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High Density Lipoproteins for the Systemic Delivery of short interfering RNA

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Abstract

Introduction—RNA interference (RNAi) is a powerful mechanism for gene silencing with the potential to greatly impact the development of new therapies for many human diseases. Short interfering RNAs (siRNAs) may be the ideal molecules for therapeutic RNAi. However, therapeutic siRNAs face significant challenges that must be overcome prior to widespread clinical use. Many efforts have been made to overcome the hurdles associated with systemic administration of siRNA; however, current approaches are still limited. As such, there is an urgent need to develop new strategies for siRNA delivery that have the potential to impact a broad spectrum of systemic diseases.

Areas covered—This review focuses on the promise of siRNA therapies and highlights current siRNA delivery methods. With an eye toward new strategies, this review first introduces high density lipoproteins (HDL) and their natural functions, and then transitions into how HDLs may provide significant opportunities as next generation siRNA delivery vehicles. Importantly, this review describes how synthetic HDLs leverage the natural ability of HDL to stabilize and deliver siRNAs.

Expert Opinion—HDLs are natural nanoparticles that are critical to understanding the systemic delivery of therapeutic nucleic acids, like siRNA. Methods to synthesize biomimetic HDLs are being explored and data demonstrate that this type of delivery vehicle may be highly beneficial for targeted and efficacious systemic delivery of siRNAs.

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Declaration of Interest:

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Keywords

Biomimetic; high density lipoprotein (HDL); nanoparticle; RNA interference (RNAi); short interfering RNA (siRNA); targeted delivery

Introduction

RNA interference (RNAi) is a potent mechanism for regulating target gene expression. RNAi takes advantage of natural mechanisms of post-transcriptional RNA regulation where RNA sequences can specifically suppress target gene expression. RNAi can be achieved using several molecular forms of RNA, including short interfering RNA (siRNA), antisense RNA (ASRNA), short hairpin RNA (shRNA), microRNAs (miRNA), and ribozymes.¹ Molecules that mediate RNAi have inherent differences in target specificity and potency; however, all provide specific gene targeting that could be leveraged to develop new drug therapies, especially, for targets previously considered undruggable. However, and despite the potential for RNAi to drastically expand the armamentarium of therapies for nearly any disease, the critical bottleneck has been targeted systemic delivery.¹⁻³ The focus of this review is to build a rationale for ways that HDLs, natural nucleic acid delivery vehicles, may be used to overcome many of the challenges to systemic siRNA delivery.

Body

1.1 siRNA Mediated RNAi

siRNAs are short, double-stranded RNAs typically 19-23 nucleotides (nt) in length.² The sense and antisense strands of mature siRNA duplexes are referred to as the passenger and the guide strand, respectively. Mature siRNA duplexes often include a 2 nt overhang on the 3'-end of each strand.⁴ Substituting DNA overhang nt bases increases siRNA stability to nuclease degradation.⁵ The 5'-end of the guide strand is usually modified with a phosphate group. The 5' terminal modification is critical for positioning the guide strand so that it engages the argonaute protein, AGO2, within the RNA induced silencing complex (RISC) for optimal target mRNA binding and cleavage.⁶⁻⁸ siRNAs are perfect complements to target messenger RNA (mRNA) sequences and mediate initial site-specific cleavage followed by complete mRNA sequence degradation.

Therapeutic siRNAs target complementary mRNAs located in the cytoplasm of target cells. As such, delivery of siRNA to the cytoplasm plays a critical role in therapeutic efficacy. In the cytoplasm, siRNA is loaded into the RISC. In addition to several other proteins, the most critical protein in the RISC is AGO2, which facilitates siRNA duplex loading, guide strand positioning, removal of the passenger strand, and catalytic cleavage of complementary mRNA targets.⁹ Once annealed to the guide strand, target mRNA is cleaved at the nt opposite the 10th nt from the 5' end of the guide strand.^{10, 11} This site-specific cleavage is useful for demonstrating the mechanism-of-action of siRNA-based therapies using 3' or 5' rapid amplification of cDNA ends (RACE) assays. Ultimately, the targeted mRNA sequence is degraded, effectively silencing mRNA translation and target protein expression. The

silencing effect can last for several days or weeks depending upon the rate of cell division.¹² (Figure 1)

1.2 siRNA Therapeutics: Summary of Developmental Challenges

The enticing ability to therapeutically target any gene of interest has given rise to tremendous development of siRNAs as drugs. In a few cases, siRNA therapies have made it into the clinic and gained FDA approval, and there are many that are under pre-clinical or Phase I, II, or III evaluation.¹³ For instance, clinical trials have been conducted on diseases amenable to local treatment, such as age related macular degeneration, respiratory infections, and chronic myelogenous leukemia.¹⁴⁻¹⁶ In general, though, systemic delivery of siRNA to specifically targeted cell types has been stunted due to numerous biological hurdles that must be overcome before widespread success can be achieved.^{11, 17} (Figure 2) Foremost, it is critical that specific siRNAs are chosen that potently regulate the expression of relevant target genes that have the highest chance of producing a clinically meaningful therapeutic response. Also, naked siRNAs are short-lived in serum due to rapid clearance by the kidney and their vulnerability to serum exo- and endo- nucleases leading to degradation. Moreover, the polyanionic nature and size of siRNAs make cell membrane penetration difficult.¹⁸ Collectively, these two limitations have obligated chemical modifications of siRNAs and formulation of siRNA with delivery vehicles that both protect and aid in cellular delivery of siRNAs.^{2, 19} Next, there are many ways in which cellular biology and the immune system are geared to clear exogenous RNAs and delivery vehicles. For instance, upon cellular uptake, siRNAs may be sequestered in the endolysosomal cell compartment limiting engagement with cytoplasmic mRNA targets. Additionally, siRNAs may stimulate non-specific immune responses due to endolysosomal toll-like receptor (TLR) binding or non-TLR-mediated cytoplasmic recognition of duplex RNAs.¹⁷ Furthermore, systemically administered delivery vehicles, the vast majority of which are foreign particulates to natural biological systems, can be rapidly sequestered within macrophages of the reticuloendothelial system (RES). Finally, delivery vehicles, especially cationic ones whose purpose is to engage and penetrate negatively charged cell membranes, may be inherently cytotoxic.²⁰

1.3 Non-HDL Nucleic Acid Delivery Strategies: Progress on All Fronts

Significant advancements have been made to address each of the previously mentioned hurdles. Several chemical modifications have been developed to tailor the siRNA phosphate backbone, ribose sugar, and/or terminal ends to increase serum stability, minimize unwanted immune responses, and reduce off-target effects.²⁰ Additionally, delivery vehicles have been designed to conjugate or encapsulate siRNA sequences to protect from nuclease degradation and enhance cellular uptake. Finally, development of delivery vehicles provides an opportunity to incorporate targeting moieties designed to deliver of siRNAs to specific cell types.

Chemical modifications of siRNA—The *in vivo* half-life of unmodified siRNA is as short as several minutes; however, the half-life of siRNA can be improved to several hours through chemical modification.²¹ Importantly, chemical modification of siRNA sequences can enhance biological stability without altering the ability of siRNAs to silence target genes. In some cases, modifications can increase the hybridization strength between guide

and target sequences allowing for a reduction in dose required for gene silencing. Furthermore, chemical modifications can minimize immune response and reduce untoward off-target side effects. Modifications to siRNA sequences can be accomplished by chemically altering the ribose sugar, the phosphate backbone, or the 5' or 3' terminal end of either one or both sequences.⁹ Below this review highlights some of the more common chemical modifications made to siRNA sequences. (Table 1) For a more detailed review, please refer to the following references: ^{9, 12, 18}.

Ribose sugar modifications. Modifications of the ribose sugar are widely used and are typically made to the 2' position of the ribosyl ring. Modifications include replacing the 2' hydroxyl (2'-OH) group with a 2'-O-methyl (2'-OMe), 2'-Fluoro (2'-F), 2' halogen, 2'-amine, or a locked nucleic acid (LNA).^{18, 20} 2'-OMe RNA naturally occurs in mammalian ribosomal and transfer RNA molecules and increases RNA stability (T_m). In addition, 2'-OMe groups provide protection from non-specific immune activation and are more resistant to nuclease degradation.¹² Interestingly, Jackson et al demonstrated that minimal chemical modifications, in fact a single 2'-OMe modification, is sufficient to reduce off-target effects by nearly 70% without reducing the silencing of intended targets.²² Furthermore, their data demonstrated that the 2'-OMe modification is position specific, such that placing the 2'-OMe at the 2nd nt from the 5' end of the guide strand provided maximal target gene knockdown and minimal off-target effects.²² On the other hand, heavy 2'-OMe modification of siRNA sequences, especially the antisense strand, can reduce RNAi activity.^{23, 24} Regarding 2'-F modifications, Layzer et al found a significant increase in siRNA stability when comparing siRNAs with and without 2'-F modifications when exposed to human serum. In addition 2'-F modification can reduce non-specific immunoactivation.¹² However, siRNAs without 2'-F modifications are just as effective at silencing target genes as their modified siRNA counterparts both *in vitro* and *in vivo*. Thus, despite an increase in stability, 2'-F modified sequences do not enhance or prolong the inhibitory activity of target gene expression suggesting that 2'-F modifications alone do not enhance *in vivo* efficacy and argue for the need to develop targeted delivery vehicles.²¹ Finally, combining 2'-OMe purine and 2'-F pyrimidine modified bases substantially increases stability to nuclease degradation and prohibits non-specific immunoactivation.²⁵

Phosphate backbone modifications—Direct modification of the phosphate backbone can enhance siRNA stability. Replacing a non-bridging oxygen with a sulfur (phosphorothioate, PTO), boron (boranophosphate), nitrogen (phosphoramidate), or methyl (methylphosphonate) group increases nuclease resistance.¹² For many reasons PTO modifications have become the main backbone modification used in therapeutic siRNAs. This is due to data demonstrating that PTO modification increases siRNA stability to nuclease degradation and can be safely used *in vivo*.¹² In addition, Overhorr and Sczakiel demonstrate that PTO modified siRNAs have enhanced cellular uptake into human cells.²⁶ However, it is important that PTO modifications can also enhance opsonization by serum proteins, which may reduce siRNA efficacy. Furthermore, increasing the amount of PTO modifications in an siRNA sequence can reduce efficacy and increase unwanted side effects, such as toxicity.²⁷⁻³⁰ Boranophosphate modifications impart a 300-fold increase in nuclease resistance as compared to unmodified siRNA counterparts without hampering siRNA

function.²⁷ Hall et al, upon direct comparison in cultured cells, found that boranophosphate-modified siRNAs were more effective at target gene silencing than native siRNA and siRNAs with PTO modification.²⁷ Despite these potential benefits, widespread use of boranophosphates is limited due to difficulty synthesizing siRNAs with this modification.¹²

