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Advanced siRNA delivery in combating hepatitis B virus: mechanistic insights and recent updates

Linh Nguyen[1], Tiep Tien Nguyen[1*], Ju-Yeon Kim[1*] and Jee-Heon Jeong[1*]

Abstract

Hepatitis B virus (HBV) infection is a major health problem, causing thousands of deaths each year worldwide. Although current medications can often inhibit viral replication and reduce the risk of liver carcinoma, several obstacles still hinder their effectiveness. These include viral resistance, prolonged treatment duration, and low efficacy in clearing viral antigens. To address these challenges in current HBV treatment, numerous approaches have been developed with remarkable success. Among these strategies, small-interfering RNA (siRNA) stands out as one of the most promising therapies for hepatitis B. However, naked siRNAs are vulnerable to enzymatic digestion, easily eliminated by renal filtration, and unable to cross the cell membrane due to their large, anionic structure. Therefore, effective delivery systems are required to protect siRNAs and maintain their functionality. In this review, we have discussed the promises of siRNA therapy in treating HBV, milestones in their delivery systems, and products that have entered clinical trials. Finally, we have outlined the future perspectives of siRNA-based therapy for HBV treatment.

Keywords siRNA, Hepatitis B, Delivery system, Conjugation, Lipid

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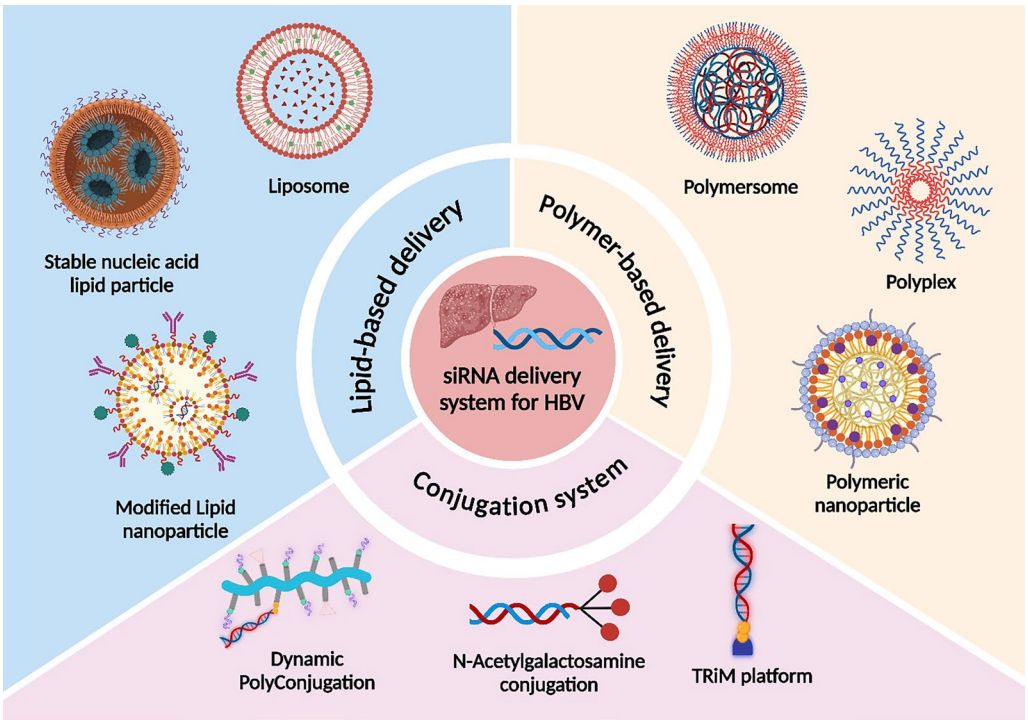
Graphical Abstract



Introduction

Chronic hepatitis B (CHB) is a major health problem, affecting over 250 million individuals and causing nearly 0.9 million deaths annually [1–4]. Patients with CHB are at risk for hepatocellular carcinoma (HCC), liver fibrosis, and/or other decompensated liver diseases [5–10]. Current therapies for CHB include nucleoside or nucleotide analogs (NUCs) and interferon (IFN) therapies [5]. Despite their various clinical benefits, these treatments have significant limitations. NUC-based therapies have minimal or no impact on viral antigens (HBsAg, HBeAg, and HBcAg) and often require long-term treatment. IFN-based therapies pose high risks for patients with liver decompensation or HCC, resulting in an incomplete cure for CHB [11–14]. Although combining NUCs and IFN therapies seems to enhance synergistic effects, none have demonstrated superior efficacy compared to monotherapy in achieving sustained responses [15, 16]. Therefore, novel treatments must be developed to achieve higher efficacy with minimal side effects or even aim for a complete cure. In recent years, siRNA-based therapy has gained significant attention for treating viral diseases [17–21]. In HBV management, siRNA delivery strategies have shown remarkable therapeutic effects, addressing the limitations of current therapies [22–25]. However, effective siRNA delivery systems still need improvement. Many scientists have dedicated substantial effort to developing siRNA delivery approaches for HBV treatment [26]. This paper will provide an overview of CHB pathology, mechanisms of siRNA therapies in CHB, and challenges associated with siRNA delivery. Subsequently, we have focused on some common carriers for siRNAs in CHB treatment and discussed future perspectives on siRNA therapy.

Hepatitis B virus life cycle

HBV is a non-cytopathic, hepatotropic DNA virus belonging to the family Hepadnaviridae [27–29]. It has a 3.2-kb relaxed circular DNA genome (rcDNA), which is packaged with polymerase inside nucleocapsids and covered by viral envelope proteins [27, 30, 31].

The life cycle of HBV is illustrated in Fig. 1. Briefly, viruses recognize heparan sulfate proteoglycan (HSPGs) on hepatocytes via low-affinity binding and subsequently bind to hepatocyte receptor–sodium taurocholate co-transporting polypeptide (NTCP) by a high-affinity interaction to enter the cell under the support of epidermal growth factor receptor (EGFR) [32]. Afterwards, HBVs import their genomes (rcDNA) into the nucleus. Once inside the nucleus, the rcDNA transforms into

