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Review article

Chemical modulation of siRNA lipophilicity for efficient delivery

Wanyi Tai[a,b,*]

[a] Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Wuhan University), Ministry of Education, China
[b] Wuhan University School of Pharmaceutical Sciences, Wuhan, Hubei 430071, China

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ABSTRACT

siRNA holds tremendous promise to knockdown the disease-promoting gene in any cell type that it can be delivered. The biggest challenge facing the clinical translation of siRNA is to overcome the barriers that impede the *in vivo* delivery. siRNA is a highly hydrophilic macromolecule with poor pharmacological properties. The critical requirement for broadening the systemic siRNA delivery is to confer on siRNA the 'drug-like' properties such as the lipophilicity. In comparison with unmodified siRNA, lipophilic siRNA has a better cell-membrane compatibility and permeability. The lipophilic siRNA can also hijack the lipid trafficking pathway and natural drug carrier albumin to achieve an advanced systemic delivery, with properties including the tissue targeting, long circulation half-life, the improved pharmacokinetic profile, bioavailability and *in vivo* safety. In this review, we summarized the recent progress on lipophilic siRNA development, in particular about the chemical methods to manipulate siRNA lipophilicity. The covalent and noncovalent methodologies, including backbone modification, lipid conjugation, chelation method and others, are discussed. We place particular emphasis on the most clinically advanced method, the lipid siRNA conjugation. The application of lipophilic siRNA on nanoparticle delivery system is also covered.

1. Introduction

Short interference RNA (siRNA) is of considerable current interest in medicine because of its unique capability in probing a broad range of gene functions and addressing some undruggable disease-targets that are inaccessible by chemical drugs [1]. Because of the reliance on base sequence for bioactivity, siRNA has the advantages over conventional medicine (chemical drugs and antibodies) in that it is easy to design, able to target every-disease-promoting gene, and promising with unprecedented specificity, potency and duration of effect [2,3]. Despite the huge therapeutic potential, the clinic application of siRNA remains challenging due to the difficulty of *in vivo* delivery [4]. Recent advances in bioengineering and nanotechnology have produced a number of nonviral siRNA carriers, such as cationic lipids, cell-penetrating peptides (CPP), polymers, inorganic nanoparticles and cationic proteins [5–7]. These cationic carriers and derivatives have been widely used for *in vitro* gene silencing and show potential for *in vivo* siRNA delivery in some studies. It is especially notable that one siRNA carrier (Patisiran) has be approved under orphan drug act by the Food and Drug Administration (FDA) [8,9]. However, there is ongoing appreciation that cationic toxicity is a limiting factor of *in vivo* siRNA delivery, which would cripple the advance toward clinical translation [10]. In order to reduce the toxicity, some researchers have developed biodegradable cationic polymer that is capable of specific endosomal degradation into low molecular weight, less toxic polycations [11–14]. Hybrid nanoparticles, formed by enveloping polymer/siRNA nanoparticle into lipid vesicles, have also been fabricated for systemic delivery with minimum - toxicity [15–17]. Despite the intriguing proof-of-concept design and ongoing evolution of capability, some characteristics including heterogeneity of size, complexity of formulation and technically challenges on large-scale fabrication, make them recalcitrant for meeting the rigorous demands of drug manufacturing and clinical trials.

siRNA is a large (~14 kDa), highly hydrophilic and polyanionic (~42 negatively charged phosphates) macromolecule that possess intrinsically poor pharmacological properties. Unmodified siRNA shares a similar chemical nature to mRNA that is feasible to be attacked by ubiquitous ribonuclease. Unmodified siRNA is highly water-soluble and reluctant to bind with any serum protein during blood circulation, eventually being quickly excreted by renal filtration and exhibiting a plasma half-life of < 10 min [18,19]. From all the aforementioned aspects, unmodified siRNA is a problematic macromolecule drug that has little 'drug-like' perspective. siRNA has to be chemically modified or modulated to improve its bioavailability *in vivo*.

Lipophilicity is one of the most important physical properties that

* Corresponding author at: Key Laboratory of Combinatorial Biosynthesis and Drug Discovery Ministry of Education, School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, China.
E-mail address: wanyi-tai@whu.edu.cn.

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correlates with the drug-likely behavior of a compound. It is often represented by the descriptor logP (the partition coefficient) to help predict *in vivo* permeability of active compounds in drug discovery. Drug with appropriate logP binds with natural drug-carrier proteins (such as albumin) in blood and exhibits a prolonged circulatory half-life, eventually improving the biodistribution profile and therapeutic index. siRNA is extremely hydrophilic molecule and almost has no lipophilicity. An attractive concept is to chemically modulate siRNA into lipophilic and confer the drug-likely properties. The 'drug-likely' properties, such as serum stability, cellular permeability, targetability, circulation half-life and tissue bioavailability, are the critical requirements to achieve systemic RNAi *in vivo*. Compared to unmodified siRNA, lipophilic siRNA exhibits a better membrane compatibility and permeability due to its lipophilic nature [20,21]. It also can take advantage of the natural lipid trafficking pathway to achieve the targeted delivery to specific tissues *in vivo*, which is valuable and incomparable by chemical drugs [22]. After lipophilic modulation, siRNA not only can harness the natural drug carriers in blood circulation for an improved pharmacokinetic, but also allow scientists to formulate it into the conventional drug formulations (such as liposome, PLGA nanoparticles) for better delivery. In this review, we describe the current aspects on chemical modulation of siRNA lipophilicity by covalent and noncovalent methods. We place particular emphasis on the most clinically advanced method, the lipid siRNA conjugation. Other modulation methods such as backbone modification, chelation and incorporation with nanoparticles are also covered.

2. Backbone modification to improve lipophilicity

Clinical translation of siRNA is hampered by siRNA's comprehensive poverty on drug-likely properties, which necessitates the lipophilic modulation. The most straightforward method to modulate lipophilicity is to graft lipophilic groups onto the siRNA backbone. Backbone modification has several advantages: a) plenty of free groups available for modification (such as 2′-OH, phosphates and bases on siRNA chain, Fig. 1A), b) flexibility to choose the modification position, thus control the distribution of lipophilicity on the siRNA linear structure.

Over the past decade, this approach has been extensively explored by many groups, initially with the synthesis and evaluation of 2′-OMe modification in which the 2′-position of the ribose moiety (2′-OH) were masked by methyl groups to give rise to a slightly hydrophobic and thermostable siRNA (Fig. 1B). Modification by such a small alkyl group was shown to successfully preserve the capability of siRNA to dock into the RNA-induced silencing complex (RISC) and engage the RNAi pathway, but fail to promote the carrier-free delivery, probably due to features of inadequate lipophilicity. However, 2′-OMe modification dramatically enhances nuclease resistance and avoids the innate immune activation, which encourage researchers to explore some larger lipophilic groups. The modifications with 2′-O-MOE and 2′-O-allyl structures (Fig. 1B) was explored but it was later found that their steric clashes with Ago2 prevent the modified siRNA loading into RISC and eventually cause an attenuated silencing activity [23,24]. More recently, Debart group has demonstrated a prodrug concept that siRNA is modified by bulky groups while at the same time it enables to preserve the RNAi potency [25]. This siRNA prodrug contains bulky lipophilic moieties chemically tethered to 2′-OH *via* a bioreversible linker. Once enter cytoplasm, the linker would be cleaved off by cytoplasmic esterase and the prodrug was converted to wild-type siRNA to allow efficient target-gene suppression (Fig. 1C). Some lipophilic groups, such as pivaloyloxymethyl (PivOM) and phenylisobutyryloxymethyl (PiBuOM) (Fig. 1B and Fig. 1C) have been selected to modify siRNA [20,26]. The modification successfully transform siRNA to highly lipophilic, and provides efficient intracellular delivery. Moreover, the greater lipophilicity of PiBuOM relative to its PivOM counterpart appears to be favorable for transmembrane delivery, which necessitate the lipophilic modulation for efficient siRNA delivery [20]. Biscans et al. extended the application scope of PiBuOM modified siRNA to target EWS-Fli1 gene for the treatment of Ewing's sarcoma. EWS-Flil activity

