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Hepatic RNA Interference: Delivery by Synthetic Vectors

Matthew Haynes and **Leaf Huang**[*]

The Center for Nanotechnology in Drug Delivery, Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill NC 27599, USA

Abstract

Though the pharmaceutical industry's infatuation with the therapeutic potential of RNA interference (RNAi) technology has finally come down from its initial lofty levels,[1] hope is by no means lost for the once-burgeoning enterprise, as recent clinical trials are beginning to show efficacy in areas ranging from amyloidosis to hypercholesterolemia to muscular dystrophy. With such resurgence comes a more informed perspective on the needs of such therapeutics: a renewed focus on true RNA drug development, and a desire for enhanced site-specific delivery.[2] In this review, we will discuss the latter with regard to hepatic targeting by synthetic vectors, covering the implications of organ and cellular physiology on conjugate structure, particle morphology, and active targeting. In presenting efficacy in a variety of disease models, we emphasize as well the extraordinary degree to which synthetic formulation improves upon and coordinates efforts with oligonucleotide development. Such advances in the understanding of and the technology behind RNAi have the potential to finally stabilize the long-term prospects RNA therapeutic development.

Keywords

nanoparticle; siRNA; hepatic; liver; *in vivo*; therapy

RNAi: A Brief Introduction

Much of the initial excitement over RNAi technology came from its unlimited intracellular applicability – even the most challenging therapeutic targets, met so often with failure by traditional pharmaceutical approaches, now present with a straightforward mechanism for potent and specific expression silencing.[3] In general, one can abrogate protein synthesis in a variety of manner, ranging from transcriptional repression to mRNA degradation to translational arrest, and as such, the specific mechanisms of action involved present different challenges in delivery. Traditional antisense oligonucleotide delivery involves nuclear translocation of therapeutic single-stranded DNA and subsequent binding to pre-mRNA, after which RNAse recruitment initiates enzymatic degradation and thus abrogates gene expression at the transcriptional level. RNAi, however, progresses through either short interfering RNA (siRNA) or micro-RNA (miRNA) to mediate post-transcriptional gene silencing, which presents a simplified approach in requiring only cytosolic delivery and function. Delivery of siRNA will be primarily emphasized herein due to greater specificity in gene knockdown, thus providing a stronger basis for formulation comparison. Further, such silencing allows for more straightforward control over dose-response as compared with that supported by gene expression vectors.[4, 5]

[*]Correspondence: Leaf Huang; Phone: (919) 843-0736; leafh@unc.edu.

Conflict of Interest

Matthew Haynes and Leaf Huang declare that they have no conflict of interest.

Within the cytosol (Figure 1), immature dsRNA for RNAi are processed by the enzyme Dicer (along with cofactors), followed by loading onto the RNA-induced silencing complex (RISC). Either cleavage of the siRNA sense strand or an unwinding of the miRNA sense strand produces an antisense strand-RISC complex which serves to locate and bind to complementary sequences in cytosolic mRNA. In cases of perfect base-pair homology, Argonaute 2 will actively degrade mRNA upon binding in the coding region; though most often exploited with siRNA, miRNA of perfect complement can support such degradation as well. Such complexes can catalyze the cleavage of multiple mRNA as well, supporting long-term (3–4 weeks) expression silencing *in vivo* when dealing with non-proliferative cells such as hepatocytes.[6–8] Minor degrees of mismatch are permitted at the 3′ end of miRNA, however, wherein binding instead in the 3′ untranslated region of mRNA initiates translational arrest through transcript degradation in cellular processing bodies (P-bodies) by decapping enzymes.[8] Mechanistically, siRNA aims to target a specific gene product with dramatic expression knockdown, whereas miRNA is believed to produce a more moderate effect across an entire gene network; such a discrepancy could provide significant flexibility in drug development.[3, 9]

Endogenously, multiple intranuclear and cytosolic pre-processing steps occur in the synthesis of mature si/miRNA; synthetic therapeutics, however, typically represent either the substrate of or product from Dicer and avoid such processing, though considerations of potency and immunogenicity with either selection have been met with debate.[10, 11] Large cellular interferon responses typically occur when delivering larger (>30 bp) dsRNA, but smaller synthetic products can still stimulate immune response,[11, 12] often in a sequence-specific manner.[3] Further, one must consider potential off-target effects due to intracellular processing in RNAi drug development; for example, the sense strand (particularly at positions 2–8), generally assumed to be non-functional, may be able to provide miRNA-like translational repression. This type of off-target silencing has been shown possible under scenarios of homology of as little as six to eight complementary nucleotides.[3, 13, 5] Thus, a thorough observation of any system's biological output would be recommended in ascertaining one's true therapeutic effects.

Unlike with most small molecules and certain proteins, RNAi therapeutics are too large and too negatively charged to cross cellular membranes,[14] necessitating novel delivery mechanisms which include direct ligand conjugation and nanoparticle encapsulation (however, recent evidence of hepatic cell-to-cell transmission of siRNA, in a cell-contact-independent manner partially mediated by exosome exchange, has been reported [15]). These synthetic systems offer significant potential over alternative methods; for instance, significant concerns for toxicity with regard to hydrodynamic injection[16] and immunogenicity in viral vector development limit their potential viability in scenarios of repeated administration in a clinical setting. Herein, we will discuss the implications of systemic, hepatic organ, and cellular physiology on conjugate structure, particle morphology, and active targeting, while presenting efficacy in a variety of disease models.

Systemic Delivery: Overcoming Rapid Clearance

As bioavailability remains limited for RNA therapeutics delivered via the oral route,[17, 18] intravenous and subcutaneous injection present as the most viable routes of administration. However, rapid clearance of naked dsRNA remains one of the most fundamental barriers toward clinical development, in part necessitating exceedingly large doses in order to attain desired efficacy.[14, 19, 20] Upon injection, various physiological complications in the circulation against effective hepatic delivery arise for both free oligonucleotides and nanoformulations, including vector aggregation with serum proteins, uptake by the mononuclear phagocyte system (MPS), off-target distribution or clearance, and nuclease-

mediated degradation. These considerations also provide fundamental bases by which simple synthetic transfection systems, such as coacervates with polyethyleneimine (PEI), can show strong efficacy *in vitro* and in specific scenarios of local delivery *in vivo*, [21, 22] but largely fail under the more demanding circumstances of systemic delivery.

