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Synthetic High-Density Lipoprotein-Like Nanoparticles as Cancer Therapy

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Abstract

High-density lipoproteins (HDL) are diverse natural nanoparticles that carry cholesterol and are best known for the role that they play in cardiovascular disease. However, due to their unique targeting capabilities, diverse molecular cargo, and natural functions beyond cholesterol transport, it is becoming increasingly appreciated that HDLs are critical to cancer development and progression. Accordingly, this chapter highlights ongoing research focused on the connections between HDL and cancer in order to design new drugs and targeted drug delivery vehicles. Research is focused on synthesizing biomimetic HDL-like nanoparticles (NP) that can be loaded with diverse therapeutic cargo (e.g. chemotherapies, nucleic acids, proteins) and specifically targeted to cancer cells. Beyond drug delivery, new data is emerging that HDL-like NPs may be therapeutically active in certain tumor types, for example B cell lymphoma. Overall, HDL-like NPs are becoming increasingly appreciated as targeted, biocompatible, and efficient therapies for cancer, and may soon become indispensable agents in the cancer therapeutic armamentarium.

1 Structure and Composition of Natural High-Density Lipoproteins (HDL)

Natural high-density lipoproteins (HDL) range in size from 7 to 13 nanometers in diameter. HDLs are dynamic nanostructures with regard to size, shape, and molecular composition. As HDLs biologically mature, they interact with cell receptors, enzymes, and other proteins. Apolipoprotein A-I (apoA-I) is the main protein associated with HDLs and represents approximately 70% of the protein content associated with HDLs [1]. ApoA-I is an

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amphipathic scaffold protein, which binds lipids and defines the ultimate size and shape of HDL species [2]. ApoA-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 apoA-IV, apoC-I, apoC-II, apoC-III, apoD, apoE, apoJ, apoL, and apoM [3, 4]. Additionally, a number of other proteins, lipids (e.g. phosphatidylcholines), free cholesterol, and esterified cholesterol contribute to the heterogeneity of HDL species [5].

HDLs are the smallest and densest of the plasma lipoproteins. HDLs are constantly remodeled in the blood stream through interaction with other lipoproteins, enzymes, and contact with target cells. These interactions result in significant particle heterogeneity. For example, HDLs can be classified into sub-populations by density, size, shape, composition, and surface charge. HDLs are classified into two main sub-fractions based on density: HDL_2 ($1.063 < d < 1.125$ g/mL), which are relatively large in size, lipid-rich, and more buoyant than HDL_3 species ($1.125 < d < 1.21$ g/mL), which are smaller in size [1, 6]. These sub-fractions can be further categorized into five distinct sub-populations by using methods such as electrophoresis and ultracentrifugation [1, 4]: HDL_{2b} (9.7 – 12.9 nanometers [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) [6]. Further, two-dimensional electrophoresis can separate HDL subpopulations by charge and size, ultimately resulting in 5–10 distinguishable HDL species [1, 6]. 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 [7], and it is important to keep these parameters in mind when developing therapeutic agents based upon HDLs.

HDL biosynthesis is initiated by the secretion of apoA-I from hepatocytes and enterocytes of the liver and small intestine, respectively [8]. Following synthesis, apoA-I is lipid-poor, but 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. Nascent HDLs contain two anti-parallel molecules of apoA-I wrapped in a belt-like fashion around a solubilized central core of phospholipids oriented as a bilayer [9–11]. These nascent HDLs are the smallest of formed HDLs and only contain small amounts of cholesterol which is mainly interdigitated in the ~160 phospholipids present in the core of the disc [12]. Self-assembly of nascent HDL is moderated through the interaction of apoA-I with ATP binding cassette receptor A1 (ABCA1), a transmembrane protein that mediates the transfer of phospholipids and free cholesterol to apoA-I. Free cholesterol transferred to HDL becomes esterified by the serum enzyme lecithin cholesterol acyltransferase (LCAT). LCAT catalyzes the esterification of free cholesterol associated with HDL and increases its hydrophobicity. Cholesteryl esters (CE) are then driven into the lipid bilayer creating a core of hydrophobic CEs surrounded by a monolayer of phospholipids. The transfer of CE into the core results in an increase in HDL size and a change in morphology from the disc-like structure of nascent HDL into a maturing spherical nanostructure, while creating a gradient enabling more free cholesterol to move onto the particle surface. Further, as the particle matures it begins to interact with additional receptors known to participate in cellular

cholesterol flux to HDLs, including ATP-binding cassette receptor G1 (ABCG1) and scavenger receptor type B-1 (SR-B1). Additionally, other lipoproteins aid in the maturation process of HDLs. For instance, low-density lipoproteins (LDLs) transfer triglycerides to HDLs in exchange for CE, a process 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. However, HDLs do not consist solely of apolipoproteins, cholesterol, and phospholipids. It is becoming increasingly appreciated that HDLs are highly diverse in their chemical composition with regard to phospholipids, small molecules, proteins [3, 13], small RNAs [14, 15], hormones, carotenoids, vitamins, and bioactive lipids [16].

