Purification and analysis of circulating lipid particles

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Running Title: Circulating lipid particles

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Abstract

Lipid particles found in circulating extracellular fluids such as blood or lymph are essential for cellular homeostasis, metabolism and survival. Such particles provide essential lipids and fats which enable cells to synthesize new membranes and regulate different biochemical pathways. Imbalance in lipid particle metabolism can cause pathological states such as atherosclerosis. Here, elevated low-density lipoprotein (LDL) accumulation leads to fat-filled lesions or plaques in arterial walls. In this chapter, we provide a detailed set of protocols for the rapid and safe purification of lipid particles from human blood using high-speed ultracentrifugation. We provide a detailed set of assays for further analysis of the biochemical and cellular properties of these lipid particles. By combining these assays, we can better understand the complex roles of different lipid particles in normal physiology and disease pathology.

Key words Lipid particles, LDL, VLDL, HDL, Gradient purification, SDS-PAGE, human umbilical vein endothelial cells (HUVECs), human embryonic kidney 293T (HEK293T) cells

1 Introduction

Lipid particles are used by many biological organisms as a source of fuel, nutrients and building blocks. In higher eukaryotes, different types of lipid particles are found in extracellular circulating fluids such as lymph and blood. Many lipid particles are synthesized *de novo* but are dependent on the ingestion of dietary lipids and triglycerides via the intestine. This can give rise to a variety of lipid particles in circulating fluids such as blood. Transport of such lipid particles via circulatory systems enables delivery of these building blocks to cells, tissues and organs for metabolism into different molecules, and to provide energy. The different lipid particles have distinct functional properties: they share features such as a unilamellar lipid monolayer around a hydrophobic core, with overlapping constituents such as lipoproteins, lipids and fatty acids. Generally, each class of lipid particle is recognized by one or more specific membrane-bound receptors which facilitate internalization and processing by cells and tissues.

The largest class of lipid particle is the chylomicron (CM) which is irregular in shape and ~100-600 nm in diameter. Chylomicron biogenesis occurs in the endoplasmic reticulum of small intestine cells

termed enterocytes [1]. These chylomicrons are transported through the enterocyte secretory pathway into the lymph. The chylomicron can undergo lipolysis to generate smaller lipid particles *in vivo*, whilst its remnants can be endocytosed via non-receptor and receptor-mediated mechanisms for uptake and processing by different cells and tissues. One potential receptor for chylomicron remnants is the low-density lipoprotein receptor-related protein 1 (LRP1) [2].

The liver is a major site for the processing, metabolism and synthesis of different lipoproteins and lipid particles. Very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high-density lipoprotein (HDL) are different single lipid monolayer particles with hydrophobic cores and distinct functional properties. These lipid or lipoprotein particles can have common molecular components, especially apolipoproteins, triglycerides, cholesterol and cholesteryl esters. However, the amount and functional significance of such components are unique to each class of lipid particle. VLDL, IDL and LDL are classed as pro-atherogenic factors where elevated levels of these particles in circulatory fluids are indicative of, or associated with, increased cholesterol levels and the risk of pathological conditions such as heart attacks, strokes and peripheral arterial disease [3]. LDL metabolism is a well-established process in which LDL particles are recognized by the LDL receptor (LDL-R), a type I membrane glycoprotein widely expressed on many mammalian cells and tissues. Interestingly, LDL-R can bind both LDL and VLDL but enables each class of lipid particle to be internalized and trafficked through different pathways [4]. A VLDL receptor (VLDL-R) which bears significant homology to LDL-R, facilitates recognition and internalization of VLDL particles, which are ~30-80 nm diameter [5]. Targeting VLDL uptake and metabolism decreases risk of arterial disease *in vivo* [6]. VLDL synthesis occurs within the liver; the removal of triglycerides from VLDL by lipoprotein lipase results in both IDL (~25-35 nm diameter) and LDL (~22-28 nm diameter) species which are smaller and denser lipid particles with a higher concentration of cholesterol esters [7]. IDL has an intermediate density between VLDL and LDL. The *Apolipoprotein E* (*ApoE*) gene encodes at least 3 protein isoforms, ApoE2, ApoE3 and ApoE4. Each ApoE isoform is associated with different lipid particle populations. Genetic polymorphisms within the *ApoE* locus are linked to Alzheimer's Disease and hypercholesterolemia [8].

High-density lipoprotein (HDL) are the smallest class of particles, ~5-15 nm diameter. HDL has the highest protein:lipid ratio, thus explaining why HDL is the most dense of the 5 different classes of lipid particles. In contrast to other types of lipid particles, HDL is often referred to as 'good cholesterol' where higher HDL levels correlate with decreased risk of vascular disease and other pathological conditions. Interestingly, HDL recognition is facilitated by novel membrane proteins termed Class B scavenger receptors (SR-B1, CD36). The exact role of HDL is open to much current debate: one hypothesis is that HDL functions as a cholesterol sink and subsequent HDL delivery to the liver enables cholesterol removal, mixing with bile salts and excretion through the gut [9]. The separation and purification of these different lipid particles for biochemical, cellular and physiological studies *in vitro* and *in vivo* is thus of much interest, and importance, in understanding lipid metabolism with implications for understanding different disease states and designing specific therapeutic strategies. Here, we provide a rapid and effective protocol for the purification and analysis of different lipid particles from human blood using biochemical and cellular assays.

2 Materials

2.1. Blood taking and plasma purification

1. Butterfly needles for venipuncture.
2. Blood collection tubes containing sodium heparin anticoagulant (*see* **Note 1**).
3. Alcohol swabs.
4. Adhesive plaster.
5. Tourniquet.