1. Nuclease Degradation and Immune Recognition

To combat the extensive degradative potential posed by various endo- and exonucleases toward free oligonucleotides in circulation, minor modifications in RNA structure (*e.g.* 2′ ribose fluorination[23] and methylation[24]) as well as complete synthetic reproductions (hexitol nucleic acids[25] and peptide nucleic acids[26]) have been established, though nanoparticle encapsulation remains the most effective strategy by which to impart stability in the circulation. Further, certain of the toll-like receptors (TLRs) have been shown to recognize single-stranded RNA (TLR7/8) and double-stranded RNA (TLR3) and activate inflammatory responses against RNA therapeutics.[27] 2′ ribose methylation of the nucleotide backbone has proven successful in this regard as well, improving RNA affinity[28] while decreasing off-target effects[5] through structural changes to molecular conformation.

Nanoformulations, however, still present challenges in achieving circulation longevity, as various monocytes and macrophages contribute significantly to particle clearance as well as the clearance of free oligonucleotides. Further, the pervasive use of cationic materials to promote nucleic acid encapsulation, cellular interaction, and endosomal lysis (*e.g.* via buffering capacity[29] and/or ion-pair formation;[30] see “Endosomal Escape”) can elicit undesirable aggregation with and adsorption of serum proteins, as well as toxicities and immune responses at increased doses.[5] Typically, surface modification with antifouling materials such as polyethylene glycol (PEG) are utilized to provide a stealth-like steric recognition barrier between the nanoparticles and the MPS cells, as well as to reduce serum adsorption of various types of proteins, termed opsonins, which contribute to MPS recognition and uptake. We will describe the theoretical mechanism and impact of PEGylation on nanoparticles in a later section.

2. Renal Filtration and Systemic Distribution

Physiological limitations on size present significant obstacles for effective delivery for RNAi as well, considering both naked dsRNA and formulated nanovectors. The upper size limits for renal filtration and normal vascular permeation are approximately 5–10 nm and 5 nm, respectively;[31] such conditions contribute to the potential for rapid clearance and distribution for free oligomers, with half-lives generally on the order of minutes in circulation. Thus, RNA therapeutics are often further chemically modified with plasma protein binding in mind, serving to minimize the degree of such passive filtration effects.[17] Synthetic coupling of molecular species (*e.g.* lipids,[32] PEG[33]) to oligonucleotides can promote similar effects, though 5′ antisense strand modifications are not well-tolerated in maintaining pharmacological activity and as such should be avoided.[34] With regard to hepatic targeting, the coupling of cholesterol as well as various bile acids and long-chain fatty acids presents as an interesting therapeutic modification.[35, 20] Cholesterol-coupled siRNA showed enhanced binding to human serum albumin (K_d = 1 μM), contributing to an enhanced elimination half-life (95 min) compared to unconjugated siRNA (6 min) and effective liver apolipoprotein B (ApoB) gene silencing capabilities in C57BL/6 mice, though requiring extremely high dosing levels (three daily injections at 50 mg/kg).[20] Similarly high doses were required for ApoB silencing with siRNA conjugates to various bile acids and fatty acids, if silencing occurred at all, and poor endosomal release has been implicated as the likely barrier against stronger efficacy.

At the other end of the spectrum, free passage of nanoparticle less than ~200 nm in diameter through the splenic filtration system has been discussed previously,[3, 36] thus establishing a general range of nanoparticle sizes which can be employed to minimize off-target distribution. However, such consideration typically limits solid, spherical formulations in a manner which more flexible vectors may be able to avoid. For instance, certain hydrogel microparticles[37] of relatively low modulus exhibit dramatically increased circulation longevity; such developments may provide potential support for novel particle distribution patterns which are less stringently dependent on size.

An exciting platform in delivery vector development, in which the exploration of the above considerations has only just begun, is that of the nucleic acid nanoassembly (NAn). Through the intrinsic nanoscale architecture possessed by both natural and synthetic polymeric nucleotides, the high specificity provided by Watson-Crick base pairing, and an understanding of the underlying structural biology, a variety of exotic, size-specific, and monodisperse three-dimensional NAn have been developed, including nanotubes, icosahedra, “buckyballs,” prisms, and nanocubes. Though the specifics are outside of the scope of this review, the authors point to the following review for a more thorough perspective on the structural development of such assemblies and the potential for site-specific functionalization of such NAn with small molecules, proteins, and inorganic materials.[38] A variety of such systems have shown potential as site-specific nanocontainers for therapeutic species as well, from cytochrome C[39] to gold nanoparticles[40] and siRNA.[41] For instance, a reconfigurable DNA tetrahedron was recently developed which can change shape “precisely and reversibly in response to specific molecular signals,” providing great perspective for controlled-release applications.[42] Proper pharmacokinetic and biodistribution studies have only begun to be conducted for such systems,[43] but many have shown improved stability under conditions wherein traditional oligonucleotides fail (*e.g.* nuclease-mediated degradation).[38]

Hepatic Macrophysiology: Influence on Vector Design

For endeavors in which targeted biodistribution is desired, proper perspective on organ physiology can be immeasurable in value. The hepatic artery, by which intravenously-injected nanoparticle technologies will be delivered, accounts for only 20% of the hematological flux in the liver, with the other 80% of blood flow coming from the portal circulation.[44] Such components pass through hepatic arterioles and meet at the sinusoidal endothelium, the primary barrier to hepatic delivery of vectors (Figure 2). Varying in size from approximately 100–175 nm, hepatic sinusoidal fenestrations act both as a size-limiting filter for the parenchyma of the liver and as a highly endocytic “scavenger system” for the uptake of various macromolecular materials.[45] Through such pores (“sieve plates”) in the sinusoidal epithelium, nanoparticles enter what is known as the space of Disse, the primary nexus of lymphatic collection in the liver, and can from there gain exposure to the target hepatocytes.[44] Thus, for full parenchymal exposure, ideal particle formulations for hepatic delivery should readily penetrate such endothelial fenestrations. *In vivo* distribution data corroborates such findings, with by far the highest degrees of hepatic distribution for particles less than 70 nm in size;[36] however, particles hundreds of nanometers in size have been shown to penetrate as well through a sort of physiological extrusion process.[46] Thus, both particle size and particle deformability can play significant roles in effective distribution to the liver.