Due to the complex synthesis and remodeling of HDL there are significant structural differences between HDL sub-species and individual particles. These differences result in a myriad of different HDL functions. Two key factors that determine the function of HDL in the human body are HDL size and composition. Below we discuss the functions of natural HDLs to outline their natural mechanisms-of-action and also to highlight opportunities for targeting specific diseases, such as cardiovascular disease and cancer. Ultimately, designing synthetic HDL-like nanoparticles with biologically functional modifications may provide targeting mechanisms to cell types critical to disease pathogenesis [17].

2 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); however, emerging data show that measuring HDL-C as a biomarker for CHD may not be adequate [18]. Yet cholesterol associated with HDL has been termed “good” cholesterol and suggests that HDLs directly protect against CHD. The process of reverse cholesterol transport (RCT) is believed to be the primary explanation for this phenomenon. During RCT cellular cholesterol is removed from lipid-laden macrophages (foam cells) in atherosclerotic lesions by HDLs via interactions with ABCA1, ABCG1, and SR-B1 [19]. Cholesterol-rich HDLs are then trafficked to the liver, delivering their cholesterol cargo to hepatocytes through SR-B1 in an apoA-I-dependent process. Cholesterol becomes incorporated into bile and excreted in the feces. In addition, HDL may transfer CE to LDLs in exchange for triglycerides. LDLs loaded with cholesterol are then taken up by hepatocytes through the LDL receptor further promoting RCT.

2.1 Non-Cholesterol Transport- HDL and Coronary Heart Disease

While RCT is believed to be the primary means by which HDL reduces CHD risk, HDLs are implicated in multiple other cardio-protective mechanisms. For instance, HDLs reduce inflammation at the site of atherosclerotic lesions [4]. HDLs also have antioxidant and antithrombotic effects that maintain endothelial integrity and promote repair [1, 4, 20].

2.1.1 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 tumor necrosis factor alpha (TNF-α), which stimulate the expression of adhesion molecules such as intercellular adhesion molecule-1

(ICAM-1) and vascular cell adhesion molecule-1 (VCAM-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 growing 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 [21–23].

2.1.2 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 [24, 25]. HDLs have antioxidant properties that can inhibit the accumulation of peroxidation products on the surface of LDLs and mitigate damage. This is accomplished by several mechanisms. First, methionine residues (112 and 148) of apoA-I reduce LOOH into redox-inactive lipids. In addition, apoA-I protein sequesters LOOHs from LDLs [26]. Third, HDLs, particularly the smaller HDL_3 sub-populations, have a unique proteome that plays a critical role in protection against LDL oxidative stress [26]. Also, the transfer of LOOH species from oxLDL to HDL_3 aids in the reduction of free radical species in atherosclerotic lesions [27]. 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 [28].

2.1.3 Antithrombotic and anticoagulant activity of HDLs—Finally, HDLs demonstrate protective effects through antithrombotic and anticoagulant activity through direct and indirect interactions with endothelial cells. Endothelial-derived nitric oxide (NO) is critical for vasodilation and maintains the integrity of vascular endothelial and smooth muscle cells [29]. Reduced NO production is a hallmark of atherosclerosis and results in increased neutrophil adherence to the endothelium, smooth muscle cell proliferation, and enhanced platelet aggregation and adhesion. HDL is capable of activating NO synthesis through endothelial nitric oxide synthase (eNOS) upon binding to endothelial cell SR-B1 [30]. Endothelial cell apoptosis contributes to arterial atherothrombosis by the release of “microparticles” that carry pro-thrombotic factors, such as P-selectin [31]. 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 TNF-α-mediated apoptosis and by suppressing growth factor deprivation [32]. Furthermore, it has been demonstrated that HDLs enhance prostacyclin synthesis in endothelial cells. Prostacyclin acts synergistically with NO to induce vascular smooth muscle (VSM) relaxation [32], inhibits platelet activation, and represses the release of growth factors responsible for local proliferation of VSM cells [29]. 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 [33]. This phenomenon is not limited to CHD as recombinant HDLs were shown to reduce platelet aggregation in individuals with type 2 diabetes mellitus [34].

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. However, the utility of HDLs extends beyond treating cardiovascular disease. It has recently been discovered that HDLs can bind, transport, and deliver a range of cargoes, including small molecules, photothermal reactive agents, and nucleic acids. Understanding the mechanisms by which HDLs perform this function will provide new opportunities to develop biomimetic, synthetic HDL-like nanoparticles for targeted delivery of therapies for disease treatment. One such disease is cancer, as cancer cells increase cellular cholesterol pools not only by increasing cholesterol synthesis, but also by increasing HDL uptake. Thus HDL-based delivery methodologies have great potential for specific and effective delivery of cargo directly to cancer cells, with minimal off-target effects.

