



LCAT-Remodeled Structural Transition of Reconstituted HDL Enhances SR-BI Binding

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Abstract: Reconstituted high-density lipoprotein (rHDL) particles adopt distinct structural states, yet how these states influence receptor recognition remains incompletely understood. Here, we compared the structural features of discoidal rHDL and LCAT-remodeled spherical-like rHDL model particles and evaluated their interactions with scavenger receptor class B type I (SR-BI). Recombinant apolipoprotein A-I (ApoA-I) was expressed and used to assemble discoidal rHDL, which was subsequently converted into spherical-like rHDL through lecithin-cholesterol acyltransferase (LCAT)-mediated remodeling. Agarose gel electrophoresis, transmission electron microscopy, and ^1H - ^{15}N HSQC NMR spectroscopy confirmed that the two rHDL species differed in electrophoretic mobility, morphology, size distribution, and ApoA-I spectral properties. In particular, ApoA-I in discoidal rHDL exhibited greater NMR visibility than in LCAT-remodeled spherical-like rHDL, consistent with distinct conformational and dynamic states. Biolayer interferometry further showed that LCAT-remodeled spherical-like rHDL model particles bound SR-BI with substantially higher apparent affinity than discoidal rHDL. Together, these findings demonstrate that the structural state of rHDL strongly influences its interaction with SR-BI and support a model in which the LCAT-remodeled structural transition of rHDL is accompanied by ApoA-I reorganization that favors receptor binding. This study provides mechanistic insight into how structural changes in model rHDL particles modulate receptor engagement.

Keywords: ApoA-I; reconstituted HDL; SR-BI; biolayer interferometry

1. Introduction

High-density lipoprotein (HDL) is widely regarded as an anti-atherogenic lipoprotein because higher HDL-cholesterol levels are generally associated with a lower risk of cardiovascular disease [1]. HDL plays a central protective role in reverse cholesterol transport (RCT), a process in which excess cholesterol is removed from peripheral tissues, particularly macrophages in the arterial wall, and delivered to the liver for metabolism or excretion [2,3]. Through this pathway, HDL helps limit foam cell formation and suppress the development of atherosclerosis. Apolipoprotein A-I (ApoA-I), the major structural and functional protein component of HDL, is essential for both HDL assembly and maturation. In addition to maintaining particle integrity, ApoA-I activates lecithin-cholesterol acyltransferase (LCAT), which catalyzes the esterification of free cholesterol (FC) and promotes the conversion of nascent discoidal HDL into mature spherical HDL particles [4,5]. ApoA-I is thus a key determinant of HDL structure and function.

Beyond its role in cholesterol transport, HDL has also been reported to exert anti-inflammatory, antioxidant, and endothelial-protective effects [4]. These multiple biological activities highlight the importance of understanding how HDL structure influences its interaction with receptors involved in lipid transport and signaling, particularly scavenger receptor class B type I (SR-BI) [6].

HDL exists in at least two major structural states during its metabolic lifecycle: nascent discoidal HDL and mature spherical HDL [7,8]. Discoidal HDL is generated during the early stage of HDL biogenesis through the association of lipid-poor ApoA-I with cellular phospholipids and cholesterol exported by ATP-binding cassette transporter A1 (ABCA1) [7]. Structurally, discoidal HDL contains a phospholipid bilayer-like disc, with ApoA-I surrounding the particle edge through its amphipathic α -helices [9,10]. This configuration is well suited for accepting free cholesterol and phospholipids from peripheral cells, thereby initiating the RCT pathway [11,12]. During HDL maturation, LCAT converts free cholesterol into cholesteryl ester, which accumulates within the



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particle core and drives the transition from a discoidal to a spherical architecture [12]. This remodeling process is accompanied by substantial reorganization of both lipid packing and ApoA-I conformation, suggesting that HDL structural state may modulate particle recognition by its receptors.

SR-BI is a high-affinity HDL receptor expressed predominantly in the liver and steroidogenic tissues, and is also present in macrophages, endothelial cells, and other cell types [13–15]. Its best-characterized function is to mediate the selective uptake of HDL-derived cholesteryl esters and free cholesterol without requiring complete particle internalization [13–15]. In the liver, this pathway contributes to the terminal steps of RCT by facilitating cholesterol delivery for metabolism and excretion. Genetic and physiological studies have demonstrated the importance of SR-BI in HDL metabolism: hepatic overexpression of SR-BI lowers circulating HDL-cholesterol and reduces atherosclerosis [16], whereas SR-BI deficiency causes abnormal HDL enlargement and impaired cholesterol handling [17]. In humans, functional variants in SCARB1 are also associated with altered HDL levels and atherosclerosis risk [18].

Although HDL binding to SR-BI has been extensively studied, it remains unclear whether SR-BI recognizes discoidal and spherical HDL particles with different binding properties. Previous *in vitro* studies have shown that recombinant HDL (rHDL) particles containing ApoA-I, ApoA-II, or small amounts of ApoC-III can bind SR-BI [19]. In addition, changes in apolipoprotein composition can alter receptor affinity and cholesterol uptake efficiency [20]. However, these studies have mainly focused on apolipoprotein composition rather than particle morphology, and a systematic comparison of the interactions of discoidal and spherical rHDL with SR-BI is still lacking [19,20]. In particular, the extent to which HDL maturation-associated structural remodeling alters receptor binding has not been clearly defined.

Because native HDL is intrinsically heterogeneous in both size and composition, it is difficult to isolate the specific contribution of particle structure to receptor interaction. To address this issue, we established two defined rHDL models representing discoidal and spherical HDL. Discoidal rHDL was assembled with ApoA-I and phospholipids, and spherical rHDL was generated through LCAT-mediated remodeling of the discoidal precursor. We then characterized the two particle types using agarose gel electrophoresis, transmission electron microscopy, and NMR spectroscopy, and compared their binding to SR-BI by biolayer interferometry. By using compositionally simplified and structurally defined rHDL particles, this study aims to clarify how HDL structural state influences SR-BI recognition and to provide a biophysical framework for understanding HDL–receptor interactions in cholesterol transport.

