Chapter

Liposomes for Targeting RNA Interference-Based Therapy in Inflammatory Bowel Diseases

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Abstract

The discovery of RNA interference (RNAi) in mammalian cells in 2001 opened up a new class of candidate therapeutics for hard-to-cure diseases like inflammatory bowel diseases. The main challenge for the development of RNAi-based therapeutics is the efficient and safe delivery of RNAi since the RNAi machinery is housed in the cytoplasm. Among the various approaches to active targeting, liposome-based delivery systems are innovative and promising systems to transport and control RNAi molecules release and overcome some of their limitations. Many RNAis in lipid formulations have progressed through various stages of clinical trials, with the measurable improvements in patients and no side effects. For colon targeting, liposomes can be manipulated by different methods. This chapter discusses the progress in delivering RNAi molecules to the colon using liposomes.

Keywords: liposomes, targeted delivery, ligand, antibody, RNAi, mRNA, siRNA, inflammatory bowel diseases

1. Introduction

The discovery of RNA interference (RNAi) in mammalian cells in 2001 opened a new class of candidate therapeutics for hard-to-cure diseases like inflammatory bowel diseases (IBD). IBD refers to a group of immune-mediated chronic remission and relapse bowel diseases. IBD is classified into two subtypes: Crohn's disease and ulcerative colitis, with different etiologies and unknown causes. Crohn's disease causes ulcers and granuloma in the small and large intestines, as well as inflammation in the alimentary canal from the mouth to the anus. Ulcerative colitis causes an inflammatory response as well as subsequent ulcers and abscesses in the colonic mucosa. IBD patients are at a higher risk of developing colon cancer because of the emergence of chronic inflammation characterized by massive immune filtration and immune-mediated tissue destruction [1]. The prevalence of IBD has recently increased significantly. IBD has become the third most common disease in the world due to the development of chronic inflammation and a large number of immune cell filtration as well as immune cell-mediated organ destruction. IBD affects over 5,000,000 people worldwide. Currently, approximately 25 per million people yearly (developed countries) and 5 per one million people yearly (developing countries) are living with this

chronic inflammatory and debilitating disease that necessitates lifelong treatment, resulting in a massive financial burden and healthcare system support [2].

Anti-inflammatory and immunosuppressive agents are the most commonly used therapeutic approaches. Nonsteroidal anti-inflammatory agents (such as mesalazine or olsalazine) are primarily used to treat mild attacks and to keep ulcerative colitis in remission. Unfortunately, the use of nonsteroidal anti-inflammatory drugs has been linked to a variety of side effects including nausea, diarrhea, cramping, headaches, fever, flatulence, rashes, and, in some cases, nephritis, pancreatitis, hair loss, and pancytopenia. In the treatment of moderate-to-severe IBD, steroidal anti-inflammatory drugs (such as prednisolone) are more effective. However, steroids' adverse drug reaction profile, which includes Cushing's syndrome, infection, adrenal suppression, sleep disorders, osteoporosis, and renal function impairment, limits their use in long-term therapy. Immunosuppressive drugs (such as azathioprine, 6-mercaptopurine, methotrexate, and calcineurin inhibitors) and the most important biological agents (such as infliximab, adalimumab, and certolizumab pegol) play an important role in the treatment of advanced disease stages. However, the use of immunotherapies is always constrained by a number of factors. For example, repeated administration of immunomodulators at high doses is always required, which may result in a series of autoimmune-mediated side effects, such as flu-like reactions and vascular leak syndrome, with significant individual variation [3, 4]. Clinical challenges include the drugs' limited efficacy, the high cost of antibody drugs, and the side effects or adverse reactions of corticosteroids and biological therapy [1, 5]. As a result, the development of new therapeutic strategies, such as the neutralization of proinflammatory cytokines, the use of anti-inflammatory cytokines, and the inhibition of neutrophil adhesion or T cell signaling, is critical [6, 7].