Terminal modifications—Numerous nucleotide modifications have been used to stabilize RNA sequences including the incorporation of DNA nucleotides, abasic residues, and inverted bases.¹² More important to this review are molecules conjugated to the ends of siRNAs used to enhance the efficacy of systemically delivered siRNAs. Most often molecules are conjugated to the 5' or 3' end of the sense strand leaving the antisense strand free to engage RISC and target sequences.⁹ Some examples include: lipophilic molecules, such as cholesterol³¹⁻³⁴; cell penetrating peptides, like the HIV-derived transactivating transcriptional activator (TAT) peptide³⁵; folic acid conjugates^{36, 37}; and aptamer ligands.^{38, 39} Notably, cholesteryl-modified siRNAs have proven efficacious in silencing apolipoprotein B in mouse liver and jejunum to effectively lower total cholesterol levels.³⁴ Cholesteryl modification increases siRNA binding to serum components, such as albumin and high and low density lipoproteins, which subsequently enhance stabilization, prolong circulating half-life, and provide for specific tissue targeting. Further and in attempts to enhance targeting, others have designed terminal modifications that take advantage of tumor-specific receptor expression. For example, the folate receptor is over-expressed in ovarian, colorectal, and breast cancers and minimally expressed in normal tissues. Thus, folic acid conjugation to siRNAs has been explored as a potential delivery route for *in vivo* siRNA therapy.³⁷ Similarly, prostate specific membrane antigen (PSMA), a cell surface receptor expressed by prostate cancer cells and tumor associated endothelial cells, has been explored as a means of targeted delivery. Aptamers conjugated to siRNA sequences have been designed to target PSMA and have had some success in targeting siRNAs to cancer cells that overexpressing PSMA.³⁹

Overall, sequence modifications provide opportunities to improve nucleic acid stability and circumvent immune stimulation. However, these modifications are not sufficient to overcome all of the hurdles required for successful targeted systemic siRNA delivery. Described below are methods used to deliver nucleic acids to target cells to increase the potency of siRNA therapy. In many cases, chemical modifications are used in conjunction with a delivery vehicle in attempts to further optimize efficacy.

Lipid-based Delivery Vehicles—In addition to modifying the nucleic acid with lipids, lipids have long been used as delivery vehicles to encapsulate nucleic acids into self-assembling micro- and nano-particulates, commonly referred to as liposomes. Liposomes are lipid vesicles composed of one or more lipids. Historically, cationic lipids have been used due to the favorable electrostatic interaction between the lipid head groups and the negatively charged phosphate backbone of siRNAs.¹⁹ However, cationic lipids are cytotoxic, which limits their utility. As such, other negatively charged, neutral, and fusogenic lipids have been explored as synthetic constituents of liposomes and lipid-based delivery vehicles. Most common is the combinatorial use of different lipids to avoid cytotoxicity, maximize siRNA binding, and maintain cellular delivery. Liposomes self-

assemble with nucleic acids into spherical, phospholipid bilayer bound vesicles and multilamellar vesicles. Through electrostatic interactions, liposomes encapsulate and adsorb negatively charged siRNAs. Electrostatic interactions between the phosphate backbone and cationic lipid head groups mask the negative charges of the phosphate backbone aiding in the delivery of nucleic acids through cellular membranes and allowing for penetration through tissues *in vivo*. To facilitate tissue specific targeting, mainly liver targeting, a number of moieties have been incorporated into the lipid layers of liposomes, such as galactose, phage peptides, and vitamins.^{40, 41}

The newest generation of lipid-based carriers are diverse, and include ones that utilize polymers or lipids to bind, stabilize, and deliver siRNAs. Stable nucleic-acid-lipid particles (SNALP) are one example. In SNALPs, siRNA is typically mixed with cholesterol, cationic lipids, and fusogenic lipids whereupon self-assembly results in particles that are approximately 120 nm in diameter.⁴² Upon systemic administration, SNALPs opsonize apolipoprotein E (Apo E) in serum, which enhances targeted siRNA delivery to hepatocytes.⁴³ The acidic environment of the endosome promotes destabilization of the particle and siRNA is released from the SNALP. Using this approach, Zimmermann et al reported success in reducing Apo B production from hepatocytes in non-human primates resulting in a reduction in serum cholesterol and low density lipoprotein levels.⁴⁴ Furthermore, SNALP-siRNA therapies proved successful in a study by Geisbert et al, in which siRNA delivered by SNALPs protected non-human primates from lethal injections of Ebola virus.⁴⁵ Multiple pre-clinical studies have demonstrated that lipid nanoparticles can be effective siRNA delivery vehicles for targets in the liver.¹³ Similar to SNALPs, solid lipid nanoparticles (SLNP) are composed of a hydrophobic core of biodegradable lipids that are solid at body temperature. To incorporate siRNA, cationic lipids are complexed with siRNA and then mixed with the hydrophobic lipid core materials, which binds the siRNA/cationic lipid complex and incorporates it into the solid lipid center. The surface of the resultant particle is decorated with polyethylene glycol conjugated lipids and lecithin, which increases the circulating half-life of the delivery vehicle.⁴⁶ Lobovkina et al designed a SLNP that resulted in slow sustained release of siRNA, which may lead to a reduction in frequency and dosage of SLNP-siRNA needed to produce an effective therapeutic response.⁴⁶

Nanoparticle Delivery Vehicles—Nanotechnology has greatly contributed to the development of nucleic acid delivery vehicles. In general, nanotechnology provides an opportunity to use organic and inorganic building blocks to develop new methods of siRNA delivery that are able to avoid immune recognition, penetrate cells and tissues, and maximize siRNA loading. Additionally, nanomaterials hold great promise for improving gene therapy efforts due to the potential of low toxicity, biodegradability, and biocompatibility.²⁰ Below is a brief summary of some nanoscale materials that have been used for siRNA delivery.

Polymeric Nanoparticle Delivery Vehicles—Polymeric nanoparticles are gene delivery vehicles synthesized using natural and synthetic polymers. Commonly used natural polymers are cyclodextrin, chitosan, and atelocollagen.^{47, 48} Synthetic polymers include, polyethyleneimine (PEI), poly (lactic-co-glycolic acid) (PLGA), and dendrimers.⁴⁹

Cyclodextrin nanoparticles with siRNA targeting the M2 subunit of ribonucleotide reductase (R2) have demonstrated success in a phase I human clinical trial in patients with melanoma refractory to standard-of-care therapies.⁵⁰ Chitosan, naturally occurring polysaccharide, has been studied for delivery of siRNA *in vitro* and *in vivo*. However, siRNA delivery using chitosan-based particulates is limited due to inherent cytotoxicity.⁵¹ Likewise, PEI-siRNA conjugates are relatively effective; however, they are limited as siRNA delivery vehicles due to significant cytotoxicity.⁵¹ PLGA, biodegradable glycolic and lactic acid polymer, has been approved by the FDA for human use. PLGA-siRNA conjugates are stable, internalized by target cells through endocytosis, penetrate human tissue, and have low toxicity. However, significant challenges exist with regard to the low electrostatic interaction between PLGA and siRNA, inefficient endosomal escape of siRNA, and non-specific cell delivery.⁵² Dendrimers are highly branched monodisperse, symmetrically spherical macromolecules.⁵³ Polycationic dendrimers complexed with siRNA have similar toxicity to cationic delivery vehicles. On the other hand, poly(propylenimine) (PPI) dendrimers have shown effective gene silencing, and less inherent cytotoxicity.⁵⁴

Inorganic Nanoparticles—Several inorganic nanoparticles are being developed for therapeutic nucleic acid delivery, including carbon nanotubes (CNTs); metal oxides, such as, iron oxide; semiconductor quantum dots (QDs); and metal nanoparticles, for instance, gold nanoparticles (AuNPs).⁵⁵ CNTs can be single-walled (SWCNT) or multi-walled (MWCNT). Both have used to deliver siRNA *in vitro* and *in vivo* delivery.⁵⁶⁻⁵⁸ Toxicity of CNTs is a concern; however, like other nanoparticles surface functionalization is critical to the observed toxicity profile.⁵⁹⁻⁶¹ Iron oxide particles functionalized with siRNA can be utilized both as magnetic resonance imaging agents and delivery vehicles.^{62, 63} In general their toxicity profile and capacity for siRNA delivery is dependent on the size and charge of the particle.⁶⁴ Semiconductor QDs, materials with unique and optical properties, have been used as siRNA delivery vehicles. However first generation QDs posed great challenges due to toxicity concerns regarding their compositions (e.g. cadmium, selenium, and tellurium).⁶⁵ More recently, Subramaniam et al developed a library of nontoxic ZnS-AgInS₂ QDs. These materials are excellent imaging agents and may also find utility as siRNA delivery vehicles.⁶⁶ Finally, gold nanoparticles (AuNPs) have been explored as delivery vehicles for siRNA due to their biocompatibility, stability, synthetic ease, and the ability to functionalize the AuNP surface.⁶⁷ Because the gold core is essentially inert and non-toxic⁶⁸, spherical AuNPs decorated with nucleic acids, including siRNAs, have been used to regulate target gene expression *in vitro*^{69, 70} and *in vivo*.⁷¹ In addition, other groups have demonstrated that AuNPs (i.e. with cationic ligands), either spheres or rods, can be used to deliver siRNA.^{72, 73}

Despite substantial advancements in nucleic acid chemistry and the development of delivery vehicles, much work remains for successful systemic siRNA administration. Many recent strategies, some that combine nucleic acid modification with a vehicle, result in the delivery of nucleic acids to the liver. Certainly, there is a significant need to develop vehicles that can deliver siRNA to specific cell types other than liver cells following systemic administration. High density lipoproteins (HDLs) are natural nanostructures that bind, stabilize, and deliver nucleic acids. Accordingly, combining nanotechnology with HDL biology may provide significant opportunities for siRNA delivery. In order to fully appreciate why there is

interest in HDLs, we first highlight the functional properties of HDLs, followed by natural mechanisms of nucleic acid delivery, and finally highlight emerging strategies for the synthesis of biomimetic HDLs for siRNA delivery.

