

Supporting Information for:

Structural and Functional Changes in High-Density Lipoprotein Induced by
Chemical Modification

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Experimental section

Materials

POPC was obtained from NOF Corp. (Tokyo, Japan). Amberlite XAD-2 was purchased from Sigma-Aldrich (St. Louis, MO, USA). TRITC was purchased from Fluka AG (St. Gallen, Switzerland). NAP-5 columns were purchased from GE Healthcare UK Ltd. (Little Chalfont, UK). Spectra/Por Dialysis membranes (molecular weight cut-off, 50 kDa) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). Ham's F-12 medium, Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), LysoTracker® Green DND-26, and MitoTracker® Green FM were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Accumax cell dissociation solution was obtained from Innovative Cell Technologies, Inc. (San Diego, CA, USA). Cell culture dishes were obtained from BD Biosciences Co. (San Jose, CA, USA). Triple-well glass bottom dishes were purchased from Asahi Glass Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

rHDL preparation

For HDL reconstitution, we used the mutated (m)-apoA-I protein containing a deletion of 43 N-terminal amino acids, because it could be obtained at a higher purity than full-length apoA-I using our one-step chromatography system.¹ rHDL particles were prepared with m-apoA-I as described previously.¹ Briefly, lyophilized or TRITC-labeled m-apoA-I samples were solubilized in phosphate-buffered saline (PBS, pH 7.4) containing 4 M urea, and then mixed with POPC at the molar ratio of 1/100 (protein/POPC) in PBS containing 30 mg/mL sodium cholate. After overnight

incubation at 4°C, the mixtures were then dialyzed against PBS at 4°C for 24 h. rHDL particles were further treated with Amberlite XAD2 to remove residual cholate.²

rHDL modification with TRITC

The isothiocyanate group of TRITC reacts with the primary amine groups of the protein to form an amide linkage. TRITC stored in DMSO (10 mg/mL) at -30°C was used in all experiments except those described in Fig. S7. m-apoA-I (pre-modification) or rHDL (post-modification) were reacted with TRITC at the ratios of 0.01/1, 0.04/1, or 0.1/1 (TRITC/protein, w/w) in 0.1 M NaHCO₃ at room temperature for 24 h. The mixtures were then dialyzed against deionized water (m-apoA-I) or PBS (rHDL) at 4°C overnight and purified by gel filtration chromatography using NAP-5 columns to remove unconjugated TRITC. For pre-modification samples, the eluate containing TRITC-labeled m-apoA-I was lyophilized and used to prepare pre-rHDL particles as described above. The eluate containing TRITC-labeled rHDL was used as post-rHDL samples.

Preparation of POPC liposomes and cholate/POPC micelles

To prepare POPC liposomes (mean hydrodynamic diameter, 116 nm), dried POPC film was hydrated in PBS, and extruded using an Avanti® Mini-Extruder and a 100-nm pore membrane (Avanti® Polar Lipids, Inc.; Alabaster, AL, USA). Cholate/POPC micelles were prepared according to the HDL reconstitution procedure without protein addition. The two types of lipid nanoparticles were labeled with TRITC according to the post-modification procedure described above.

Size-exclusion chromatography

TRITC-labeled samples were analyzed using an Agilent 1260 high-performance liquid chromatography system (Agilent Technologies; Santa Clara, CA, USA) equipped with a TSKgel Lipopropak XL column (TOSOH; Tokyo, Japan); TSKgel eluent LP-1 (TOSOH) was used at a flow rate of 0.6 mL/min. Alternatively, an AKTAprime plus liquid chromatography system (GE Healthcare) equipped with a Superdex 200 prep grade 16/60 column (GE Healthcare) was used; elution buffer containing 10 mM Tris-HCl, 100 mM NaCl, 2 mM ethylenediaminetetraacetate (EDTA), 2 mM ethylene glycol bis(2-aminoethylether)tetraacetate (EGTA), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was applied at a flow rate of 1 mL/min.

Cell culture

Chinese hamster ovary (CHO) cells stably expressing hamster scavenger receptor class B type I (SR-BI) were kindly provided by Dr. Hiroyuki Arai of the University of Tokyo. CHO-SR-BI cells were maintained in Ham's F-12 medium supplemented with 10% (v/v) FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM glutamine, and 50 µg/mL gentamycin (G418) as a selective antibiotic, at 37°C in a humidified 5% CO₂/95% air incubator. Cell cultures were passaged every 3–4 days.