RNA interference (RNAi) is a common natural phenomenon that can be induced by exogenous RNA oligonucleotides (e.g., small interfering RNA or siRNA) and endogenous small RNA species such as microRNA (miRNA) and piwi-interacting RNAs. Since Fire and Mello's discovery of RNAi, new mechanisms of gene silencing and gene regulation have been elucidated, providing new tools for biological research and the development of new pharmacological strategies. Currently, the majority of RNAi research is focused on siRNA and miRNA. siRNAs are double-stranded RNA fragments of 21–25 nucleotides that can inhibit the expression of specific proteins by inducing the enzymatic cleavage of perfectly complementary target mRNAs. miRNA is a double-stranded endogenous noncoding molecule with 21–25 nucleotide segments and two nucleotide 3′ terminal overhangs. The RNAi technique modulates the expression of susceptibility genes as well as the secretion of proinflammatory cytokines associated with IBD, resulting in the therapeutic effects of mucosal restoration and immune balance recovery in disease sites. The RNAi pathway works by increasing the degradation of unwanted messenger RNA (mRNA) sequences and thus decreasing their translation. The main RNAi molecules being researched for IBD therapy are miRNA and siRNA. Both RNAi molecules interact with the RNA-induced silencing complex (RISC), resulting in the separation of RNA double strands by a RISC complex component (the endonuclease argonaute 2 protein, AGO2). The sense strand of RNA (passenger strand) degrades in the cytoplasm, whereas the antisense strand of RNA (guide strand) directs the RISC complex and binds to the target mRNA. In the case of siRNA, the antisense strand binds to fully complementary mRNA, resulting in mRNA cleavage. In the case of mRNA, the antisense strand binds to target mRNAs that are partially complementary to it, resulting in target gene silencing *via* translational repression, cleavage, and/or degradation (**Figure 1**) [8–10].

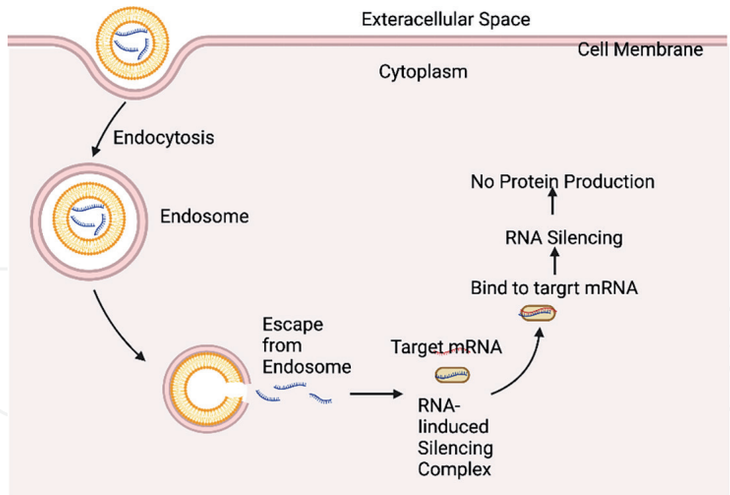

Figure 1.
Mechanism of RNAi molecule delivery via liposomal delivery system and RANi molecule gene silencing mechanism. Created with BioRender.com.

Formulation components	Target gene	Model	References
Lipofectamine 2000 (Invitrogen)	TNF-alpha	Murine	[17]
Lipidoids, cholesterol, DSPC, PEG2000-DMG	GAPDH	Caco-2 cells	[18]
Lipidoids, cholesterol, DSPC, PEG2000-DMG	GAPDH	Mice	[19]
DODAB, DSPG, HSPC, CF-PE	Model protein	Rats	[20]
DPPE, protamine, hyaluronan, antibody FIB504	Cyclin D1	Mice	[21]
HSPC, cholesterol, mPEG2000-PE, calcein, antibody (KN2/NRY or irrelevant human IgG), or haptoglobin	Cyclin D163	Mice	[22]
Ginger-derived lipids	IL-6, TNF-alpha, and IL-1β	Mice	[23]
Ginger-derived lipids	CD98	Mice	[24]

DSPC, distearoyl L-3-phosphatidylcholine; PEG2000-DMG, 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000; DODAB, dimethyl-dioctadecylammoniumbromide; DSPG, 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]; HSPC, Hydrogenated soybean phosphatidylcholine; CF-PE, cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-carboxyfluorescein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DPPE, dipalmitoylphosphatidylethanolamine; mPEG2000-PE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt); TNF-alpha, tumor necrosis factor-alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin; IL-1β, interleukin-1β.