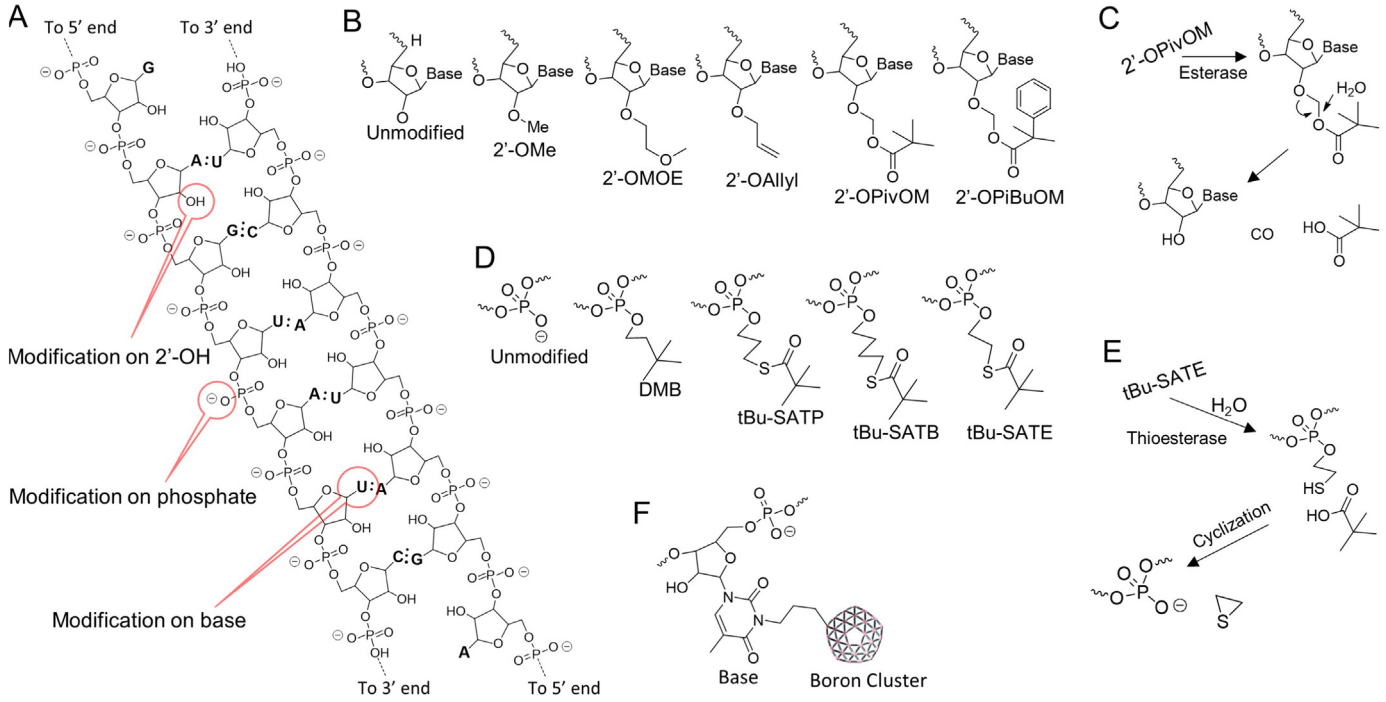

Fig. 1. Chemical modification of siRNA backbone with lipophilic moieties. A) The structure of siRNA duplex and the corresponding modification positions on 2'-OH of sugars, phosphate and base. Note: the marked position represents just one of the many same groups. B) Lipophilic moieties modified at the 2'-OH groups of siRNA backbone. C) Conversion of 2'-OPivOM into wild-type 2'-OH by cytoplasmic esterase. D) Chemical modification of phosphate group into phosphotriester by SATE and other lipophilic groups. E) Cleavage by thioesterase deprotects tBu-SATE, and converts siRNA backbone into wild-type phosphodiester. F) Modification of siRNA base with hydrophoblic boron cluster.

represents an attractive target to regulate cell proliferation, differentiation, and apoptosis of bone tumors. A dose/response effect, with a maximum of 60% knockdown of EWS-Flil mRNA, was obtained when A673 cells were treated by a PiBuOM modified siRNA containing eight consecutive nucleotide modification at the 5′-terminus of the sense strand [20].

The negatively charged phosphate groups on the phosphodiester linkage are potential sites for siRNA modification (Fig. 1D). Chemical conjugation of lipophilic group onto the phosphate would mask the inherent negative charges of siRNA and presumably promote the cellular uptake. The feasibility of this strategy has already been demonstrated decades ago with oligo DNA in which the phosphate backbone was replaced by charge-neutral phosphotriester group [27]. More recently, Meade et al. expanded this concept to siRNA [28]. The bioreversible phosphotriester group containing S-acyl-2-thioethyl (SATE) was placed on RNA building blocks and synthesized into siRNA on column, which finally give a lipophilic siRNA prodrug, namely short interfering ribonucleic neutral (siRNN). siRNN can be activated by cytoplasmic thioesterase and convert into wild-type siRNA by a rapid two-step reaction (Fig. 1E). Similar to chemical drugs, lipophilic siRNN, but not unmodified siRNA, avidly bound serum albumin, thus prolonging the retention of siRNN in blood circulation. Single intravenous injection of GalNAc-siRNN (25 mg/kg) induced strong ApoB RNAi response in liver at 72 h. In contrary, only weak response was observed within the group treated by the charged GalNAc-siRNA (unmodified siRNA). A kinetic analysis of the RNAi response over time reveals the strong correlation between prodrug-hydrolysis dynamics and RNAi activity. This observation implicates that the siRNN prodrug can convert into wild-type siRNA in cytosol and induce robust RNAi responses *in vivo*. Unfortunately, siRNN fails to permeate through cell membrane, and a TAT peptide has to be conjugated to promote the delivery in the study [28]. Considering the sparseness of the modified sites on siRNN, the hurdle to carrier-free delivery of siRNN might be surmounted by increasing siRNN's lipophilicity or expanding the chemical diversity of SATE groups.

