Received: 31 July 2019 | Revised: 5 November 2019 | Accepted: 6 November 2019

DOI: 10.1002/wnan.1606

FOCUS ARTICLE

WIREs NANOMEDICINE AND NANOBIOTECHNOLOGY WILEY

Insight into the siRNA transmembrane delivery—From cholesterol conjugating to tagging

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Funding information
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Abstract

Small interfering RNA (siRNA), combining the features of unprecedented potency, target-specificity, and the unique sequence-based disease-intervention model, has received immense considerations over the past decades in the academia and pharmaceutical industry. siRNA fits the criteria of being drug-likely enough to meet with the therapeutic purpose, but its clinical translation has been impeded for a long time by the poor efficiency of in vivo delivery. To reach the cytosol where the RNA interference (RNAi) takes place, siRNA delivery faces a serial of systemic and cellular barriers, especially the endosomal sequestration that would prevent the majority of siRNA from cytosol entry. Transmembrane delivery of siRNA represents a new avenue for efficient delivery by bypassing the endosomal pathway. This rationale is bolstered by the high efficiency of viral entry by membrane fusion, but rarely pursued by artificial siRNA delivery systems. Here, this article provides an opinion of transmembrane delivery by hydrophobic modulation of siRNA. We give a brief introduction of the current siRNA delivery modes, including the hydrophobic cholesterol siRNA conjugates. The cholesterol tagging technology is design on the rationale of hydrophobic siRNAs approach, but hydrophobic modulation throughout the whole siRNA backbone for efficient membrane fusion and transmembrane delivery. The challenge and potential of this technology for preclinical development are also discussed.

This article is categorized under:

Nanotechnology Approaches to Biology > Nanoscale Systems in Biology
Therapeutic Approaches and Drug Discovery > Emerging Technologies
Biology-Inspired Nanomaterials > Lipid-Based Structures
Biology-Inspired Nanomaterials > Nucleic Acid-Based Structures

KEYWORDS

cholesterol conjugate, cholesterol tagging, hydrophobic siRNA, siRNA delivery, transmembrane delivery

1 | INTRODUCTION

Since Fire and Mello's ground-breaking discovery of RNA interference (RNAi), small interfering RNA (siRNA)-based therapeutics have witnessed an explosion in the academia and industry (Chakraborty, Sharma, Sharma, Doss, & Lee, 2017; Fire et al., 1998). Different from chemical drugs and antibodies that require a blockage of the activity pocket of a protein, the

bioactivity of siRNA relies on the base sequences to complementarily match the mRNA of target and induce its degradation (Wilson & Doudna, 2013). This disease-intervention model gives siRNA the incomparable advantages such as easiness to design, ability to target almost every disease-promoting gene, and promises with unprecedented specificity, potency, and duration of effect (Dowdy, 2017; Vaishnaw et al., 2010). The sequence-based therapeutics also possess the modality with the ability to quickly evolve the siRNA sequence at the same pace of disease progression, in particular valuable in the treatment of genome-instable diseases such as cancer and pandemic viral infections (Dowdy, 2017). Looking at it enthusiastically, siRNA holds tremendous promise to deal with almost every gene-promoted disease in any cell type that it can be delivered (Tai, 2019a, 2019b). However, the enthusiastic upside of the therapeutic potential comes with the opposite downside that the delivery problem has remained as the major barriers over decades to impede the clinical production of siRNA drugs (Khvorova & Watts, 2017).

2 | ANALYSIS OF THE siRNA DELIVERY MODES

siRNA is a highly negative-charged macromolecule. It shares the same RNA chemistry to mRNA, and thus just like mRNA, it is particularly water-soluble and unstable. Cell membrane seals the mRNA inside cells for cell-life functions, and at the same time it serves as a boundary to prevent the access of extracellular materials including exogenous siRNA. Due to the Coulomb force, siRNA molecule is repulsed by the same negative-charged cell membrane (Figure 1a). Moreover, the hydrophilic nature of siRNA backbone is incompatible to the hydrophobic interior of lipid bilayer. Therefore, siRNAs themselves, including some modified siRNAs such as 2′-F or 2′-OMe siRNA, are unable to cross the membrane or uptake by cells. siRNA has to be delivered into cells by the assistance of siRNA delivery carriers.

