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Chemical, Structural, Advances and Hurdles to Clinical Translation of RNAi Therapeutics

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Abstract

RNAi can wipe out the disease-causing proteins from being translated. Now RNAi technique has become a powerful tool for basic research to selectively knock down gene expression in vitro and in vivo. At the same time, both scientific and industrial communities started to develop RNAi therapeutics as the next class of drugs for treating a variety of genetic disorders, such as cancer and other diseases that are particularly hard to be addressed by current treatment strategies.

Small interfering RNA (siRNA), a 21–23 nt double-stranded RNA responsible for post-transcriptional gene silencing, has attracted great interests as promising genomic drugs, due to its strong ability to silence target genes in a sequence-specific manner. In order to develop siRNAs as therapeutic agents for cancer treatment, delivery strategies for siRNA must be carefully designed and potential gene targets carefully selected for optimal anti-cancer effects. In this review, various modifications and delivery strategies for siRNA delivery are discussed. In addition, we present current thinking on target gene selection in major tumor types.

Introduction

The discovery of RNAi in the late 1990s unlocked a new realm of therapeutic possibilities by enabling potent and specific silencing of theoretically any desired genetic target. Better elucidation of the mechanism of action, the impact of chemical modifications that stabilize and reduce nonspecific effects of siRNA molecules, and the key design considerations for effective delivery systems has spurred progress toward developing clinically-successful siRNA therapies. A logical aim for initial siRNA translation is local therapies, as delivering siRNA directly to its site of action helps to ensure that a sufficient dose reaches the target tissue, lessens the potential for off-target side effects, and circumvents the substantial systemic delivery barriers. While locally injected or topically applied siRNA has progressed into numerous clinical trials, an enormous opportunity exists to develop sustained-release, local delivery systems that enable both spatial and temporal control of gene silencing.

In 1993, pioneering observations on RNA-mediated gene silencing were first reported in plants by John Lindbo and Bill Dougherty [1]. Half a decade later, RNA-mediated gene silencing known as RNA interference (RNAi) was made famous by Andrew Fire and Craig Mello's break-through study which has decisively proven the RNAi mechanism working as an antiviral defense mechanism in Caenorhabditis elegans [2].

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Small interfering RNAs (siRNAs) therapeutics for cancer treatment

The discovery of RNA interference (RNAi) has opened doors that might introduce a novel therapeutic tool to the clinical setting [3]. For many decades, small molecules have been developed and utilized in cancer therapy; however, critical problems, such as undesirable toxicity against normal tissues due to a lack of selectivity, still remain today. Using RNAi as a therapeutic tool will allow targeting previously unreachable targets with its potential to silence the function of any cancer causing gene [4].This unique advantage is made possible by utilizing the biological functions of double-stranded RNA molecules (dsRNA). Endogenous dsRNA is recognized by a ribonuclease protein, termed dicer, and cleaved into small double stranded fragments of 21 to 23 base pairs in length with 2-nucleotide overhangs at the 3′ ends. The cleaved products are referred to as small interfering RNAs (siRNAs). The siRNAs consist of a passenger strand and a guide strand, and are bound by an active protein complex called the RNA-induced silencing complex (RISC). After binding to RISC, the guide strand is directed to the target mRNA, which is cleaved between bases 10 and 11 relative to the 5′ end of the siRNA guide strand, by the cleavage enzyme argonaute-2. Thus, the process of mRNA translation can be interrupted by siRNA [5–7].

The therapeutic application of siRNA has the potential to treat various diseases including cancer [8,9]. Cancer is a genetic disease caused by the generation of mutated genes within tumor cells; multiple gene mutations both activate disease driving oncogenes and inactivate tumor suppressor genes in cancer [10–12]. Small interfering RNAs that can inactivate specific cancer driving genes have shown great potential as novel cancer therapeutics. Several anti-cancer siRNA based drugs have entered clinical trials, and many are actively sought after in preclinical research [13–15].

Even though the usage of siRNA as therapy has shown promise in the treatment of cancer, many obstacles that hinder the ultimate functionality of siRNAs in the clinic remain to be solved [16,17]. In order to make this therapy effective, the first and most crucial step is to ensure the delivery of siRNA to the tumor cells from the injection site. In practice, siRNAs face physiological and biological barriers that prevent their delivery to the active site when administered systemically [18–20]. These barriers include, but are not limited to, intravascular degradation, recognition by the immune system, renal clearance, impediments to tumor tissue penetration and uptake into tumor cells, endosomal escape once in tumor cells, and off-target effects [21–23]. Delivery formulations as well as chemical modification of siRNA are required to overcome these challenges and facilitate siRNAs in reaching their target cells [24]. Furthermore, selection of gene targets in cancer is also crucial in designing siRNA therapeutic strategies. Discoveries of mechanisms in cancer provide innovative targets for siRNA therapy that in many cases cannot be targeted with conventional drugs. However, the particular gene pool that drives cancer varies depending on the origins and types of the tumors. Thus, careful selection of gene targets according to their cancer type is essential in siRNA therapeutic strategies.

Materials and methods

To summarize, target discovery in cancer leads to the selection of siRNA gene targets, followed by their incorporation of the siRNAs into suitable delivery systems that allow access to the desired sites. Once therapeutic effect is observed, further application in varying organs and tissues can be anticipated as shown in Fig. 1. This chapter examines current thoughts on the therapeutic potential of siRNA delivery strategies and the optimal targets for siRNA in major cancer types.

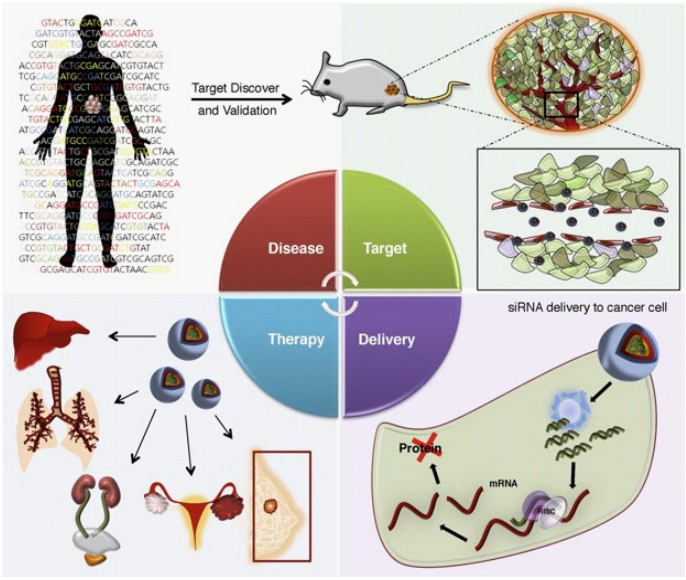

Figure 1: Development process of siRNA therapeutics for cancer treatment.

Small interfering RNA (siRNA), a 21–23 nt double-stranded RNA responsible for post-transcriptional gene silencing, has attracted great interests as promising genomic drugs, due to its strong ability to silence target genes in a sequence-specific manner. Despite high silencing efficiency and on-target specificity, the clinical translation of siRNA has been hindered by its inherent features: Poor intracellular delivery, limited blood stability, unpredictable immune responses and unwanted off-targeting effects. To overcome these hindrances, researchers have made various advances to modify siRNA itself and to improve its delivery. In the present study, first we briefly discuss the innate properties and delivery barriers of siRNA. Then, we describe recent progress in (1) chemically and structurally modified siRNAs to solve their intrinsic problems and (2) siRNA delivery formulations including siRNA conjugates, polymerized siRNA, and nucleic acid-based nanoparticles to improve in vivo delivery.

