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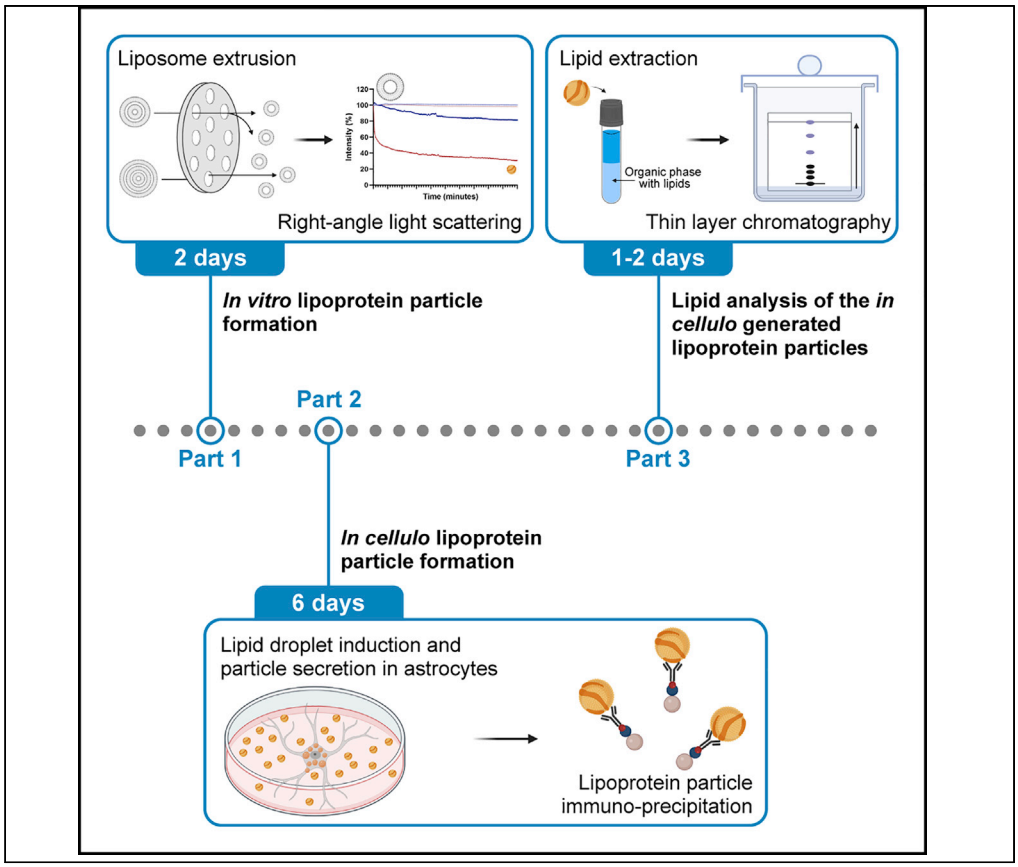
In vitro and *in cellulo* ApoE particle formation, isolation, and characterization



Apolipoprotein E (ApoE) particles are responsible for packing and transporting lipids throughout aqueous environments. We detail steps to assess *in vitro* particles forming from artificial membranes using right-angle light scattering and to measure their size using dynamic light scattering. We further describe how to generate *in cellulo* ApoE particles containing triacylglycerol under fatty-acid-induced stress. We also detail steps to isolate them from cell secretome by immunoprecipitation and analyze their lipid cargo by thin-layer chromatography.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Collection of protocols that enable the analysis of ApoE lipoprotein particles

Characterization of ApoE particles generated *in vitro* using light scattering

ApoE lipidation in lipid droplet-loaded cultured cells

Isolation of particles and characterization of lipid cargo by thin layer chromatography

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In vitro and *in cellulo* ApoE particle formation, isolation, and characterization

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SUMMARY

Apolipoprotein E (ApoE) particles are responsible for packing and transporting lipids throughout aqueous environments. We detail steps to assess *in vitro* particles forming from artificial membranes using right-angle light scattering and to measure their size using dynamic light scattering. We further describe how to generate *in cellulo* ApoE particles containing triacylglycerol under fatty-acid-induced stress. We also detail steps to isolate them from cell secretome by immunoprecipitation and analyze their lipid cargo by thin-layer chromatography.

For complete details on the use and execution of this protocol, please refer to Lindner et al. (2022).[1]

BEFORE YOU BEGIN

The first part of the protocol describes the *in vitro*-lipidation of ApoE by exposure to artificial large unilamellar vesicles (LUVs; ~ 100 nm in diameter) and the assessment of the kinetics of particle formation, showing how certain lipid species and membrane features influence the formation of lipoprotein particles. The formation of lipoprotein particles can be validated at the end of the kinetics experiment by electron microscopy. An example of lipoprotein particles generated *in vitro* from phosphatidylinositol bisphosphate (PIP_2)-containing LUVs with the *APOE3* protein purified from *Escherichia coli* will be highlighted here. *In vitro* formation of lipoprotein particles from membranes made of lipids extracted from human brain and membranes mimicking mouse brain compositions with all three bacterial- and mammalian-purified ApoE isoforms (*APOE2*, *APOE3*, *APOE4*) or truncated versions of ApoE (N-termini) have also been performed,[1] but will not be further discussed in this protocol.

Furthermore, the protocol describes the specific steps for isolating and characterising the lipid cargo of ApoE-containing lipoprotein particles from the secretome of CCF-STTG1 astrocytes treated chronically (72 h) with 250 μM oleic acid (OA) loaded on bovine serum albumin (BSA) at a 5:1 ratio. The OA treatment was used to induce a metabolic stress in astrocytes. However, other cell lines could be used and cellular metabolism can be perturbed by other factors (e.g., increasing reactive

oxygen species levels.[2] This protocol has also been applied to isolate particles i) at earlier time points (24 h or 48 h), ii) after enhancing ApoE secretion with a hormone (progesterone) or liver X receptor (LXR) inducer (TO901317)[1] and iii) from cerebrospinal fluid of an Alzheimer's disease patient (unpublished results), but these conditions will not be covered in this protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat biotinylated anti-human ApoE antibody (20 μg antibody / 200 μL bead slurry)	Meridian Life Science	Cat# K74180B; RRID: AB_150544
Chemicals, peptides, and recombinant proteins		
DPBS	Sigma	Cat# D8537
RPMI 1640 Medium (ATCC modification)	Gibco	Cat# A1049101
Fetal bovine serum (heat inactivated)	Gibco	Cat# 10500-064
LipidSpot 610	Biotium	Cat# 70069
Hoechst 33342	Abcam	Cat# ab228551
Streptavidin-coated agarose beads	Sigma-Aldrich	Cat# S1638
Fatty-acid-free BSA	Sigma-Aldrich	Cat# A3803; CAS:9048-46-8
Trizma base	Sigma	Cat# T1503 CAS:77-86-1
Sodium chloride	Acros organics	Cat# 207790010 CAS:7647-14-5
Sodium hydroxide	Carlo Erba reagents	Cat# 480507
Methanol	Merck	Cat# 1060091000; CAS:67-56-1
Chloroform	Merck	Cat# 1024451000; CAS:67-66-3
Hexane	Sigma-Aldrich	Cat# 296090; CAS:110-54-3
Acetic acid	Merck	Cat# 1000621000; CAS:64-19-7
Acetone	Sigma	Cat# 650501 CAS: 67-64-1
85% ortho-phosphoric acid	Merck	Cat# 1005731000; CAS:7664-38-2
Ammonium hydroxide solution 25%	Sigma-Aldrich	Cat# 30501; CAS:1336-21-6
Diethyl ether	Thermo Fisher Scientific	Cat# 10263230; CAS:60-29-7
Copper (II) sulfate pentahydrate ($CuSO_4$)	Sigma-Aldrich	Cat# 12849; CAS:7758-99-8
His-ApoE2 full length/N-terminus	(Petros et al., 2019)[3]	N/A
His-ApoE3 full length/N-terminus	(Petros et al., 2019)[3]	N/A
His-ApoE4 full length/N-terminus	(Petros et al., 2019)[3]	N/A
Critical commercial assays		
Apolipoprotein E Human ELISA Kit	Thermo Fisher Scientific	Cat# EHAPOE
Experimental models: Cell lines		
CCF-STTG1 cell line	ECACC	Cat# 90021502; RRID:CVCL_1118
Software and algorithms		
GraphPad Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/; RRID: SCR_002798
BioRender	BioRender	https://www.BioRender.com
Malvern Zetasizer software (v7.13)	Malvern Panalytical	https://www.malvernpanalytical.com/en/support/product-support/zetasizer-range/zetasizer-nano-range/zetasizer-nano-zs#software
Other		
Extruder set with holder/heating block	Avanti	Cat# 610000
Filter supports, polyester, 10 mm diameter	Avanti	Cat# 610014
1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC)	Avanti	Cat# 850345
L-α-Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$; Brain; porcine; ammonium salt)	Avanti	Cat# 840046