2. Materials and Methods

2.1. Expression Vector Construction

The coding sequence of full-length human apolipoprotein A-I (ApoA-I, UniProt: P02647) was codon-optimized for expression in *Escherichia coli*. An N-terminal 6×His tag was introduced, and *Nco*I and *Bam*HI restriction sites were incorporated at the 5' and 3' ends, respectively. The optimized gene was synthesized and inserted into the pET-28a (+) vector by restriction digestion and ligation, generating the recombinant plasmid pET-28a (+)-His₆-ApoA-I. For BLI assays, a second construct, pET-28a (+)-Strep-ApoA-I, was generated by replacing the N-terminal His₆ tag with a Strep tag to avoid interference with Ni-NTA biosensors.

The coding sequence of full-length human lecithin–cholesterol acyltransferase (LCAT, UniProt: P04180) was codon-optimized for mammalian expression. A C-terminal 6×His tag was introduced, and *Eco*RI and *Bam*HI restriction sites were inserted at the 5' and 3' ends, respectively. The optimized gene was synthesized and cloned into pcDNA3.1(+) to generate the recombinant plasmid pcDNA3.1(+)-LCAT-His₆ [21].

The gene encoding full-length human scavenger receptor class B type I (SR-BI, UniProt: Q8WTV0) was synthesized and subcloned into the pcDNA3.1(+) vector with a C-terminal His₁₀–Strep tag, generating the construct pcDNA3.1(+)-SR-BI-His₁₀-Strep. All gene synthesis and plasmid construction were performed by GenScript Biotech Corporation (Nanjing, China), and the final construct was verified by DNA sequencing.

2.2. Protein Expression and Purification

ApoA-I. *E. coli* strain BL21 (DE3) cells were transformed with pET-28a (+)-His₆-ApoA-I plasmid and cultured in LB medium supplemented with kanamycin (30 µg/mL) at 37 °C. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the culture reached an optical density at 600 nm (OD₆₀₀) of 0.6 to 0.8 and cells were further cultured for 4 h at 37 °C. For preparation of ¹⁵N-labeled ApoA-I, cells were grown in M9 medium supplemented with ¹⁵NH₄Cl (1 g/L, Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). Cells were harvested by centrifugation at 12,000× g for 30 min at 4 °C. The cell pellet from 1 L of culture was resuspended in 30 mL lysis buffer containing 100 mM Tris-HCl, 150 mM NaCl (pH 8.0), 2 mM

phenylmethanesulfonyl fluoride (PMSF), and a protease inhibitor cocktail (Complete Mini EDTA-free). Cells were disrupted by sonication on ice for three cycles (6 min per cycle, 1.5 s on/1.5 s off, 60% power). The lysate was clarified by centrifugation at $12,000\times g$ at 4 °C and the supernatant was collected for purification. Recombinant ApoA-I was purified by Ni-NTA affinity chromatography (GenScript, Nanjing, China). Briefly, the supernatant was incubated with Ni-NTA resin pre-equilibrated with ApoA-I binding buffer (100 mM Tris-HCl, 150 mM NaCl, pH 8.0) for 1 h at 4 °C with gentle agitation, and then loaded onto a gravity flow column (Econo-Pac Column, Bio-Rad). The resin was washed sequentially with (1) ApoA-I binding buffer containing 1% Triton X-100; (2) ApoA-I binding buffer containing 50 mM sodium cholate; (3) ApoA-I binding buffer alone; and (4) ApoA-I binding buffer containing 20 mM imidazole. Bound ApoA-I was eluted with ApoA-I elution buffer (100 mM Tris-HCl, 150 mM NaCl (pH 8.0), 500 mM imidazole). Finally, the eluted protein was dialyzed against phosphate buffered saline (PBS) (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4) and stored at -80°C until ready for use. Expression, cell harvest, and lysis of Strep-ApoA-I were carried out as described above for His₆-ApoA-I. The clarified lysate was then purified using a StrepCaptureXT affinity column (GenScript, Nanjing, China) in place of Ni-NTA column, with subsequent washing and elution performed according to the manufacturer's protocol.

LCAT. Human embryonic kidney 293S (HEK293S) cells were transiently transfected with the pcDNA3.1(+)-LCAT-His₆ plasmid using polyethyleneimine (PEI) in SMM 293-TI expression medium at a DNA:PEI ratio of 1:3, and conditioned media was collected 4 days after transfection. The medium was clarified by centrifuging at $3000\times g$ for 10 min, followed by centrifugation at $12,000\times g$ for 30 min at 4 °C. The pH of the supernatant was adjusted to 7.5 using 1 M HEPES. For purification, the clarified medium was incubated with Ni-NTA resin pre-equilibrated with LCAT binding buffer (20 mM HEPES, 300 mM NaCl, pH 7.5) for 1 h at 4 °C with gentle agitation, and then loaded onto a gravity-flow column. After washing with LCAT washing buffer (20 mM HEPES, 300 mM NaCl, 20 mM imidazole, pH 8.0), bound LCAT was eluted with the LCAT elution buffer (20 mM HEPES, 300 mM NaCl, 200 mM imidazole, pH 8.0). Purified LCAT was dialyzed into imidazole-free PBS, aliquoted, and stored at -80°C until use.

SR-BI. HEK293S cells were transiently transfected with the SR-BI construct using PEI. Cells were harvested 72 h post-transfection. Cell pellets were thawed and resuspended in SR-BI lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% (w/w) Lauryl Maltose Neopentyl Glycol (LMNG), DNase I, and protease inhibitors. Cells were lysed by gentle rotation for 2–3 h at 4 °C. Insoluble material was removed by centrifugation at $20,000\times g$ for 60 min at 4 °C. The clarified supernatant was incubated with StrepCaptureXT resin for 2 h at 4 °C. The resin was washed with SR-BI washing buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.002% LMNG), and bound protein was eluted with SR-BI washing buffer supplemented with 50 mM biotin. The eluate was concentrated and further purified by size-exclusion chromatography (SEC) on a Superose 6 Increase 10/300 GL column equilibrated in SR-BI washing buffer.