Recent progress in bioengineering and nanotechnology has produced numerous nonviral siRNA carriers that utilize the polycations to condense the siRNA and promote interaction with the opposite-charged cell membrane (Tatiparti, Sau, Kashaw, & Iyer, 2017). The interaction triggers the cellular activation of the endocytic pathway and finally seal the siRNA/carrier complex in the lipid bilayer vesicles called endosome/lysosome (Sahay et al., 2013) (Figure 1b). Most of the endosome-trapped siRNA is degraded along with the pH decrease and enzyme activation during lysosome maturation. The endocytic compartment is a fundamental safeguard system in protecting cells from the invading RNA or exogenous materials. To deliver siRNA into cells, it is necessary to tackle with this defense system that has a billion-year evolutionary history. The improved understanding of the endosome-escape mechanism and ongoing progress of carrier development has enabled to deliver siRNA into the cytosol for gene function manipulation using agents such as Lipofectamine or clinic approved carrier Patisiran (Adams et al., 2018; Kristen et al., 2019). Nevertheless, all these cationic carriers exclusively take the route of endocytic pathway and only 1–2% of the siRNAs internalized by cells can reach the cytosol (Choi, Zhang, & Choi, 2018; Dowdy, 2017). Although this extremely low efficiency is bearably enough and effective to knockdown the disease-promoting gene, it is somehow more appealing to promote the delivery efficiency to a higher, at least moderate level, for the benefits such as reduced dosage, better safety and lower cost.

One possible solution is to take the shortcut and circumvent the endosomal sequestration by direct delivery across cell membrane. Of all siRNA carriers so far, cell-penetrating peptide (CPP) offers an exceptional model of transmembrane delivery, in which CPP/siRNA complex causes a transit pore on cell membrane and translocate itself into the cytosol (Allolio et al., 2018; Tai & Gao, 2017). However, the translocated siRNA only accounts for a tiny portion of the internalized siRNA. The majority remains trapped by the endocytic pathway and finally are degraded inside endosome/lysosome (Madani, Lindberg, Langel, Futaki, & Graslund, 2011). In nature, several viruses can fuse their membrane with cell plasma membrane and directly release the virion contents into host cytoplasm (Harrison, 2015; Poranen, Daugelavicius, Ojala, Hess, & Bamford, 1999; Figure 1c). This capability confers the possibility to infect a cell with just one virus particle (100% efficiency in delivery). However, the membrane fusion of viral entry is drive by a complicate biological process, which so far remains impossible to imitate by artificial delivery systems.

3 | THE SEEKING TO HYDROPHOBIC MODULATION

The inspiration of hydrophobic modulation comes from the thought about the chemical drugs, which have a low molecular weight (<1 kDa), low to no charges and enough hydrophobicity to allow them to gently cross the cell membrane. siRNA is a type of large (~14 kDa), polyanionic and hydrophilic macromolecules that do not show any ability to cross cell membrane. Of all these impeders (size, charge, and hydrophilicity), the lack of hydrophobicity might be the main limiting factor to impede