2. High Density Lipoproteins: A Multifunctional Natural Nanostructure

2.1 Structure and Composition of Natural HDL

High density lipoproteins are dynamic natural nanostructures ranging from 7-13 nanometers in diameter. HDLs are composed of multiple biological molecules and interact with several receptors, enzymes, and proteins during their progressive maturation. (Figure 3) Apolipoprotein A-I (Apo AI), the main protein associated with HDLs, represents approximately 70% of the protein content of HDLs.⁷⁴ Apo AI is an amphipathic scaffold protein, which readily binds lipids and, ultimately, defines the ultimate size and shape of HDL species.⁷⁵ Apolipoprotein A-II, the second most common protein associated with HDL, makes up approximately 20% of the total protein. Although less studied, multiple other apolipoproteins associate with HDLs, such as: A-IV, C-I, C-II, C-III, D, E, J, L, and M.^{76, 77} Additionally, a number of other proteins, lipids (e.g. phosphatidylcholines), free cholesterol, and esterified cholesterol contribute to the heterogeneity of HDL species.⁷⁸

Of the plasma lipoproteins, HDLs are the smallest and densest. HDLs undergo constant remodeling in the blood stream through interaction with other lipoproteins, enzymes, and by engaging target cells. These interactions result in significant particle heterogeneity. HDLs are classified into many sub-populations by density, size, shape, composition, and surface charge. With regard to density, HDLs are classified into two main sub-fractions: HDL₂ ($1.064 < d < 1.125$ g/mL), which are relatively large in size, lipid rich, and more buoyant than HDL₃ species ($1.25 < d < 1.21$ g/mL), which are smaller in size and denser. HDL₂ and HDL₃ can be further categorized into five distinct sub-populations.^{74, 77} Using non-denaturing gradient polyacrylamide gel electrophoresis or isopycnic density gradient ultracentrifugation, HDL₂ and HDL₃ can be separated into the following size fractions: HDL_{2b} (9.7 – 12.9 nm), HDL_{2a} (8.8 – 9.7 nm), HDL_{3a} (8.2 – 8.8 nm), HDL_{3b} (7.8 – 8.2 nm), and HDL_{3c} (7.2 – 7.8 nm).⁷⁹

Two-dimensional electrophoresis separates HDL subpopulations by charge and size. Ultimately, 5-10 HDL species can be distinguished.^{74, 79} The smallest population consists of precursor HDLs (pre- β 1, 2, 3) that contain Apo AI and phospholipids. These species are discoidal in shape and ~5.6 nm in diameter. Next, α -4 HDL particles are somewhat larger at ~ 7.4 nm in diameter, are also discoidal, and consist of Apo AI, phospholipids, and free cholesterol. Small spherical HDLs (α -3 HDLs, ~8.0 nm in diameter) are more mature and contain Apo AI, Apo AII, phospholipids, free cholesterol, cholesteryl esters, and triglycerides. Medium-sized HDLs are larger (α -2 HDL, ~9.2 nm) and are compositionally similar to α -3 HDLs. Large spherical HDLs, α -1 HDLs, contain the same components as α -2 and α -3 HDL, except they almost exclusively contain Apo AI. Last, pre- α species (pre- α 1, 2, 3) are similar in size and have similar constituents as α -2 and α -3 particles, but are less prevalent in serum and do not contain Apo AII.^{74, 79} Finally, a number of other techniques can be used to categorize HDL into sub-populations based on protein content. In short, HDLs are highly dynamic structures that have great variability with regard to size,

shape, and surface chemistry. Each of these parameters is known to greatly modulate *in vivo* HDL function.⁸⁰

HDL biosynthesis is initiated in hepatocytes and enterocytes of the liver and small intestine, respectively, as these cells make and secrete Apo AI.⁸¹ Initially lipid poor, Apo AI begins to sequester phospholipids and some free cholesterol. Acquisition of these molecular components results in nascent HDLs that have a discoidal shape, 8 nm in diameter. Importantly, Apo AI self-assembles with phospholipids and free cholesterol through its interaction with ATP binding cassette receptor A1 (ABCA1). ABCA1 is a cell membrane protein that mediates the transfer of phospholipids and free cholesterol to Apo A1. In this formation, two anti-parallel molecules of Apo AI wrap in belt-like fashion around a solubilized central core of phospholipids oriented as a bilayer.⁸²⁻⁸⁴ Nascent HDLs are the smallest and most cholesterol poor of the formed HDLs and free cholesterol that is associated with nascent HDL is mainly interdigitated in the ~160 phospholipids present in the core of the disc.⁸⁵

By engaging ABCA1, Apo AI and nascent HDLs adsorb cholesterol from peripheral tissues. Free cholesterol transferred to HDL becomes esterified by interacting with the serum enzyme, lecithin cholesterol acyltransferase (LCAT). LCAT catalyzes the esterification of free cholesterol associated with HDL. This increases the hydrophobicity of cholesterol and drives cholesteryl esters (CE) into the lipid bilayer creating a core of hydrophobic CEs surrounded by a monolayer of phospholipids. The esterification of free cholesterol and movement into the core increases the size and transforms nascent HDL from a disc-like structure into a maturing spherical nanostructure. This process also ensures that there is a gradient for free cholesterol to move onto the particle surface. As the particle matures, it engages additional cholesterol efflux receptors including ATP-binding cassette receptor G1 (ABCG1) and scavenger receptor type B-1 (SR-B1). Additionally, other lipoproteins, such as low density lipoprotein (LDL), aid in the maturation process of HDLs. For instance, LDLs transfer triglycerides to HDLs in exchange for CE. This transfer is catalyzed by the serum protein cholesteryl ester transfer protein (CETP). Together these interactions increase the size of HDL and contribute to the heterogeneity of HDLs. It is important to note that HDLs are quite simplified in the context above. It is becoming increasingly appreciated that HDLs are highly diverse in their chemical composition with regard to phospholipids, small molecules, proteins^{76, 86}, R RNAs^{87, 88}, hormones, carotenoids, vitamins, and bioactive lipids.⁸⁹ (Figure 4)

2.2 Function of Natural HDL

Due to the complex synthesis and remodeling of HDL there are significant structural differences between HDL sub-species and individual particles. Of course, structural differences endow HDLs with a myriad of functions. Two key factors that determine the function of HDLs in the human body are (1) size and (2) composition. Below we discuss the functions of natural HDLs in order to comprehensively understand their natural mechanisms-of-action and also to highlight opportunities for targeting specific diseases, such as cardiovascular disease and cancer. Ultimately, formulating synthetic HDLs with siRNAs may provide targeting mechanisms to cell types critical to disease pathogenesis.⁹⁰

2.3 Cholesterol Transport- HDL and Coronary Heart Disease

Several epidemiological studies demonstrate that plasma concentrations of HDL-cholesterol (HDL-C) inversely correlate with the risk of coronary heart disease (CHD). Therefore, cholesterol associated with HDL has been termed “good” cholesterol. This correlation has led to the hypothesis that HDLs directly protect against CHD. Currently, the most accepted mechanism of HDL-mediated cardio-protection is the process of reverse cholesterol transport (RCT). RCT is the process by which cellular cholesterol is removed from lipid-laden macrophages (foam cells) in atherosclerotic lesions by HDLs. HDLs acquire cholesterol from macrophages by interacting with ABCA1, ABCG1, and SR-B1.⁹¹ Cholesterol-rich HDLs, then, traffic to the liver where cholesterol is delivered to hepatocytes through SR-B1 for excretion in the feces. Free cholesterol sequestered by HDLs may undergo esterification by LCAT. Apo A1 is necessary for mediating HDL binding to SR-B1. Cholesterol returned to the liver by HDLs is excreted into the bile and expelled from the body through the feces. In addition, HDLs promotes RCT by transferring CE to LDLs in exchange for triglycerides. LDLs loaded with cholesterol are taken up by hepatocytes through the LDL receptor. RCT is believed to be the primary mechanism by which HDLs contribute to reduce the risk of CHD. However, several other properties of HDLs may contribute to reducing CHD risk.

2.4 Non-Cholesterol Transport- HDL and Coronary Heart Disease

HDLs are thought to reduce CHD risk by multiple other mechanisms. For instance, HDLs reduce inflammation at the site of atherosclerotic lesions. HDLs also have antioxidant and antithrombotic effects that maintain endothelial integrity and promote repair.^{74, 77, 92} Taken together, HDLs have many functions beyond RCT for preventing CHD.

Anti-inflammatory properties of HDLs—Chronic inflammation develops as atherosclerotic plaques mature. Pro-inflammatory stimuli contribute to the release of circulating cytokines, such as interleukin-1 and TNF- α (tumor necrosis factor alpha). The release of cytokines stimulates the expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), P-selectin, and E-selectin. These molecules recruit and tether leucocytes and monocytes to endothelial cells overlying lipid-rich atheromas. Upon recruitment, the captured cells undergo molecular transformation and differentiate into macrophages that contribute to the atherosclerotic lesion. *In vitro* and *in vivo* studies demonstrate that HDLs reduce the expression of endothelial adhesion molecules and prevent the recruitment of monocytes to the arterial wall.⁹³⁻⁹⁵

HDLs combat oxidative damage—Oxidative stress is a risk factor associated with premature atherosclerosis and cardiovascular disease. Oxidized LDL (oxLDL) is the main mediator of oxidative arterial damage and promotes a pro-inflammatory and pro-atherogenic phenotype that contributes to endothelial cell dysfunction and apoptotic cell death. oxLDL contains a number of free radical-induced lipid hydroperoxides (LOOH), LOOH-derived short-chain oxidized phospholipids, and oxidized sterols. HDLs have antioxidant properties that attenuate the damaging effects of oxLDLs. Specifically, HDLs inhibit the accumulation of peroxidation products on the surface of LDLs. This is accomplished by several

mechanisms. First, methionine residues (112 and 148) of Apo AI reduce LOOH into redox-inactive lipids. In addition, Apo AI protein sequesters LOOHs from LDLs.⁹⁶ Second, HDLs, particularly the smaller HDL₃ sub-populations, have a unique proteome that plays a critical role in protection against LDL oxidative stress. Also, the transfer of LOOH species from oxLDL to HDL₃ aids in the reduction of free radical species in atherosclerotic lesions.⁹⁷ Lastly, enzymes associated with HDLs, such as paraoxonase 1 (PON1), platelet activating factor-acetyl hydrolase (PAF-AH), and LCAT contribute to the antioxidant function of HDLs by hydrolyzing pro-inflammatory short-chain oxidized phospholipids.

