Published in final edited form as:

Nanoscale. 2017 December 21; 10(1): 366–377. doi:10.1039/c7nr06295h.

Core Hydrophobicity Tuning of a Self-Assembled Particle Results in Efficient Lipid Reduction and Favorable Organ Distribution

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Abstract

Atherosclerosis, the deadliest disease in the United States, arises due to the building up of plaques in the arteries as a result of excessive cholesterol deposition and impaired cholesterol removal process. High density lipoproteins (HDL), popularly known as “good cholesterol”, are naturally occurring nano-sized particles that, along with apolipoproteins are deployed to maintain cholesterol homeostasis in the body. Both cholesterol efflux, from the fat-laden macrophages in the arteries, and intracellular lipid transport, to deliver cholesterol to the mitochondria of liver cells for metabolism, hold key responsibilities to maintain healthy lipid levels inside the body. We designed a library of nine mitochondria targeted polymer-lipid hybrid nanoparticles (NPs), comprised of completely synthetic yet biodegradable components, that are capable of performing HDL-like functions. Using this library, we optimized a superior mitochondria targeted NP candidate which can show favourable organ distribution, therapeutic potential, and non-toxic properties. Two targeted NP formulations with optimum NP size, zeta potential, and cholesterol binding and release properties were identified. Lipid reduction and anti-oxidative properties of these two NPs demonstrated cholesterol removal ability. *In vivo* therapeutic evaluation of targeted-

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S.D. designed the research, supervised experiments; B.B., R.W., S.M., A.K., N.K. performed research; B.B., R.W., and S.M. contributed reagents; N.K. helped in designing experiments and supervised R.W. in initial experiments; A.K. assisted in experiments with Balb/c and apoE^{-/-} mice; E.W.H. performed and analyzed histopathology studies; B.B., R.W., S.M., N.K., E.W.H., and S.D. analyzed the data; B.B., N. K., R.W., and S.D. wrote the paper. All authors discussed the results and commented on the manuscript.

Electronic Supplementary Information (ESI) available: Description of materials and methods, and additional data are described in Supporting Information. See DOI: 10.1039/x0xx00000x

Conflicts of interest

There are no conflicts to declare.

NP formulation in apolipoprotein E knockout (apoE^{-/-}) mice indicated lipid reduction and anti-inflammatory properties compared to non-targeted NPs. This synthetic targeted NP with potential abilities to participate in both extra- and intracellular cholesterol transport might potentiate therapeutic interventions for heart diseases.

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Optimization of a self-therapeutic mitochondria targeted nanoparticle for lipid reduction and atherosclerosis.

Introduction

Atherosclerosis is a global threat to human race.¹ Ingestion of cholesterol by macrophages within the arteries and formation of foam cells initiate and drive the process of atherosclerotic plaque formation.² Maladaptive inflammatory responses promote plaque progression; mitochondrial dysfunctions, oxidative stress, and pro-inflammatory signals. Macrophage derived foam cells filled with high cholesterol and cholesteryl ester (CE) influence the stability of plaques and their progression.³ Cholesterol removal from foam cells by cellular cholesterol efflux is an orchestrated pathway operated by ATP-binding cassette (ABC) transporters, apolipoproteins such as apoA-I, apoE, and high-density lipoproteins (HDL).⁴ The extracellular cholesterol efflux by ABC transporters, apoA-I, and HDL is well studied.⁵⁻⁶ However, efficient removal of cholesterol from foam cells depends on the intracellular lipid transport and is not well understood.⁷ The intracellular cholesterol transport regulates the sterol contents in organelles such as mitochondria.⁷ Steroidogenic acute regulatory protein (StAR)-related lipid transfer domain with a hydrophobic lipid binding pocket participate in maintenance of cellular cholesterol.⁸ Thus, the relative contribution of extra- and intracellular cholesterol plays a significant role in removal of lipids from foam cells.

The cleavage of cholesterol side chain by P-450 side chain cleavage enzyme is the initiation step of steroid biosynthesis and this reaction takes place at the inner mitochondrial membrane (IMM).^{8–9} The rate of this reaction is determined by the efficiency of StAR protein-mediated delivery of cholesterol to the mitochondria of liver cells.^{10–11} Under the conditions of atherosclerosis, the limited amounts of StAR proteins are incapable of tackling the enormous amounts of cholesterol. Mitochondrial matrix contains less cholesterol since the outer mitochondrial membrane (OMM) contains more cholesterol than the IMM.¹² Thus, under atherosclerotic conditions, an external agent capable of delivering cholesterol directly to the mitochondria bypassing the StAR dependent pathway could significantly enhance the rate of cholesterol metabolism. Here, we report a completely synthetic, scalable, biodegradable, and mitochondria-targeted nanoparticle (NP) with potential abilities to maintain both extra and intracellular lipid homeostasis to provide a unique therapeutic strategy for coronary heart diseases (CHDs) (Scheme 1).