6. 50 ml sterile conical screw cap tubes.
7. Low-speed refrigerated centrifuge with swing-out rotor.
8. Class 1 hood for blood handling.
9. Disposable sterile plastic 10 mL pipettes and pipette controller.
10. Digital balance.
11. Dialysis tubing of 12000 Da molecular weight cutoff (MWCO).
12. Phosphate buffered saline (PBS): 1.37 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 for dialysis.
13. Retort stand and boss.

2.2. Ultracentrifugation

1. Sealable ~5 mL ultracentrifuge tubes capable of withstanding 543,000 *g*.
2. HEPES buffered saline (HBS): 0.85% (w/v) sodium chloride, 10 mM HEPES, pH 7.4.
3. OptiPrep™ Density Gradient Medium (Sigma-Aldrich).
4. Narrow barreled glass Pasteur pipettes and pipette controller.
5. Benchtop ultracentrifuge capable of 543,000 *g*.
6. Fixed angle ultracentrifuge rotor capable of 543,000 *g*.

2.3. Agarose gel electrophoresis

1. Electrophoresis grade agarose powder.
2. 0.5X TBE (Tris-Borate-EDTA) buffer: 80 mM boric acid, 90 mM Tris-HCl, 3 mM EDTA, pH 8.3.
3. Horizontal agarose gel electrophoresis gel rig.
4. 1.5 mL microcentrifuge tubes.
5. 6X Agarose Gel Loading Dye: 15% Ficoll®-400, 50 mM EDTA, 19.8 mM Tris-HCl, 0.48% (w/v) SDS, pH 8.0.
6. Ethanol.
7. Acetic acid.
8. Sudan Black B, a high purity lipid stain. Dissolve 0.5 g of Sudan Black in a 37°C solution of 60% (v/v) ethanol. Stir for 2-5 h to ensure maximum dye saturation and then filter using a funnel lined with filter paper to remove precipitates. Treat 100 mL of Sudan black solution with 0.2 mL of 25% (w/v) sodium hydroxide as a preservative for long-term storage at room temperature, protect from light by storage in a dark bottle and wrap aluminum foil around the bottle.
9. Agarose gels were visualized using any digital-based PC-based gel documentation system capable of transmitted light imaging where data is collected as 16-bit images in .tif files.

2.4. SDS-PAGE

1. 10 % (w/v) Ammonium persulfate (APS) solution.
2. N,N,N′,N′-tetraethylmethylenediamine (TEMED).
3. 30% Acrylamide solution (37.5:1 ratio).
4. 5X SDS PAGE sample buffer: 250 mM Tris pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol.
5. 1X SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
6. 20 x 10 cm vertical gel electrophoresis system containing loading rig, glass plates and buffer tank.
7. SDS-PAGE pre-stained polypeptide markers ranging from 200-10 kDa.

8. 20% SDS-PAGE resolving gel: 6.7 mL 30 % (v/v) acrylamide, 1.25 mL 3 M Tris–HCl pH 8.8, 1.75 mL water, 100 µL 10 % (w/v) SDS, 200 µl of 10% (w/v) APS, 8 µl of TEMED for 10 ml of resolving gel (sufficient for one gradient gel). Add APS and TEMED when the gel needs to be poured.
9. 6% SDS PAGE resolving gel: 2.0 mL 30 % (v/v) acrylamide, 1.25 mL 3 M Tris–HCl pH 8.8, 6.45 mL water, 100 µL 10 % (w/v) SDS, 200 µl of 10% (w/v) APS, 8 µl of TEMED for 10 ml of resolving gel (sufficient for one gradient gel). Add APS and TEMED when the gel needs to be poured.
10. 5% SDS PAGE stacking gel: 1.67 mL 30 % (v/v) acrylamide, 1.25 mL 1 M Tris–HCl pH 6.8, 7.0 mL water, 100 µL 10 % (w/v) SDS, 100 µl of 10% (w/v) APS, 20 µl of TEMED for 10 ml of stacking gel (sufficient for one gradient gel). Add APS and TEMED when the gel needs to be poured.
11. Sterile plastic 25 mL pipettes.
12. Propanol.
13. Filter paper.
14. Heating block set at 95℃.
15. Coomassie blue protein staining solution: Dissolve 2.5 g of Brilliant Blue R in a solution of 450 ml water, 450 ml methanol, 100 ml acetic acid for 1 L of stain. Stir for 1-2 days to ensure maximum dye saturation and then filter using a funnel lined with filter paper (*see* **Note 2)**.
16. Coomassie destaining solution: 10% methanol, 10% glacial acetic acid.
17. SDS-PAGE gels were visualized using a digital PC-based gel documentation system which generates 16-bit images as .tif files.

2.5. Protein and Cholesterol assay

1. BCA assay kit
2. HDL and LDL/VLDL Quantitation assay Kit (Sigma-Aldrich) including: cholesterol assay buffer, 2X LDL/ VLDL precipitation buffer, cholesterol probe in DMSO, enzyme mix, cholesterol esterase, 2 µg/ µL cholesterol standard (*see* **Note 3)**.
3. 96-well plastic plates.
4. Multichannel pipette.
5. Phosphate-buffered saline (PBS): 1.37 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4. Autoclave at 121°C for 20 min on standard sterilization cycle.
6. Variable wavelength absorbance plate reader.

2.6. Lipid particle labeling with fluorescent DiI

1. $DiIC_{18}(5)$-DS (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine-5,5'-Disulfonic Acid (Dil) or $DiIC_{18}(5)$ (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DID) (see **Note 4).**
2. Dimethyl sulfoxide (DMSO).

2.7. Cell culture and cell lysis

1. Complete Dulbecco's modified Eagle medium (DMEM) with 25 mM glucose supplemented with 10 % (v/v) fetal bovine serum, 1% (v/v) of 100X MEM Non-Essential Amino Acids Solution, 1% (v/v) of 200 mM L-Glutamine and 1% (v/v) of 10,000 U/mL penicillin-streptomycin.
2. Serum-free medium.