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hamilton syringes	Hamilton	N/A
Clear CD vials, 9-mm screw thread, with blue cap	Supelco	Cat# 29307-U
Wheaton tubes	Wheaton	Cat# 358646
Argon gas	Carbagas	EG# 231-147-0
Millex 0.22 μm pore, 33 mm diameter, PVDF filter	Merck	Cat# SLGV033RS
Nuclepore Track-Etch membrane of 0.1 μm, 19 mm diameter (pore membrane)	Avanti	Cat# 800309
Zetasizer nano-ZS	Malvern Panalytical	N/A
Chemidoc MP imaging system	Bio-Rad	N/A
HPTLC sampler ATS4	CAMAG	N/A
UV-Cuvette micro	Brand	Cat# 759210
Vacuum chamber	N/A	N/A
Amicon Ultra – 15, Ultracel 10K	Merck	Cat# UFC901008D
Oleic acid (OA)	Sigma	Cat# O1383; CAS: 112-80-1
Sphingomyelin (SM)	Sigma	Cat# S0756; CAS: 85187-10-6
DOPC	Avanti	Cat# 850375; CAS: 4235-95-4
DOPE	Avanti	Cat# 850725; CAS: 4004-05-1
Triacylglycerol 18:1 (TAG)	Sigma	Cat# 41679; CAS: 122-32-7
Cholesterol	Sigma	Cat# C8667; CAS: 57-88-5
Cholesteryl oleate (CE)	Sigma	Cat# C9253; CAS: 303-43-5
Fluorimeter (RALS)	JASCO	FP-8500
QS high precision cell quartz cuvette, 3 × 3 mm path, 15 centre	Hellma Analytics	Cat# 105-251-15-40
Cuvette washer	Sigma	C1295
Triton X-100	Sigma	Cat# T9284; CAS:9036-19-5

MATERIALS AND EQUIPMENT

High-performance thin-layer chromatography (HPTLC) is an analytical method by which different lipid classes can be separated based on their hydrophobicity. The separation is acquired by using specific solvent systems:

First HPTLC solvent system	
Reagent	Amount
Chloroform	65 mL
Methanol	25 mL
Ammonium hydroxide 25%	4 mL
Total	**94 mL**
Prepare fresh. Final solvent ratio should be chloroform: methanol: ammonium hydroxide = 65: 25: 4 (v/v/v).	

Second HPTLC solvent system	
Reagent	Amount
Hexane	80 mL
Diethyl ether	20 mL
Acetic acid	2 mL
Total	**102 mL**
Prepare fresh. Final solvent ratio should be hexane: diethyl ether: acetic acid = 80: 20: 2 (v/v/v).	

Preparation of stocks for the physiological buffer used during liposome extrusion and the particle kinetics assay:

Tris 1 M stock	
Reagent	Amount
Trizma base	121.14 g
Water	800 mL
Adjust pH to	7.4
Fill up with water to	1 L
Total	**1 L**
Store at 20°C–25°C for up to one year.	

NaCl 1 M stock	
Reagent	Amount
NaCl	58.44 g
Fill up with water to	1 L
Total	**1 L**
Store at 20°C–25°C for up to one year.	

Physiological buffer: 50 mM Tris pH 7.4, 25 mM NaCl	
Reagent	Amount
Tris 1 M stock	2.5 mL
NaCl 1 M stock	1.25 mL
Water	46.25 mL
Total	**50 mL**
Prepare fresh, adjust the pH to 7.4, and filter-sterilise.	

⚠ CRITICAL: All the reagents used for the HPTLC solvent systems are harmful to the skin or by inhalation and should be handled with extreme caution with chemical-resistant gloves and under a ventilated hood.

⚠ CRITICAL: Use glassware for pipetting organic solvents, because plastic accessories are degraded by organic solvents.

Alternatives: The first HPTLC solvent system separates more hydrophilic lipids, such as phospholipids, and migrates more hydrophobic lipids in its front. The second solvent system separates more hydrophobic lipid species like cholesterol, triacylglycerols and cholesteryl esters without migrating more hydrophilic lipids any further. For the separation of other lipid classes, use appropriate HPTLC solvent systems.[4]

STEP-BY-STEP METHOD DETAILS

Part 1. *In vitro* lipoprotein particle formation

Timing: 2 days

The following steps describe the lipoprotein particle formation *in vitro* from artificial membranes with a defined lipid composition.