Dynamic light scattering analysis

The hydrodynamic (volume-based) diameter of rHDL particles was determined using a Nanotracs UPA-EX250 particle size analyzer (Nikkiso Co., Ltd.; Tokyo, Japan). Zeta potential of rHDL particles in 10-fold diluted PBS was determined using the Zetasizer Nano ZS system (Malvern Instrument; Malvern, UK).

Confocal microscopy

Cultured CHO-SR-BI cells were detached from the substrate using Accumax solution, seeded at a density of 1×10^5 cells/mL, and cultured for 2 days. The cells were treated with 10 μ g protein/mL of pre-rHDL or post-rHDL particles (TRITC/protein, 0.1/1) in G418-free medium at 37°C for 1 h, and then washed once with the medium. Late endosomes/lysosomes or mitochondria of live cells were stained with LysoTracker® Green DND-26 or MitoTracker® Green FM, respectively, according to the manufacturer's protocols. Confocal images were acquired using an Olympus confocal laser-scanning microscope (FV10i-LIV, Olympus Corp.; Tokyo, Japan).

FACS analysis

CHO-SR-BI cells were plated as described above and treated with 10 and 15 μ g protein/mL of pre-rHDL and post-rHDL samples, respectively (to adjust for TRITC content) in the presence or absence of 100 μ g protein/mL non-labeled rHDL in Ham's F-12 medium supplemented with 0.2% bovine serum albumin at 37°C for 1 h. The cells were then detached using Accumax solution, fixed with 2% paraformaldehyde in PBS at 4°C for 15 min, and subjected to FACS analysis using a Guava easyCyte™ flow cytometer (EMD Millipore, Merck KGaA; Darmstadt, Germany).

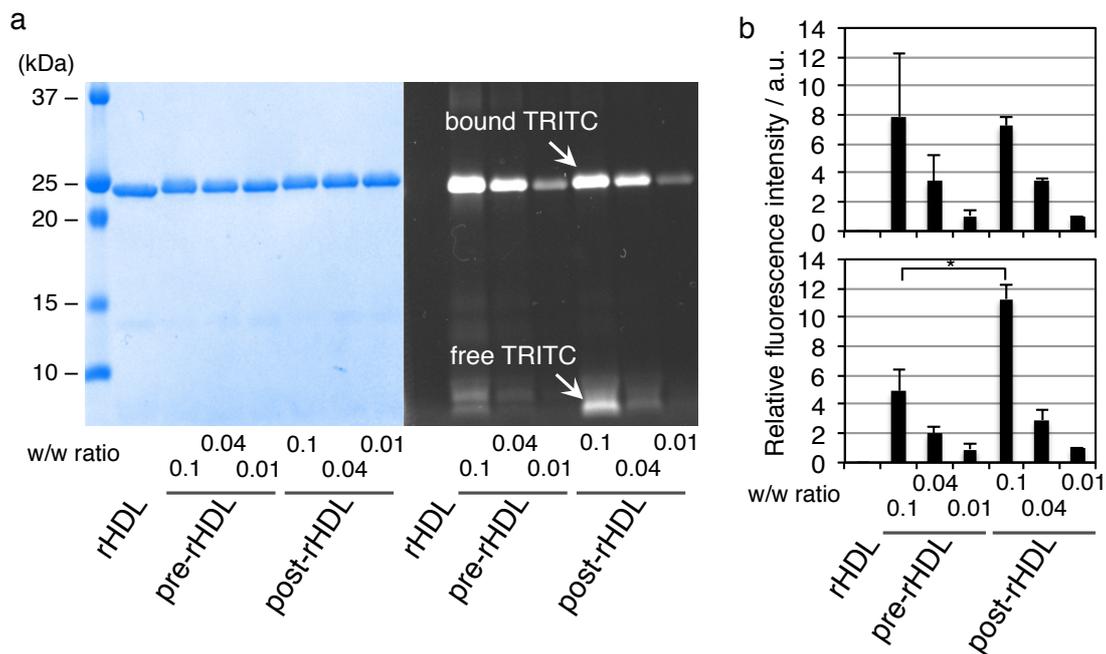


Fig. S1 (a) Coomassie Brilliant Blue staining (left) and fluorescence (right) images of a representative SDS-PAGE gel. Non-modified rHDL (rHDL) was used as a standard. (b) Densitometry analysis of TRITC bound to m-apoA-I (upper panel) or dissolved in the lipid bilayer (lower panel). Fluorescence intensity was normalized to that of post-rHDL prepared at the TRITC/protein (w/w) ratio of 0.01/1. More unconjugated TRITC was trapped in the lipid bilayers of post-rHDL particles than in pre-rHDL particles, while protein modification efficiency was comparable between these samples. The data represent mean \pm standard deviation (SD) for at least three gels. * $p < 0.05$ (two-tailed Student's t-test)