From another angle, nanoparticle surface modification by PEG has been widely accepted as not only an effective means by which to prevent nanoparticle aggregation, but a successful mechanism by which to achieve long-circulating properties in the nanoformulation. However, such a broad generalization can only be applied to a relative range of particle sizes

(approximately 70–200 nm); smaller liposomes show no long-circulating capabilities at all, as they penetrate quite effectively through the fenestrations of the liver and accumulate to a predominant degree of the injected dose.[36, 45] Nevertheless, high degrees of PEGylation still can present utility in such formulations via particle distribution at the cellular level. Hepatic macrophages, known as Kupffer cells, make their home both within the hepatic sinusoids and the space of Disse.[44] These cells of the reticuloendothelial system (RES; more modernly classified as the mononuclear phagocyte system, or MPS) act as the primary phagocytes of the liver, actively clearing a variety of vascular and interstitial components (including nanoparticles). Further, the liver is populated with a variety of lymphocytes: $CD8^+$ and $CD4^+$ T cells, which present an activated phenotype, as well as natural killer cells (also identified as "pit cells" in rodents).[44] In a variety of states of disease and inflammation, one can observe clear shifts in the lymphocyte populations; for example, $CD8^+$ T cell populations can increase in number and/or more heavily invade the hepatic sinusoids, as observed in mouse models of *Propionibacterium acne* infection,[47] hepatitis C,[48] lymphocytic choriomeningitis,[49] and fatty liver disease.[50] Methods by which to avoid non-hepatocyte uptake and clearance of nanoparticle delivery systems are similar to those previously described for systemic delivery, and will be elaborated upon in subsequent sections along with targeting for particle uptake and mechanisms for endosomal release (Figure 3).

Alternative hepatic targets to hepatocytes may be considered as well. For example, the hepatic stellate (HS) cell is a perisinusoidal cell responsible in part for vitamin A storage in the liver.[51] Under conditions of oxidative and/or cytokine-mediated stress, HS cells can be transformed into myofibroblasts which produce the procollagen that leads to advancing fibrosis in the liver.[52] Further, a variety of disease states, including Leishmaniasis, present as parasitic infections of the reticuloendothelial system,[53] and Kupffer cells have also been implicated in the pathophysiological development of steatohepatitis, progressive fibrosis, and cirrhosis through enhanced TNF-α-mediated signaling.[54] In this regard, one may in fact wish to minimize (or even exclude) PEGylation in (from) their formulation, as MPS uptake and thus overall silencing effect may be reduced by such a material.

PEGylation: Theory and Considerations

To reduce the adsorption of opsonins, it has been postulated that the rapid exploration of the tethered PEG chain of the free volume at the nanoparticle surface constitutes a sort of "conformational cloud" which prevents opsonization in that local region.[55] Mathematical and computational models of PEGylated surfaces have been developed which describe such findings as a "mushroom" vs. "brush" conformational description.[56] At sufficiently large distances from each other, the conformational probability distribution of a single PEG chain's spatial location resembles a hemisphere ("mushroom"). As one chain comes into the proximity of another, conformational overlap begins between the two. Theoretically, when the distance between any two closest chains in a grafted sample is less than the Flory radius of the polymer chains, such extensive conformational overlap between the chains drives conformational exploration vertically ("brush"). In this manner, an increased surface density of PEG, at sufficient chain lengths, can help to minimize protein adsorption and increase circulation half-life by sterically preventing proteins from accessing the particle surface. However, traditional liposomes present stability issues at higher fractions of PEG-lipid, experiencing breakdown into micelles at fractions (above ~5 mol%) far below what is necessary to achieve a brush-like conformation.[3]

Our lab has performed extensive work in the area of PEGylated lipid nanoparticles, and the interested reader is directed to the following review article for a more detailed reference on PEG conformation on curved surfaces, shedding from vesicles, and pharmacokinetic impact

on lipid nanoparticles.[3] Briefly, while conventional liposomes disintegrate at higher PEGylation densities, should the lipid membrane be supported internally by a solid matrix, higher densities can be achieved. This phenomenon has been observed in both of our parent systems, liposome-polycation-DNA (LPD)[57] and liposome-calcium-phosphate (LCP)[58] (as well as in the many derivatives produced by our lab in recent years), with maximum PEGylation densities of approximately 10 mol% and 20 mol% of outer leaflet lipids, respectively.[59, 60] Such densities have shown the capability of fully evading Kupffer cell uptake; however, recent data on LCP PEGylation utilizing small-angle neutron scattering measurements has shown it to be unlikely that a full brush conformation is achieved, with the surface grafts resembling more of an interpenetrating polymer network.[60] Thus, extensive functional selection away from the MPS may be achieved without establishing a true “brush” density on the nanoparticle surface.

Further, PEGylation no longer remains the only viable option for achieving such properties, or even the best option in many cases, and issues with regard to its potential toxicity, stability, and immunogenicity are only beginning to be appreciated (the authors point toward the following excellent review, which presents a thoroughly objective perspective on PEG and its potential alternatives, for more detail[61]). For example, the accelerated blood clearance (ABC) phenomena has been shown in PEGylated liposomal formulations, drastically reducing subsequent dose circulation longevity, and has recently been linked to IgM responses specifically to PEG conjugates of hydrophobic chains.[62] To properly combat this issue, poly(hydroxyethyl-L-asparigine) (PHEA)-coated liposomes have been shown to drastically reduce the ABC effect at various total lipid concentrations.[63] Further, a number of alternative polymers, such as poly(amino acids), polyglycerol, and polyoxazolines have shown strong potential, through inherent biodegradability / bioabsorbability, increased elimination half life, and/or decreased degradative toxicity, to replace PEG as a preferred polymeric coating for immune evasion.[61]