Liposome formation by extrusion

Timing: 3–4 h

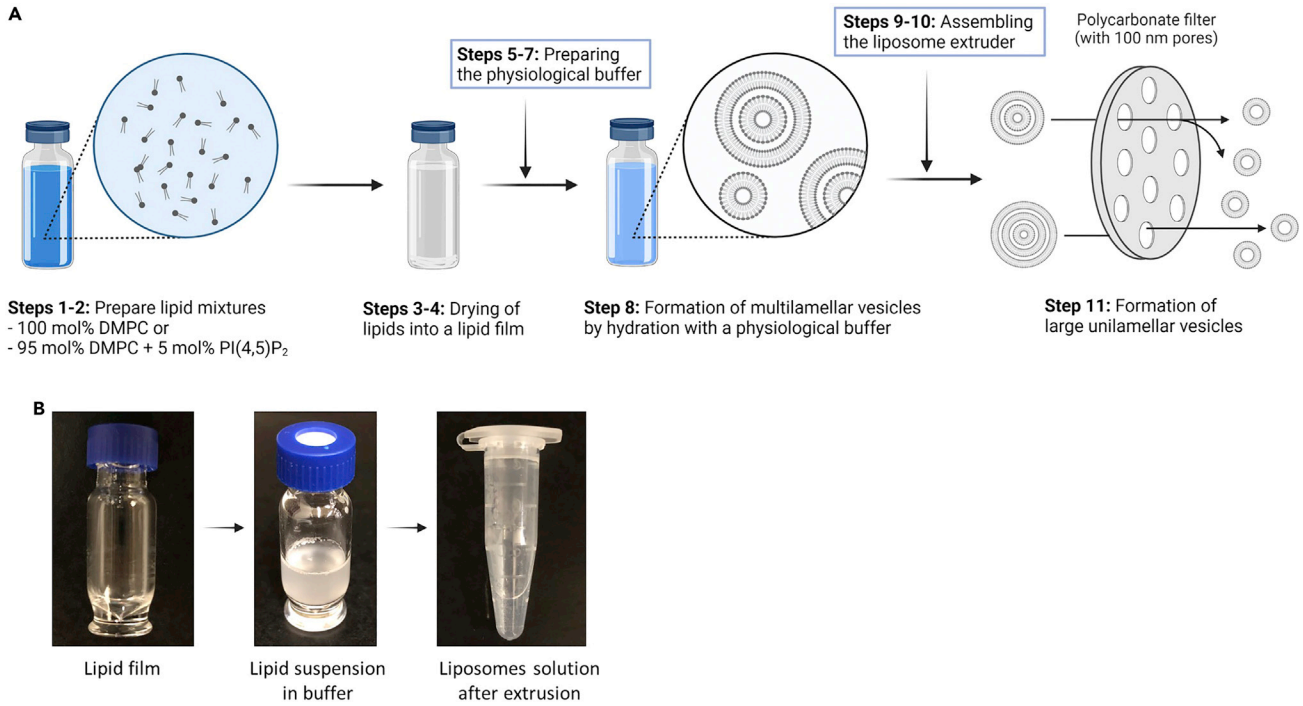

Figure 1. Liposome extrusion of PI(4,5)P_2-containing large unilamellar vesicles

(A) 100 mol% DMPC- and 95 mol% DMPC + 5 mol% PI(4,5)P_2-containing large unilamellar vesicles are made by liposome extrusion. First, the lipids are mixed and the organic solvents are evaporated by rotationally drying down the lipids with an inert gas, thereby creating a lipid film. The lipids are rehydrated in a pre-heated physiological buffer (50 mM Tris pH 7.5, 25 mM NaCl) to form multilamellar vesicles. The multilamellar vesicles are then extruded through a 100 nm pore polycarbonate filter to reshape them into large unilamellar vesicles. Created with Biorender.com.

(B) Example of pictures for the dried lipid film, lipid suspension in physiological buffer and the final liposomes solution after extrusion. See also Methods videos S1, S2 and S3.

By liposome extrusion, large unilamellar vesicles (LUVs) with a defined size and composition are formed. These LUVs will be the membrane mimetic from which ApoE will extract lipids to form lipoprotein particles *in vitro* (Figure 1A).

Note: Use glass vials and Hamilton syringes for the handling of lipids. Wash the Hamilton syringes three times with chloroform:methanol 1:1 (v/v) in between each pipetting step.

Note: Perform steps 1–3 under a flow hood.

Optional: Use printed labels to label the glass or cover the writing with scotch or parafilm. A single drop of solvent will otherwise wipe out the labelling on the glass vials.

1. Calculate the amount of lipids needed for DMPC liposomes (100 mol%) and DMPC-PI(4,5)P_2 liposomes (95 and 5 mol%, respectively) at a final concentration of 1 mg/mL for a total volume of 500 µL.

   Note: In this assay, the DMPC liposomes have a membrane defect and therefore helps facilitate the lipids pick-up from the liposomes by ApoE. Extrusion of liposomes for other purposes should use a more stable backbone, such as POPC (the most abundant phosphatidylcholine in mammalian cells) instead of DMPC.

2. Pipette the calculated amounts of lipids in the glass vials.

Note: The volume of the pipetted lipid mixture at this step is not relevant. The lipids will be dried down and resuspended in 500 μL of a physiological buffer.

Note: Thaw the lipid stocks to facilitate pipetting. Lipid stocks are typically stored at a concentration of 10 mg/mL or 1 mg/mL in its corresponding solvent. After using the lipid stocks, the lipids should be overlayed with an inert gas and stored at −20°C.

3. Dry the lipids rotationally under an inert gas stream to form a thin layer of lipids on the wall of the tube (Figure 1B, Methods video S1).

⚠ CRITICAL: Use a low flow of inert gas to dry the lipids. If the flow is too strong, the solution will splash while drying. The gas stream should be barely sensed on the skin. The wrists are sensitive areas that will allow a good check of the flow strength (Methods video S1).

4. Put the glass vials without a cap in a vacuum chamber for 30 min to remove residual solvents.
5. Prepare freshly 50 mL of 50 mM Tris-Cl pH 7.5, 25 mM NaCl (physiological buffer).
6. Filter-sterilise the solution with a 0.22 μm pore filter.
7. Heat the physiological buffer to 65°C in a water bath.

Note: The physiological buffer is dependent on the protein that is used in the assay. The most suitable buffer for the protein should also be used during the liposome extrusion. In case the buffer is changed from what is stated here, it is important to check the quality of the liposomes after extrusion.

Note: The temperature of the buffer is set slightly higher than the extruder temperature to account for the cooling down of the sample during sample loading.

8. Add 500 μL of pre-heated physiological buffer to each glass vial and heat it to 65°C in a water bath for 1 h.

Note: To bring the lipids in suspension in the physiological buffer, heat is required. The temperature at which the lipid mixtures are heated should be above the phase transition temperature of each lipid in the composition.[5] Out of convenience and for comparability we often stick to a high temperature for the buffer and extruder, especially when neutral and sterol lipids are present in the mixture.

Note: The mixture should become slightly cloudy due to the formation of multilayer sheets. Vortexing the vials will help solubilize the lipids in the physiological buffer (Figure 1B).

⚠ CRITICAL: The temperature should not be above 80°C. Temperatures above 80°C are incompatible with the gas-tight syringes of the extruder for longer periods of time.

9. Assemble the extruder and let the extruder get to a temperature of 62°C on a heating block (roughly 5–10 min before extruding) (Methods video S2).

   a. Wet 4 filter supports and one pore membrane in physiological buffer.
   b. Place two filter supports on top of each other on each internal membrane support.
   c. Place the pore membrane on top of the filter supports.
   d. Clamp the pore membrane in between the O-rings of the internal membrane supports, place it inside the extruder outer casting, screw the retainer nut on the extruder outer casting, and place it in the extruder holder on top of a heating block.
   e. Fill one of the gas-tight syringes that are part of the extruder kit (blunt end needle) with physiological buffer and insert both syringes at each end of the extruder.

f. Push the physiological buffer halfway into the extruder to ensure that the membranes stay wet during the heating equilibration and to reduce the dead volume in the extruder.

Note: For extra support on the assembly of the extruder, see the Avanti manual of the mini-extruder.[6]

10. Remove the gas-tight syringes from the extruder.
11. Load the sample into one of the gas-tight syringes and push the sample 21 times through the pore membrane (Methods video S3).

Note: Vortex the sample before loading it into one of the gas-tight syringes. The suspension should look slightly cloudy at first due to the multilaminar sheets that formed and clear up when the sample is pushed through the pore membrane (Figure 1B, Methods video S3).

Note: Always extrude an uneven number of times through the pore membrane. One of the gas-tight syringes will serve as the input syringe and the opposite syringe as the collecting syringe. By extruding an uneven number of times, any residual free or aggregated lipids are not collected together with the formed LUVs.

Note: For extra support on the extrusion technique, see the Avanti manual for extrusion.[7]

12. Transfer the extruded liposomes to an Eppendorf tube and let the liposomes cool down to 20°C–25°C (Figure 1B, Methods video S3).
13. Store the liposomes for up to 3–4 days at 4°C.