RNA interference (RNAi) is highly effective regulatory mechanism of gene expression in post-transcriptional level [2,25]. Small double stranded RNA, called as small interfering RNA (siRNA), is responsible for RNAi-based gene silencing. When siRNA is generated via Dicer processing of long double-stranded RNA or synthetic siRNA is delivered into cytoplasmic region, it is incorporated into RNA-Induced Silencing Complex (RISC) and then removes sense strand by the action of Argonaute-2. The activated RISC recognizes target mRNA with sequence homology and cleaves them RNA at the opposite of position 10 from the 5′ end of antisense strand [26]. siRNA has been considered as a promising gene therapeutics because it can down-regulate the expression of virtually all the genes, including previously undruggable targets. In past decades, the therapeutic potentials of siRNA have been proven in the treatment of genetic diseases, virus infections, and cancer [27,28].

Determination of clinical trials of RNAi therapeutics.

Up to date, several RNAi therapeutics, including naked siRNA and siRNA/carrier complexes, have been undergoing clinical trials for the treatment of ocular disorders, kidney disorders, and cancers (Table 1). However, most of them yet stays in Phase I (safety test), and very few ones have entered or are planning to enter Phase II/III (efficacy test). The first Phase III entering RNAi drug, Bevasiranib by Opko Health, was withdrawn due to the low therapeutic efficacy. The development

of siRNA drugs still suffers from the practical problems, such as easy degradation of siRNA in vivo, unwanted off-target effects, and immunogenicity [3]. Delivery of the nucleic acids to desired tissues and cells is also one of the critical concerns [29,30]. Modification of siRNA itself, both chemically and structurally, and development of efficient delivery carrier can be considered as promising strategies to dissolve such problems. Because the chemically or structurally modified siRNA can contribute to solve both intrinsic and delivery problems of siRNA, herein, we focused on recent advances of siRNA modification strategies. For a detailed description about the development of delivery carriers, please refer to recent review articles [31,32].

In this study, we describe the current challenges of siRNA for clinical applications in details; intrinsic properties of siRNA itself and many concerns in siRNA delivery are discussed. Because siRNA modifications can be beneficial to achieve the clinical goal, several siRNA modification strategies are also summarized. First, we introduce chemically modified siRNAs or siRNA structural variants, which have been developed to overcome its inherent problems. Secondly, development of siRNA conjugate systems or polymerized siRNA for enhancing delivery efficacy is described. It is generally known that gene carriers could increase the blood circulation time of siRNA, provide targeting moieties, and improve cellular uptake. The direct conjugation of siRNA and carriers or the use of polymerized siRNA can improve the loading efficiency of siRNA into gene carriers. Finally, recent advances in nucleic acid nanoparticle systems for efficient siRNA delivery are highlighted.

Results

Challenges in clinical applications of siRNA

Inherent properties of siRNA

The inherent properties of siRNA, which should be adjusted before the clinical applications, are mainly categorized into three groups: in vivo instability, off-target effects, and immunogenicity (Figure 2). These shortcomings may reduce the therapeutic efficacy of siRNA and cause unexpected toxicity. In this section, detailed mechanisms of easy degradation, unwanted gene silencing, and immune stimulation of siRNA are described, and the strategies to overcome these weaknesses are proposed.

Table 1: Clinical trials of RNAi therapeutics.

Drug name	Target sequence	Target disease	Phase	Status	Company
siG12D LODER	KRAS	Pancreatic tumor	I	Completed	Silenseed Ltd.
15NP	P53	Acute renal failure	I	Completed	Quark Phamaceuticals
Atu027	PKN3	Advanced solid tumors	I	Completed	Silence Therapeutics GmbH
TD101	Keratin6A	Pachyonychia congenital	I	Completed	Pachyonychia Congenita Project
AGN 211745	VEGF	Age-related macular degeneration	I/II	Completed	Allergan
siRNA-EphA2-DOPC	EphA2	Advanced cancers	I/II	Not yet recruiting	M.D. Anderson Cancer Center
CALAA-01	RRM2	Solid tumor	I	Terminated	Calando Pharmaceuticals
TKM-PLK1	PKL1	Hepatic metastases	I	Completed	Tekmira Pharmaceuticals Corporation
TKM-ApoB	ApoB	Hypercholesterolemia	I	Terminated	Tekmira Pharmaceuticals Corporation
ALN-VSP02	VEGF, KSP	Solid tumor	I	Completed	Alnylam Pharmaceuticals
EZN-2968	HIF-1	Liver metastases	I	Completed	Sataris Pharma and Enzon Pharmaceuticals
PF-04523655	PTP-801	Choroidal neovascularization, diabetic macular edema	II	Completed	Quark Phamaceuticals
Bevasiranib	VEGF	Age-related macular degeneration	III	Withdrawn	OPKO Health, Inc

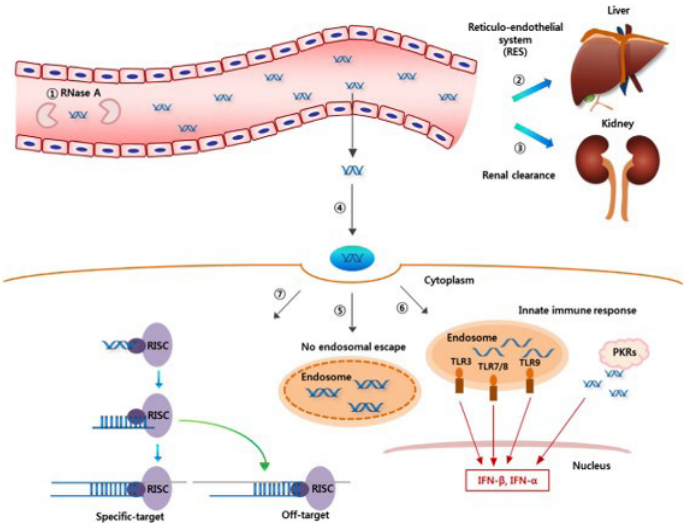

Figure 2: Inherent problems and delivery barriers of synthetic siRNA in vivo. (1) siRNAs are influenced by enzymatic degradation in the blood system. Also, siRNAs can be rapidly eliminated from the blood circulation by (2) reticuloendothelial system (RES) and (3) renal clearance. In the siRNA delivery system, the current main drawbacks include (4) poor transport across cell membranes and (5) endosomal entrapment. (6) siRNAs trapped in endosome can induce unwanted TLR-mediated immune responses, and external cytoplasmic RNAs activate the immune cells through PKRs. Finally, (7) siRNA can cause off-targeting mRNA degradation, leading to unintended transcription and translation suppression.

The easy degradation of siRNA under in vivo physiological conditions has been considered as one of the main problems for its clinical applications. Native siRNA has a short half-life less than ~15 min in serum due to their vulnerability toward nuclease activity. It has been reported that siRNA is susceptible to RNase A family enzyme in serum [33,34]. Thus chemical modification of specific dinucleotide motifs, substrates for RNase A-like activity, can improve the serum stability. In addition, intracellular siRNA degradation occurs via the activity of the 3′ exonuclease Eri-1 [35], and chemical modified siRNA showed the improved resistance to Eri-1 [36]. Alternatively, the use of gene carriers can prevent the access of nucleases to siRNA, subsequently leading to the enhancement of siRNA stability.