3 HDL and Cancer

A sustained and increased supply of cholesterol is essential for cancer cell proliferation and tumor progression [35–41]. Cancer-related anomalies of cholesterol metabolism have been implicated in angiogenesis, metastasis, and drug resistance [38]. On a cellular level, cholesterol is a crucial component of membranes and modulates their fluidity, stability, and overall architecture [42]. Additionally, cholesterol is known to accumulate in discrete regions of the membrane, termed lipid rafts, endowing these areas with very unique properties. Lipid rafts serve as assembly platforms for molecules involved in signaling cascades, including those associated with cancer development [35, 43–45]. On an organismal level, cholesterol serves as a precursor for steroid hormones, known regulators of cell proliferation and differentiation, and are critical in the progression of breast and prostate cancer [35].

Lipoproteins play a role in cancer progression through delivery of cholesterol to malignant cells. Consistently, many cancer patients exhibit reduced levels of cholesterol in the blood, which are restored to normal values upon successful cancer treatment [38, 46, 47]. Interestingly, it is the levels of HDL, not LDL, that are most affected in patients suffering from cancer [5, 38]. Exogenous addition of HDL has been shown to promote the growth of breast cancer cells *in vitro* and promote aggressiveness of tumors *in vivo* [48–50]. HDLs also exhibit anti-inflammatory, anti-oxidant, anti-microbial and pro-immunity properties [51, 52] and carry non-lipid cargo including microRNA, hormones, vitamins and metabolites [14, 16]. All of these characteristics play an important functional role for HDL in cancer progression and tumor cell survival.

Many malignant cells have been shown to overexpress SR-B1 [5, 38, 42, 53–57]. For instance, among 50 human ovarian epithelial cancers, 96% of the tumors highly expressed SR-B1 [54]. Further, prostate cancer cells have been shown to express SR-B1 for the uptake of cholesterol-rich HDLs to support endogenous androgen biosynthesis [53]. Also, HDL binding to SR-B1 can trigger downstream signaling cascades, such as through Akt, that promote breast cancer progression [57]. Thus, there is a great deal of evidence supporting increased HDL uptake via SR-B1 in cancer cells. This presents a unique opportunity to exploit this system for the targeted delivery of cancer therapeutics.

3.1 Cancer Therapies: Drug Hurdles and Emerging Drug Potential

Most anti-cancer drugs are inherently non-specific in their bio-distribution and diffuse into both healthy and cancer tissues. Further, the mechanism-of-action of most cancer drugs affects all rapidly diving cells rather than cancer cells specifically. Solubility is also a major limitation as ~40% of new anti-cancer drugs are characterized by poor water-solubility [58]. Targeted drug delivery vehicles soluble under physiologically relevant conditions have great potential to overcome these issues. By preferentially increasing accumulation of the therapeutic agent in malignant rather than healthy cells, the therapeutic index of a drug can be significantly improved and undesirable side effects reduced. HDLs are an attractive tool for the targeted delivery of antineoplastic substances due to their central role in promoting cancer proliferation by providing a constant supply of cholesterol to malignant cells. A number of research groups are therefore focused on the development of synthetic nanostructures that imitate natural HDLs in regards to function and structure. For the remainder of the chapter we will refer to these artificial structures as HDL-like nanoparticles (HDL-like NPs).

The preferential accumulation of such HDL-like NPs in cancer cells is achieved by both passive and active targeting. On the one hand, tumor tissues are characterized by a leaky vasculature and low lymphatic drainage, leading to differences in interstitial pressure between the center of a tumor and its periphery. This pressure difference allows for the preferential retention of particles between 10–100 nm in the tumor, a passive targeting phenomenon known as the enhanced permeability and retention (EPR) effect [59, 60]. On the other hand, synthetic HDL-like NPs are, like natural HDL, are actively targeted to cancer cells by specific interaction with SR-B1 [42, 53–57]. Importantly, SR-B1 facilitates the uptake of cholesterol esters and anticancer drugs from spherical HDLs and HDL-like NP to the cytosol via a non-endocytic pathway [61, 62], avoiding lysosomal degradation of particle cargo.

3.2 Synthetic HDL-like NP Composition

In contrast to natural HDL, synthetic HDL-like NPs are highly customizable, providing researchers with the unique opportunity to control many of the particles' structural and compositional features and to endow these particles with tailored and unique functions. Examples of the structural diversity of HDL-like NPs are provided in Table 1.1. Because HDL-like NPs are designed to mimic their natural counterparts in composition, surface properties, and size, many of them were shown to be non-immunogenic, capable of avoiding clearance by the reticuloendothelial *system*, and to have relatively long circulation times [59, 63].