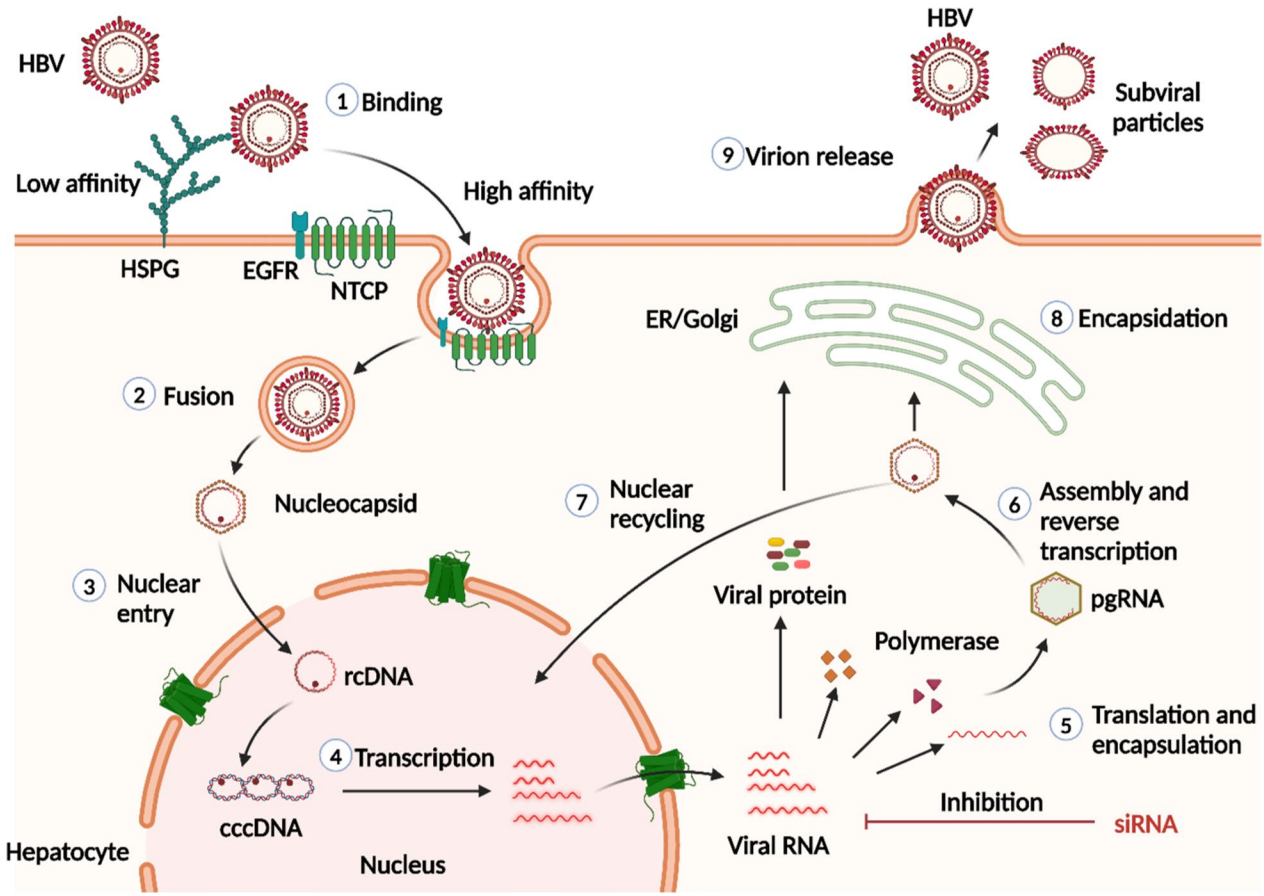


Fig. 1 HBV lifecycle and roles of siRNA therapy. (**1**) HBV enters hepatocytes through interactions with HSPG and NTCP receptors on hepatocytes [39, 40]. (**2, 3**) Once inside the hepatocytes, the viral envelopes are uncoated, transporting the genome (rcDNA) into the host nucleus. (**4**) HBV utilizes host genomic machinery to synthesize cccDNA from rcDNA [41]. (**5**) The virus then produces various types of RNAs through the transcription of cccDNA and exports them to the cytosol. (**6**) pgRNA serves as a template for reverse transcription of HBV DNA, whereas other mRNAs are templates for viral antigens, such as HBsAg or HBeAg [5]. (**7**) Assembled HBV DNA re-imports back into the nucleus, contributing to the cccDNA pool. This pool is persistent and continuously replenished through new infections or recycling of nucleocapsids [42]. (**8, 9**) Finally, the viral capsids, containing the HBV genome, incorporate with surface proteins to generate new infectious viral particles, which then infect other hepatocytes [43, 44]. Created with Biorender.com

covalently closed circular DNA (cccDNA). The formation of cccDNA is a complex process, in which rcDNA enters the DNA "repair" process utilizing the host machinery and enzyme activities [33, 34]. This process requires the removal of covalently bound HBV polymerase by tyrosyl-DNA phosphodiesterase and terminal redundancy sequence 'r' by flap endonuclease 1 in the minus strand of rcDNA, the cleavage of RNA primer in plus strand, the completion of DNA synthesis using host DNA polymerase and the ligation of both strands by DNA ligase to form cccDNA [35, 36]. Subsequently, cccDNA enters chromatinization with histone and non-histone proteins to form cccDNA minichromosome, which stably persists in infected cells and serves as transcriptional templates for viral RNAs, including pregenomic RNA (pgRNA) and other messenger RNAs (mRNAs). The pgRNA undergoes reverse transcription and assembles with viral proteins (derived from mRNAs) to form new nucleocapsids. Some of these nucleocapsids return to the nucleus, whereas others combine with viral surface proteins and exit the cell as infectious viruses [37, 38].

The cure for HBV has been categorized into complete, functional, and partial cure. A complete or sterilizing cure is classified as the complete eradication of all viral traces, which is an optimal but unrealistic goal due to the persistence of cccDNA. The favorable and realistic endpoint for CHB is a functional cure, considered as stable loss of HBsAg and unquantifiable HBV DNA for 6 months after treatment regardless of seroconversion to anti-HBs. A partial cure is defined as low HBsAg (< 100

IU/mL), undetectable HBV DNA, and the absence of HBeAg after treatment discontinuation. Partial cure is only considered as an intermediate endpoint due to the lack of HBsAg loss [11, 45–47]. However, current medications with NUCs and IFNs hardly reach functional cure in patients (< 1%) [48–54]. Besides, NUC therapy may face viral resistance due to long-term treatments [55–58], while IFN therapy possibly causes several adverse effects [56, 59].

Innovative approaches for a more effective HBV cure are extensively required. Some have been demonstrated significant promise in preclinical studies, including therapies using entry inhibitors, gene editing tools (CRISPR/Cas), nucleic acid polymers (NAPs), capsid assembly modulators (CAMs), and siRNAs. However, there are no clinical records for CRISPR/Cas in HBV treatment, while NAPs and entry inhibitors necessitate a combination treatment and their monotherapy in clinic did not exhibit apparent effects. Entry inhibitors seem to be more suitable for vaccination rather than treatment. Though some CAM candidates have entered phase II clinical trials, they are less effective in reducing viral antigens and may face the risk of resistance [46, 60–64]. SiRNA therapy is likely to be the most promising approach with many candidates have been in clinical trials [24]. Among the latest clinical trials, two siRNA candidates stand out: HRS-5635 and TQA3038, though their detailed information has not been released yet. They have completed phase I without serious adverse events and will soon enter phase II in late 2024.

SiRNA-based therapies for CHB

Mechanistic effect of siRNA

Each short fragment (20–30 nucleotides), known as siRNA, consists of two strands: sense (or passenger) and antisense (or guide) strands [65–67]. The guide strand has specific sequences that target the desired mRNAs. After entering hepatocytes, siRNAs interact with Dicer (RNase III endonuclease), RNA-binding co-factor, and RNase Argonaute to form RNA-induced silencing complex loading complex (RLC) [25, 68–70]. Subsequently, RLC removes sense strand, resulting in the formation of a mature RNA-induced silencing complex (RISC) [71–73]. The mature RISC can selectively recognize and inhibit translation and trigger the degradation of the target mRNA [74, 75]. This RNA interference mechanism of siRNAs shares some common machinery (e.g. Dicer and RISC) with endogenous micro-RNA (miRNA) in the cell, but they differ in their origin, structure and specificity of action [76, 77]. This understanding is important in the design of siRNA to avoid a common off-target effect of siRNA—so called miRNA like off-target effect, in which case, siRNA acts like miRNA, resulting in degradation of multiple unwanted mRNAs. SiRNAs containing "seed region" of miRNA can compete with endogenous miRNA to block normal miRNA pathway in the cell leading to detrimental effects [78]. Thus, careful design of siRNA is the initial important step for the success of siRNA-based therapy.

Various siRNA candidates have been developed to treat CHB [79–81]. One notable advantage of siRNA is that one siRNA could be designed to reduce all viral proteins and transcripts derived from cccDNA and integrated HBV DNA since all HBV transcripts from both genomes share the common coding region. Thus, siRNA could rapidly decline HBsAg and viral replication, which then indirectly decreases the cccDNA reservoir [82]. Moreover, research has shown that biogenesis of cccDNA relies on many host factors (e.g. DNA repair machinery and enzymes) (section "Hepatitis B virus life cycle"), thereby inhibiting these factors by siRNAs possibly decreases cccDNA level [35]. In addition, recent preclinical studies have demonstrated that employing siRNA to knockdown important proteins such as DOCK1 (a host factor dedicator of cytokinesis 11), BRD4 (Bromodomain-containing protein 4), or HBx could significantly reduce the amount of cccDNA [60, 83–86]. DOCK11 is involved in promoting HBV endocytosis, trafficking and cccDNA biogenesis that are important for viral infection and replication [87]. The primary role of HBx is to degrade the structural maintenance of the chromosome 5/6 complex which functions as an inhibitor of cccDNA transcription [85]. Therefore, targeting these proteins with siRNAs could potentially reduce cccDNA, contributing to a sterilizing cure for CHB.

Challenges in siRNA delivery

Like other nucleic acid molecules, naked siRNAs struggle to reach the target organs after administration due to their instability, short duration in blood circulation, and off-target effects [88–90]. In systemic circulation, siRNAs are prone to degradation by endogenous enzymes and rapid excretion via renal filtration, resulting in a short lifetime in the body [91–93]. In addition, their low permeability through cell membranes is a significant challenge due to their negative charges from phosphate groups and highly hydrophilic nature [94–97]. Off-target effects also pose a serious issue for siRNAs. Without targeting moieties, naked siRNAs are rarely internalized into the desired organs, leading to severe toxicities [71, 98, 99]. Furthermore, endosomal entrapment after uptake by endocytosis needs to be addressed to optimize siRNA delivery.

