Conformational Changes in High-Density Lipoprotein Nanoparticles Induced by High Payloads of Paramagnetic Lipids

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ABSTRACT: High-density lipoprotein (HDL) nanoparticles doped with gadolinium lipids can be used as magnetic resonance imaging diagnostic agents for atherosclerosis. In this study, HDL nanoparticles with different molar fractions of gadolinium lipids (0 < \( x_{\text{Gd-lipids}} < 0.33 \)) were prepared, and the MR relaxivity values (\( r_1 \) and \( r_2 \)) for all compositions were measured. Both \( r_1 \) and \( r_2 \) parameters reached a maximal value at a molar fraction of approximately \( x_{\text{Gd-lipids}} = 0.2 \). Higher payloads of gadolinium did not significantly increase relaxivity values but induced changes in the structure of HDL, increasing the size of the particles from \( d_{\text{HDL}} = 8.2 \pm 1.6 \) to \( 51.7 \pm 7.3 \) nm. High payloads of gadolinium lipids trigger conformational changes in HDL, with potential effects on the in vivo behavior of the nanoparticles.

INTRODUCTION

Atherosclerosis, the accumulation of lipids and immune cells in arterial walls, is the underlying cause of many cardiovascular diseases. A new generation of treatments for atherosclerosis is particularly focused on the use of drugs against vessel wall inflammation. Recent studies in the field have shown that nanomedicine-based theranostic agents can work more efficiently in targeting the disease sites than the use of bare therapeutics. In this sense, direct targeting of atherosclerotic sites by nanosystems that exploit natural plaque homing mechanisms is a very promising approach to treat this disease. In this sense, high-density lipoprotein (HDL) naturally interacts with lipid-laden plaque macrophages through adenosine \( S'-\)triphosphate-binding cassette transporters A1/G1 and scavenger receptor B1A. Those interactions lead to the transfer of cholesterol from the cells to the nanoparticle and its reverse transport back to the liver.

In this field, the effective use of reconstituted HDLs for therapeutic and/or diagnostic purposes has been broadly reported. Nascent HDL is a small disklike nanoparticle with a diameter of 8–12 nm that comprises an amphiphilic apolipoprotein embracing a planar bilayer of phospholipids. Typically, to generate HDL biomimetic nanoparticles, apolipoprotein APOA1 is incubated with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1-myrstoyl-2-hydroxy-sn-glycero-3-phosphocholine (MHPC), although the use of alternative lipids and peptides has also been reported for HDL-like nanoparticles.

Atherosclerosis

The introduction of paramagnetic magnetic resonance imaging (MRI) probes (e.g., gadolinium ions) in molecules of biomedical interest is an extended practice in the field of molecular imaging, allowing the tracking of the in vivo fate of the molecules by noninvasive means. Paramagnetic compounds influence the physicochemical environment of nearby water molecules, shortening the magnetic resonance longitudinal (\( T_1 \)) or transversal (\( T_2 \)) relaxation times (i.e., increasing the relaxation rates, defined as \( R_1 = 1/T_1 \) and \( R_2 = 1/T_2 \)). However, the intrinsic low sensitivity of \( T_1 \) contrast on MR images demands the use of effective amplification strategies like the preparation of nanoparticles that include high payloads of gadolinium, to increase the longitudinal relaxivity (\( r_1 \)) per particle. In the case of self-assembling systems (e.g., micelles, liposomes, or rHDL), it is frequent to use commercially

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available lipids containing gadolinium chelates, such as Gd-DTPA–DSA\(^{16}\) and Gd-DTPA–DMPE\(^{17−20}\) (diethylenetriami-
neptanecetic acid Gd[III] salts of, respectively, distearlamide
and 1,2-dimyristoyl-sn-glycero-3-phosphethanol amine-N-di-
ethylenetriamine), among others.

Gadolinium phospholipids are incorporated into the particles
during the assembly phase, together with other phospholipids,
thus avoiding the need for chemical synthesis for the
introduction of the probe and allowing the detection of HDL
by MRI techniques.