⚠ CRITICAL: Never freeze liposomes! Liposomes should not be vortexed to prevent fusion. To homogenize liposomes in a solution, flick the tube or carefully pipet up and down with a plastic tip.

⚠ CRITICAL: Putting freshly extruded liposomes directly in the fridge can cause these unstable liposomes to fuse/break down. Let the liposomes slowly cool down to 20°C–25°C and afterwards store them at 4°C.

Note: Be very careful with the liposomes in general and especially with liposomes having this composition. The DMPC causes a membrane packing defect that makes these liposomes more fragile.

14. Clean the extruder.
    a. Disassemble the extruder and syringes as much as possible.
    b. Put the parts that were in contact with the sample in hot demiH_2O (e.g., the O-rings, internal membrane supports and disassembled gas-tight syringes).
    c. After 5 min, transfer the parts to acetone.
    d. After 5 min in acetone, discard the acetone and let the parts dry for 12–20 h under a flow hood on tissue paper.

Note: Exchange the pore membrane in between different samples. In the case of 100% DMPC and 95% DMPC, 5% PI(4,5)P_2 liposomes, it suffices to rinse the parts with physiological buffer. First extrude 100% DMPC to ensure no cross-contamination of PI(4,5)P_2 in the 100% DMPC liposomes occurs.

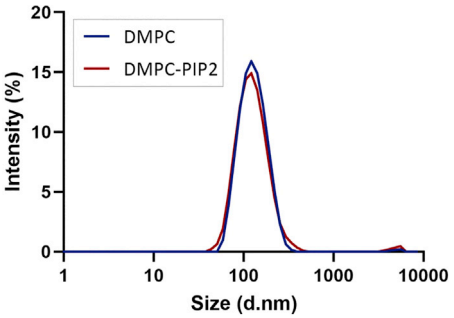

Figure 2. Quality check of liposomes with dynamic light scattering

100 mol% DMPC- and 95 mol% DMPC + 5 mol% PI(4,5)P_2-containing large unilamellar vesicles (LUVs) are 16× diluted and measured for their size in dynamic light scattering. 100 mol% DMPC liposomes: Z-average = 115.8 nm diameter, PDI = 0.122, attenuator = 8. 95 mol% DMPC + 5 mol% PI(4,5)P_2-containing liposomes: Z-average = 119.5 nm diameter, PDI = 0.179, attenuator = 8.

Note: If the lipid compositions are strongly different from each other, it is recommended to clean the extruder thoroughly before extruding the next composition, as explained in step 14.

Quality control of liposomes with dynamic light scattering

Timing: 45 min for starting up and 10 min per sample

To ensure that the liposomes are of good quality, dynamic light scattering (DLS) is performed to measure the size of the liposomes (Figure 2).

Note: Ideally, the quality of the liposomes is checked directly after extrusion and shortly before the lipoprotein particle formation assay to ensure that the liposomes have not fused.

15. Let the liposomes equilibrate to 20°C–25°C.
16. Turn on the DLS machine (Zetasizer nano-ZS) 30 min before starting.
17. Filter the physiological buffer through a 0.22 μm pore filter.
18. Dilute the sample 10–20× in physiological buffer (100 μL total volume) and transfer the sample into a disposable cuvette.
19. Set up a DLS method with the following parameters:
    a. Choose "size" measurement mode.
    b. Set the refractive index for the physiological buffer.
    c. Set the temperature to 24.8°C.
    d. Set: 5 s per run, 11 runs per measurement, and 3 measurements per sample.
    e. Choose as cell type: Disposable cuvettes – ZEN0040.
20. Put the cuvette in the direction indicated on the machine (arrow to the front) in the machine and run the method.
21. Take the average values of the 3 measurements to depict the Z-average diameter in nm.
22. Check the polydispersity index and attenuator to ensure good quality measurement.

Note: Reliable sizes need to show a polydispersity index (PD) <0.5. When these liposomes are correctly formed, the PD falls within 0.05–0.2.

Note: The attenuator is the correction factor that is calculated based on the concentration of the particles that are being measured. An attenuator of 10–11 indicates that the sample is too diluted, whereas an attenuator of 5–6 indicates that the sample is too concentrated. An attenuator of 8–9 is ideal. Usually, this is achieved by diluting the liposomes 10–20 times.

Note: The size of the liposomes is dependent on the fluidity of the membrane. Extruding with a 100 nm pore size will lead to liposomes of 115–130 nm in size.

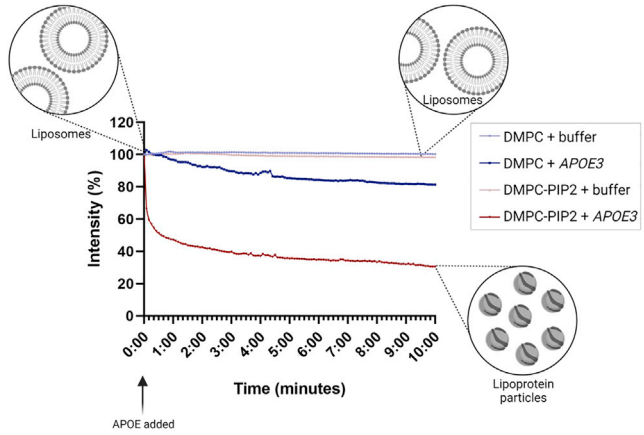

Figure 3. Lipoprotein particle formation by addition of ApoE to liposomes detected by right-angle light scattering

Lipoprotein particles are formed upon the addition of *APOE3* to 100 mol% DMPC- and 95 mol% DMPC + 5 mol% PI(4,5)P_2-containing large unilamellar vesicles (LUVs), as shown by the decrease in light intensity. The presence of PI(4,5)P_2 in the LUVs increases the speed at which lipoprotein particles are formed. As a negative control, DMPC and PIP(4,5)P_2-containing LUVs are incubated with buffer and the liposomes stay intact, as indicated by the stable level light intensity. y-axis: light intensity represented as a percentage relative to time 0 (t/t0 of corresponding liposomes with buffer alone). Created with BioRender.com.

Kinetics of the in vitro *ApoE lipoprotein particle formation by right-angle scattering*

Timing: 30 min starting up and 45 min per sample

Right-angle light scattering allows to capture the particle formation kinetics (10–20 nm diameter) when ApoE is incubated with LUVs (100 nm). Larger particles reflect more light than smaller particles in this assay. This technique captures the kinetics of the particle formation by the decrease in size, and thus light intensity (Figure 3).

23. Turn on the Jasco FP-8500 fluorimeter 30 min before measuring.
24. Pre-incubate the liposomes and protein separately at 24°C for at least 30 min.

Note: Liposomes can be kept at 24°C throughout the day, but the protein should be incubated at this temperature for only 30 min. Ideally, pre-incubate an aliquot of the protein equivalent to the amount needed for the next sample at 24°C and keep the remainder on ice.

25. Set up the method on the fluorimeter with the following settings:
    a. Go to the program ''Time-course''.
    b. Photometric mode: Em intensity.
    c. Ex bandwith: 2.5 nm.
    d. Em bandwith: 2.5 nm.
    e. Response: 1 s.
    f. Sensitiviy: ''Low'' or ''Medium''.
    g. Measurement range: 0–600 s.
    h. Data interval: 5 s.
    i. Ex wavelength: 600.0 nm.
    j. Em wavelength: 600.0 nm.
    k. Intensity normalisation: off.
    l. Blank correction: off.
    m. Auto gain: off.
    n. Shutter control: Open only for measurement.

o. Light source: Xe lamp.
p. Filter: Not used.
q. Temperature: 24°C.