3. Endothelial Cell Growth Medium (ECGM) supplemented with 0.4 % (v/v) endothelial cell growth supplement, 0.1 ng/ ml recombinant epidermal growth factor (EGF), 1 ng/ ml recombinant basic fibroblast growth factor (bFGF), 1 μg/ml hydrocortisone, 90 μg/ml heparin (PromoCell).
4. MCDB131 medium (no glutamine) supplemented with 0.2% (w/v) BSA.
5. Primary human umbilical vein endothelial cells (HUVECs).
6. HEK293T cell line.
7. 0.1% (w/v) sterile filtered pig skin gelatin.
8. 0.01% (w/v) poly-L-lysine solution.
9. Trypsin solution e.g. (1 mg/ml porcine trypin in PBS, pH 7.4), filter-sterilized through a 0.22 μm filter.
10. T75 flasks for cell maintenance.
11. 24 well plates to house coverslips.
12. 13 mm #1.5 round glass coverslips.
13. Glass microscopy slides.
14. 3% (w/v) paraformaldehyde (PFA).
15. 4',6-diamidino-2-phenylindole (DAPI).
16. Fine metal tweezers.
17. Anti-fade medium for mounting glass coverslips for long-term storage.
18. Clear nail varnish.

2.8. Confocal microscopy

1. Variable wavelength confocal microscope capable of multichannel fluorescence imaging.
2. Microscope software for visualization of images.

3 Methods

3.1 Blood collection

1. Collect blood from human volunteers under institutional local ethical approval using butterfly needles for venipuncture based on the size of the volunteer's vein.
2. Constrict the blood flow above the vein in the arm using a tourniquet. Wipe the area around the vein using a sterile disposable alcohol swab. Insert the butterfly needle carefully into a vein in the arm and collect ~20 mL of human blood into a blood collection tube containing sodium heparin (*see* **Note 1**). The tourniquet was removed during the blood taking.
3. Wipe the arm with cotton wool after taking blood and place an adhesive plaster over the punctured vein.

3.2 Lipid particle purification

1. Immediately centrifuge blood at 1500x *g* for 10 min at 4°C in a centrifuge with a swing out rotor. This step will separate blood into a lower dark red layer (erythrocytes and platelets) from a fuzzy white interface (buffy coat layer of lymphocytes) and the top straw or yellow colored layer (plasma).
2. Carefully transfer the clear yellow layer (blood plasma) to a sterile 50 mL tube in a Class I hood.
3. Re-centrifuge the plasma again at 1500x *g* for 10 min at 4°C to remove any remaining red and white blood cells.
4. Mix with iodixanol (OptiPrep™) in a 4:1 ratio using disposable sterile 10 mL plastic pipettes. This will give a 12% (v/v) iodixanol concentration in the final gradient.

5. Carefully load ~5ml sealable ultracentrifuge tubes with 1 mL of HBS.
6. Carefully layer the plasma-Optiprep solution from **step 4** under the HBS in the ultracentrifuge tubes using a narrow-barreled glass Pasteur pipette and pipette controller until tubes are at their rated capacity.
7. Seal tubes and balance to 2 decimal places using a digital balance. Place into a fixed angle ultracentrifuge rotor.
8. Place the rotor in the ultracentrifuge.
9. Centrifuge the plasma-Optiprep mixture at 543,000 *g* for 3 h at 16°C.
10. Remove the ultracentrifuge tubes from the rotor and clamp the tube on a retort stand (*see* Figure 1).
11. Collect 250 µL fraction volumes from the top of the gradient for subsequent analysis (*see* **Note 5**). Lipid fractions were dialyzed overnight using dialysis tubing (MWCO 12000 Da) against 1000 volumes of PBS at 4°C.
12. Determine the protein content of lipid particle (e.g. by BCA assay).

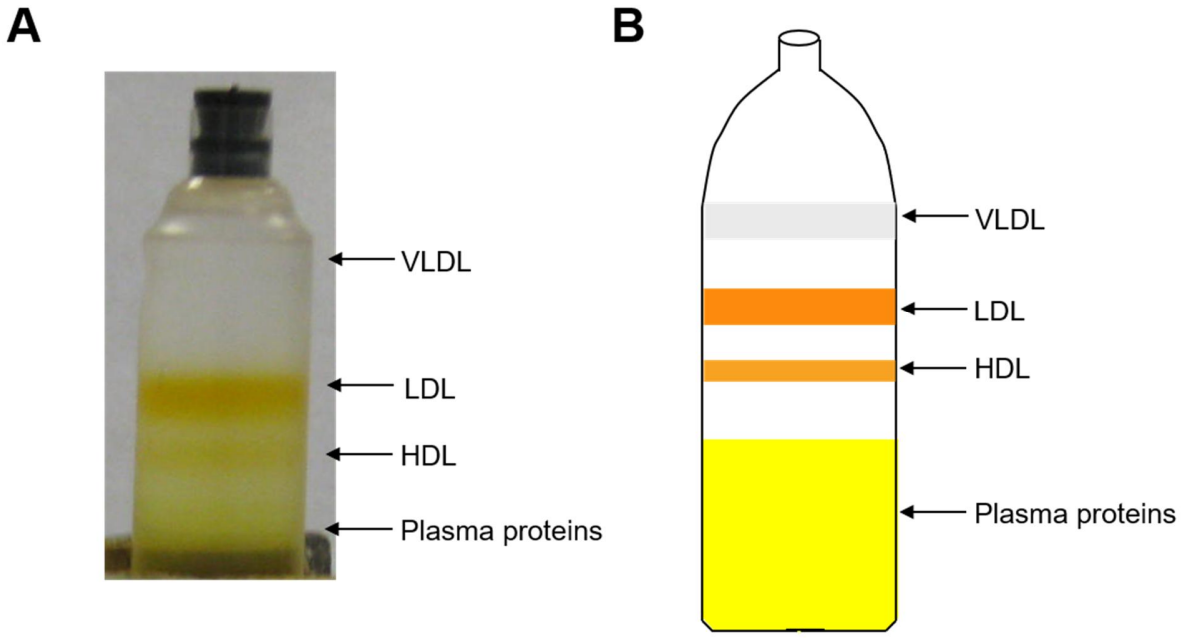

Figure 1: Fractionation of plasma lipoproteins using an iodixanol gradient and ultracentrifugation. *(A) Blood plasma mixed with iodixanol was subjected to ultracentrifugation. This causes lipoproteins to separate according to their relative density. VLDL forms an almost indistinct grey band near the top of the tube. LDL appears as a large orange band below this, a third of the way down the tube. HDL forms an orange-yellow band between LDL and the yellow plasma proteins at the base of the tube. 250 µl fractions were taken from the top of the tube. (B) Schematic representation of lipid particle populations.*