HDLs are nano-sized lipoprotein particles composed of hydrophobic lipid core that is surrounded by phospholipid and apolipoproteins.¹³ This lipoprotein which are known as “good cholesterol” performs the reverse cholesterol transport (RCT).¹⁴ Recent studies explored the possibility of raising HDL levels in the body with the aim to reduce atherosclerosis symptoms. The laboratories of Fayad and Mulder have pioneered the use of reconstituted HDL (rHDL) in the heart diseases.^{15–22} Various other groups around the globe also contributed towards the use of NPs to handle different aspects of atherosclerosis and other diseases.^{23–26} There are also few examples of synthetic NPs which can potentially show HDL like properties.^{27–29} To the best of our knowledge, we are the first to explore the possibility of using synthetic NP with potential abilities to participate in both extracellular cholesterol removal and intracellular cholesterol metabolism pathways for atherosclerotic conditions. In 2013, we reported our preliminary studies on the design and synthesis of a mitochondria targeted nano-sized NP which can functionally mimic HDL.³⁰ In this work, we describe complete composition optimization of such a NP platform for maximum therapeutic gain

Results and Discussion

Mitochondria Targeted Self Therapeutic NP

Based on the composition and design of naturally occurring HDLs, we recently developed a mitochondria-targeted HDL-mimicking polymer-lipid hybrid NP. This mitochondria targeted NP (T-HDL-NP) is composed of a hydrophobic core containing cholesteryl oleate (CO) and biodegradable and FDA approved poly(lactic-co-glycolic acid) (PLGA) polymer.³⁰ This core is surrounded by a 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-polyethylene glycol (PEG) lipid layer embedded with apoA-I mimetic L-4F peptide with Ac-FAEKFKAEVAKDYFAKFW sequence.³¹ The phospholipid shell also contained stearyl-triphenylphosphonium (TPP) bromide (stearyl-TPP). The TPP cation utilizes its delocalized lipophilic positive charge to enable the construct to demonstrate mitochondria association property by taking advantage of the significant negative mitochondrial membrane potential (Ψ_m) that exists across the mitochondrial membranes (Fig. 1).^{30, 32–33} For mitochondria non-targeted control NP (NT-HDL-NP), poly vinyl alcohol (PVA) was used instead of

stearyl-TTP. These NPs were in the size range of 100–150 nm as determined by dynamic light scattering (DLS), a range which is ~10 times higher than that of natural HDL. The larger size of these synthetic NPs can be advantageous to avoid rapid clearance. As a result, these NPs demonstrated long circulation lifetime with favorable biodistribution (bioD) and pharmacokinetic (PK) parameters.³⁰ Furthermore, studies demonstrated that larger HDL might be able to lower the risk of cardiovascular diseases due to enhanced cholesterol efflux properties.³⁴ T-HDL-NPs demonstrated unique abilities to target mitochondria of macrophages and within the mitochondria, T-HDL-NPs were localized mainly in the mitochondrial matrix and the intermembrane space (IMS)³⁰ indicating that this T-HDL-NP has the potential to be able to participate in intracellular cholesterol transport pathway. Preliminary binding studies with 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesterol-3 β -ol or NBD-cholesterol indicated that T-HDL-NPs were capable of binding cholesterol with high efficiency.³⁰

Each of the components in the T-HDL-NP construct serves unique purpose which can result in therapeutic potential of these NPs. Thus, we attempted to determine optimal composition in order to tune and achieve maximum therapeutic potential. We synthesized a library of T-HDL-NPs by varying the ratio of CO to PLGA (Fig. 1A). This library of NPs follows a trend in terms of CO loading, size, zeta potential, and cholesterol binding efficiencies (Fig. 2A). As the percent feed of CO was increased, %CO loading increased, %CO encapsulation efficiency (EE) decreased (Fig. 2A, Table S1). The size and zeta potential trends of this NP library indicated that only by adding suitable amount of PLGA (0.5 mg in total NP), we are able to achieve stable NPs with suitable size and charge (Fig. 2A, Table S1). At a low percent feed of CO such as NPs synthesized with 10–20% CO feed demonstrated large diameter and heterogeneous population (Table S1, Fig. 2A, Fig. S1). Further, transmission electron microscopy (TEM)-based morphological analyses also indicated that NPs at a low CO percent feed demonstrated some degree of aggregation (Figs. S2). A library of NT-HDL-NPs was constructed from PLGA-COOH, CO, PVA, DSPE-PEG-COOH, and 4F peptide through a modified nanoprecipitation as described for T-HDL-NPs by varying the ratio of PLGA to CO (Table S2, Fig. S1). NT-HDL-NPs demonstrated similar size ranges as seen with T-HDL-NPs and had negative zeta potential. Morphology of T-HDL-NPs and NT-HDL-NPs were confirmed by transmission electron microscopy (TEM) (Fig. 2B, Figs. S2–S3). A comparison of morphology of T and NT-HDL NPs indicated similar morphological pattern. Stability studies performed with the T-CO₄₀-HDL and T-CO₇₀-HDL NPs by storing the aqueous solutions at 37 °C and analyses of the NPs in terms of their size, zeta potential indicated that these are highly stable at least over a period of 3 weeks (Fig. S4).