Note: Determine the sensitivity modus of the method based on the liposomes incubated with buffer. This will be the highest intensity measured and should not be close to saturating light intensity levels.

26. Clean the quartz cuvette with a cuvette washer and dryer in the following order: 1× milliQ water, 1× 70% ethanol, 2× milliQ water.

Alternatives: Squirt milliQ water in the quartz cuvette and shake the liquid out above a sink. Do the washing with milliQ water and 70% ethanol in the same order as with a cuvette washer. Remove the remaining liquid at the last step with a pipette.

27. Wash the quartz cuvette once with filter-sterilised buffer (50 mM Tris, pH7.5, 25 mM NaCl; same buffer as used during liposome extrusion).
28. Clean the outside of the quartz cuvette with an ethanol-soaked Kimtech wipe to remove any dust particulates on the outside of the cuvette.
29. Mix 50 μg liposomes with 50 μg ApoE in a total volume of 100 μL in the quartz cuvette while the quartz cuvette is in the fluorimeter.
30. Pipette the mixture five times up and down.
31. Start the measurement.
32. After the measurement, pipette the sample out of the quartz cuvette.
33. Repeat steps 26–32 for the next sample. After the last sample measurement, repeat only step 4 and store the quartz cuvette in a dust-free environment.

Part 2. *In cellulo* lipoprotein particle formation

Timing: 6 days

The following steps describe an *in cellulo* ApoE lipidation by forcing the endogenous ApoE to be loaded with lipids (containing triacylglycerol) after lipid droplet induction with a fatty acid treatment.

Oleic acid conjugation to fatty-acid-free BSA

Timing: 1 h

BSA can accommodate and transport up to 7 fatty acids. In this assay, oleic acid is loaded on BSA in a molar ratio of 5:1 to mimic a pathologic condition.[8]

34. Prepare a 50 mM sodium oleate solution.
    a. Prepare a NaOH 0.1 M solution by dissolving 0.2 g NaOH in 50 mL ultrapure water. Filter the solution with a 0.22 μm pore filter.
    b. Dissolve 15.7 μL OA (equivalent to 0.014 g OA; ρ=0.89 g/mL) in 1 mL NaOH 0.1 M to obtain a final concentration of 50 mM OA. Mix in a 1.5 mL Eppendorf at 70°C while shaking until OA is dissolved (a few seconds until no oily phase remains at the surface).
    c. Transfer the solution to a glass vial, overlay with an inert gas (argon or nitrogen) and store at −20°C until use.

⚠ CRITICAL: Oleic acid and the sodium oleate stock solution must be kept in glass vials under an inert gas atmosphere to prevent lipid oxidation.

Note: The sodium oleate solution should be thawed at 20°C–25°C for 15–20 min before use. If an oily phase appears at the surface, it should be briefly vortexed to homogenise.

35. Prepare a 10% fatty acid-free BSA solution.
    a. Dissolve 1 g BSA in 10 mL phosphate-buffered saline (PBS) and mix well until the solution is clear.
    b. Sterilise the solution by filtering it through a 0.22 μm filter.

Note: Add PBS and BSA in this order for better and faster solubilisation.

36. Load the OA on fatty acid-free BSA.
    a. Mix 0.2 mL sodium oleate solution with 1.2 mL 10% fatty acid-free BSA at 37°C for 15 min in a 2 mL Eppendorf, while shaking at 600 rpm.
    b. Add 0.6 mL PBS for a final concentration of 5 mM OA.
    c. Filter the solution with a 0.22 μm filter under a sterile hood used for cell culture purposes and store it at 4°C for up to one month.

Lipid droplet induction and lipoprotein particle formation in astrocytes

Timing: 5 days

This step describes the treatment of astrocytes with OA-BSA 5:1, which leads to cellular triacylglycerol accumulation and lipid droplet formation. After chronic exposure, astrocytes reroute stored triacylglycerol to secretion on ApoE-containing lipoprotein particles (Figure 4).

37. Plate CCF-STTG1 cells at 0.026 million cells/cm^2 in media containing fetal bovine serum (FBS) for 24 h in a 150 cm^2 flask and in a 35 mm Petri dish for microscopy, to allow cell adherence.
38. Induce chronic lipid droplet formation for 72 h. Wash the cells thoroughly and treat them with 1.7 mL OA-BSA 5:1 solution in 32.3 mL media without additives for the 150 cm^2 flask or 100 μL OA-BSA 5:1 solution in 1.9 mL media for the 35 mm Petri dish. This will lead to a final OA concentration of 250 μM.
39. Collect the conditioned media and visualise lipid droplet formation.
    a. Collect the conditioned media from the 150 cm^2 flask. Remove the cell debris by centrifugation at 18,400 × *g* for 10 min at 4°C and store the supernatant at 4°C.
    b. Perform live-cell imaging on the astrocytes in the 35 mm Petri dish. Wash the cells extensively and incubate them in serum-free media for 30 min with 1:1000 LipidSpot 610 and 1 μM Hoechst 33342 at 37°C before imaging.

Isolation of lipoprotein particles formed in cellulo *by immunoprecipitation*

Timing: 1 day

Lipoprotein particles are isolated by immunoprecipitation from the cellular secretome in a detergent-free environment. This assay is particularly useful to characterise the lipid cargo of the particles.

40. Couple the biotinylated anti-ApoE antibody with streptavidin-agarose beads (Figure 5A).
    a. Wash the streptavidin-agarose beads slurry.
        i. Pipette 200 μL slurry/condition in an Eppendorf tube and wash the beads 4× with 500 μL cold PBS.
        ii. Centrifuge the tubes at 1,000 × *g* for 2 min at 4°C and discard the supernatants between washes.
    b. Resuspend the beads in PBS to the slurry starting volume (200 μL).