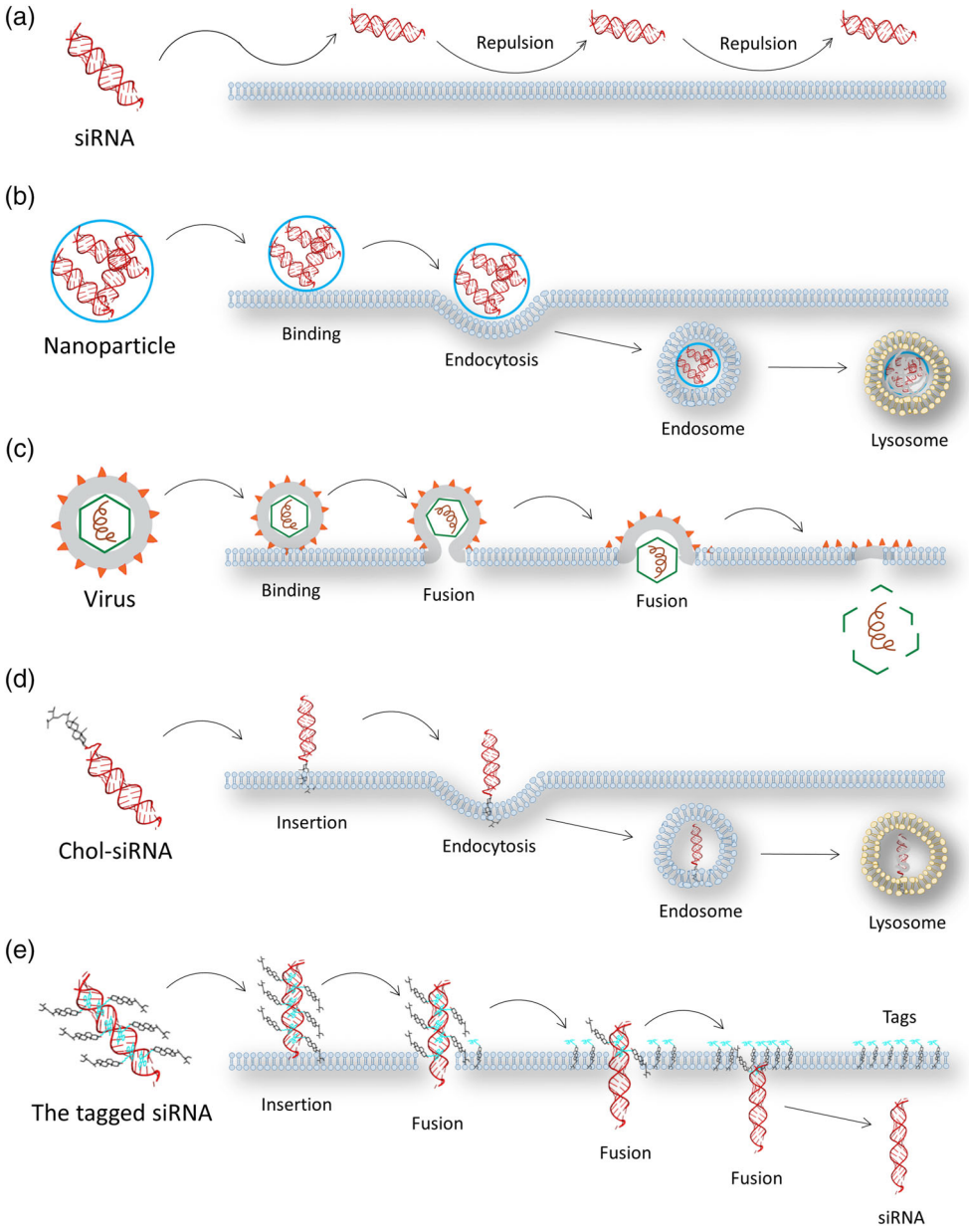

FIGURE 1 The comparison of small interfering RNA (siRNA) delivery modes. (a) siRNA is unable to cross the cell membrane due to the repulsive force from the hydrophobic and anionic lipid bilayer. (b) The cationic siRNA nanoparticle is uptake by cells through the endocytic pathway, but most of the cargoes are degraded during lysosome maturation. (c) Some viruses are able to directly fuse their membrane with cell membrane without being internalized by endocytosis. (d) Chol-siRNA inserts its cholesterol tail into membrane and triggers the endocytosis. (e) The tagged siRNA is able to fuse with cell membrane because of the hydrophobic modulation throughout the siRNA surface. The tagged siRNA eventually slips into the cytosol and leave the tags on the cell membrane

the membrane crossing. The siRNA molecule is highly hydrophilic and thus strongly repulsed by the hydrophobic interior of cell membrane. The aforementioned studies encourage to explore the possibility of transmembrane delivery by hydrophobic manipulation of siRNA molecules. By the hydrophobicity manipulation, for example, decoration of siRNA with hydrophobic molecules, siRNA would be more compatible to cell membrane, thus may overcome the hydrophobic barrier of lipid bilayer and reach the cytosol directly.

The exploration of hydrophobic siRNA began decades ago, known as the famous "cholesterol siRNA conjugate" (chol-siRNA, Figure 2a). Scientists at Alnylam Pharmaceuticals Inc. conjugated cholesterol molecule to the 3′ end of sense strand of siRNA by a short trans4-hydroxyproinol (Hyp) linker (Lorenz, Hadwiger, John, Vornlocher, & Unverzagt, 2004; Soutschek et al., 2004; Wolfrum et al., 2007). The terminal modification allows siRNA to remain capable of docking into the RNA-induced silencing complex and preserves the gene silencing potency. At the same time, the cholesterol conjugation increases the hydrophobicity and confer the "drug-likely" properties to siRNA, which promote the binding with albumin (Kd ~ 1 μM), extend serum half-life (90 min vs <10 min of siRNA), and increase the hepatic delivery by harnessing the lipid trafficking pathway (Wolfrum et al., 2007). The targeted delivery of siRNA by lipid transport pathway is a unique advantage of chol-siRNA. Hydrophobic modification of siRNA has markedly improved the pharmacokinetic profile and therapeutic index of siRNA, which allow chol-siRNA to be delivered in vivo without the aid of any carriers (Dowdy, 2017). Nevertheless, it is