2.3. Reconstitution of SR-BI into Nanodiscs

Membrane Scaffold Protein (MSP) purification and SR-BI nanodisc reconstitution were performed as previously described [22]. Briefly, fractions corresponding to the SR-BI peak were pooled, concentrated, and mixed with purified MSP1E3D1 and detergent-solubilized lipids at a molar ratio of SR-BI: MSP1E3D1: lipid = 1:5:500. The mixture was incubated for 2 h at 4 °C, followed by overnight incubation with pre-washed Bio-Beads at 4 °C to remove detergent. After removal of Bio-Beads, the sample was incubated with StrepCaptureXT resin for 1 h at 4 °C to isolate SR-BI-containing nanodiscs. The resin was washed with SR-BI washing buffer (50 mM Tris (pH 7.4), 150 mM NaCl), and SR-BI-containing nanodiscs were eluted with SR-BI washing buffer supplemented with 50 mM biotin. The eluate was further purified by SEC on a Superdex 200 Increase 10/300 GL column equilibrated in SR-BI washing buffer. Fractions corresponding to the SR-BI nanodisc peak were pooled and used immediately for BLI analysis.

2.4. Preparation of Discoidal and Spherical rHDL

Discoidal rHDL (d-rHDL) were prepared using a modified sodium cholate dialysis method [23] with 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC, Aladdin, purity $\geq 99\%$), free cholesterol (Macklin, purity $\geq 99\%$), and His₆-ApoA-I. Briefly, 10 mg of POPC and 0.57 mg of unesterified cholesterol dissolved in 1 mL of chloroform (CHCl_3) were added to a glass tube, and the solvent was evaporated under a stream of nitrogen to form a uniform lipid film on the inner wall of the tube. Residual organic solvent was removed by overnight vacuum-drying. A 100 mM sodium cholate solution was then added to obtain a final POPC concentration of 13.2 mM, and the mixture was vortexed thoroughly and sonicated in a water bath until transparent. His₆-ApoA-I was subsequently added, and the

mixture was adjusted with 100 mM sodium cholate and PBS to a final sodium cholate concentration of 20 mM. The final molar ratio of POPC: free cholesterol: His₆-ApoA-I was 360:40:3. The mixture was incubated at 4 °C for 2 h. Sodium cholate was removed by incubation with hydrated Bio-Beads (1 g Bio-Beads per mL emulsion) for 12 h at 4 °C, after which the Bio-Beads were removed and the supernatant containing d-rHDL was collected.

LCAT-remodeled spherical-like rHDL (s-rHDL) was generated by incubating d-rHDL with LCAT (molar ratio His₆-ApoA-I: LCAT = 10:1), bovine serum albumin (BSA, 0.75% w/v), and β-mercaptoethanol (1 mM) at 37 °C with shaking for 24 h. LCAT was added in two separate doses to maintain catalytic activity during reaction. rHDL for BLI experiments were prepared following the same protocol except that strep-ApoA-I was used in place of His₆-ApoA-I. All other parameters, including lipid composition, protein-to-lipid ratio, and assembly procedure, remained unchanged. The resulting discoidal and spherical rHDL particles were further purified by size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated with PBS and run at a flow rate of 0.5 mL/min. Fractions corresponding to the major particle peaks were collected for subsequent analyses. After SEC purification, the ApoA-I-equivalent concentration of each rHDL preparation was determined by measuring absorbance at 280 nm using a NanoDrop spectrophotometer and the theoretical extinction coefficient of ApoA-I. Because ApoA-I was the only intentionally retained protein component in the SEC-purified rHDL fractions, the measured protein concentration was used to calculate the ApoA-I-equivalent molar concentration.

2.5. Agarose Gel Electrophoresis

The electrophoretic mobility of d-rHDL and s-rHDL was analyzed by agarose gel electrophoresis. Each rHDL sample (15 µL) was mixed with 5 µL loading buffer containing 60% sucrose and 0.1% bromophenol blue in double distilled water, and then loaded into a 0.8% agarose gel prepared in Tris-borate-EDTA (TBE) buffer (pH 8.3). Electrophoresis was carried out horizontally at 90 V for 1 h at 25 °C in TBE running buffer (pH 8.3). Purified lipid-free ApoA-I was included as a marker corresponding to preβ-HDL-like mobility.

Following electrophoresis, proteins were transferred to a PVDF membrane for 1 h in an ice bath. The membrane was blocked in TBS containing 5% (w/v) non-fat dry milk powder for 1 h at room temperature, and then incubated for 1 h with a mouse anti-His monoclonal antibody (1:50,000). After washing three times with TBS containing 0.1% (v/v) Tween-20 (TBST), the membrane was incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000). The membrane was then washed three times with TBST (5 min each), and immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagents according to the manufacturer's instructions.

2.6. NMR Spectroscopy

Two-dimensional ¹H-¹⁵N TROSY-HSQC spectra were acquired at 308 K on a Bruker Avance 600 MHz spectrometer (Bruker, USA) equipped with a cryogenic probe. For all samples, including lipid-free ApoA-I, d-rHDL, and s-rHDL, the ApoA-I-equivalent concentration was adjusted to 250 µM, and identical acquisition parameters were used throughout. Data were collected with 2048 complex points in the ¹H dimension and 200 data points in the ¹⁵N dimension, using 16 scans per increment. The spectral width was 14 ppm in the ¹H dimension, centered at 4.7 ppm, and 20 ppm in the ¹⁵N dimension, centered at 116.5 ppm. NMR data were processed using NMRPipe [24] and analyzed with Sparky software (University of California, San Francisco, CA, USA).

2.7. Transmission Electron Microscopy

The morphology of purified rHDL particles was examined by negative stain transmission electron microscopy (TEM). Carbon-coated copper grids (CF400-Cu-50, 400 mesh) were glow-discharged using a Pelco easiGlow device (Ted Pella, Redding, CA, USA) to improve even sample spreading. A 5 µL aliquot of each sample was applied to the grid and allowed to adsorb for 1 min, after which excess liquid was removed using filter paper. The grid was then stained with 5 µL of 1% uranyl acetate for 1 min, and excess stain was blotted away. This staining step was repeated three times, followed by air drying.

Specimens were imaged on a transmission electron microscope (Thermo, Hillsboro, OR, USA) equipped with a field emission gun and operated at 120 kV. Representative fields were recorded at a nominal magnification of 36,000×. Particle diameters were measured from the micrographs to assess the size and morphology of the rHDL particles.

