

Recent Progress in Chemically Modified siRNAs

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Abstract: RNA interference technology has become a powerful laboratory tool to study gene function. Small interfering RNAs (siRNAs) have provided unprecedented opportunities for the development of new therapeutics in human diseases. Unfortunately, siRNA duplexes are not optimal drug-like molecules. The problems for their effective application are fundamentally delivery, stability and off-target effects. Chemical modification provides solutions to many of the challenges facing siRNA therapeutics. In this review, we recapitulate and discuss the development of the latest described chemical modifications of siRNAs, with a special focus on novel chemical modifications of siRNA structure, architecture and siRNA conjugates.

Keywords: RNAi, siRNAs, chemical modification, siRNA conjugates.

INTRODUCTION

The completion of human genome sequencing project and the elucidation of many molecular pathways that are important in diseases have made the use of nucleic acid-based inhibitors of gene expression an immense attractiveness in functional genomics and therapeutic strategies. The increase in Oligonucleotide-Mediated Gene Silencing [1] (OMGS) is largely a result of improvements in the rational design of ONs and in the methods used for synthesizing oligonucleotides with relatively inexpensive cost. The concept underlying nucleic acids based technology is relatively straightforward: the use of a sequence that recognizes a specific mRNA through Watson-Crick base pair hybridization inhibits gene expression at either the transcriptional or post-transcriptional level [2]. The major classes of inhibitory agents are Antisense Oligonucleotides (AS-ONs or ASOs) and more recently Small Interfering RNA (siRNAs).

From Antisense to siRNA

The notion that gene expression could be modified through the use of small ODNs derives from studies by Paterson *et al.* [3], who first used singlestranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977. The following year, Zamecnik and Stephenson [1, 4] showed that a short (13-mer) DNA that was ANTISENSE to the Rous sarcoma virus could inhibit viral replication in culture. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was shown [5, 6]. Since then, the antisense strategy has enjoyed exponential gains in interest and has been the subject of thousands of published reports. The basic concept and the mechanisms of inhibition of AS-ONs have been characterized and discerned in two main mechanisms: steric-blockage and RNase-H activation [7, 8]. The

steric-blocker AS-ONs physically prevent or inhibit the progression of splicing or the translation machinery, while the RNase H dependent oligonucleotides induce the degradation of mRNA. Most of the oligonucleotides capable of inhibiting splicing are not RNase-H dependent [9-11]. In spite of the theoretical simplicity, the molecular mechanism of action of AS-ONs has critical drawbacks such as poor stability versus nuclease activity *in vitro* and *in vivo*, low intracellular penetration and low bioavailability [12, 13]. Synthetic nucleic acids can be chemically modified to improve their general pharmacodynamic properties for their therapeutic use. Hundreds of different modifications have been studied for utility in antisense applications [14-16]. Three generations of chemically modified ASOs have been developed to enhance nuclease resistance, prolong tissue half-life, reduce off-target effects and increase affinity and potency [7, 17-24]. The most notable discovery was the introduction of a phosphorothioate backbone in oligonucleotides, leading to a significant increase in their stability without major changes in ability to hybridize their target mRNA [7]. Other chemical modifications, including the development of DNA/RNA mixed-backbone oligonucleotides [25] and other oligonucleotides barely resembling DNA (such as peptide nucleic acid (PNA) and locked nucleic acid (LNA) structures) [7], have been made to increase the efficacy and stability of the antisense molecules. Although a few first generation ASOs have shown promising therapeutic performances, newer generations have entered into clinical trials.

RNA interference (RNAi) is the most recent explosion of interest in antisense world following the discoveries of Mello and colleagues [26] that double-stranded RNAs (dsRNAs) elicit potent degradation of targeted mRNA sequences in *C. elegans* and in mammalian cells [27, 28]. The active components of the RNAi are dsRNAs, small interfering RNA (siRNAs), that typically contain 19-21 bp and 2-nt 3'-overhangs. These short species are naturally produced by Dicer-mediated cleavage of larger dsRNAs and they are functional in mammalian cells. Synthetic siRNAs can also be introduced into cells exogenously in order to experimentally activate RNAi [27]. When an exogenous 19-21 bp siRNA is intro-

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duced into a mammalian cell the 5'-end is phosphorylated. The duplex is assembled to form the RNA-Induced Silencing Complex (RISC), a multiprotein complex including Argonaute 2 (AGO2), Dicer, TRBP (HIV-1 TAR RNA binding protein) and PACT (a dsRNA-binding protein), as well as other proteins, some of which are yet unknown [29]. The RISC proteins facilitate searching through the genome for RNA sequences that are complementary to one of the two strands of the siRNA duplex. One strand of the siRNA (the sense or passenger strand) is lost from the complex, while the other strand (the antisense or guide strand) is matched with its complementary RNA. In particular, the targets of siRNA-loaded RISC are mRNAs presenting a perfect sequence complementarity. When siRNA-mediated silencing occurs, after the cleavage, release and degradation of the products, RISC-complex can interact with other molecules from the mRNA pool [30]. It was shown that endogenously expressed small RNA molecules, named microRNAs (miRNAs), elicit gene-silencing in higher organisms [31, 32]. MiRNAs are not involved in the pathway that leads to the production of a protein, instead they regulate the expression of mRNAs. The targets of miRNA-loaded RISC are mRNAs presenting a perfect sequence complementarity with nucleotides 2-7 in the 5' region of the miRNA (the so-called seed region), and additional base pairings with its 3'-region. RISC mediates downregulation of gene expression by cleavage or translational inhibition of the target mRNA. The choice between these two modes of action is dictated by the degree of complementarity between miRNA and its target. Near-perfect complementarity produces cleavage of the mRNA, as well as siRNAs, followed by its complete degradation, whereas partial complementarity causes translational inhibition [33]. In vertebrates, most of the time, the consequence is an inhibition of translation. In addition to repressing translation, miRNA interactions can promote the deadenylation or decapping, leading to rapid mRNA decay [34]. MicroRNA target sequences are usually located in the 3'-UTR of mRNAs. A single miRNA is expected to target at least 100 transcripts from various genes, and one mRNA may be targeted, at its 3'-end, by different miRNAs [35]. In mammalian cells, long double strand RNAs (> 30 nt) induce immune responses [36] resulting in global gene silencing and in certain cases cell death [37, 38]. This fact prevented the application of RNAi to mammalian cells until it was discovered that 21-mer siRNA duplexes were capable of effecting potent and specific gene knockdown without inducing of the interferon response [27]. However, the immunogenic activity of short RNA duplexes is more significant than it was originally thought. Recent findings report off-target effects resulting from immunostimulation caused by dsRNAs that are shorter than 30 bp [39-46]. Immune response activation by short RNAs is a complex process. Briefly, RNAs are recognized by three main types of immunoreceptors: Toll-like receptors (specifically TLR3, TLR7 and TLR8), protein kinase R (PKR) and helicases such as RIG-I and MDA5. Recognition by these receptors can lead to a number of cellular responses including release of cytokines and changes in gene expression [42]. The receptors involved are expressed on cell surfaces (TLR3) [47], in endosomes (TLR3/7/8) [48-50] and in the cytoplasm (RIG-I, MDA5, PKR) [49]. Given its reliability and ease of use, RNAi has become the most widely used