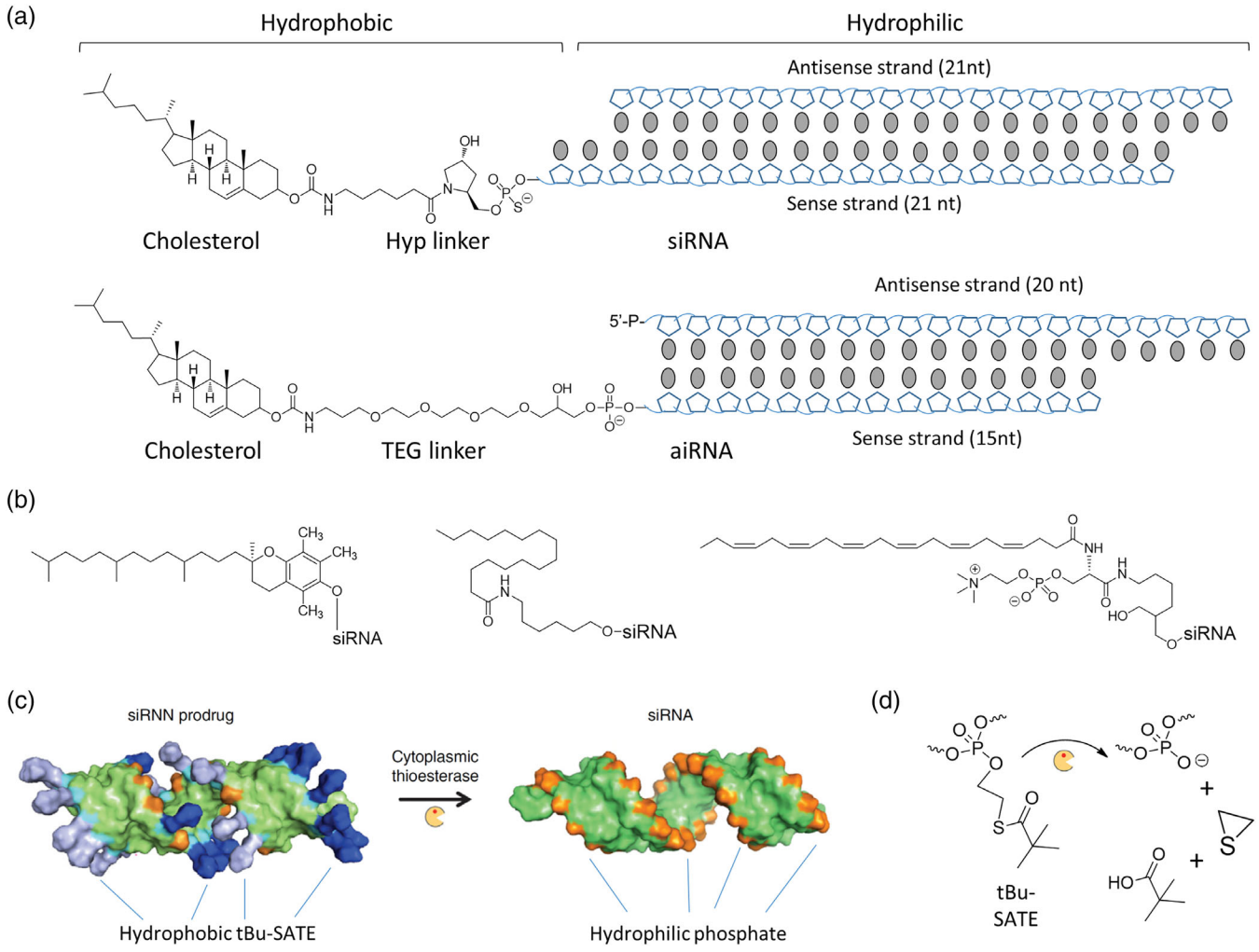

FIGURE 2 Hydrophobic modification of small interfering RNA (siRNA). (a) Structures of chol-siRNA (upper panel) and sd-rxRNA (bottom panel). (b) siRNA conjugated with α-tocopherol (left), palmitic acid (middle) and PC docosahexaenoic acid (right). (c) siRNA prodrug by bioreversible modification on phosphate groups of siRNA backbone. (Reprinted with permission from Meade et al. (2014). Copyright 2014 Nature Publishing Group) (d) The phosphate groups of siRNA are unmasked by thioesterase in the cytoplasm

worth noting that chol-siRNA can only elicit a maximum of ~50% silencing effect in vitro and in vivo, even at extremely high dose or concentration (Chernikov et al., 2017; Petrova et al., 2012). The comprehensive analysis leads to the question about the "endosomal escape" problem associated with chol-siRNA uptake. It appears that only an extremely low percentage (often as low as <0.01%) of the chol-siRNA can escape from endosome compartment, thus dramatically limiting the therapeutical potential of chol-siRNA (Dowdy, 2017). Many chemical enhancers have been developed to promote the escape of chol-siRNA from endosome/lysosome compartment (Gilleron et al., 2015; Wooddell et al., 2013). However, most of these enhancers are either too weak or too toxic, thus showing no therapeutic window for clinical use.