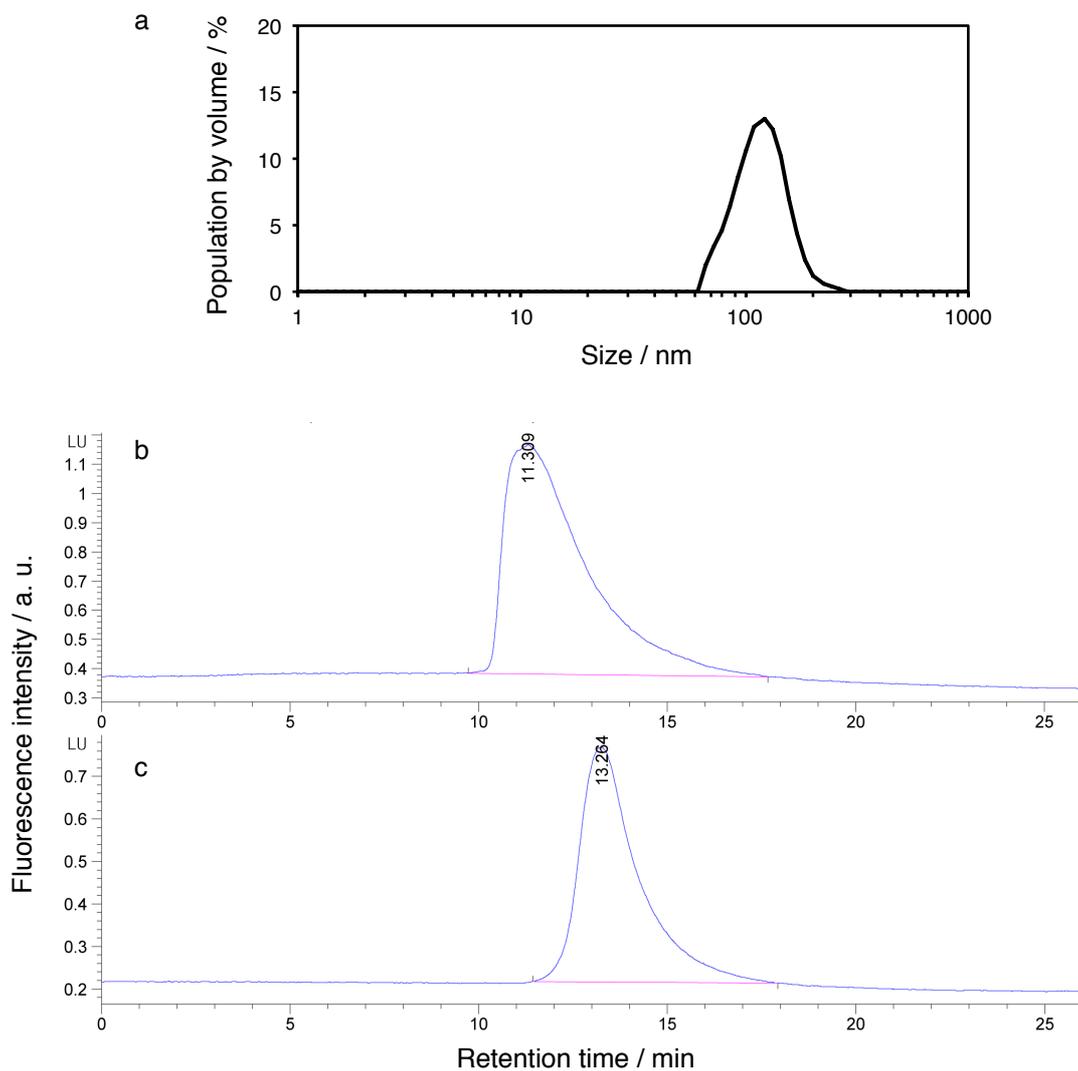


Fig. S2 Size distribution of TRITC-embedded 116-nm POPC liposomes (a); fluorescence-based size-exclusion chromatography of POPC liposomes (b); and post-rHDL particles (c) using a TSKgel Lipopropak XL column (excitation at 529 nm, emission at 596 nm).