It has been known that synthetic siRNA induces unwanted gene silencing, called as off-target effects, via two pathways: (1) miRNA like pathway and (2) sense strand-mediated pathway. MiRNA suppresses the translation of genes, which contains partial homology with 3′-Untranslated Region (UTR) of the target mRNA, in nature [37]. Similarly, seed region of siRNA antisense strand (position 2 ~ 8 from 5′ end) can interact with 3′-UTR of mRNA with partial homology. The translational suppression owing to imperfect matching with 3′-UTR, rather than mRNA degradation by Argonaute-2, consequently leads to unintended off-targeting [38,39]. miRNA target prediction, the use of the lowest dose of siRNAs, and the multiple siRNA pools can minimize the unwanted gene silencing via miRNA-like pathway. Sense strand of siRNA can participate in gene silencing mechanism [40]. Incorporation of sense strand in RISC may cause down-regulation of non-target gene expression. The improved selection of antisense strand in RISC via chemical modification at 5′-end of sense strand can overcome this type of off-target effects.

The innate immune response to exogenous siRNA is categorized as Toll-Like Receptor (TLR)-mediated and non-TLR-mediated immune responses. Three types of TLR (TLR3, TLR7, and TLR8) among 13 TLRs are involved in the TLR-mediated immune response. TLR3 shows the length-dependent activity [41]; dsRNAs longer than 21–23 nt can stimulate the TLR-mediated immune response, though the length threshold is dependent on cell type [42]. TLR7 and TLR8 sense the nucleotide sequence; both of themare stimulated by GU-rich motifs, whereas AU-rich motifs primarily activate TLR8 [43,44]. The latter group of immune responses includes the activation of dsRNA-dependent Protein kinase R (PKR) and retinoic acid inducible protein (RIG-1). PKR is activated by dsRNA longer than 30 bp in a sequence-independent manner, though it can interact with short dsRNA containing 11 bp [45]. The activation of RIG-1 is not sequence-specific but length dependent [46]. Further the siRNA overhang can reduce the RIG-1- mediated immune response [47]. The modification of TLR-activatable motifs or the alteration of the interactions between RNA and immune-related proteins has been proposed as potential strategies to avoid siRNA-triggered immune reactions.

Barriers to siRNA delivery

To play a role as therapeutic agent, siRNA should be delivered to target tissue, be internalized into specific type of cells, and be placed at the site of action (cytosol). However, siRNA delivery has troubled with short blood circulation, lacking of target specificity, and difficulty to cellular uptake. The endosomal escape is also one of main barriers in siRNA delivery (Figure 2).

Short blood circulation time has been considered as a critical barrier to clinical applications of siRNA therapeutics, and it may be caused by enzymatic degradation, renal clearance, and capture by Reticuloendothelial System (RES). As aforementioned, siRNA is rapidly degraded in blood stream by RNase A-like nucleases [33,34]. The rapid renal clearance of naked siRNA occurs upon systemic administration because small molecules less than 50k Da are excreted through the kidney [30]. Phagocytic cells in RES also contribute to remove the foreign nucleic acids as well as gene carriers [48]. The pharmacokinetics of siRNA can be improved by chemical modification of siRNA itself, through inhibition of enzymatic degradation, and incorporation with efficient delivery vehicles, through siRNA protection from nuclease attack and prevention of renal clearance and phagocytosis in RES.

It is important to deliver the gene therapeutics into specific target tissue, but siRNA itself does not have any targeting moieties. To provide tissue targeting efficacy, the introduction of nano vehicle system, specific cell targeting molecules, or both to siRNA have been widely studied. When siRNA is transported by using nanocarriers, the resulting nanoparticles can be diffused into liver tissue through the fenestrated blood vessels or into tumor tissue through immature leaky endothelia. Inefficient lymphatic drainage in tumor tissue attributes to the retention of nanovehicles; this is termed as ‘enhanced permeability and retention (EPR) effects’ [49]. The incorporation of targeting molecules, such as antibodies, aptamers, and ligands for cell surface receptors, enables to recognize specific types of cells [31].

The intracellular entrance of siRNA is hampered by large size (~15 kDa) and highly negative charge [30]. Positive charged carrier helps to not only nanosized particle formation but also crossing the negatively charged cell membrane. Receptor-binding ligands improve the receptor-mediated endocytosis, and cell Penetrating Peptides (CPPs) have been also widely used to enhance cellular uptake of siRNA [50].When siRNA is endocy-

tosed, it should escape from the endosomes before the relocation to lysosomes, which contain nucleases. Considering the acidic environment of late endosomes, gene carriers having pH-responsive proton sponge effects or membrane disruption activities allow the endosomal escape of siRNA [51].

Development of efficient siRNA delivery system

For the use of siRNA in the clinic, aforementioned intrinsic and delivery problems of siRNA should be overwhelmed. The inherent properties of siRNA, including easy degradation against serum nucleases, unwanted off-target effects, and immunogenicity, can be somewhat conquered by chemical modification at the specific position or sequence of the nucleic acids and by structural alteration [52]. As will be described latter, diverse chemical modification strategies and siRNA structural variants have been developed and are applicable to improve serum stability, to minimize off-target effects, and to reduce immune stimulation.

The weaknesses of siRNA in delivery issues, such as short blood circulation time, lack of targeting moieties, and difficulty to subcellular localization, can be overcome by using effective gene carriers. Delivery vehicle may protect siRNA from nuclease attack and from detection by macrophages. Nanoparticle formation itself by gene carriers and further introduction of targeting molecules can improve the delivery efficiency of siRNA into specific target tissues and cells. Gene carriers can be adopted by two strategies: direct conjugation of carrier with siRNA and complex formation between carrier and siRNA [32,53]. The former generally include covalent linkage between siRNA and carriers. Lipophilic molecules or polymers prolong the blood circulation of siRNA, and aptamers are used to provide targeting efficacy to siRNA. The latter groups of siRNA delivery system have been extensively focused to use cationic carriers. Considering the stiff structure and low charge density of siRNA, however, the enhancement of physicochemical properties of siRNA has been required to improve the interaction between siRNA and gene carriers; thus polymerization strategies of siRNA has been proposed in past decade.

Chemical modification of siRNA

Common chemical modification strategies

The most common backbone modification of siRNA is the substitution of non-bridging phosphate oxygen to sulfur (phosphorothioate, PS) (Figure 3). According to the previous studies, PS modification of antisense oligonucleotides resulted in the improved nuclease resistance and favorable pharmacokinetics [54]. Similarly, siRNA with PS modification exhibited high serum stability and high blood concentration at the early time post-injection [55]. Moderate PS modification of siRNA improved gene silencing activity although the effects are highly position-dependent; PS modified siRNA at the position of 3, 5, and 17 from the 5′-end of sense strand showed the high silencing effects by improvement of RISC loading of antisense strand [56]. However, high degree of PS modification led to severe toxic effects, presumably attributing to the non-specific binding to cellular membrane proteins; siRNA with 50% PS content (PS modification in every second nucleotide) showed cytotoxicity and reduced cell growth [57]. The substitution of two non-bridging oxygen atoms with sulfurs, called as phosphodithioate (PS2), also resulted in the enhanced serum stability and the higher gene silencing activity, which is position-dependent [58].

Alternative backbone modifications include boranophosphate substitution, obtained by introduction of BH3 group in place of non-bridging phosphate oxygen. This modification thermodynamically destabilizes siRNA with the decrease of Tm (0.5–0.8 °C per modification) [59]. Boranophosphate modification resulted in siRNA potency when seed region of antisense strand was not modified, and serum stability of siRNA was enhanced after this modification [60].