Table 1.
Lists of some liposomal formulations for colon-targeted RNAi delivery.

The use of RNAi in IBD models results in mucosal healing and the restoration of immune balance at the site of inflammation. RNAi techniques have high selectivity for intestinal tissues, a simple preparation method, and a low cost when compared with other IBD therapies [11, 12]. The main challenges for the development of RNAi-based therapeutics are efficient and safe delivery of RNAi molecules, endosomal escape, and entry into the cytoplasm. Moreover, RANis have a short half-life in the circulation, a

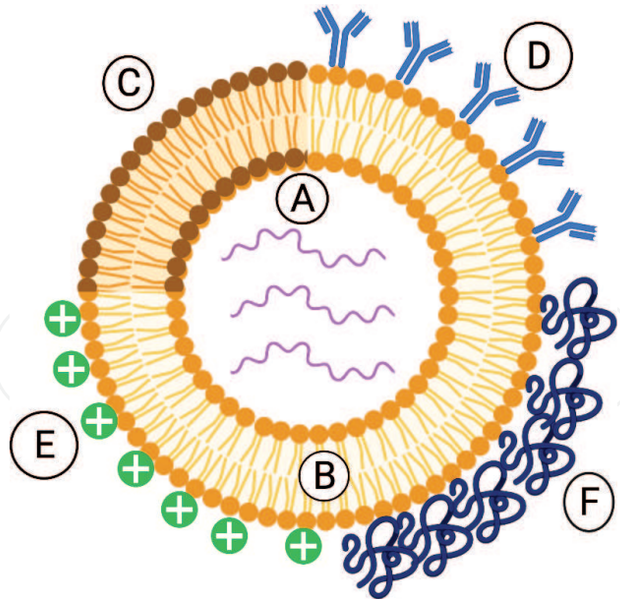

Figure 2.
Strategies for surface modification of liposomal formulations for colon-targeted RNAi delivery. A) RNAi molecules, B) lipid bilayers, C) natural lipids, D) ligand or antibody, E) coating with cationic polymers, F) coating with pH sensitive polymers. Created with Biorender.com.

relatively large size (13 kDa), an overall negative charge due to its phosphate backbone and degrade rapidly *in vivo*, necessitating targeted delivery to the site of action [13].

Among the various approaches to active targeting, liposomes-based delivery systems are innovative and promising systems to transport and control RNAi molecule release and overcome some of their limitations [11, 14, 15]. Many RNAis in lipid formulations have progressed through various stages of clinical trials, with the measurable improvements in patients and no side effects [16]. Alnylam Pharmaceuticals recently developed an FDA-approved first-of-its-kind intravenous dosage of siRNA-lipid nanoparticles, ONPATTRO® (patisiran), for the treatment of polyneuropathy [17]. Furthermore, 10 nanocarriers made with lipids are used in 12 ongoing clinical trials involving gene delivery, demonstrating the high potential of lipids nanocarriers for RNAi delivery [18]. Lipidic systems for RNAi molecules delivery have many advantages: The cell membrane is primarily composed of phospholipids (e.g., phosphatidylcholine) and cholesterol, making these natural lipids biocompatible. They have the ability to interact with the cell membrane and efficiently deliver the payload into the cell and can be purified or synthesized in large quantities [19]. For colon targeting, liposomes can be manipulated by different methods (**Table 1**, **Figure 2**). This chapter discusses the progress in delivering RNAi molecules to the colon using liposomes.

2. Targeting RNAi-encapsulated liposomes to the colon

2.1 Size and surface charge-dependent liposomes

The design of the liposomal surfaces in relation to the size, surface charge, and injury of the intestinal wall is the major challenge in the development of oral liposome-based carriers. As a result, a variety of modified liposome-based carriers are being tested in experimental colitis to determine the efficiency of accumulation and the improvement of clinical symptoms. There have been numerous studies that