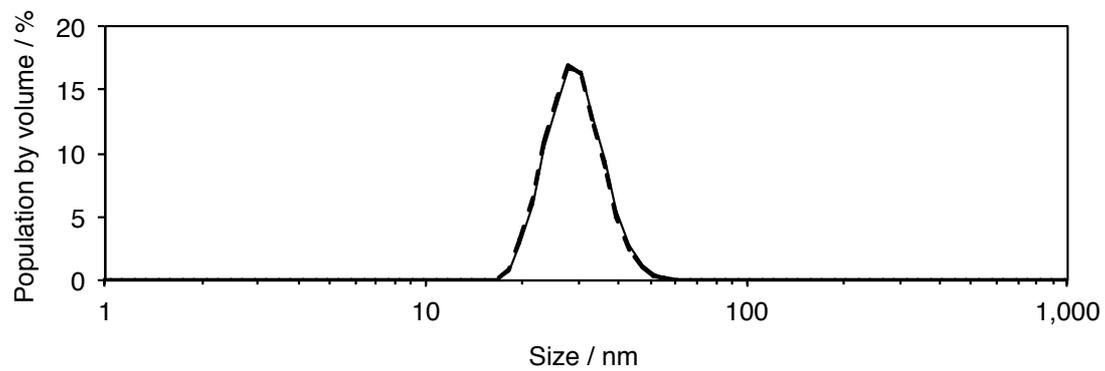


Fig. S3 Size distribution of cholate/POPC micelles before (---) and after (—) mixing with TRITC.

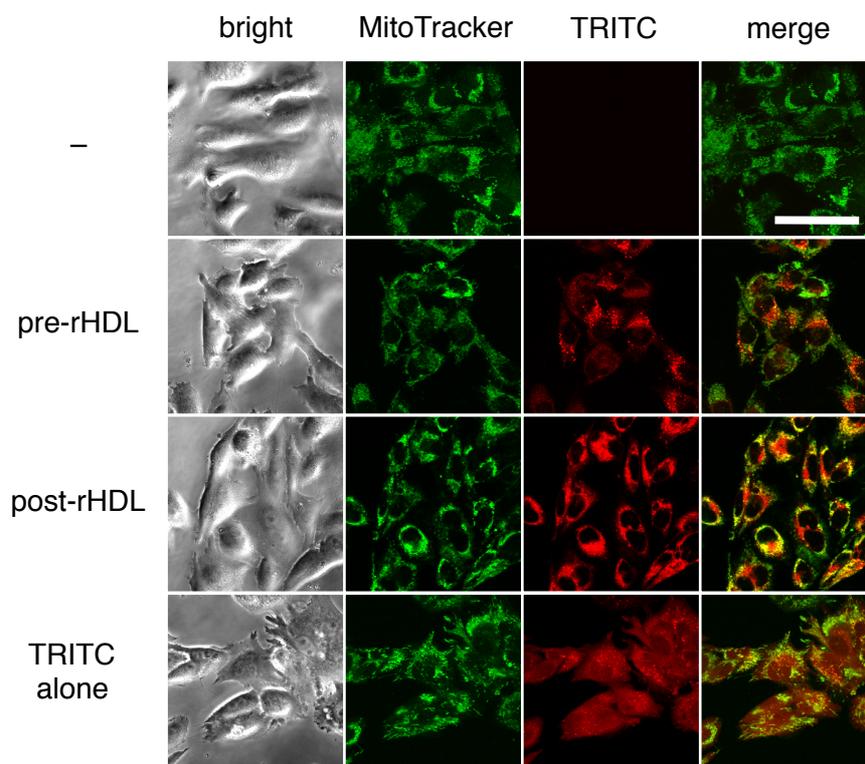


Fig. S4 Confocal images of CHO-SR-BI cells treated with pre-rHDL or post-rHDL. Cells were incubated with TRITC-labeled pre-rHDL or post-rHDL particles (10 μ g protein/mL) at 37°C for 1 h and stained with MitoTracker (green) to reveal mitochondria; subcellular localization of rHDL particles was analyzed by TRITC fluorescence (red). In contrast to pre-rHDL, a considerable portion of post-rHDL particles were localized in the mitochondria (merge). Given that free TRITC showed a tendency to accumulate in the mitochondria (TRITC alone, merge), a significant presence of free TRITC in the lipid bilayer of post-rHDL particles may account for their mitochondrial localization. Scale bar, 50 μ m.