2.8. Biolayer Interferometry Analysis

Binding of rHDL to SR-BI was analyzed by biolayer interferometry (BLI) using an Octet RED96e instrument (ForteBio, Fremont, CA, USA). Purified d-rHDL and s-rHDL were diluted in PBS containing 1% BSA to final

starting concentrations of 14.4 μM and 4.8 μM , respectively, and then subjected to three-fold serial dilution to final lowest concentration of 0.18 μM and 0.06 μM , respectively. SR-BI reconstituted into nanodiscs was prepared in PBS. Ni-NTA biosensors were loaded with SR-BI nanodisc solution for 5 min to immobilize SR-BI on the sensor surface. After loading, the sensors were equilibrated in PBS buffer containing 1% BSA for 60 s and then transferred to wells containing serially diluted d-rHDL and s-rHDL for 10 min to record the association phase. Dissociation was monitored by transferring the sensors to PBS buffer containing 1% BSA for 10 min. All measurements were performed at 25 °C. Binding signals were double-referenced to correct for background and nonspecific signal contributions. The resulting sensorgrams were fitted using a 1:1 binding model as an empirical approximation to estimate the apparent equilibrium dissociation constant ($K_{D,app}$). Because the assay involved a membrane protein reconstituted in nanodiscs and lipoprotein particle analytes, the interaction was likely to include multivalent and structurally heterogeneous components. Therefore, the fitted $K_{D,app}$ values should be interpreted as model-dependent apparent affinities rather than intrinsic microscopic binding constants.

2.9. Statistical Analysis

Quantitative data are presented as mean $\pm$ SD unless otherwise stated. For TEM analysis, particle diameters were measured from 100 particles per sample, and comparisons between d-rHDL and s-rHDL were performed using unpaired two-tailed Student's t-test as appropriate.

NMR peak volume, peak height and linewidth, and values from selected matched resonances are presented as descriptive comparisons unless otherwise indicated. Statistical analysis was performed using Origin.

For BLI analysis, each analyte concentration was measured in three technical replicates per independent experiment, and apparent $K_{D,app}$ values were obtained separately for each independent experiment. $K_{D,app}$ values were used for statistical comparison between d-rHDL and s-rHDL.

3. Results

3.1. Expression and Purification of Recombinant ApoA-I and LCAT

To prepare rHDL particles, human ApoA-I was expressed in *E. coli* and LCAT was expressed in HEK293S cells, and both proteins were purified for subsequent reconstitution. SDS-PAGE analysis of ApoA-I showed a single major band at approximately 28 kDa, consistent with the expected molecular weight of mature ApoA-I (Figure 1A). Typical yields of purified ApoA-I were 13–15 mg per liter of bacterial culture.

The observed molecular weight of purified LCAT on SDS-PAGE was approximately 69 kDa (Figure 1B), higher than the 49 kDa predicted from its amino acid sequence. Upon deglycosylation with PNGase F, the molecular weight decreased to 49 kDa (Figure S1B), confirming that the higher molecular weight of the purified LCAT is due to post-translational glycosylation during secretion by HEK293S cells [25].

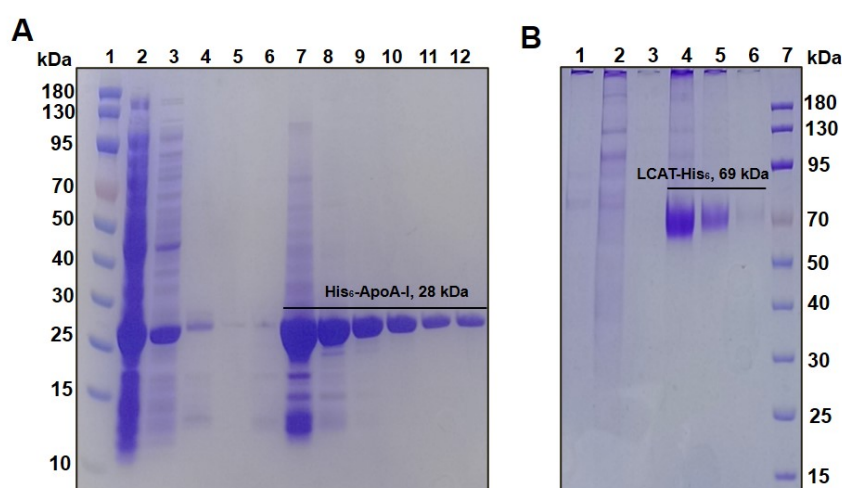


Figure 1. SDS-PAGE analysis of recombinant ApoA-I and LCAT. Representative Coomassie-blue-stained 4–12% SDS-PAGE gels showing the expression and purification of ApoA-I and LCAT. (A) Purification of His₆-ApoA-I. Lane 1, molecular mass marker; lane 2, flow-through; lanes 3–6, wash fractions; lanes 7–12, elution fractions. His₆-ApoA-I migrated at approximately 28 kDa. (B) Purification of LCAT-His₆. Lane 1, flow-through; lanes 2–3, wash fractions; lanes 4–6, elution fractions; lane 7, molecular mass marker. The major glycosylated LCAT-His₆ band migrated at approximately 69 kDa.

3.2. Assembly and Structural Validation of Discoidal and Spherical-Like rHDL

We prepared two types of rHDL: discoidal rHDL (d-rHDL), composed of ApoA-I, POPC and free cholesterol, and LCAT-remodeled spherical-like rHDL (s-rHDL). Although LCAT-mediated remodeling is expected to convert free cholesterol into cholesteryl ester, cholesteryl ester formation was inferred from the LCAT reaction and the observed structural transition from discoidal to spherical rHDL, rather than directly confirmed by lipid composition analysis. To evaluate particle assembly and compare their electrophoretic behavior, d-rHDL and s-rHDL were analyzed by agarose gel electrophoresis. As shown in Figure 2A, both particle preparations migrated as single discrete bands, consistent with relatively homogeneous populations and with no obvious aggregates or detectable unincorporated ApoA-I. Notably, d-rHDL migrated more slowly than s-rHDL. This difference in electrophoretic mobility indicates that the two particle types differ in their surface properties, likely reflecting differences in lipid organization and ApoA-I arrangement [26]. Together, these results support the successful assembly of two structurally distinct rHDL species.