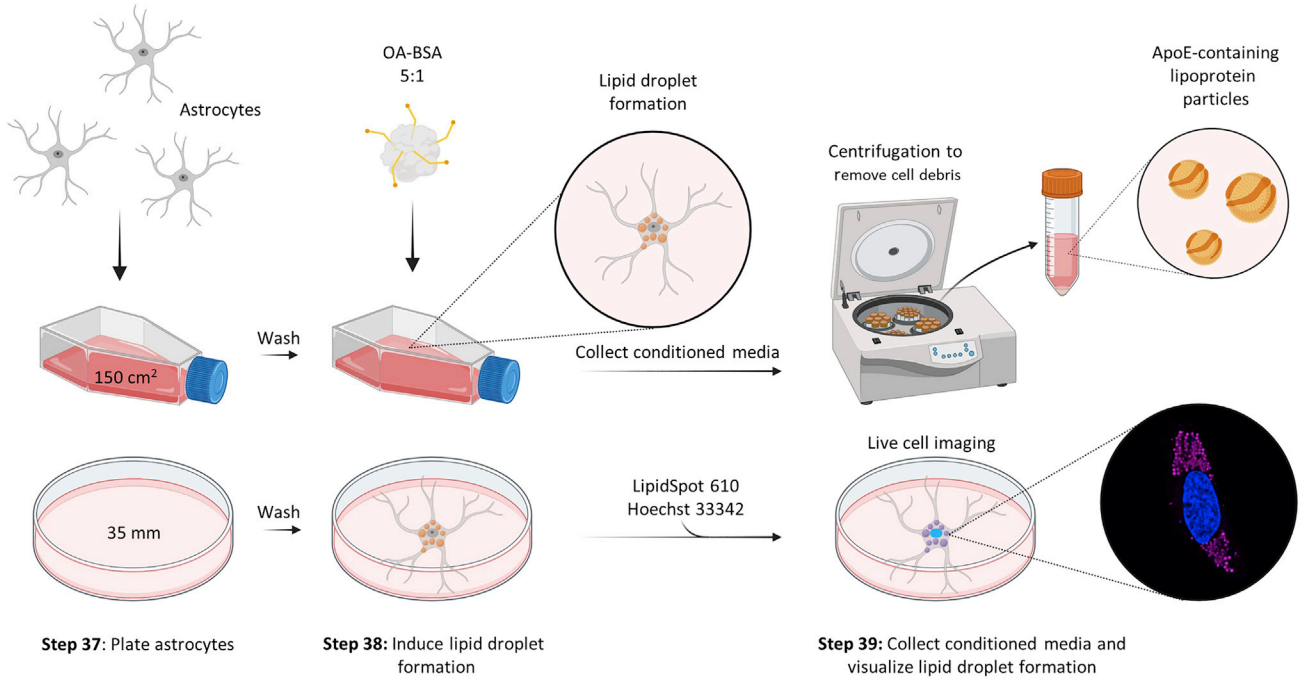

Figure 4. ApoE-containing lipoprotein particle formation *in cellulo*

CCF-STTG1 astrocytes are plated in a 150 cm^2 flask and a 35 mm Petri dish in media containing FBS for 24 h. After extensive washing, OA-BSA 5:1 is added in FBS-free media for 72 h to induce chronic accumulation of lipid droplets. The cell supernatant containing lipoprotein particles formed by ApoE is collected from the 150 cm^2 flask and centrifuged to remove the cell debris. Live-cell imaging is performed on cells in the 35 mm Petri dish that are stained with LipidSpot 610 (lipid droplets) and Hoechst 33342 (nuclei). Created with BioRender.com.

c. Incubate the beads slurry with 20 µg anti-ApoE antibody for 2 h at 20°C–25°C on a rotating wheel.
d. Wash the antibody-coupled beads 3× with 500 µL cold PBS to remove the unbound antibody and store at 4°C.

⚠ CRITICAL: For lipidomic analysis of the lipoprotein particles, it is important to not use any detergents during the immunoprecipitation process.

Note: Cut the tip diagonally off the pipette tips to pipette the beads slurry.

41. Concentrate the cell supernatant on a 10 kDa cut-off ultracentrifugation device to approximately 1 mL final volume (Figure 5B).
42. Immunoprecipitate the ApoE-containing lipoprotein particles (Figure 5C).

Note: Use a specific method to detect the amount of immunoprecipitated bait protein. For example, for ApoE we use a commercial ELISA kit (see key resources table). Typically, we immunoprecipitate 90 ng (normal protein secretion) to 1.5 µg ApoE (under specific pharmacological induction with TO901317) from 34 mL of CCF-STTG1 cells conditioned media.

a. Incubate the concentrated supernatant with the antibody-coupled beads for 12–20 h at 4°C on a rotating wheel.
b. Centrifuge the tube at 1,000 × *g* for 2 min at 4°C and keep the flow-through.
c. Wash the beads 4× with 1 mL cold PBS.

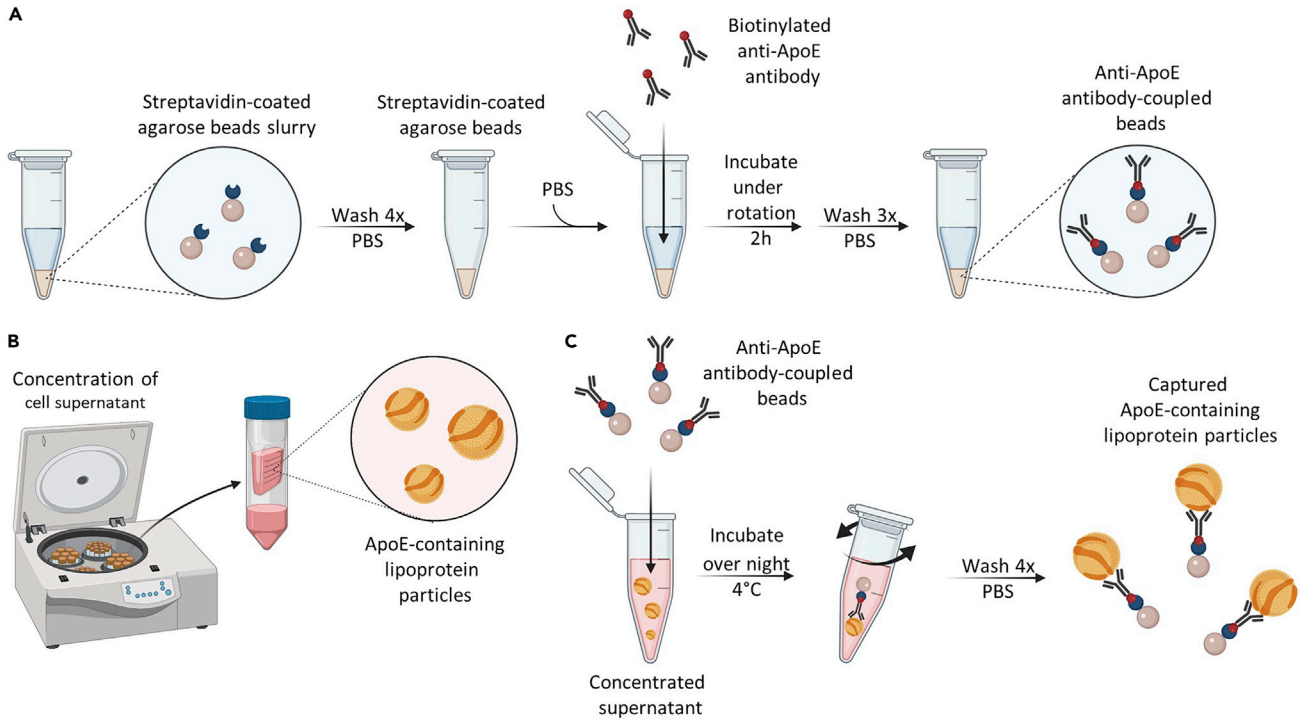

Figure 5. Immunoprecipitation of ApoE-containing lipoprotein particles

(A) Conjugation of streptavidin beads with biotinylated anti-ApoE antibody. Streptavidin-coated agarose beads are washed four times and resuspended in PBS. They are incubated with a biotinylated anti-ApoE antibody for two hours on a rotating wheel. The final anti-ApoE antibody-coupled beads are washed three times and resuspended in PBS.

(B) Cell supernatant concentration. Cell supernatant containing lipoprotein particles formed by ApoE is concentrated in an ultracentrifugation device.

(C) Isolation of ApoE-containing lipoprotein particles. Anti-ApoE antibody-coupled beads (A) are incubated with concentrated cell supernatant (B) for 12–20 h under rotation at 4°C. The beads are washed four times and should contain the lipoprotein particles formed by ApoE. Created with BioRender.com.

⚠ CRITICAL: Analyse in parallel a control antibody-coupled beads sample (processed in similar conditions) to assess the presence of potential lipid contaminants.