Direct modification on the bases of siRNA is an alternative strategy, but it is rarely reported in literatures. The reason might be that base modification has negative impact on the A-form helix geometry of duplex while the A-form pattern is essential to siRNA's RNAi activity [29]. Kwiatkowska et al. modified the thymidine of siRNA at two positions with a lipophilic boron cluster ($C_2B_{10}H_{11}$) (Fig. 1F). The helical structure of the modified siRNA remained unchanged upon boron cluster introduction. However, the lipophilic modification does not facilitate the cellular uptake of the siRNA duplex, probably due to the poor hydrophobicity of boron cluster and the weak affinity to the cell membrane [30]. Instead, Peel et al. incorporated a cholesterol group at a uracil base of siRNA backbone. The gene silencing studies revealed that the modification is well tolerated within the RNAi machinery. siRNA with one cholesterol-modified pyrimidine substituent offers effective silencing of firefly luciferase at lever > 80% in the absence of a carrier or transfection reagent [31].

3. Lipid siRNA conjugate for carrier-free delivery

3.1. Cholesterol siRNA conjugate

In exploring potential route to siRNA delivery *in vivo*, scientists at Alnylam Pharmaceuticals Inc. discovered that cholesterol conjugated siRNA (chol-siRNA) showed an increased uptake to liver cells *in vitro* and a substantial silence (around 50%) of an endogenous gene apolipoprotein (apoB, a gene clinically relevant with hypercholesterolaemia) *in vivo* [18,22,32]. Cholesterol was tethered to the 3′ end of sense strand of siRNA molecule by a short trans-4-hydroxyprolinol linker (Hyp) (Fig. 2A). The terminal conjugation preserves the capability of siRNA loading into RISC and results in only a slight loss of gene silencing activity *in vitro* [18]. The lipophilic chol-siRNA was injected in mice to knockdown the endogenous apoB mRNA expression. The treatment brought about a significant reduction of apoB expression in both liver (57 ± 10%) and jejunum tissue (73 ± 10%), leading to the pharmacological effect of reduction on total cholesterol absorption (Fig. 2B) [18]. The *in vivo* silencing efficacy of chol-siRNA was found to be dependent on presence of the cholesterol moiety. Cholesterol binds and associates with lipoprotein to form lipoprotein/chol-siRNA particle (Fig. 2C), that hijacks the natural lipid transport pathway to deliver chol-siRNA into liver cells [22]. siRNA delivery by lipid transport pathway is a unique advantage of chol-siRNA. Compared to the cationic nanoparticle systems that rely on electrostatic interaction for siRNA condensation and targeting, chol-siRNA, administrated without complex formulation, forms a stable particle with endogenous lipoprotein and selectively be delivered to liver by taking the advantage of natural lipid transport pathway, with expectation of minimum toxicity. Both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) play important role in the delivery of chol-siRNA, but it appears that their uptake involves in different lipid transport pathways and they preferentially deliver chol-siRNA to different tissues. More specifically, LDL particle mediates delivery of chol-siRNA predominately into liver, whereas HDL-bound chol-siRNA are accumulated in many other tissues, including adrenal, ovary, kidney and liver [22]. It is worth mention that the plasma clearance of lipoprotein particle containing chol-siRNA ($t_{1/2}$, 1–2 h) is markedly faster than that of lipoprotein itself ($t_{1/2}$, 2.6–4.8 days). Because of the short serum half-life, repeated injection of high doses of chol-siRNA is necessary for target gene silencing [18].

Cholesterol conjugation on siRNA has markedly improved the pharmacokinetic property of siRNA. Despite priory associated with lipoprotein, chol-siRNA is also avidly bound with serum albumin in circulation ($K_D \sim 1\ \mu M$) (Fig. 2C) [18]. Especially in the situation of high dose *i.v.* injection when the lipoprotein is saturated, the majority of chol-siRNA in blood is carried by the endogenous albumin. Albumin is the most abundant protein in serum and it has a blood circulation half-life of > 20 days [33]. It is a natural carrier of many chemical drugs and can significantly prolong the plasma residence time of drugs [34]. Presumably because of the albumin binding, chol-siRNA administrated by *i.v.* injection showed an elongated elimination half-life ($t_{1/2}$–95 min) in comparison to unconjugated counterpart ($t_{1/2}$–6 min) [18]. The elongated blood retention helps to broaden the tissue biodistribution of chol-siRNA beyond the liver, that enables therapeutic targeting of diseases in extrahepatic organ such as muscle disorders and autoimmune encephalomyelitis [35,36]. It is aware that the uptake of chol-siRNA by these extrahepatic tissues might be mediated by a mechanism independent of lipid trafficking pathway, as no expression of lipoprotein receptor on these tissues. Unpublished data revealed that LDL receptor-knockout and ApoE-knockout mice remained responsive to the chol-siRNA treatment, suggesting that chol-siRNA uptake by liver only partially rely on the lipoprotein [36]. The extrahepatic delivery of chol-siRNA is likely related to the albumin. Albumin binding elongates the blood half-life of chol-siRNA and at same time provides a mean for it to reach the extrahepatic organs. It is envisaged that the dynamic and reversible binding between albumin and chol-siRNA give a chance for chol-siRNA to directly contact with cell membrane, insert its cholesterol tail and internalize through endocytic pathway similar to the *in vitro* cellular uptake mode of chol-siRNA [37].

It was reported that the carrier-free cellular uptake and gene silencing efficiency of chol-siRNA was strongly affected by the linker length between siRNA and cholesterol [38]. It is optimal to have cholesterol and siRNA conjugated by an oligomethylene linker lengthing from 6 to 10 atom. The shortening or elongating of the linker was founded to either reduce the cell uptake of chol-siRNA or attenuate the gene silencing efficacy [38]. Nevertheless, it is worth noting that a maximally around 50% silencing effect can be obtained *in vitro* and *in vivo*, even treated by the chol-siRNA of best linker length at high dose [18,22]. It was reported that chol-siRNA enter cells *in vitro* through a two-step mechanism with a rapid membrane binding and a following