technology in functional genomic studies *in vitro* and in several model organisms. To translate this potential into a broad new family of therapeutics, it is necessary to optimize the efficacy of the RNA-based drugs. As discussed in this review, it might be possible to achieve this optimization using chemical modifications that improve, just like for ASOs, their *in vivo* stability, cellular delivery, biodistribution, pharmacokinetics, potency, and specificity. Previous experience with antisense oligonucleotides is directly relevant to the clinical progress of siRNAs. Indeed, siRNAs and antisense oligonucleotides have more similarities than differences. ASOs and siRNAs are natural phosphodiester compounds, they are short (~20 base) nucleic acids and are large, negatively charged molecules [51, 52]. Similar protocols exist for the large-scale synthesis of nucleic acid drugs in amounts needed for clinical trials. Such as ASOs, siRNAs do not readily cross the cellular membrane because of their negative charge and size [53]. There are biological barriers that stand between initial administration of oligonucleotides and their final actions within cells. One the first biological barriers encountered by administered naked ASOs and siRNAs is represented by the nuclease activity in plasma and tissues. Single-stranded nucleic acids are rapidly degraded in serum or inside cells. Double-stranded nucleic acids, including siRNAs, are more stable than their single stranded counterparts, but are still degraded and must be protected from nuclease attack [54]. The systemic administration of ASOs and siRNAs results in primary localization to liver while their local administration is an option for many diseases. They have a common target: siRNAs are also intended to bind by Watson-Crick base-pairing complementary mRNA, and they can induce target RNA destruction. Both ASOs and siRNAs can incorporate chemical modifications in order to improve their *in vivo* properties. SiRNAs and ASOs, because of synthetic nature can be recognized as "foreign" by the innate immune system. In addition, for siRNAs both strands have the potential to trigger unwanted side effects [55 and cited references]. Finally, antisense and siRNA molecules are involved in ongoing testing in multiple clinical trials. There are also significant differences between antisense oligonucleotides and siRNAs. siRNAs contain two strands, which must be synthesized separately and then hybridized. Each strand has a molecular weight of approximately 7000 Da and the large size, as well as the charged backbone, discourage easy passage of RNA duplex through cell membranes. The target recognition of siRNA is mediated through the RISC complex. Moreover, very few molecules of siRNA are needed to inhibit gene expression [56]: siRNAs co-opt a natural silencing pathway and the RISC proteins facilitate efficient recognition of target sequences by siRNAs, whereas ASOs must find their targets unassisted. Because endogenous duplex RNAs control important physiologic processes, introducing synthetic RNA may perturb this native machinery. ASOs have one strand that can block RNA by a steric mechanism or form a DNA-mRNA hybrid that recruits RNase H. Other mechanisms described for the ASOs include interference with mRNA processing and transport, and formation of a triplex directly with DNA into nucleus (Triplex Forming Oligonucleotides, TFO) [57, 58]. The mechanism by which antisenses exert their effects, largely depends on their structure and chemistry: morpholino, PNA and 2'-O-alkyl modi-

fied ASOs are capable of acting by other mechanisms e.g. they can inhibit intron excision, a key step in the processing of mRNA. Splicing occurs into nucleus during the maturation step of pre-mRNA and can be inhibited by hybridization of an oligonucleotide to the 5' and 3'-regions involved in this process. ASOs can act like splicing regulator while siRNAs cannot target nuclear RNAs or introns [59]. When chemical modifications are introduced into siRNAs it is important to underline that modified siRNAs work through an only pathway and must interact with a number of different cellular proteins. Many or all of these proteins may be sensitive to changes in siRNA structure caused by the modifying group. SiRNA duplexes have been modified in a wide variety of ways and the reported results seem to contradict one another.

The better and recent understanding of both ASOs and siRNA properties have raised an important discussion about the future of antisense and siRNA in clinic applications. But the theoretical discussion of the relative value of siRNA and antisense oligonucleotides can only be resolved by clinical development. In fact, the widespread and rapid adoption of siRNA in cell culture, due to the potency of the natural mechanism of RNAi, has supported the conclusion that siRNA strategy has advantages for gene silencing respect to ASOs approach. However, further work will be needed to improve *in vivo* potency, biodistribution and toxicity of siRNAs. Certainly the future of siRNA as therapy will involve advanced medicinal chemistry to improve both biodistribution to tissue and uptake by specific cell types to reduce off-target effects and enhance the efficiency of silencing by those duplexes that ultimately reach target cells.

A logical starting point for the reaching of this goal has lied with the previous generation of research on the properties of ASOs. Because of this head start, progress that required 10-15 years for antisense oligonucleotides has required just 2-4 years for siRNA. The lesson learned from antisense drug clinical development has provided insights into how to choose target genes, into experimental pitfalls and the criteria for designing well-controlled experiments in drug based RNAi. In conclusion, the chemical modifications that have been developed to optimize the properties of ASOs, have had a major impact on the properties and efficacy of siRNAs.

Functional Anatomy of siRNA: The Rationale for Chemical Modification of siRNAs

On the basis of analyses of a small number of silenced genes in mammalian cells, a set of empirical guidelines have already been proposed for siRNA design. These rules require the generation of RNA duplexes containing a 19-nt duplexed region, symmetric 2-3-nt 3' overhangs, 5'-phosphate and 3'-hydroxyl groups targeting a region in the gene to be silenced. In most cases, only one of the two strands of a siRNA enters the RISC with high efficiency. This so-called guide strand is selected on the basis of thermodynamic bias and is responsible for mediating knockdown of target genes. The targeted mRNA is then cleaved by activated RISC at a single site that is defined with regard to where the 5'-end of the antisense strand is bound to the mRNA target sequence [60, 61]. For RNAi-mediated mRNA cleavage and degradation to be suc-

cessful, the double helix of the antisense-target mRNA duplex must be in the A-form [62]. On the basis of these criteria, an effective siRNA has high stability (high content of G or C) at the 5'-terminus on the sense strand, in order to block incorporation of sense strand into RISC, lower stability at the 5'-terminus of antisense (AU-richness) to promote incorporation of antisense strand into RISC, and at the cleavage site (U at position 10 of sense strand), to help RISC-AS-mediated cleavage of mRNA and the RISC-AS-complex release. Effective gene silencing by RNAi machinery requires complete understanding of the elements that influence siRNA functionality and specificity. These include, as well as only just described structural and sequence features required, sequence space restrictions that define the boundaries of siRNA targeting. The sequence space is defined as the region of a gene that can be targeted for effective gene knockdown. Actually, targets are limited to regions of the gene that are transcribed: the 5' and 3' UTRs (untranslated regions) regions within 75 bases of the start codon and sequences with > 50% C+G content and the ORF (open reading frame). Many computer programs are available for identifying the optimal target sequences for a given gene [63, 64]. The abundance of 3'UTR seed complements in off-target gene [65] suggested that, in the future, bioinformatic techniques that minimize off-target effects may be a standard component in siRNA design. There are multiple types of chemical modifications that are introduced into siRNAs in order to enhance thermodynamic and nuclease stability, to increase the half-life of the siRNA duplexes *in vivo*, to improve the biodistribution and pharmacokinetic properties of siRNAs, to target small RNAs to certain cell types and to improve the potency of siRNAs in terms of target binding affinity, and finally to reduce off-target effects. The synthesis and the properties of siRNAs bearing phosphodiester, carbohydrate and nucleobase modifications have been revived extensively [66, 67]. Consequently, this review particularly covers the recent advances in this area that have been published over the past recent years, with particular attention on modified structure, architecture and conjugated siRNAs.

CLASSICAL AND NOVEL CHEMICAL MODIFICATIONS OF siRNAs

The current and most popular approach in the synthesis of oligoribonucleotides is based on phosphoramidite chemistry which was originally developed for the synthesis of DNA oligonucleotides by Beaucage and Carutens in the early 1980 [68]. Most siRNAs used in research today are made by chemical synthesis using phosphoramidite building blocks as single-stranded form. This approach permits incorporation of a wide variety of modifications into the siRNAs. A rational design of effective chemically modified siRNA must consider as general principle that the two strands of a siRNA function differently and as practical hint that the nucleotides are different according to positions and nature. It has been recently demonstrated that novel sense and/or antisense strand modifications can greatly enhance siRNA specificity targeting, distinct and separate the events in interference cycle [69, 70].

In this section, we will briefly review the most significant siRNA modifications described in literature, drawing attention to those that have improved siRNA performance. We

will point out the useful and universal modifications as well as the most innovative modifications to move siRNA toward the clinic.

Base Modifications

Base-modified siRNAs (Fig. 1) have been investigated so far to a very limited extent [71, 72] despite the central involvement in target recognition of nucleobases. The report by Parrish *et al.* describe induction of RNAi in *C. elegans* by dsRNAs containing 4-thiouridine, 5-bromo-, 5-iodo-, 5-(3-aminoallyl)-uridine, or inosine [71]. While these experiments did not demonstrate any remarkable differences in silencing activity regardless of the introduced modifications, the work by Chiu and Rana [72] describes reduced silencing activity of duplexes containing 5-bromouridine, 5-iodouridine, 2,6-diaminopurine, and N-3-methyl-uridine. The patent literature also claims several base-modified nucleosides as components of small interfering nucleic acids, albeit no details are provided with regard to properties of such modified siRNAs [73]. In other studies [74], Sipa *et al.* evaluated the thermodynamic stability and gene-silencing activity of base-modified siRNAs containing three modified nucleosides: 2-thiouridine (s^2U), pseudouridine (ψ), and dihydrouridine (D). They demonstrated that these three naturally occurring modified nucleosides, when introduced into a siRNA duplex, modulate its silencing potency. The extent of this effect depends on the modification position and is most advantageous when the s^2U , ψ , and D nucleosides participate in an enhancement of the siRNA duplex asymmetry. More recently, Kool *et al.* have evaluated steric and stability effects on RNAi activity by use of siRNAs modified with propynyl and methyl functionalities at the C-5 position of pyrimidine nu-