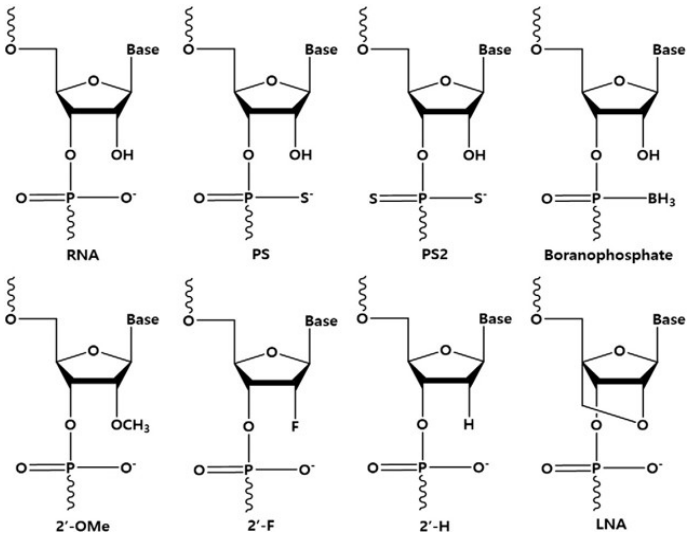

Figure 3: Popular chemical modifications of siRNA. RNA, ribonucleic acid; PS, Phosphothioate; PS2, Phosphodithioate; 2′-OMe, 2′-O-methyl; 2′-F, 2′-fluoro; 2′-H, 2′-deoxy; LNA, locked nucleic acid.

Ribose 2′-OH is one of the most attractive modification sites because 2′-OH is not required for recognition by RNAi machinery or for mRNA cleavage process by activated RISC [61]. Chemical modification of ribose 2′-OH involves the substitution of 2′-OH to other chemical group, such as 2′-O-methyl (2′-OMe), 2′-F, and 2′-H (Figure 3). 2′-OMe modification improved the resistance to enzymatic digestion and thermal stability (0.5-0.7 °C increase in T_m permodification) [62]. When antisense strand or both strands of siRNA was fully modified with 2′-OMe, the RNAi activity was completely abolished, whereas same modification in sense strand did not modulate the gene silencing efficacy [63]. In contrast, the substitution of 2′-OH with fluorine (2′-F) can be accepted in both antisense and sense strands without loss of gene silencing activity [61,64]. 2′-F modification enhanced serum stability and the binding affinity of siRNA duplex (~1 °C increase in T_m permodification) [61,65,66]. 2′-H modification, DNA itself, is also well-tolerated in siRNA duplex, particularly in the sense strand and at the end region (3′- overhangs or 5′-end of antisense strand) [67,68].

Intramolecular linkage of 2′-oxygen to 4′-carbon is the alternative strategy for 2′-OH modification. The bridged nucleic acids contain the linkage between 2′ and 4′ positions of ribose ring via methylene bridge (Locked nucleic acid, LNA) or ethylene bridge (Ethylene-bridged nucleic acid, ENA) (Figure 3). LNA modification locked the sugar ring in 3′-endo conformation, which increases in Tm by 2-10 °C per modification [69]. Further, this modification is highly position-sensitive; the introduction of LNA modification at 10, 12, and 14 positions of antisense strand abolishes RNAi activity due to the steric and conformational change near the cleavage site [70]. LNA modifications at 3′-overhangs protect siRNA from the 3′ exonucleases, subsequently leading to improve serum stability [70].

With current bioorganic techniques, oligonucleotides can be synthesized and modified as single strands, then annealed into the desired double stranded material. Customizable oligonucleotide synthesis incorporating artificial modifications enhances the potential of RNA therapeutics by overcoming problems associated with administration of naked siRNA. In particular, unmodified siRNA exposed in the bloodstream stimulates the innate the immune response and is readily degraded by serum nucleases. One of the methods to increase stability in serum and potency of gene silencing efficacy is to employ chemical modifications on the RNA-backbone of siRNA. A wide variety of chemical modifications, listed in Figure 4 have been proposed to overcome existing challenges of siRNA therapeutics.

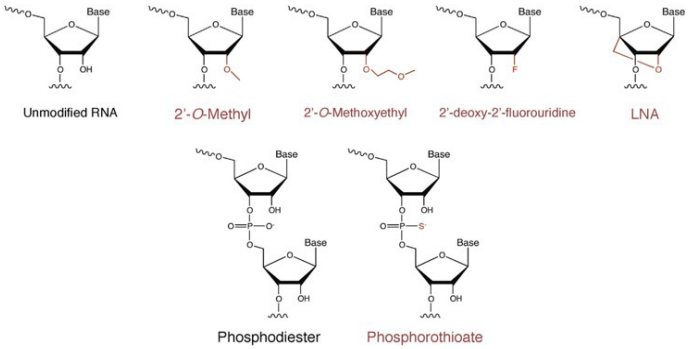

Figure 4: Chemical modifications and siRNA.

One of the most common alterations of RNA is modification of the 2′ position on the ribose backbone. These modifications include 2′-Omethyl, 2′-O-methoxyethyl, 2′-deoxy-2′-fluorouridine, locked nucleic acid (LNA), and many more [69,71-73]. These chemical modifications increase stability against nucleases and improve thermal stability. As a naturally occurring RNA variant, 2′-O-methyl RNA has shown reduced potency or even inactivation in siRNA activity in the RNAi pathway upon heavy modification [64]. The 2′-fluoro modification is compatible with siRNA function and lends stability in presence of nucleases. Combined modification with 2′-fluoro pyrimidines and 2′-O-methyl purines results in highly stable RNA duplexes in serum and improved in vivo activity [74]. The 2′-O-methoxyethyl RNA modification has also shown significant nuclease resistance as well as increased thermal stability (T_m). Nevertheless, this modification is not generally used as frequently as the 2′-O-methyl and 2′-Fluoro RNAs. LNA contains a methylene bridge that connects the 2′-O with the 4′-C positions of the ribose backbone. This causes the siRNA to have “locked” sugar that results in higher stability with increased Tm. Though incorporation of LNA also interferes with the siRNA activity, limited modification retains the functionality [64].

In addition to the sugar modifications, variations in phosphate linkage of siRNA are also accepted as an alternative strategy to overcome functional limitations. The Phosphorothioate (PS) linkage, perhaps the most commonly modified linkage in siRNA, often displays cytotoxicity when used extensively; however, PS incorporation does not appear to have a major effect on biodistribution of siRNA [75].

A part from modifications made on the backbone, chemical modifications are also made on other parts of siRNA to facilitate delivery to the target site. One of the hurdles in siRNA delivery is that weak negative charge and high molecular weight makes the nucleic acid more prone to serum degradation and capture by the Reticuloendothelial System (RES). In order to form more stable delivery complexes, polymerized siRNA can be synthesized, resulting in greater electrostatic interactions and facilitating incorporation into nanoparticles. Lee et al. developed polymerized siRNA using a thiol group to form a stable complex with glycol chitosan via not only electrostatic interaction but also disulfide bond crosslinking. Polymerized siRNA synthesized with thiol groups was also shown to form stable complexes with PEI, albumin, transferrin, hyaluronic acid, and other nanoparticles [76–81]. This delivery reagent was shown to have an anti-tumor effect in xenograft cancer models when systemically injected.

Other chemical alterations of siRNA include base modification, change in overhangs and termini of the RNA duplexes, and varying tertiary structure of the siRNA. In an attempt to develop siRNA for use in clinical trials as drugs, various chemical modifications are being investigated to improve qualities such as serum stability, siRNA potency, low immunostimulation, off-target effects, and target organ/cell delivery [82].