3.3 Agarose gel electrophoresis

1. Melt a suspension of 0.5% (w/v) agarose in 0.5X TBE buffer in the microwave and cool to 55-60°C.
2. Pour the melted agarose solution into a horizontal gel electrophoresis rig and allow to set with plastic comb in place (*see* **Note 6**).

3. Carefully remove the plastic comb and place the gel within the electrophoresis tank in 0.5X TBE buffer.
4. Mix 3 µL of each 250 µL centrifugation fraction with 6X gel loading dye at a ratio of 5:1.
5. Load samples onto the 0.5% agarose gel and carry out electrophoresis at 80 V for 60 min.
6. Carefully remove the gel and place in a sealable plastic box. Immerse the gel in a fixation solution of ethanol-acetic acid (75% ethanol, 5% acetic acid) and gently rock at room temperature for 15 min. Discard fixation solution.
7. Incubate the agarose gel with the Sudan Black solution and allow to gently agitate at room temperature for 30 to 180 min with frequent monitoring until saturated.
8. Discard the Sudan Black solution and rinse the gel twice briefly in 50% (v/v) ethanol.
9. Leave the gel to de-stain in 50% (v/v) ethanol overnight at 4°C in an air-tight dark container until lipid bands are distinct.
10. Digitally analyze the gel using a gel documentation system capable of transmitted light imaging and collect images as .tif files (*see* Figure 2A and **Note 7**).

3.2.2 Protein analysis of lipid particles using gradient SDS-PAGE

1. Assemble the polyacrylamide gel rig as per manufacturer's instructions. With a pen, mark the outer glass plate up to where the resolving gel will be added (roughly 1 cm below where the comb will be inserted).
2. In two separate 50 mL sterile screw cap tubes, prepare 6% and 20% polyacrylamide gel mixtures omitting APS and TEMED until immediately prior to pouring the gel.
3. Using a 25 ml serological pipette, take up 8 mL of the 6% solution followed by 8 mL of the 20% solution (*see* **Note 8**).
4. Take up one bubble of air into the serological pipette which will mix the two gel solutions forming a gradient.
5. Pipette the solution into the gel rig between the two glass plates, moving along the length of the gel casing.
6. Carefully add a layer of propanol to sit above the gel mixture in a visibly separate layer. Allow the gel to set for 20 to 30 min. Gently tilt to ensure the gel is visibly solid below the still liquid propanol.
7. Pour off the propanol and rinse with distilled water three times. Use filter paper to remove any residual water, without touching the gel itself.
8. Make 10 mL of 5% stacking gel and pipette on top of the set resolving gel until flush with the top of the gel plates. Immediately insert the well comb, ensuring not to trap any bubbles in the wells. Allow to set for 30 min.
9. Place the gel rig in the electrophoresis running tank and fill internal reservoir with 1x SDS PAGE running buffer.
10. Prepare lipoprotein fraction samples by combing 20 µg of each centrifugation fraction with 5X SDS sample buffer in a 4:1 ratio (*see* **Note 9**).
11. Incubate protein samples for 10 min at 95°C to denature proteins.
12. Pipette pre-stained molecular weight markers into the first well followed by fraction samples in order.
13. Begin electrophoresis at 120 V at room temperature until the SDS sample buffer dye bands have migrated off the gel after approximately 1.5 h.
14. Dismantle the gel rig and remove the polyacrylamide gel from between the glass plates.
15. Incubate the gel with Coomassie protein stain for 4 h to overnight with gentle agitation.
16. Briefly wash the gel with distilled water 3X to remove excess stain.
17. Incubate the gel in a de-staining solution of 10% methanol, 10% acetic acid for 3-6 h, changing the solution upon saturation, until bands become visibly distinct (*see* Figure. 2B).