One common approach to enhance the stability in blood circulation and therapeutic efficiency of siRNA is through structural modification of the siRNA

molecules [75, 100–103]. Although chemical modifications can improve the siRNA stability to some extent, they are insufficient to overcome all practical challenges. It is still necessary to avoid clearance by undesired organs, ensure entry into the intended tissues, and facilitate the uptake by targeted cells without causing detrimental immune responses [26, 104, 105]. Therefore, the development of rational delivery systems for siRNAs in HBV treatment could allow to achieve such purposes.

Approaches for delivering siRNA for treating CHB

To overcome the remaining challenges, it is necessary to design appropriate vehicles for siRNA. Non-viral delivery structures are often preferred for nucleic acid delivery, particularly for treating cancer and infectious diseases [106–114]. In CHB treatment, the most frequently used materials are lipid-based carriers, polymeric carriers, and conjugation systems (Table 1).

Conjugation system

Design and fabrication

Conjugation strategies currently represent the most promising approach for hepatic delivery. As previously mentioned, targeting remains a significant hurdle in siRNA delivery for HBV treatment. To ensure therapeutic effects, siRNAs must successfully internalize into hepatocytes, hence, labeling them with targeting ligands presents a reasonable solution [124–129].

Initially, siRNAs were reversibly connected to a backbone polymer, to which masking agents and targeting ligands were attached via carboxylate dimethyl maleic acid bonds. These linkages are broken under acidic conditions within endosomes and lysosomes, facilitating endo-lysosomal swelling to release siRNA (known as the proton sponge effect) [130, 131]. This platform, developed by Arrowhead, was named Dynamic Poly Conjugation (DPC) first generation. In HBV treatment, DPC consists of an amphipathic polymer, such as PBAVE, decorated with Polyethylene glycol (PEG) (masking agent), *N*-acetyl galactosamine (GalNAc or NAG; hepatocyte

Table 1 Clinical trials on siRNA therapy for hepatitis B treatment

Product	Delivery system	Inventor	Target	Status	Therapeutic effects	Clinical trial	Refs
ARC-520	Dynamic PolyConjugate (DPC)	Arrowhead Pharmaceuticals	HBV X region	Terminated	Significantly reduced HBsAg in both HBeAg-positive and -negative patients but moderately declined absolute levels	NCT02452528 NCT02577029 NCT02604212	[115, 116]
JNJ-3989	GalNAc conjugation	Arrowhead and Janssen	HBV S and X region	Phase I, IIa	Well-tolerated with no safety concerns; in combination with nucleic acids provides a sustained decrease in all HBV traces	NCT05275023 NCT04667104 NCT04439539	[117]
ARB-1467	LNP	Arbutus Biopharma	HBV S and X region	Phase IIa	Significantly reduced serum HBsAg levels	NCT02631096	[118, 119]
ALG-125755	GalNAc conjugation	Aligos Therapeutics	HBV S region	Phase I	N/A	NCT05561530	
VIR-2218	GalNAc conjugation	Alnylam Pharmaceuticals	HBV X region	Phase II	Interfered transcription of both cccDNA and HBV DNA; persistently reduces HBsAg	NCT05484206 NCT03672188 NCT02826018	[120]
AB-729	GalNAc conjugation	Arbutus Biopharma	HBV RNA	Phase IIa	Targeted all transcripts in HBV to suppress viral replication and all antigens	NCT04980482 NCT04820686 NCT04847440	[121]
RG-6346	GalNAc conjugation	Dicerna Pharmaceuticals	HBV S region	Phase Ib, IIa	Significantly and consistently reduced HBsAg in a long-term pattern regardless of HBeAg status with safe and well-tolerated properties	NCT04225715	[122]
RBD-1016	GalNAc conjugation	Suzhou Ribo Life Science	HBV X region	Phase II	Rapidly reduced serum HBsAg; is safe and well-tolerated	NCT05961098	[123]

targeting ligand), and siRNAs via reversible linkages (Figs. 2b) [132, 133]. The core polymer is selected based on its membrane lytic capacity. PEG aims to shield the polymer to control its lytic activation. SiRNA is incorporated into the polymer via a disulfide bond, a pH-sensitive linkage, that enables siRNA cleavage from the polymer. Upon entering hepatocytes via NAG, the bonds between PEG and the polymer break under acidic conditions, triggering endosome disruption by the polymer to release siRNA into the cytosol [132, 134]. The underlying mechanism relies on pH-sensitive linkers responding to pH changes in the endosome [135–137]. In subsequent years, a second generation of DPC was developed, co-injected with siRNA–cholesterol. Cholesterol conjugation facilitates hepatocyte targeting while DPC promotes endosomal escape [133]. However, the observed toxicity has hindered their clinical implementation, prompting a shift toward the use of the TRiM™ platform.

In TRiM™, siRNA is directly conjugated to the targeting moiety via covalent linkages, with structural modifications to enhance the pharmacokinetic activity. This platform is designed with optimized gene sequences to maximize silencing efficiency in the liver and minimize off-target accumulation in other tissues, thus, reducing toxicity [137]. Unlike DPC, which relies on third party agents for stabilization and targeting, TRiM™ directly conjugates siRNAs with high affinity targeting ligands and modified structures to improve pharmacokinetics and stability [138]. Its uniqueness lies in its ability to efficiently screen ideal components for optimal pharmacokinetics, enabling the development of a personalized siRNA therapy [139]. This advancement in ligand-mediated siRNA delivery has led to the development of the most potent ligand–siRNA conjugate for HBV treatment, known as *N*-acetylgalactosamine–siRNA (GalNAc–siRNA).

GalNAc and its derivatives have emerged as effective options for enhancing the accumulation in the liver and internalization of siRNA [140–144]. To express their effects, siRNAs are conjugated with GalNAc, targeting the asialoglycoprotein receptor (ASGP-R), which is predominantly expressed on hepatocytes rather than other cells [93, 124, 145–147]. The mechanism underlying the therapeutic effects of GalNAc–siRNA conjugation was illustrated in Fig. 3. In brief, GalNAc–siRNAs bind to ASGPR on hepatocytes, facilitating their entry into cells via endocytosis. During the late endosome stage, the chemical bonds between GalNAc and siRNAs degrade under acidic conditions, allowing siRNAs to escape from the endosome and enter the cytosol, where they can exert their functions [124, 148]. However, the initial form of GalNAc–siRNA may potentially interact with off-target transcripts, necessitating higher doses to achieve the desired effectiveness. Therefore, advancements in chemical modifications have been developed in conjugation with GalNAc conjugation. One notable modification is the enhanced stabilization chemistry, so-called ESC, which improves the stability, efficacy, and safety of siRNA

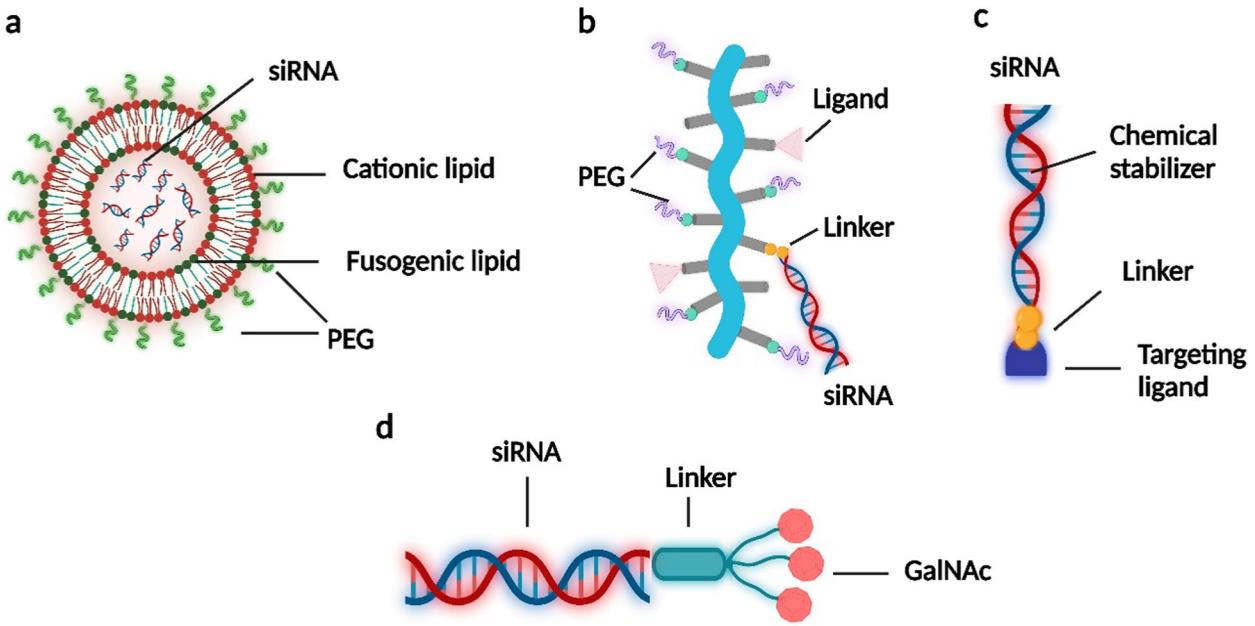


Fig. 2 Various advanced technologies for siRNA delivery include: **a** Stable nucleic acid lipid particles (SNALPs), **b** Dynamic PolyConjugate (DPC) system, **c** TRiM platform, and **d** *N*-acetylgalactosamine (GalNAc) conjugation. Created with BioRender.com