3.3 HDL-like Nanoparticles for Drug Delivery

3.3.1 Discoidal HDL NPs for drug delivery—Both discoidal and spherical HDL-like NPs have been shown to target SR-B1 [81] and have therefore both been employed for targeted drug delivery to cancer cells. While spherical HDL-like NPs are more similar in shape to the more mature versions of their natural counterparts, discoidal HDL-like NPs are more reminiscent of nascent HDL. Like natural, nascent HDL, discoidal HDL-like NPs are prone to undergo maturation in the blood stream due to interaction with LCAT [82–85]. This

biological maturation of discoidal HDLs can lead to unwanted leakage of drug cargo [69]. To combat this problem, researchers used monocholesterylsuccinate (CHS) instead of cholesterol to prevent particle interaction with LCAT, and anchored apoA-I on the particle by covalently linking it to CHS [76, 86]. The authors employed a CHS-modified discoidal HDL-like NPs, termed recombinant HDL (rHDL), and loaded it with paclitaxel, a mitotic inhibitor used to treat a variety of cancers. Studies in rats showed improved drug levels in the blood over extended periods of time both compared to drug-loaded, but unmodified particle (P-d-rHDL), as well as compared to free or liposome-loaded drug [86]. Further, paclitaxel-loaded CHS-modified particles (cP-d-rHDL) showed increased cytotoxicity and uptake in the human cancer cell line MCF-7 compared to P-d-rHDL. In breast tumor bearing mice, cP-d-rHDL were superior in regards to both tumor targeting as well as limiting tumor growth compared to P-d-rHDL, liposome-loaded drug or free drug [76].

3.3.2 Spherical HDL-like NPs for drug delivery—Delivery of hydrophobic antineoplastic agents by HDL-like NPs is often achieved by encapsulation of the drug in the hydrophobic core. Recently, Lacko and co-workers showed that inclusion of highly water-insoluble drugs, like valrubicin, into spherical HDL-like NPs composed of phosphatidyl choline, apoA-I, free cholesterol and cholesteryl oleate led to increased toxicity in SR-B1 expressing, malignant prostate and ovarian cancer cell lines as compared to the free drug [62, 78]. Further, off-target cytotoxic effects in non-malignant epithelial prostate and ovarian cell lines with low SR-B1 expression were decreased for the drug-containing particle compared to the free drug [62, 78]. Encapsulation of valrubicin into HDL-like NP may expand the therapeutic spectrum of the drug, which had previously been used exclusively for the treatment of bladder cancer. The authors also used a similar NP construct to target the therapeutic agent fenretinide to two different neuroblastoma cells lines *in vitro* [78]. Compared to the free drug, cytotoxicity in the malignant cell lines was significantly increased, while cytotoxicity in retinal pigment epithelial cells, a control cell line for off-target fenretinide toxicity, was reduced [62, 78]. Feng and coworkers used a similar strategy of drug encapsulation to target the chemotherapeutic doxorubicin to hepatocellular carcinoma and hepatoma cells lines [73, 77]. In this work, HDL-like NPs were composed of egg phospholipids, apoA-I, and doxorubicin and showed increased cytotoxicity, apoptosis induction, and conjugate accumulation in target cells compared with the free or liposome encapsulated drug [73]. The drug-loaded HDL-like NPs also decreased tumor growth in a metastatic model of human hepatocellular carcinoma (HCC) in nude mice and reduced hemolysis-related side effects [77].

To better understand the uptake of lipophilic cargo from spherical HDL-like NPs through SR-B1, Lin et al developed multifluorophore-labeled HDL-mimicking peptide phospholipid scaffold (HPPS) nanoparticles [80]. Although apoA-I is critical for determining the shape of HDLs and allowing for specific interactions with cellular receptors, the protein can be replaced by short (<20 amino acids) peptides that show no sequence similarity to the full-length protein [87]. These peptides mimic the amphipathic helical structure of apoA-I, including its receptor and lipid binding abilities [87]. Lin et al [80] generated a variety of different cholesteryl oleate/phosphocholine HDL-like NPs with either fluorescent compounds in the particle core, fluorescently labeled apoA-I peptides and phospholipids on

the surface, or combinations thereof. Through sequential inhibition studies, they found that after initial interaction of the HDL-like NP with SR-B1, the particle bound to a specific sub-domain of the receptor, leading to particle dissociation and internalization of the hydrophobic cargo into the cytosol by a lipid-raft/caveolae-like mechanism. Phospholipids and apoA-I-mimetic peptides were mainly retained on the cell surface. It is important to note that for natural, mature HDL, selective influx of CE payload does not require particle catabolism [88, 89], suggesting that the cellular fate of specific HDL-like NPs might be dependent on their specific composition and determined by additional factors that warrant further investigation.