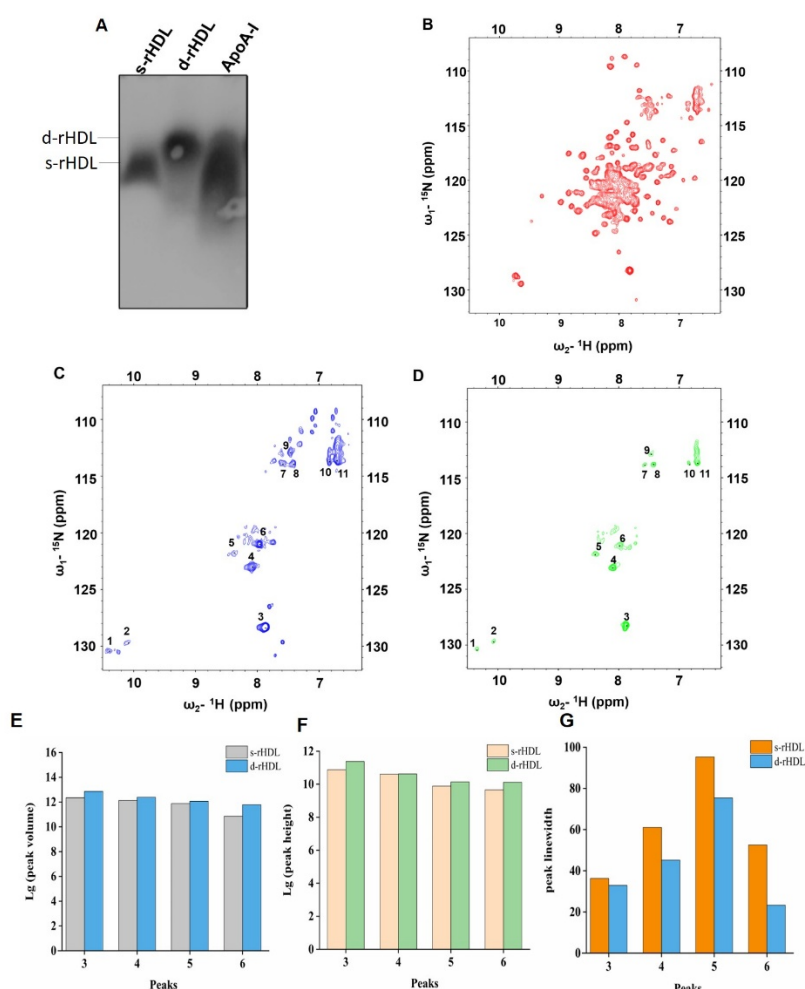


Figure 2. Structural validation of discoidal and spherical rHDL particles. (A) Agarose gel electrophoresis of rHDL showing the distinct electrophoretic mobility of s-rHDL, d-rHDL, and lipid-free ApoA-I. Lane 1, s-rHDL; lane 2, d-rHDL; lane 3, lipid-free ApoA-I. (B–D) Representative ^1H - ^{15}N TROSY-HSQC spectra of ^{15}N -labeled ApoA-I in the lipid-free state and after incorporation into rHDL particles: (B) lipid-free ApoA-I, (C) d-rHDL, and (D) s-rHDL. The 11 selected resonances used for quantitative comparison are indicated by numbers in panels (C) and (D). (E–G) Conservative peak-based comparison of cross-peaks 3–6 between d-rHDL and s-rHDL spectra, showing differences in (E) peak volume, (F) peak height and (G) linewidth.

^1H - ^{15}N HSQC spectra further revealed clear differences between d-rHDL and s-rHDL. As a reference, lipid-free ApoA-I displayed a large number of well-dispersed resonances (Figure 2B). In both d-rHDL and s-rHDL, the number of observable peaks was markedly reduced, and many resonances were broadened beyond detection, consistent with lipid association and reduced overall mobility (Figure 2C,D). To provide a descriptive quantitative comparison between the two rHDL particles, 11 well-resolved resonances were selected from the ^1H - ^{15}N HSQC

spectra for analysis (Figure 2C,D, indicated by numbers). These peaks were chosen because they were clearly observable in both d-rHDL and s-rHDL spectra, exhibited relatively high signal-to-noise ratios (SNR), and showed comparable chemical shift positions between the two samples, as assessed using Sparky. Because resonance assignments were not available for the rHDL-bound states, peak selection was based on these empirical spectral criteria. Considering the chemical shift positions of the selected resonances, the main peak-based comparison was restricted to cross-peaks 3–6, whereas peaks 1–2 and 7–11 were retained only as descriptive supplementary Materials because they may include Trp H ϵ and Asn/Gln side-chain resonances. For cross-peaks 3–6, peak volume, peak height, and linewidth were measured in d-rHDL and s-rHDL (Figure 2E–G), while the complete 11-peak measurements are provided in Supplementary Tables S1 and S2. Compared with d-rHDL, ApoA-I in s-rHDL exhibited lower peak intensity and volume together with broader linewidths (Figure 2E–G) for these selected resonances. These spectral changes indicate that ApoA-I adopts distinct conformational and dynamic states in the two rHDL particles. In particular, the reduced NMR visibility observed for s-rHDL is consistent with its larger particle size and the presence of a hydrophobic core, both of which would slow molecular tumbling and enhance transverse relaxation [27,28]. Under these conditions, only the more flexible regions of ApoA-I are expected to remain observable in the spherical particles.

3.3. Direct Visualization and Size Determination by TEM

To obtain homogeneous rHDL preparations for structural analysis, the reconstituted particles were purified by SEC to remove heterogeneous species and free components. The morphology and size of the purified rHDL particles were then examined by negative-stain TEM. For each sample, TEM images were collected from 5 non-overlapping fields of view, and 20 particles were selected from each field using Velox software (Thermo Fisher Scientific, Waltham, MA, USA), yielding a total of 100 particles measured per sample. Particle diameter measurements were not performed in a blinded manner, because the distinct morphologies of d-rHDL and s-rHDL made sample identity readily apparent during image analysis.

d-rHDL appeared as thin, disk-shaped particles and frequently displayed a characteristic stacked arrangement in the micrographs (Figure 3A,B), a feature commonly observed for discoidal HDL under negative-stain conditions. Measurement of particle diameters showed that d-rHDL had an average diameter of 13.82 ± 2.23 nm (For detailed values, see Supplementary Figure S2A). In contrast, s-rHDL appeared as uniformly rounded particles without obvious stacking (Figure 3C,D). These spherical particles were substantially larger, with an average diameter of 22.88 ± 3.45 nm (For detailed values, see Supplementary Figure S2B). Unpaired two-tailed Student's t-test revealed a statistically significant difference between the two groups ($p < 0.001$, $n = 100$ particles per sample). Particle diameters were estimated from negatively stained TEM images and may therefore represent an upper-bound approximation of the actual particle size. The larger size of s-rHDL is consistent with the formation of a neutral lipid core during LCAT-mediated remodeling, which increases particle dimensions relative to the discoidal precursor. Together, the TEM data further support the structural distinction between discoidal and spherical rHDL.