Khvorova group optimized the strategy comprehensively by conjugating cholesterol with a smaller, less-charged asymmetric interfering RNA (aiRNA), annotated sd-rxRNAs technology that has already progressed into clinic trials (Figure 2a; Ly et al., 2017; Ly, Paz, Hunstad, & Cauwenbergh, 2015). sd-rxRNA is a hydrophobic siRNA evolved from chol-siRNA technology. It consisted of cholesteryl siRNA module, but with a short and flexible TEG linker, and a fully chemically modified aiRNA (Ly et al., 2017). The aiRNA is a siRNA alternative but has a smaller and less-charged scaffold (Chang et al., 2009; Chu & Rana, 2008; Sun, Rogoff, & Li, 2008). The reduction in size and charge is in favor of the transmembrane delivery. More importantly, the asymmetric scaffold contains a 5-nt single-strand tail (PS tail) that can be phosphorothiolated to promote cellular internalization by a cellular-uptake mechanism similar to that of fully phosphorothiolated antisense oligonucleotide (Geary, Norris, Yu, & Bennett, 2015; Ly et al., 2017). The uptake of sd-rxRNA is quick and instant, generally within minutes of exposure to cells (Ly et al., 2017). It was demonstrated that the uptake starts with a rapid membrane association

followed by the formation of vesicular structures and internalization, a typical endosomal route but by a specific class of early endosomes related to epidermal growth factor trafficking pathways (Ly et al., 2017). sd-rxRNA technology has progressed into clinic trials to evaluate the ability to treat scarring diseases. RXI-109, one of the sd-rxRNAs, was tested in a phase II trial study participated with 16 patients (Ly et al., 2015). The treatment by RXI-109 improved the visual appearance of the revised scars after scar revision surgery in comparison to control. The development of this hydrophobic siRNA may offer a clinically meaningful benefit for the treatment and prevention of hypertrophic scars in the future.

Nishina and Kubo et al. replaced the cholesterol with α-tocopherol and palmitic acid to expand the modulation to a broad-range selection of hydrophobicity (Figure 2b; Kubo, Takei, Mihara, Yanagihara, & Seyama, 2012; Nishina et al., 2008). However, despite hydrophobic features, endocytosis remains the only route for all these hydrophobic siRNAs when uptake by cells (Gilleron et al., 2015). In other words, it is still problematic for them to deliver directly across cell membrane. It was reported that the cellular uptake of these hydrophobic siRNAs is a two-step process, with rapid membrane association (insertion of cholesterol into membrane) followed by internalization through a selective, saturable subset of the endocytic process (Figure 1d; Ly et al., 2017). In consideration of the amphiphilic diblock structure of chol-siRNA, it is envisaged that the cholesterol tail fuses into cell membrane, but the hydrophilic siRNA is still repulsed by the lipid bilayer, thus being stuck at cell membrane and leading to cellular internalization by endocytosis. Special discussion on these diblock hydrophobic siRNAs has been reviewed elsewhere (Tai, 2019a, 2019b).

The aforementioned efforts were endeavoring to enhance hydrophobicity by terminal conjugation, in which only one cholesterol molecule is conjugated to siRNA (Benizri et al., 2019; Chernikov, Vlassov, & Chernolovskaya, 2019; Jeong, Mok, Oh, & Park, 2009). Previous studies have revealed that this diblock structure is disadvantageous to the transmembrane delivery (Tai, 2019a, 2019b). Meade et al. (2014) developed a chemical method to modulate the hydrophobicity of the whole siRNA by tethering the hydrophobic molecules onto the phosphate groups of siRNA backbone (Figure 2c). The modification generates a siRNA prodrug with the bioreversible phosphotriester linker *t*-butyl S-acyl-2-thiothethyl (tBu-SATE) that can be activated by cytoplasmic thioesterase and reverse into wild-type siRNA once entry into cytoplasm (Figure 2d). In addition, the modification on the phosphate groups masks the inherent negative charges of siRNA and presumably promote the cellular uptake of the siRNA prodrug. Due to the chemical recalcitrance of phosphate groups, Meade et al. were only able to synthesize a lipophilic siRNA prodrug containing 26 scattered modifications on nucleotides. Nevertheless, the siRNA prodrug exhibits the apparent hydrophobicity, with features including the avid bound with albumin and the elongated half-life in blood circulation. Unfortunately, the hydrophobic siRNA prodrug is unable to diffuse across the cell membrane despite the extensive modification. A cationic CPP TAT was conjugated to promote the cellular delivery of the siRNA prodrug in the study. The siRNA prodrug reported by Meade et al. represents an example that manipulates the hydrophobicity evenly on the whole siRNA backbone, a mode supposed to encourage the membrane crossing superior to the diblock scaffold of chol-siRNA. The failure of membrane crossing might be attributed to the weak hydrophobicity of tBu-SATE groups, or may due to the covalent linkage that prevent siRNA release from cell membrane once siRNA prodrug fuses into the membrane.