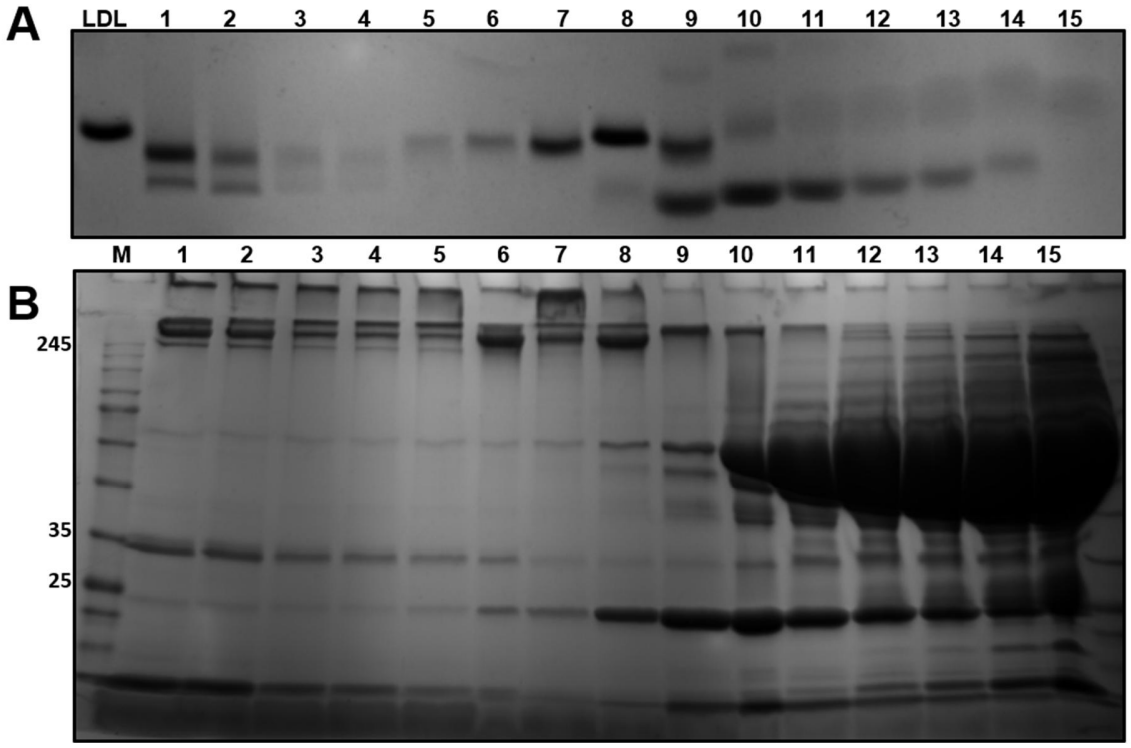

Figure 2: Gel electrophoresis analysis of fractionated lipid particles. *(A) Agarose gel electrophoresis. Samples (3 µl) of each 250 µl fraction (#1-15) were analyzed on 0.5% (w/v) agarose gels and stained with Sudan black. As a reference, purified LDL collected by centrifuge tube needle puncture was analyzed in the first lane (LDL). As the smallest and most dense lipoprotein particle, HDL, migrates furthest. LDL shows the slowest mobility as although it is smaller than VLDL, it contains far fewer apolipoproteins so has an overall lower negative charge (see* ***Note 7)****. In the later fractions (#10 onwards), high levels of serum proteins affect HDL migration properties. (B) SDS-PAGE analysis of samples (20 µg) of each 250 µl fraction (#1-15) analyzed on 6-20% gradient gels and stained with Coomaisse. ApoB-48 (MW ~241 kDa) is indicative of chylomicrons (CM) and ApoB-100 (MW ~512 kDa) are difficult to detect by conventional SDS-PAGE. ApoA1 (MW ~28 kDa) is indicative of HDL and initially appears in fraction #6, becoming more abundant in fraction #8 onwards. ApoA1 migrates at an apparent MW ~24 kDa.*

3.3. Cholesterol assay of lipid particle fractions

We here present a generic protocol for determining the cholesterol content of serum samples using a commercial cholesterol assay kit. This protocol provides an overview of the stages involved however the specific details of the manufacturers protocol should be adhered to (*see* **Note 3**).

1. Select an assay kit for measuring blood serum cholesterol levels, in this case assaying the centrifugation fractions in place of whole serum.
2. Prepare the kit provided cholesterol standards by diluting 20 µL of the cholesterol standard solution with 140 µL of the cholesterol assay buffer to generate a 0.25 µg/ µL solution.
3. In duplicate add 0, 4, 8, 12, 16, and 20 µL of this solution to a 96-well plate.
4. To separate HDL from LDL and VLDL in the centrifugation fractions mix 100 µL of each fraction (50 µL per duplicate) with 100 µL of 2X precipitation buffer in a microcentrifuge tube.
5. Incubate for 10 min at room temperature.
6. Centrifuge at 2,000 *g* for 10 min.
7. Carefully pipette the clear supernatant containing the HDL fraction into a separate microcentrifuge tube leaving the precipitated LDL/ VLDL fraction.

8. Centrifuge at 2,000 *g* for 10 min again and remove any residual HDL containing supernatant (*see* **Note 10**).
9. Resuspend the LDL/ VLDL precipitate in 200 µL of PBS.
10. In duplicate add 50 µL of the HDL supernatant or resuspended LDL/ VLDL precipitate to the 96-well plate for each fraction (*see* **Note 11**).
11. Assemble the kit provided assay reaction mix as instructed per well: 44 µL cholesterol assay buffer, 2 µL cholesterol probe, 2 µL cholesterol enzyme mix, 2 µL cholesterol esterase.
12. Using a multichannel pipette add 50 µL of the reaction mix to each well containing either cholesterol standard or fraction sample and mix well by pipetting.
13. Incubate the plate for 60 min at 37 °C protected from light.
14. Measure the colorimetric absorbance at 570 nm using a 96-well plate reader to determine the presence of cholesterol in each fraction. This will generate absorbance values for the cholesterol standards as well as HDL and VLDL/ LDL populations for each fraction.
15. Calculate the mean absorbance by averaging the values received from the duplicated wells for each condition.
16. From all averaged results, subtract the value received from the zero cholesterol standards condition to adjust for the baseline.
17. Using the averaged absorbance readings from the kit-provided cholesterol standards, construct a standard curve of absorbance (y) versus known µg quantity of cholesterol (x) as defined by the kit.
18. Generate a line of best fit for the standard curve and calculate the straight-line equation for the correlation of cholesterol mass and resultant signal in the form $y = m x + c$
19. Use the standard curve calculated m and c values alongside the experimentally derived absorbance value y to calculate the mass of cholesterol in a given fraction: $x = (y – c) / m$
20. Divide the value received by the µl quantity of fraction sample that was added to wells initially (50 µl) and multiply by any dilution factor in the assay (2X dilution factor here) to give a concentration in µg/µl.
21. Plot fraction number (x) against the cholesterol concentration (y) for LDL, HDL and the sum of the two as total cholesterol (*see* Figure 3).