cleobases [75]. Their results suggest that at the 5'-half of the guide RNA, large increases in the size of the functionality at the position 5 of pyrimidine nucleobases can be detrimental to RNAi activity. Despite the strong stabilizing effects of the 5-propynyl modification, this bulky substitution caused negative effects in RNAi activity, probably due to disruption of interactions in the major groove of the active RISC complex. However, the smaller 5-methyl substitution did not adversely affect gene silencing activity. Surprisingly, some atypical base structures have been used in siRNA [76]. SiRNA duplexes containing the 2,4-difluorotoluy ribonucleoside (rF) were synthesized to evaluate the effect of non-canonical nucleoside mimetics on RNA interference. 5'-Modification of the guide strand with rF did not alter silencing relative to unmodified control. Internal uridine to rF substitutions were well-tolerated and thermal melting analysis showed that the base pair between rF and adenosine (A) was destabilizing relative to a uridine-adenosine pair, although it was slightly less destabilizing than other mismatches. siRNAs with the rF modification effectively silenced gene expression and offered improved nuclease resistance in serum. Base modifications can also help to reduce immune activation: modification with pseudouracil or 2-thiouracil prevents the RIG-I-mediated immunostimulation due to a 5'-triphosphate [40], and 5-methyl-C, N⁶-methyl-A pseudouridine prevents recognition of RNA by TLR3, TLR7 and TLR8 [77].

Sugar Modifications

The siRNA modifications on the sugar moiety are the most widely described (Fig. 2). The milestone study reporting the pioneering analysis of chemical modified siRNA

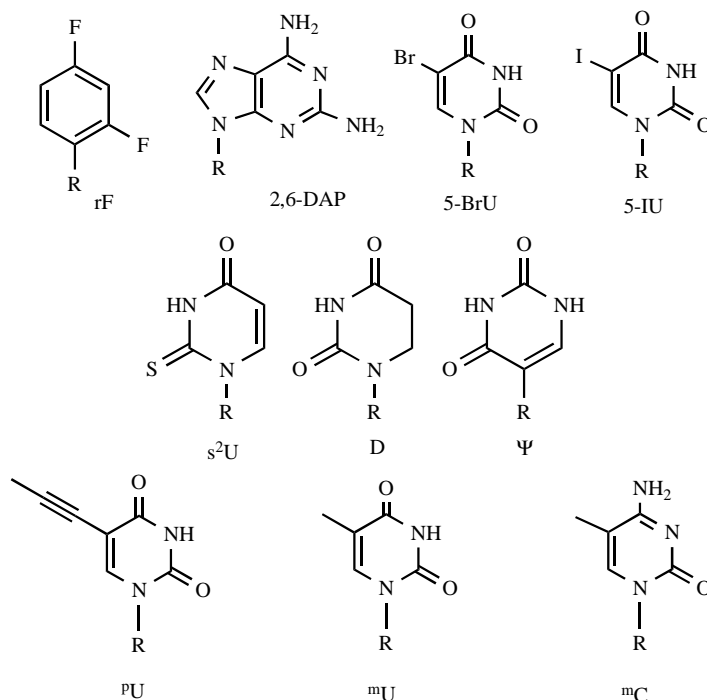


Fig. (1). Nucleobase modifications in siRNA. rF: 2,4-difluorotoluy ribonucleoside; 2,6-DAP: 2,6-diaminopurine; 5-BrU: 5-bromouridine; 5-IU: 5-Iodouridine; s^2U : 2-thiouridine; D: dihydrouridine; ψ : pseudouridine; pU : 5-propynyluridine; mU : 5-methyluridine; mC : 5-methylcytosine

function [72] showed that while A-form helix is required for the mechanism of RNAi, the 2'-OH function was not an essential requirement for interfering activity. This study strongly indicates that RNAi machinery does not require the 2'-OH for recognition of siRNAs and catalytic ribonuclease activity of RISC does not involve the 2'-hydroxyl group of guide antisense of RNA. Therefore, the 2' position of ribose has been heavily modified. Modification of the 2' position of the ribose can increase T_m of duplex and confers varying degrees of nuclease resistance. It may also provide protection from immune activation. Briefly, we list here the most extensively studied 2' modifications in siRNA effective molecules. 2'-fluoro (2'-F) was among the first modifications that were independently tested by several groups and they result well tolerated in siRNA applications [71, 72, 78-80]. 2'-O-methyl (2'-O-Me) was extensively tested in siRNA by different groups since 2003 [72, 79-87]. Partial modification of siRNA strands with 2'-O-Me (less than 8 nt) led to improved performance of siRNA in cell [86], but siRNAs completely made of 2'-O-Me lost interference activity [72]. Position-specific 2'-O-methylation of siRNAs reduces "off target" transcript silencing [69]. The sharp position dependence of 2'-O-Me modification contrasts with the broader position dependence of base-pair within the seed region, suggesting a role for position 2 of the guide strand distinct from its effects on pairing to target transcripts. Besides aforementioned stability, 2'-O-Me siRNAs exhibit increased potency [88] and reduced immunostimulatory activity [41, 89], with the exception 2'-O-Me cytidine [90]. Introduction of 2'-O-Me uridine or guanosine into one strand of the siRNA duplex generates noninflammatory siRNAs. 2'-O-Me siRNAs, containing less than 20% modified nucleosides and targeting apolipoprotein B (apoB), can mediate potent silencing of its target mRNA and cause significant decreases in serum apoB and cholesterol. This is achieved at therapeutical viable siRNA doses without cytokine induction, toxicity or off target effects [90]. It was been showed that 2'-O-Me RNAs act as potent antagonists of immunostimulatory RNA and they are able significantly to reduce both interferon- α , and interleukin-6 induction by small-molecule TLR7 agonist loxoribine in human peripheral blood mononuclear cells, murine Flt3L dendritic cells and *in vivo* mice [89]. Further, 2'-O-methylation selectively protects the particularly vulnerable 5'-end of the guide strand against exonucleases in human blood serum [91]. Specific chemical modification thus resolves the asymmetric degradation of the guide and sense strands, which is inherent to the thermodynamic asymmetry of the siRNA termini as required for proper utilization of the guide strand in RNAi pathway. The 2'-O-(2-methoxyethyl) residues (2'-O-MOE) can be incorporated into siRNAs much like 2'-O-Me or 2'-F. The 2'-MOE modification in the antisense strand resulted in less active siRNA constructs regardless of placement position in the construct. The incorporation of modified residues in the sense strand did not show a strong positional preference [85]. This modification is not generally available for use but a successful gene target *in vivo* is reported [92]. Likewise, 2'-O-allyl modification is well tolerated in the 3'-overhangs but not at most positions in siRNA duplexes [87]. Another class of 2' modification is 2'-deoxy-2'-fluoro- β -D-arabino nucleic acids (FANA). FANA nucleotides have the stereochemistry at the 2' posi-

tion inverted relative to that found in 2'-O-Me and 2'-F RNA. siRNAs that have completely FANA-substituted sense strands are 4-fold more potent than unmodified siRNAs and have a longer half-life in serum. The antisense strand is less tolerant of the FANA modification [93, 94]. DNA itself represents a further modification in which there is not the electronegative OH-group in 2' position. The use of DNA in 3'-overhangs of synthetic siRNAs is well known [27]. Substitution with double stranded DNA in the 8-bp region at the 5'-end of the guide strand gives active duplexes with reduced off-target effects [95]. Interestingly, 2'-deoxy bases have recently been reported to also block immune detection, particularly T or dU bases [96]. Locked nucleic acids is a family of conformationally locked nucleotide analogues containing a methylene bridge between the 2' and 4' carbons of the ribose ring [97]. This linkage constrains the ribose ring, "locking" it into the 3'-*endo* conformation close to that formed by RNA after hybridization. The incorporation of LNA in siRNAs enhances serum half-life substantially and a few LNA units at the 5'-end of the sense strand improves thermodynamic bias, reduces immunostimulatory and off-target effects [39, 98]. Particular LNA based siRNAs were designed and developed against the highly structured 5'-UTR of coxsackievirus B3 (CVB-3) [99]. The best siLNA improved viability of infected cells by 92% and exerted good antiviral activity in plaque reduction assays. 2'-O-(2,4-dinitrophenyl) RNAs were among the 2' modification that have been more recently tested in the context of RNAi. DPN-ssRNA and DPN-siRNA outperform previous generation of antisense and unmodified siRNAs in gene silencing potency, stability, hybridization affinity and delivery maintaining specific binding properties. Poly-2'-O-(2,4-dinitrophenyl)-oligoribonucleotide (DNP-RNA) represents a promising new gene silencing platform. DNA-RNAs with a DNP nucleotide molar ratio of about 0.7 can spontaneously cross viral envelopes and mammalian cell membranes. DNP-RNAs are resistant to degradation by ribonucleases, including RNases A, B, H, S, T 1 and 2 and phosphodiesterase I and II [100] and hybridize with their complementary RNA. Interestingly, 2'-O-DNP modified siRNA and its single stranded counter part have shown similar silencing activities [101-103]. The ring oxygen has also been modified: 4'-thio modified nucleosides have a sulfur atom at the 4 position of the ribose ring. Hoshika *et al.* report that 4'-thio RNAs form a thermally stable duplex with the complementary RNA and show high nuclease resistance, despite the 2'-OH groups. In addition, structural analysis by CD spectra indicates that the 4'-thioRNA:RNA duplex adopts an A-form conformation as does the natural RNA duplex. 4'-S-RNA is very well tolerated near the termini of siRNA duplexes [104-106]. Modifications at the sense-strand preserve RNAi activity of modified siRNA, except for full modification with 4'-thioribonucleoside and the activity of siRNAs modified at the antisense-strand was dependent on the position and the number of modifications with 4'-thioribonucleosides [104]. For example, the 5'-end of the antisense strand could be modified with a few 4'-S-RNA inserts, without significant loss of potency [105]. Furthermore, Dande *et al.* describe that significant improvements in siRNA activity and plasma stability were achieved by judicious combination of 4'-thioribose with 2'-O-Me and 2'-O-MOE modifications. It is