Antithrombotic and anticoagulant activity of HDLs—Finally, HDLs demonstrate protective effects through antithrombotic and anticoagulant activity. By directly and indirectly interacting with endothelial cells, HDLs reduce thrombosis and actively inhibit platelet aggregation. Endothelial-derived nitric oxide (NO) is critical for vasodilation and maintains the integrity of vascular endothelial and smooth muscle cells.⁹⁸ As atherosclerosis develops, NO production is reduced leading to increased neutrophil adherence to the endothelium, smooth muscle cell proliferation, and enhanced platelet aggregation and adhesion. HDL binding to the endothelial cell receptor SR-B1 enhances NO production by stimulating endothelial nitric oxide synthase (eNOS), leading to NO-dependent relaxation of the aorta.⁹⁹ Endothelial cell apoptosis contributes to arterial atherothrombosis by the release of “microparticles” that carry prothrombotic factors, such as P-selectin.¹⁰⁰ Along with the release of microparticles, apoptotic endothelial cells enhance adhesion between unactivated platelets and leukocytes, promoting coagulation. HDLs directly contribute to endothelial protection by inhibiting oxLDL and tumor necrosis factor alpha (TNF- α) mediated apoptosis and by suppressing growth factor deprivation.¹⁰¹ Furthermore, it has been demonstrated that HDLs enhance prostacyclin synthesis in endothelial cells. Prostacyclin acts synergistically with NO to induce vascular smooth muscle (VSM) relaxation¹⁰¹, inhibits platelet activation, and represses the release of growth factors responsible for local proliferation of VSM cells.⁹⁸ In addition to HDLs acting on endothelial cells to reduce coagulation, an inverse correlation between platelet aggregation and HDL levels in humans has been reported.¹⁰² This phenomenon is not limited to CHD as recombinant HDLs were shown to reduce platelet aggregation in individuals with type 2 diabetes mellitus.¹⁰³

Collectively, HDLs function in many ways to protect against cardiovascular disease. Developing a more complete understanding of HDL structure-function relationships will continue to provide new insights into the mechanisms of atheroprotection. Beyond cardiovascular disease, it has recently been discovered that HDLs bind and transport nucleic acids. Understanding the mechanisms by which HDLs perform this function is providing new opportunities to develop biomimetic, synthetic nanoparticles for nucleic acid delivery. Moreover, HDLs target a diverse number of cell types and tissues, which may have implications that go beyond cardiovascular disease.

3. Natural HDL for Systemic Delivery of siRNA

The concept of HDLs as delivery vehicles for nucleic acids has emerged from three key pieces of evidence. First, data demonstrated that siRNAs terminally modified with lipophilic moieties, like cholesterol, results in siRNA-specific gene silencing in cultured cells and in

the liver following systemic administration.^{34, 104, 105} This prompted investigations to better understand how lipophilic siRNAs were being productively delivered to the liver. Toward this end, Wolfrum et al demonstrated that systemically injected lipid-modified siRNAs bound to lipoproteins in the serum (i.e. HDL and LDL). As such, lipoprotein-bound siRNAs were then delivered to tissues that express receptors for specific lipoproteins. In the case of HDL-bound siRNAs, delivery was mediated by SR-B1.³³ These data suggest that HDLs are delivery vehicles for exogenous siRNAs that overcome many of the hurdles to systemic siRNA delivery. The third important piece of evidence was provided by Vickers et al. Data showed that HDLs naturally bind and stabilize nucleic acids for transport, and that HDLs deliver and scavenge functional microRNAs to and from cells that express SR-B1, respectively.⁸⁷ This was the first demonstration of HDLs as mediators of endogenous nucleic acid transport. Collectively, these pieces of evidence strongly support HDLs as nucleic acid delivery vehicles that may overcome the hurdles associated with systemic siRNA delivery. As such, there has been a significant focus on the development of synthetic HDL vectors for siRNA therapies.

4. Synthetic HDL-Like Nanoparticles for Nucleic Acid Delivery

A number of research groups are focused on the development of synthetic HDL nanostructures and, in some cases, using them for nucleic acid delivery. Routes to synthetic HDLs provide an opportunity to control many of the structural and compositional features, which may impart tailored and unique functions. Below are highlights clearly demonstrating the power and potential of this approach for delivering nucleic acids. (Figure 5)

4.1 Spherical HDL Biomimetic: Gold Nanoparticle Scaffold

Our group developed a synthetic HDL nanostructure that closely mimics the size, shape, and surface chemistry of natural, mature spherical HDLs.¹⁰⁶ A five nm gold particle (AuNP) was used as a scaffold to control the size, shape, and composition of synthetic, spherical HDLs. Data demonstrated that high density lipoprotein nanoparticles (HDL NPs) are similar in size to their natural counterparts and have a similar surface composition: ~3 copies of Apo AI and an outer phospholipid layer of zwitterionic 1-2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC).¹⁰⁶ Much like natural HDLs, HDL NPs were found to tightly bind cholesterol ($K_d = 3.8$ nM).¹⁰⁶ Further, our group also demonstrated that tailoring the size, lipid attachment strategy, and presence of Apo A1 heavily impact the function of HDL NPs in the context of cholesterol binding and efflux from lipid-laden macrophages *in vitro*.¹⁰⁷

Because HDL NPs tightly bind cholesterol and natural HDLs spontaneously associate with lipidated siRNAs after systemic administration, our group hypothesized that the HDL NP platform could be utilized to adsorb cholesterylated antisense DNAs (chol-DNA) for delivery to cells that express SR-B1 to regulate target gene expression.¹⁰⁸ Data demonstrated that chol-DNAs bound to HDL NPs (chol-DNA-HDL NPs) appear to overcome many of the cellular hurdles associated with *in vitro* nucleic acid delivery. For instance, HDL NPs stabilize chol-DNA against nuclease degradation. Further, chol-DNA-HDL NPs do not exhibit cellular toxicity and are efficient conjugates for regulating target gene expression. Following cell treatment, transmission electron micrographs suggested that chol-DNA-HDL NPs bypass endolysosomal sequestration, a major biological hurdle in

nucleic acid delivery. Collectively these data suggest that synthetic, spherical HDLs are an efficient delivery vehicle for systemic nucleic acid delivery.

4.2 HDL-Mimicking Peptide-Phospholipid Scaffold (HPPS) Nanoparticles for siRNA Delivery

Yang et al constructed an HDL biomimic using a peptide-phospholipid scaffold (HPPS).¹⁰⁹ The particles consisted of phospholipids, cholesteryl oleate, and amphipathic α -helical peptides, which mimic Apo AI. These components self-assembled into structures similar to plasma-derived HDL.¹¹⁰ Direct incubation of HPPS particles with cholesteryl conjugated siRNAs (chol-siRNA) targeting the oncogene bcl-2 (chol-si-bcl-2) resulted in final constructs with a hydrodynamic diameter of (25.3 ± 1.2) nm and contained an average of 8 chol-siRNAs per particle. The surface charge of the particle shifted from (-2.7 ± 1.9) mV to (-15.2 ± 4.8) mV when loaded with chol-siRNA, consistent with RNA loading. Data showed that HPPS particles delivered siRNA cargo to the cytosol of SR-B1 expressing cells and regulated target gene expression. Treatment of KB cells expressing a high degree of SR-B1 resulted in $(35 \pm 9\%)$ reduction of bcl-2 protein as compared to the control, and a 2.5 fold increase in apoptosis was measured when compared to the chol-si-bcl-2 siRNA alone. Importantly, HPPS chol-si-bcl-2 particles were not effective in knocking down bcl-2 in HT1080 cells that express minimal SRB1. Furthermore, and consistent with SR-B1 expression, HPPS particles efficiently deliver siRNA cargo to the cytoplasm of target cells bypassing endolysosomal sequestration.¹¹⁰ These results suggest that HPPS chol-si-bcl-2 particles utilize SR-B1 for efficient siRNA delivery and gene knockdown.

4.3 Reconstituted HDL (rHDL) for siRNA Delivery

In 2002, Lacko et al reported that rHDL nanoparticles could be used to deliver anticancer drugs, such as chemotherapeutics, to cancer cells that express SR-B1. rHDLs are synthetic forms of human HDL, essentially composed of phospholipids, Apo AI, cholesterol, and cholesteryl esters.¹¹¹ For drug delivery, Shahzad et al incorporated siRNA into rHDL nanoparticles. The particles were approximately 10 nm in diameter, possessed a net neutral charge (-3.2) mV, and demonstrated efficient delivery of siRNA to target cells. Further rHDL siRNA conjugates were shown to regulate target gene expression after systemic administration in orthotopic ovarian and colorectal cancer models.¹¹² To explore SR-B1 as the mechanism of delivery, Shahzad tested the expression of SR-B1 in multiple cell lines and human tumors and found SR-B1 to be highly expressed in liver tissue and in multiple cancer cell lines. Among 50 human ovarian epithelial cancers, 96% of the tumors highly expressed SR-B1. Moreover, several other cell lines were tested and all were found to express SR-B1 to a high degree as compared to normal tissues. Accordingly, and consistent with these findings, Shahzad et al found that rHDLs loaded with fluorescently labeled siRNA showed preferential uptake in mouse tissues that highly expressed SR-B1 (i.e. tumor and liver), with minimal fluorophore-labeled siRNA in other tissues such as the brain, heart, lung, kidney, and spleen.¹¹² Importantly, this work provides insight into cell specific targeting *in vivo*, especially in tumor bearing mice, suggesting efficient delivery to tumor cells that express SR-B1.