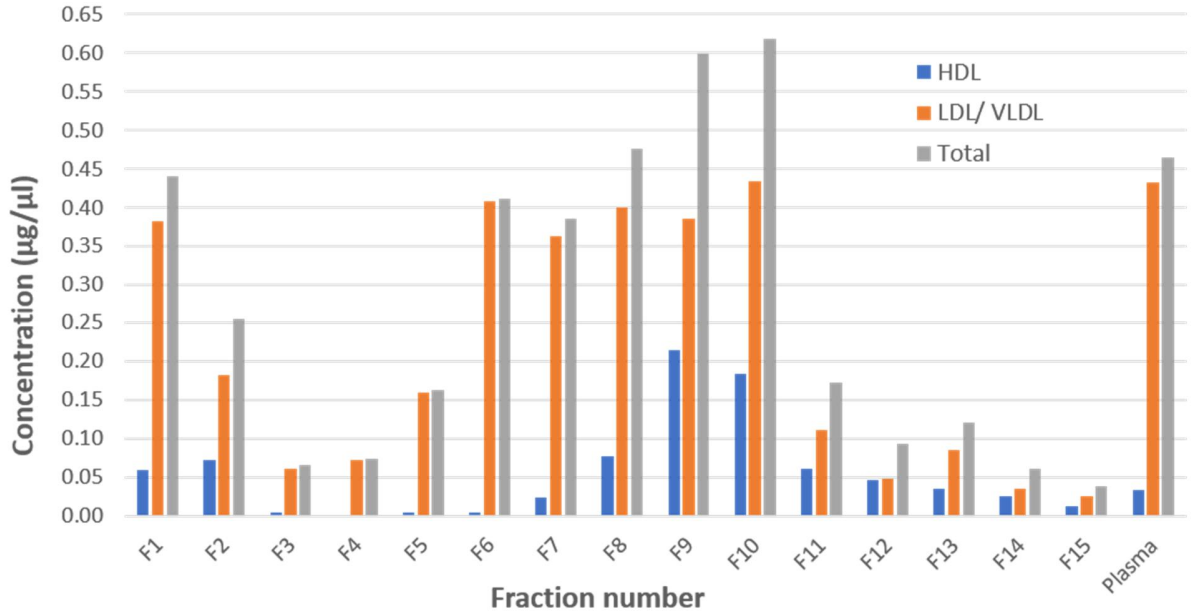

Figure 3: Cholesterol quantification in plasma fractions. *HDL and LDL/VLDL profiling using a cholesterol assay. 50 µl of each lipid fraction was first separated into HDL and LDL/VLDL fractions followed by enzymatic*

determination of cholesterol concentration. VLDL signal is apparent in fractions #1 and #2. LDL signal appears in fractions #6-10. HDL signal appears most strongly in fractions #8-10. If HDL and LDL/ VLDL are imperfectly separated during the first stage of this assay, then they may be reported as the other population during the cholesterol quantification stage.

3.4. Lipid particle labelling with fluorescent DiI derivatives

1. From gel analyses for lipid and protein content (*see* **Note 12**), select fractions corresponding to the lipid particles and lipoproteins of interest.
2. Aliquot 50-100 µl of each fraction into a 1.5 ml microcentrifuge tube.
3. Using a 3 mg/ml stock of DiI derivative (in DMSO) add 300 µg DiI derivative per 1 mg of lipoprotein particles and mix by gentle pipetting.
4. Protect the solution from light by wrapping in aluminum foil and incubate for 16-20 h (overnight) at 37°C.
5. Dialyze the lipoprotein particles against >1000 volumes of PBS for 24 h at 37°C, changing the buffer at least once, to remove unincorporated DiI derivative and DMSO (*see* **Note 13**).
6. Recover fluorescent lipid particles after dialysis and store in 1.5 mL microcentrifuge tubes at 4°C protected from light.

3.5. HUVEC and HEK293T cell culture and stimulation of DiI-labeled lipoprotein uptake

All steps are carried out in a laminar flow hood working at Biosafety Level 1. All media are pre-warmed to 37°C prior to use

1. Sterilize 13 mm diameter round glass coverslips by dipping in 100% ethanol and allowing to air dry in a well of a 24 well plate.
2. Coat coverslips with 500 µl of cell adhesion stratum, as appropriate for each cell type, and incubate for 1 h at room temperature. For HUVECs, use sterile filtered 0.1% (w/v) pig skin gelatin in PBS. For HEK293T cells, use 0.01% (w/v) poly-L-lysine.
3. Aspirate the solution and wash coverslips three times with PBS. Allow coverslips and plates to air dry for 1 h.
4. Using standard protocols, split HUVECs (*see* **Note 14**) [10] and HEK293T cells (*see* **Note 15**) [11] at 40% confluence onto the coverslips and incubate overnight in complete growth medium to allow cells to adhere to the coverslips.
5. The following day, replace medium with starvation medium and incubate for 3 h. HUVECs should be starved in MCDB131, 0.2% (w/v) BSA. HEK293T cells should be starved in Opti-MEM.
6. Aspirate starvation medium and replace with fresh starvation medium supplemented with 10-50 µg/ml DiI-labeled lipid particles and place in a humidified tissue culture incubator at 37°C (*see* **Note 16**).
7. Aspirate the medium, wash once with 1 mL of ice-cold PBS and replace with pre-warmed complete medium. Place in a humidified tissue culture incubator at 37°C for 55 min.
8. Aspirate medium and wash cells three times with 1 mL of ice-cold PBS.
9. Add 0.5 mL of 3% (w/v) paraformaldehyde (PFA) to each well and incubate for 15 min at 37 °C.
10. Aspirate fixative and wash cells 3 times with 1 mL of PBS (*see* **Note 17**).
11. Incubate the coverslips in their 24-well plate with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 2 h at room temperature protected from light.
12. Aspirate medium and wash with three times with PBS.
13. On a single glass microscopy slide, dispense a drop (~ 25 µl) of mounting medium.