show the presence of macrophages and dendritic cells in IBD, and these can lead to liposome capture to a greater extent than tablets and solutions [20]. As a result, the size of liposomes is an important factor in drug delivery in IBD. Furthermore, electrostatic interactions allow nanocarriers of opposite charge to specifically target the charged surface of inflammatory tissues in IBD. Unlike the healthy regions, the mucosal composition of the inflamed colonic epithelium has a dysregulated mucous layer, a high degree of cationic proteins (transferrin, ferritin, bactericidal, or permeability-enhancing proteins (BMPs)), and accumulation of antimicrobial peptides (AMPs). This led to cationic charge build-up at the colitis surface. Thus, anionic nanocarriers as a delivery system can favorably adhere to the cationic-inflamed surfaces, release the drug locally, and prolong drug residency [21]. It has been demonstrated that positively charged liposomes better adhered to healthy mucosa, whereas negatively charged liposomes showed an increased adhesion to inflamed mucosa [3]. However, cationic liposomes have been the standard for siRNA transfection. These liposomes are used in commercially available transfection carriers such as lipofectamine. Lipofectamine was introduced in 1993 for DNA transfection and has since been optimized for siRNA transfection (oligofectamine, lipofectamine RNAiMAX). The phospholipid bilayer of the liposome allows it to cross the cell membrane and deliver its hydrophilic core of siRNA to the cytoplasm. When lipofectamine transfected, unmodified anti-TNF-siRNA prevented experimental colitis in mice following rectal administration [22]. However, cationic liposome delivery is complicated by toxicity concerns and requires efficacy improvement. Possible explanations include cationic liposomes made of cationic lipids, which are known to be membrane active. When incubated with cells, cationic lipids can disrupt the cell's or subcellular compartments' membrane function and integrity, resulting in toxicity. Another cause of toxicity could be the presence of the pH-sensitive lipid 1,2-dioleoyl-sn-glycero-3- phosphoethanolamine (DOPE) in the liposome. The intracellular fate of the complexes following uptake into the cells determines transfection success; the majority of the complexes are degraded in the lysosomes. DOPE may increase cationic lipid toxicity by destabilizing the lysosomal membrane due to the formation of an inverted hexagonal phase at acidic pH, which is typical of lysosomes. Furthermore, cationic lipids can become cytotoxic by interacting with important enzymes like protein kinase C. According to recent research, many cholesterol derivatives with tertiary or quaternary nitrogen headgroups can inhibit protein kinase C activity [23].

Many interesting types of liposomes with various physicochemical properties were prepared and tested in cell culture and experimental colitis models with varying degrees of success. An *ex vivo* study on neutral, positively charged, and negatively charged liposomes to target colitis induced by dinitrobenzene sulfonic acid (DNBS) revealed that anionic liposomes adhered to inflamed colonic mucosa twice as well as neutral or cationic liposomes. This adherence was dependent on the presence of 12,dimyristoyl-sn-glycerol-3-(phosphor-rac-(1-glycerol)) (DSPG, negatively charged) on the liposomes, whereas cationic and neutral liposomes did not significantly bind to the inflamed intestinal mucosa [24]. In a rat colitis model, negatively charged liposomes accumulated more in the inflammatory regions than cationic or neutral charged liposomes. These findings demonstrate that liposomes, whether positively or negatively charged, can interact with components in the GI tract, providing specificity in drug delivery. Unwanted electrostatic interactions, however, continue to be a problem in these systems. Charged liposomes have the potential to interact with oppositely charged GI tract components such as soluble mucins and bile acids [24, 25].

Despite the fact that anionic liposomes have been found to be specific in drug delivery, additional approaches are required to improve bioavailability in the colon.

Ball et al. prepared lipoid nanoparticles from amphiphilic lipid-like materials that form nanoparticles when complexed with cholesterol, distearoyl-sn-glycerol-3-phosphocholine (DSPC), and PEG-lipid. Three lipoids from a library of synthesized lipoids were chosen for their ability to target intestinal epithelial cells. The ability of one lipid nanoparticle, 306O13, to silence the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in Caco-2 cells *in vitro* was then chosen [26]. Further studies show that 306O13 lipid nanoparticle were effective at gene silencing in HeLa cells *in vitro* across a pH range of 1–9, indicating that they may be stable across the pH range found in the GI tract. Pepsin and bile salts were found to reduce lipid nanoparticle GAPDH silencing in Caco-2 cells after they were subjected to simulated GI digestion conditions. Pancreatin and low pH (1–2) had little effect on silencing efficacy. Mucin at a concentration of 2% w/v in Caco-2 cell buffer was also found to significantly reduce silencing potential (90–40%). Lipid nanoparticles were found in mouse intestinal cells for 8 hours after delivery, and fluorescently labeled siRNA was found; however, gene silencing of GAPDH *in vivo* was not statistically significant. The low *in-vivo* efficacy could be attributed to uneven uptake. Working on uniform delivery across more epithelial cells may therefore yield better results [26, 27].