3.3.3 HDL-like NPs for The Delivery of Photothermal Therapeutics—HDL-like NPs have also been used to for the delivery of photothermal agents, which facilitate infrared light-induced temperature changes in tumor tissues leading to tissue necrosis. Mathew et al generated an HDL-like NP composed of phosphocholine, apoA-I fused to a trans-activating transcriptional activator peptide (TAT-peptide, for enhanced cell internalization), and a water-insoluble gadolinium bis(naphthalocyanine) sandwich complex (as a photothermal compound) [90]. Using this particle, the authors achieved photothermal killing of human lung cancer cells in a near infrared light-irradiation-dependent manner. In addition, delivery of the photothermal compound was dependent upon the expression of SR-B1 by target cells. Also, by conjugating pyropheophorbide, a reduced porphyrin, to lysophospholipids, Ng et al created a phototherapeutic fluorescent pyro-lipid, which self-assembled with apoA-I into nanodiscs [65]. The authors used these particles to treat Chinese hamster ovary (CHO) cells that were transfected to express SR-B1. The fluorescence of the photosensitizer was quenched in intact particles; however, upon cellular uptake fluorescence of the particle became un-quenched, likely due to disruption of particle structure. Importantly, transfected SR-B1-expressing cells exhibited a dose-dependent decrease in survival when treated with 660 nm light, whereas SR-B1 negative, untransfected CHO cells neither took up the particles or were affected by light treatment [65]. To our knowledge, these and other constructs are awaiting efficacy testing in murine models.

3.3.4 Modification of HDL-like NP for Uptake via Alternate Receptors—HDL-like NPs can also serve as scaffolds for the attachment of alternative targeting ligands for binding receptors beyond SR-B1. Corbin et al [91] exploited the fact that over 90% of non-mucinous ovarian cancers overexpress folate receptor-α (FR-α), as previously reported [92]. The authors first synthesized HDL-like NPs composed of apoA-1, egg yolk phosphatidylcholine, and cholesteryl oleate and then covalently attached folate to the lysine residues of apoA-I. Folate modification abolished the ability of the particle to interact with SR-B1, therefore re-routing the HDL-like NP to FR-α. The researchers used this technique to specifically deliver a near-infrared fluorescent dye to ovarian tumors in mice, enabling *in vivo* tumor imaging [75, 91]. Current studies are underway to utilize these particles to deliver therapeutic antineoplastic agents as well.

3.3.5 Natural HDLs for Systemic Delivery of siRNA—The potential of HDLs and HDL-like NPs as delivery vehicles for nucleic acids emerged from a few key studies. The first data demonstrating the interaction between natural HDLs and siRNAs focused on

siRNA sequences terminally modified with lipophilic moieties, like cholesterol. Addition of lipophilic siRNAs resulted in siRNA-specific gene silencing in cultured cells *in vitro* and in the liver following systemic administration [93–95]. These observations prompted investigations to better understand how lipophilic siRNAs were being productively delivered to target cells and the liver. Toward this end, Wolfrum et al demonstrated that systemically injected lipid-modified siRNAs spontaneously bound 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 [96].

In addition, upon extraction of natural HDLs from human plasma, Vickers et al demonstrated that HDLs naturally bind and stabilize microRNAs [14]. Further, they found distinct microRNA profiles from HDL of healthy human samples as compared to subjects with hypercholesterolemia. For example, the most abundant microRNA associated with HDLs in healthy subjects was hsa-miR-135a, whereas hsa-miR-223 was the most abundant microRNA found in hypercholesterolemic patients. To determine if HDL had the capacity to load microRNAs *in vivo*, rHDLs free of RNA were intravenously injected into wild-type or apoE null mice. After six hours of incubation, rHDLs were isolated from mouse plasma. rHDLs isolated from healthy mice produced 110 unique miRNAs while rHDLs isolated from the apoE null mice were found to contain 162 microRNAs. These results suggest that HDL has the capacity to bind microRNAs *in vivo*. In addition, Vickers et al determined that HDL transfers microRNA to recipient cells though ABCA1. By increasing the expression of ABCA1 in J774 murine macrophage cells using a liver-X-receptor-α agonist (LXRα), they were able to demonstrate an increase in abundance of miR-223 associated with rHDL in the presence of the LXRα. Finally, to determine the importance of SR-B1 mediated microRNA delivery, baby hamster kidney (BHK) cells were stably transfected with an inducible human SR-B1 vector to increase the expression of SR-B1. Upon treatment of induced BHK cells with native HDL and native HDL pre-complexed with miR-223, induced BHK cells resulted in a 69-fold increase in intracellular levels of hsa-miR-223 as compared to induced BHK cells treated with native HDL. These data suggest that HDL delivered microRNA depends upon SR-B1 expression. These data demonstrate that natural HDLs bind, stabilize, and productively deliver RNAs to cells that express SR-B1.