Targeted Delivery

Even after successful distribution to the liver and passage through the sinusoidal fenestrae, there still remains a variety of cell types of which one must be selectively targeted. Often, the target cells are the hepatocytes, and in this manner, exogenous carbohydrate-based ligands (*e.g.* galactose)[64–66] have often been conjugated for active targeting via asialoglycoprotein receptor (ASGPR)-mediated endocytosis. A galactose derivative, N-acetylgalactosamine (NAG), has been utilized as well,[67] as have apolipoprotein E[68] and lactose[69]. The appeal of NAG lies in that it presents a 50-fold higher binding affinity for ASGPR as compared with galactose.[70] Further, multivalent galactose of up to the tetra-antennary level, as well as sugar residue spacing of approximately 15 angstroms, has been shown to optimize receptor-ligand interaction.[70, 71]

Rather than specific receptor-ligand targeting, another hepatocyte-targeting pathway involves ligand relationship with lipid metabolism. Linoleic acid (LA), administered as a free compound, has been known to accumulate in hepatocytes,[72] and has been utilized as a targeting mechanism for internalization into hepatocytes as well.[73, 74] Such a highly hydrophobic moiety presents potential for use in developing an amphiphilic carrier development, and a considerable amount of work has been conducted in this regard in developing chitosan-linoleic acid nanoparticles.[74, 75] Dual grafting of both LA and poly (β-malic acid) to chitosan has been described in order to properly balance hydrophilicity and hydrophobicity in such nanoparticles.[75]

Such ligand-metabolism relationships can be taken a step further from the perspective of endogenous targeting. Apolipoprotein E (ApoE), a major player in hepatic lipoprotein

uptake and clearance, has the potential to be acquired by non-ApoE-containing lipid nanoparticles in circulation, providing what is described as endogenous targeting to liver receptors such as the low-density lipoprotein receptor.[76] Such methods present significant potential for non-ionic and ionizable lipids (see below) which present limited interactive potential with anionic cell membranes at physiological pH as compared with cationic lipids. Further, endogenous targeting to hepatic stellate (HS) cells can be carried out in a similar manner through conjugation of vitamin A to an RNAi delivery system.[51]

Beyond simply driving cellular internalization, active targeting has been exploited to provide further advantage by shifting the balance of uptake away from the MPS. Though the Kupffer cells of the liver possess galactose-particle receptors (GPRs) which can recognize both galactose, NAG, and their derivative ligands, preferential uptake appears to be size-dependent.[70] Utilizing tri-antennary, galactose-bearing unilamellar liposomes, *in vivo* results show the upper nanoparticle size limit for hepatocyte uptake to be about 70 nm for ASGPR-mediated endocytosis.[70] GPR-mediated internalization via Kupffer cells, however, has been presented as relatively slow at such sizes (~80 nm); more rapid uptake was shown with liposomes sized to be 500 nm.[77] For a more narrow size comparison, galactose-targeted nanoparticles at sizes of around 50 nm are preferentially taken up by hepatocytes, whereas similarly-targeted 140-nm particles show greater uptake by Kupffer cells.[78]

Endosomal Escape

Upon internalization, rationally-designed nanoparticle delivery systems must avoid eventual endosomal trafficking to the lysosome. A variety of mechanisms have been proposed and exploited for effective endosomal escape, the most well-known being the “proton-sponge effect,” or effective buffering of endosomal pH. After endocytosis, endosomal acidification through active proton transport begins, complete with counterion for maintenance of charge neutrality. It is postulated that strongly buffering materials at mildly acidic pH (around 5–7) [3] can drive a large ion-counterion excess into the endosome, creating an osmotic gradient by which the endosome ruptures and releases its contents. Such a theory has been experimentally tested[29] by monitoring endosomal volume, pH, and [Cl^-] after administration of polyplexes of polyethyleneimine (PEI) and polyamidoamine (PAM), both of which possess strong buffering capacity, versus a polylysine (PL) control. Both PEI and PAM showed increased [Cl^-] and a decreased rate of acidification as compared with PL, culminating in an endosomal volume increase greater than 125% as well as observable endosomal rupture for both PEI and PAM polyplexes, versus merely 20% without rupture for polyplexes of PL.[29] However, the role of the proton-sponge effect in endosomal release from polyplexes has recently been questioned; for example, in the use of various chain lengths of PEI, proton pump inhibition was shown to “only partially [attenuate] the transfection efficiency.”[79]

Materials such as cationic lipids, however, employ a process known as ion-pair complex formation to mediate endosomal escape. Within the endosome, electrostatic interactions between the cationic lipids of the delivery system and the anionic endosomal membrane serve to disrupt the endosome through exclusion of surface-bound water,[3, 80] further supporting the concomitant breakdown of the delivery vector and release of encapsulated components. Such a mechanism has been observed experimentally as well with a variety of cationic and anionic species, which conform into what is known as the nonbilayer inverted hexagonal (H_{II}) phase.[30] Similar exclusion of surface water and endosomal disruption has been shown with free positively charged molecules.[3] Cationic polymers may also lyse the endosome membrane by a similar mechanism.

Vector Delivery *in Vivo*: Physiology, Pathology, and Efficacy

A variety of conditions can directly affect or establish their origins in the liver, including dyslipidemia, malaria, and cirrhosis, and can mediate further complications such as the development of hepatocellular carcinoma. This section summarizes some of the most prominent hepatic disease states, as well as the vectors utilized for *in vivo* gene silencing either in relation to such diseases or in animal models of such diseases. An organized complement of study parameters and results can be found in Table 1.