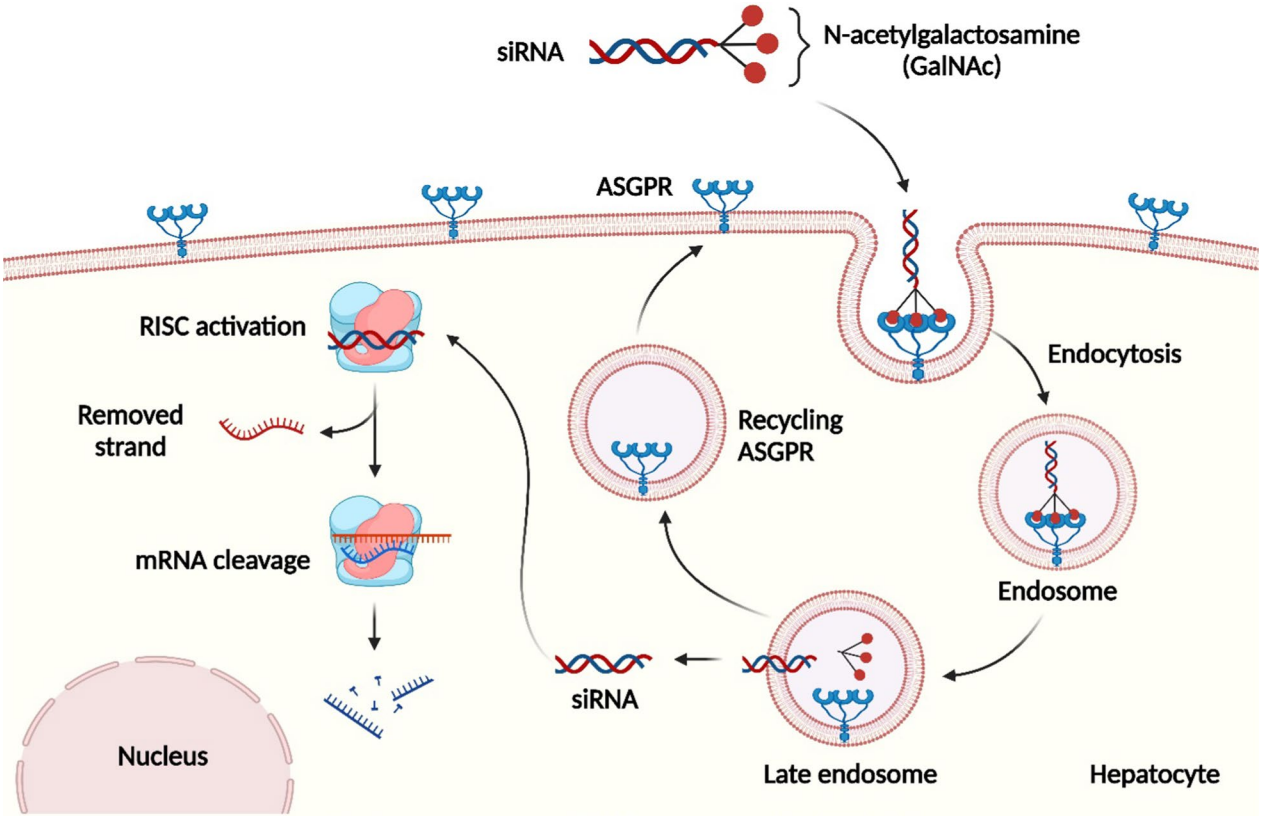


Fig. 3 GalNAc facilitates gene silencing through a specific mechanism. Initially, GalNAc–siRNAs bind to ASGPR and enter cells via endocytosis. Within endosomes, the linkers between GalNAc and siRNA degrade, releasing siRNA molecules into the cytosol where they induce desired alterations at the target gene site. Subsequently, free ASGPR molecules revert to their original form and reintegrate into the cell surface. Created with BioRender.com

[148]. The modification could be either 2′-O-methylation, phosphorothioate linkages, deoxyribonucleotides, or a combination of these modifications. They have been demonstrated to endow nucleic acids with nuclease resistance, thus preventing the degradation of siRNA in systemic circulation. In addition, 2′-O-methylation could enhance affinity to intended mRNA, reducing off-target effects [137, 149]. Further advancements such as ESC + have minimal off-target effects, improved safety and specificity of siRNAs [150, 151].

Current preclinical studies and clinical candidates

Most current clinical trials for siRNA-mediated therapies in CHB employ the GalNAc–siRNA platform. The first siRNA therapy in clinical trials for hepatitis B, ARC 520 from Arrowhead Pharmaceuticals, was developed based on this approach [134]. ARC 520 utilized two types of siRNAs targeting the X and S open reading frames (ORFs) in the viral genome, modified to minimize off-target effects [135]. Arrowhead designed a delivery system that utilizes cholesterol-conjugated siRNA and DPC technology [132, 133, 152]. DPC included melittin-like peptide (a core membrane active peptide) conjugated with hepatocyte-targeting ligands (NAG) (Fig. 4).. Data from phase I clinical studies demonstrated that a single dose of ARC 520 was well-tolerated with mild adverse effects in healthy patients [82]. Phase II trials showed a considerable decrease in absolute HBsAg levels for 0.38 and 0.539 log IU/mL in HBeAg-negative and -positive patients receiving a high dose of ARC 520 (2 mg/kg) [116]. These effects remained for almost 85 days after the final dose of ARC 520 [153, 154]. However, the candidate was discontinued due to long-term liver toxicity [115, 116].

JNJ-3989, also known as ARO-HBV, is a siRNA product that utilizes GalNAc conjugation for targeted delivery to hepatocytes. Compared to ARC-520, JNJ-3989 has been enhanced by incorporating the TRiM™ platform with GalNAc conjugation to improve delivery efficacy [156]. JNJ-3989 consists of two siRNAs targeting HBV S-ORF and X-ORF from both integrated DNA and ccc-DNA. In 2019, Janssen Research and Development Company combined JNJ-3989 with a novel class N capsid assembly modulator (JNJ-6379) and NUC to inhibit

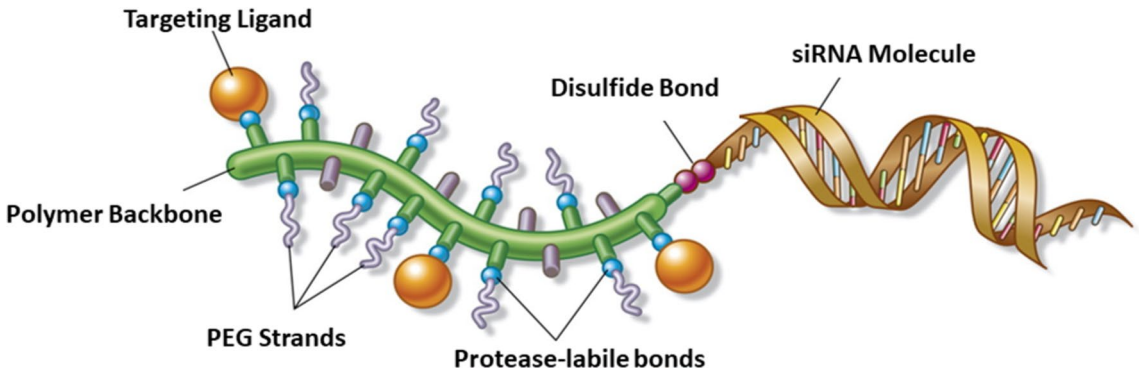


Fig. 4 Illustration of the structure of ARC-520. The structure of DPC includes a polymer backbone, targeting moiety, and PEG strands. The polymer is linked to siRNAs through disulfide linkages. Reprinted from Rozema et al. [155] with permission from Elsevier

HBV replication and robustly reduce all HBV genome levels, including cccDNA formation [157]. Following treatment, approximately 90% of HBsAg levels decreased (from − 1.01 to − 2.2 $\log_{10}$ IU/mL) in all patients. No deaths or severe complications were reported during the extensive study period [158]. A phase IIa clinical study demonstrated that 97.5% of participants who received this triple combination achieved an HBsAg reduction of ≥ 1 $\log_{10}$ IU/mL throughout the treatment. Around 75% of patients had HBsAg levels < 100 IU/mL on day 112 and this reduction was maintained in 38% of patients for 336 days post-treatment [159]. No severe adverse events were observed throughout 392 days, either with single or combination treatment of JNJ-3989 [159, 160].

