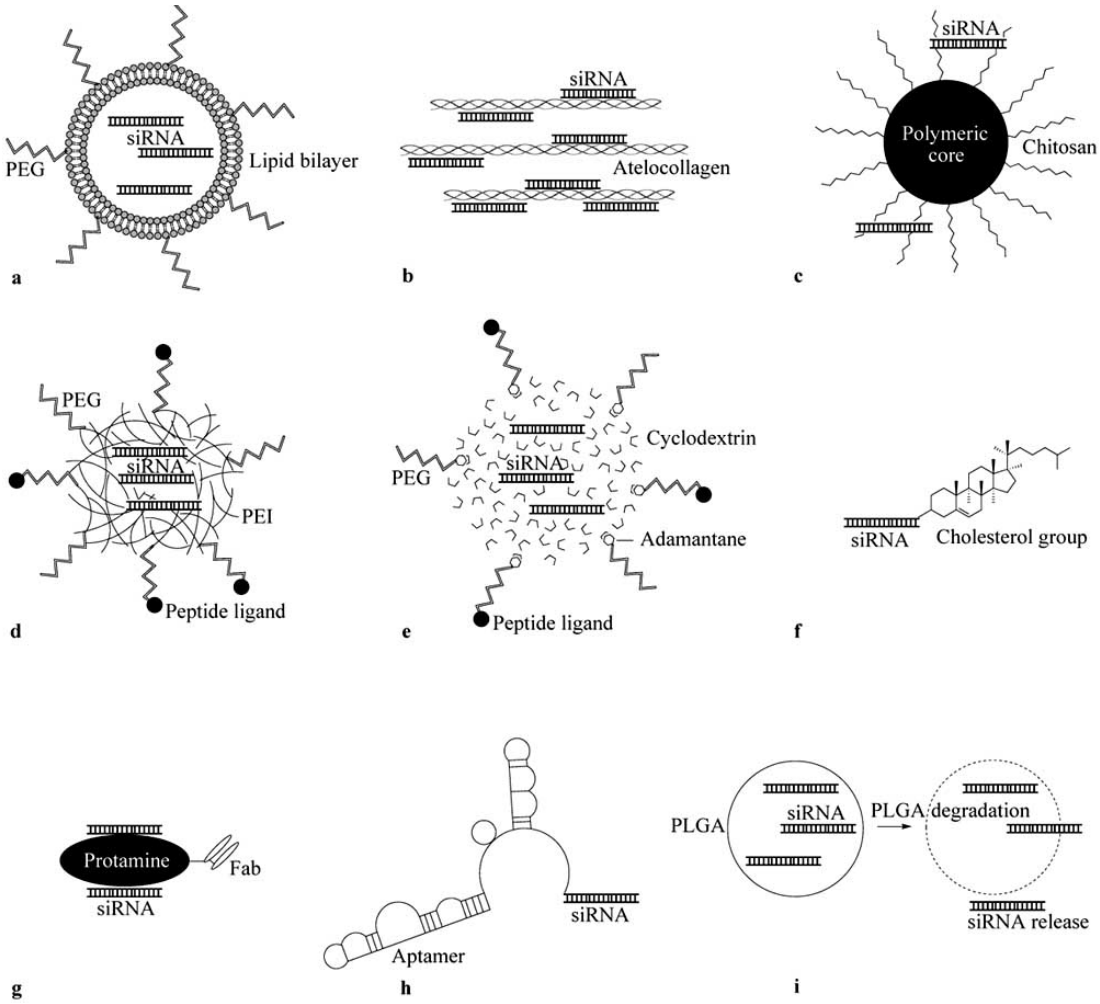


**Fig. (3).** Schematic representation of the different *in vivo* siRNA delivery formulations. **a**) Liposomal delivery system with a PEGylated lipid bilayer entrapping siRNAs. **b**) Atelocollagen, which is positively charged forms complexes with the negatively charged siRNAs. **c**) Chitosan coated nanoparticles enable noncovalent binding of siRNAs to the positively charged chitosan. **d**) Polymeric nanoparticle composed of PEI noncovalently complexed with siRNAs. PEI is PEGylated and a peptide ligand is coupled to PEG, allowing cell type specific delivery. **e**) Cyclodextrin-containing polycation nanoparticle with PEG linked to the cyclodextrins through adamantane. A protein-targeting ligand on the distal end of PEG enables cell type specific delivery. **f**) Chemical conjugation of cholesterol to the 3'end of the siRNA sense strand. **g**) Antibody Fab fragment-protamine fusion protein; a targeted delivery system for siRNAs. **h**) Aptamer-siRNA chimaeras are capable of cell type-specific binding. Dashes in the structure represent base pairs. **i**) Delivery with PLGA biodegradable microspheres provides sustained release of siRNA molecules through degradation of the polymeric microspheres.