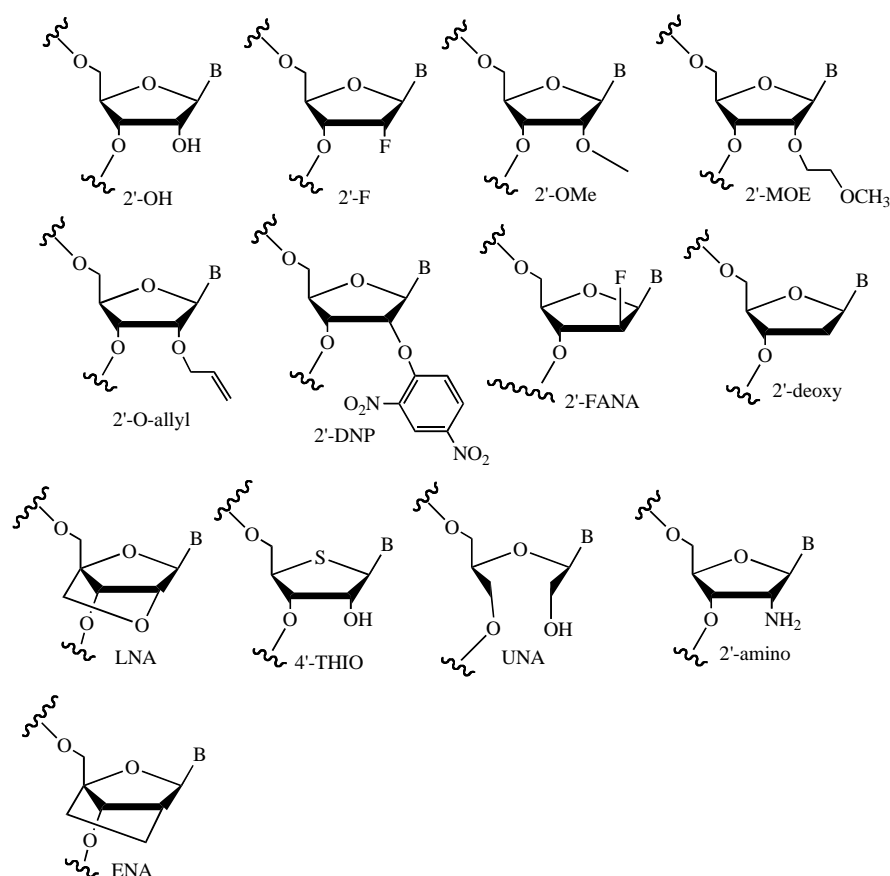


Fig. (2). Sugar modifications in siRNA. 2'-F: 2'-fluoroRNA; 2'-OMe: 2'-O-Methyl RNA; 2'-MOE: 2'-O-(2-methoxyethyl)RNA; 2'-O-allyl: 2'-O-allyl RNA; 2'-DNP: 2'-O-(2,4-dinitrophenyl) RNA; 2'-FANA: 2'-deoxy-2'-fluoro- β -D-arabino nucleic acids; 2'-deoxy: 2'-deoxyribonucleotide; LNA: locked nucleic acid; 4'-Thio: 4'-thioribonucleoside; UNA: unlocked nucleic acid; 2'-amino: 2'-amino RNA; ENA: ethylene-bridge nucleic acid.

also possible to combine 4'-thio substitution with FANA nucleotides, and 4'-S-FANA-modified siRNAs have been shown to possess potencies for silencing in cell culture that were similar to unmodified siRNAs [95]. 2',3'-Secunucleosides, also known as unlocked nucleic acids [107], are a class of modifications whereby the ribose ring becomes acyclic by removing the bond between the 2' and 3' carbon atoms [108-111]. This change in sugar structure increases the nucleic acid analog flexibility, and, when incorporated into duplexes, can reduce duplex stability as observed by lower thermal denaturation temperatures [74, 108, 110, 112, 113]. Recently, UNAs were incorporated into the guide strand of siRNAs designed against GFP, at positions 3, 6, 12 and 18 and found to have target specific mRNA degradation [113]. In a recent study, it has systematically evaluated UNA substitutions throughout the guide strand of a siRNA targeting the ApoB transcript. Modifying at positions 1-3, 9-12, 14 and 16, siRNA-mediated mRNA degradation in cells was decreased when compared to an unmodified siRNA [107]. After chemical addition of phosphate at 5'-OH of the UNA 1 modified siRNA the interference activity was recovered in cell and in mice [107].

Other 2'-modifications such as 2'-amino [99], 2'-O,4'-C-ethylene thymidine (eT), which is a component of ethylene-

bridge nucleic acids (ENA) [114], have also been preliminarily tested within the siRNA scheme.

Backbone Modifications

The simplest approach to increase nuclease stability has been to directly modify the internucleotide phosphate linkage (Fig. 3). Replacement of a non-bridging oxygen with sulphur (PS), boron (boranophosphate), nitrogen (phosphoramidate), or methyl (methylphosphonate) groups provides nuclease resistance and has been used to stabilize single-stranded antisense oligonucleotides. Phosphorothioate (PS) modifications can be easily placed in the RNA duplex at any desired position and will prolong the life of the duplex when exposed to serum or other nuclease sources. Limited PS modification preserves potency of the siRNA; however, extensively modified duplexes show reduced potency and/or toxicity [73, 78, 80, 87]. Phosphorothioate modified nucleic acids are sulfated polyanions that are "sticky" and can not specifically bind to a variety of cellular proteins, potentially causing unwanted side effects [115], so it may be preferable to limit the use of this modification to the ends of the siRNA where exonuclease resistance is probably most important. Another backbone modification tested for siRNA is the boranophosphate linkage. Boranophosphate modification makes the efficacy of RNA oligos 300 fold higher than that