In the particular case of T1-based contrast enhancement in
MRI, and due to the specific physical mechanisms implicated in
its generation,\(^{15,21−23}\) the use of large amounts of gadolinium
units may not necessarily ensure a high value for the relaxivity
of the molecule,\(^{22,24}\) due to effects such as a disproportionate
increase in the transverse relaxivity (\(r_2\)) with respect to the
longitudinal one (\(r_1\)), limited water accessibility to highly
packed gadolinium ions, the existence of particular regimes of
rotation, or translation and diffusion correlation times of
molecules. As such, T1 relaxivity is strongly dependent on the
composition, stability, and size of the self-assembled nano-
particles. Thus, the introduction of gadolinium-laden lipids as
T1 contrast agents for MRI into self-assembling nanoparticles,
such as HDL, is not a trivial issue, and the proportion of those
lipids in the composition of the particle has to be carefully
adjusted for each particular nanosystem.\(^{24}\)

## RESULTS AND DISCUSSION

The initial aim of this work was to study the effects of the
presence of different loads of Gd-DTPA–DSA lipids in HDL
particles. For this purpose, we synthesized eight different sets of
HDL nanoparticles, with an increasing molar fraction of
\(x_{\text{Gd-DTPA–DSA}} = 0.0, 0.098, 0.175, 0.207, 0.237, 0.263, 0.287,
and 0.333\). For each set of nanoparticles, 10 identical batches (\(n
= 10\)) were prepared, achieving a total of \(n = 80\) samples for our
study (see the Materials and Methods section for further
details).

Five dilutions (1.0, 0.75, 0.5, 0.25, 0.125) of solutions
containing ca. 1.5 mM of total amount of lipids were prepared
for each of the \(n = 80\) samples of HDL (eight compositions, \(n
= 10\) batches each), and the longitudinal (\(T_1\)) and transversal
(\(T_2\)) relaxation times of each of these dilutions were
measured. After calculation of the respective longitudinal (\(R_1 = 1/T_1\))
and transversal (\(R_2 = 1/T_2\)) relaxation rates, these parameters were
plotted versus the total concentration of lipids in mM (see
Figure S1 in the Supporting Information (SI)), revealing strong
linear correlations for all plots. The slopes of the linear
regressions in the plots of the relaxation rates versus the
concentration represent the longitudinal (\(r_1\)) and transversal
(\(r_2\)) MR relaxivities of the particles. MR relaxivities for naive
HDL (no gadolinium) were virtually equal to zero (\(r_1 = 0.00 \pm
0.00\) mM\(^{-1}\) s\(^{-1}\), \(r_2 = -0.04 \pm 0.03\) mM\(^{-1}\) s\(^{-1}\)). The values
determined for the longitudinal relaxivities were \(r_1 = 6.54 \pm
1.95, 7.85 \pm 0.65, 8.34 \pm 0.51, 8.19 \pm 1.24, 8.14 \pm 0.67, 8.61 \pm
0.54, and 8.68 \pm 0.69\) mM\(^{-1}\) s\(^{-1}\), and the values determined for
the transversal relaxivities were \(r_2 = 7.92 \pm 3.24, 10.96 \pm 0.69,
12.17 \pm 1.14, 11.67 \pm 1.68, 12.18 \pm 0.84, 12.71 \pm 0.94, and
12.81 \pm 0.70\) mM\(^{-1}\) s\(^{-1}\), for the HDL compositions containing
molar fractions of gadolinium lipids of \(x_{\text{Gd-DTPA–DSA}} = 0.098,\)
0.175, 0.207, 0.237, 0.263, 0.287, and 0.333, respectively.

When values were plotted versus the molar fraction of
gadolinium lipids included in each set of particles (Figure 1),
we observed that the addition of high payloads of gadolinium
lipids in the composition did not result in a significant increase
in \(r_1\) and \(r_2\) MR relaxivities.

In this plot, a plateau is reached at values of \(r_1 \approx 8\) mM\(^{-1}\) s\(^{-1}\)
and \(r_2 \approx 12\) mM\(^{-1}\) s\(^{-1}\). These values are of the same order of
magnitude as the published values of those parameters for HDL
particles doped with Gd-DTPA–DSA.\(^{16,17,25}\)

Furthermore, the size and shape of the nanoparticles were
measured by using dynamic light scattering (DLS) and
transmission electron microscopy (TEM) techniques. The
mean hydrodynamic diameter measured for naïve HDL
particles (no gadolinium) was \(d_H = 8.4 \pm 1.1\) nm, in agreement
with the 7–12 nm range of sizes reported in the literature.\(^{26,27}\)