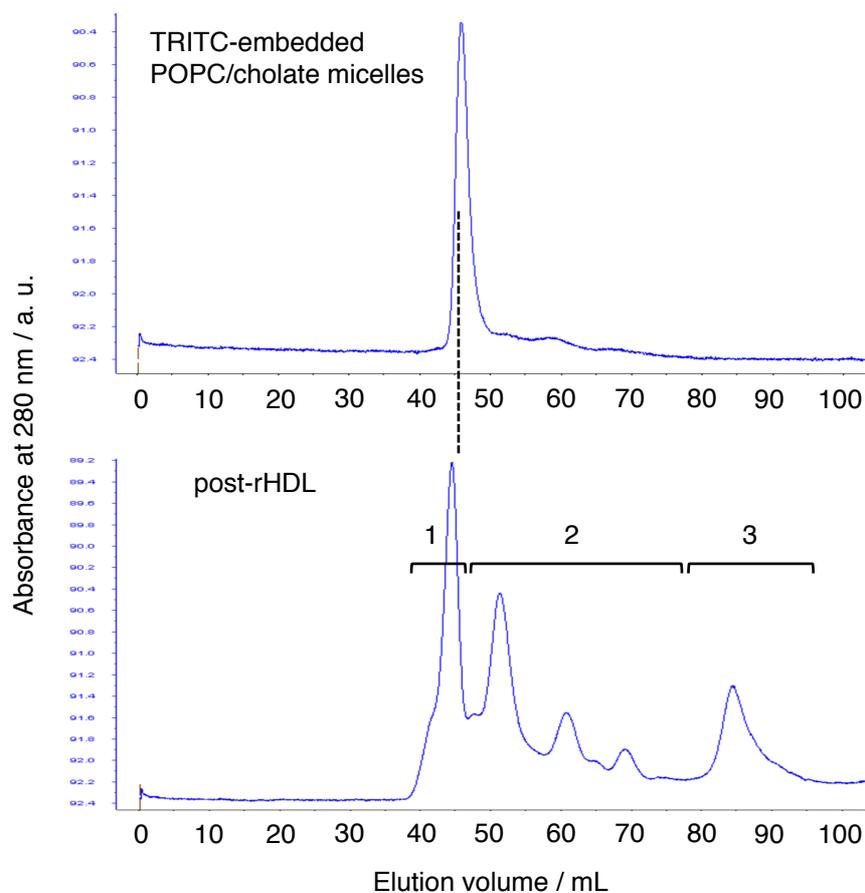


Fig. S5 Size-exclusion chromatography of TRITC-embedding POPC/cholate micelles (upper graph) and post-rHDL particles (lower graph) using a Superdex 200 prep grade 16/60 column. Each fraction was solubilized with 2% SDS and subjected to fluorescence analysis (excitation at 529 nm/emission at 596 nm). TRITC content was 20%, 80%, and 0% in fractions 1, 2, and 3, respectively. Fraction 1 of the post-rHDL sample was identified as POPC/cholate micelles. Fraction 3 would be non-rHDL because m-apoA-I was detected only in fraction 2 by SDS-PAGE (data not shown).