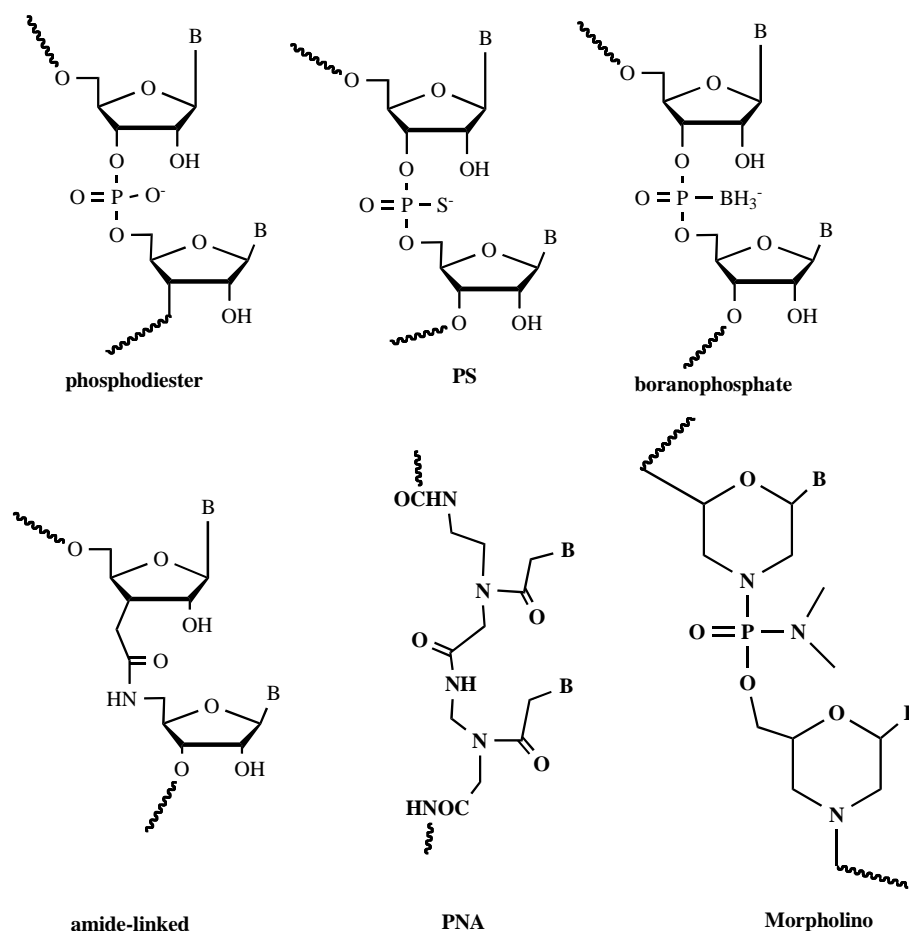


Fig. (3). Backbone modifications in siRNA. PS: phosphorothioate linkage; PNA: peptide nucleic acid.

of native form and is two fold more stable than the classic phosphorothioate [82] especially when incorporated at C position. Advantage of the boranophosphate is its low cytotoxicity in comparison to phosphorothioate. Uncharged nucleic acid mimics are represented by Peptide Nucleic Acids (PNA) [21, 116] and morpholino oligomers [117]. PNA and morpholino derivatives can be chemically introduced in RNA oligomers creating modified siRNAs with improved performances [21, 116, 117, 118]. SiRNAs modified with morpholino nucleoside analogues [118] were synthesized and their biological properties were examined in details. The gene silencing abilities of modified siRNAs were correlated to the positions of the modifications, some of which appeared to be more potent than the native siRNA. The 3'-end modification improved the stability of siRNAs in serum significantly. Furthermore, the dose-response and timecourse experiments demonstrated that the siRNAs with 3'-end modification have potent gene silencing activity at lower concentration and prolonged action time. It is noteworthy to underline that certain modification patterns can alter the potency of the siRNA and these effects vary with sequence (or site of modification introduction). Besides, Davis *et al.* demonstrated that a number of bulkier 2'-sugar and backbone modifications correlate with poor activity as siRNA passenger strands and with excellent activity as "anti-miRNA oligonucleotides" (anti-miRNA ASOs) [119]. Further studies [120] show

there are multiple mechanisms of miRNA inhibition by ASOs without degradation of the miRNA. It was also reported a different AMO approach (anti-miRNA antisense oligodeoxyribonucleotide) in which multiple antisense units are engineered into a single unit that is able to simultaneously silence multiple-target miRNAs (the multiple-target AMO or MTg-AMO) [121]. This approach may find its broad application for exploring biological processes involving multiple miRNAs and multiple genes.

Architecture Modifications

Chemical synthesis provides a useful tool to modify also RNA duplex architecture (Fig. 4). Alternative designs are in use for both research and drug development including asiRNA [122, 123], sisiRNA [124], dgrNAs [125], ss-siRNA [79], short-hairpin-siRNA [126] and dumbbell-shaped RNA [127]. Some of these compounds directly load into RISC whereas others are substrates for DICER and are processed into shorter species before RISC loading. Doré-Savard *et al.* have demonstrated that low dose of 27-mer Dicer-substrate siRNA (DsiRNA) silences targets related to CNS disorders such as pain states [128]. Particularly, DsiRNA formulated with the cationic lipid i-Fect, was highly efficient in reducing the expression of the neurotensin receptor-2 in rat spinal cord through intrathecal administration. Further, blunt-end 20-bp siRNA with full 2'-O-Me

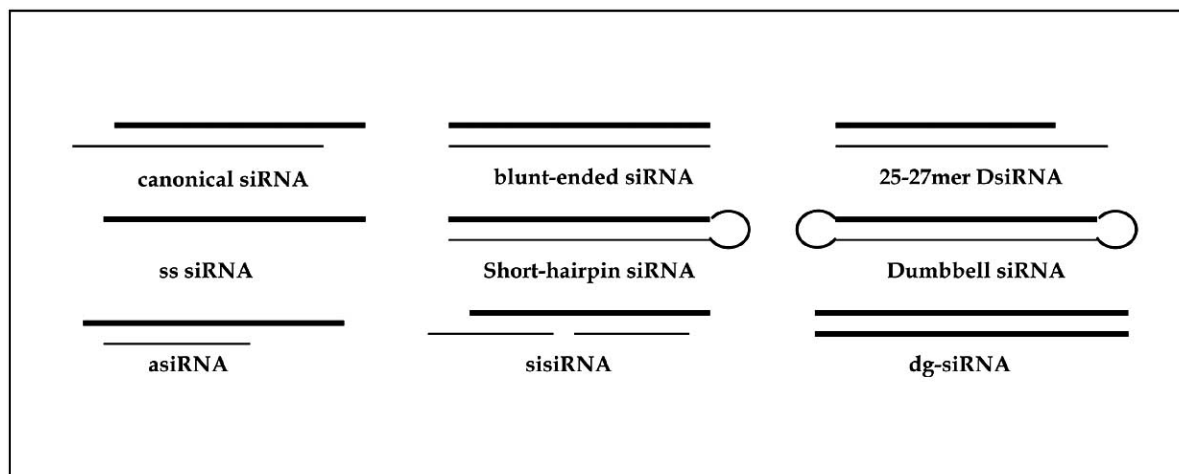


Fig. (4). Duplex architecture modifications. (Bold line: antisense strand, thin line: sense strand).

modified sense strand exhibits better performance when compared to the analogous 19-bp 3'-TT against PTEN target [129]. Asymmetric siRNAs (aiRNAs) are siRNAs with deletions at the passenger and/or guide strand that preserve an efficacious silencing activity [122, 123]. AiRNAs were designed to determine the essential region of the dsRNA helix needed to induce effective RNAi and to reduce the off-target effects of the sense strand. In the study published last year by Sun *et al.* [122], the authors designed asymmetric siRNAs of various length and found that silencing required at least 19-nt of complementary target sequence. The observed activity suggested that aiRNA characterized by long guide and short passenger stands, represents a novel and effective scaffold structure for siRNAs. They also found that aiRNAs, because of their short 15-nt passenger strand, reduce the incorporation of the sense strand into RISC causing less off-target silencing compared with standard siRNAs. Like Sun *et al.*, Chu and Rana identified a short (16-nt) passenger strand as a RNAi trigger [123] but they found that deletions were also tolerated by the guide strand and even better when both strands of siRNA were of 16-nt. Also interesting in this frame, is the study from Bramsen *et al.* [124] showing that intact sense strand is not required for efficient RNAi. The "three stranded construct" termed small internally segmented interfering RNAs (sisiRNA) and composed of an intact antisense strand complemented with two shorter 10-12-nt sense tracts, is highly functional. In sisiRNA only the guide strand is recognized and loaded by RISC thereby significantly reducing unwarranted off-target effects. Precedently, Hossbach *et al.* [125] eliminate the passenger strand and synthesize siRNA duplexes consisting of two guide strands (dgRNAs). This strategy needed further inside but the crucial benefit is to minimize the non-specific effects from the passenger strand. Single-stranded antisense siRNAs (sssiRNAs) are also effective in silencing genes in HeLa cells, especially when 5'-phosphorylated [130]. Successively, Hollen *et al.* found that maximum depletion of target mRNA occurs faster with antisense siRNA than with double strand siRNA, possibly due to the direct incorporation into RISC requiring less time to exert its activity [79]. Short hairpin-type duplexes (shRNAs) are made from a single strand and represent an optimized Dicer substrate [126]. Chemically synthesized

shRNAs with 29-base-pair stems and 2-nt 3'-overhangs were converted, almost quantitatively, into siRNAs comprising the 22 bases at the 3'-end of the stem by Dicer. It was found that synthetic shRNAs are more potent than siRNAs achieving maximal inhibition of target gene at lower concentration. Because of their high stability, intramolecular hairpin structure with various loop units has been reported. Hairpin oligonucleotides can be obtained by introduction of non-nucleosidic molecules at the loop position such as cholane-3,24-diol moiety and 18 β -glycyrrhetic acid derivative [131, 132]. Intramolecular structures with foreign loop molecules showed better properties such as structural stability [133], nuclease resistance [134] and therapeutic ability [135]. The shRNAs bearing similar modified loop could suppress their target gene expression without losing their RNAi efficiency. Dumbbell-shaped nanocircular RNAs withstand enzymatic degradation and offer prolonged RNAi activity because of the shape of the molecule [127]. As shRNAs, circular RNAs can be cleaved by Dicer enzyme to form and slowly release dsRNAs. Maximum suppression was induced by dumbbell with a stem length of 23 and 27-nt.