Optimization of Self Therapeutic NPs for Cholesterol Binding and Efflux

In vitro cholesterol-binding assay using NBD-cholesterol with the NP libraries provided insight pertaining to the optimized CO:PLGA ratio so as to maximize the efficiency of cholesterol binding ability of T-HDL-NPs (Fig. 2C, Fig. S5). A time dependent cholesterol binding assay indicated that at low %CO feed with respect to PLGA, the T-HDL-NPs demonstrated highest cholesterol binding abilities. As the %CO feed was increased, cholesterol-binding affinity of the NPs was reduced (Fig. S6). Binding constants (k_d) were calculated by analyzing the binding curves obtained from T-HDL-NP library at a constant

time point of 6 h with “one site total binding” function in GraphPad Prism 5.0 software using the equation: $RFU = (B_{max} * [NBD\text{-}cholesterol]) / (K_d + [NBD\text{-}cholesterol])$. We used incremental concentrations of NBD-cholesterol keeping the concentration of NPs constant. The high K_d values obtained for the NPs with low CO feed imply that NPs were capable of binding large amounts of NBD-cholesterol. Moderately high values for K_d were obtained for the NPs in the range of 40–70% CO feed (Fig. 2C, Fig. S6). NBD-cholesterol binding ability of human HDL (hHDL) was also studied to serve as a positive control (Fig. 2C, Fig. S7). The binding constant for hHDL, when compared to that of T-CO₄₀-HDL-NP, indicated that the binding affinity of NBD-cholesterol towards 40% CO feed T-HDL-NP is significantly higher ($P < 0.0026$). The K_d value for the T-CO₇₀-HDL-NPs was comparable to that for hHDL and significantly lower than the T-CO₄₀-HDL-NP ($P < 0.026$).

The NT-HDL-NPs, on the other hand, demonstrated significantly reduced cholesterol binding properties compared to T-HDL-NPs (Figs. S8–S9). This observation led us to raise a question whether the negatively charged NBD-cholesterol is getting adsorbed on the positively charged surface of T-NPs. Control binding experiments performed with a cationic molecule TPP-(CH₂)₅-COOH and micelles constructed from stearyl-TPP demonstrated that neither stearyl-TPP nor the micelles bind to NBD-cholesterol efficiently (Fig. S10). This led us to conclude that positive surface charge on the NPs is not the sole factor that determines efficacious cholesterol binding. We believe that the positive charge plays significant role in attracting the cholesterol followed by incorporation of cholesterol into the hydrophobic core and lipid layer of this NP. Thus, it is combination of positive charge, core hydrophobicity, and unique composition comprising of lipid layer and apoA-I mimetic peptide presence which contribute to efficacious cholesterol binding abilities of T-HDL-NPs. NPs with 40–70% CO feed showed optimum NP size and differential cholesterol binding kinetics, therefore taking these parameters in account, we decided to use NPs with 40% (T-CO₄₀-HDL-NP) and 70% (T-CO₇₀-HDL-NP) CO feed to carry out *in vitro* and *in vivo* therapeutic studies. The corresponding non targeted NPs were used as controls.

We investigated the release kinetics of bound NBD-cholesterol from the T-HDL-NPs (Fig. 2C). Under physiologically relevant conditions (pH 7.4 and 37 °C temperature), release of bound NBD-cholesterol from T-CO₄₀-HDL-NPs, which had less CO content, was found to be in a more controlled fashion compared to T-CO₇₀-HDL-NPs that had 70% CO feed (Fig. 2C). From the cholesterol release kinetics, it was clear that these NPs release bound cholesterol in a controlled fashion at 37 °C, hence we next assessed the cholesterol binding kinetics at this temperature and compared the binding efficiencies with room temperature (RT) data (Fig. 2D). It was evident that both the formulations demonstrate enhanced cholesterol binding and better release of bound cholesterol at 37 °C when compared to room temperature.