Applications of chemically modified siRNA

As stated above, chemical modification can be used to overcome the inherent problems of siRNA. Considering that several nucleases catalyze the nucleophilic attack of 2′-OH and the hydrolysis of the interphosphate linkage in siRNA, modification of ribose 2′-OH position enable to improve serum stability. Particularly, 2′-OMe and 2′-F modification of nuclease-sensitive regions, such as UA and CA motifs, dramatically enhanced the resistance to nuclease digestion [33,62,83]. Combination of different modification strategies results in the highly stable siRNA in vivo; successful example was the modified siRNA consisting of sense strand with 2′-F on pyrimidine, 2′-H on purines, and 5′ and 3′-inverted abasic end caps and antisense strand with 2′-OMe on purines and PS at 3′-terminus [84]. The 3′-overhangs are also susceptible to exonuclease attack; the chemical modified 2 nt 3′- overhangs, such as LNA modification, reduced the siRNA degradation in serum [70].

Off-target effects of siRNA via miRNA-like pathway are dependent on the seed region homology with 3′-UTR of mRNA. Although in silico siRNA-mRNA sequence matching prediction may reduce this type of off-target effects, it cannot be fully avoided. According to the previous literature, chemical modification of antisense strand modulated the undesired gene silencing effects; 2′-OMe modification at position 2 of antisense strand and introduction of 8 DNA in antisense strand seed region reduced the down-regulation of non-target gene expression [21,85]. Increase in the incorporation selectivity of antisense strand into RISC can modulate off-target effects, resulted from the contribution of sense strand in gene silencing process. Considering that phosphorylation of 5′-terminus is required to RISC activation [86], 5′-end modification of sense strand via 5′-OMe or LNA reduced its participating in RNAi mechanism [87,88].

Short RNA exhibits immunostimulatory properties; mediated by TLR family or PKR, and these exogenous siRNA-triggered immune responses can be decreased by applying several modification strategies. Concerning the incorporation of U-richmotif in TLR activation, the modification at ribose 2′-OH position of uridine residue enabled to minimize the siRNA immunogenicity [89]. Introduction of alternating 2′-OMe modification in sense strand reduced the cytokine induction without loss of gene silencing activity [90]. LNA modification of sense strand also blocked the TLR activation [91]. The activation of cytoplasmic PKR after intracellular delivery of siRNA can be abrogated by the reduction of hydrogen bonding between RNA minor groove

and PKR domain; chemical modification for hydrogen bonding alteration, such as 2'-H or 2'-F modification, reduced PKR activation [92].

siRNA structural variants

A diverse of structural variants of RNAi-based therapeutics with its own advantages and disadvantages has been reported to improve intrinsic properties of siRNAs. It suggests that the nature's RNAi pathway machinery can tolerate various structurally different mediators of gene silencing. The first successful sequence specific gene silencing using chemically synthesized exogenous siRNA in mammalian cells was demonstrated by Tuschl and colleagues without causing innate immune responses [93,94]. The siRNA has a 19 base paired duplex with 3'-end 2 nt overhangs at both sense and antisense strand (19 + 2 traditional siRNA) and are the most widely used siRNA (Figure 5A). The 19 + 2 siRNA has a structural similarity to the nature's Dicer product.

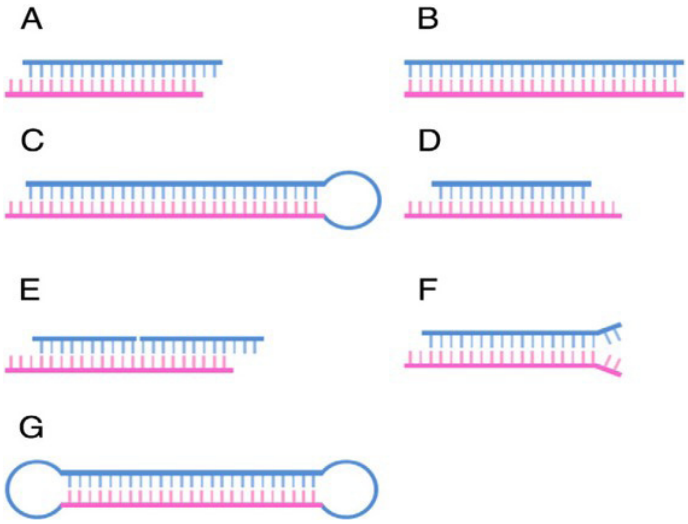

Figure 5: Schematic diagrams of structural variants of siRNA. (A) Conventional siRNA of 19 + 2. (B) 27-mer long dsRNA Dicer substrate. (C) 29-mer short hairpin RNA (D) Asymmetric siRNA. (E) Internally segmented siRNA. (F) Fork-shaped siRNA. (G) Dumbbell-shaped circular RNA.

RNAi triggers with increased potency

Designing highly potent gene suppressing RNAi triggers is one of the important properties for successful clinical application of RNAi therapeutics. In efforts to find RNAi triggers that work efficiently at a low concentration, Dicer substrates were found by two groups. Kim et al. found that long synthetic 27-mer duplex RNA without overhangs can be substantially more efficient in gene silencing than the corresponding traditional 21-mer siRNA (Figure 5B) [95]. In company with this report, Siolas et al. also identified a synthetic short hairpin RNA (shRNA) as a potent mediator of RNAi (Figure 5C) [96]. The shRNAs composed of 29 base-pared stems with 2 nt 3'-overhangs and 4 nt loops. Both 27-mer RNA duplex and 29-mer shRNA were processed to 21- or 22-mer siRNA by Dicer (RNase III-family endonuclease) in vitro. It was reported that Dicer are involved in not only cleaving long double stranded RNAs but also RISC loading of processed RNA and RICS assembly [97,98]. The improved potency of these RNAi triggers may be attributed to the fact that they are Dicer substrates. siRNA of ~21 nt in length was produced when recombinant Dicer was treated to both 27-mer duplex RNA and 29-mer shRNA. Dicer processing may enhance the loading and incorporation of siRNA into RISC, thereby increasing gene silencing efficiency. Furthermore, the Dicer substrates RNA did not induce innate immune responses, such as interferon production and PKR activation.

Exogenously introduced high concentration of siRNA can cause a saturation of cellular RNAi proteins, which would hinder the RNAi pathway and cause toxicity [99]. In addition, extents of unwanted offtarget effects were proportional to the siRNA treatment concentration [100]. Therefore, these highly potent effectors of gene silencing will facilitate clinical translocation of RNAi therapeutics.

RNAi triggers with reduced off-target effect

Incorporation of sense strand into RISC is a main undesired off targeting effect of RNAi-based therapeutics [101]. Novel designs of siRNA structural variants to reduce the off-target effects were suggested by Sun et al. [102]. They investigated whether asymmetric RNA duplexes with various lengths could induce gene silencing and found that an asymmetric RNA duplexes with short 15 nt sense strand having both 3' and 5' antisense overhangs could mediate gene silencing (Figure 5D). The asymmetric interfering RNA (aiRNA) was incorporated into RISC effectively than inhibited target gene expressions sequence specifically. More importantly, the sense strand mediated off-target effects were reduced compared with conventional siRNAs, which may be attributed to the nature of structural asymmetry. They speculated that the asymmetric structure leads to preferential incorporation of antisense strand into RISC than short sense strand, which resulted in reduced off-target effects.

Along with the aiRNA, a novel design of small internally segmented interfering RNA (sisiRNA) also showed decreased off-target effects caused by loading of sense strand into RISC (Figure 5E) [103]. The sisiRNA had an intact antisense strand and a sense strand which was divided into two segments. Because incorporation of segmented sense strand into RISC was excluded and only antisense strand could be loaded into RISC, this structural siRNA variant showed reduced off-target effects and increased target specificity.