Part 3. Lipid analysis of the *in cellulo*-generated lipoprotein particles

Timing: 1 day

Here we describe an example of lipid extraction and analysis for *in cellulo*-generated lipoprotein particles, but the protocol could also be adapted to the analysis of *in vitro* lipoprotein particles or particles coming from other sources.

Lipid extraction of in cellulo*-generated lipoprotein particles*

Timing: 1/2 day

The lipids carried by the lipoprotein particles were isolated by organic solvent extraction using a modified Bligh and Dyer protocol[9] (Figure 6). Prepare 3 Wheaton tubes for lipid extraction per sample.

⚠ CRITICAL: Use glassware for lipid extraction and Hamilton glass syringes for pipetting, because plastic accessories are degraded by organic solvents.

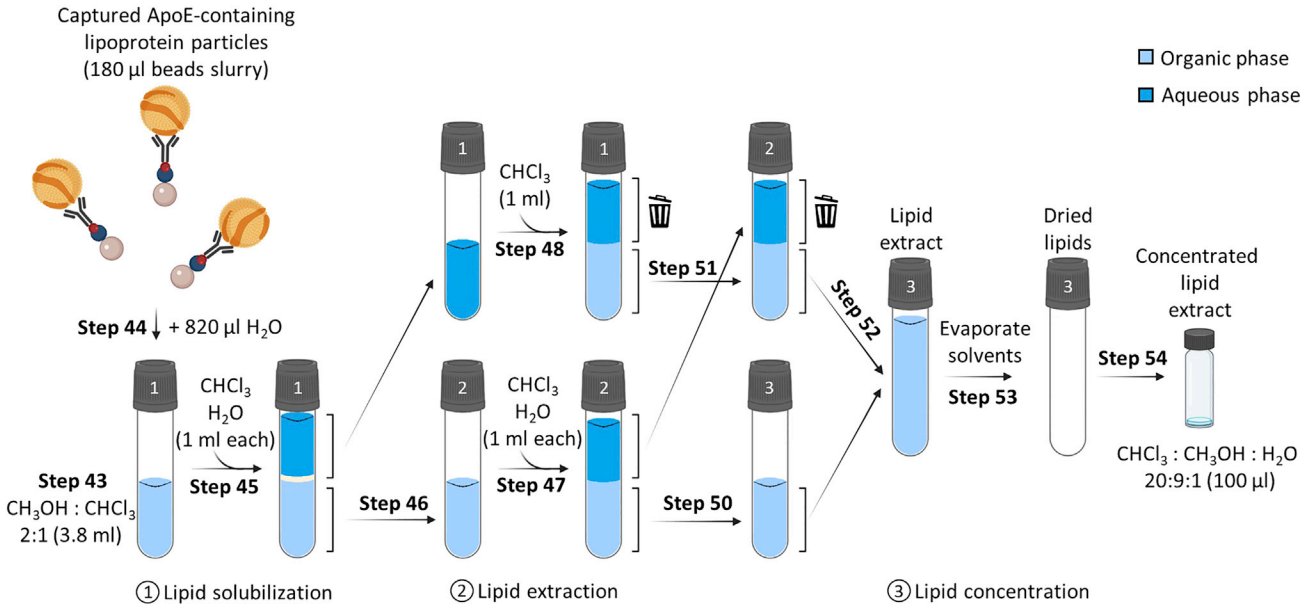

Figure 6. Lipid extraction using a Bligh and Dyer protocol

A methanol:chloroform 2:1 (v/v) solution is added in a first Wheaton tube (1), followed by the beads slurry containing lipoprotein particles formed by ApoE obtained in Figure 4C, resuspended in water. Chloroform and water are added, followed by thorough vortexing after each addition. Tube (1) is centrifuged and the lower (organic) phase is transferred into tube (2) and is topped up with chloroform and water. The upper (aqueous) phase remaining in tube (1) is reextracted with chloroform. Tubes (1) and (2) are vortexed and centrifuged to allow phase separation. The lower phase from tube (2) is transferred to a final tube (3). The upper phase from tube (2) is mixed with the lower phase from tube (1). After vortexing and centrifugation, the lower phase is transferred to tube (3). The solvents from tube (3), containing the total lipid extract, are evaporated. Dried lipids are resuspended in a small volume of chloroform:methanol:water 20:9:1 (v/v/v) to obtain the concentrated lipid extract. Created with BioRender.com.

43. Add 3.8 mL methanol:chloroform 2:1 (v/v) to a Wheaton tube no. 1.
44. Resuspend 180 µL beads slurry in 820 µL ultrapure water and transfer the suspension in tube no. 1. Vortex thoroughly.
45. Add 1 mL chloroform and 1 mL water in tube no. 1, followed by vortexing thoroughly after each addition. Centrifuge the tube at 900 × *g*, 2 min, at 4°C in a swinging bucket rotor.

Note: The agarose beads will remain at the interface between the two liquid phases.

46. Transfer the lower phase in a Wheaton tube no. 2.
47. Add 1 mL chloroform and 1 mL water in tube no. 2, followed by vortexing thoroughly after each addition.
48. Add 1 mL chloroform to the remaining solution in tube no. 1 and vortex thoroughly.
49. Centrifuge tubes no. 1 and no. 2 at 900 × *g*, 2 min, at 4°C in a swinging bucket rotor.
50. Transfer the lower phase of tube no. 2 to a final Wheaton tube no. 3.
51. Transfer the lower phase of tube no. 1 to the remaining solution in tube no. 2. Vortex thoroughly and centrifuge tube no. 2 at 900 × *g*, 2 min, at 4°C in a swinging bucket rotor.
52. Transfer the lower phase of tube no. 2 to tube no. 3.
53. Evaporate the organic solvents from tube no. 3 under vacuum at 20°C–25°C or under a stream of nitrogen.

Note: The lipid film remaining after solvent evaporation is transparent (Figure 1B). If white precipitates or impurities are present, repeat the extraction steps 43–53.

54. Resuspend dried lipids in 100 µL chloroform:methanol:water 20:9:1 (v/v/v) and overlay them with an inert gas.

Note: The resuspension solvent mix should be chosen based on the solubility of the lipid of interest. Chloroform:methanol:water 20:9:1 (v/v/v) solvent mix is allowing the solubilization of both polar and non-polar lipids.

Lipid analysis of lipoprotein particles by HPTLC

Timing: 1/2 day

The concentrated lipid extract is separated by HPTLC. This allows the separation of the different lipid classes and their identification and quantification based on known standards.

55. Lipid spotting on an HPTLC plate (Figure 7A, Methods video S4).

    a. Pre-wash the HPTLC plate by covering it with chloroform:methanol 1:1 (v/v). Remove the solvent excess and let it air dry.
    b. Spot 20 µL lipid extract or the corresponding lipid standards using an automated HPTLC sampler (CAMAG ATS 4).

    Alternatives: The lipids can alternatively also be spotted on the TLC plate by hand with a Hamilton syringe. Make sure that all samples are spotted on a line so that the migration of the lipids is not shifted between samples.

56. HPTLC plate development (Figure 7B, Methods video S4).
    a. Develop the plate in the first solvent system, chloroform:methanol:ammonium hydroxide 65:25:4 (v/v/v), for 5 cm and dry it briefly.
    b. Re-develop the plate in the second solvent system, hexane:diethyl ether:acetic acid 80:20:2 (v/v/v), for 9 cm.
    c. Dry the plate under vacuum for 30 min at 20°C–25°C.