Effect of T-HDL-NPs in Cardiomyoblasts

We previously reported the mitochondrial association properties of T-HDL-NPs.³⁰ The current formulations, T-CO₄₀-HDL-NP and T-CO₇₀-HDL-NP, were tested in H9C2 cardiomyoblasts for determination of their effects on mitochondrial respiration (Fig. 2E, Fig. S11). H9C2 cells are rat ventricular cardiomyoblasts that have hyperpolarized mitochondrial

membrane potential. MitoStress analyses on H9C2 cells in presence of T/NT-CO₄₀-HDL-NP or T/NT-CO₇₀-HDL-NP using Seahorse analyzer suggested that these NPs do not cause any inhibition of mitochondrial respiration up to a concentration of 20 µg/mL (Fig. 2E). At a higher concentration of 50 µg/mL, T-CO₄₀-HDL-NPs demonstrated some degree of inhibition of mitochondrial respiration (Fig. S11).

Therapeutic Potential of Targeted Self Therapeutic NPs using Macrophage Derived Foam Cells

Cholesterol accumulation in arterial wall macrophages resulting in foam cell formation is one of the initial steps of atherogenesis. During foam cell formation, the balance between cholesterol influx and efflux in macrophages becomes dysfunctional.³⁵ Under well-maintained cholesterol homeostasis environment, there exists a balanced equilibrium between cholesterol influx through scavenger receptors and efflux through RCT. We investigated athero-protective effects of T-HDL-NPs on RAW 264.7 macrophage-derived foam cells^{36–38} formed by oxidized LDL (oxLDL). RAW macrophages were treated with oxLDL (100 µg/mL) to induce foam cell formation (Fig. 3, Fig. S12).³⁶ Foam cells derived from macrophages in the presence of oxLDL demonstrate inflammatory signaling, cholesterol accumulation, and oxidative stress similar to the hallmark of early atherosclerotic lesions. To examine therapeutic potential, T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs were used, NP treatment was carried out prior or after addition of oxLDL to gain insight about both preventive and therapeutic potential of these NPs. As controls, hHDL isolated from blood bank produced human plasma was used under both therapeutic and preventive settings. For preventative treatment, T-CO₄₀-HDL-NPs, T-CO₇₀-HDL-NPs, or hHDL were added and allowed to internalize for 6 h. The media was changed and fresh RPMI was added supplemented with Ox-LDL. The cells were further incubated for 24 h. For therapeutic foam cell treatment, after 24 h treatment with lipid deprived media, the media was replaced with RPMI supplement with Ox-LDL and the cells were incubated for 12 h. After which, the media was removed and NPs (T-CO₄₀-HDL-NPs, T-CO₇₀-HDL-NPs) or hHDL were added. Effects on total cholesterol, triglyceride, and anti-oxidative effects of these NPs and hHDL on foam cells were studied (Fig. 3 and 4).

Intracellular triglyceride contents were stained using AdipoRed and live cell imaging was performed to analyze the lipid droplets in foam cells in the absence and presence of T-HDL-NPs or hHDL (Fig. 3). Both methods of treatment indicated that T-CO₄₀-HDL-NPs have the ability to reduce intracellular lipid levels in macrophages (Fig. 3). Lipid reduction abilities of T-CO₇₀-HDL-NP were found to be better under preventive settings compared to abilities seen under therapeutic treatment (Fig. 3). Native hHDL demonstrated more efficient lipid reduction profiles under therapeutic treatment (Fig. 3). Quantification of intracellular triglyceride contents using AdipoRed assay was used to calculate the extent of macrophage transformation into foam cells in the presence of oxLDL (Fig. 4A). Both methods of treatment indicated that T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs have the abilities to reduce macrophage derived foam cell formation (Fig. 4A). Native hHDL was able to the extent of this transformation only under therapeutic settings (Fig. 4A). Quantification of the reactive oxygen species (ROS) in the foam cells demonstrated an elevated oxidative stress levels in cells compared to normal macrophages (Fig. 4B). Treatment of foam cells with T-

CO₄₀-HDL-NPs or T-CO₇₀-HDL-NPs or hHDL attenuated the increased ROS levels in foam cells. The extent of ROS reduction by the NPs was comparable to the effects shown by natural hHDL (Fig. 4B).