Fork shaped siRNA having 1–4 nt mismatch at the 3'-end of sense strand was another structure that showed increase target specificity while maintaining gene silencing activity (Figure 5F) [104]. The section suggested that the mismatch part may render antisense strand more favored to be incorporated into RISC. It was reported that thermodynamically less stable 5'-end of siRNA was preferentially incorporated into RISC during the strand selection [105,106]. Although chemical modifications to reduce off-target effects have been reported, structure based asymmetry provides another way to overcome the problems [21].

RNAi triggers with increased stability

Natural RNAs are rapidly degraded in biological fluid. Chemical modification can enhance siRNA stability, although it often causes toxicity or decreases gene silencing activity [107]. A method to increase RNA stability using natural RNA was proposed by Abe et al. [108]. One more loop was added into shRNA using T4 RNA ligase, which resulted in dumbbell-shaped circular RNA structure (Figure 5G). Due to the endless structure of the dumbbell-shaped RNA, it showed higher stability when treated with exonuclease, compared with linear form of siRNA. In addition, the RNA dumbbell was processed more slowly and exhibited prolonged RNAi activity.

A variety of structural variants of siRNA has been reported with improved features including higher potency, reduced off-target effects, and increased stability. There is substantial structural flexibility of gene silencing mediators in nature although more detailed structural, biochemical, and biological studies in RNAi mechanism are demanded. Optimization of the siRNA structure will provide safety and efficacy for clinical applications of RNAi therapeutics.

siRNA conjugate system

Lipophile-siRNA conjugates

The introduction of lipophilic molecules, such as cholesterol and α-tocopherol, can improve the pharmacokinetic properties as well as cellular uptake of siRNA (Figure 6A) [109]. Cholesterol-conjugated siRNA (Chol-siRNA) exhibited the prolonged blood circulation time ($t_{1/2}$ of 95 min), compared to naked siRNA ($t_{1/2}$ of 6 min), after systemic administration, presumably attributing to the enhanced binding to serum proteins [24]. This enhanced pharmacokinetics resulted in the increase of specific gene silencing efficacy; Chol-siRNA against apolipoprotein B (apoB) led to the down-regulation of target mRNA in liver and jejunum and the decreased level of plasma apoB protein and serum cholesterol. Since lipoprotein particles, including both High-Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL), play a critical role in cholesterol transport in vivo, the delivery of Chol-siRNA may be facilitated when Chol-siRNA was preassembled with HDL or LDL.

According to the previous results, Chol-siRNA with HDL was delivered into liver, gut, kidney, and steroidogenic organs, whereas LDL directed the Chol-siRNA primarily into liver [110]. The binding with HDL or LDL further enhanced the cellular uptake of Chol-siRNA via HDL- or LDLreceptor, respectively.

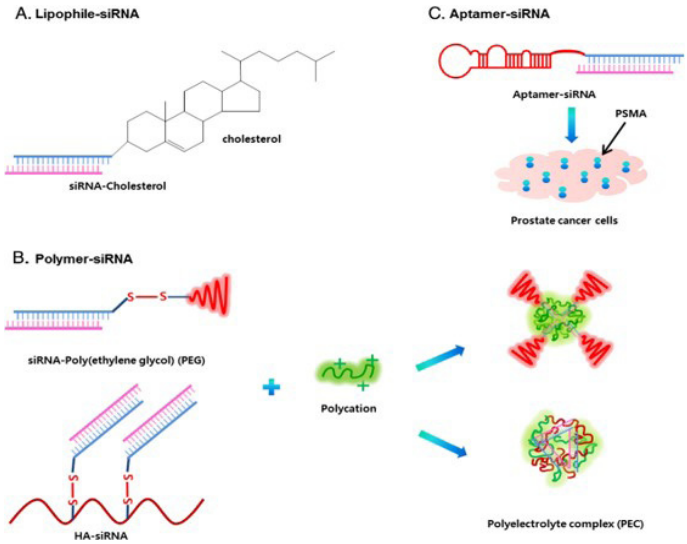

Figure 6: Schematic diagrams of siRNA conjugate system. (A) Cholesterol-siRNA conjugate. (B) Polymer-siRNA conjugate, containing bioreducible disulfide linkage. The polymer-siRNA conjugate can form a stable polyelectrolyte complex with polycation. (C) Aptamer-siRNA chimera. PSMA-specific A10 aptamer is linked to siRNA.

The conjugation of lipophilic molecules can be used as a targeting moiety for siRNA delivery to specific tissue. Considering that α-tocopherol (vitamin E) is incorporated into lipoproteins and traveled into liver for hepatic uptake, α-tocopherol-conjugated siRNA (TocsiRNA) was delivered into liver [111]. Toc-siRNA targeting apoB achieved the reduction of liver apoB mRNA expression and serum triglyceride/cholesterol level. Furthermore, neither interferon induction nor other side effects were observed after systemic administration of Toc-siRNA.

Polymer-siRNA conjugates

The introduction of poly(ethylene glycol) (PEG) provided the stealth functionality, which avoids capture by RES, consequently resulting in prolonged blood circulation time. The half-life of siRNA was increased from5 min to 1 h after conjugation with 20 kDa PEG, and the distribution of PEG-siRNA in liver, kidney, spleen, and lung was observed without significant degradation [112]. When PEG-siRNA conjugate was complexed with Polyethylenimine (PEI), negatively charged siRNA and positively charged PEI formed polyelectrolyte core and hydrophilic PEG was present on the particle surface (Figure 6B) [113,114]. The resultant PEG-siRNA/PEI nanocomplex exhibited the enhanced serum stability and excellent tumor targeting efficacy without any induction of interferon. When siRNA targeting Vascular Endothelial Growth Factor (VEGF) was used, the local or systemic administration of PEG-siRNA/PEI complex achieved the reduction of micro vessel formation and the suppression of tumor growth [114]. When the polymer end was decorated with targeting moiety, such as folate and lactose, the delivery of PEG-siRNA into specific cell type was further improved [115,116].

Hyaluronic Acid (HA), a natural polymer having biocompatibility and binding affinity to CD44, has also been widely used as siRNA carrier. Recently, HA-siRNA conjugates containing bioreducible disulfide bonds were developed [117,118]. The HA conjugation led to the enhancement of serum stability and the compact nanocomplex formation with cationic carriers (Figure 6B). Further, HA-siRNA/PEI nanocomplex was successfully internalized into cells via receptor-mediated endocytosis and downregulated the target gene expression in CD44-expressing cancer cells. Attributing to the abundance of HA receptors in liver, HA-siRNA conjugate was delivered specifically into liver and silenced the target gene expression after systemic administration [118].