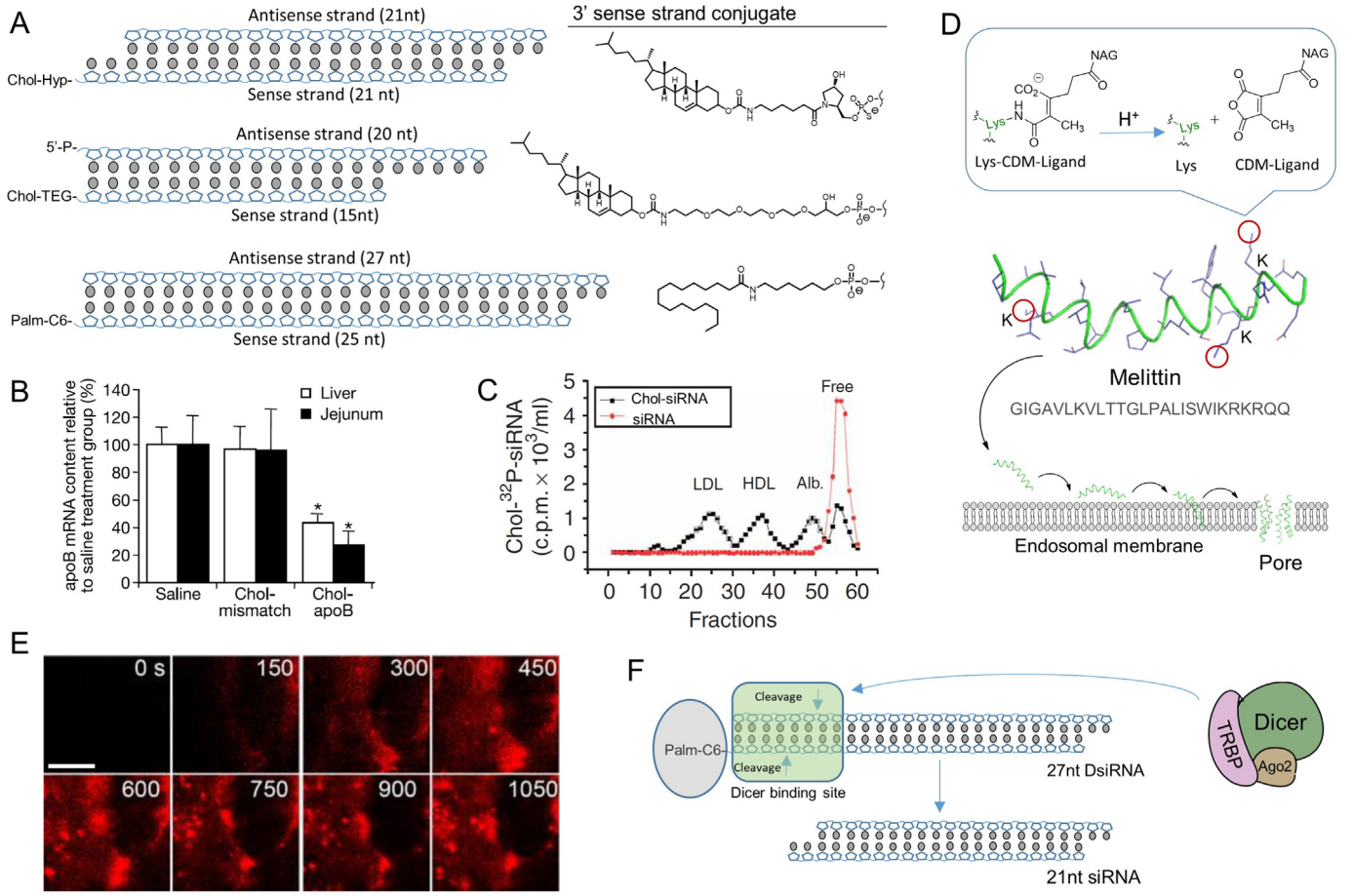

Fig. 2. Delivery siRNA by lipid conjugation. A) lipid conjugated with siRNA of three scaffolds. Upper, cholesterol conjugate with canonical 21 nt siRNA (chol-siRNA); middle, cholesterol conjugated with asymmetric siRNA. The whole structure was annotated as hsiRNA or sd-rxRNA; bottom, palmitic acid tethered at 5′ end of sense strand of DsiRNA. B) Intravenous administration of cholesterol conjugated anti-apoB siRNA (chol-apoB) induces *in vivo* silencing in the liver and jejunum of wild-type mice. Adapted with permission from *Nature* 2004; 432: (7014). 173–178. C) Chol-siRNA associates with lipoproteins and albumin in blood. Plasma from C57BL/6 mice injected with 32P-labeled chol-siRNA was separated by size exclusion gel filtration. Adapted with permission from *Nat. Biotechnol.* 2007; 25(10). 1149–1157. D) Chemical enhancer, the modified melittin, promotes the endosomal release of chol-siRNA by forming pore on the endosomal membrane. pH sensitive group CDM masks the amino residues of three lysines in melittin, thus suppresses the toxin's pore-forming activity. The liver tropic ligand NAG guides the melittin conjugate into the liver and enters endosome together with chol-siRNA. The CDM protecting groups unmask from the melittin amino acids in the endosomal acidic environment. The melittin peptides subsequently contact with lipids, insert into membrane, tetramerize into a nanopore and eventually lysis the endosomal vesicles. Red circle indicates the residues of lysine (K) in melittin peptide. E) Quick internalization of the DY574 (red) labeled hsiRNA (250 nM) into COS-7 cells. Images were visualized by total internal reflection (TIRF) microscopy. Scar bar: 10 μm. Adapted with permission from *Nucleic Acids Res* 2017; 45: (1). 15–25. F) Dicer binds at the proximal end of the lipid conjugated, and processes the 27 nt DsiRNA into 21 nt canonical siRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cellular internalization, whereas the *in vivo* uptake of chol-siRNA by hepatic cells was mediated by a lipid transport mechanism [22,39]. The comprehensive analysis of the weak gene-silencing efficacy leads to the question of endosome escape after cellular uptake of chol-siRNA. It appears that the exceedingly low rate of siRNAs escaping from endosomes (often as low as < 0.01%), significantly limits the potential of RNA-based therapeutics [3]. A classic approach is to use endosomal escape enhancer that help chol-siRNA escape into cytosol. Work form Zerial group has focus on identifying small chemical enhancers from chemical library to improve gene silencing efficiency of chol-siRNA [40]. However, these enhancers act on the endocytic pathway at concentration as high as 10 μM, which has no therapeutic window for clinical use. Arrowhead Inc. focuses on a derivative of natural pore-forming peptide melittin (bee venom) for endosomal escape. Feature of this enhancer include pH-sensitive protecting groups (CDM) that are reversibly masked on peptide chain in order that the pore-forming activity of melittin is only revealed in the acidic environment of the endosome (Fig. 2D). In a proof of concept study, simple coinjection of chol-siRNA with the melittin peptide enhancer targeted to hepatocytes by the NAG ligand results in around 25-fold improvement in the efficacy compared with injection of chol-siRNA alone, without toxicity in mice and nonhuman primates [41]. This enhancer-assisted chol-siRNA has been developed as a RNAi therapeutic for the treatment of chronic hepatitis B virus (HBV) infection, which was later promoted into clinic evaluation under the name of ARC-520. In the phase I study consisting of 54 healthy volunteers, single injection of ARC-520 was well tolerated within an intravenous dose of 0.01–4.0 mg/kg [42]. The adverse-event frequency was same to placebo and no serious adverse events were observed. A phase 2a clinic trial revealed that ARC-520 can reduce the expression of s-antigen, e-antigen and correlated antigen in human after a single dose injection, a positive sign correlating to the pharmacological outcome of siRNA mediated HBV protein suppression [43]. The use of potent endosomal enhancer like melittin to promote chol-siRNA delivery has proved to be helpful, but this exogenous toxin invites concerns on immunogenicity and toxicity, which eventually led to the clinical hold on all these trials by FDA in 2017.