2.2 pH-dependent liposomes

Another approach for protecting liposomes from the harsh gastrointestinal environment is to coat liposomal surfaces with layers of polymers such as enteric polymers. Enteric coatings are well known for preventing liposome disintegration in the stomach, which improves absorption by allowing more liposomes to survive and be exposed in the small intestine [28, 29]. Liposomes are frequently coated with pH-dependent coating polymers such as methacrylic acid co-polymers (Eudragit®) for oral delivery. The liposomes coated with Eudragit® S100 exhibit appropriate pH response release characteristics when the polymer retains liposomal drug release at pH levels of 1.4 and 6.3 (resembling the stomach and small intestine, respectively), but releases the drug similar to plain liposomes NPs at pH 7.8 (ileocecal junction) [30]. Though pH-dependent liposomes have demonstrated excellent results in preclinical studies, the variability of pH in the colons of IBD patients suggests that a colonic drug delivery system based solely on gastrointestinal pH would be unreliable [31]. Despite the fact that pH-dependent liposomes have shown excellent results in preclinical studies, the variability of pH in IBD patients' colons suggests that a colonic drug delivery system based solely on gastrointestinal pH would be unreliable [1].

2.3 Active targeting-dependent liposomes

Polymers used to coat liposomal formulations have improved drug delivery to the colon after oral administration *via* pH-dependent release and mucoadhesive properties. These formulations, however, have a limited effect on the specificity of targeting to diseased versus healthy colon tissue. Surface modifications of liposomes with the coupling of ligands play a key role in drug delivery to more specific targeting to regions within the colon by exploiting disease-induced cellular changes in cell-surface receptors and proteins. Also, one of the more versatile ligands that can be affixed to

liposome surfaces is the coupling of antibodies, particularly monoclonals, to create immuno-liposomes [32]. Veiga et al. used an ASSET (Anchored Secondary scFv Enabling Targeting) method, in which anti-Ly6c mAb is linked to liposomes, to target Ly6c + inflammatory leukocytes. The authors tested this strategy in a dextran sodium sulfate (DSS) colitis mouse model of inflammatory bowel disease using anti-Ly6c mAb coated or isotype control liposomes-formulated IL-10 mRNA, and they found that the liposomes-mRNA-targeted approach was more effective than the nontargeted approach [33].

Transferrin is a glycoprotein that transports ferric ions throughout the body. The transferrin receptor protein was found to be highly expressed in the basolateral and apical membranes of enterocytes in the colonic mucosa of IBD patients, as well as in the colonocytes of rats induced with colitis [34]. Transferrin receptor-mediated endocytosis is a normal physiological process that transports iron to cells. To create pendant-type PEG-liposomes, transferrin was coupled to the distal terminal of the PEG chains of PEG-liposomes. After that, transferrin -PEG-liposomes were injected intravenously into tumor-bearing mice. Transferrin-PEG-liposomes extravasate from the blood circulation and are followed by specific binding and internalization of transferrin -PEG-liposomes into tumor cells, leading to the delivery of their content into the cytoplasm *in vivo* [35]. Anti-transferrin receptor immune liposomes were found in higher concentrations in the mucosa of rats with dinitrobenzensulfonic acid (DNBS)-induced colitis than nonconjugated immunoliposomes in *ex vivo* binding studies [34].