Cholesterol Efflux property of Mitochondria Targeted Self Therapeutic NPs in RAW 264.7 and Smooth Muscle Cells

RCT is a process of cholesterol transport from lipid-laden macrophages to liver tissues.³⁹ Thus, cholesterol efflux capacity is an important factor to evaluate the therapeutic efficacy of HDL-mediated atheroprotection *via* RCT.^{40–41} NBD-cholesterol undergoes hydrolysis to about 8% in 24 h.^{42–43} Thus, NBD-cholesterol can be used for analysis of cholesterol efflux capacity when conducted within 24 h.^{42, 44} We conducted a cholesterol efflux study in RAW 264.7 macrophages in presence of T/NT-CO_{40/70}-HDL-NPs (Fig. 5A). NBD-cholesterol treated cells when incubated with T-HDL-NPs, showed excellent cholesterol efflux property (Fig. 5A). The ratio of cholesterol present in the media to that retained by the cells gives a measure of cholesterol efflux property of the NPs. The results indicated that the T-CO₄₀-HDL-NPs were the best in removing cholesterol from RAW264.7 macrophages when compared to T-CO₇₀-HDL-NPs or the non-targeted analogues (Fig. 5A). Similar trends in cholesterol efflux property of the T-HDL-NPs were also observed in smooth muscle cells (SMCs) isolated from normal mouse aorta (Fig. 5A). Contractile nature of the SMCs was ensured by staining the α -smooth muscle actin and imaging the stained cells under a confocal microscope (Fig. 5B). These results further established the potential of the mitochondria targeted self-therapeutic NPs in the context of anti-atherosclerotic treatment.

Biodistribution, Immunogenicity, and Lipid Modulation Properties of T/NT-CO_{40/70}-HDL-NPs in Normal Mice

We previously reported pharmacokinetic (PK) and bioD properties of T-CO₇₅-HDL-NPs in Sprague Dawley rats, which demonstrated favorable profiles.³⁰ The T-CO₇₅-HDL-NPs were mostly distributed in the heart. The ability of therapeutic agents to distribute in the target organs *in vivo* is of importance in order to assess the therapeutic potentials. We set out to determine the bioD properties T/NT-CO_{40/70}-HDL-NPs in BALB/c albino mice. T-CO₄₀-HDL-NP or T-CO₇₀-HDL-NPs or their non targeted controls containing quantum dots (QDs) for detection were administered in BALB/C albino mice at a dose of 10 mg/kg with respect to total NP (0.24 mg/kg for T-CO₄₀, 0.28 mg/kg for T-CO₇₀, 0.31 mg/kg for NT-CO₄₀, and 0.33 mg/kg for NT-CO₇₀ with respect to Cd) by intravenous injection. We used a polymer conjugated QD, PLGA-PEG-QD in these NP formulations.^{30, 32, 45} Detailed characterizations of these NPs are listed in Table S3. Schematic representations of T/NT-CO_{40/70}-HDL-QD-NPs are shown in Fig. 6A, **top row**. QD incorporated HDL-NPs can be seen from un-stained TEM images (Fig. 6A, middle row). Monodispersed populations of T/NT-CO_{40/70}-HDL-QD-NPs can be predicted from size distribution pattern obtained from dynamic light scattering (DLS) measurements and surface charge of these NPs corresponded to positively charged T-NPs and negatively charged NT-NPs (Fig. 6A, bottom row). Normal mice were intravenously injected with 10 mg/kg of T/NT-CO_{40/70}-HDL-QD-NPs and after 24 h treatment; serum samples were analyzed for triglyceride, total cholesterol, and LDL; bioD was followed by Inductively coupled plasma mass spectrometry (ICP-MS). BioD profiles for the NPs in BALB/c mice indicated that the targeted NPs (T-CO_{40/70}-QD-HDL-

NPs) have an inherent tendency to localize in the heart, aorta and liver tissues (Fig. 6B). The T-CO₄₀-QD-HDL-NPs localize predominantly in the aorta and liver whereas the T-CO₇₀-QD-HDL-NPs distribute mostly in the heart and liver (Fig. 6B). The non-targeted NPs (NT-CO_{40/70}-QD-HDL-NPs) distributed mostly in the lungs, liver, and spleen (Fig. 6B). These observations make T-CO₄₀-QD-HDL-NPs ideal for anti-atherosclerotic applications as these NPs are capable of localizing predominantly in the aorta where plaques are localized. Thus, these NPs will be able to collect lipids from the plaques which are mainly located in the aortic wall and transport the lipids to the liver for lipid metabolism since the clearance of these NPs possibly happen through the liver. The reason why the T-CO₄₀-QD-HDL-NPs mostly accumulate in the aorta and the exact fate of the NPs in circulation, in the aorta, and liver need further investigations.

The lipid level analyses of the blood serum of T-CO₄₀-QD-HDL-NP treated mice showed significant reduction in triglyceride (saline vs. T-CO₄₀-QD-HDL-NPs, $P < 0.0001$) and cholesterol (saline vs. T-CO₄₀-QD-HDL-NPs, $P < 0.0001$) (Fig. 6C). Since these are normal mice and are not challenged with high amounts of lipid, the T-CO₄₀-HDL-NPs were able to get rid of the body lipids within as quickly as 24 h. The LDL levels in blood serum of T-CO₄₀-QD-HDL-NP treated group mice were lower than that in the saline treated animals whereas the HDL, the good cholesterol levels were similar in all the groups (Fig. 6C).