Collectively, it has been demonstrated that HDLs are natural nucleic acid delivery vehicles with high potential for overcoming hurdles associated with systemic siRNA delivery. As such, there has been a significant focus on the development of synthetic HDL-like vectors for the delivery of nucleic acids. Importantly, and described below, synthetic HDL-like platforms vary with regard to size, charge, and surface chemistry. The goal of these efforts is to understand the parameters of natural HDLs that lead to RNA binding, stabilization, and productive delivery to target cells, like cancer cells, that express receptors for HDL.

3.4 Synthetic HDL-Like Nanoparticles for Nucleic Acid Delivery

3.4.1 Spherical HDL gold nanoparticles for delivery of nucleic acids—Our research group pioneered the synthesis of HDL-like NPs using a gold nanoparticle (AuNP) core template. We demonstrated that these particles tightly bind cholesterol (K_d = 3.8 nM)

[97] and function to modulate cholesterol metabolism in target cells through the same receptors as natural HDLs [64, 98]. Because our HDL-like AuNPs tightly bind cholesterol, and natural HDLs spontaneously associate with lipidated siRNAs after systemic administration, we hypothesized that our HDL-like AuNP platform could be utilized to adsorb cholesterol modified nucleic acids for delivery to target cells that express SR-B1. Initially, we tested this hypothesis using cholesterylated antisense DNAs (chol-DNA) for delivery to prostate cancer cells that express SR-B1 [71]. As in our previous work, we developed a synthetic HDL-like nanostructure that closely mimics the size, shape, and surface chemistry of natural, mature spherical HDLs [97]. A five nm AuNP was used as a scaffold to control the size, shape, and composition of synthetic, spherical HDL-like AuNPs. We termed these high-density lipoprotein-like gold nanoparticles (HDL-like AuNPs). They are similar in size to their natural mature spherical HDL counterparts and have a similar surface composition: approximately 3 copies of apoA-I and an outer phospholipid layer of zwitterionic 1-2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) [97][64]. Moreover, chol-DNAs bound to HDL-like AuNPs (chol-DNA-HDL-like AuNPs) can overcome many of the difficulties associated with *in vitro* nucleic acid delivery. For instance, HDL-like AuNPs stabilize chol-DNA against nuclease degradation. Further, chol-DNA-HDL-like AuNPs do not exhibit off-target cellular toxicity and are efficient conjugates for delivering nucleic acid to regulate target gene expression. Following cell treatment, transmission electron micrographs suggested that chol-DNA-HDL-like AuNPs bypass endolysosomal sequestration, a major biological hurdle in nucleic acid delivery. This manuscript was the first to demonstrate synthetic, biomimetic HDL-like AuNPs as a delivery vehicle for nucleic acids [71].

In addition, our group recently reported the ability of HDL-like AuNPs to deliver cholesteryl-modified single stranded RNA (RNAi-HDL-like AuNPs) for modulating gene expression in the context of neovascularization and angiogenesis, both *in vitro* and *in vivo* [99]. RNAi-HDL-like AuNPs were functionalized with antisense RNA targeting vascular endothelial growth factor receptor 2 (VEGFR2), the receptor for vascular endothelial growth factor (VEGF), a key regulator of neoangiogenesis. The RNAi-HDL-like AuNPs were delivered to cultured vascular endothelial cells, which express SR-B1, and are responsible for the formation of new blood vessels. RNAi-HDL-like AuNPs effectively reduced VEGFR2 expression in target endothelial cells. In addition, treatment with RNAi-HDL-like AuNPs also reduced VEGF-induced endothelial cell survival and morphogenesis. Importantly, knockdown of SR-B1 expression by cultured endothelial cells reduced the uptake of RNAi-HDL-like AuNPs and drastically reduced VEGFR2 knockdown. Accordingly, SR-B1 expression, like for natural nucleic-acid-carrying HDLs, was shown to be required for the internalization and function of RNAi-HDL-like AuNPs. Most importantly, *in vivo* data showed that RNAi-HDL-like AuNPs reduce neovascularization induced by VEGF after local and systemic injection of the conjugates. Further, systemic administration of RNAi-HDL-like AuNPs targeting VEGFR2 significantly reduced target gene expression, tumor volume, and tumor weight in Lewis lung carcinoma tumor xenografts, a tumor model well-known for its high degree of vascularity. Collectively, these data suggest that synthetic, spherical HDL-like AuNPs are an efficient delivery vehicle for

systemic nucleic acid delivery to cells and tissues involved in carcinogenesis both *in vitro* and *in vivo*.