Modifications to Overhangs and Termini

The extremities of the siRNA duplex are critical determinants of its capacity to interfere with the unwinding of the siRNA duplex, incorporation of the siRNA into RISC and/or the rate of target cleavage and product release. The overhangs of the sense and antisense strands of siRNA can be modified in various ways. Synthetic siRNAs usually have two deoxy-thymidines at the 3' termini of both strands. However, RNA units also work well [136]. In mammalian cells synthetic double-stranded siRNAs without specific nucleotide overhangs are highly efficient in gene silencing [86]. When the 3' end of the antisense strand and both termini of the sense strand were modified with either an inverted deoxy abasic residue or an amino group attached through a 6-carbon linker no reduction in activity was observed. Blunt-ended siRNAs 19-mers [86, 129] blunt 25-mers and 27-mers [33, 137], have also been tested. An expanded set of synthetic RNA duplexes of varying length containing 3' and/or 5' overhangs or blunt ends were tested by Kim *et al.* for their relative potency [33]. RNA duplexes 25-

30-nt in length can be up to 100-fold more potent than corresponding 21-mer siRNA without interferon induction. However, they are more immunogenic than duplexes with 3'-overhangs. It is important to keep the length below 30-nt to avoid triggering the interferon response [36]. Blunt-ended duplexes are more resistant to 3'-exonucleases and one study reports a greater tolerance to chemical modifications in combination with blunt-ended duplexes [129]. Sticky overhangs (ssiRNAs) enhance siRNA-mediated gene silencing. siRNA "gene like" with short complementary A₅₋₈/T₅₋₈ 3'-overhangs increase the stability of siRNA with non-viral vectors in delivery strategy [138]. Free ssiRNAs in the cytoplasm doesn't induce antiviral responses and show interference activity. As it has been shown that in the RNAi machinery, the 2-nt 3'-overhang are accommodated into an hydrophobic binding pocket in the PAZ domain, Ueno *et al.* have recently synthesized siRNAs possessing aromatic compounds at their 3'-overhangs. These modified and nuclease-resistant siRNAs are more potent than the siRNAs without the 3'-overhangs and their silencing activity is almost equal to those of siRNAs with natural nucleosides at the 3'-overhang region [139]. Recently, it has been synthesized RNA-3'-PNA chimeras and their effects on siRNA functioning and stability were tested [140]. It was shown that the PNA introduction in siRNA is compatible with the RNAi machinery, because all the PNA-modified siRNAs can efficiently mediate specific gene silencing in mammalian cells. Furthermore, the modification on the sense strand of siRNA resulted in an increased persistence of the activity, whereas modification on both strands resulted in enhanced nuclease resistance in serum. Previously, it has also been modified the 3'-overhang region with a partial replacement of phosphodiester linkage: siRNAs having thymidine dimers consisting of a carbamate or urea linkage at 3'-end provide a gene silencing activity 78 and 37 times more efficiently than that with the natural linkage respectively [141]. As well as the others 3'-termini modified siRNAs, these novel siRNAs result more resistant to exonuclease degradation.

5'-phosphorylation status of the siRNA strands acts as an important determinant for strand selection [142]. 5'-O-methylated siRNA duplexes were examined for their biases in siRNA strand selection. 5'-O-methylation of sense strand of siRNA duplexes controls guide strand selection and targeting specificity without affecting interfering activity [137]. The loss of silencing activity of duplex siRNAs in which only the antisense or both strands were 5'-O-methyl-modified was observed. These results strongly indicate that chemical phosphorylation of the 5'-end of the antisense strand is suggested particularly when the strand is modified.

Conjugations

The concept behind siRNA conjugates is simple. One part of the conjugate is dsRNA which provides specificity for the target mRNA and the other part of the conjugate is a foreign molecule designed to improve thermodynamic and nuclease stability, biodistribution and pharmacokinetic profiles of siRNAs, specific cell target. Increased binding to serum proteins might extend the circulation time and tissue distribution of conjugate, while compounds that bind to cell surface proteins can facilitate specific tissue targeting. It is important to underline that the site of conjugation must be

carefully selected to not compromise the siRNA performance. The safest sites for conjugation are the 5' and 3'-end of the sense strand. Theoretically, most of the same conjugations are also applicable to the 3' and 5' termini of the antisense strand of the siRNA but the 5'-end of the antisense strand is much more restricted area. Anyway, these positions are considered off-limits because of the introduction of chemical moieties in these regions interferes with 3'-overhang recognition by the PAZ domain [143, 144] and/or 5' phosphate recognition by the PIWI domain of RISC [145].

Along these lines, a plethora of works has already been reported in the medicinal chemistry of ASOs that have been directly applied to siRNAs. Various molecules can be covalently linked to the ends of synthetic siRNA duplexes and can include groups that increase enzymatic stability (e.g. inverted deoxy abasic residue) [41, 146] that allow biochemical studies (fluorescent dyes [78] or biotin [62]), that realize specific tissue targeting [147]. Certain terminal conjugates (cholesterol, lithocolic acid, lauric acid or long alkyl branched chains, TAT peptide) [148, 149] have been reported to improve cellular uptake without perturbing gene silencing activity. Most of the above-mentioned conjugations are not tolerated at 5'-end of the antisense strand; it is surprising the relatively bulky fluorescein is somehow tolerated in this site [78].

Considered by many as the most important remaining hurdle to the widespread therapeutic use of RNAi, selection and formulation of effective siRNA with an biocompatible delivery system is necessary for improving targeted cell uptake. Although transfection efficiency of siRNAs into cultured cells is satisfactory for most *in vitro* applications, its therapeutic applications *in vivo* present an altogether more daunting challenge. Ideally, a delivery system would be capable of binding siRNA in a reversible manner to ensure subsequent release of the siRNA in target cells, protect siRNAs from nuclease degradation in circulation, escape from endosomal compartment, be biocompatible and avoid rapid clearance by the liver and kidney. The success of siRNA therapeutics is highly dependent on the delivery vector which can be generally categorized into viral and non-viral vector [150]. Viral vectors are highly efficient but some show a limited loading capacity, are difficult to produce in large scale and most importantly arise severe safety risks due to their oncogenic potential and inflammatory and immunogenic effect. To overcome these limitations, non-viral vectors have been developed as a promising alternative for gene delivery. Several types of synthetic vectors have been investigated for gene-silencing applications. siRNA formulations include electrostatic complexes of siRNA with cationic lipids (lipoplex) or polycations (polyplex) [151-153], lipopolyplexes [154-156], liposomes encapsulating siRNA [157]. A common theme amongst these vectors is their positive charge, which facilitates both complex formation with the polyanionic nucleic acid and interaction with the negatively charged cell membrane. However, some charged molecules can disrupt such complexes before they reach the target cell and can often result toxic. A full description of these complexes is beyond the scope of this Review that focuses on the description of latest siRNA chemically conjugated to a variety of bioactive molecules, such as lipids, polymers, pep-

tides, aptamers and inorganic nanostructured materials with or without forming nanoconstructs (Table 1) [158-160].

Most of the conjugates employ cleavable linkages including acid-labile and reducible bonds between siRNA and conjugation partner to facilitate the release of intact siRNA inside cells. The acid-labile and disulfide linkages are cleaved in the acidic endosome compartments and the reductive cytosolic environment, respectively.