Induction of immunogenic response *in-vivo* by the NPs was studied by analyzing pro-inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and IL-1 β in serum using enzyme-linked immunosorbent assay (ELISA). There was no significant difference in the levels of TNF- α , IL-6, and IL-1 β among serum samples when treated with T/NT-CO₄₀-HDL-NPs or T/NT-CO₇₀-HDL-NPs when compared to saline control group (Fig. 6D). Analyses of monocyte chemoattractant protein-1 (MCP-1), the key chemokine which is related to migration and infiltration of monocytes or macrophages, in the NP treated serum samples indicated no enhancement or decrease of this chemokine (Fig. 6D). These studies show the non-immunogenic nature of these T/NT-HDL-NPs at the doses used in this mice study.

Therapeutic Potential of T-CO₄₀-HDL-NPs in ApoE^{-/-} Mice

To evaluate the therapeutic potential of T-CO₄₀-HDL-NP towards atherosclerosis treatment, we chose apoE^{-/-} mouse model as it would be a clinically relevant model for the study. A cohort of apoE^{-/-} mice were fed with normal Chow diet and treated with saline (n = 3), T-CO₄₀-HDL-NPs (n = 3), or NT-CO₄₀-HDL-NPs (n = 3) to understand the lipid reduction profiles of mitochondria-targeted self-therapeutic NPs in contrast to the non-targeted NPs (Table S4 for NP characterization). Saline, T-CO₄₀-HDL-NPs, or NT-CO₄₀-HDL-NPs (10 mg/kg with respect to total NP) were injected intravenously *via* tail vein twice weekly for a total of 7 weeks. Blood samples were collected before starting the treatment to determine the serum lipid levels in all the animals. Serum samples were isolated on predetermined days; total cholesterol, triglyceride, and LDL levels were quantified. NT-CO₄₀-HDL-NPs and saline treated animals served as control groups. Predose total cholesterol, triglyceride, and LDL levels were comparable in the animals from different groups (Fig. 7A). Quantification of serum lipid levels from different groups at the end of the study indicated that T-CO₄₀-

HDL-NP treated animals have significantly reduced levels of triglyceride, total cholesterol, and LDL compared to the control group injected with saline (Fig. 7A). The total cholesterol level in the T-CO₄₀-HDL-NP treated group was significantly less compared to the NT-CO₄₀-HDL-NP group, the animals which were treated with non-targeted NPs also demonstrated reduced total cholesterol compared to the saline group (Fig. 7A). A comparison of predose total cholesterol and LDL with those of saline, T-CO₄₀-HDL-NP, and NT-CO₄₀-HDL-NP treated groups on day 18 clearly demonstrate the efficacy of the targeted NPs to reduce lipid levels (Fig. 7A, **bottom**).

Multifocal accumulations of macrophages lining aortic valve and aorta have enlarged vacuolated cytoplasm that stained positive for lipid (e.g., triglyceride) by Oil Red O staining was observed with the saline treated group (Fig. 7B). Photographs of the aortic valve with Oil Red O positive macrophages on the surface can be seen with the saline treated group. However, there were little to no foam cells or Oil Red O staining along the endothelium of either the T-CO₄₀-HDL-NP or NT-CO₄₀-HDL-NP treated groups (Fig. 7B). Within the myocardium, there was linear Oil Red O staining of the small vessel lumens and myocytes contained minute positive lipid droplets in all the groups.

Caspase 3 plays major role in execution of cascade of events during cellular apoptosis and has been used for apoptosis detection in tissues.^{46–47} Apoptosis of macrophages in plaques is actively involved in the status and progression of atherosclerosis in all stages⁴⁸. Apoptosis staining of caspase 3 was carried out to interpret the apoptotic status of early lipid laden macrophages. Cytoplasmic staining for caspase 3 (brown) was moderate in lipid laden macrophages adhered to the aortic valve (long arrow; left photo) but staining was not evident in adjacent endothelial cells (short arrow; left photo). Within the myocardium (right photo) there is severe staining, mostly cytoplasmic but also nuclear, of endothelial cells (dark brown) lining small vessels within the myocardium (Fig. 7C). At the arrow, the dark staining may be partially due to plaque deposition. Saline treated animals showed most lipid laden macrophages in comparison to the NP treated group. The difference between T and NT-HDL-CO₄₀-NP treated was not very significant from this staining. A larger group of animals might reveal more statistically relevant results. Cytokine analysis in serum samples showed significant reduction in TNF- α with T-HDL-CO₄₀-NP treated group in comparison to saline and NT-NPs treated mice (Fig. 7D). No change in IL-6 level was observed with respect to saline. Further, body weight data indicated that there is no toxicity associated with multiple dosing of T and NT-HDL-CO₄₀-NP (Fig. 7E). Histopathological examinations are shown in Fig. 7F. Hematoxylin and eosin staining of major tissues further supported no toxic effects from the NP treatments.