1. Cardiovascular Stress: Hypercholesterolemia and Thrombogenesis

Dyslipidemia can be briefly described as an imbalance in normal lipid levels in the circulation. Elevated plasma concentrations of cholesterol and/or triglycerides are more specifically delineated as hypercholesterolemia or hyperlipidemia, and such conditions have been implicated as risk factors in the development of progressive atherosclerosis[81] as well as coronary heart disease.[82] In the increasingly obese populations of the developed world, prevalence remains high, and common therapeutic strategies include the assortment of "-statin" drugs which intend to limit hepatic cholesterol production through inhibition of HMG-CoA reductase. RNA interference, however, provides the opportunity to directly diminish serum low-density lipoprotein (LDL) levels in a variety of manner; for instance, by silencing the gene for Apolipoprotein B (ApoB), the primary lipoprotein of LDL. Decreased levels of proprotein convertase subtilisin/kexin type 9 (PCSK9), a regulator of LDL receptor (LDLR) expression, as been shown to increase liver LDLR levels and reduce serum LDL levels as well.[83, 84] Further, loss of X-box binding protein 1 (XBP-1), involved in hepatic fatty acid synthesis, can lead to decreased serum lipid levels without a concomitant induction of hepatic steatosis.[85, 86] Conversely, from the perspective of hypolipidemia, peroxisome proliferator-activated receptor alpha (PpaR-α) proves an interesting target in its function to increase hepatic triglyceride content and reduce triglyceride availability for VLDL assembly.[87] Platelet activity increases under conditions of hypercholesterolemia as well,[82] and high levels of LDL cholesterol have been implicated in platelet activation *in vitro*,[88] providing a basis for ischemia via thrombotic proclivity; thus, depletion of the thrombotic mediator Factor VII could be exploited with the intent of decreased ischemic incidence. A variety of delivery platforms have been utilized to explore the silencing of each of these targets:

- Interfering nanoparticles (iNOPs), or lysine-based dendrimer-like nanoparticles terminally-functionalized with lipid chains, were used to deliver siApoB which was chemically modified for both stability and ApoB targeting. Chemical modifications included 2′-fluorination and phosphorothiolation. Maximum silencing in mice was observed dosing at 1 mg/kg, with 50% ApoB mRNA silencing, greater than 70% serum ApoB reduction, and a 34% reduction in total cholesterol levels. Histological toxicities were not observed.[89]
- Stable nucleic-acid-lipid particles (SNALPs), composed simply of cationic, nonionic, and PEGylated lipids, have been utilized to strong efficacy with an siApoB cross-reactive to mouse, human, and cynomolgus monkey ApoB genes. Liver ApoB mRNA knockdown of roughly 80% was observed after a single 2.5 mg/kg dose in mice, with a concomitant reduction in serum ApoB by 72%. Such protein reduction was maintained for approximately nine days. Similar effects were observed in monkeys, with a 2.5 mg/kg dose eliciting 90% mRNA knockdown, maintaining knockdown for 11 days, and reduced serum cholesterol by over 60%. [90]
- A combinatorial library of synthetic lipids was produced through the Michael addition of alkyl acrylates/acrylamides and amides for formulation as siRNA

delivery vectors, and screened for gene silencing in a high-throughput fashion *in vitro*. The optimized lipidoid variant $98N_{12}$-5 was complexed with siApoB and siFVII for efficacy studies in rats and monkeys. Significant increases in prothrombin time in rats due to siFVII delivery were observed at the 5 mg/kg dosing level, at which roughly 90% of the hepatic Factor VII mRNA had been silenced. Splenic enlargement was observed at doses of 10 mg/kg/week in rats; however, no other organ toxicities were observed. Administration in cynomolgus monkeys (highest dose: 6.25 mg/kg) reduced ApoB mRNA expression up to 85% and protein expression up to 74%, reducing LDL cholesterol by more than 50% at two days post-administration and maintaining such knockdown for approximately two weeks. No significant hematological changes were observed in either species. [91] Further work with $98N_{12}$-5 was conducted in silencing PCSK9 as well, producing reductions in PCSK9 mRNA levels at 5 mg/kg siPCSK9 of over 50% in mice and rats which supported an over 60% decrease in serum cholesterol levels. [84]

- A poly (butyl/amino) vinyl ether (PBAVE) polymer was reversibly linked, both via disulfide to siRNA and via maleamate to PEG and NAG, to create a siRNA Dynamic PolyConjugate for hepatocyte-targeted delivery. The most promising results in mice were produced from dosing siApoB at 2.5 mg/kg, eliciting ApoB mRNA silencing by 87% with protein levels reflecting such reductions and serum cholesterol reduced by approximately 42%. Such reductions remained significant for 2 and 8 days more for cholesterol and mRNA levels, respectively. Further, similar delivery of siPpaR-α elicited a significant increase in serum triglyceride levels. Serious histological and hematological abnormalities were not observed.[67]

  However, unconjugated co-injection of the targeted and endosomolytic PBAVE with cholesterol-modified siRNA has provided only slightly less effective results in reduction of both serum FVII activity and ApoB protein levels by in mice and nonhuman primates.[92] Further, use instead of a targeted melittin-like peptide (NAG-MLP) in a similar manner reduced FVII activity by at least 97% at a 0.1 mg/kg chol-siFVII dose in mice and a 2 mg/kg chol-siFVII dose in monkeys.[93] Such materials do not complex prior to injection, and separate injections did not effectively limit gene silencing or hepatic co-localization; thus, both materials likely distribute to and are taken up by hepatocytes effectively, and then can coordinate at the intracellular (co-endosomal) level to promote strong cytoplasmic siRNA bioavailability.

- Recently, a separate combinatorial lipidoid library based on epoxide-amine chemistry was developed and screened *in vitro* for luciferase silencing activity. siFVII delivery was conducted utilizing a variety of top-performing lipidoids, of which C12-200 was selected as the optimal formulation candidate. Such a lipidoid-cholesterol-PEG formulation presented with remarkable efficacy, eliciting Factor VII mRNA knockdown of 80% at 0.03 mg/kg dosing in mice, and transthyretin mRNA knockdown of 75% at 0.03 mg/kg dosing in cynomolgus monkeys. Such low-dose silencing was presented as a desirable multi-arm treatment basis, where concomitant knockdown of five separate gene products (including FVII, ApoB, PCSK9, and XBP-1) in a pooled formulation (less than 1 mg/kg total siRNA) was observed as well.[86]