3.2. hsiRNA, and cholesterol conjugated with siRNA of noncanonical scaffolds

hsiRNA, also called sd-rxRNA, is a novel class of cholesterol siRNA conjugate that has noncanonical and asymmetric siRNA scaffold (Fig. 2A). Chu and Chang et al. reported that asymmetric siRNA with 16 nt sense strand and 22 nt antisense strand showed similar silencing potency to the canonical siRNA (21 nt, 19 nt duplex plus 2 nt 3′ overhangs) [44,45]. More importantly, the asymmetric scaffold includes a 6 to 8-nt single strand tail (PS tail) that can be phosphorothioated and promote cellular internalization by a mechanism similar to that of fully phosphorothiolated antisense oligonucleotide [39,46]. Khvorova group first conjugated asymmetric siRNA with cholesterol and generated the hsiRNA scaffold that efficiently enter cells *in vitro* and *in vivo* without aid of any delivery carriers [47]. The uptake of hsiRNA is quick and the internalization happens within minutes of exposure to cells (Fig. 2E), which is attributed by synergic uptake effect of the cholesterol and PS tail [39]. Compared to canonical chol-siRNA scaffold, hsiRNA reduces the number of overall charges on siRNA while maintains the RNAi activity, which is beneficial to the siRNA's deliverability and stability [3]. Beyond the new siRNA structure, Khvorova group capped the 5′ end of the guide strand with 5′-vinylphosphonate group and thus stabilized the phosphorylation state of hsiRNA by protecting it from phosphatase hydrolysis [48]. The modification confers better exonuclease resistance and longer silencing duration in tissues, thereby enables efficient silencing genes in brain, placenta, kidney and heart [49–51]. RXI-109, a lipophilic siRNA powered by hsiRNA technology, has advanced into several clinical evaluation of the ability to reduce hypertrophic scarring [52]. RXI-109 targets to the connective tissue growth factor (CTGF) gene, a key regulator of the dermal scarring pathway, as a means to reduce hypertrophic scarring and keloid formation. The initial phase II study on 16 patients indicated that RXI-109 improved the appearance of the scars with no instances of significant side effects or dose limiting toxicity [52]. In other clinic trials, reduction of CTGF by RXI-109 shows the clinical potential for the treatment of age-related macular degeneration and retinal scarring as well [53].

Dicer substrate siRNA (DsiRNA) is the short dsRNA of 27 nt in length that is much more powerful effectors of gene-specific silencing than canonical 21 nt siRNA [54]. Unlike the canonical siRNA that directly loads into RISC, DsiRNA is first processed into 21 nt siRNA and subsequently passed over to RSCI by Dicer. It is believed that the tandem biological processes and the secondary role of Dicer are responsible for the enhanced silencing effect (up to 100-fold more potent than 21 nt siRNA targeting same sequence) [55]. Works from Seyama and Yokota groups focus on chemical modulation the lipophilicity of DsiRNA (Fig. 2A). It was found that DsiRNA sequence can preserve the cleaving efficiency to Dicer after it was tethered with bulky lipidoid structure (Fig. 2F). The modification improves the stability, facilitates the membrane permeability and has no negative impact on RNAi potency [56–58]. Due to the necessity of Dicer processing, DsiRNA can't tolerate the fully chemical modification using the advanced version of an alternating 2'-F, 2'-OMe pattern [59]. This full modification technology has been used on hsiRNA and improves its efficacy by 100 times in various tissue *in vivo* [60]. From aspect of stability, lipophilic DsiRNA may has less therapeutic potential than hsiRNA in clinic.

3.3. The influence of lipidoid structure on targeting and delivery

Lipophilicity is an important factor that control the *in vivo* biodistribution of lipid siRNA conjugate. The liver-tropic chol-siRNA (and hsiRNA) has been extensively investigated [22]. The targeted delivery of this lipophilic siRNA *in vivo* is mediated by hijacking the natural lipid transport pathway and delivering to liver or other tissues that express lipoprotein receptors [22]. The delivery pattern and efficacy are highly dependent on interaction between lipid and lipoprotein, and the interaction is driven by lipophilicity of lipid moiety (Fig. 3A). It was

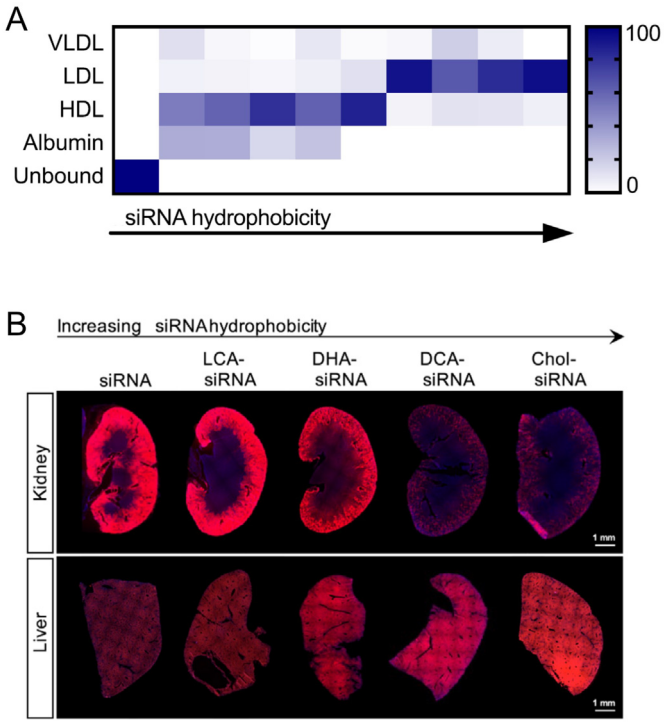

Fig. 3. The lipophilicity of the lipid impacts the protein binding profiles and tissue biodistribution. A) The summary of protein binding profiles *vs* the hydrophobicity of the lipid siRNA conjugate. Highly hydrophobic lipid conjugate prefers binding with LDL, whereas lipid siRNA conjugate with moderate hydrophobicity binds with HDL and albumin. Unmodified siRNA can't bind with any protein. B) Kidney and liver distribution is governed by the hydrophobicity of the lipidized siRNA. Adapted with permission from *Nucleic Acids Res* 2019; 47: (3). 1070–1081.

revealed that siRNA conjugated with cholesterol, lithocolic-oleyl (C43, Fig. 4)) and all other highly lipophilic lipid (such as C32) has high affinity to LDL. Its delivery follows the LDL mediated lipid transport pathway and predominately deposited in the liver [22,32]. Shorter chain lipids or less lipophilic moieties such as lithocholic acid (LCA), prefer binding with HDL and albumin. The uptake of these protein/siRNA complex is mediated by different transport pathway and siRNA is finally delivered to widespread tissues including liver, kidney, ovary, intestine and others (Fig. 3B) [22,61]. These evidences suggest that the lipophilicity of siRNA can affect the selectivity of *in situ* incorporation to endogenous lipoprotein pathway, which eventually governs the *in vivo* biodistribution.