Conclusions

The NP platform presented here could decrease plaque formation directly by locally accumulating HDL mimic in plaque macrophages, working at the intracellular cholesterol metabolism pathways by targeting the mitochondria. This study indicated the therapeutic potential of T-CO₄₀-HDL-NPs and also highlighted the advantages of mitochondria targeted HDL mimicking NPs over the non-targeted formulation. The rate at which cholesterol is transferred to the mitochondria determines the rate of for cholesterol degradation in the liver.

Therefore, mitochondria targeted T-HDL-NPs will be able to carry excess cholesterol from cytosol to the mitochondria for cholesterol metabolism, thus maintaining proper equilibrium for intracellular lipids. This technology uses biocompatible polymers and lipids to create a synthetic yet biodegradable HDL mimic. The technology presented here is synthetic, biodegradable, non-toxic and the mitochondria targeted HDL mimicking NP has the potential in participating in intracellular lipid homeostasis.

Experimental

Materials and Methods

Description of materials and all methods are described in Supporting Information.

Animals

Balb/c Albino mice (4 weeks old) were purchased from Jackson Laboratory. ApoE^{-/-} mice age five weeks of age (Jackson Laboratory, Bar Harbor, ME) were fed a normal chow diet. All animals were handled in accordance with “The Guide for the Care and Use of Laboratory Animals” of American Association for Accreditation of Laboratory Animal Care (AAALAC), Animal Welfare Act (AWA), and other applicable federal and state guidelines. All animal work presented here was approved by Institutional Animal Care and Use Committee (IACUC) of University of Miami Miller School of Medicine (Protocol# 16–141) and University of Georgia (AUP# A2012 01-011-Y1-A0). All housing, surgical procedures, and experimental protocols were approved by the IACUC Committee of UM and UGA. Animals had free access to chow diet and water during all experiments.

Statistics

All data were expressed as mean $\pm$ S.D (standard deviation). Statistical analyses were performed using GraphPad Prism[®] software v. 5.00. Comparisons between two values were performed using an unpaired Student t test. A one-way ANOVA with a post-hoc Tukey test was used to identify significant differences among the groups.

Cell Lines and Cell Culture

Mouse macrophage RAW 264.7 cells were procured from American type culture collection (ATCC). H9C2 cardiomyocytes was given as a generous gift from Prof. Mark Anderson, University of Iowa. Both RAW 264.7 cells and cardiomyocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM L-glutamine, 1.5 g/L sodium pyruvate, 4.5 g/L glucose, 1% penicillin/streptomycin, and 10% fetal bovine serum. Cells were passed every 2–3 days and restarted from frozen stocks upon reaching pass 20. The mouse aortic smooth muscle cells were cultured in DMEM: nutrient mixture F-12 (DMEM/F-12) media supplemented with 4 mM L-glutamine, 1.5 g/L sodium pyruvate, 4.5 g/L glucose, 1% penicillin/streptomycin, and 5% fetal bovine serum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported, in whole or in part, by the National Institutes of Health Grant R56HL121392 from National Heart, Lung, and Blood Institute and American Heart Association National Scientist Award 14SDG18690009 to S.D., and Sylvester Comprehensive Cancer Center. We thank Lei Song for providing us with SMCs and Dr. Roberto Vazquez-Pardon for useful discussions.

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Fig. 1.

(A) Schematic representation of possible structural orientation of T-HDL-NPs. (B) Various components and their structure used in the construction of synthetic T-HDL-NPs. (C) A cartoon demonstrating variation in the components to generate library of T-HDL-NPs.

Fig. 2.

(A) Diameter, PDI, and zeta potential as determined by dynamic light scattering; % loading and %EE of CO in T-HDL-NP library as determined by the Amplex Red assay, and cholesterol binding constants in the library of HDL mimicking NPs constructed by varying the ratio of CO to PLGA in the core. (B) TEM of 40% and 70% CO feed T-HDL-NPs. (C) Top: Comparison of NBD-cholesterol binding constants at 6 h of T-CO₄₀-HDL-NP, T-CO₇₀-HDL-NP, NT-CO₄₀-HDL-NP, NT-CO₇₀-HDL-NP, and hHDL. Bottom: Release of bound NBD-cholesterol from T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs under physiological pH of 7.4 at 37 °C. (D) Comparison of NBD-cholesterol binding kinetics with T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs at room temperature and 37 °C. (E) Effect of T/NT-CO₄₀-HDL-NPs and T/NT-CO₇₀-HDL-NPs (20 µg/mL) on the mitochondrial respiration of H9C2 cardiomyocytes as determined by MitoStress assay using a Seahorse analyser.