A similar approach to delivering siRNA using a rHDL nanoparticle has been demonstrated by Ding et al. Here, rHDL nanoparticles were synthesized using a mixture of phospholipids, apolipoprotein AI, cholesterol, and cholesteryl esters. In contrast to the previously cited work, Ding et al incorporated cholesteryl modified siRNA sequences into the rHDL nanostructure. This approach yielded a rHDL/chol-siRNA complex that was ~90 nm in diameter with a near-neutral charge of (-4.2) mV. Consistent with the previous findings, the rHDL structure aids in the protection of siRNA from nuclease degradation and is effective in silencing gene expression *in vitro* and *in vivo*. Most importantly systemic administration of rHDL/chol-siRNA particles complexed with siRNA against pokémon demonstrated a reduction in tumor volume of HepG2 xenografts.¹¹³

Conclusion

The discovery of RNAi provided an opportunity to develop potent, specific therapies for any protein target of interest and for nearly any disease process. Significant progress has been made to realize the therapeutic potential of RNAi; however, more work remains with regard to identifying methods to systemically deliver molecules that mediate RNAi, such as siRNAs, for efficient gene silencing in target cells. Toward this end, the first wave of innovation occurred at the RNA sequence level, greatly increasing siRNA duplex stability. Ribose sugar, phosphate backbone, and terminal modifications greatly increased siRNA resistance to endo- and exonucleases upon systemic administration, reduced untoward immune stimulation, and increased the circulating half-life. Further, delivery vehicles have been developed that protect siRNAs from nuclease degradation and provide for cell specific targeting, in some cases. Though much progress has been made, targeted cell delivery outside of the liver has proven difficult and toxicity issues have hindered some of these efforts. Looking forward, nanotechnology has imparted a new wave of delivery vehicles that may play a fundamental role in the systemic application of siRNA in the clinic. The conjugation capability and the particle tailorability make these delivery vehicles good candidates for future directions.

Importantly, and most recently appreciated, is the ability to incorporate biomimicry into siRNA delivery efforts. The discovery that natural HDLs bind and deliver microRNAs to cells that express SR-B1, suggests that synthetic HDLs may be leveraged for siRNA delivery. Thus far, there are a number of synthetic HDL platforms that have emerged as promising ones for mediating target gene silencing *in vitro* and *in vivo*. As such, synthetic HDLs hold great promise as future generation delivery vehicles, because they have demonstrated biodistribution similar to that of natural HDL and are internalized by cells through a mechanism that bypasses endolysosomal sequestration. Therefore, synthetic HDLs may represent the next generation of siRNA delivery vehicles with the capacity to overcome many of the biological hurdles that stand in the way of successful clinical translation and target tissues beyond the liver.

Expert Opinion

Nanoscale delivery vehicles will be critical to the success of nucleic acid therapeutics. Due to their small size, nanoscale particles are able to better overcome some of the biological

hurdles prohibiting successful systemic nucleic acid delivery. For instance, some nanoparticles are able to bypass sequestration by cells of the reticuloendothelial system (RES), avoid immune stimulation, and many formulations have proven non-cytotoxic. Furthermore, developing synthetic methods to impart biomimetic features to nanoparticle delivery vehicles appears to hold significant promise. Biomimicry offers opportunities to develop delivery vectors, like HDLs, that are biocompatible and offer inherent mechanisms to overcome well-known challenges to siRNA delivery.

The ultimate goal is to develop optimal biomimetic delivery vehicles for potent siRNAs that target cell types critical to specific disease processes. For HDLs, significant opportunities exist for targeting cells that make up solid tumors, endothelial cells, and macrophages, among others. As discussed, these cells express the cell surface receptor, SR-B1, which may enable efficient uptake of particles carrying siRNAs. As such, biomimetic HDL delivery vehicles provide opportunities to realize therapeutic success across a broad spectrum of diseases.

Developing strategies for synthetic HDLs whereupon the size, shape, surface chemistry, and drug loading can be controlled is critical. Understanding the function of these structures in their natural environment will be important for synthetic strategies and maximal drug delivery. Understanding how synthetic nanoparticles interact with other lipoproteins and serum components will likely play a critical role in adequate stabilization and delivery of siRNA cargo.

The future of HDL mediated siRNA delivery is bright and continued advances may provide real opportunities for siRNA therapies to be translated to the clinic, where patients will ultimately benefit from this entire class of therapeutic molecules. Looking forward, it is likely that other natural nanoparticles will emerge as siRNA delivery vehicles. Certainly lipoproteins are not the only biological structures that interact with nucleic acids. One critical example that has already been shown is the use of natural exosomes for the delivery of nucleic acid cargoes. Due to their inherent targeting capacity, exosomes will certainly play a large role in the development of next generation therapies for targeted drug delivery, including siRNAs.

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References

- 1**. Guo PX, Coban O, Snead NM, Trebley J, Hoeprich S, Guo SC, et al. Engineering RNA for Targeted siRNA Delivery and Medical Application. *Advanced drug delivery reviews*. Apr 30; 2010 62(6):650–66. [PubMed: 20230868] [A thorough review of siRNA for therapeutic use.]
2. Weinstein S, Peer D. RNAi nanomedicines: challenges and opportunities within the immune system. *Nanotechnology*. Jun 11.2010 21(23):232001. [PubMed: 20463388]

3. Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nature reviews Molecular cell biology*. Jan; 2007 8(1):23–36.
4. Watts JK, Deleavey GF, Damha MJ. Chemically modified siRNA: tools and applications. *Drug Discov Today*. Oct; 2008 13(19-20):842–55. [PubMed: 18614389]
5. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. May 24; 2001 411(6836):494–98. [PubMed: 11373684]
6. Kenski DM, Cooper AJ, Li JJ, Willingham AT, Haringsma HJ, Young TA, et al. Analysis of acyclic nucleoside modifications in siRNAs finds sensitivity at position 1 that is restored by 5'-terminal phosphorylation both in vitro and in vivo. *Nucleic acids research*. Jan; 2010 38(2):660–71. [PubMed: 19917641]
7. Wang Y, Sheng G, Juranek S, Tuschl T, Patel DJ. Structure of the guide-strand-containing argonaute silencing complex. *Nature*. Nov 13; 2008 456(7219):209–13. [PubMed: 18754009]
8. Wang Y, Juranek S, Li H, Sheng G, Tuschl T, Patel DJ. Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature*. Dec 18; 2008 456(7224):921–6. [PubMed: 19092929]
9. Gaglione M, Messere A. Recent progress in chemically modified siRNAs. *Mini reviews in medicinal chemistry*. Jun; 2010 10(7):578–95. [PubMed: 20500149]
10. Manoharan M. RNA interference and chemically modified small interfering RNAs. *Curr Opin Chem Biol*. Dec; 2004 8(6):570–79. [PubMed: 15556399]
11. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nature reviews Drug discovery*. Jan; 2010 9(1):57–67.
12. Behlke MA. Chemical Modification of siRNAs for In Vivo Use. *Oligonucleotides*. Dec; 2008 18(4):305–19. [PubMed: 19025401]
13. Schutz CA, Juillerat-Jeanneret L, Mueller H, Lynch I, Riediker M. Therapeutic nanoparticles in clinics and under clinical evaluation. *Nanomedicine (Lond)*. Mar; 2013 8(3):449–67. [PubMed: 23477336]
14. Nguyen T, Menocal EM, Harborth J, Fruehauf JH. RNAi therapeutics: an update on delivery. *Current opinion in molecular therapeutics*. Apr; 2008 10(2):158–67. [PubMed: 18386228]
15. Bumcrot D, Manoharan M, Kotliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nature chemical biology*. Dec; 2006 2(12):711–9.
16. Vaishnaw AK, Gollob J, Gamba-Vitalo C, Hutabarat R, Sah D, Meyers R, et al. A status report on RNAi therapeutics. *Silence*. 2010; 1(1):14. [PubMed: 20615220]
17. Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. RNA interference in the clinic: challenges and future directions. *Nature Reviews Cancer*. Jan; 2011 11(1):59–67.
18. Higuchi Y, Kawakami S, Hashida M. Strategies for in vivo delivery of siRNAs: recent progress. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy*. Jun; 2010 24(3):195–205.
19. Miele E, Spinelli GP, Miele E, Di Fabrizio E, Ferretti E, Tomao S, et al. Nanoparticle-based delivery of small interfering RNA: challenges for cancer therapy. *International journal of nanomedicine*. 2012; 7:3637–57. [PubMed: 22915840]
20. Gao Y, Liu XL, Li XR. Research progress on siRNA delivery with nonviral carriers. *International journal of nanomedicine*. 2011; 6:1017–25. [PubMed: 21720513]
21. Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. In vivo activity of nuclease-resistant siRNAs. *RNA*. May; 2004 10(5):766–71. [PubMed: 15100431]
22. Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, et al. Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA*. Jul; 2006 12(7):1197–205. [PubMed: 16682562]
23. Chiu YL, Rana TM. siRNA function in RNAi: A chemical modification analysis. *Rna-a Publication of the Rna Society*. Sep; 2003 9(9):1034–48.
24. Czauderna F, Fechtner M, Dames S, Aygun H, Klippel A, Pronk GJ, et al. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic acids research*. Jun 1; 2003 31(11):2705–16. [PubMed: 12771196]