The chemical structures of lipid also has a profound effect on siRNA delivery *in vivo*. α-Tocopherol is a fat-soluble natural molecule (vitamin E) which is highly tolerable for administration. Its siRNA conjugate (Toc-siRNA, Fig. 4) can bind with lipoproteins (LDL and HDL) just like chol-siRNA and take advantage of lipid transport pathway for systematic delivery, but it also associates with the α-tocopherol-associated proteins in serum and mediates the hepatic uptake in another pathway, thus resulting in the different hepatic delivery efficiency compared to chol-siRNA [58]. Similarly, it was observed that, after *i.v.* administration, DCA-siRNA is preferentially deposited in heart, whereas EPA-siRNA is mainly accumulated in lung, and PC-DCA-siRNA in muscle, fat and adrenal gland [62]. Although there is no hypothesis to explain the relationship between the lipid structure and organ tropism, the data demonstrated the feasibility of extra-hepatic delivery of lipophilic siRNA by screening particular lipid that enable to accumulate highest siRNA at target tissues. Besides the organ tropism, lipid structure can highly affect the cell-style distribution in organs as well. Take the distribution pattern in kidney for example, LCA-siRNA is only accumulated

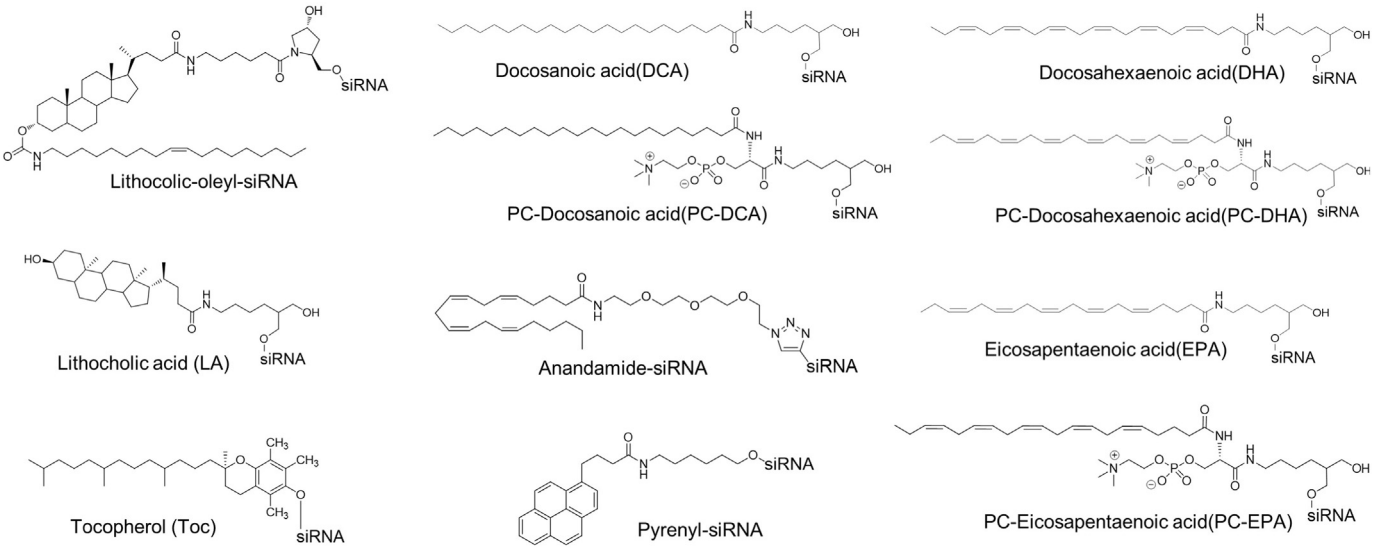

Fig. 4. The structures of lipids conjugated with siRNA.

in epithelial cells, whereas EPA-siRNA (and DHA-siRNA) can reach to the glomerulus. In contrary, its derivative PC-EPA-siRNA (and PC-DHA-siRNA) shows no accumulation in the glomerulus [62]. The selectivity of lipid to a specific cell type might be attributed to the specific affinity of a lipid structure to the membrane of target cells. This mechanism has been demonstrated by Willibald et al. with an anandamide modified siRNA (Anandamide-siRNA, Fig. 4) targeting to neuronal and immune cells [63].

Some efforts have been made to explore the lipid structure for better local delivery of lipophilic siRNA. Chol-siRNA was the first pilot compound to examine the local delivery efficacy of siRNA to eyes [47]. The cholesterol conjugated siRNA can efficiently silence the target mRNA in retinal, but it was found that chol-siRNA exhibit a steep gradient of diffusion from the injection site, which might be attributed to the high lipophilicity of cholesterol [64]. Several groups has explored to use smaller and less hydrophobic lipids to improve the local delivery efficacy. Nikan et al. intrastriatally inject DHA-siRNA, a less lipophilic siRNA, into mouse brain and found that DHA-siRNA show widespread distribution in brain [64]. Nikan et al. later introduced a phosphocholine to DHA lipid (PC-DHA-siRNA) and believed that it will further reduce the lipophilicity, while more closely resemble the natural structure of endogenous DHA, hoping to improve the activity and safety profile of DHA-siRNA. However, PC-DHA-siRNA didn't outperform DHA-siRNA in delivery and gene-silencing efficiency. Instead it shows a sign of adverse effect which is believed to relate with the local high-dose toxicity of PC-DHA-siRNA [65]. Seyama group circumvented the route by conjugating siRNA (and DsiRNA) with small chemicals such as aromatic compound (Pyrenyl-siRNA, Fig. 4) or palmitic acid. It was shown that the modification provides sufficient lipophilicity for delivery and at same time well preserves the gene silencing efficacy of siRNA [56,57,66]. However, this type of lipophilic siRNAs has never been evaluated in the test of local delivery.

3.4. siRNA conjugated with multiple cholesterol molecules

Previous reports have demonstrated that higher lipophilicity promote the incorporation within lipoprotein and enhance delivery efficacy [22]. However, most of the current effort was devoted to the terminal modification, in which only one cholesterol molecule (or other lipid) is conjugated with siRNA (either 5′ or 3′ end of sense strand). Tethering multiple cholesterol molecules with siRNA is a promising strategy because it can further increase the lipophilicity of siRNA and better promote carrier-free delivery. Lorenz et al. conjugated the 5′ ends of both sense and antisense strand with cholesterol, which give a lipophilic siRNA with two cholesterol copies (chol2-siRNA) [32]. Despite higher lipophilicity, Chol2-siRNA showed an attenuated gene suppression ability, which might be attributed to the modification at antisense strand [67]. Salim et al. developed a chemical method that incorporated cholesterol within the central region of sense strand and claimed that, this modification bypassed the antisense strand and was compatible with RNAi pathway [68]. The new methodology enables the incorporation of multiple cholesterol moieties at varying positions of sense strand, thus can tune the siRNA lipophilicity precisely [31]. A triple cholesteryl modified siRNA (chol3-siRNA) has been synthesized and evaluated in animal model by Bienk et al. [69]. This new lipophilic siRNA exhibits an extended blood circulatory half-life, liver accumulation and gene silencing of target mRNA compared to chol-siRNA, which likely correlated with the enhanced affinity to serum albumin (K_d ~ 0.1 μM *vs* 1 μM). The relevance of therapeutic potential to the number of cholesterol molecules offers exciting versatility in chemical modulation of siRNA lipophilicity.