(PEG = poly ethylene glycol, PEI = polyethylenimine, PLGA = poly(lactic-co-glycolic) acid).

of siRNA-mediated immunostimulation [140]. Tissue specific uptake of siRNA/PEI complexes can be enhanced by adding peptide-conjugations. This was shown *in vivo* in a mouse tumour model, demonstrating tumour specific complex uptake, target specific downregulation, and a 90% reduction in tumour growth rate upon intravenous injection of siRNAs complexed with PEI. The PEI was PEGylated with an RGD peptide ligand, attached to the distal end of PEG (Fig. (**3d**)), targeting tumour-specific integrins, thereby providing tissue specific delivery [141]. Targeted nanoparticles have also been used for systemic delivery of siRNAs in non-human primates and mice, for this a cyclodextrin-containing polycation with a transferrin protein-targeting ligand (Fig. (**3e**)) for delivery to transferrin receptor-expressing cells was used [142, 143]. Several studies report high toxicity for PEIs, however toxicity is related to the exact composition (length, charge density [144], and primary amine groups [145]) of the used PEI. Therefore, for the successful *in vivo* application of small RNA/PEIs, it is crucial to analyze PEI structure-toxicity. Also, next to chitosan, cationic polymers like PEI have been known to have intrinsic anti-tumour effects [138], which have to be taken into account when developing PEIs for the delivery of small RNAs.

## Conjugations

Alternatively to siRNA pre-complexation and incorporation into liposomes, certain siRNA conjugations have also shown to increase small RNA stability and enhance cellular uptake. More importantly, tissue specific delivery can be facilitated. This has been realized through chemical conjugation of cholesterol to the 3'-end of the siRNA sense strand *via* a pyrrolidine linker (Fig. (**3f**)). Chol-siRNAs showed improved *in vivo* pharmacokinetic properties as compared to unconjugated siRNAs, presumably because of enhanced binding to human serum albumin. The *in vivo* elimination half-life was prolonged to approximately 95 minutes, compared to 6 minutes for unmodified siRNA, after an intravenous injection of 50 mg/kg body weight into rats and resulted in an approximate 60% knockdown of the target mRNA in the liver. More importantly, cholesterol attachment improved efficacy and specificity in liver and jejunum tissue uptake [146]. A major advantage of cholesterol conjugated siRNA is that the modification is minor and does not significantly alter the chemical and biological properties of the siRNA formulation, as is seen for siRNA-lipid and -polymer complexes [147]. Although this approach has high potential for *in vivo* rodent studies, one potential problem remains when extrapolating the rodent data to human clinic; the high dosage required for a desired effect would be very expensive.

A very promising method for cell type specific delivery of small RNAs is antibody mediated delivery. In 2005 it was shown that systemic delivery of a siRNA-protamine-antibody conjugate (Fig. (**3g**)) improved efficacy and specificity in tissue uptake in mice with subcutaneously injected gp160 expressing tumour cells, and caused a significant anti-tumour effect [148]. The fragment antibody, which is linked to protamine, targets the HIV-1 envelope protein gp160. The efficiency of this study is proven by the use of much lower amounts of siRNA to achieve significant target downregulation, with 2 to 2.5 mg/kg body weight. Another advantage of this technique is the flexibility, ease-of-use and preparation, since no specialized chemistry is involved. Given the large availability of humanized monoclonal antibodies, this method can be easily adapted to target nearly any given cell type. However, reaching certain cell types still remains a challenge.

An alternative method for cell type-specific binding and delivery of small RNAs is the use of aptamer-siRNA chimaeras (Fig. (**3h**)). Intratumoural injections of siRNA-aptamers in mice resulted in a marked reduction in tumour size only in tumours that expressed the aptamer binding ligand. Moreover, siRNA aptamers were non-toxic, and the effect was siRNA specific [149].

One drawback of all these small RNA formulations is the lack of long-term sustained release as in vector-mediated delivery. Although small RNA stability has increased enormously, with cells being exposed to the effect of the small RNA molecule for longer periods, the amount of small RNA gradually decreases. One method using poly(lactic-co-glycolic) acid (PLGA) biodegradable microspheres has shown to provide sustained release of siRNA molecules (Fig. (**3i**)) at the site of administration in mice even after 7 days [150]. Recently, the use of PEI as a carrier was added to this delivery system, and siRNA release was shown to last for over one month in a pH 7.4 buffered phosphate solution [151].