Imdusiran or AB-729 from Arbutus Biopharma has entered phase IIa clinical trials in May 2024 and shown impressive results regarding both potent activity and safety [161]. In AB-729, a single siRNA–GalNAc was used to block all HBV RNA transcripts [162]. Clinical results demonstrated that a single dose of AB-729 (90 mg) could maintain HBsAg and HBV DNA below baseline levels for up to 48 and 44 weeks, respectively. All HBV RNAs also declined from − 0.64 to − 1.98 $\log_{10}$ U/mL after treatment with AB-729 [162]. A clinical long-term follow-up study has just started from July 2024 to observe the durability of therapeutic effect of Imdusiran on HBV parameters.

Another candidate, ALN-HBV from Alnylam Pharmaceuticals, was designed to target all main transcripts of viral mRNA. ALN-HBV passed preclinical evaluations for both efficacy and safety in rats and nonhuman primates. However, this candidate failed in a phase I clinical study due to increased alanine aminotransferase (ALT) levels observed in healthy volunteers. The issues were attributed to the off-target binding of siRNAs to a seed region of the guide (or antisense) strand. To address these problems, an advancement of ALN-HBV, known as VIR-2218, was developed by incorporating GalNAc–siRNA with ESC + technology [164, 165]. In VIR-2218, siRNAs were modified using ESC + and subsequently linked with GalNAc groups for targeted hepatic delivery (Fig. 5). VIR-2218 has entered a phase II clinical trial since 2021. Based on research from 2023, 50% of participants have achieved an absolute HBsAg concentration below 100 IU/mL. Notably, HBsAg reductions were more stable at higher doses, with mean decreases of 0.75 and 0.87 $\log_{10}$ IU/mL after 48 weeks in the 100 mg and 200 mg cohorts, respectively [163]. GalNAc conjugation and chemical modification with ESC + are primarily attributed to these beneficial effects. Indeed, while ESC + technology significantly contributes to stability of VIR-2218 in systemic circulation, GalNAc–siRNA conjugation effectively targets the liver, with more than 77% of siRNA located in the liver [149].

Different from the above candidates, Dicerna Pharmaceuticals has developed a proprietary GalXC – siRNA, so-called RG6346, aims to leverage siRNA therapy to the next generation. GalXC platform in RG6346 employs a tetra antennary GalNAc, instead of common tri-antennary pattern, to interact with siRNA, which then provides high efficacious in targeting liver with long remaining effectiveness for up to one year [122, 124, 166].

In 2023, RBD-1016, developed by Ribo Life Science, entered a phase II clinical trial. This product utilizes liver-targeting technology, RIBO-GalSTAR™, based on mechanical GalNAc–siRNA conjugation. It has demonstrated excellent sustained effectiveness in reducing HBsAg in preclinical research and well-tolerated safety properties in phase Ia clinical study [123].

In the most recent update, TQA3038, another GalNAc – siRNA candidate, has just completed its phase I clinical trial with no serious adverse incidents and will soon enter phase II study.

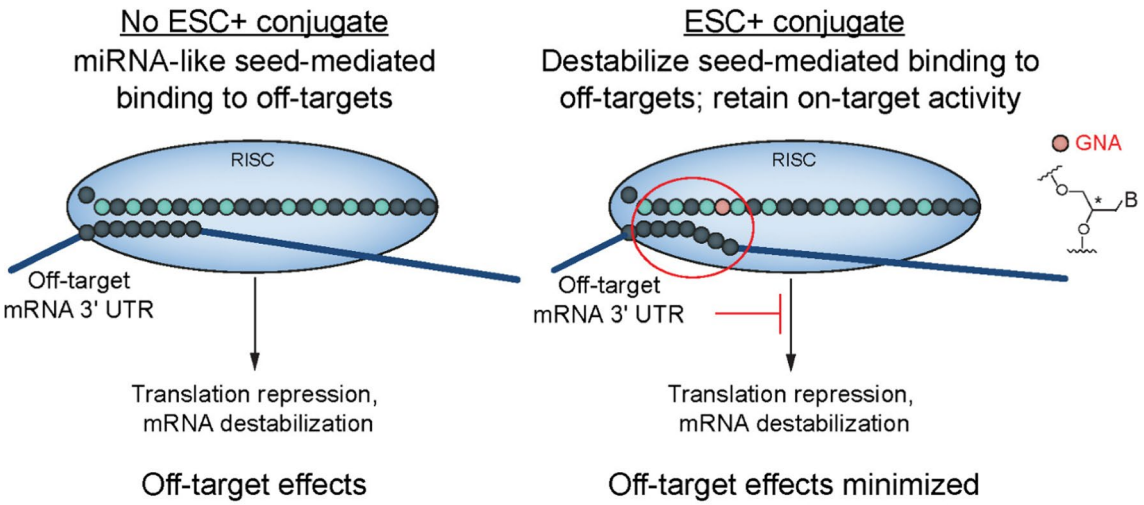


Fig. 5 Schematic illustration shows the ESC + structure of VIR2218. Reprinted from Gane et al. [163] with permission from Elsevier

Lipid-based delivery

Technological design

Lipid-based delivery systems generally resemble the structure of the cytomembrane, making them ideal for siRNA delivery [167]. They protect siRNA from degradation and can rapidly pass through the cell membrane and are relatively simple to produce. Despite their numerous advantages, lipid carriers have shortcomings related to lower targeting efficacy and potential toxicity that need to be considered.

Liposomes were the first lipid-based formulations used to load siRNA, with their components binding to negatively charged siRNAs via electrostatic interactions. However, these formulations may induce high toxicity, low stability, and poor scalability, hindering their clinical implication [168, 169]. Initially, cholesterol and phosphatidylcholine were used, but the entrapment efficiency was low, leading to the addition of cationic lipids (lipoplexes). Although cationic lipids can enhance the encapsulation of siRNAs, clinical toxicities and instability are major concerns [170, 171]. Consequently, current development has led to the next generation of lipid-based carriers for siRNA delivery known as lipid nanoparticles (LNPs) (Fig. 6). LNPs are constructed from ionizable lipids, phospholipids, PEGylated lipids, and cholesterol [93]. The key component is ionizable lipids (40–50% molar ratio of LNP), which determine the loading efficiency, extended retention in circulation, and enhanced cytosolic release of siRNA in hepatocytes. This is due to their special ability to change their charge based on the lipid pKa and pH of the environment. With proper pKa (6.0–6.5), ionizable lipids turn to be positively charged under acidic conditions to maximize electrostatic interaction with siRNA during preparation. In physiological environments with pH 7.4, they become almost neutral to prevent the adsorption of serum proteins and rapid clearance by immune cells. Subsequently, these lipids turn positive again in the acidic environment of endosomes, promoting endosomal escape, and finally release siRNA under neutral pH of the cytosol to exert effects. Phospholipids (e.g. DSPC, DOPE) account for a 10–15% molar ratio in LNP formulation to support the cohesion and cell internalization of LNPs due to their chemical structure. Cholesterol is added to maintain membrane integrity, avoid nucleic acid leakage, prolong retention in circulation, and affect internalization into cells. Finally, PEGylated lipids with small proportion, help to reduce fast clearance by the immune system and improve stability of LNPs [172–174].

To enhance the targeted delivery of siRNAs with high potential for endosomal escape, an advanced system called MEND (multifunctional envelope-type nano device) was investigated. In MEND, siRNA is encapsulated within a cationic lipid envelope having pH-sensitive properties. This lipid envelope possesses higher fusogenic ability and improved endosomal escape capability, resulting in improved gene silencing efficiency [175, 176].