For the other seven sets of HDL particles containing Gd-
DTPA–DSA (\(x_{\text{Gd-DTPA–DSA}} = 0.098, 0.175, 0.207, 0.237, 0.263,
0.287, and 0.333\)), mean measured sizes were, respectively,
\(d_H = 8.3 \pm 1.5, 13.2 \pm 14.1, 19.3 \pm 20.0, 23.4 \pm 21.7, 35.4 \pm 20.6,
44.9 \pm 20.2, and 58.5 \pm 21.1\) nm. A plot of the size of the
particles versus their contents in gadolinium lipids, expressed as
molar fraction (\(x_{\text{Gd-DTPA–DSA}}\)), is presented in Figure 2.

Whereas the sizes of HDL particles containing a 0.098 molar
fraction of gadolinium lipids are included within the 7–12 nm
range reported for naïve HDL particles, HDL particles
containing higher molar fractions of Gd-DTPA–DSA revealed
larger hydrodynamic diameters and very high standard
deviation values.

To further investigate the cause of the high standard
deviations observed for the particle sizes, we plotted the
value of each individual sample versus its content in gadolinium
(Figure 2).

This plot suggests the existence of two different particle
populations (delimited by boxes A and B), with defined mean
sizes of \(d_H(A) = 8.2 \pm 1.6\) nm (\(n = 143\)) and \(d_H(B) = 51.7 \pm
7.3\) nm (\(n = 86\)) and reasonably reduced standard deviations.

Type A particles correspond to the typical 7–12 nm disklike
structure of naïve HDL, whereas type B particles are sixfold
larger structures.

To confirm these results, a sample of type A particles (batch
of \(x_{\text{Gd-DTPA–DSA}} = 0.098\)) and a sample of type B particles (batch

![Figure 1](https://example.com/figure1.png)

Figure 1. Variation of the longitudinal (\(r_1\), hollow circles) and
transversal (\(r_2\), filled circles) MR relaxivities for HDL formulations vs
their contents in Gd-lipids (expressed as molar fraction). Each point
represents the mean (±SD) of \(n = 10\) different batches of particles.
Dashed lines correspond to values calculated with a developed model
that explains the experimental results (see SI for details).
of $x_{Gd}$ = 0.333) were characterized by TEM. TEM micrographs of both samples are presented in Figure 3.

The sample of particles with a low ($x_{Gd}$ = 0.098) molar fraction of gadolinium lipids (Figure 3, top) shows small typical disklike structures, characteristic of natural nascent HDL nanoparticles. Disks appear piled back to back, a feature also characteristic of TEM images of natural nascent HDL. On the other hand, similar to DLS measurements, TEM micrographs of the particles with a high molar fraction of gadolinium lipids revealed the presence of two populations: discoidal HDL and (up to sixfold) large nanoparticles (Figure 3, bottom).

As the main goal of this work consisted in the optimization of gadolinium-laden HDL-like particles, we did not further investigate the nature and properties of the larger aggregates. Future work should confirm that functional properties of this sort of larger aggregates are quite different from nascent HDL particles, as it can be suspected by the structural differences revealed by DLS and electron microscopy studies.

Our experimental findings allowed us to develop a model that explains the experimental behavior observed for the mean relaxivity (Figure 1) and mean particle sizes (Figure 2) with respect to the molar fraction of gadolinium lipids used for the preparation of MRI-labeled HDL nanoparticles. The principles of this model are schematically represented in Figure 4, and the model is fully developed in the SI.

The suggested model proposes the following:

1. The use of increasing molar fractions of gadolinium lipids ($x_{Gd}$) in the preparation of HDL induces an increase in the MR relaxivity values of the particles (both $r_1$ and $r_2$), while maintaining the mean size of the particle at $d_H = 8.2 \pm 1.1$ nm, characteristic of the disklike structure of naive HDL.

2. Once a certain critical value of $x_{Gd}$ is reached, the preparation of a batch of particles by a combination of DMPC, MHPC, Gd-DTPA-DSA, and APOA1 can lead to the formation of either HDL-like particles ($d_H =$
8.2 ± 1.1 nm) or a different type of particle with a sixfold mean size ($d_{H} = 51.7 \pm 7.3$ nm). Once formed, both types of particles are stable.