- As cationic lipids present significant concerns in circulation for aggregation, adsorption, nonspecific uptake, and dose-dependent toxicity and immunogenicity, the design of lipid molecules whose pKa lies below 7 (known as “ionizable lipids”) allows one to present little to no cationic surface charge at circulation pH (~7.4) but

still mediate the charge interactions in the acidic environment of the endosome which elicits endosomal release via the reverse hexagonal phase. From such a mindset, a small subset of lipids were designed and screened based on linker and head group modifications of DLinDMA, a highly effective lipid utilized in SNALP formulations. The best performing of such lipids, DLin-KC2-DMA, formulated in a SNALP for siFVII delivery, was able to support knockdown of Factor VII protein expression greater than 80% with as little as 0.03 mg/kg in mice.[94]

2. Parasitic Infection: HBV and Malaria

Infectious disease remains an area of therapy in which RNAi can make a profound impact, with a common perpetrator from the viral subcategory being hepatitis B. With approximately 350 million carriers in the world and 1 million carriers in the United States, hepatitis B presents as a serious mediator of chronic liver disease. Sexual transmission remains the predominant mode of infection in many developed countries, although hepatitis B also represents “the most commonly transmitted blood-borne virus in the health care setting” through scenarios such as needle stick, dialysis, or transplantation (even of extrahepatic organs). Complications of hepatitis B virus (HBV) infection include, but are not limited to, hepatitis, cirrhosis, acute liver failure, and hepatocellular carcinoma.[53] Though vaccination will remain the focal point in HBV therapy, disease management through antiviral therapy remains a necessary endeavor, especially in immunocompromised and transplant patients, where interferon treatment may not be well-tolerated,[95] and under conditions of drug resistance to nucleoside analogues such as entecavir and emtricitabine.[96] RNAi provides such opportunities in that one can directly target and inhibit multiple and more conserved regions of the viral transcriptome, limiting the potential for the development of resistance.[97] In such a perspective, SNALPs encapsulating siRNA against conserved sites in the HBV RNA sequence were tested *in vivo* in an HBV-induced mouse model. Via three daily intravenous injections of 3 mg/kg, approximately order-of-magnitude reductions in serum HBV DNA and HBsAg (an HBV surface antigen) were shown three and seven days, respectively, after the first administration.[98] Co-injection of NAG-MLP with one of various synthetic chol-siHBV (0.25 mg/kg siHBV dose) improves upon such results as well, eliciting over 90% HBsAg and HBV DNA knockdown in each case.[93]

Protozoal infections impact the liver in diverse and toxic manner as well, with the most well-known example being malaria. The global pathological impact of malaria outpaces all others as “the world’s most prevalent fatal parasitic disease.” Two million lives per year are lost to malaria, and though treatment strategies (*e.g.* intravenous quinine) exist, the capacity for malaria to develop drug resistance brings about similar therapeutic problems in comparison to viral infection.[53] To satisfy such needs, the silencing of heme oxygenase 1 (Hmox1), which has been accomplished utilizing lipidoid technology, “may represent a potential approach for the treatment of malaria infection and disease progression,”[14] as the degree of Hmox1 expression has been correlated with increased parasitic burden in the liver.[99]

3. Chronic Hepatic Insult: Steatosis and Cirrhosis

A variety of liver diseases involve lipid accumulation within the liver, including alcoholic steatohepatitis, diabetes, Wilson disease, Weber-Christian disease, and lipodystrophy.[53] Such conditions often present as either microvesicular or macrovesicular steatosis (lipid retention), largely due to imbalanced lipid synthesis, transport, and/or export from the liver, and can over time elicit hepatic injury through lipoperoxidative stress.[100] CD36 has been implicated in both the uptake and metabolism of circulating fatty acids,[101–103] and its expression has been shown to increase in a mouse model of hypercholesterolemia. Our recent work (unpublished) utilizes Liposome Calcium Phosphate (LCP), an amorphous

nanoprecipitate coated with an asymmetric and galactose-PEGylated lipid membrane, to deliver siCD36 in a mouse model of non-alcoholic fatty liver disease. Such a formulation showed significant ability to down-regulate hepatic CD36 protein expression (64±6%; dosed four times at 0.5 mg/kg) and drastically reduced lipid accumulation in the liver. Little to no Kupffer cell uptake was observed, nor was any noticeable organ toxicity present.

Further, cirrhosis is a common complication involved in the progression of a variety of chronic hepatic diseases. Cirrhosis physiologically represents an advanced and extensive degree of fibrosis and scarring for which there still remains a dire need for therapies, and can be driven extensively by alternative hepatocellular targets. The HS-mediated procollagen synthesis in such a progression is regulated in part by heat shock protein 47, which ensures proper triple-helix formation in the endoplasmic reticulum and presents as a potential target for the delivery of siRNA therapeutics.[51] Lipotrust, or liposomes targeted to HS cells through conjugation of vitamin A, was utilized to deliver an siHSP47 analog in rats (siRNAgp46) for the purposes of combating fibrosis. Treatment (0.75 mg/kg/treatment) almost completely abolished fibrosis in rats induced in a variety of manner towards lethal cirrhosis (dimethylnitrosamine – five total treatments, three treatments per week; CCl_4 – six total treatments, two treatments per week; bile duct ligation – five total treatments, every other day) in a dose- and duration-dependent manner.[51] Kupffer cell-mediated TNF-α signaling has been therapeutically targeted as well, through the development of submicron (800–900 nm) particles composed of the acid-sensitive polyketal PK3, the endosome-disrupter chloroquine, and DOTAP-siTNFα complexes. Such donut-shaped particles (PKCNs) were taken up selectively by Kupffer cells, and elicited 96% reduction in serum TNF-α and 59% reduction in alanine aminotransferase (ALT) levels at a dramatically low dose of 3.5 μg/kg.[104]