Conjugation with lipids may enhance siRNAs uptake by an increased membrane permeability or *via* receptor-mediated endocytosis. Cholesterol was covalently conjugated to siRNA (chol-siRNA) at 3'-end through aminocaproic acid-pyrrolydine noncleavable linker [147] or at 5'-end [159] by reducible disulfide linker of sense strand. The 3'-chol-siRNA induces intracellular RNAi without significant loss of gene silencing activity and exhibits higher cellular transfer efficiency in cultured cells without the aid of transfection agents. In animal experiments, after intravenous injection the 3'-chol-siRNA is localized in liver and jejunum and induces silencing of apoB gene that results in the decrease of total cholesterol and plasma apoB protein levels. In the study of Moschos *et al.* [159], it has been also shown that siRNA-mediated knockdown of p38 MAP kinase mRNA in mouse lung is influenced by conjugation to the nonviral delivery vector cholesterol. Conjugation to cholesterol improved upon the duration but not the magnitude of mRNA knock-

down. In addition, several lipophilic siRNA conjugates, e.g. with bile acids and lipids, were synthesized [161, 162]. They interacted with lipoproteins in the blood serum, lipoprotein receptors and trans-membrane proteins, affecting tissue distribution and uptake properties of the siRNA conjugates [161]. The hydrophobicity, directly related to the length of the alkyl chain can be the major determinant for the affinity of lipid-siRNA conjugate to lipoproteins. Recently, an alternative steroid conjugate, α -tocopherol-siRNA, has been reported [163]. Vitamin-E has its own physiological transport pathway to most of the organs. The α -tocopherol was covalently bound to the antisense strand of 27/29-mer siRNAs, designed to be cleaved by Dicer, at the 5'-end (toc-siRNA). After the intracellular delivery the toc-siRNA conjugate produced a mature form of siRNA after releasing vitamin E moiety. The intravenous administration of the conjugate achieved efficient reduction of endogenous apoB mRNA in the liver without induction on interferons or other side effects.

Cell penetrating peptides (CPP), also called protein transduction domain (PTD), represent an assortment of fairly unrelated sequence essentially characterized by a high content of basic aminoacids and a length of 10-30 residues [159, 164-178]. Numerous sequences have been reported to show cell-penetrating properties and it has become apparent that endocytosis is a major route of internalisation. CPPs are able

Table 1

siRNA conjugate	site of conjugation on siRNA	target siRNA
Cholesterol-siRNA	3'-end of sense strand ^a 5'-end of sense strand	Apo-B-1 siRNA p38 MAP kinase siRNA
α -tocopherol-siRNA	5'-end of antisense strand ^a	Apo-B-1 siRNA
Lipid-siRNA	3'-end of sense strand ^a	Apo-B-1 siRNA
TAT-siRNA	3'-end amine of antisense strand ^b 5'-end thiol of sense strand	GFP siRNA/CDK9 siRNA p38 MAP kinase siRNA
Penetratin-siRNA	5'-end thiol of sense strand 5'-end thiol of sense strand	Luciferase siRNA p38 MAP kinase siRNA
Transportan-siRNA	5'-end thiol of sense strand ^b	Luciferase siRNA
PEG-siRNA	3'-end amine of sense strand ^b 5'-end thiol of sense strand	Luciferase siRNA/RecQL1 siRNA
Peptide analogue of insulin-like growth factor 1-siRNA	5'-end amine of sense strand	Insulin receptor substrate 1 siRNA
Antibody-siRNA	3'-end biotin of sense strand	Luciferase siRNA
Aptamer-siRNA	5'-end of sense strand 5'-end of sense strand	Lamini A/C siRNA Bcl-2 siRNA/PLK1 siRNA
Quantum dot-siRNA	5'-end thiol of sense strand	GFP siRNA
Iron oxide nanoparticle-siRNA	5'-end thiol of antisense strand	GFP siRNA/survivin siRNA
Carbon nanotube-siRNA	5'-end thiol of sense strand	Luciferase siRNA

^aChemical modification of backbone nucleotide: partial 2'-O-methyl sugar modification, phosphorothioate linkage.

^bReaction with duplex siRNA.

to mediate the cellular uptake of hydrophilic macromolecules like peptides and nucleic acids. TAT (trans-activator protein from HIV-1) was conjugated to the 3'-terminus of the antisense strand of a siRNA using bifunctional sulfo-succinimidyl-4-(*p*-maleimidophenyl)-butyrate cross-linker [179]. The TAT-siRNA conjugate demonstrate a dramatic improvement of delivery and a significant downregulation of the target protein. The presence of peptide did not seriously interfere with the process of RNAi despite of noncleavable thioether linkage between CPP and siRNA. However, it is also preferred the using of cleavable linkers such as the disulfide bond to minimize potential reduction of siRNA activity because of the presence of highly charged peptide [159, 180]. The disulfide linkage in Penetratin and Transportan siRNA conjugates was formed by oxidation of thiol groups in the presence of oxidant diamide or disulfide exchange [159]. Significant uptake of siRNAs and efficient suppression of the target gene expression were observed in a variety of cell lines, e.g. in primary mammalian neuronal cells [167]. However, not improvement in CPP-siRNA stability respect to naked siRNAs was recorded. Besides, some peptide siRNA-conjugates elicited undesirable immune responses [159]. More recently, it was investigated whether the covalent conjugation of a signal peptide for transmembrane transport to lamin A/C-directed siRNA had any effect on its intracellular localization and release [181]. Chemical coupling of the N-bromoacetyl peptide to the 3'-thiopropyl terminus of the guide and passenger strand was carried out as described [181]. This study strongly suggests that the increased efflux of the siRNA-peptide conjugate from the ER-specific perinuclear sites leads to improved target inhibition after its PS-stimulated delivery [182]. The conjugation of siRNAs with peptide was also used to regulate small RNA functions [183]. Yamayoshi *et al.* have developed a novel peptide, antagonist against the PIWI box, conjugated to oligonucleotide complementary to the guide strand of siRNA demonstrating the regulatory effects on RISC activity of antago_{piwi} siRNA conjugates.

Several other types of conjugate have also been tested. siRNA conjugated to polyethylene glycol (PEG) [158, 168] and other polymers. PEG, a biocompatible, hydrophilic and non-ionic polymer, was conjugated to siRNA *via* a reducible disulfide linkage [60]. The PEG-siRNA conjugate is often complexed with cationic polymers or peptides as condensing agent to form colloidal nanoparticles, so-called polyelectrolyte complex micelles (PEC-micelles). The PEG-siRNA conjugate itself showed much higher levels of siRNA stability than the naked siRNA in the presence of 50% serum [60]. In order to overcome the risk of polyplex dissociation in extracellular environment, siRNA was covalently incorporate into a pH- and redox- responsive conjugate. The novel siRNA conjugate described by Meyer *et al.* consists of polylysine (pLL) as RNA binding and protecting polycation, PEG as solubilizing and shielding polymer, the lytic peptide melittin masked by dimethylmaleic anhydride (DMMAN) removable at endosomal pH, and siRNA [184]. The covalent linkage was realized by bioreducible disulphide bond between the 5'-end of the sense strand of thiolated siRNA and mercaptopropionic acid-modified pLL. Despite the *in vitro* biocompatibility and efficient gene silencing, *in vivo* toxicity studies revealed that this conjugate remains to be optimized

for therapeutic application. Further, Nothisen *et al.* have recently developed a stepwise automated synthesis of RNA-oligospermines by using of a DMT-spermine-phosphoramidite [185] in addition to the four nucleic bases synthons. This strategy allows incorporation of any number of spermine residues at any position of oligoribonucleotide [186]. In summary, these cationic spermine conjugated siRNAs enter Human cells and perform gene silencing in the submicromolar concentrations.

An intriguing recent approach to deliver siRNAs includes a variety of supramolecular nanocarriers such as quantum dot nanoparticles (quantum dot NPs), iron oxide nanoparticles, gold nanoparticles, carbon nanotubes that are employed for the development of multifunctional nanoconjugates. This strategy is attractive for several reasons as peculiar pharmacokinetics including minimal renal filtration, high surface to volume ratios enabling modifications with surface functional groups that can mediate the specific targeting of therapeutic agents. They can also serve for dual diagnostic imaging and therapeutic drug delivery. Polyvalent ON-Au NPs have several unique properties such as sharp and elevated melting temperatures, enhanced binding properties and distance-dependent optical properties [187]. Gold NPs densely functionalized with synthetic RNAs take advantage from the dense surface functionalization of oligonucleotides, increase the stability and efficacy of the bound RNA, while retaining the ability to act in the RNAi pathway. In Mirkin's laboratory, a straightforward synthetic approach was developed to obtain the first polyvalent RNA-gold nanoparticle conjugate, as RNAi trigger [188]. Duplexes composed of a 27-base strand and 25 base complement terminated with an ethylene glycol space and alkylthiol were hybridized and added to the Au-NPs, where they were allowed to chemisorb *via* thiol-gold bond. The novel conjugate results a potent material for cellular uptake, serum stability and genetic regulation without auxiliary transfection agents or other chemical modifications [188]. Gold nanoshell-siRNA conjugates represent a temporal and spatial controlled material for laser-activated cell delivery [189]. It was demonstrated the pulsed near-infrared (NIR) laser-dependent release of siRNA from coated 40 nm gold nanoshells. TAT-lipid coating mediates the cellular uptake of the conjugate at picomolar concentration. The release was found to be power- and time- dependent through surface-linker bond cleavage, while the escape of the siRNA from endosomes occurs above a critical pulse energy due to the local heating and cavitation. In an original work, Bradley's group reports the attachment of siRNA to 200 and 500 nm polystyrene microspheres *via* cleavable (disulfide) and noncleavable (amide) linkages and demonstrates the efficient silencing of EGFP, stably expressed in HeLa cells [190]. The authors underline the particular suitability of this construct because of the non-toxic nature of the microspheres along with their facile functionalization and controllability over cellular loading. Carbon nanotube-siRNA conjugate was also reported [191]. Single-walled carbon nanotubes (SWNT) have unique electronic, mechanical and structural properties as well as chemical stability that make them ideal nanomaterials for applications in materials science and medicine. A novel and versatile functionalization scheme was described for single-walled carbon nanotubes (SWNTs) to afford nanotube-biomolecule conjugates with the incorpo-