14. Using a pair of fine tweezers, carefully remove coverslips from the 24-well plate and invert with cells 'facing down' onto the drop of mounting medium. Leave to dry overnight protected from light.
15. The following day, seal the edges of the coverslip using clear nail varnish (*see* **Note 18)**.

3.6. Confocal microscopy

1. Collect images with the confocal microscope using the Plan-Apochromat 63x/1.40 oil objective lens (*see* Figure 4).
2. Excite detection of nuclear DNA staining using DAPI with a 405 nm laser and emission at 435 nm (filter range 0-585 nm). Excite the fluorescent DiI derivative with a 555 nm laser line and detect emission at 585 nm (filter range 560-1000 nm). Frame-scan channels to reduce the risk of signal overlap.
3. Visualize lipid particle uptake by the appearance of a punctate pattern of red 'dots' where lipid particles have been endocytosed and delivered to specific intracellular compartments such as endosomes and lysosomes (*see* Figure 4). There are also some differences between the staining patterns, especially that for HDL (*see* **Note 19**).

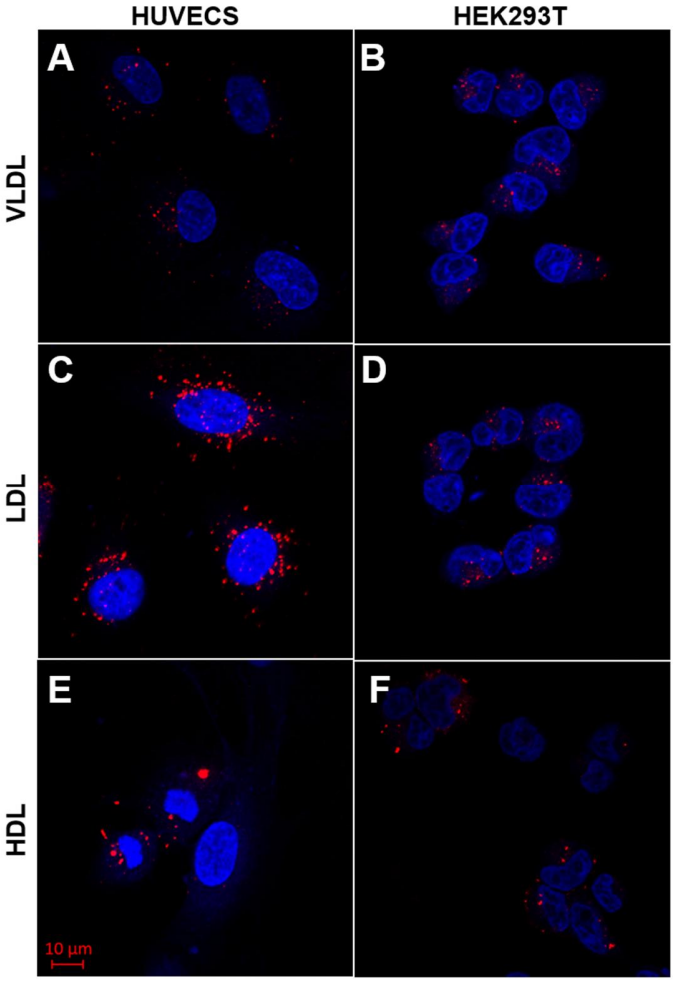

Figure 4: Confocal microscopy on fluorescent labelled lipid particle uptake in primary and immortalised human cells. *Primary human endothelial cells (A, C, E) or immortalised HEK293T (B, D, F) cells were serum starved and then incubated with 10 µg/ml fluorescent labelled lipid particles for 5 min before chase for 55 min (see section 3.5). The only exception being that cell incubation with 50 µg/ml labelled HDL was necessary to detect signal for HEK293T cells. VLDL and LDL show a punctate pattern (red) surrounding the DAPI stained nucleus (blue) indicative of clustering in late endosomes. This patten appears irregular when cells were treated with fluorescent labelled HDL. Scale bar, 10 µm.*

4 Notes

Note 1. Vacutainer tubes pre-coated with anticoagulant (sodium heparin, EDTA, sodium citrate ect.) can be selected for blood collection, dependant on their interaction with downstream experiments. Sodium heparin is the suggested anticoagulant for use in this work due to its compatibility with the HDL and LDL/ VLDL Quantitation Kit (Sigma-Aldrich) used in this work. If not performing this step, then other anticoagulants could be used. Alternatively, provided that blood is mixed immediately after taking the sample, solutions of liquid anticoagulant can be used. Commonly we mix 18 ml of blood with 2 ml of 3.8% sodium citrate (9:1 ratio) pre-added to a 50 ml screw cap tube.

Note 2. There are several commercially available Coomassie stains which claim shorter staining times, reduced background staining and do not require a de-staining step which could be pursued at the operator's discretion. Here we present a generic method using an in-house stain for widely applicable use. It should however be noted that Figure 2b was generated using a commercial Coomassie stain.

Note 3. There are many commercially available cholesterol assay kits designed for blood plasma analyses that can be selected according to the specific requirements of the operator. Many of these assays employ the approach of providing the user with cholesterol standards to generate a concentration curve of absorbance/ fluorescence readings from which to determine the cholesterol concentration of experimental samples. Here a HDL and LDL/VLDL quantitation kit was selected for its ability to separate HDL from VLDL/ LDL prior to cholesterol quantification as another method of delineating lipoprotein particle species. This assay kit precipitates both LDL and VLDL, separating them from HDL before a generic cholesterol determination step. There is little danger of mistaking LDL and VLDL cholesterol as they are spatially separated after ultracentrifugation with little or no overlap. If using a different assay kit then follow the manufacturers protocols.