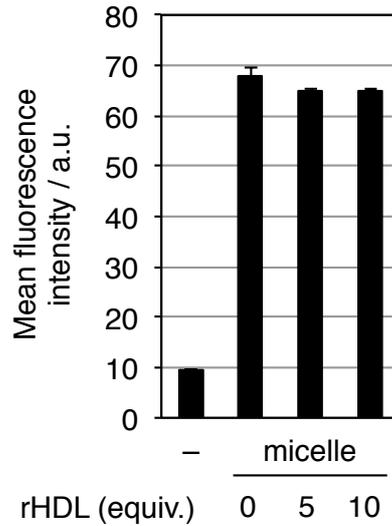


Fig. S6 Binding of TRITC-labeled POPC/cholate micelles to CHO-SR-BI cells. Cells were incubated with TRITC-labeled POPC/cholate micelles in the presence or absence of non-labeled rHDL (rHDL in the x-axis) at 37°C for 1 h and subjected to FACS analysis. Micelle concentration was adjusted to TRITC equivalent in pre-rHDL and post-rHDL samples; non-labeled rHDL concentration corresponded to protein equivalent (0, 5, and 10) in the post-rHDL samples. The binding efficiency of TRITC-labeled POPC/cholate micelles was comparable to that of pre-rHDL particles, and there was no competition for binding with rHDL. The data represent the mean \pm standard deviation of triplicate measurements.

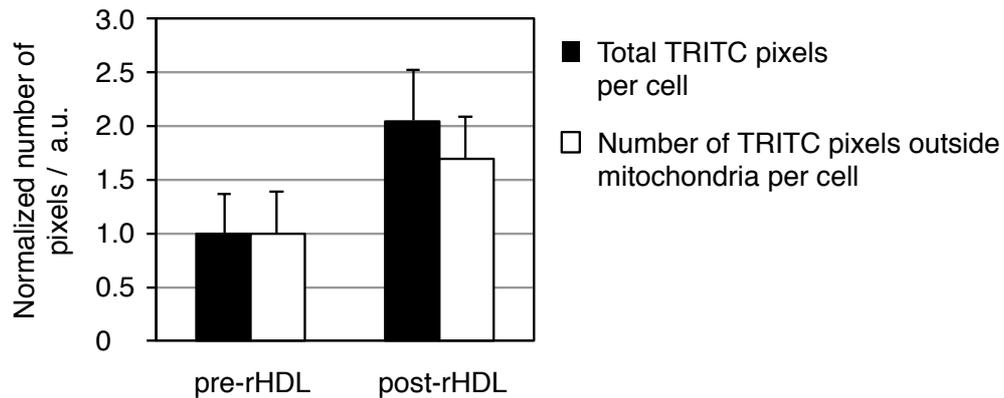


Fig. S7 Effect of free TRITC embedded in the post-rHDL lipid bilayer on the increase of post-rHDL binding to SR-BI. TRITC contribution was analyzed by calculating the total TRITC pixels (red + yellow in Fig. S4) and the number of TRITC pixels colocalized with MitoTracker (yellow in Fig. S4) for both pre-rHDL and post-rHDL in each of the five independent images. Total TRITC pixels per cell for post-rHDL was 2.1-fold that for pre-rHDL, which is consistent with the 2.6-fold difference revealed by FACS analysis. The colocalization ratio calculated as yellow pixels/(red + yellow pixels) was slightly higher for post-rHDL (43%) than for pre-rHDL (31%), indicating the increase of TRITC signal in mitochondria of post-rHDL-treated cells. This increase was due to the 2-fold higher TRITC content in the lipid bilayer of post-rHDL compared with that in pre-rHDL particles (Fig. S1b) and to the high TRITC affinity for mitochondria. TRITC bound to m-apoA-I was calculated as the number of TRITC pixels outside mitochondria (red) by subtracting the number of MitoTracker-colocalized TRITC pixels (yellow) from total TRITC pixels (red + yellow) in each cell. The ratio of TRITC bound to m-apoA-I in post-rHDL to that in pre-rHDL was 1.7, which was not significantly different from 2.1, indicating that the elevated TRITC content in the lipid bilayer did not significantly contribute to the increased binding of post-rHDL to CHO-SR-BI cells (Fig. 4).