3.4.2 HDL-Mimicking Peptide-Phospholipid Scaffold (HPPS) Nanoparticles for siRNA delivery—Other HDL-like NPs have been used to deliver nucleic acids. Yang et al [100] designed an HDL-like NP using a peptide-phospholipid scaffold, termed HPPS particles. The particles consisted of phospholipids, cholesteryl oleate, and amphipathic α-helical peptides, which mimic apoA-I. The components self-assembled into structures similar to plasma-derived HDL. Direct incubation of HPPS particles with cholesteryl conjugated siRNAs (chol-siRNA) targeting the oncogene B-cell lymphoma-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 chol-siRNA was added, which is 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 SR-B1 resulted in a 35 ± 9% reduction of BCL-2 protein as compared to the control, and a 2.5 fold increase in apoptosis was measured when compared to 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 SR-B1. Furthermore, and consistent with SR-B1 expression, HPPS particles efficiently deliver siRNA cargo to the cytoplasm of target cells, bypassing endolysosomal sequestration. These results suggest that HPPS chol-si-bcl-2 particles utilize SR-B1 for efficient siRNA delivery and target gene knockdown.

3.4.3 Reconstituted HDL for siRNA delivery—As mentioned above, reconstituted HDLs (rHDLs, distinct from the HDL-like NPs described in section 3.3.1) are synthetic forms of human HDL, essentially composed of phospholipids, apoA-I, cholesterol, and esterified cholesterol [63]. For siRNA delivery, Shahzad et al [101] incorporated siRNA into rHDL nanoparticles. The particles were approximately 10 nm in diameter, had 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. This group 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. 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 an rHDL nanoparticle has been demonstrated by Ding et al [102]. Here, rHDL nanoparticles were synthesized using a mixture of phospholipids, apoA-I, 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 previous findings, the rHDL protected siRNA from nuclease degradation and rHDL/chol-siRNA was effective in silencing gene expression *in vitro* and *in vivo*. The siRNA sequence was targeted to

Pokemon. Pokemon is a proto-oncogene overexpressed in a number of human cancers and induces carcinogenesis by repressing two key tumor suppressive pathways: one, the alterative reading frame (ARF)-multiple murine double minute gene 2 (HDM2)-p53 pathway, and two, the retinoblastoma (Rb)-early-region-2 transcription factor (E2F) pathway. Systemic administration of rHDL/chol-siRNA particles complexed with siRNA targeting Pokemon demonstrated a reduction in tumor volume of HepG2 xenografts.

3.4.4 ApoE lipopeptide nanoparticles for siRNA delivery—In addition to apoA-I, apoE has been used as a targeting moiety for HDL-like NPs. Dong et al [68] generated a library of NPs composed of phospholipids, cholesterol, polyethylene glycol-lipid, siRNA, and different lipopeptides – lipids with constituent groups conjugated to different amino acids, peptides, and polypeptide head groups. The library was generated to evaluate combinations of lipoamino acid derivatives designed to mimic apolipoproteins associated with natural lipoproteins. They initially screened the lipopeptide nanoparticle's (LNP) capacity to silence Factor VII (a blood clotting factor) in mice following intravenous injection. Upon further screening, a lead compound, CKK-E12, was used to evaluate the silencing of phosphatase and tensin homolog (PTEN) in different tissues after intravenous administration in mice. Data showed significant silencing of PTEN in the liver as compared to the lung, spleen, kidney, heart, and brain. Upon further examination of the liver, CKK-E12 LNPs silenced over 80% of PTEN in hepatocytes; however, it showed no significant silencing in endothelia cells and leukocytes in the liver with comparison made to a luciferase siRNA-formulated CKK-E12 LPN, as a control. To study the effects of cell uptake and gene silencing with regard to apolipoprotein species, CKK-E12 LNPs were mixed with 11 isoforms of apoA, apoB, apoC, apoE and apoH. Upon transfection of HeLa cells, results showed that apoA, apoC, and apoH did not show significant gene silencing; however, four apoE isoforms showed improved luciferase silencing and the apoE-3 isoform increased cellular uptake and endolysosomal escape when added to cultured cells upon treatment with CKK-E12 LNPs. Collectively, these data demonstrate that particle optimization can significantly improve specificity and efficacy, and improvements such as these offer the potential to broaden the therapeutic application of siRNA therapeutics. Further, the incorporation of apoE, instead of apoA-I into HDL-like particles appears to be superior in silencing gene targets in the liver [66]. ApoE-conjugated HDL-like NPs more efficiently delivered chol-siRNA to hepatocytes compared to apoA-I-conjugated particles, presumably due to the employment of a different uptake mechanism (LDL receptor for apoE vs. SR-B1 for apoA-I) [66].