Current preclinical studies and clinical candidates

Several studies have explored various lipids to enhance siRNA delivery to hepatocytes in HBV treatment. ARB-1467, developed by Arbutus Biopharma, utilizes a lipid nanoparticle to deliver three different RNAs to hepatocytes, reducing all HBV transcriptions. In preclinical studies, a single dose of ARB-1467 demonstrated sustained decreases in HBsAg and HBV DNA levels without safety concern [177, 178]. During a phase IIa clinical trial, multi-dose treatment with ARB-1467 resulted in HBsAg

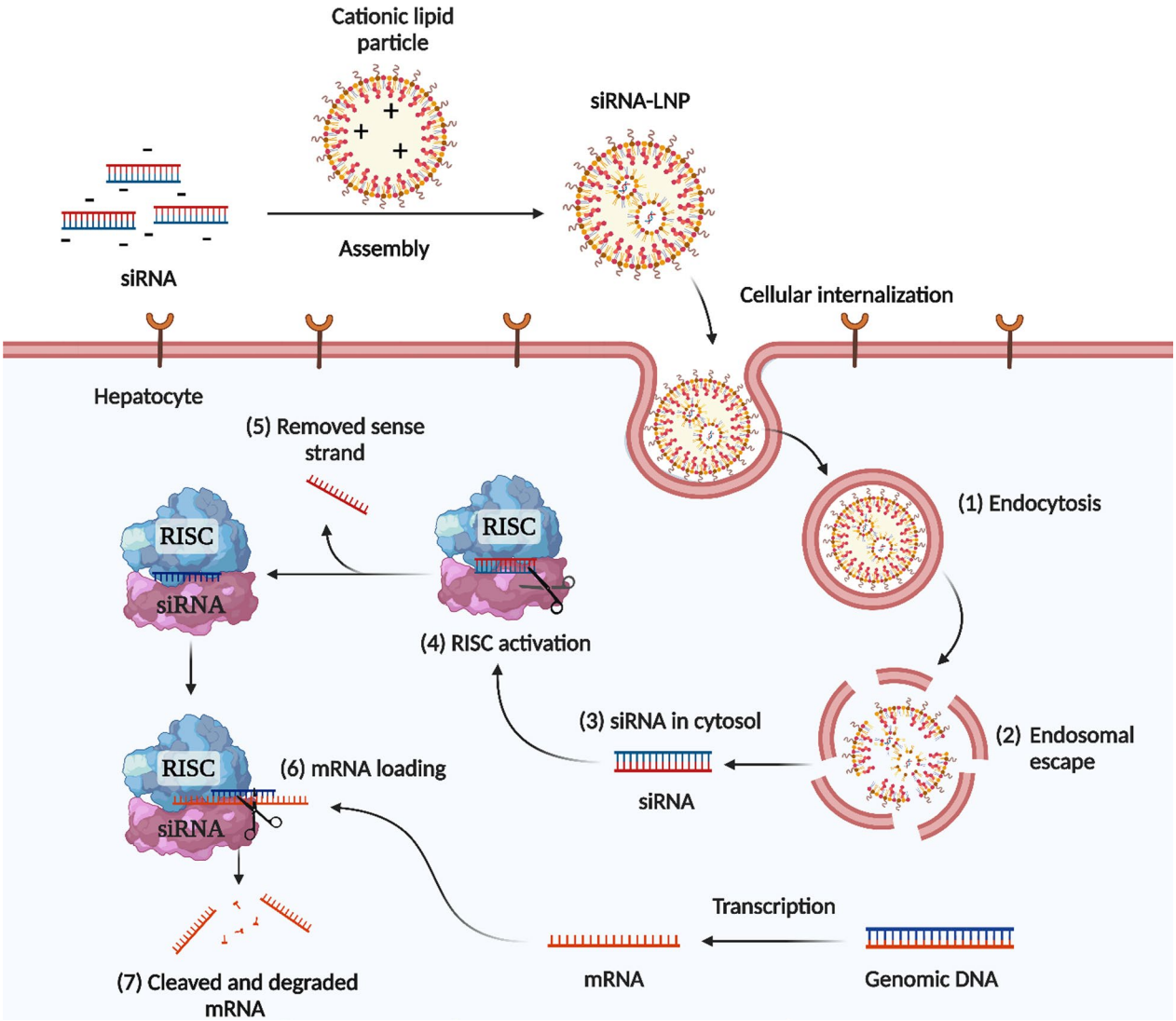


Fig. 6 Schematic illustration of LNP formulation and their delivery mechanism. LNPs are assembled through the electrostatic interaction of siRNA and lipid components. They may enter hepatocytes through phagocytosis or endocytosis pathways. Due to the special properties of the cationic lipid components, LNPs can escape from the late endosome stage to release siRNA into the cytosol, where they exert their functions. Created using BioRender.com

reductions of up to 0.9 log in HBeAg-negative patients and 0.7 log in HBeAg-positive patients, with no significant ALT increases [118, 119].

Most recently, Hikari Okada et al. utilized a LNP formulation, composed of Dlin-MC3-DMA (ionizable lipid), DSPC, PEG2000-C-DMG, and cholesterol [179] to encapsulate modified siRNA targeting a host factor dedicator of cytokinesis 11 (DOCK11). Research has shown that DOCK11 could activate CDC42 for promoting actin polymerization, which in turn triggers signaling pathway to facilitate the DNA repair for the conversion of rcDNA into cccDNA. DOCK11 also controls retrograde trafficking of HBV into nucleus via association with AGAP2 [87, 180]. Thus, inhibiting DOCK11 could lead to reduction of cccDNA in HBV treatment. In this study, siRNA targeting this protein was first modified with 2'-O-methylation and phosphorothioate before loading into the LNPs. Chemical modification along with the LNP platform significantly promotes the stability and efficacy of DOCK11-siRNA, leading to the reduction of more than 50% of cccDNA in an AAV8-HBV1.3mer infection mouse model [84].

In 2022, a novel ionizable lipid-like material (lipidoid), named LC8, was developed and incorporated with 16:0 PEG2000 PE and cholesterol to create a lipidoid nanoparticle known as RBP131 for delivering siRNA to the

liver [181]. A lead siRNA against HBV was encapsulated in RBP131 to form RB-HBV008 (Fig. 7a). LC8 had a pKa value within the range 6.0–6.5, facilitating the endosomal escape of siRNA. In the C57BL/6J-TgN 44Bri/J model (a partial gene-integrated transgenic mouse model), RB-HBV008 significantly suppressed X-gene expression with 94.4% efficiency at a dose of 1 mg/kg siRNA (Fig. 7b). In the C57BL/6J-M-Tg (HBV C1.0) model, a reduction of 2.10 log or 99.2% in HBsAg (ng/mL) was observed in animals receiving 1.0 mg/kg of RB-HBV008, which was retained for four weeks following a single dose (Fig. 7c, d). Another animal model demonstrated that RB-HBV008 induced a robust decrease in HBsAg levels in a dose-dependent manner (46.9%, 91.6%, and 99.3% knockdown of HBsAg expression were achieved with doses of 0.3, 1, and 3 mg/kg, respectively) (Fig. 7e). Moreover, no significant differences in blood urea nitrogen, liver histology, and creatinine levels were observed in RB-HBV008-treated mice.

To improve targeting ability, some LNPs have been anchored with moieties or ligands targeting receptors on hepatocytes. Research has demonstrated the critical role of HBV PreS1 protein in attaching the virus to the NTCP receptor on hepatocytes. Drawing from this, in 2022, Gao et al. developed a PreS/2–21-directed siRNA-loaded liposomal nanoparticles (PSN), in which PreS/2–21