25. Morrissey DV, Lockridge J, Shaw L, Blanchard K, Jensen K, Breen W, et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Hepatology*. Oct; 2005 42(4):576A–76A.
26. Overhoff M, Sczakiel G. Phosphorothioate-stimulated uptake of short interfering RNA by human cells. *Embo Rep*. Dec; 2005 6(12):1176–81. [PubMed: 16170302]
27. Hall AHS, Wan J, Shaughnessy EE, Shaw BR, Alexander KA. RNA interference using boranophosphate siRNAs: structure-activity relationships. *Nucleic acids research*. 2004; 32(20): 5991–6000. [PubMed: 15545637]
28. Harborth J, Elbashir SM, Vandenburgh K, Manninga H, Scaringe SA, Weber K, et al. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic A*. Apr; 2003 13(2):83–105.
29. Amarzguioui M, Holen T, Babaie E, Prydz H. Tolerance for mutations and chemical modifications in a siRNA. *Nucleic acids research*. Jan 15; 2003 31(2):589–95. [PubMed: 12527766]
30. Braasch DA, Jensen S, Liu YH, Kaur K, Arar K, White MA, et al. RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry*. Jul 8; 2003 42(26):7967–75. [PubMed: 12834349]
31. Chen QM, Butler D, Querbes W, Pandey RK, Ge P, Maier MA, et al. Lipophilic siRNAs mediate efficient gene silencing in oligodendrocytes with direct CNS delivery. *J Control Release*. Jun 1; 2010 144(2):227–32. [PubMed: 20170694]
32. DiFiglia M, Sena-Esteves M, Chase K, Sapp E, Pfister E, Sass M, et al. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proc Natl Acad Sci U S A*. Oct 23; 2007 104(43):17204–9. [PubMed: 17940007]
- 33*. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, et al. Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nature biotechnology*. Oct; 2007 25(10): 1149–57. [This paper shows that systemically administered lipophilic siRNAs bind to lipoproteins, including HDLs, where they are stabilized and delivered to cells that naturally express receptors for lipoproteins. In the case of HDLs, siRNAs are targeted to a myriad of cell types, including but not exclusively, to the liver and taken up through SR-B1.]
- 34*. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*. Nov 11; 2004 432(7014):173–78. [PubMed: 15538359] [One of the first papers demonstrating the systemic administration of siRNAs and regulation of target gene expression.]
35. Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, et al. Lung delivery studies using siRNA conjugated to TAT(48-60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjugate chemistry*. Sep-Oct; 2007 18(5): 1450–9. [PubMed: 17711319]
36. Arima H, Yoshimatsu A, Ikeda H, Ohyama A, Motoyama K, Higashi T, et al. Folate-PEG-Appended Dendrimer Conjugate with alpha-Cyclodextrin as a Novel Cancer Cell-Selective siRNA Delivery Carrier. *Mol Pharmaceut*. Sep; 2012 9(9):2591–604.
37. Yu B, Zhao X, Lee LJ, Lee RJ. Targeted delivery systems for oligonucleotide therapeutics. *The AAPS journal*. Mar; 2009 11(1):195–203. [PubMed: 19296227]
38. Zhou JH, Rossi JJ. Therapeutic Potential of Aptamer-siRNA Conjugates for Treatment of HIV-1. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy*. 2012; 26(6):393–400.
39. McNamara JO, Andrechek ER, Wang Y, D Viles K, Rempel RE, Gilboa E, et al. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nature biotechnology*. Aug; 2006 24(8):1005–15.
40. Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, et al. Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proc Natl Acad Sci U S A*. Aug 7; 2007 104(32):12982–7. [PubMed: 17652171]
41. Nishina K, Unno T, Uno Y, Kubodera T, Kanouchi T, Mizusawa H, et al. Efficient in vivo delivery of siRNA to the liver by conjugation of alpha-tocopherol. *Molecular therapy : the journal of the American Society of Gene Therapy*. Apr; 2008 16(4):734–40. [PubMed: 18362929]

42. Rossi JJ. RNAi therapeutics: SNALPing siRNAs in vivo. *Gene therapy*. Apr; 2006 13(7):583–4. [PubMed: 17526070]
43. Akinc A, Querbes W, De S, Qin J, Frank-Kamenetsky M, Jayaprakash KN, et al. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Molecular therapy : the journal of the American Society of Gene Therapy*. Jul; 2010 18(7):1357–64. [PubMed: 20461061]
44. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. *Nature*. May 4; 2006 441(7089):111–4. [PubMed: 16565705]
45. Geisbert TW, Lee AC, Robbins M, Geisbert JB, Honko AN, Sood V, et al. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet*. May 29; 2010 375(9729):1896–905. [PubMed: 20511019]
46. Lobovkina T, Jacobson GB, Gonzalez-Gonzalez E, Hickerson RP, Leake D, Kaspar RL, et al. In vivo sustained release of siRNA from solid lipid nanoparticles. *ACS nano*. Dec 27; 2011 5(12):9977–83. [PubMed: 22077198]
47. Yuan X, Naguib S, Wu Z. Recent advances of siRNA delivery by nanoparticles. *Expert opinion on drug delivery*. Apr; 2011 8(4):521–36. [PubMed: 21413903]
48. Inaba S, Nagahara S, Makita N, Tarumi Y, Ishimoto T, Matsuo S, et al. Atelocollagen-mediated Systemic Delivery Prevents Immunostimulatory Adverse Effects of siRNA in Mammals. *Molecular Therapy*. Feb; 2012 20(2):356–66. [PubMed: 22031237]
49. Wang Y, Li Z, Han Y, Liang LH, Ji A. Nanoparticle-based delivery system for application of siRNA in vivo. *Current drug metabolism*. Feb; 2010 11(2):182–96. [PubMed: 20359287]
- 50*. Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. Apr 15; 2010 464(7291):1067–70. [PubMed: 20305636] [First paper demonstrating systemic administration of a targeted siRNA-containing nanoparticle and target gene regulation in human.]
51. Alameh M, Dejesus D, Jean M, Darras V, Thibault M, Lavertu M, et al. Low molecular weight chitosan nanoparticulate system at low N:P ratio for nontoxic polynucleotide delivery. *International journal of nanomedicine*. 2012; 7:1399–414. [PubMed: 22457597]
52. Singha K, Namgung R, Kim WJ. Polymers in small-interfering RNA delivery. *Nucleic acid therapeutics*. Jun; 2011 21(3):133–47. [PubMed: 21749290]
53. Kesharwani P, Tekade RK, Gajbhiye V, Jain K, Jain NK. Cancer targeting potential of some ligand-anchored poly(propylene imine) dendrimers: a comparison. *Nanomedicine : nanotechnology, biology, and medicine*. Jun; 2011 7(3):295–304.
54. Taratula O, Garbuzenko OB, Kirkpatrick P, Pandya I, Savla R, Pozharov VP, et al. Surface-engineered targeted PPI dendrimer for efficient intracellular and intratumoral siRNA delivery. *J Control Release*. Dec 16; 2009 140(3):284–93. [PubMed: 19567257]
55. Lee JM, Yoon TJ, Cho YS. Recent Developments in Nanoparticle-Based siRNA Delivery for Cancer Therapy. *Biomed Res Int*. 2013
56. Zhang Z, Yang X, Zhang Y, Zeng B, Wang S, Zhu T, et al. Delivery of telomerase reverse transcriptase small interfering RNA in complex with positively charged single-walled carbon nanotubes suppresses tumor growth. *Clin Cancer Res*. Aug 15; 2006 12(16):4933–9. [PubMed: 16914582]
57. Podesta JE, Al-Jamal KT, Herrero MA, Tian B, Ali-Boucetta H, Hegde V, et al. Antitumor activity and prolonged survival by carbon-nanotube-mediated therapeutic siRNA silencing in a human lung xenograft model. *Small*. May; 2009 5(10):1176–85. [PubMed: 19306454]
58. Liu Z, Tabakman S, Welsher K, Dai HJ. Carbon Nanotubes in Biology and Medicine: In vitro and in vivo Detection, Imaging and Drug Delivery. *Nano Res*. Feb; 2009 2(2):85–120. [PubMed: 20174481]
59. Shvedova AA, Kisin ER, Porter D, Schulte P, Kagan VE, Fadeel B, et al. Mechanisms of pulmonary toxicity and medical applications of carbon nanotubes: Two faces of Janus? *Pharmacology & therapeutics*. Feb; 2009 121(2):192–204. [PubMed: 19103221]
60. Firme CP 3rd, Bandaru PR. Toxicity issues in the application of carbon nanotubes to biological systems. *Nanomedicine : nanotechnology, biology, and medicine*. Apr; 2010 6(2):245–56.

61. Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nature nanotechnology*. Jul; 2008 3(7):423–8.
62. Medarova Z, Pham W, Farrar C, Petkova V, Moore A. In vivo imaging of siRNA delivery and silencing in tumors. *Nature medicine*. Mar; 2007 13(3):372–7.
63. Lee JH, Lee K, Moon SH, Lee Y, Park TG, Cheon J. All-in-one target-cell-specific magnetic nanoparticles for simultaneous molecular imaging and siRNA delivery. *Angew Chem Int Ed Engl*. 2009; 48(23):4174–9. [PubMed: 19408274]
64. Yu Y, Sun D. Superparamagnetic iron oxide nanoparticle ‘theranostics’ for multimodality tumor imaging, gene delivery, targeted drug and prodrug delivery. *Expert review of clinical pharmacology*. Jan; 2010 3(1):117–30. [PubMed: 22111537]
65. Su Y, He Y, Lu H, Sai L, Li Q, Li W, et al. The cytotoxicity of cadmium based, aqueous phase - synthesized, quantum dots and its modulation by surface coating. *Biomaterials*. Jan; 2009 30(1): 19–25. [PubMed: 18848354]
66. Subramaniam P, Lee SJ, Shah S, Patel S, Starovoytov V, Lee KB. Generation of a library of nontoxic quantum dots for cellular imaging and siRNA delivery. *Adv Mater*. Aug 2; 2012 24(29): 4014–9. [PubMed: 22744954]
67. Ghosh P, Han G, De M, Kim CK, Rotello VM. Gold nanoparticles in delivery applications. *Advanced drug delivery reviews*. Aug 17; 2008 60(11):1307–15. [PubMed: 18555555]
68. Connor EE, Mwamuka J, Gole A, Murphy CJ, Wyatt MD. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. *Small*. Mar; 2005 1(3):325–27. [PubMed: 17193451]
69. Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AKR, Han MS, Mirkin CA. Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. *Science*. May 19; 2006 312(5776): 1027–30. [PubMed: 16709779]
70. Giljohann DA, Seferos DS, Prigodich AE, Patel PC, Mirkin CA. Gene Regulation with Polyvalent siRNA-Nanoparticle Conjugates. *Journal of the American Chemical Society*. Feb 18.2009 131(6): 2072. [PubMed: 19170493]
71. Zheng D, Giljohann DA, Chen DL, Massich MD, Wang XQ, Iordanov H, et al. Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. *P Natl Acad Sci USA*. Jul 24; 2012 109(30):11975–80.
72. Kim ST, Chompoosor A, Yeh YC, Agasti SS, Solfiell DJ, Rotello VM. Dendronized Gold Nanoparticles for siRNA Delivery. *Small*. Nov 5; 2012 8(21):3253–56. [PubMed: 22887809]
73. Bonoiu AC, Mahajan SD, Ding H, Roy I, Yong KT, Kumar R, et al. Nanotechnology approach for drug addiction therapy: Gene silencing using delivery of gold nanorod-siRNA nanoplex in dopaminergic neurons. *P Natl Acad Sci USA*. Apr 7; 2009 106(14):5546–50.
74. Camont L, Chapman MJ, Kontush A. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. *Trends in molecular medicine*. Oct; 2011 17(10):594–603. [PubMed: 21839683]
75. Xu S, Laccotripe M, Huang X, Rigotti A, Zannis VI, Krieger M. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *Journal of lipid research*. Jul; 1997 38(7):1289–98. [PubMed: 9254056]
76. Warnick GR, McNamara JR, Boggess CN, Clendenen F, Williams PT, Landolt CC. Polyacrylamide gradient gel electrophoresis of lipoprotein subclasses. 26. *Clin Lab Med*. Dec. 2006 4(4):803.
77. Tabet F, Rye KA. High-density lipoproteins, inflammation and oxidative stress. *Clin Sci*. Jan; 2009 116(1-2):87–98. [PubMed: 19076062]
78. Damiano MG, Mutharasan RK, Tripathy S, McMahon KM, Thaxton CS. Templated high density lipoprotein nanoparticles as potential therapies and for molecular delivery. *Advanced drug delivery reviews*. Aug 17.2012
79. Rosenson RS, Brewer HB, Chapman MJ, Fazio S, Hussain MM, Kontush A, et al. HDL Measures, Particle Heterogeneity, Proposed Nomenclature, and Relation to Atherosclerotic Cardiovascular Events. *Clin Chem*. Mar; 2011 57(3):392–410. [PubMed: 21266551]