Aptamer-siRNA chimeras

Aptamer is single-stranded nucleic acids having high affinity to target molecules; thus it has been extensively studied as a targeting molecule in biomedical fields. Aptamer-siRNA chimera exhibited high specificity to adhere against target protein-expressing cells. For example, Prostate Specific Membrane Antigen (PSMA) targeting A10 aptamer specifically delivered siRNA into PSMA-expressing cell and tumor (Figure 6C) [119,120]. A10 aptamer-siRNA chimera bound to PSMA on cell surface and mediated cellular uptake. After intracellular translocation, A10 aptamer-siRNA chimera was processed by Dicer and released the active siRNA. When A10 aptamer-siRNA targeting polo-like kinase 1 (plk1) was systemically administrated, the gene silencing and tumor growth inhibition were observed in PSMA-expressing tumor [120]. The incorporation of PEG into A10 aptamer-siRNA chimera further enhanced the pharmacokinetic and pharmacodynamic properties of the chimera [119]. Similarly, nucleolin-targeting aptamer has been considered as a promising tumor targeting moiety due to the high expression of nucleolin on various cancer cells [121]. Combined treatment of nucleolin aptamer-siRNA chimeras against snail family zinc finger 2 (SLUG) and neuropilin 1 (NRP1) synergistically suppressed the invasion of lung cancer cells and tumor-induced angiogenesis.

siRNA polymerization

Efficient and safe delivery of siRNA to target tissues and cells is one of the most critical problems for therapeutic application of siRNAs. A diversity of siRNA delivery carriers based on polymers or lipid, and nanoparticles have been devised to improve delivery of siRNA [122]. The cationic polymers can form condensed polyelectrolyte complexes with anionic nucleic acid by electrostatic interactions. Furthermore, because synthetic cationic polymers have merits of the facile introduction of functional moieties and modification of its structure and molecular weight, they have been wildly used as nucleic acid delivery carriers [123].

However, siRNA delivery is much more difficult than plasmid DNA delivery due to their different intrinsic physicochemical properties. The persistence length of dsRNA is approximately ~70 nm (corresponding to ~260 bp) [124], therefore, siRNA of ~21 bp behaves like a rigid rod. Furthermore, having ~ 42 negative charges per molecule, siRNA has much low spatial charge density than plasmid DNA which has more than several thousand negative charges per molecule. The rigidity and low charge density of siRNA make it difficult to form small, stable, and condensed complexes through efficient electrostatic interactions with cationic polymers.

The shape, size, and surface properties of nanoparticles significantly affect the cellular uptake and in vivo biodistribution of nanoparticles [125]. It has been reported that stable, compact, and small nanoparticles were more efficiently taken up by cells [126]. Therefore, making stable, compact, and small nanocomplexes of siRNA/cationic polymers is prerequisite of successful siRNA delivery systems. Although it is possible to make more stable and compact complexes if large amount of high molecular weight cationic polymeric carriers are used, the toxicity is also depends on concentration and structure of polymers [127]. Recently, structural modification of siRNA itself has been reported along with chemical modification of carries to develop efficient delivery systems without causing toxicities and immune responses.

Sticky siRNA

A gene like structure was constructed by connecting several siRNAs together to increase the charge density of siRNAs [128]. Short additional complementary A_{5-8}/T_{5-8} overhangs were introduced into 3′-ends of siRNA (called sticky siRNA) and siRNA concatemers were constructed by hybridizing the sticky siRNAs in aqueous solution (Figure 7A). When complexed with PEI, a wildly used cationic polymer, the sticky siRNA concatemer showed increased complex stability and protection of siRNAs. The sticky siRNA/PEI complexes resulted in enhanced gene silencing in cultured A549 cells and in vivo mouse lung. The enhanced stability and delivery efficacy of the complexes were attributed to increased charge density of sticky siRNAs which enabled more efficient electrostatic interactions with PEI. This study demonstrated the concept that increasing size of siRNA like pDNA could enhance complex stability and delivery efficacy even though there was stability concern of the sticky siRNA concatemers with A8/T8 overhangs having low melting point (T_m < 10 °C).

Multi/poly-siRNA

Chemical crosslinking of several siRNAs together to increase the size was reported by two independent groups [129,130]. Mok et al. synthesized multimerized siRNA (multi-siRNA) using a dithio-bismaleimidoethane (DTME), a cleavable chemical crosslinker (Figure 7A) [129]. Thiol-modified sense and antisense strands at 3′ end were reacted with DTME to construct dimeric sense and antisense strands. The prepared dimeric sense and antisense strands were hybridized by complement base paring to produce multi-siRNAs. In company with this report, Lee et al. also prepared polymerized siRNA (poly-siRNA) without using chemical cross linkers. Poly-siRNA was obtained by direct oxidation of siRNAs thiol-modified at 5′-end of both sense and antisense strands (Figure 7A) [130].

The synthesized multi-siRNA showed ladder like band patterns on polyacrylamide electrophoresis analysis, which implied that mixtures of multi-siRNAs with various degree of crosslinking were obtained. When incubated with reducing agents of dithiothreitol or glutathione, multi-siRNA was cleaved into monomeric-siRNAs which are active component of RNAi. It is well known that intracellular cytosol is more reductive than extracellular space [131]. Therefore, it was anticipated that monomeric-siRNA would be generated by cleavage of disulfide bonds in the reductive cytosolic environments after cellular uptake.

According to the morphology analyses, multi-siRNA formed more stable and compact nanocomplexes with linear PEI (LPEI), more biocompatible cationic carrier than branched PEI (bPEI), whereas monomeric-siRNA/LPEI complexes showed unstable, large, and loose aggregates. The more stable and compact complex formation was largely attributed to increased charge density and introduction of flexible linkage of multi-siRNA, which enabled more efficient electrostatic interaction and entanglement with cationic polymers. Thus, much larger amount of multi siRNA/LPEI complexes were entered into cells and thereby significantly enhanced gene silencing were observed, compared tomonomeric-siRNA/LPEI complexes, in vitro PC3 cell and in vivo PC3 xenograft mouse model (Figure 7).

Nonspecific immune responses should be considered for clinical application of siRNAs since it could be induced by long double stranded RNAs [132]. The multi-siRNA/LPEI complexes did not elicit significant undesired INF-α induction when treated into peripheral blood monocyte cell or ICR mice. Furthermore, sequence-specific mRNA degradation was confirmed through reverse transcription-polymerase chain reaction (RT-PCR) and Rapid Amplification of cDNA Ends (RACE). It was suggested that cleavable chemical linkage between siRNAs and regeneration of short monomeric-siRNA after cellular uptake of multi-siRNA prevented induction of immune responses.

siRNA microhydrogel

Linear gene like structural modification of siRNA further proceeded to construct three-dimensional siRNA structures. Although many 3D structures made of DNA have reported, few of them have biological activities [133,134]. Microhydrogels composed of networked siRNAs were introduced by Hong et al. (Figure 7B) [135]. The 3D siRNA microhydrogels were prepared by annealing Y-shaped sense strand with Y-shaped antisense strand. A trifunctional chemical cross linker, tris-(2-maleimidoethyl)amine (TMEA), was reacted with thiol group at 3′-end of sense or antisense strand to prepare Y-shaped single-stranded RNAs. Dimeric sense or antisense strands were also synthesized by reacting 3′ thiol-modified sense or antisense strand with bifunctional chemical crosslinker, 1, 8-bis (maleimidodiethylene) glycol ($BM(PEG)_2$). By controlling the ratios of Y-shaped and dimeric RNAs, several micrometered siRNA hydrogels with different pore size were obtained in aqueous solution through

complement base paring.

The siRNA-based microhydrogels were readily condensed to stable,~100 nm of nanocomplexes upon interacting with a cationic polymer LPEI. Furthermore, the nanoscale complexes showed not only superior cellular uptake but also greatly enhanced gene silencing activity in breast cancer cells (Figure 7). Significantly increased charges as well as flexibility of the siRNA microhydrogels enabled efficient condensation with cationic polymers. Even though no cleavable bond was introduced in siRNA microhydrogels, the construct was processed by Dicer and the processed product would participate in the sequence-specific gene inhibition.