(3) Above this critical $x_{Gd-DTPA-DSA}$ value, the probability of obtaining large particles increases linearly with the molar fraction of gadolinium lipids added to the system.

It is important to remark that, according to this model, there is a critical value of $x_{Gd-DTPA-DSA}$ that should not be exceeded for preparing a batch of Gd-labeled HDL particles, to ensure the formation of typical $\approx 8$ nm disklike structures. If larger amounts of gadolinium are used, there is a probability (whose frequency increases with the amount of gadolinium lipids) of obtaining another kind of larger structure, instead of HDL disks. In fact, this is not the first report of changes in size and morphology of APOA1 stabilized disks after doping the molecules with large amounts of gadolinium lipids.15

This model represents a simple interpretation of the experimental data. When more precise modeling of the data is desired, other effects, like potential “saturation” of $T_1$ in the case of restricted water access to Gd ions at high concentrations, should be taken into account, and more complex models, like the one proposed by Strijkers et al.,28 should be considered.

For the particular case of Gd-DTPA−DSA, we have estimated a value of $x_{critical} = 0.148$ (see the SI for details). However, for other gadolinium lipids this value will most likely vary depending on the nature and properties of the lipids used for labeling HDL. Indeed, it is reasonable to believe that the formation of larger aggregates is not an exclusive event for gadolinium-doped lipids, and it can be expected that other lipids without paramagnetic label cause a similar effect. In any case, the critical value of the molar fraction at which larger aggregates are formed should be estimated for each particular case.

It has been reported that, in solution, disklike structures are thermodynamically more stable than the coexistence of a mixture of APOA1 and separated larger lipid vesicles.26,27

The substitution of a fraction of DMPC lipids by gadolinium lipids, with different structures and properties, may affect the packaging of lipids by APOA1, altering the balance between the enthalpy and entropy of formation of disklike structures, forcing in this way the formation of thermodynamically more favored structures. In this case, it is logical to believe that the use of increasing amounts of gadolinium lipids during synthesis would increase the probability of formation of larger aggregates and that there is a critical point for the amount of gadolinium lipids that can be introduced into the structure of HDL before triggering a conformational change of HDL particles to larger structures.

Furthermore, several authors have suggested a high activation barrier for the formation of disks (sonication is used for disk formation, see Figure 4), but also for the degradation of disks, ensuring the stability of disklike structures in solution, once formed.25,26,29 In other words, spontaneous rearrangements between the pre-existing structures A and B seem unlikely (see the scheme in Figure 4).

Further research should be done to reveal whether the use of alternative MRI probes to Gd-DTPA−DSA, with a composition and structure more similar to DMPC, would induce changes in the morphology of the particles, and at which molar fraction this event would eventually take place.

Figure 4. Schematic representation of the model. Under sonication (energy), a mixture of DMPC, MHPC, and APOA1 in solution yields HDL particles. Low amounts of Gd-DTPA−DSA lipids can be packed in disklike conformations (A). Above a certain threshold, alternative molecular rearrangements (B particles) are more stable. The large aggregate shown here is an example for purely descriptive purposes and the factual structure may be different.
CONCLUSIONS

In conclusion, the labeling of HDL for MRI detection purposes is a very promising feature for in vivo applications of this molecule. However, the use of gadolinium lipids must be cautiously analyzed to avoid conformational changes of the particles, which would yield larger nanosystems with a potentially different in vivo behavior.

MATERIALS AND METHODS

Synthesis of HDL Nanoparticles. Phospholipids: DMPC, MHPC, and Gd-DTPA–DSA (gadolinium diethylenetriamine pentaacetatediethylamidine) were purchased from Avanti Polar Lipids (Alabaster, AL). Apolipoprotein A-I solution (APOA1) was donated by CSL Ltd. (Parkville, Australia). Phosphate buffered saline (PBS; 1x) was obtained from Fisher Scientific (Pittsburg, PA).