80. Asztalos BF, Tani M, Schaefer EJ. Metabolic and functional relevance of HDL subspecies. *Curr Opin Lipidol*. Jun; 2011 22(3):176–85. [PubMed: 21537175]
81. Robert, O.; Bonow, ea, editors. Braunwald's Heart Disease - A Textbook of Cardiovascular Medicine. 9th Edition.. Elsevier Saunders; Philadelphia, PA: 2012. 9th Edition
82. Ikonen E. Cellular cholesterol trafficking and compartmentalization. *Nature reviews Molecular cell biology*. Feb; 2008 9(2):125–38.
83. Calabresi L, Gomaschi M, Rossoni G, Franceschini G. Synthetic high density lipoproteins for the treatment of myocardial ischemia/reperfusion injury. *Pharmacology & therapeutics*. Sep; 2006 111(3):836–54. [PubMed: 16960983]
84. Silva RAGD, Huang R, Morris J, Fang J, Gracheva EO, Ren G, et al. Structure of apolipoprotein A-I in spherical high density lipoproteins of different sizes. *P Natl Acad Sci USA*. Aug 26; 2008 105(34):12176–81.
85. Segrest JP, Li L, Anantharamaiah GM, Harvey SC, Liadaki KN, Zannis V. Structure and function of apolipoprotein A-I and high-density lipoprotein. *Curr Opin Lipidol*. Apr; 2000 11(2):105–15. [PubMed: 10787171]
86. Cheung MC, Albers JJ. Distribution of High-Density Lipoprotein Particles with Different Apoprotein Composition - Particles with a-I and a-Ii and Particles with a-I but No a-Ii. *Journal of lipid research*. 1982; 23(5):747–53. [PubMed: 6811682]
87. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. Apr; 2011 13(4):423–U182. [PubMed: 21423178]
88. Liu X, Suo R, Xiong SL, Zhang QH, Yi GH. HDL drug carriers for targeted therapy. *Clinica chimica acta; international journal of clinical chemistry*. Jan 16.2013 415:94–100.
- 89**. Vickers KC, Remaley AT. Functional Diversity of HDL Cargo. *Journal of lipid research*. Mar 26.2013 [Data demonstrate that HDLs are endogenous carriers of microRNAs and that cargo microRNAs are delivered to target cells via SR-B1.]
90. Pirillo A, Norata GD, Catapano AL. High-Density Lipoprotein Subfractions - What the Clinicians Need to Know. *Cardiology*. 2013; 124(2):116–25. [PubMed: 23428644]
91. Rader DJ, Alexander ET, Weibel GL, Billheimer J, Rothblat GH. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *Journal of lipid research*. Apr; 2009 50(Suppl):S189–94. [PubMed: 19064999]
92. Gomaschi M, Ossoli A, Vitali C, Calabresi L. HDL and endothelial protection: examining evidence from HDL inherited disorders. *Clin Lipidol*. Jun; 2013 8(3):361–70.
93. Nicholls SJ, Dusting GJ, Cutri B, Bao S, Drummond GR, Rye KA, et al. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periaarterial collar in normocholesterolemic rabbits. *Circulation*. Mar 29; 2005 111(12):1543–50. [PubMed: 15781735]
94. Cockerill GW, Rye KA, Gamble JR, Vadas MA, Barter PJ. High-Density-Lipoproteins Inhibit Cytokine-Induced Expression of Endothelial-Cell Adhesion Molecules. *Arterioscl Throm Vas*. Nov; 1995 15(11):1987–94.
95. Navab M, Imes SS, Hama SY, Hough GP, Ross LA, Bork RW, et al. Monocyte Transmigration Induced by Modification of Low-Density-Lipoprotein in Cocultures of Human Aortic-Wall Cells Is Due to Induction of Monocyte Chemotactic Protein-1 Synthesis and Is Abolished by High-Density-Lipoprotein. *Journal of Clinical Investigation*. Dec; 1991 88(6):2039–46. [PubMed: 1752961]
96. Kontush A, Chapman MJ. Antiatherogenic function of HDL particle subpopulations: focus on antioxidative activities. *Curr Opin Lipidol*. Aug; 2010 21(4):312–18. [PubMed: 20581677]
97. Davidson WS, Silva RAGD, Chantepie S, Lagor WR, Chapman MJ, Kontush A. Proteomic Analysis of Defined HDL Subpopulations Reveals Particle-Specific Protein Clusters Relevance to Antioxidative Function. *Arterioscl Throm Vas*. Jun; 2009 29(6):870–U234.
98. Mineo C, Deguchi H, Griffin JH, Shaul PW. Endothelial and antithrombotic actions of HDL. *Circulation research*. Jun 9; 2006 98(11):1352–64. [PubMed: 16763172]

99. Yuhanna IS, Zhu Y, Cox BE, Hahner LD, Osborne-Lawrence S, Lu P, et al. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nature medicine*. Jul; 2001 7(7):853–7.
100. Furie B, Furie BC. Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. *Trends in molecular medicine*. Apr; 2004 10(4):171–8. [PubMed: 15059608]
101. Farmer JA, Liao J. Evolving concepts of the role of high-density lipoprotein in protection from atherosclerosis. *Current atherosclerosis reports*. Apr; 2011 13(2):107–14. [PubMed: 21380938]
102. Naqvi TZ, Shah PK, Ivey PA, Molloy MD, Thomas AM, Panicker S, et al. Evidence that high-density lipoprotein cholesterol is an independent predictor of acute platelet-dependent thrombus formation. *The American journal of cardiology*. Nov 1; 1999 84(9):1011–7. [PubMed: 10569655]
103. Calkin AC, Drew BG, Ono A, Duffy SJ, Gordon MV, Schoenwaelder SM, et al. Reconstituted high-density lipoprotein attenuates platelet function in individuals with type 2 diabetes mellitus by promoting cholesterol efflux. *Circulation*. Nov 24; 2009 120(21):2095–104. [PubMed: 19901191]
104. Bijsterbosch MK, Rump ET, De Vruh RLA, Dorland R, van Veghel R, Tivel KL, et al. Modulation of plasma protein binding and in vivo liver cell uptake of phosphorothioate oligodeoxynucleotides by cholesterol conjugation. *Nucleic acids research*. Jul 15; 2000 28(14):2717–25. [PubMed: 10908328]
105. Lorenz C, Hadwiger P, John M, Vornlocher HP, Unverzagt C. Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. *Bioorganic & medicinal chemistry letters*. Oct 4; 2004 14(19):4975–77. [PubMed: 15341962]
106. Thaxton CS, Daniel WL, Giljohann DA, Thomas AD, Mirkin CA. Templated spherical high density lipoprotein nanoparticles. *Journal of the American Chemical Society*. Feb 4; 2009 131(4):1384–5. [PubMed: 19133723]
107. Luthi AJ, Zhang H, Kim D, Giljohann DA, Mirkin CA, Thaxton CS. Tailoring of biomimetic high-density lipoprotein nanostructures changes cholesterol binding and efflux. *ACS nano*. Jan 24; 2012 6(1):276–85. [PubMed: 22117189]
- 108*. McMahon KM, Mutharasan RK, Tripathy S, Veliceasa D, Bobeica M, Shumaker DK, et al. Biomimetic high density lipoprotein nanoparticles for nucleic acid delivery. *Nano letters*. Mar 9; 2011 11(3):1208–14. [PubMed: 21319839] [First manuscript to demonstrate nucleic acid delivery on a synthetic, mature spherical HDL nanoparticle. Also, data suggested that the conjugates avoid endolysosomal uptake.]
109. Zhang ZH, Chen J, Ding LL, Jin HL, Lovell JF, Corbin IR, et al. HDL-Mimicking Peptide-Lipid Nanoparticles with Improved Tumor Targeting. *Small*. Feb 5; 2010 6(3):430–37. [PubMed: 19957284]
110. Yang M, Jin HL, Chen JA, Ding LL, Ng KK, Lin QY, et al. Efficient Cytosolic Delivery of siRNA Using HDL-Mimicking Nanoparticles. *Small*. Mar 7; 2011 7(5):568–73. [PubMed: 21370456]
111. Lacko AG, Nair M, Paranjape S, Johnson S, McConathy WJ. High density lipoprotein complexes as delivery vehicles for anticancer drugs. *Anticancer research*. Jul-Aug; 2002 22(4):2045–49. [PubMed: 12174882]
112. Shahzad MM, Mangala LS, Han HD, Lu C, Bottsford-Miller J, Nishimura M, et al. Targeted delivery of small interfering RNA using reconstituted high-density lipoprotein nanoparticles. *Neoplasia*. Apr; 2011 13(4):309–19. [PubMed: 21472135]
113. Ding Y, Wang W, Feng MQ, Wang Y, Zhou JP, Ding XF, et al. A biomimetic nanovector-mediated targeted cholesterol-conjugated siRNA delivery for tumor gene therapy. *Biomaterials*. Dec; 2012 33(34):8893–905. [PubMed: 22979990]

- siRNAs are powerful molecules for regulating target gene expression and offer tremendous opportunities for the development of new therapies
- Significant challenges have prevented siRNAs from becoming mainstay therapies for systemic diseases
- siRNA delivery has improved from the development of sequence modifications and delivery vehicles
- Lipid and polymer based nanoparticles effectively target siRNAs to the liver, in part, by taking advantage of natural trafficking of lipoproteins
- High density lipoproteins (HDL) are natural nanoparticles that bind, stabilize, and transport nucleic acids to target cells that express scavenger receptor type B-1
- Synthetic HDLs are potentially powerful vehicles for systemic siRNA delivery

Figure 1.