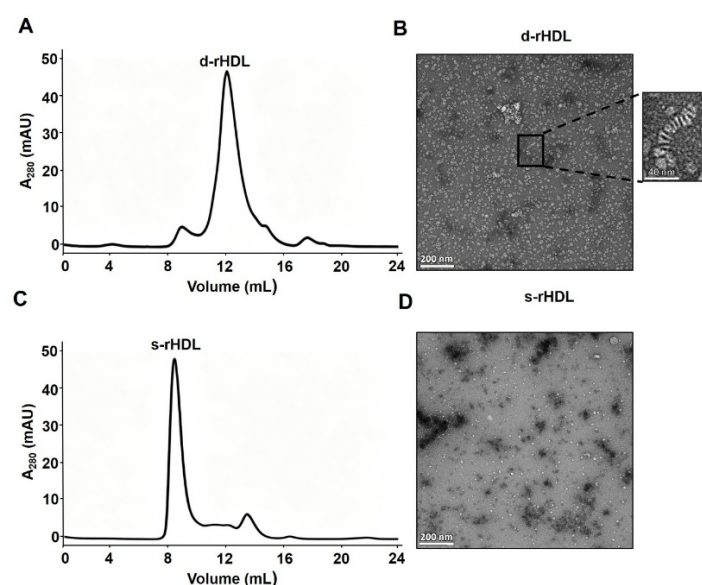


Figure 3. Purification and morphological characterization of discoidal and spherical rHDL by SEC and TEM. (A) Representative SEC profile of discoidal rHDL (d-rHDL). (B) Negative-stain TEM image of purified d-rHDL, showing

disc-like particles with typical stacked morphology; the inset shows a magnified view of a representative stacked particle assembly. (C) Representative SEC profile of LCAT-remodeled spherical-like rHDL (s-rHDL). (D) Negative-stain TEM image of purified s-rHDL, showing rounded particles without obvious stacking. Scale bars, 200 nm.

3.4. Binding Kinetics of rHDL to SR-BI Measured by BLI

Having established two structurally defined rHDL models, we next examined their interactions with SR-BI by BLI. Recombinant SR-BI was purified, reconstituted into nanodiscs, and its purity was verified by SDS-PAGE (Figure S1). Purified SR-BI nanodiscs were immobilized on Ni-NTA biosensors, and binding to increasing concentrations of d-rHDL and s-rHDL was monitored in real time. SR-BI nanodisc loading was standardized by applying a fixed loading time of 5 min and using sensors with similar loading responses (approximately 0.5 nm), thereby reducing variation in receptor loading on the sensor surface. Specifically, nonspecific binding of rHDL to Ni-NTA biosensors was assessed by incubating the analytes with BSA-blocked sensors in the absence of SR-BI. These nonspecific signals were subtracted from all binding sensorgrams before curve fitting. For d-rHDL, concentrations ranged from 0.18 μM to 14.4 μM (three-fold serial dilution), whereas for s-rHDL, the concentration range was 0.06 μM to 4.8 μM . The lower concentration range was used for s-rHDL because preliminary experiments showed that higher concentrations ($\geq 14.4 \mu\text{M}$) led to abnormal binding curves, likely due to signal saturation or nonspecific aggregation at the high-affinity interface [29]. Therefore, the concentration range for each particle was optimized individually to ensure well-resolved association and dissociation phases. Both d-rHDL and s-rHDL particles bound to SR-BI in a concentration-dependent manner (Figure 4A,B). Fitting of the sensorgrams yielded an apparent equilibrium dissociation constant ($K_{D,app}$) of $(2.75 \pm 0.03) \times 10^{-6} \text{ M}$ for d-rHDL, $R^2 = 0.9479$. In contrast, s-rHDL exhibited substantially stronger binding, with an apparent $K_{D,app}$ of $(2.36 \pm 0.02) \times 10^{-7} \text{ M}$, $R^2 = 0.9530$, corresponding to an approximately 10-fold higher apparent affinity. These values were used here as model-dependent comparative measures of binding strength under the present assay conditions. These results indicated that spherical rHDL interacted more strongly with SR-BI than discoidal rHDL under the present assay conditions.

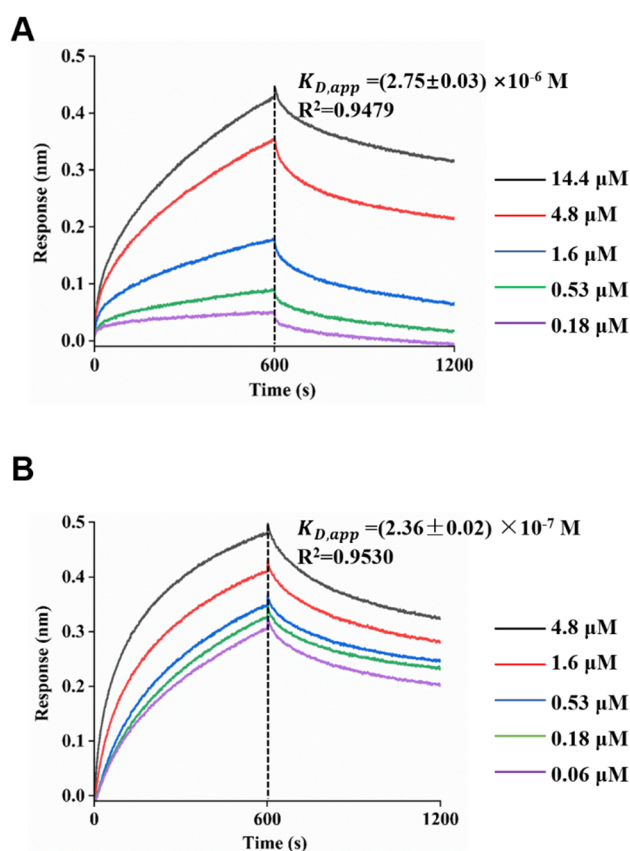


Figure 4. BLI analysis of the interactions between rHDL particles and SR-BI. (A) Representative biolayer interferometry (BLI) sensorgrams showing concentration-dependent binding of discoidal rHDL (d-rHDL) to immobilized SR-BI. The concentrations shown are 14.4, 4.8, 1.6, 0.533, and 0.178 μM (reported as ApoA-I-equivalent concentration). The dashed line indicates the transition from association to dissociation phase. Sensorgrams were fitted with an empirical 1:1 model to estimate the apparent equilibrium dissociation constant $K_{D,app}$. (B) Representative