4 | THE CHOLESTEROL TAGGING TECHNOLOGY

By the comparison of the virus's membrane fusion and the aforementioned examples, we hypothesized that two prerequirements are essential for transmembrane siRNA delivery: (a) hydrophobic decorations throughout the double helix (beyond modification of one of the two termini) should enhance siRNA attraction to cell membrane and (b) upon fusion with the cell membrane, siRNA capable of shedding off the hydrophobic compounds might be able to slip into the cytosol rather than being stuck on the membrane. This hypothesis leads to our new concept of cytosolic siRNA delivery, namely 'cholesterol tagging technology', to bypass the endocytic sequestration (Figure 1e; Tai & Gao, 2018). Our cholesterol tag consists of a bipartite chemical, with an ethidium molecule serving as a siRNA intercalator and a terminus of cholesterol molecule for membrane fusion. The tag utilizes the ethidium-siRNA interaction to decorate the cholesterol molecule onto siRNA backbone, thus transforming siRNA into membrane-compatible and -permeable (Figure 3a,b). We screened a small library of chemical tags that represent a broad-spectral selection of lipids and hydrocarbons. As expected, the cholesterol, one of the major lipid components of cell membrane, outperforms all other lipids, and be selected for siRNA tagging. From the confocal microscopic images, we are pleasantly surprised that the cholesterol-tagged siRNA completely bypasses the endosomal sequestration and enters the cytosol exclusively by membrane translocation. In contrast, the chol-siRNA is trapped in the endocytic compartments. The endocytic uptake is an energy-dependent and quickly saturable route because it takes hours for the internalized endocytic vesicle components to be recycled and resynthesized. Only a tiny amount of chol-siRNA would be internalized by

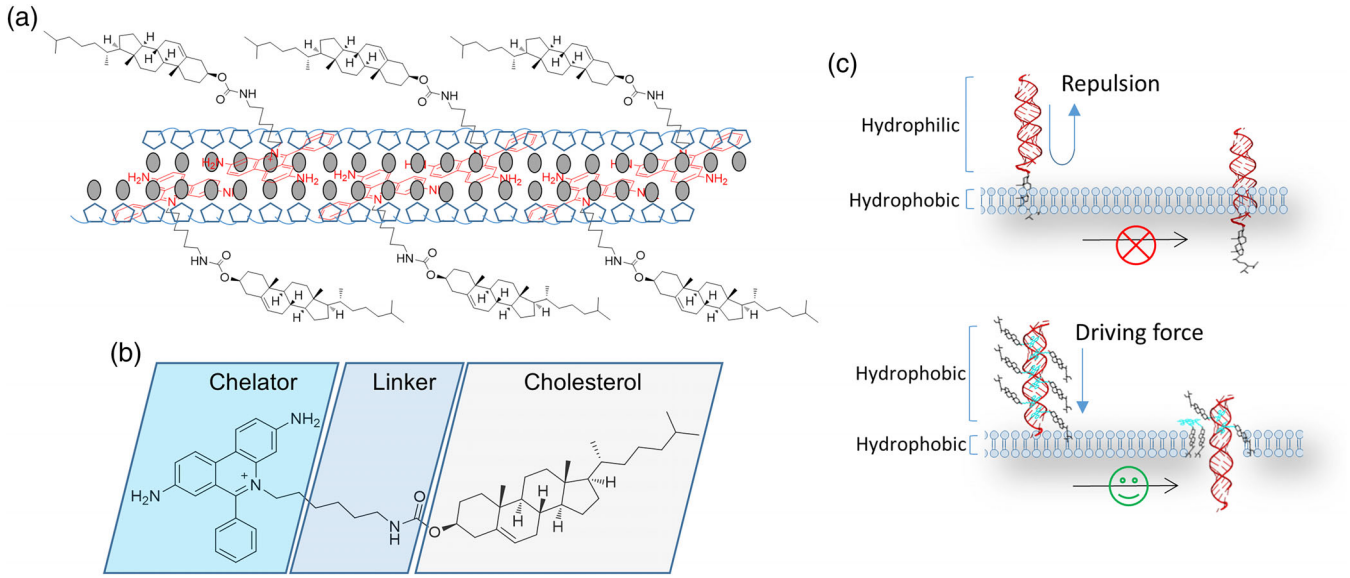

FIGURE 3 The cholesterol tagging technology for cytosol delivery of small interfering RNA (siRNA). (a) The tagging technology decorates siRNA with six copies of cholesterol molecules by chelation. It eventually transforms the hydrophilic siRNA into membrane compatible and permeable. (b) The chemical structure of tags. (c) The amphiphilic chol-siRNA fails to fuse into cell membrane after it inserts its cholesterol tail into lipid bilayer, because the hydrophobic interior of lipid bilayer is repulsive to the hydrophilic and polyanionic siRNA block (upper panel). Membrane fusion of the tagged siRNA is initiated by the insertion of its first cholesterol tail into cell membrane. Other tails outside of membrane provide a driving force to pull siRNA into cells because of the attraction of these cholesterol tails to lipid bilayer (bottom panel). The driving force disappears until the whole siRNA is slipping into the cytosol

cells in hours, whereas the uptake of the tagged siRNA is continuously increasing with the incubation time, which eventually leads to the nearly 30 times higher cellular uptake of the tagged siRNA than that of chol-siRNA in 2 hr.