In addition, Fischer et al [70] explored the bio-distribution, toxicity, and potential of human cloned apoE4 containing NPs to deliver a variety of different compounds, including cholesterol modified single stranded DNA (ss-DNA) [70]. The authors used a series of modifications to conjugate additional cargo to their apoE-HDL-like NPs; namely, nickel-chelating lipids for binding His-tagged proteins, highly hydrophobic molecules like cholesterol for covalently linkage to cargo (like chol-ssDNA), and covalent conjugation of proteins to lipophilic moieties for binding to the lipid bilayer. The resulting suite of HDL-like NPs was stable in complex biological matrices and were shown to be non-cytotoxic *in vitro* at concentrations up to 320 mg/ml. Administration of the particles *in vivo* did not cause

weight loss, organ specific toxicity or overt immunogenicity. Biodistribution of the particles varied by route of administration, with preferred accumulation of the particles in kidney and liver after intravenous, intraperitoneal, intramuscular or subcutaneous administration.

3.5 HDL-like Nanoparticles: Intrinsically Therapeutic Agents

More recently, HDL-like AuNP constructs have shown intrinsic therapeutic activity without requiring additional molecular drug cargo [98]. The HDL-like AuNPs are similar to the nanoparticles our group employed for cholesterylated nucleic acid formulation, as previously discussed. The structural conformation and number of apoA-I molecules, as well as phospholipid number, particle size, and negative particle charge are consistent with natural HDLs [71, 99]. Our data demonstrate that the HDL-like AuNPs directly compete with natural HDLs to target SR-B1, a high-affinity HDL receptor expressed by lymphoma cells. Uniquely, the AuNP template at the core of the HDL-like AuNP enables differential modulation of cholesterol flux as compared with natural HDL. Data show that binding of SR-B1 and the manipulation of cholesterol homeostasis in B lymphoma cells upon treatment with the HDL-like AuNP leads to a selective induction of apoptosis. Furthermore, upon HDL-like AuNP treatment to mice bearing B-cell lymphoma xenografts, data show inhibition of B cell growth as compared to mice treated with human HDL or saline. Collectively, these data suggest that HDL-like AuNPs may provide opportunities as stand-alone therapies due to cooperative SR-B1 targeting and manipulation of cellular cholesterol homeostasis in B cell lymphoma [98].

In addition to our findings, Zheng et al found that their SR-B1-targeted HPPS NP inhibited motility and colony formation of nasopharyngeal carcinoma (NPC) cells [67, 86, 103]. The NPs were active in nude mice bearing subcutaneous tumors. This effect involved neither tumor cell necrosis nor apoptosis, suggesting an alternative tumor-shrinking mechanism that is related to HPPS-induced inhibition of NPC cell motility and colony formation. ApoA-I-mimetic peptides have been shown to exhibit anti-tumor activities by reducing plasma levels of lysophosphatidic acid, a stimulator of cell migration, invasion and colony formation [104]. While apoA-I-peptide may be involved in the anti-tumor mechanism of HPPS, the exact mechanism is still unknown.

In conclusion, a significant body of evidence demonstrates that biomimicry may play an important role for next generation cancer therapies. Synthetic HDL-like NP mimics are appealing as new treatments, due to their inherent active targeting to cancer cells and their ability to deliver diverse therapeutic cargo. Importantly, synthetic HDL-like NPs can be manipulated such that they are able to bind and deliver small molecules, photothermal reactive agents, nucleic acids, and may also be stand-alone entities that are intrinsically active. Furthermore, HDL-like NPs can also serve as delivery vehicles for imaging agents. Research directed at these intriguing nanostructure conjugates has only begun. Further pre-clinical and clinical development of these new approaches based upon HDL biology may offer tremendous new therapeutic opportunities for patients with cancer.

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Table 1.1

Examples of the structural diversity of HDL-like NPs for drug delivery.

Structural feature	Examples
Composition of particle core	Cholesterol esters [54] Inorganic scaffolds (Au) [64]
Shape	Discoidal [65] Spherical [64]
Protein associated with particle	Full-length apoA-I [66] ApoA-I-mimetic peptide [67] ApoE [68]
Phospholipids	Phosphatidylcholine (PC) [69], 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) [70] 1,2-dimyris-toyl-sn-glycero-3-phosphocholine (DMPC) [70] 1,2-dioleoyl-sn-gly- cero-3-((N(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl)(- nickel salt) (NiLipid) [70] 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (DSPE-PEG2000-folate) (PF) [70] 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate] (PDP PE) [5,71] Dipalmitoylphosphatidylcholine (DPPC) [5,71] Pyropheo-phorbide conjugated to the glycerol backbone of lysophospholipids [65]
Lipid layer surrounding the particle	Monolayer [72] Bilayer [64]
Mechanism of drug loading	Covalent attachment [70] Encapsulation [73] Integration in lipid layer [74]
Additional targeting moieties	Folic acid, covalently linked to lysine residues of apoA-I [75] Lipid-conjugated folate (1,2-distearoyl-sn-glycero-3- phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (DSPE-PEG2000-folate) (PF) [70]
Incorporated drug	Paclitaxel [76] Doxorubicin [77] Valrubicin [62] Fenretinide [78]
Incorporated fluorophore	Fluo-BOA [79] Lipid-conjugated Rhodamine B [80]