ration of cleavable bonds to enable controlled molecular releasing from nanotube surfaces. Transporting, enzymatic cleaving and releasing of DNA from SWNT transporters and subsequent nuclear translocation of DNA oligonucleotides in mammalian cells occurred. Magnetic nanoparticles have been intensively investigated for their potential biomedical applications, such as magnetic resonance imaging (MRI), hyperthermia, magnetofection and drug delivery [192]. It is possible to immobilize siRNA onto the surface of magnetic nanoparticles [193] in order to obtain siRNA delivery with concomitant visualization of its localization *in vivo*. The iron oxide nanoparticles were conjugated to the thiol group of the antisense strand of siRNA after activation of the primary amine groups using a heterofunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). Magnetic nanoparticles were also functionalized with near-infrared Cy5.5 dye (NIRF) and membrane translocation peptides. MRI and NIRF image has been used to visualize the accumulation of conjugates in tumor tissues and silencing activity was observed despite siRNA was conjugated onto magnetic nanoparticles *via* a noncleavable bond.

Targeted delivery of siRNAs to the desired cells and tissues has been considered as an attractive way to bring siRNA drugs to clinical applications. The targeted siRNA delivery minimizes the potential adverse effects and reduces the amount of dose required for therapeutic effects. Specific ligands (aptamers, antibodies, peptides, sugar molecules, vitamins and hormones) conjugated to siRNA lead to cell recognition by specific interaction between the targeting ligand and its cellular membrane receptor. A technology for specific targeted delivery is based on aptamer-siRNA chimeras [194]. Aptamers are *in vitro*-evolved, synthetically prepared nucleic acids that selectively bind specific ligands. The nucleic acid-based aptamers offer more synthetic accessibility, convenient modification, chemical versatility, stability and lack immunogenicity [195]. An aptamer targeting prostate-specific membrane antigen (PSMA), a cellular receptor abundantly expressed in prostate cancer cells was directly conjugated by biotin-streptavidin interaction to siRNA for cell specific delivery [196]. Zhou *et al.* have successfully identify several 2'-F substituted RNA aptamers that bind to the HIV1_{BAL} gp 120 protein. Two different types of dual inhibitory function anti-gp 120 aptamer-siRNA conjugate were constructed [197]. One of these is a covalent aptamer siRNA chimera and the other is an aptamer with a GC-rich bridge. The authors demonstrate that the selected aptamers not only provide lead inhibitors for potential anti-HIV therapeutical applications but also act as effective and potent delivery vehicles for anti-HIV siRNAs into infected cells. Particular oligonucleotide sequences, CpG motif, are well-known agonist of Toll-like receptor (TLR)9. SiRNA synthetically linked to CPG sequence silences genes in TLR9⁺ myeloid and B cells [198]. Although both proof-of concept studies suggested the potential use of aptamers for the targeted delivery of siRNA drugs, nuclease susceptibility of aptamers may limit their systemic application. More robust results the antibody-mediated targeted drug delivery system due to superior stability during systemic circulation and high selectiveness towards a target protein on the cell surface. siRNA was delivered to brain *in vivo* with the combined use of a receptor-specific monoclonal antibody delivery system, and

avidin-biotin technology. The siRNA was mono-biotinylated on either terminus of the sense strand, in parallel with the production of a conjugate of the targeting MAb and streptavidin. The intravenous administration of the siRNA caused a 69-81% decrease in luciferase gene expression in the intracranial brain cancer *in vivo* [199].

A conjugation of a siRNA to vitamin A coupled liposomes succeeded in delivering antifibrotic siRNAs to hepatic stellate cells which are produced in response to liver damage [200]. IGF1 [*D*-(Cys-Ser-Lys-Cys)] is a peptide mimetic that was conjugated by activated carboxylic acid group to an amine function of the 5'-sense strand of siRNA [178]. Without the use of transfection reagent, IGF1-siRNA conjugate abolishes about 60% the gene expression of the target insulin receptor substrate 1. The siRNA-PEG-LHRH conjugate was synthesized by coupling the terminal carboxyl group of 2-pyridyl disulfide-NH-PEG-COOH derivative with the ϵ -amino group of a lysine residue in LHRH peptide [201]. The luteinizing hormone-releasing hormone (LHRH) peptide analogue was introduced as a cancer targeting ligand. The siRNA-PEG- LHRH conjugate self-assembled to form nanosized PEC micelles, upon mixing with PEI. The LHRH receptor targeted siRNA formulation enhanced the intracellular delivery of siRNA for A2780 cells, due to LHRH-specific receptor ligand interaction and specifically inhibited VEGF expression in cancer cells. In a multifunctional platform and using a PEGylated quantum dot core as scaffold, siRNA and tumor- homing peptides (F3) were conjugated to functional groups of the particle surface. The homing peptide was required to target the internalization in tumor cells [202]. Strategies targeting tumor cells appear to hold promise for the treatment of tumors because they focus the potential therapeutic benefits and reduce the adverse side effects of the cancer therapy. Targeted therapeutics require the introduction of molecules that specifically recognize tumor cells. For example, the folate receptor is overexpressed in many human tumors, while its normal tissues distribution is minimal [203]. The covalent conjugation of small-molecular-weight, high-affinity ligands, such as folic acid and DUPA (2-[3-(1,3-dicarboxy propyl)-ureido]pentanedioic acid), to siRNA realize an efficient receptor mediated targeting of the siRNA to malignant cells and tissues *in vitro* and *in vivo* [204]. Oishi *et al.* have constructed a lactosylated-PEG-siRNA conjugate *via* an acid cleavable β -thiopropionate linkage. The lactose moiety was used as a targeting ligand for the asialoglycoprotein receptors in hepatoma cells [158, 205]. The lactosylated-PEG-siRNA conjugates form PEC micelles by interacting with polylysine and inhibit the growth of spheroids formed from human hepatocarcinoma cells.

CONCLUSIONS

The demonstration that synthetic siRNAs can be used to specifically silence genes in mammals has catalyzed an explosion of research in the field of chemically modified siRNAs. The chemical approach, as in antisense strategy, can help to overcome the problems of stability, delivery and specificity in therapeutic applications of RNAi. Potential siRNA drugs are now in clinical trials. These include chemical modified siRNAs [206]. Despite the technique's youth, the list of human diseases for which siRNAs are being tested

as a therapeutic agents is extensive and includes Parkinson's disease, diabetic macular oedema, wet age-related macular degeneration, HIV infection, hepatitis B, respiratory syncytial virus infection and solid tumors [207] but the success of siRNA as drug cannot be obtained without improving the safety, effectiveness and reliability of RNAi-trigger delivery systems. The use of targeted delivery strategies will be a big step towards fulfilling this difficult task. Calando Pharmaceuticals (Pasadena, California), meanwhile, has initiated a phase I clinical trial for solid tumours using an siRNA that targets a subunit of ribonucleotide reductase (RRM2), an enzyme required for the synthesis of DNA building blocks. Importantly, this trial is the first to utilize receptor-mediated delivery of siRNAs, which are encapsulated in cyclodextrin particles decorated with transferrin. This results in uptake by cells expressing the transferrin receptor, which is highly expressed on cancer cell surfaces. In most of the siRNA modification research, people tend to use a single type of chemical modifications to derivatize the siRNA in order to evaluate the compatibility of each of the modifications with RNAi machinery. Up to now, there is no single best or universal modification pattern to recommend; rather, choice of the precise modification pattern used can vary with the design, application and the delivery systems of the siRNA. The best modified siRNA consist of a combination of multiple chemical modifications [105, 41, 88, 208] including architecture modification that can further benefit from improved delivery vehicle designs. A creative and rational use of chemistry tools will make a key contribute in the development of siRNA therapeutics.

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