Intracellular degradation of target mRNA by siRNA. Upon entering the cell cytoplasm, exogenous siRNA assembles into the RNA-induced silencing complex (RISC), which involves AGO2 and other cellular proteins. For simplicity, only AGO2 is shown. Upon RISC loading, the antisense strand (guide) engages with AGO2 for optimal mRNA target binding and the sense strand (passenger) is degraded. The complex then begins to screen the cytoplasm for complementary mRNA targets. Once bound, AGO2 mediates the catalytic cleavage of the mRNA target.

Requirements for Successful Systemic siRNA Delivery		
siRNA Sequence / Delivery Vehicle	Cellular	Organism

<ul style="list-style-type: none"> • siRNA sequence stability • Optimal mRNA target specificity • Cell-specific targeting • No inherent cytotoxicity • No untoward immunoactivity • Enters target cells 	<ul style="list-style-type: none"> • Endolysosomal escape to engage target mRNA in cytosol • Limit non-specific cell uptake • siRNA engagement in the RISC • Saturation of RNAi machinery 	<ul style="list-style-type: none"> • Avoid renal filtration • Avoid aggregation • Avoid rapid uptake by RES in liver and spleen • Stable to endo- and exonuclease degradation • Avoid untoward Immune stimulation • Potent mRNA knockdown in target cells

Figure 2.
Requirements for successful systemic siRNA Delivery.

HDL Maturation		Receptor	Function	Target Cell
Apo AI		ABCA1	Efflux of free cholesterol and phospholipids	Hepatocyte, Enterocyte, Macrophage
Nascent HDL		ABCA1	Efflux of free cholesterol and phospholipids	Hepatocyte, Enterocyte, Macrophage
		ABCG1	Efflux of free cholesterol	Macrophage
Mature Spherical HDL (2a, 2b, 3a, 3b, 3c)		ABCG1	Efflux of free cholesterol	Macrophage
		SR-B1	Influx and Efflux of cholesterol	Hepatocyte, Macrophage, Adrenocortical Cells, Gonadal Cells, Tumor Cells, Platelets, Endothelial Cells
			Anti-inflammatory	Macrophages, Endothelial Cells
			Anti-thrombotic	Platelets
			Nucleic acid delivery	Tumor Cells, Hepatocytes, ????
			Enhances cell proliferation and migration	Endothelial Cells

Figure 3.
HDL maturation and targeting.

Figure 4. HDL components. Data demonstrate the molecular complexity of spherical HDLs with regard to nucleic acid, protein, small molecule, and lipid cargoes.

Figure 5.
Natural and Synthetic HDL Properties.

Table 1

Chemical Modifications to siRNA

Modification	Advantages	Caveats	References
Ribose Sugar Modifications			
2' Fluoro	Increases duplex stability, increases nuclease stability, tolerated at the site of AGO2 cleavage, reduces immune activation	No enhanced efficacy <i>in vivo</i>	Gao, Layzer
2' O-Methyl	Increases duplex stability, increases nuclease stability, reduces off-target effects, reduces immune activation, enhances siRNA potency	Heavy modification reduces potency, site specific modification	Gao, Higuchi, Braasch, Chiu, Cazauderna, Gaglione
2' O-MOE	Increases duplex stability, increases nuclease stability	Antisense modifications reduces siRNA potency	Behlke, Gaglione
FANA	Increases nuclease stability, enhances siRNA potency, increases serum half-life	Antisense modifications reduces siRNA potency	Gao, Gaglione
LNA	Increases duplex stability, increases nuclease stability, reduces immune activation, reduces off-target effects, increases serum half-life	Generally reduces siRNA efficacy, site-specific modification, dose-dependent toxicity	Gao, Gaglione, Behlke
Backbone Modifications			
Phosphorothioate	Increases nuclease stability, reduces immune activation, increases serum half-life	Heavy modification reduces potency and results in toxicity, end modifications cause sticky ends leading to nonspecific protein binding	Gaglione, Czech
Boranophosphate	Increases nuclease stability, enhances siRNA potency, low cytotoxicity compared to phosphorothioate modification	Not easily prepared by chemical synthesis, site-specific modification, antisense modification reduces potency	Gaglione, Manoharan
Terminal Modifications (3' or 5'*)			
3' Cholesterol	Increases serum half-life, enhances binding to serum components (i.e. albumin, HDL, LDL), improves cellular uptake, enhances delivery to selective tissues		Gaglione, Manoharan
3' - 2 nucleotide overhang (deoxyribose bases)	Increases nuclease stability, important for RISC loading		Behlke, Gaglione
5' Phosphorylation of antisense strand	Important for proper strand selection in RISC		Gaglione
Lipophilic molecules (bile acids, long alkyl branched chains, lithocholic acid)	Improves cellular uptake, enhances delivery to selective tissues		Manoharan
TAT peptide	Improves cellular uptake	Need high concentration to produce potent response	Gaglione, Manoharan
Folic acid	Improves cellular uptake, enhances cell-specific targeting	Large assembled complex limits potential cell-specific targeting	Kim, Zhou and Lee
Aptamers	Improves cellular uptake, enhances cell-specific targeting	Endolysosomal mediated uptake	Zhou and Rossi

Abbreviations: 2' O-MOE, 2'-O-(2-methoxyethyl); FANA, 2'-deoxy-2'-fluoro-β-D-arabino nucleic acids; LNA, locked nucleic acid; TAT, transactivating transcriptional activator peptide

Table 2**Liposome and Polymer Based Delivery Vehicles**

Vehicle Type	Advantages	Caveats	Reference
Liposomes			
Cationic Lipid Liposomes			
• DOTMA	Stabilizes RNA by electrostatic interaction, increases serum half-life, enhances cellular uptake	Dose dependent toxicity, induce type I and II interferon response, pulmonary inflammation, short serum half-life, rapid clearance by the RES	Miele
• DOTAP	Stabilizes RNA by electrostatic interaction, increases serum half-life, enhances cellular uptake, tissue specific targeting with incorporation of targeting moieties (mainly liver targeting)	Dose dependent toxicity, induce type I and II interferon response, pulmonary inflammation, enhanced permeability and retention effect, short serum half-life, rapid clearance by the RES	Miele, Guo
Neutral Lipid Liposomes			
• DOPC and DOPE	Stabilizes RNA, enhances internalization by membrane fusion or receptor mediated uptake, low toxicity, low immunogenicity	Low transfection efficiency, rapid clearance by the RES, some liver toxicity	Gao, Miele, Weinstein, Guo
Polymers			
Synthetic Polymers			
• PEI	Strong buffering capacity for enhanced endosomal escape, enhances cellular uptake by endocytosis, increases nuclease stability, enhances biocompatibility	TLR-mediated immune response, non-specific cell and protein binding, high molecular weight PEI has low transfection efficiency,	Higuchi, Guo, Singha
• PLGA	Stabilizes and protects RNA from nuclease degradation by encapsulating RNA, enhances cellular uptake through endocytosis, biodegradable, can incorporate targeting moieties, controlled release of RNA	Encapsulation difficulty due to the lack of electrostatic interactions with RNA, low RNA cargo loading capacity, lack of endosomal escape	Singha
• Dendrimers	Stabilizes RNA by electrostatic interaction, protects RNA from nuclease degradation, can incorporate targeting moieties (mainly liver targeting)	Size dependent and charge dependent toxicity, larger size and charge the greater the toxicity, surface modifications are necessary to circumvent cytotoxicity	Guo, Singha
Natural Polymers			
• Cyclodextrin	Enhances bioavailability, biodegradable, low toxicity, increases nuclease stability, low immunogenicity, can incorporate targeting moieties	Lack endosomal escape	Guo, Singha
• Chitosan	Enhances bioavailability, biodegradable, non-allergenic, biocompatible	Low solubility and inefficient endosomal buffering capacity, lack endosomal escape, high viscosity can limit <i>in vivo</i> applications	Guo, Singha
• Atelocollagen	Stabilize RNA by electrostatic interaction, enhances siRNA delivery due to EPR effect, low toxicity	Passive targeting	Weinstein, Inaba

Abbreviations: DOTMA, (N-[1-(2,3-dioleoyloxy) Propyl]-N,N,N trimethyl ammonium chloride; DOTAP, (1,2-dioleoyl-3-trimethylammonium-propane); DOPC, (1,2-dioleoyl sn-glycero-3-phosphatidylcholine; DOPE, (1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine); PEI, polyethyleneimine; PLGA, poly-(lactic-co-glycolic acid)

Table 3**Nanoparticle Delivery Vehicles**

Vehicle Type	Advantage	Caveat	Reference
Organic			
SNALP	Stabilizes RNA by electrostatic interaction, increases serum half-life, enhances cellular uptake	Passive targeting, endolysosomal uptake, some formulations of SNALPs show cytokine induction and increase in liver enzymes, biodistribution limited to liver and spleen	Weinstein, Guo
SLNP	Stabilizes RNA by electrostatic interaction, increases serum half-life, enhances cellular uptake, tissue specific targeting with incorporation of targeting moieties (mainly liver targeting), slow release	Passive targeting, endolysosomal uptake, biodistribution limited to liver and spleen	Lobovkina
Inorganic			
Carbon Nanotubes			
• SWCNT	Easily functionalized, low toxicity with proper functionalization, optical properties	Biodistribution limited to liver and spleen, toxic when unfunctionalized	Liu
• MWCNT	Easily functionalized, low toxicity with proper functionalization, optical properties	Biodistribution limited to liver and spleen, toxic when unfunctionalized, size dependent toxicity	Liu
Iron Oxide Particles	Large surface area for functionalization, imaging properties	Biodistribution and toxicity are highly dependent on size; particles < 10 nm result in rapid renal clearance, particles > 200 nm are sequestered by the liver and spleen, certain surface coatings can result in toxicity (e.g. Gd)	Lee, Yu
Quantum Dots			
• Cd, Se, Te	Optical properties	Toxic	Lee, Yu
• ZnS-AgInS ₂	Optical properties, low toxicity		Lee, Subreamaniam
Gold Nanoparticles	Biocompatible, non-toxic, easily synthesized and functionalized, large surface area to volume ratio, increases nuclease stability of RNA, enhances cellular uptake, some constructs demonstrate receptor mediated uptake and endolysosomal escape	Some formulations are sequestered by endolysosome	Lee, Ghosh, McMahon, Connor, Giljohann

Abbreviations: SNALP, stable nucleic acid lipid particle; SLNP, solid lipid nanoparticle; SWCNT, single-walled carbon nanotube; MWCNT, multi-walled carbon nanotube; Cd, cadmium; Se, selenium; Te, tellurium