Clinical Trials

As the field remains in its infancy compared with a variety of other therapeutic modalities (*e.g.* small-molecule organics, proteins and peptides, synthetic polymers), limited development in the clinic has been observed thus far for RNA-based therapeutics; however, promising results can be gleaned from current efforts. A prior Phase I study from Tekmira in targeting ApoB through a SNALP formulation presented two patients with 16.3% and 21.1% reductions in circulating LDLc;[105] nevertheless, the observation of immune stimulation during dose escalation proved cause for trial termination.[3] However, their lead siRNA targeting Polo-like Kinase 1 for treatment of metastases to the liver has been shown to be generally well tolerated, presenting dose-proportional kinetics with confirmation in biopsy samples of successful RNAi. (http://finance.yahoo.com/news/tekmiras-tkm-plk1-promising-solid-113000178.html) Positive results from Alnylam's Phase I trial considering their intravenous formulation for hepatic transthyretin silencing (ALN-TTR02, for the treatment of amyloidosis) have been released recently; up to 94% knockdown of serum protein levels were observed in a dose-dependent and well-tolerated manner, with silencing maintained at nearly 80% for one month. Phase II trials have begun in Europe, with Phase I trials for subcutaneous administration (ALN-TTRsc) beginning as well. (http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/TTR-Amyloidosis.php) Further, Alnylam recently completed safety and pharmacokinetics studies in PCSK9 silencing (ALN-PCS), in which well-tolerated and consistent serum protein reductions of up to 84%, along with LDLc reductions of up to 50%, were observed therein. (http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/Hypercholestoralemia.php). Such results expand their successes on top of their dose-escalation trial in 2011 administering ALN-VSP02, which simultaneously targets vascular endothelial growth factor and kinesin spindle protein for the purposes of primary and metastatic cancer therapy.[105] Though it remains to be seen whether such attractive preliminary results will translate into successful Phase III

campaigns, the necessary proof-of-principle for RNAi in humans has been thoroughly confirmed.

Conclusions

Since the seminal research of Fire and Mello,[106] RNAi has made serious progress from bench to bedside. A greater awareness in drug development is being placed on not only efficacious, but also safe and specific, RNA therapeutics, and a variety of delivery systems are now available for potent and specific targeting to various cell lines of the liver. Society has only just begun to observe the clinical potential for such therapeutic oligonucleotides and delivery platforms. Though the field has experienced its share of growing pains, renewed motivation and recent successes shine a new light on the prospects of RNAi as a revolutionary therapeutic development strategy.

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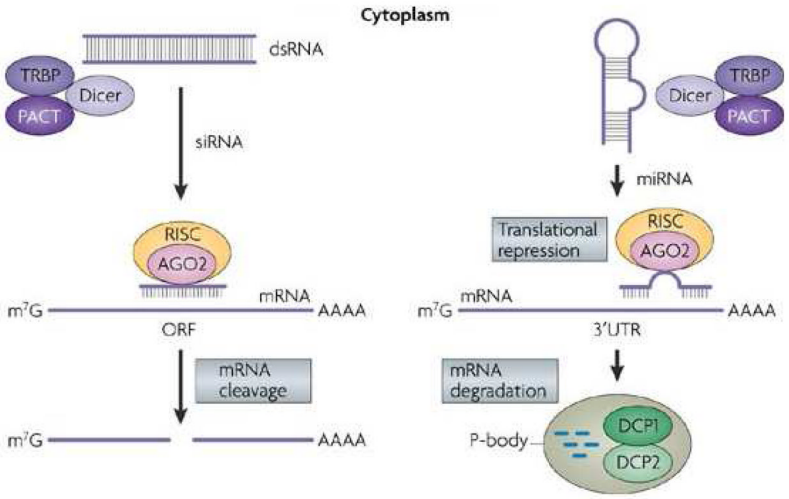

Fig. 1.

Cytosolic mechanisms of action involving siRNA and miRNA. The enzyme Dicer processes these interfering RNA for loading onto RISC, after which removal of the sense strand allows for the silencing of gene expression through mRNA-antisense binding. The mechanism of mRNA degradation depends strongly on the degree of sequence homology. *Adapted and reproduced with permission from [8]*

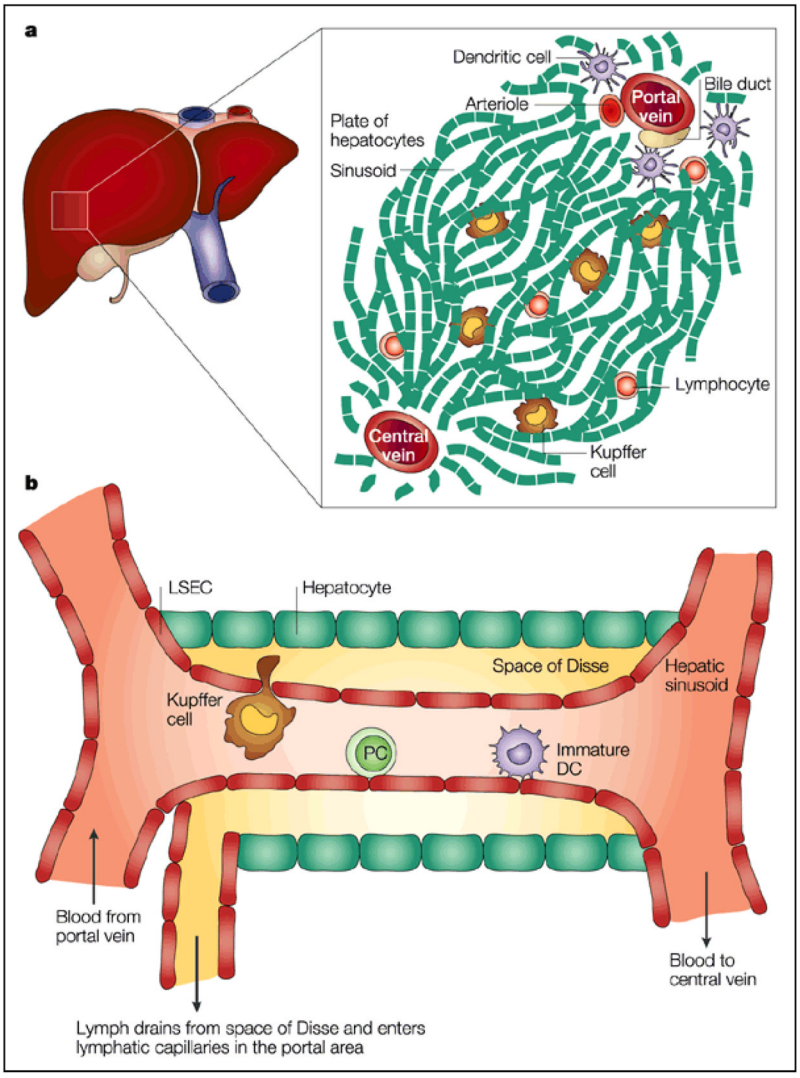

Fig. 2.