Preliminary animal experiments reveal that the tagged siRNA show higher hepatic accumulation than the untagged siRNA. After i.v. injection, the untagged siRNA is subjected to renal clearance due to its small size (~14 kDa) and the reluctance to albumin binding. The cholesterol tagging alters siRNA's distribution and results in an elevated accumulation in the liver. The liver deposit of the tagged siRNA might be attributed by hijacking the albumin carrier and lipid trafficking pathway, a similar route that has been well demonstrated by chol-siRNA transportation in vivo (Wolfrum et al., 2007). Nevertheless, it is worth noting that the diffuse fluorescent signal of siRNA was also observed in the kidney and intestine. The accumulation of the tagged siRNA in the kidney and intestine led us to speculate that a portion of the tagged siRNA might dissociate during circulation and clear from the body through two typical elimination organs of "nake" siRNA: kidney and intestine (Huang et al., 2011). Although the absence of other nucleic duplexes in blood for bound competence, the relatively weak affinity of ethidium to siRNA duplex (K_d ~1.3 μM) might be not sufficient to maintain the stability of the intact complex of 6:1 (tag:siRNA ratio). An updating tag technology, such as the dimeric ethidium, would increase the tagging stability in vivo and give an improved biodistribution profile.

The ethidium molecule in the tag invites a concern to the potential toxicity, in particular the risk of genetic mutation. It was reported that ethidium bromide would not only act as a mutagen in microorganism, but also might induce mutations in higher plants and mammals (Dujardin & Dujon, 1979; Hayakawa et al., 1998; Levy & Ashiri, 1975). Primary cultured neurons treated by ethidium bromide (EtBr) present the consequences of mitochondrial DNA (mtDNA) depletion, which dramatically reduces mitochondrial function and can be used as a method of mouse mtDNA mutagenesis for modeling human pathological conditions (Fayzulin et al., 2015; Warren, Aicher, Fessel, & Konradi, 2017; Yu et al., 2007). Our preliminary experiment indicated that our siRNA tagging technology does not show any cytotoxicity on PC-3 cell, even at very high tagging concentration (10 μM of the tags). It is also worth to mention that ethidium bromide has been used as veterinary medicine to treat the trypanosome for decades (Roy Chowdhury et al., 2010). However, ethidium is labeled as mutagenic and thus been considered carcinogenic in most laboratories. Alternative siRNA-intercalating chemicals will be, therefore, more feasible for clinical evaluation. It is noting that ethidium used in this tag is just a model for siRNA chelation. Our lab has already trodden the path of seeking a better siRNA binder to extend our tagging technology to preclinical evaluation. We have already screened eight siRNA binders (entries 1–7 and entry 9 in Table 1) using fluorimetri titration method (Tai & Gao, 2018). It revealed that only two binders, ethidium and acridinium, shows strong intercalation activity with siRNA, although all of them can bind and chelate DNA very well. DNA and dsRNA, despite similar duplex structure, preferentially exhibit the B- and A-form double

TABLE 1 List of some typical nucleic acid chelators

No.	Name	Target[a]	Binding mode	Fluorophore?	Application[b]
1	Acridines	DNA, dsRNA, ssRNA	Intercalator	Yes	Dye
2	Ethidium	DNA, dsRNA	Intercalator	Yes	Dye and veterinary medicine
3	TO-PRO-1	DNA	Intercalator	Yes	Dye
4	Doxorubicin	DNA, dsRNA	Intercalator	Yes	Anticancer drug
5	Sybr-Green	DNA, dsRNA, ssRNA	Intercalator	Yes	Dye
6	Acdi 1	DNA	Intercalator	Yes	Dye
7	Methylene Blue	DNA	Intercalator	No	Dye
8	Ellipticine	DNA	Intercalator	No	Natural product
9	DAPI	DNA	Groove binder	Yes	Dye
10	Distamycin	DNA	Groove binder	No	Antibiotic
11	Netropsin	DNA	Groove binder	No	Antibiotic and antiviral
12	Hoechst	DNA	Groove binder	Yes	Dye
13	Carbocyanines	DNA, dsRNA	Groove binder	Yes	Dye
14	Cisplatin	DNA	DNA crosslinker	No	Anticancer drug
15	Bleomycin	DNA	DNA cleavage	No	Anticancer drug
16	Neocarzinostatin	DNA	DNA cleavage	No	Antibiotic
17	Dynemicin A	DNA	DNA cleavage	No	Anticancer drug

[a]Some chelators are tested only on DNA, but they may also bind with dsRNA.
[b]Indication of a typical application.

helical structures, respectively. Moreover, A-form helix is around 20% wider and 1/5 shorter than B-form in size (Lipfert et al., 2014). Some DNA chelators may not bind siRNA as well as DNA. Nevertheless, the family of nucleic acid chelators includes a large number of small molecules that interact with DNA or RNA by diverse mechanisms (Table 1). Any small-molecule alternative that binds with siRNA should work similarly. We pay particular attention to some FDA-approved binders, such as doxorubicin (entry 4 in Table 1) and netropsin (entry 11 in Table 1). Utility of these chemical drugs could improve the clinical relevance of the cholesterol tagging technology.