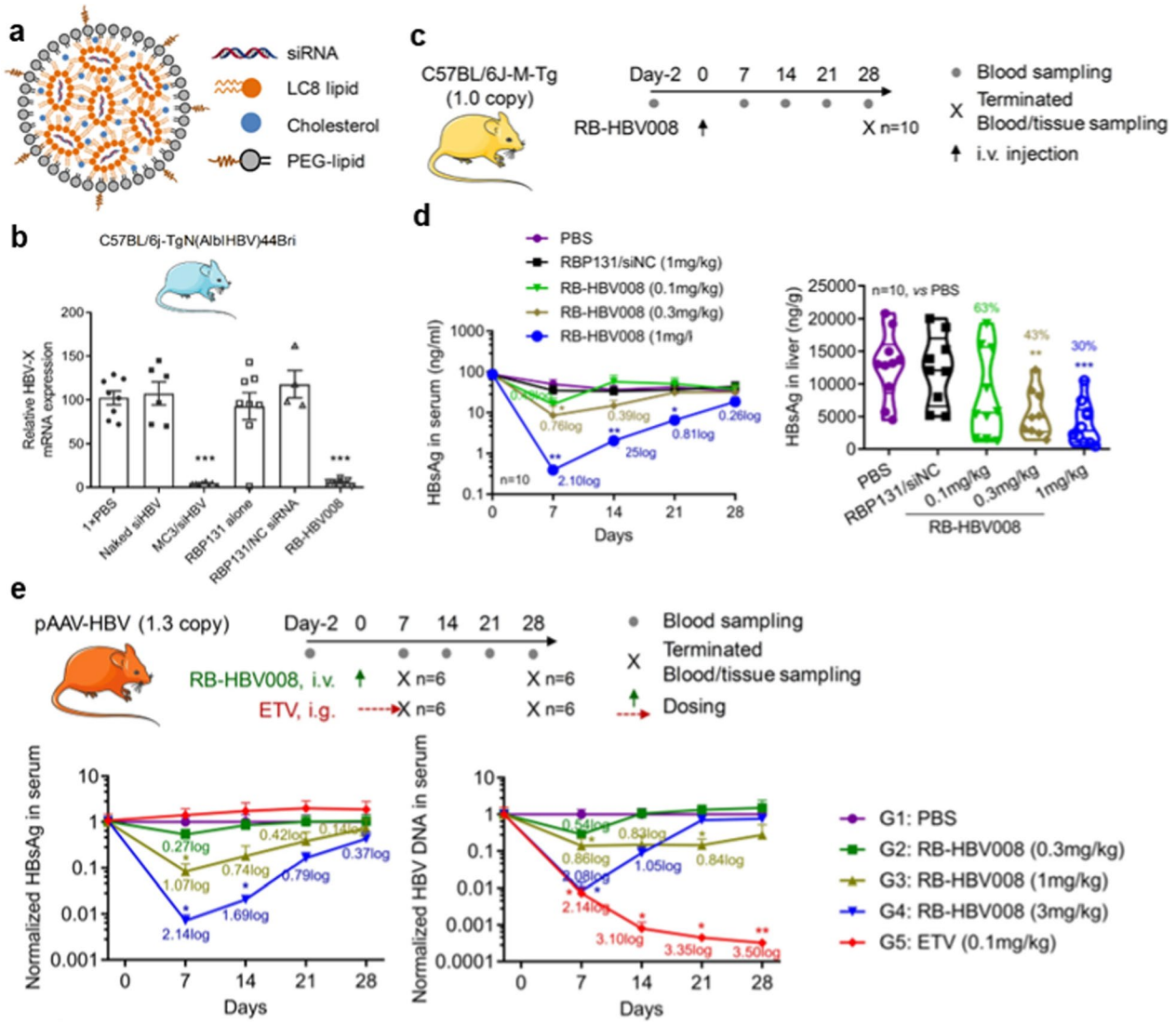


Fig. 7 Effectiveness of RB-HBV008 in various mouse models. **a** Formulation details of RB-HBV008. **b** Inhibition of *HBV* gene by RB-HBV008 in C57BL/6J-TgN (AlblHBV)-44Bri mouse model. **c**, **d** Efficacy demonstrated in a transgenic animal model with HBV 1.0 copy. **e** Therapeutic effects of RB-HBV008 in the pAAV-HBV model. Reprinted from Huang et al. [181] with permission from Springer Nature

was employed as a directing molecule to deliver siRNA into hepatocytes [182]. In this study, siRNA-targeting X gene of HBV was incorporated with TT3; cholesterol; 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC); PreS/2–21; and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N[maleimide(polyethyleneglycol)-2000] (Mal-PEG2000-DSPE) to fabricate PSN (Fig. 8). Results in mice indicated that PSN reduced HBeAg, HBsAg, cccDNA, and HBV DNA levels by 55.45%, 38.79%, 28.93%, and 24.43%, respectively. Intriguingly, PSN exhibited inhibitory effects on both intracellular HBV replication and new viral invasion, which are superior compared to PreS/2–21-modified nanoparticles and siRNA nanoparticles that only act on viral entry inhibition or prevention of viral replication, respectively.

Lipoplex is another vehicle for siRNAs, where cationic liposome or micelles blend with negatively charged nucleic acid to form a complex [183]. Typically, lipoplexes consist of cationic lipids along with neutral co-lipids, such as dioleoyl-l -α-phosphatidylethanolamine (DOPE) and cholesterol, to interact with siRNA via electrostatic bonds [184]. Their ability to deliver siRNAs for HBV treatment has been investigated in several studies [185].

Other kinds of carriers for anti-HBV siRNA have also been reported. Yamamoto et al. have introduced a novel siRNA carrier, called MEND/siRNA, which contained a pH-responsive cationic lipid (YSK13-C3), mPEG2000-DMG (PEGylated lipid), cholesterol and siRNAs. While YSK13-C3 enabled siRNAs to escape endosomes due to the fusogenic properties with bio-membranes, cholesterol and mPEG2000-DMG were added to enhance stability, extend circulation time and improve cellular internalization of MEND/siRNA (Fig. 9) [175]. MEND/siRNA has emerged as a promising carrier for targeting hepatocytes, with 80% of it accumulating in the liver, thereby exerting a stronger gene silencing effect (Fig. 9b). Antiviral activities were investigated both in vitro and in vivo, demonstrating notable efficacy in reducing HBsAg, HBeAg, and HBV DNA. This effect could persist for up to 14 days (Fig. 9c). Moreover, a notable feature of MEND is its versatility to deliver various types of nucleic acids and is not limited to siRNA alone.

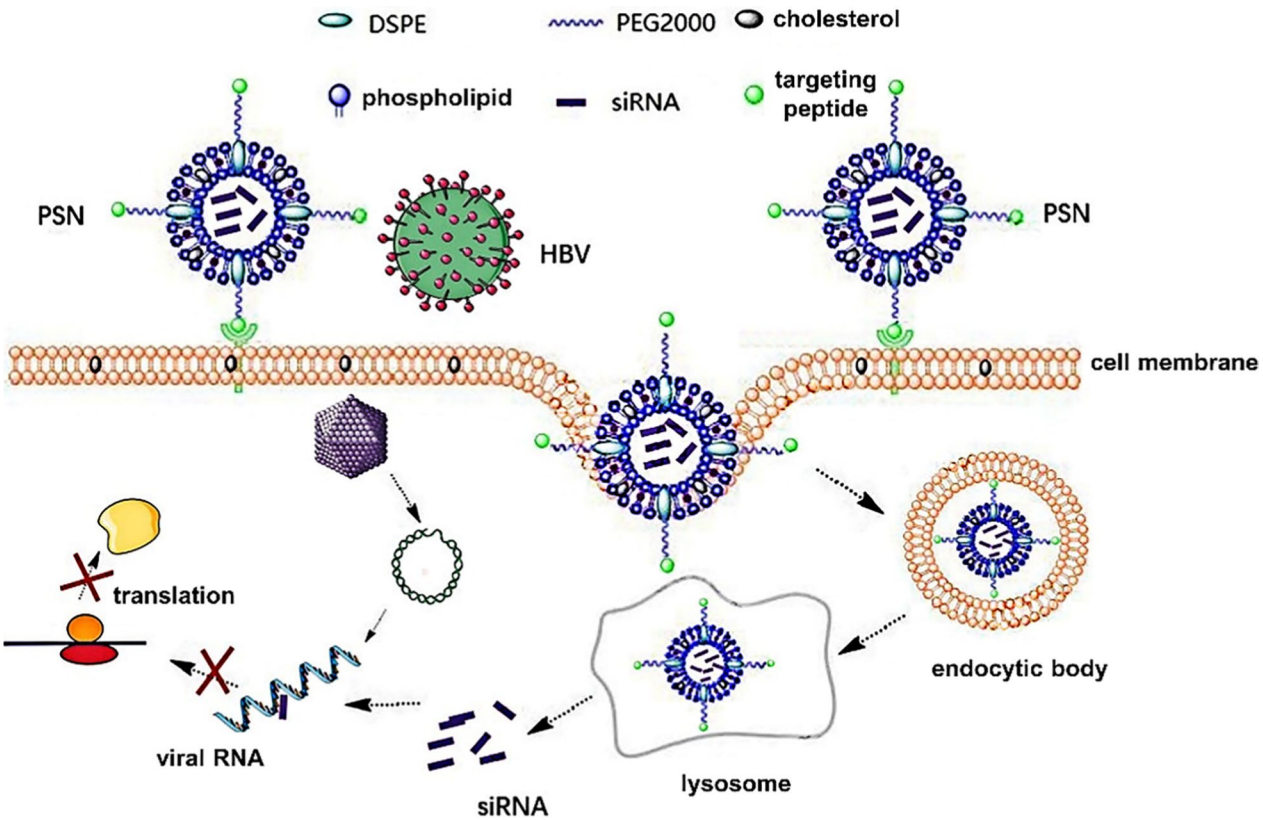


Fig. 8 Mechanism of PreS/2–21 modified nanoparticles loading siRNA (PSN) to prevent HBV. In PSN, TT3 and phospholipids are the backbone to encapsulate siRNA, while PEG and cholesterol were used to increase the stability of the particles. PreS/2–21 (targeting peptide) was conjugated on the surface of lipid nanoparticles to precisely direct siRNA delivery to the NTCP of hepatocytes. Moreover, PreS/2–21 binding to NTCP could also act as an entry inhibitor to block viral invasion. Reprinted from Gao et al. [182] with permission from Frontier