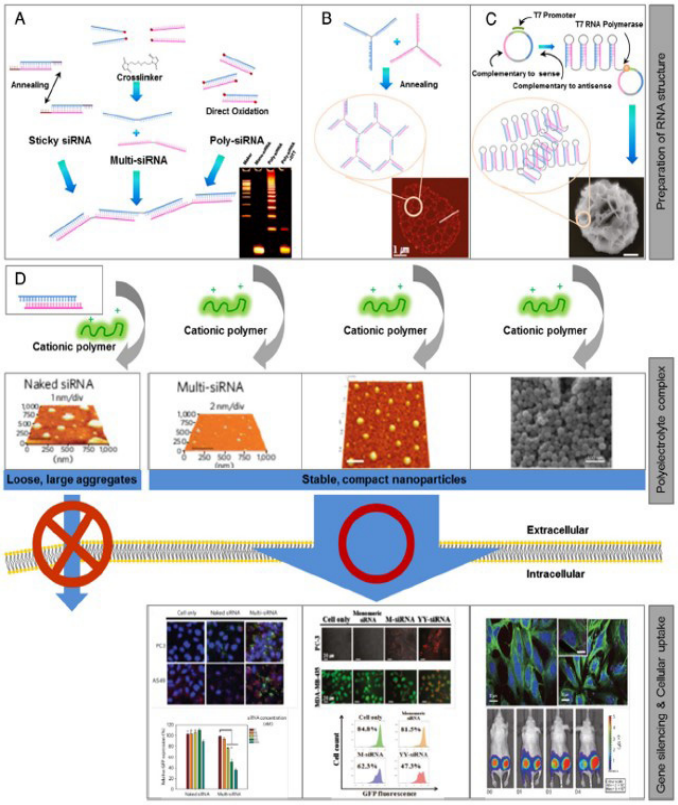

Figure 7: Schematic diagrams of siRNA conjugate system. (A) Cholesterol-siRNA conjugate. (B) Polymer-siRNA conjugate, containing bioreducible disulfide linkage. The polymer-siRNA conjugate can form a stable polyelectrolyte complex with polycation. (C) Aptamer-siRNA chimera. PSMA-specific A10 aptamer is linked to siRNA.

RNAi microsponge

Besides chemical reactions, biological enzymatic reactionswere also used to produce condensed RNA 3D structures [136]. Single-stranded circular DNA encoding both strands of siRNA was used as a template for Rolling Circle Transcription (RCT) to produce hairpin RNA polymers (Figure 7C) [137]. Owing to the in vitro RCT process, a large amount of tandem repeats of hairpin RNA transcripts could be obtained efficiently. Interestingly, the RNA tandem repeats self-assembled into pleated sheets, which subsequently formed sponge like microspheres (RNAi microsponge). The RNAi microsponge itself formed a highly dense structure without additional cationic materials.

Considering the molecular weight and concentration, approximately more than a half million copies of hairpin RNAs was included in a single RNAi microsponge. The tandem repeat of RNA was designed to generate ~21 nt siRNA under the Dicer processing, and it was confirmed that RNAi microsponge was cleaved after treatment with recombinant Dicer. Due to its much higher negative charge of RNAi microsponge, cationic PEI was readily interacted with the particle and formed ~200 nm condensed nanoparticles. The compact nanoparticle complexes exhibited superb cellular uptake and specific gene silencing (Figure 7). It is worthy of notice that extremely low numbers of RNAi/PEI particles were needed to induce similar gene silencing efficiencies, compared to conventional nanoparticle delivery systems. The RNAi microsponge siRNA delivery system provides easy method for high loading of siRNA and production of large amount RNAi triggers using biological enzymatic reactions.

Advances and hurdles to clinical translation of RNAi therapeutics

Now RNAi technique has become a powerful tool for basic research to selectively knock down gene expression in vitro and in vivo. At the same time, both scientific and industrial communities started to develop RNAi therapeutics as the next class of drugs for treating a variety of genetic disorders, such as cancer and other diseases that are particularly hard to be addressed by current treatment strategies.

As distinct advantages of RNAi therapeutics over small-molecule drugs and biologics, first it can wipe out the disease-causing proteins from being translated, thus avoiding the need to attack other downstream components in a molecular cascade, as small-molecules often do [138]. In addition, RNAi therapeutics can target even intracellular proteins hard to be reached bymonoclonal antibodies. Since RNAi is a highly conserved mechanism across mammals with the same siRNA sequences, it can also allow the animal results to be quickly translated into clinical design.

Nevertheless, the journey of RNAi therapies to the clinic didn't go as smoothly as expected. In the early stage both biotechs and major pharmaceutical companies competitively jumped into RNAi therapeutics regardless of technical challenges such as delivery. Consequently, a staggering number of early clinical studies failed to deliver patient benefit [139]. These initial failures made Big Pharma players like Roche, Pfizer and Merck halt their own RNAi programs. But thankfully, for the past few years there has been considerable technical refinement in RNA chemical structure, targeting and delivery. Recently the clinical success rate of RNAi therapeutics is increasing and now some are on the right track to gain FDA approval in the next year or two [140].

Although there still remain some challenges in translating RNAi therapeutics to the clinic and commercialization, they certainly couldn't have been able to proceed this far in developing RNAi drugs without a lot of academic and industry's attempts to design RNAi therapeutics to this day, which are delineated throughout the special issue. In this book, we highlighted the unforeseen hurdles and the potential pathways to translating RNAi therapeutics to the clinic. This book covers the discovery of valuable disease target for RNAi therapeutics (Thomas Roberts et al.), [141] chemical and structural modification of siRNA molecules (Sun Hwa Kim et al. and Hyukjin Lee et al.), cancer-targeted delivery systems for RNAi therapeutics (Xiaoyuan Chen et al. and Kanjiro Miyata et al.) and preclinical and clinical issues in RNAi therapeutics (Hyejung Mok et al. and Yoon Yeo et al.) [141]. We hope that this theme issue provides the readers of Advanced Drug Delivery Reviews great insight into the current advances and hurdles of RNAi therapeutics and accelerates

RNAi drug development from bench to bedside.

Conclusions

siRNA has been received much attention due to its sequence-specific gene silencing efficacy and universality in therapeutic target. Despite its promising potentials as gene therapeutics [142-145], a lot of limitations to clinical applications of siRNA remain to be overcome; not only the inherent properties of siRNA but also delivery barriers have been considered as serious challenges in clinical translation of siRNA drugs. There are many efforts to develop safe and efficient gene carriers, but, recently, the improvement of physicochemical properties of siRNA itself have been accompanied through chemical and structural modifications. The chemically or structurally modified siRNA could exhibit enhanced stability, reduced off-target effects, and minimized immunogenicity. Furthermore, the development of siRNA conjugates, the increase in siRNA size, and the construction of nucleic acid nanostructures could achieve the advancements in siRNA delivery properties. Thus, rational design of the modified siRNA and integrating it with efficient delivery carrier can overcome hurdles to clinical translation of siRNA therapeutics.

Finally, a combination approach using siRNA with a variety of cancer therapies such as chemotherapy, immunotherapy, radiation therapy, or photodynamic therapymay dramatically improve the efficacy of cancer therapy. In this strategy, each form of therapy can be used on targets particularly suited to the therapy type, such as small molecules inhibitors for kinase targets and siRNAs for targets that are structurally unsuited to small molecule attack. Moreover since different therapeutic modalities may trigger different forms of resistance mechanisms such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRP1, MRP2) for small molecules drugs and other yet to be determined modes for siRNAs, such multimodal therapies may be harder for tumors to circumvent. It is our strong hope that by skillful delivery and careful target selection siRNA nanoparticles may take a prominent place in the armamentarium that is being assembled to treat the many diseases that constitute cancer.

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