3.5. Safety profile of lipid siRNA conjugate

Due to the structure diversity of lipids, lipid siRNA conjugates exhibit complicate safety profiles compared to the unmodified siRNA. The toxicity of lipophilic siRNA correlates with the lipid structure, the administration route and RNA chemistry. It was reported that local administration of highly hydrophobic hsiRNA (25 μg) induce the acute toxicity in the central nervous system [64], whereas the same hsiRNA is well tolerable at the dosage as high as 20 mg/kg when administrated systematically [51]. The toxicity of local delivery is likely caused by the cholesterol structure. Cholesterol may disturb the neural membrane potential and activate glial cells, which induce a significant neuronal death at as little of 25 μg dosage [70]. In contrary, DHA-siRNA conjugate shows no evidence of neuronal toxicity and innate immune activation at all. When systmetic addministration, DHA-siRNA also has a much higher maximum tolerated dose than chol-siRNA [60]. In addition, RNA chemistry is also a contributor to the observed toxicity. The partially modified siRNA, which is degradable *in vivo*, is considered more safe and tolerable than the fully stabilized chol-siRNA [49,60]. It is worth noting that the PS tail of hsiRNA is not only responsible for the cellular uptake mechanism, but may also trigger sequence-unspecific toxicity, including apoptosis induction, endoplasmatic reticulum stress and other side effects commonly seen on phosphorothioate-modified antisense oligonucleotides [71–73]. An advanced discussion on the

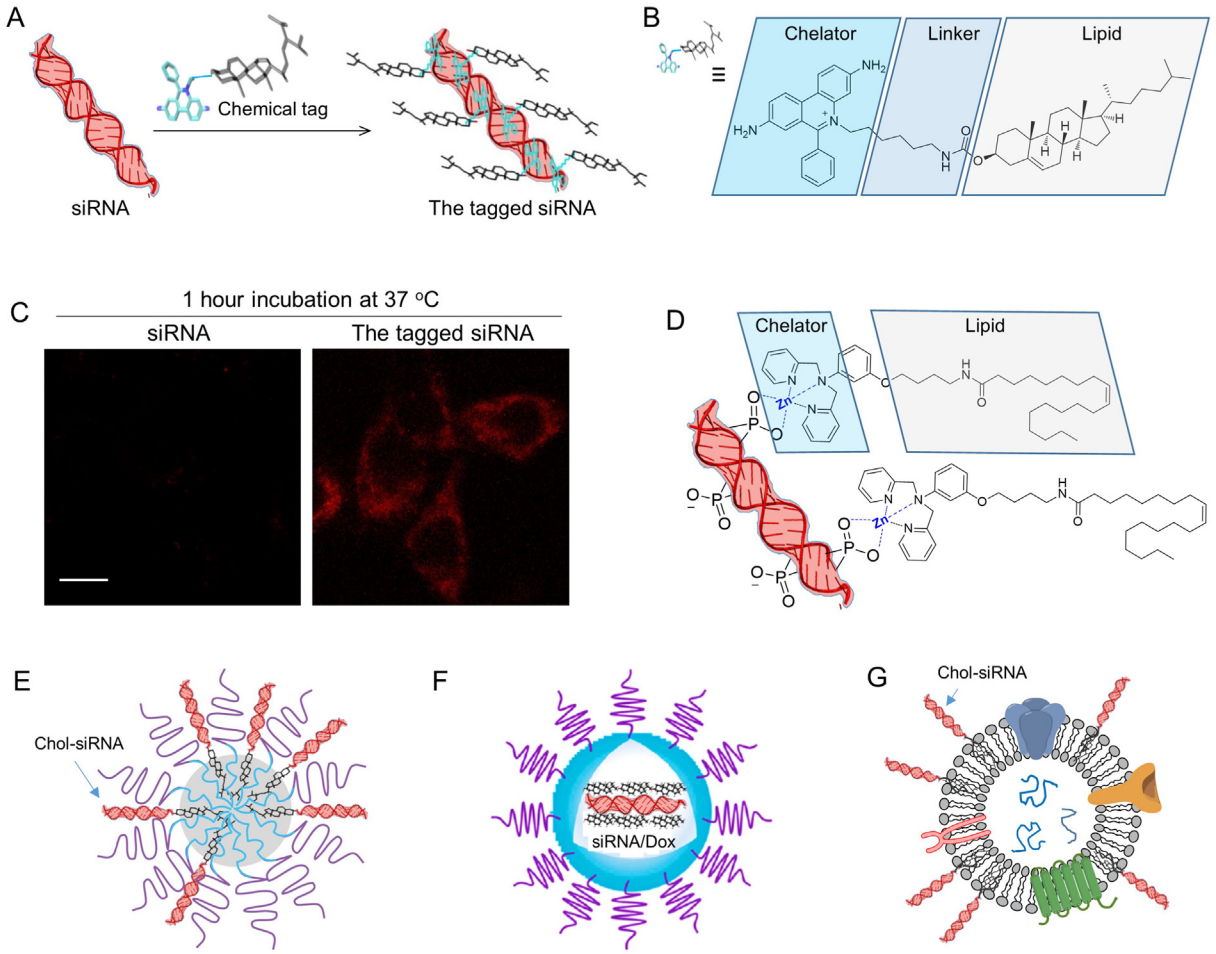

Fig. 5. A) Chemical tag intercalates its ethidium moiety (cyan) into siRNA duplex and leaves its cholesterol moiety (grey) outside, thus transform the tagged siRNA into lipophilic and membrane permeable. B) The chemical structure of the chemical tag. The functional moieties are highlighted within colour boxes. C) The tagged siRNA (red, Cy3 labeled) translocates into cytosol 1 h after co-incubation with PC-3 cells. Scar bar: 10 μm. D) Zn/DPA tag chelates with the phosphate groups of siRNA. The structure of chelator and lipid are highlighted within cyan and grey boxes representatively. E) 'Micelleplex' nanoparticle formed by incorporating Chol-siRNA into polymeric micelle. F) siRNA can form a hydrophobic complex with doxorubicin and be readily encapsulated into noncationic PEG-b-PLA micelles for systemic delivery. G) Chol-siRNA inserts its cholesterol moiety into the exosome membrane, thus enables efficient and controllable loading of siRNA onto exosome vesicles for delivery. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

toxicity of lipidized siRNA has been reviewed elsewhere [70].

4. Modulation of siRNA lipophilicity by chelation

siRNA modulation can be achieved by the reversible, noncovalent linkage such as chelation. From the aspect of bioconjugate chemistry, the covalent approach (bioconjugate method) to modulate siRNA lipophilicity is challenging and low efficient (time consuming). In order to rapidly expand the chemical diversity of lipophilic siRNA, our group investigated a conjugation-free methodology that utilizes a small chemical tag to transform siRNA lipophilic, membrane-permeable and self-deliverable (Fig. 5A) [21]. The chemical tag utilized ethidium-siRNA intercalation to attach the lipophilic moiety to the siRNA backbone surface, thus transforming siRNA lipophilic and membrane permeable. We built a library of > 10 chemical tags that present a broad variety of lipophilicity and functionality. siRNA decorated with cholesterol tag (chemical structure in Fig. 5B) is cell membrane-permeant, and the cytosolic delivery can bypass the endocytic pathway [21]. With a short period of 1-h incubation, the tagged siRNA translocates across the membrane and evenly distributes in the cytosol, without the sign of endosomal trapping (Fig. 5C). The tagging technology relies on the relatively weak affinity of ethidium to siRNA duplex, high concentration of the tagged siRNA is required to achieve efficient gene-silencing effect. Nevertheless, this new methodology enables siRNA modulation without the need of bioconjugation or chemical modification, opening a new avenue for cytosolic delivery of siRNA.

With the same concept, Kim et al. developed a zinc(II)-dipicolylamine (Zn/DPA) tag that can chelate with the phosphate groups on the siRNA backbone (Fig. 5D) [74]. Once mix with siRNA, the Zn/DPA head groups bind to the phosphate backbones while the hydrophobic tails extrude out and ready to interact with cell membrane. This Zn/DPA tag enhances the delivery efficiency of siRNA but without imparting significant cytotoxicity, in spite that it contains heavy metal ion [74].