Fig.3.

Intracellular lipid modulation by T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs in foam cells under preventive and therapeutic settings using live cell imaging. For foam cell formation, RAW 264.7 macrophages were treated with oxLDL. Treatment with hHDL, T-CO₄₀-HDL-NPs, or T-CO₇₀-HDL-NPs was carried out before addition of oxLDL under preventive setting and after treatment with oxLDL under the therapeutic setting. Intracellular lipids were stained using AdipoRed and live cell imaging was performed. Representative AdipoRed stained (red) images showed formation of foam cells from RAW 264.7 macrophages upon treatment with oxLDL. Scale bar: 25 μ m.

Fig. 4.

Lipid reduction by T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs in foam cells under preventive and therapeutic settings using AdipoRed assay. For foam cell formation, RAW 264.7 macrophages were treated with oxLDL. Treatment with hHDL, T-CO₄₀-HDL-NPs, and T-CO₇₀-HDL-NPs were carried out before addition of oxLDL under preventive settings and after treatment with oxLDL under the therapeutic settings. Intracellular triglyceride contents were stained using AdipoRed and quantified using a plate reader. (B) Increased ROS levels after foam cell formation and subsequent reduction in oxidative stress in foam cells after

treatment with T-CO₄₀-HDL-NPs or T-CO₇₀-HDL-NPs or hHDL. *, **, *** *P* value 0.01–0.05, 0.001–0.01, and <0.001, respectively. ns: non significant.

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Fig. 5.

(A) Cholesterol efflux from RAW 264.7 cells and SMCs in presence of T/NT-CO_{40/70}-HDL-NPs. (B) Contractile nature of aortic SMCs as evident by staining of α -smooth muscle actin (α -SMA).

Fig.6. (A) Schematic representation of the NP formulations used for bioD study in BALB/c mice, TEM images of the respective NPs, diameter, and zeta potentials of the NPs as determined by DLS. (B) Accumulation of T-CO₄₀-HDL-NPs in the aorta and T-CO₇₀-HDL-NPs in the heart of normal BALB/C mice. T-CO₄₀-QD-HDL-NPs or T-CO₇₀-QD-HDL-NPs (10 mg/kg with respect to total NP) were administered by intravenous injection. NPs were quantified by ICP-MS in organ samples. (C) Levels of serum total cholesterol, triglyceride, LDL, and HDL from the animals administered with T-CO₄₀-HDL-NPs and T-CO₇₀-HDL-NPs. (D) Levels of serum cytokines IL-6, TNF-α, IL-1β, and chemokine MCP-1 from the treated

animals. *, **, *** *P* value 0.01–0.05, 0.001–0.01, and <0.001, respectively. ns: non-significant.

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Fig. 7.

(A) Therapeutic efficacy of T-CO₄₀-HDL-NP and NT-CO₄₀-HDL-NP in apoE^{-/-} mouse model fed with normal diet. Animals (n = 3 for each group) were treated T-CO₄₀-HDL-NPs or NT-CO₄₀-HDL-NPs at a dose of 10 mg/kg (with respect to total NP) twice weekly *via* intravenous injection. Serum lipid levels were compared between the treatments by quantifying triglyceride, total cholesterol, LDL, and HDL. (B) Representative image of Oil Red O staining for aorta and myocardium showing no foam cells with T-CO₄₀-HDL-NP or NT-CO₄₀-HDL-NP treatment in aorta and linear staining of the small vessel lumens in myocardium. (C) Representative images of caspase 3 apoptotic staining in aorta and myocardium from apoE^{-/-} mice treated with saline, T-CO₄₀-HDL-NP or NT-CO₄₀-HDL-NP (D) Pro-inflammatory IL-6 and TNF—cytokine level in the serum at the end of the study. (E) Average body weight of apoE^{-/-} animals during the course of the study demonstrating non-toxic nature of the NPs. (F) Toxicity of targeted HDL NPs in major organs from apoE^{-/-} mouse model fed with normal diet by histopathological analyses with hematoxylin and eosin staining. Scale bar = 100 μm.

Scheme 1.

A pictorial representation of potential action of a mitochondria-targeted biodegradable self-therapeutic NP with the ability to eliminate/reduce atherosclerosis by transporting lipids from plaques to the mitochondria of liver cells for metabolism.