Hepatic organ and cellular physiology. (**a)** Diagram showing the structure of a liver lobule. Arterial, venous, and portal blood mix in the hepatic sinusoids, passing between plates of hepatocytes through fenestrations in the sinusoidal endothelium. The sinusoids contain a large population of macrophages, known as Kupffer cells. (**b)** Organization of sinusoids. The sinusoid is lined by an endothelium (liver sinusoidal endothelial cells, LSECs) that is fenestrated and lacks a basement membrane. Kupffer cells, lymphocytes (Pit cells, PCs) and immature dendritic cells (DCs) are found in the sinusoids. Kupffer cells exist mainly in the sinusoidal lumen, but they can make direct contact with hepatocytes. The sub-endothelial space, known as the space of Disse, is the region from which hepatic lymph originates. Effective nanoparticle delivery to hepatocytes requires both penetration through the sinusoidal endothelium and evasion of such mononuclear phagocytes. *Adapted and reproduced with permission from [44]*

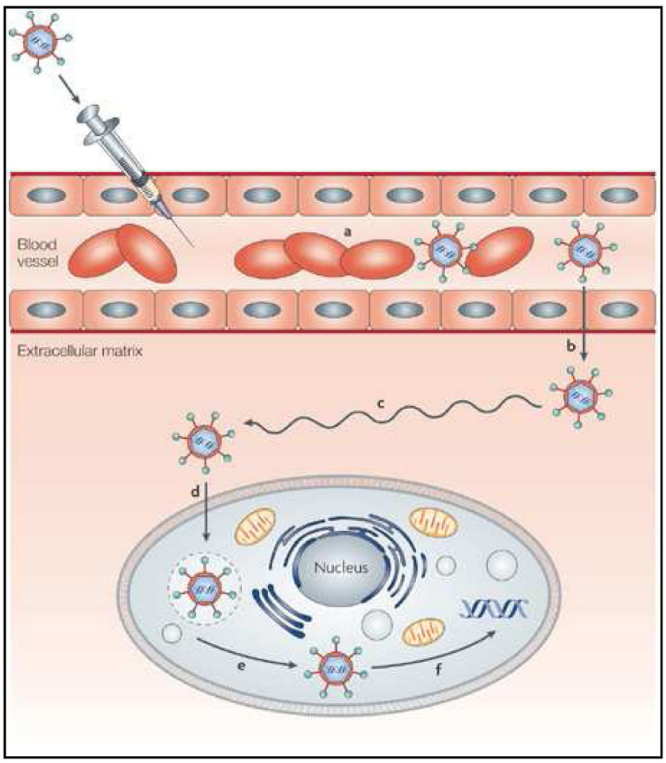

Fig. 3.

Mechanistic simplification of the structural barriers to nanoparticle delivery after intravenous injection. Through the vasculature (in this case, directed from hepatic arterioles, **a**), vectors of a sufficiently small size can pass through the 100–175 nm fenestrations in the sinusoidal epithelium (**b**) and cross the Space of Disse (**c**) to gain exposure to the hepatocytes. MPS-mediated clearance must be avoided throughout this process. Through either passive association or active targeting, effective vectors must elicit endocytosis (**d**) at the hepatocyte membrane. Nanoparticles must then break free from the endosomal compartment (**e**) to avoid sequestration in lysosomes, and effectively release their cargo (**f**) for RISC-mediated gene silencing. *Adapted and reproduced with permission from [*14*]*

Table 1

In vivo efficacy studies involving hepatic delivery of siRNA.

Physiologic Process/State/Disease	Delivery System	Therapy (animal)	$Dose_{total}$ (mg/kg)	Effects	Ref.
Hypercholesterolemia	Interfering Nanoparticles (iNOP)	siApoB (mouse)	1.0	50% ApoB mRNA silencing 34% serum cholesterol reduction	[89]
	Stable Nucleic Acid Lipid Particles (SNALP)	siApoB (monkey)	2.5	90% ApoB mRNA silencing 60% serum cholesterol reduction	[90]
	Dynamic PolyConjugate (PBAVE)	siApoB (mouse)	2.5	87% ApoB mRNA silencing 42% serum cholesterol reduction	[67]
	Acryl-amide Lipidoids	siApoB (monkey) siPCSK9 (mouse) (rat)	2.5 5.0	85% ApoB mRNA silencing >50% serum cholesterol reduction >50% PCSK9 mRNA silencing >60% serum cholesterol reduction	[91]
Thrombogenesis	Acryl-amide Lipidoids	siFVII (rat)	2.5	85% serum FVII reduction	[91]
	Epoxy-amide Lipidoids	siFVII (mouse)	0.03	80% serum FVII reduction	[86]
	Ionizable SNALP	siFVII (mouse)	0.03	>80% serum FVII reduction	[94]
Hepatitis B	SNALP	siHBV (mouse)	9.0	~90% serum HBV DNA and HBsAg reduction	[98]
	Targeted melittin-like peptide (NAG-MLP) + chol-siRNA	siHBV (mouse)	0.25	>90% serum HBV DNA and HBsAg reduction	[93]
Malaria	Lipidoids	siHmox1 (mouse)	-	-	[14]
Fatty Liver	Liposome Calcium Phosphate (LCP)	siCD36 (mouse)	2.0	65% CD36 protein reduction	-
Cirrhosis	Lipotrust	siHSP47 (rat)	~4.0	90% procollagen mRNA silencing 70% collagen protein reduction	[51]
Acute Liver Failure	Polyketal-chloroquine Nanocomposites (PKCN)	siTNFα (mouse)	0.0035	96% serum TNF-α reduction 59% serum ALT reduction	[104]