HDL nanoparticles were prepared in eight different compositions, following procedures described elsewhere. In brief, three stock solutions containing 10 mg/mL of DMPC, MHPC, or Gd-DTPA–DSA were prepared, using a 3:1 (v/v) mixture of chloroform and methanol as solvent. Then, proper volumes of these stock solutions were mixed to achieve the desired composition of HDL particles. The total amount of lipids in the final mixture was fixed to either 2.5 mg/mL (for n = 5 batches of particles per composition tested) or 5 mg/mL (in another n = 5 batches of particles per composition tested), giving a total of n = 10 batches of particles per composition tested. The mass ratio between single chained lipids (MHPC) versus double chained lipids (DMPC + Gd-DTPA–DSA) was always kept constant at 1/10 (w/w), whereas eight different DMPC/Gd-DTPA–DSA mass ratios (1/0, 1/0.2, 1/0.4, 1/0.6, 1/0.8, and 1/1 all of them w/w) were used, giving HDL nanoparticles containing molar ratios of gadolinium lipids of x_{Gd-DTPA–DSA} = 0.000, 0.098, 0.175, 0.207, 0.237, 0.263, 0.287, and 0.333. A table summarizing the composition of each particle is presented in the SI.

The solvent of each mixture of lipids was removed under reduced pressure to form a lipid film, which was thoroughly dried under vacuum overnight. Films were subsequently hydrated with 5 mL of PBS (1x) and a proper amount of ApoA1 protein (ApoA1 vs total amount of lipids in a constant mass ratio of 1 to 2.5 w/w). Solutions were then incubated at 37 °C for 3 h and then submitted to sonication for 60 min (avoiding overheating by keeping the samples on ice during the whole process). Finally, samples were centrifuged (2880 g, 30 min) to remove metal debris from the sonicator probe and centrifuged again (2880 g, 30 min) in 100 kDa molecular weight cut-off (MWCO) tubes (Vivascip; Sartorius Corporation, Edgewood, NY) to remove uncomplexed lipids. Five batches of samples per composition were incubated overnight at 25 °C (bench), whereas the other five batches of particles per composition were incubated overnight at 37 °C (incubator). For all batches, the samples were further stored at 4 °C.

Magnetic Resonance studies. For each of the n = 80 HDL samples prepared (eight compositions, n = 10 batches each), five different dilutions (1, 0.75, 0.5, 0.25, and 0.125 times the initial concentration) were prepared in PBS (1x), and T1 and T2 MR relaxation times were measured using a Minispec system (Bruker BioSpin MRI GmbH, Ettlingen, Germany), operating at 60 MHz. T1 values were acquired in duplicate with a saturation–recovery sequence of 12 exponentially distributed repetition times ranging from 100 ms to 12 s, whereas T2 values were acquired 4 times, using a CPMG sequence of 3600 points with an interpulse delay of 1 ms.

After measurement of the relaxation times (T1 and T2) for the series of five dilutions for each sample, the corresponding relaxation rates, R1 = 1/T1 and R2 = 1/T2, were calculated and plotted versus the total concentration of lipids in each sample (see Figure S1). Those plots were fitted to linear regressions, corresponding the slope of each plot to the respective ionic relaxivity of the sample under study.

DLS Studies. The hydrodynamic diameter of the particles was measured by DLS. Diluted samples of nanoparticles were prepared in distilled water and measured at 25 °C using a ZetaPALS analyzer (Brookhaven Instruments, Holtsville, NY). Particle sizes were determined as the mean of 10 cumulative acquisitions each of 1 min length. Sizes were measured just after the synthesis of the nanoparticles (t = 0) and 12 and 96 h later, observing no significant changes in size with time.

TEM. TEM microscopy images of selected samples were acquired using a Hitachi H-7650 TEM microscope (Hitachi High Technologies, Pleasanton, CA) operating at 80 kV, coupled to a Scientific Instruments and Applications digital camera, controlled by the Maxim charge-coupled device software.

HDL samples were centrifuged in (MWCO 100 000) centrifugal concentration tubes (Vivascip; Sartorius Corporation, Edgewood, NY) to replace the original buffer solution by acetate buffer (0.125 M ammonium acetate, 2.6 mM ammonium carbonate, and 0.26 mM tetrasodium ethylenediaminetetraacetate at pH 7.4). Afterward, 10 μL of HDL solution was mixed with 10 μL of 2% phosphotungstic acid for negative staining and casted on a 100-mesh Formvar-coated nickel grid (Electron Microscopy Sciences, Hatfield, PA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.6b00108.

Description of the synthesis of HDL nanoparticles, measurement and processing of MR relaxation rates, and modeling of experimental data (PDF)

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Notes

The authors declare no competing financial interest.

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