## MODULATING SMALL RNA ACTIVITY *IN VIVO:* MIRNA INHIBITION

Next to the *in vivo* gene silencing through siRNAs, small endogenous RNAs, like miRNAs, can be targeted *in vivo* to inhibit translational repression. As described earlier, many pathological conditions depend on abnormal gene expression levels, including miRNA genes. Therefore, silencing of endogenous disease-associated miRNAs may have therapeutic value. However, the delivery of miRNA inhibitors faces the same problems as siRNA delivery. Nevertheless, the *in vivo* inhibition of miRNA function can be achieved by the use of several techniques, which all act through steric blocking rather than RNAi. The first applied technique was a chemically modified (2'-O-Me-modified nucleotides, phosphorothioate linkage), cholesterol-conjugated (through a hydroxyprolinol linkage) single-stranded RNA analogue, complementary to the miRNA, termed 'antagomir'. Antagomirs were administered on three consecutive days at doses of 80 mg/kg body weight, leading to the targeted miRNA being undetectable for as long as 23 days after injection, whereas the unmodified single-stranded RNA had no effect on miRNA levels. In addition, antagomirs achieved broad biodistribution and efficiently silenced miRNAs in most tissues *in vivo* without apparent toxicities. Moreover, antagomir silencing was highly sequence specific, even discriminating between miRNAs derived from the same primary transcript [152]. It was later demonstrated that antagomirs are able to discriminate between single nucleotide mismatches of the targeted miRNA and require >19-nt in length and a significant number of phosphorothioates for highest efficiency [153]. A study on cardiac hypertrophy showed 70% lower levels of targeted miR-133 in antagomir-treated mice compared to controls after a single infusion of 80 mg/kg body weight, causing marked and sustained cardiac hypertrophy [154]. Altogether, antagomirs can effectively and specifically silence miRNAs *in vivo*, which makes them highly suitable to study gene regulation *in vivo*. Moreover, antagomirs provide a straightforward and fast method for the generation of mice lacking specific miRNAs and could potentially become a therapeutic strategy for human diseases.

Besides antagomirs, unconjugated forms of single-stranded RNA analogues have been used for the *in vivo* silencing of miRNAs as well. 2'-O-methoxyethyl-phosphorothioate-modified ASOs, targeting miR-122, an abundant liver-specific miRNA implicated in cholesterol and fatty acid metabolism as well as hepatitis C viral replication, were injected twice weekly for over 4 weeks at doses ranging from 12.5 to 75 mg/kg body weight, resulting in a specific 3-fold to over 10-fold reduction of miR-122 activity in the liver with low toxicity. This miR-122 reduction resulted in reduced plasma cholesterol levels, increased hepatic fatty-acid oxidation, and a decrease in hepatic fatty-acid and cholesterol synthesis rates [155]. 2'-O-Me modified ASOs, targeting miR-21, were delivered locally into a balloon injured carotid artery without showing toxicity. MiR-21 expression was significantly and specifically decreased, as no inhibitory effect was found on other miRNAs, resulting in inhibited neoitima formation [156]. MiR-1-targeting ASOs, containing 2'-O-Me modifications at every base and a 3' C3-containing amino linker, were pre-treated with lipofectamine 2000 and injected into the infarcted myocardium at quantities of 80 μg total ASO, resulting in significantly suppressed arrhythmias [157].

A recently described strategy for the *in vivo* inhibition of miRNAs is the use of LNA-antimiRs [158]. Here, the use of an unconjugated 16-nt mixed LNA/DNA fully phosphorothiolated oligonucleotides with two methylated cytosines complementary to the 5' region of miR-122 was explored. Single intravenous injections of LNA-antimiR for three consecutive days, at doses ranging from 2.5 to 25 mg/kg per day, led to specific and dose-dependent miRNA-122 antagonism in mice without observed hepatotoxicity. Moreover, single intravenous doses of LNA-antimiR for three consecutive days at 25 mg/kg per day resulted in over 85% reduction of miR-122 at 24 h, followed by a gradual increase in mature miR-122 with complete normalization at 3 weeks, implying that the inhibition of miR-122 by LNA-antimiR is reversible. More recently, the same group successfully demonstrated the use of their miR-122 targeting LNA-antimiRs in non-human primates [159]. LNA-antimiRs show to be a promising tool for studying the biological role of miRNAs and for identifying their targets. Additionally, the LNA modification, which has been discussed before, highly increases nuclease resistance, decreases off-target effects and does not show toxicity. This makes it a promising modification for the *in vivo* delivery of siRNAs and ASOs, possibly leading to novel therapeutic strategies for disease-associated genes and miRNAs.

## CONCLUSION

Clearly, the *in vivo* modulation of gene expression by using small RNAs has great potential, but successful *in vivo* modulation falls or stands with the efficiency of small RNA delivery into the target tissue, together with the efficiency and selectivity of long-term target silencing. Several of the siRNA and antimiR oligonucleotide modifications and formulations described in this review are efficiently delivered into their target tissue and effectively knockdown their targets. Nevertheless, non target-specific knockdown, sequence specificity problems, immune responses, and other off-target effects like siRNA competition with the miRNA pathway are less well understood and are still hurdles to tackle. For the *in vivo* and especially clinical applicability of small RNA formulations, minimally invasive delivery methods would be preferable. However, local more invasive administration will allow the use of low doses, thereby also minimizing systemic off-target effects and reducing costs. Nonetheless, the majority of diseases require treatment through intravenous or intraperitoneal injection, making systemic administration the more widely applicable strategy for the clinic, thereby requiring small RNA modifications and formulations that increase protection against serum nucleases and kidney elimination, target specific tissues and overcome biological barriers without inducing toxic and non-specific effects.

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