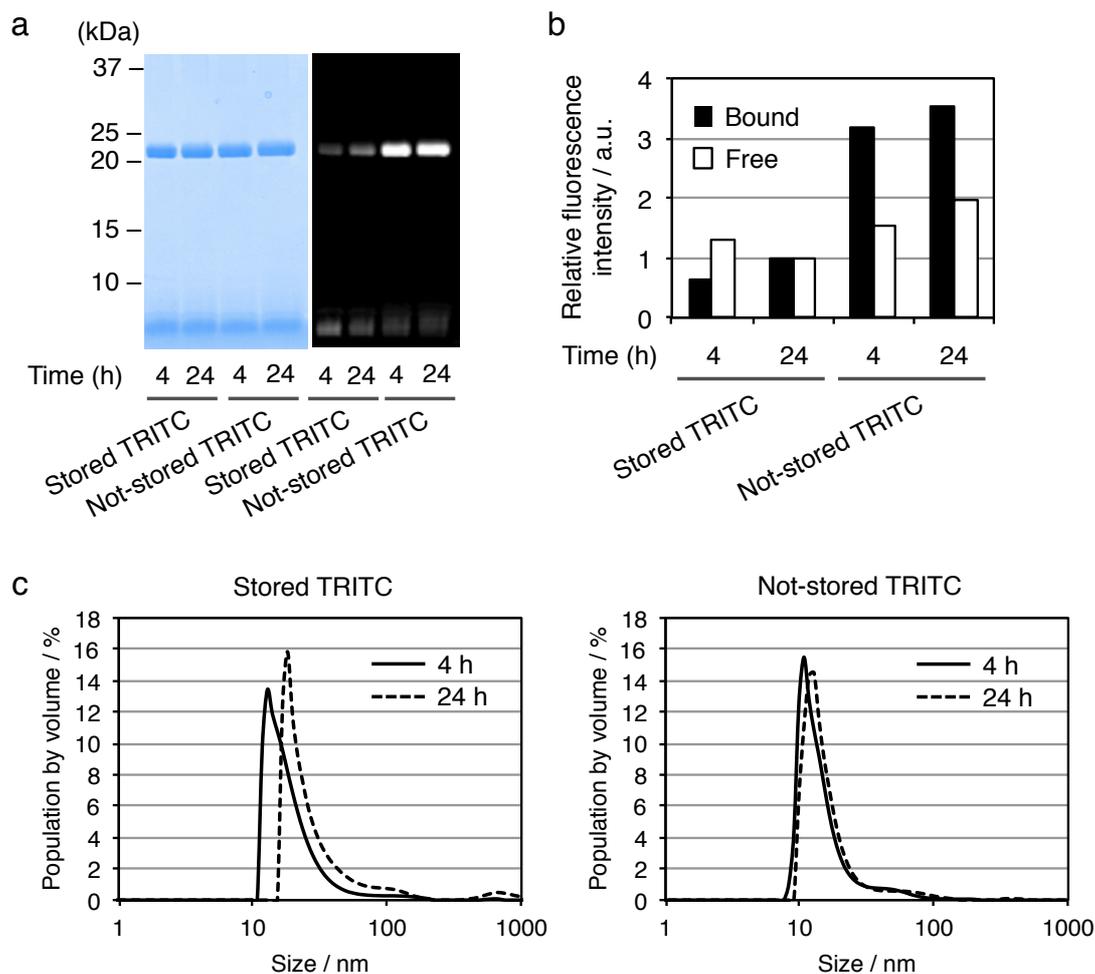


Fig. S8 The effect of TRITC modification conditions on the size of post-rHDL particles (TRITC/protein (w/w) ratio, 0.1). (a) Coomassie Brilliant Blue staining (left panel) and fluorescence (right panel) images of a representative SDS-PAGE gel. Post-reconstitution modification was performed for 4 h and 24 h with TRITC stored in DMSO at -30°C (stored TRITC) or not stored in DMSO (not-stored TRITC). (b) Densitometry analysis of fluorescent TRITC bound to m-apoA-I (black bars) or released from the rHDL lipid bilayer (white bars). Fluorescence intensity was normalized to that of post-rHDL prepared by 24-h reaction with stored TRITC. Following shortening the

reaction time with stored TRITC, protein labeling efficiency was reduced by half, whereas TRITC lipid embedding was almost unchanged. Not-stored TRITC significantly enhanced protein labeling and slightly increased lipid embedding. (c) Size distribution of post-rHDL particles under the four conditions described above. The mean particle size was decreased to 22 nm after 4-h reaction when stored TRITC was used. Meanwhile, no significant particle enlargement was observed even after 24 h when not-stored TRITC was used (mean size, <15 nm). These results suggest that increased reaction time and decreased TRITC reactivity play a role in rHDL particle enlargement.

References

- 1 T. Murakami, W. Wijagkanalan, M. Hashida and K. Tsuchida, *Nanomedicine (London, U. K.)*, 2010, **5**, 867–879.
- 2 A. Das and S. G. Sligar, *Biochemistry*, 2009, **48**, 12104–12112.