Note 4. DiI and DiD can be used interchangeably with similar spectral properties and fluorescence properties in hydrophobic environments such as lipid particles.

Note 5. A P200 pipette is best used for this purpose as the smaller pipette tip aperture is at lower risk of disturbing the boundary between layers. In some cases, as for the clearly defined orange LDL band, it is apparent that the LDL fraction often exceeds 250 µL. In this case, at the operator's discretion, more than 250 µL can be collected within a fraction. This needs to be done carefully as there is potential of overlapping LDL and HDL species due to their close proximity within the gradient (*see* Fig. 1). It is not necessary to collect all the fractions towards the bottom of the OptiSeal tube as the lower third of the gradient contains largely plasma proteins.

Note 6. Do not make the agarose gel unnecessarily thick as this may impede the Sudan Black lipid stain from penetrating effectively into the gel and reduce lipid particle detection.

Note 7. Lipoprotein particles differ in electrophoretic mobility dependent on their size and electrical charge. Chylomicrons are the largest class of lipoproteins and will not migrate out of the gel wells (*see* Figure 2A). LDL appears closest to the origin, followed by the larger particle VLDL. This is because in addition to apolipoprotein B-100 which they both carry, VLDL also contains apolipoproteins A5, C1, C2, C3 and E which increase the negative charge of VLDL and therefore electrophoretic mobility. The band for HDL appears furthest from the origin. In the later HDL fractions, the mobility of HDL appears to decrease. This is likely to be attributable to an increasing presence of human serum albumin binding to HDL and increasing the apparent size of particles.

Note 8. These volumes assume the use of the suggested 20 x 10 cm gel system. The exact volume to occupy the resolving gel portion of a particular gel rig should be worked out on a case-by-case basis. Regardless, an equal volume of 6% and 20% polyacrylamide solutions should be used.

Note 9. Some lipid particle fractions will have very low protein concentration and it may not be possible to add 20 µg of protein due to SDS-PAGE well volume restriction. In this case, add as much volume of each fraction as possible. It is possible to concentrate the fractions but given that they contain low concentrations of protein, they likely do not contain the lipoproteins of interest so this may be an inefficient use of time.
Note 10. The VLDL/LDL particles form a diffuse white to yellow precipitate above the soluble HDL fraction and separating the two can be difficult. Repeated centrifugation during the separation stage may be necessary. As the HDL and LDL/ VLDL fractions are subsequently assayed for cholesterol content by the same protocol any unsuccessfully separated material persisting in the wrong separated aliquot will be reported by the assay as the wrong type of cholesterol. If the HDL fraction remains cloudy despite repeated centrifugation, dilute the sample 1:1 in PBS and repeat the treatment with 2X precipitation buffer. **Note 11**. Commonly, these assay kits provide cholesterol standards for the operator to generate a standard curve from

which to interpret their results. Some fractions may require dilution with cholesterol assay buffer to receive signals which fit within this standard curve. This must be determined on a per fraction basis.

Note 12. Analysis of different lipoprotein particle fractions derived from human blood with subsequent biochemical analysis will enable the operator to identify specific lipid particle fractions of interest. After agarose electrophoresis and SDS-PAGE (see Figure 2), we identified fraction #2 as enriched for VLDL, #8 for LDL and #10 for HDL. It must be kept in mind, that overlap of LDL and HDL populations (Fig. 1) is unavoidable without further centrifugation or gel filtration steps.

Note 13. Some DiI derivative may remain in the lipid particle fraction as aggregates following dialysis. If so, centrifuge at 16 000 *g* for 10 min to sediment unincorporated fluorescent dye aggregates before use.

Note 14. Endothelial cells (HUVECs) are grown in complete supplemented ECGM unless otherwise stated. For maintenance fresh medium should be provided every 2-3 days. HUVECs should be passaged upon reaching ~90% confluence (every ~6 days) and split 1:3 into flasks coated with 0.1% (w/v) pig skin gelatin. To split cells first wash 2X with prewarmed 37 °C sterile filtered PBS and aspirate. Add 1 ml of Ttrypsin solution and incubate for 2-5 mins, until cells come away from the plastic. Quench the trypsin by adding 9 ml of supplemented ECGM and split cells 1:3 for cell maintenance or otherwise dependent on experimental parameters.

Note 15. Immortalized cells (HEK293T) are grown in complete supplemented DMEM unless otherwise stated. HEK293T cells should be passaged upon reaching ~80% confluence (every ~5 days) and seeded at 25% confluence. It is not necessary to pre-coat plastic but if seeding onto glass then this should be coated with 0.01% (w/v) poly-L-lysine. To split cells first wash 2X with prewarmed 37 °C sterile filtered PBS and aspirate. Add 1 ml of trypsin solution and incubate for ~3 mins, until cells come away from the plastic. Quench the trypsin by adding 9 ml of supplemented DMEM and seed cells at 25% for cell maintenance or otherwise dependent on experimental parameters.

Note 16. It may be necessary to optimize the concentration of each DiI-labelled lipid particle used depending on cell type and other experimental conditions. In this study 10 µg/ mL of DiI-labelled lipid particle usually produced the most optimal results; however, HEK293T cells required 50 µg/ mL DiI-labelled HDL.

Note 17. The operator may want to use an antibody to simultaneously label their cells for an intracellular marker or reference. If so, cells should be incubated with 0.5% (w/v) BSA in PBS for 1 h to reduce non-specific binding, prior to primary and secondary antibody incubations following manufacturer's instructions.

Note 18. Sealing with nail varnish prevents the mounting medium from drying up and causing air bubbles to form between the coverslip and glass slide.

Note 19. The labeled LDL and VLDL staining patterns are relatively similar showing a pattern of punctate dots radiating outwards from the nuclear periphery into the cell cytoplasm (*see* Figure 4). Of note, the staining pattern for HDL is more irregular and less rounded in appearance (*see* Figure 4).

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