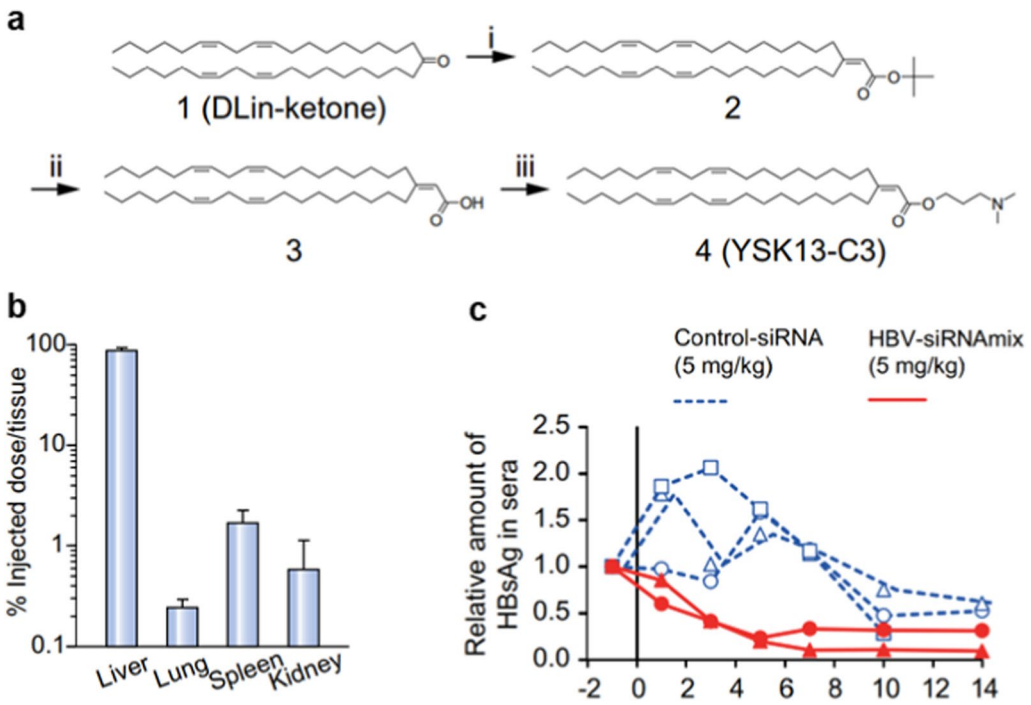


Fig. 9 Development of the novel cationic lipid YSK13-C3 for siRNA delivery. **a** Synthesis of YSK13-C3 using reagents *tert*-butyl diethylphosphoacetate (i), TFA/DCM (ii), and 3-(dimethylamino)-1-propanol (iii). **b** Biodistribution of MEND/siRNA in major organs. **c** In vivo efficacy of MEND/siRNAmix using chimeric mice. Reprinted from Yamamoto et al. [175] with permission from Elsevier

Polymer-based delivery

Polymer-based delivery system represents another approach for gene therapy [186]. Cationic polymers bind to negatively charged siRNA through electrostatic interactions, making them promising candidates for siRNA delivery [187].

Polymeric complexes: Cationic polymers interact with siRNA to form polyplexes, protecting them from degradation and aiding cellular uptake. Increasing polymer charge densities can enhance siRNA entrapment but may also lead to cytotoxicity and rapid clearance by the renal system [188], which is a major drawback of polymeric carriers in nucleic acid delivery.

One of the most widely used polymers for siRNA delivery is polyethyleneimine (PEI) [189]. PEI exhibits the proton sponge effect, facilitating endosomal escape for siRNA after endocytosis. Studies have demonstrated that PEI achieves higher gene transfection efficiency and better nucleic acid loading efficacy compared to other materials, such as chitosan. However, due to their high positive charge, PEI-coated nanoparticles can disrupt membranes and increase cytotoxicity. Consequently, the next generation of cationic polymers has been developed to address these issues. Promising novel polymers include poly[(2-dimethylamino) ethyl methacrylate] (pDMAEMA), poly(β-amino ester) (PBAE), and polyamidoamine (PAMAM) [106]. pDMAEMA and PBAE are noted for their ability to enhance endosomal escape and achieve superior transfection efficacy with reduced cytotoxicity, owing to their unique chemical structures [190].

Polymeric nanoparticles Biodegradable polymeric nanoparticles are widely used for transporting gene therapeutics due to their high safety and low cytotoxicity [191]. Commonly used polymers include poly (D,L-lactide-co-glycolide) (PLGA), chitosan, poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and polycaprolactone acid (PCL) [192]. Initially, PLGA was primarily used for DNA delivery, but its ability to escape from endo-lysosomes has expanded its application to siRNA encapsulation [193]. In some studies, PLGA nanoparticles have been combined with some cationic materials, such as chitosan, to enhance encapsulating efficacy and cellular uptake [194–196].

Conclusion and future perspective

For decades, we have witnessed a significant revolution in the development of siRNA delivery to the liver. Innovative technologies have introduced various delivery platforms that have profoundly improved the effectiveness and safety of siRNAs. The approval of some siRNA products has paved the way for novel siRNA-based therapies. In CHB treatment, using optimal carriers has effectively

enabled siRNA delivery to hepatocytes, reduced off-target effects, and enhanced stability post-administration. Currently, the GalNAc conjugation approach is considered the most promising method, with significant potential for future development [197]. This strategy offers highly potent delivery capacity, reduced off-target effects, and a simple procedure [198, 199].

However, current designs still lack the capability to completely eliminate the HBV genome, leaving a significant gap between treatment and HBV eradication in clinical settings. An emerging trend in siRNA research may come from the discovery of new targets aiming toward epigenetic regulation of cccDNA. The recent outcomes on the delivery of siRNAs targeting important protein regulators of cccDNA (i.e., HBx, BRD4 or DOCK11) have been fundamental for achieving a such purpose [83, 84, 200, 201]. We can hope for the future of complete cure of HBV using siRNA therapy. However, achieving this goal will take time. In the new era of artificial intelligence, future research should exploit this advancement to further optimize formulations and shorten the screening process for materials and siRNA candidates. By doing so, all pharmacokinetic challenges can be addressed to maximize the efficacy of siRNA therapies [202]. Long-term activity, unique mechanisms of action, and specific effects make siRNA suitable for combination therapies. Current advancements have enabled the use of multiple siRNA against different targets and the combination of siRNAs with other therapeutic approaches (entry inhibitors, capsid assembly modulators, or nucleic acid polymers) for simultaneous effects. The clinical success of these combinations needs to be investigated, but the future looks promising [203, 204]. As novel targets and optimal delivery designs continue to improve, we anticipate a complete cure for CHB using siRNAs in the near future.

Abbreviations

ASGP-R	Asialoglycoprotein receptor
cccDNA	Covalently closed circular DNA
CHB	Chronic hepatitis B
DPC	Dynamic PolyConjugate
ESC	Enhanced stabilization chemistry
GalNAc	*N*-Acetyl galactosamine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
IFN	Interferon
LNP	Lipid nanoparticles
MEND	Multifunctional envelope-type nano device
mRNAs	Messenger RNAs
NTCP	Sodium taurocholate co-transporting polypeptide
NUC	Nucleotide analog
PAMAM	Polyamidoamine
PBAE	Poly (β-amino ester)
PCL	Polycaprolactone acid
pDMAEMA	Poly (2-dimethylamino) ethyl methacrylate
PEG	Polyethylene glycol
PGA	Poly (glycolic acid)
pgRNA	Pregenomic RNA
PLA	Poly (lactic acid)
PLGA	$_{D,L}$-lactide-co-glycolide
rcDNA	Relaxed circular DNA
RISC	RNA-induced silencing complex
siRNA	Small interfering RNA
SNALPs	Stable nucleic acid lipid particles

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Author contributions
Linh Nguyen: Writing—original draft, Writing—review and editing, Conceptualization. Tiep Tien Nguyen: Writing—review and editing. Ju-Yeon Kim: Writing—review and editing. Jee-Heon Jeong: Supervision, Writing—review and editing, Conceptualization.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This review article does not require any ethical approval or allied consents for publication.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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