BLI sensorgrams showing concentration-dependent binding of LCAT-remodeled spherical-like rHDL (s-rHDL) to immobilized SR-BI. The concentrations shown are 4.8, 1.6, 0.533, 0.178, and 0.059 μM (reported as ApoA-I-equivalent concentration). Concentrations of d-rHDL and s-rHDL are expressed as ApoA-I equivalents. The reported apparent affinities ($K_{D,app}$) should therefore be interpreted as normalized to ApoA-I concentration rather than absolute particle number. The dashed line indicates the transition from association to dissociation phase. Sensorgrams were fitted with an empirical 1:1 model to estimate apparent binding parameters $K_{D,app}$. The $K_{D,app}$ and R^2 values shown in each panel were obtained from the fitted curves.

4. Discussion

In this study, we established a defined reconstituted system to compare the structural properties and receptor-binding behavior of discoidal and LCAT-remodeled spherical-like rHDL model particles (s-rHDL). By assembling d-rHDL and generating s-rHDL through LCAT-mediated remodeling, we were able to examine how HDL structural state influences SR-BI recognition under controlled conditions. Agarose gel electrophoresis, NMR spectroscopy, and TEM consistently showed that two particle types differed in electrophoretic behavior, morphology, particle size, and ApoA-I spectral properties. Most importantly, the two rHDL species exhibited markedly different binding to SR-BI. The main characteristics of discoidal and LCAT-remodeled spherical-like rHDL were summarized in Table 1.

Table 1. Comparison of discoidal and LCAT-remodeled spherical-like rHDL particles.

Particle Type	Discoidal rHDL (d-rHDL)	Spherical-Like rHDL (s-rHDL)
Preparation method	POPC+ free cholesterol+ ApoA-I (360:40:3), cholate dialysis	d-rHDL + LCAT + BSA+ β -mercaptoethanol (37 °C, 24 h)
SEC elution behavior	Single peak, elution volume 12.05 mL	Single peak, elution volume 8.26 mL
Agarose mobility	Lower mobility (upper band position)	Higher mobility (lower band position)
TEM morphology	Discoidal (disc-shaped)	Spherical (round-shaped)
Particles Diameter	$\sim 13.82 \pm 2.23$ nm	$\sim 22.88 \pm 3.45$ nm
NMR spectral visibility	Higher (more narrow peaks, stronger signals)	Lower (broader and fewer peaks, weaker signals)
$K_{D,app}$ for SR-BI	$(2.75 \pm 0.03) \times 10^{-6}$ M, $R^2 = 0.9479$	$(2.36 \pm 0.02) \times 10^{-7}$ M, $R^2 = 0.9530$
Key interpretation	Flexible ApoA-I conformation, weaker receptor binding	ApoA-I reorganization, larger size with hydrophobic core, stronger receptor binding
Main limitation	Non-native discoidal morphology	Larger size than most native HDL subclasses

The s-rHDL particles generated here had an average diameter of approximately 23 nm by negative-stain TEM, which is larger than most native plasma HDL subclasses, typically reported in the approximately 7–14 nm range depending on the analytical method. It is also larger than many previously reported LCAT-remodeled spherical rHDL particles. Therefore, the spherical particles used in this study should be regarded as LCAT-remodeled, compositionally defined model particles rather than direct size-matched mimics of native HDL subclasses. This larger size may reflect the lipid-to-protein ratio, remodeling conditions, and simplified composition of the reconstituted system. Nevertheless, because d-rHDL and s-rHDL were generated and analyzed within the same experimental workflow, this system remains useful for comparing how rHDL structural state influences ApoA-I spectral behavior and apparent SR-BI binding.

The apparent equilibrium dissociation constant ($K_{D,app}$) of s-rHDL for SR-BI was approximately one order of magnitude lower than that of d-rHDL, indicating substantially stronger binding of the spherical particle under the present assay conditions (Figure 4A,B). This difference is likely influenced by multiple structural features, including particle size, lipid organization, and the presence of a neutral lipid core. Our results are generally consistent with previous observations that larger and more lipid-rich HDL particles can display stronger interaction with SR-BI [30]. At the same time, our finding differs from an earlier report [31] showing stronger binding of discoidal rHDL. One possible explanation is the differences in experimental design: the previous study compared recombinant discoidal HDL with plasma-derived spherical HDL, whereas the present study compared two compositionally defined recombinant particles generated within the same reconstitution system. This distinction may be important because it minimizes the compositional heterogeneity that is intrinsic to native HDL and allows particle morphology and maturation state to be evaluated more directly.

Our data further indicated that LCAT-remodeled structural transition of rHDL was associated with altered ApoA-I dynamics and particle architecture, which may contribute to the differential binding to SR-BI between d-rHDL and s-rHDL. The NMR spectra showed markedly reduced signal visibility for ApoA-I in s-rHDL compared with d-rHDL, consistent with differences in conformational and dynamic behavior between the two particle states. In the spherical particle, broader lines and lower peak intensity are expected from slower overall tumbling and

enhanced transverse relaxation associated with the larger particle size and hydrophobic core [28]. Our NMR data are therefore best interpreted as supportive evidence for altered ApoA-I spectral behavior and particle dynamics. Although these data do not define the receptor-binding interface at residue-level resolution, they support the idea that LCAT-remodeled structural transition of rHDL is accompanied by redistribution of ApoA-I dynamics. Such structural reorganization may alter the presentation of ApoA-I and lipid surfaces to SR-BI and thereby modulate receptor binding. Although these observations are consistent with a role for ApoA-I reorganization in differential SR-BI binding, they do not establish the structural mechanism directly. At the current level of analysis, the present data cannot distinguish whether the observed binding difference is driven primarily by changes in ApoA-I conformation, lipid surface presentation, particle size, or a combination of these factors. Accordingly, the mechanistic interpretation proposed here should be considered a working model supported by the present biophysical data, rather than a definitive structural explanation of SR-BI recognition.