An increased risk of colorectal cancer is the common feature of IBD. The chronic inflammation caused by these diseases can disrupt the cellular cycle, causing intestinal cells to replicate uncontrollably, potentially leading to tumor formation. Russo et al. used siRNA molecules to reduce the production of cellular cycle proteins CyD1 and E2F1 in explanted Crohn's disease intestinal tissue. Commercial siRNAs for CyD1 and E2F1 inhibition were encapsulated in Invivofectamine® leading to liposome nanocarriers designed specifically to silence CyD1 and E2F1 expression. As a result, the liposomes nanocarriers were able to reduce the amount of proteins associated with intestinal cancer in the tissue [36]. In a similar approach, protamine-condensed siRNA entrapped in a liposome modified with hyaluronan and coupled with a ß7 integrin-targeting antibody reversed experimentally induced colitis after systemic administration in mice. The condensation of siRNA with protamine allowed for a high drug load per nanoparticle (4000 siRNA molecules) as well as liposome protection against siRNA-induced interferon production. Furthermore, ß7 integrin-targeting antibodies coated on the outer surface of the liposomes provided selective cellular targeting, whereas cell surface integrins proved to be effective antibody targets for both nanocarrier delivery and uptake [37]. CD163 is a hemoglobin scavenger receptor that is overexpressed in the tissues of M2 resident macrophages as well as macrophages at sites of inflammation and tumor growth [38]. Etzerodt et al. studied CD163-binding monoclonal antibodies conjugated to the surface of PEG-liposomes to target CD163 cells and macrophages. PEG-liposomes mediated by antibodies significantly increased liposome uptake in both CD163-transfected cells and macrophages. Furthermore, the PEG-liposomal doxorubicin-targeted receptor CD163 exhibited strong cytotoxic effects on CD163-expressing human monocytes. The PEG-liposome mediated by CD163-binding monoclonal antibodies is a potential approach for targeting therapeutic agents to macrophages that support inflammatory and malignant progression [39].

2.4 Natural liposomes

There are two major drawbacks to synthetic liposomes. Before clinical application, each constituent of the synthesized liposomes must be tested for potential *in vivo* toxicity, and the production scale is limited. Liposomes derived from natural sources, on the other hand, are thought to be safe and cost-effective, and they may overcome the limitations of synthetic liposomes [10]. Extracellular vesicles have recently emerged as a more complex form of liposomes with a biological origin. Extracellular vesicles are nanoparticles encased in a complex lipid bilayer. They are released as exosomes and microvesicles from viable cells either on their own or in response to certain stimuli. Exosomes are formed within the endosomal system's multivesicular bodies. Exosomes are released when the multivesicular body fuses with the plasma membrane. Exosomes are reported to be between 30 and 150 nm in size. Microvesicles, on the other hand, have been reported to have sizes ranging from 100 to 1000 nm and are released directly from the plasma membrane [40, 41]. Using this approach, Zhang et al. isolated exosome-like nanoparticles from edible plants (ginger) using an eco-friendly protocol. The study demonstrated that ginger-derived nanoparticles increased survival and intestinal epithelial cell proliferation by upregulating anti-inflammatory cytokines and reducing proinflammatory cytokines (IL-6, TNF-α, and IL-1β) *in vivo* [42]. In another study, delivery of ginger-derived nanoparticles loaded with siRNA-CD98 was tested in colon-26 cells and successfully reduced the expression level of colonic CD98. Also, the oral administration of ginger-derived nanoparticles loaded with siRNA-CD98 reduced the expression of CD98 in the ileum and colon and thus may be useful for treating ulcerative colitis. Moreover, it was found that the effective dose of siRNA-CD98 delivered *via* oral administration of ginger-derived nanoparticles is approximately 10,000 lower than that of systemically administered naked siRNA-CD98 [23]. These studies suggest liposomes derived from natural compounds have immense potential in curing IBD.

3. Conclusion and future perspectives of liposomal formulations for colon-targeted RNAi delivery

The use of modified-liposome formulations as RNAi delivery systems has greatly improved RNAi molecule stability and therapeutic effectiveness. Because of their biocompatibility, biodegradability, low cost, stability, long-circulating times (PEGylated liposomes), and high encapsulation efficiency, nanoliposomes are preferred over other nanoparticle platforms as drug carriers. Furthermore, by attaching specific targeting ligands to their external surfaces, nanoliposomes can be functionalized. Several *in vivo* preclinical studies have highlighted the potential of modified-liposomes that retain RNAi activity at the target site. Despite the obvious advantages of modified-liposomes in terms of therapeutic development, target specificity, and reproducibility, their success from bench to bedside for RNAi therapeutics remains to be seen. Many RNAi in lipid formulations have progressed through various stages of clinical trials, with the measurable improvements in patients and no side effects. The development of modified-liposomes for RNAi molecules has enormous clinical potential as next-generation drugs.

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