The most intriguing point of the tagging technology is to manipulate the lipophilicity of siRNA surface for membrane fusion, followed by slipping into the cytosol, a process that amounts to mimic the viral entry. Hydrophobic manipulation is increasingly been used to facilitate the membrane crossing. Numerous reports from different groups have demonstrated that hydrophobic nanoparticles (>1,000 kDa), in contrast to expectations, can translocate through lipid membranes by direct penetration within milliseconds (Daniel, Reznickova, Handl, Iglic, & Kralj-Iglic, 2018; Guo, Terazzi, Seemann, Fleury, & Baulin, 2016; Nakamura & Watano, 2018; Wang, Guo, Li, & Li, 2019; Yong, 2015). Although the mechanism is still in debate, there is an appreciation that the hydrophobicity of particle surface dictates the absorption behavior into the membrane and, subsequently, induce the possible translocation into the cell (Bochicchio, Panizon, Monticelli, & Rossi, 2017; Yong, 2015). Similar transmembrane behavior was also observed in hydrophobic macromolecules (Bochicchio et al., 2017; Werner & Sommer, 2015; Werner, Sommer, & Baulin, 2012), even polymer with negative charges (Abou Matar & Karam, 2018). Our tag utilizes the noncovalent approach to decorate the siRNA surface with cholesterols. Membrane fusion of the tagged siRNA is initiated by the insertion of its first cholesterol tail into cell membrane. Other tails outside of membrane provide a driving force to pull siRNA into cells because of the attraction of these cholesterol tails to lipid bilayer (Figure 3c). The excellent compatibility of cholesterol to cell membrane allows an efficient fusion of the tagged siRNA with lipid bilayer. The noncovalent feature between the tag and siRNA encourages the separation of tags from siRNA during cytosol slipping-in. The driving and tag-separation process complete until the whole siRNA is slipping into the cytosol.

5 | OUTLOOK

The tagging technology provides a simple methodology to modulate siRNA hydrophobicity for efficient cytosol delivery. The delivery is relying on the attraction of cholesterol to the hydrophobic interior of cell membrane, which is expected to show no

selectivity to the cell types. Although the tagged siRNA could hijack the lipid trafficking pathway and be delivered to the hepatic cells with some selectivity in vivo, the tagging technology would be classified as a passive targeting system that may not be able to distinguish the healthy tissue from the diseased one, just like the chemotherapeutic regimen. Grafting a ligand onto the tagged siRNA would enable a smart delivery of the hydrophobic siRNA to the diseased tissue thus avoiding the toxicity. Our tagging technology is designed based on a noncovalent chelation module that enables us to add a targeting ligand flexibly either on the siRNA or the tag. One feasible strategy might be the utilization of two-tag cocktails in which the cholesterol-ethidium tag for membrane fusion and another tag, ligand-ethidium conjugate, for active targeting.

The cytosolic delivery of siRNA by our tagging method is a drive by the compatibility of cholesterol tail to the cell membrane. Ethidium just provides a chelation force to attach cholesterol molecules on the cargo surface. By simply changing the bound mode toward other macromolecules, for example, replacing ethidium with coomassie blue for protein binding, it is expected to expand the tagging technique to deliver many other cargoes such as proteins, polymers, polysaccharides and even microbes, into the cytosol. Considering the ease of handling, the tagging method may serve as a powerful tool to transfect these important biomacromolecules into cells for their functionality study. In other words, this simple membrane-fusion and slip-in mode open a new avenue for cytosolic delivery of many biological cargoes, which is worth paying attention to and further exploring.

ACKNOWLEDGMENTS

I am thankful to Prof. Xiaohu Gao (Department of Bioengineering, University of Washington) for fruitful discussions and proof reading.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Yan Zheng: Writing-original draft, review, and editing. **Wanyi Tai**: Conceptualization; supervision; validation; writing-original draft, review, and editing.

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How to cite this article: Zheng Y, Tai W. Insight into the siRNA transmembrane delivery—From cholesterol conjugating to tagging. *WIREs Nanomed Nanobiotechnol*. 2020;12:e1606. https://doi.org/10.1002/wnan.1606