5. Lipophilic siRNA for nanoparticlization

5.1. Lipophilic siRNA complexed with proteins

It has been demonstrated that the intravenously injected chol-siRNA exhibited as several existing forms, either as lipoprotein nanoparticle associating with HDL, LDL, albumin or existing in unbound form in plasma (Fig. 2C). All these forms mediate differential uptake pathwaies in the lipid transport system. It raises the necessity of 'pure' form that can take advantages of the most effective lipoprotein and uptake route for delivery. Nanoparticles made from *ex vivo*-assembly of chol-siRNA

and reconstituted HDL exhibits a uniform nanoparticle of 90 nm, a 'right' size for the enhanced permeation and retention (EPR) effect [75]. Yokota group found that the incorporation of chol-siRNA to HDL was saturable [76]. Stoichiometric study reveals that each lipoprotein particle can incorporate up to 25 copies of chol-siRNA without changing nanoparticle morphology [77]. The lipoprotein vector is negatively charged but can be stable in mouse serum at 37 °C for at least 24 h. Moreover, this *ex vivo*-assembled chol-siRNA is more effective than the free chol-siRNA in delivery especially when the target is the hard-reachable organ such as brain, which might be attributed to the stable and homogenous structure of the *ex vivo*-assembled nanoparticle [76].

Sarett et al. expanded the protein chaperone to endogenous albumin that allow deliver siRNA to extrahepatic tissues. To leverage on the long-lived serum protein albumin, siRNA was modified with diacyl lipid (siRNA-L2), rather than cholesterol, for rapid and high-affinity binding to albumin. The albumin complex exhibited a 5.7-fold increase in circulation half-life and an 8.6-fold increase in bioavailability compared to the unmodified siRNA, which eventually results in 19-fold greater tumor accumulation and 46-fold increase in per-tumor-cell uptake in a mouse tumor xenograft [78]. Compared with the scrambled siRNA, siRNA-L2 targeting luciferase gene achieved > 60% silencing at day 1 and sustained a comparable silencing level for as long as 7 day, revealing the prolonged gene silencing capacity of the lipidized siRNA/albumin nanoparticle. On the contrast, jetPEI nanoparticle only elicited ~30% silencing at day 3, and the silencing was fully abrogated by day 7 [78].

5.2. Lipophilic siRNA incorporated with charge-neutral nanocarriers

siRNA can readily condense with cationic nanoparticles and act as an efficient route for siRNA delivery, but robust and scalable methods for loading the polyanionic siRNA cargo into charge-neutral vesicles are lacking. Modulation of siRNA lipophilicty provides a mean to incorporate siRNA into neutral nanoparticles that has a hydrophobic core or shell. It was shown that lipophilic siRNA (chol-siRNA and palm-siRNA) can incorporate into polymeric micelle and provide additional hydrophobic stabilization to the micelle structure [79,80]. The architecture of the chol-siRNA incorporated micelle is quite similar to the well-reported "micelleplex" system. The difference is that siRNA is decorated around the micelleplex core by electrostatic interaction [81–83], whereas in this system chol-siRNA (or other lipid siRNA conjugate) is appended by inserting its cholesterol tail into the hydrophobic core of micelle (Fig. 5E). Compared with conventional polyplex systems, the micelleplex embrances the modification to architecture surface. The high degree of PEG shielding in micelleplex is favorable to *in vivo* application as well [81]. In another example, Xu et al. modulated the siRNA lipophilicity by mixing siRNA with its intercalating agent doxorubicin, which form a hydrophobic clathrate that can be readily encapsulated into PEG-*b*-PLA micelle (Fig. 5F). Overall, these approaches provide a simple and general avenue for the systemic delivery of siRNA with noncationic nanoparticles [84].

Given the lipophilic nature, it was reported that cholesterol moiety of chol-siRNA is capable to anchor siRNA onto the membrane outlet of liposome and exosome (Fig. 5G) [85]. O'Loughlin et al. exploited it as a general method to load siRNA onto small extracellular vesicles (sEV, an exosome) for siRNA delivery. Method optimization revealed that the highest retention percentage was achieved at only around 15 copies of chol-siRNA per vesicle [86]. To optimize the loading strategy, Khvorova group studied the influence of linker chemistry behind siRNA conjugate and screened a panel of lipidoid moieties for best loading. Finally it demonstrated that this method is able to give efficient and controllable loading of siRNA to sEV, which yield the maximum of 3000 copies of siRNA per vesicle, without altering particle-size distribution or integrity [87,88]. The siRNA harbored by sEV particle was able to be efficiently delivered to mouse primary cortical neurons and induced dose-dependent knockdown of huntingtin mRNA and protein [89].

6. Conclusion

siRNA is being considered as potential therapeutic medicine because of its high specificity and efficiency on suppressing genes that involved in disease pathogenesis. Once inside cells, siRNA associates with RISC and locates to a complementary mRNA. The activated RISC argonaute RNase cuts the target mRNA, which eventually initiate the degradation of the mRNA. An active RISC can target multiple transcript mRNA copies, therefore a few hundred cytosolic siRNA molecules per cell would be sufficient for efficient gene knockdown [90,91]. In addition, the cytosolic RISC is stable within high catalytic status for weeks, thus the siRNA mediated therapeutic effect can sustain for even months *in vivo* [92]. Compared to small chemical medicine, another advantage of siRNA is that its activity and potency is derived mainly from the base sequence, which can be readily engineered for targeting specifically a single gene or evolving the sequence at the same pace of disease progression such as pandemic influenza [3]. Looking at it optimistically, siRNA holds tremendous promise to deal with almost every disease-promoting gene in any cell type that it can be delivered. However, for siRNA to achieve the therapeutic potential, it first need to overcome the delivery barrier, which actually has become the main obstacle impeding the clinic productivity of siRNA. Modulation of siRNA lipophilicity and conferring 'drug-likely' properties have become an promising strategy for systemic siRNA delivery. The lipophilic siRNA, especially cholesterol siRNA conjugate, avidly binds with serum proteins, greatly enhance circulation time, promote local and systemic delivery efficacy. By screening the library of diverse lipid conjugates, it is feasible to identify a specific lipidized siRNA that can harness its unique pathway to delivery siRNA to numerous extrahepatic organs including planceta, skin, lung, heart and others, dramatically expanding the therapeutic application of lipophilic siRNA.

On the other hand, lipophilic siRNA is an ideal drug for incorporation into charge-neutral, conventional drug delivery platform such as PLGA nanoparticle, liposome and others. siRNA is typically condensed with cationic nanocarriers by electrostatic interaction. The resulting nanocomplex is positively charged, thus possesses inherent toxicity issue and colloidal instability, which shows limited therapeutic potential in clinic. Modulation siRNA lipophilicity makes siRNA ready to formulate into conventional drug delivery system, thus provides a simple avenue for the systemic delivery of siRNA within noncationic nanoparticles, hoping to surmount the limitations. Taken together, lipophilic siRNA holds promise for the clinical development of a new class of siRNA therapeutics in the future. Any concept and methodology for modulation of siRNA lipophilicity represent an arena worth paying attention to and further exploring.

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