This observation is important because SR-BI functions as a major receptor involved in HDL binding and selective lipid uptake [32]. Our results suggest that SR-BI does not interact equivalently with all HDL particles, but instead is sensitive to HDL structural state. In this context, the stronger apparent binding of s-rHDL suggests that LCAT-remodeled structural transition of rHDL may generate a particle surface that is more favorable for receptor association. However, because the present study measures binding rather than receptor-mediated lipid transfer or cellular uptake, the stronger association of s-rHDL with SR-BI should be interpreted as evidence for differential receptor binding, not as direct proof of enhanced functional cholesterol transport. Future studies using cell-based lipid uptake assays, selective cholesteryl ester transfer assays, or fluorescent/radiolabeled HDL lipid-tracing approaches will be required to determine whether the observed binding difference translates into altered SR-BI-mediated lipid transport. Therefore, the present results provide a biophysical basis for differential SR-BI recognition of distinct rHDL states, while the functional consequences for cholesterol delivery remain to be directly established.

The preferential interaction of SR-BI with LCAT-remodeled spherical-like rHDL also has broader implications for HDL biology. It suggests that LCAT-remodeled structural transition of rHDL may serve two coordinated functions: first, conversion of free cholesterol into a neutral-core form that can be efficiently transported, and second, optimization of particle recognition by the receptor responsible for selective uptake. Conversely, the weaker binding of discoidal HDL may be compatible with its role as an earlier, more lipid-acquiring form of the particle. From this perspective, structural remodeling of HDL is not merely a passive consequence of cholesterol esterification, but a process that may directly shape receptor selectivity within the reverse cholesterol transport pathway.

Several limitations of this study should be acknowledged. First, the reconstitution system used here cannot fully reproduce the compositional diversity and dynamic remodeling of HDL in plasma. Although LCAT-mediated remodeling is expected to convert free cholesterol into cholesteryl ester, the cholesteryl ester content of the remodeled particles was not directly quantified in the present study. Therefore, the formation of cholesteryl ester was inferred from the LCAT reaction and the observed structural transition from discoidal to spherical-like rHDL, rather than directly confirmed by lipid composition analysis. The lipid composition, including cholesteryl ester content will be examined in future work using direct lipid analysis methods such as LC-MS, TLC, or enzymatic lipid assays. Second, the BLI measurements provide model-dependent apparent binding parameters in a simplified *in vitro* setting. Because the assay involves nanodisc-reconstituted SR-BI and lipoprotein particle analytes, the interaction likely includes multivalent and heterogeneous interfaces that are not fully captured by a 1:1 model. Third, although the NMR data reveal clear differences in ApoA-I spectral behavior between discoidal and spherical-like rHDL, they do not identify the precise structural determinants responsible for SR-BI recognition. Backbone relaxation measurements such as R_1 , R_2 , and τ_c would be valuable for directly comparing ApoA-I dynamics in d-rHDL and s-rHDL, but these experiments were not performed in the present study. Future studies integrating higher-resolution structural approaches, mutational analysis, and cell-based functional assays will be needed to define the molecular interface between SR-BI and distinct HDL particles more precisely.

5. Conclusions

In summary, this study shows that discoidal and spherical rHDL differ not only in morphology and ApoA-I dynamics, but also in their binding to SR-BI. Spherical rHDL displayed substantially stronger apparent affinity for SR-BI than discoidal rHDL, supporting the view that HDL maturation reshapes receptor recognition. These findings provide a biophysical framework for understanding how HDL structural state influences SR-BI engagement and highlight the importance of receptor discrimination between distinct HDL forms in cholesterol transport.

Supplementary Materials: The following supporting information can be downloaded at: <https://media.scilit.com/articles/others/2610151338327289/HM-26040110-SM-FC.pdf>, Figure S1: SDS–PAGE analysis of purified Strep-ApoA-I, glycosylated and deglycosylated LCAT and SR-BI samples used for BLI assays. (A) Purified Strep-ApoA-I. Lane 1, molecular mass marker; lane 2, purified Strep-ApoA-I. (B) Glycosylated and deglycosylated LCAT. Lane 1, molecular mass marker; lane 2, glycosylated LCAT; lane 3, deglycosylated LCAT. (C) SEC fractions containing purified SR-BI in micelles. Lane 1, molecular mass marker; lanes 2–9, SR-BI-containing fractions. (D) Purified SR-BI reconstituted in nanodiscs. Lane 1, molecular mass marker; lane 2, SR-BI nanodisc sample containing SR-BI (upper band) and MSP1E3D1 (lower band); Figure S2: Size distribution of discoidal and LCAT-remodeled spherical-like rHDL particles measured from negative-stain TEM images. Blue histograms show the relative frequency distributions of particle diameters, and red curves indicate Gaussian fits to the distribution. (A) Discoidal rHDL (d-rHDL), $n = 100$ particles, mean diameter = 13.82 ± 2.23 nm, median = 14.16 nm, range = 8.16–18.40 nm. (B) Spherical-like rHDL (s-rHDL), $n = 100$ particles, mean diameter = 22.88 ± 3.45 nm, median = 22.67 nm, range = 14.27–30.66 nm; Table S1: Chemical shift positions, peak volume, peak height, and linewidths of the 11 selected resonances in the ^1H – ^{15}N HSQC spectrum of d-rHDL. w1 and w2 denote the peak positions in the ^{15}N and ^1H dimensions, respectively. lw1 and lw2 denote the linewidths in the ^{15}N and ^1H dimensions, respectively; Table S2: Chemical shift positions, peak volume, peak height, and linewidths of the 11 selected resonances in the ^1H – ^{15}N HSQC spectrum of s-rHDL. w1 and w2 denote the peak positions in the ^{15}N and ^1H dimensions, respectively. lw1 and lw2 denote the linewidths in the ^{15}N and ^1H dimensions, respectively.

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