    Note: Prepare the solvent systems beforehand and let air within the chamber saturated for 30 min.

57. Lipid revelation.
    a. Prepare a $CuSO_4$ staining solution.
        i. Dissolve 5 g of $CuSO_4$ in 40 mL water.
        ii. Filter the solution with a 0.22 µm filter.
        iii. Add 4.7 mL of 85% ortho-phosphoric acid and fill up to 50 mL with water.

    Note: Prepare the staining solution freshly before use.

    b. Pour 10 mL of the staining solution on the HPTLC plate and incubate for 1 min (Figure 7C, Methods video S4).
    c. Decant the staining solution excess and dry the plate in the vacuum chamber for 15 min.
    d. The lipids are charred at 145°C for 7.5 min.

    Note: As oven characteristics might slightly differ, it is important to adapt the lipid charring time to the best lipid visualisation.

    e. The plate is visualised in visible light and at 488 nm using a ChemiDoc MP (Bio-Rad) imaging system.

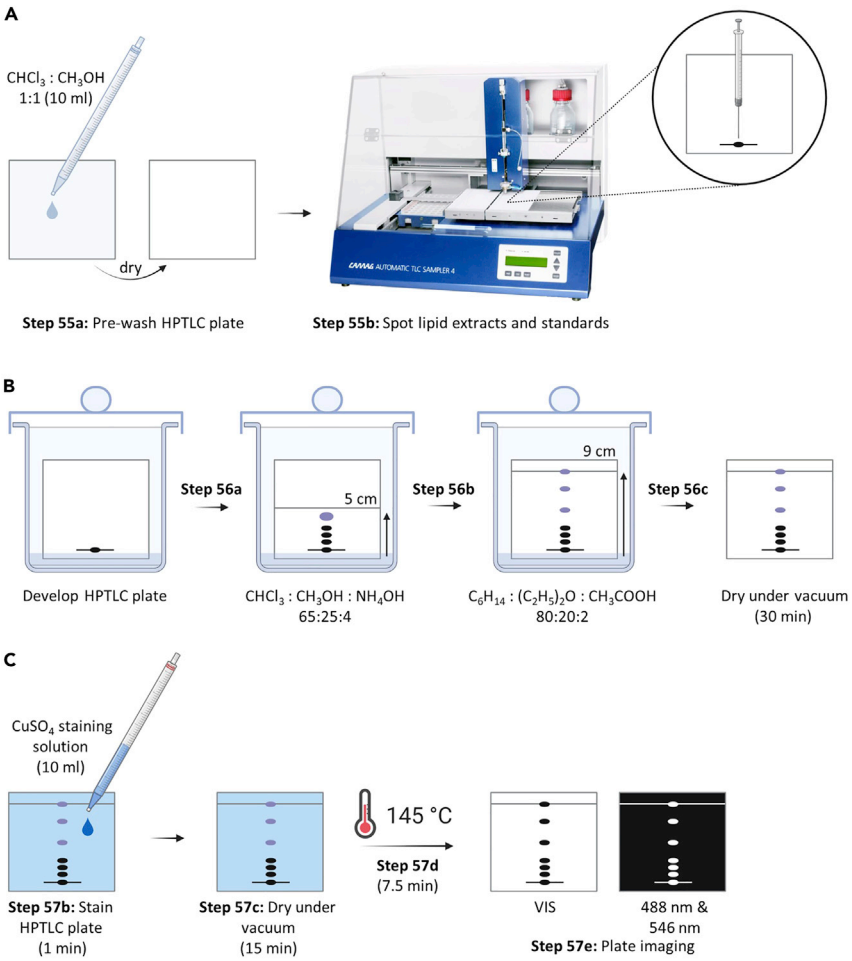

Figure 7. Lipid separation and detection by HPTLC

(A) HPTLC plate preparation and automated lipid spotting. The HPTLC plate is prewashed with an excess of chloroform:methanol 1:1 (v/v) and dried briefly. The plate is placed in the automated HPTLC sampler (CAMAG ATS4) and lipid extracts and standards are spotted.

(B) Lipid separation. The HPTLC plate from (A) is developed in a first solvent system (chloroform:methanol:ammonium hydroxide 65:25:4 (v/v/v)) for 5 cm and dried briefly. Next, the plate is developed in the second solvent system (hexane:diethyl ether:acetic acid 80:20:2 (v/v/v)) that is left to migrate for 9 cm. Finally, the plate is dried under vacuum for 30 min at 20°C–25°C. **Note**: The first solvent system separates hydrophilic lipids like phospholipids (black spots) and the second solvent system separates hydrophobic lipid species like cholesterol, triacylglycerols and cholesteryl esters (blue spots). At this step, lipids are not visible - the dots were represented in the scheme to depict the lipid separation pattern.

(C) Lipid revelation. After lipid separation, the HPTLC plate is stained with a $CuSO_4$ staining solution for 1 min. The excess solution is decanted and the plate is dried under vacuum for 15 min at 20°C–25°C. Lipids are charred at 145°C for 7.5 min and the plate is visualised in visible light or fluorescence at 488 nm or 546 nm. See also Methods video S4. Created with BioRender.com.

EXPECTED OUTCOMES

For the liposome extrusion, the liposomes are expected to have a size between 115 and 130 nm in diameter when extruded through a 100 nm pore filter, a PDI value of <0.5 and attenuator of 8 or 9. DMPC liposomes containing $PI(4,5)P_2$ tend to be a couple of nm larger than liposomes without $PI(4,5)P_2$. An example of a dynamic light scattering output graph is shown in Figure 2. In the *in vitro* lipoprotein particle formation assay with right-angle light scattering, the liposomes in buffer are expected to keep the same level of light intensity throughout the measurement duration, whereas upon the addition of ApoE, the light intensity levels should drop. An example of a

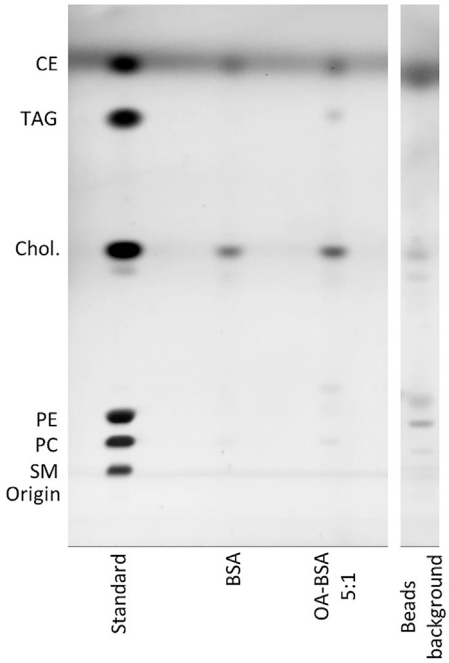

Figure 8. Lipid composition of ApoE-containing lipoprotein particles

Representative fluorescent-inverted image of an HPTLC plate showing the lipids carried by immunoprecipitated ApoE particles isolated from OA-BSA 5:1 treated cells or their respective BSA control for 72 h. Lipid standards (left): 1 μg SM, 1 μg PC, 1 μg PE, 0.1 μg cholesterol, 1 μg TAG, 0.1 μg CE. The lipid background of the immunoprecipitation beads is also shown (right). Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Chol., cholesterol; CE, cholesteryl ester.

right-angle light scattering output graph is shown in Figure 3. An example of an TLC plate is shown in Figure 8.

QUANTIFICATION AND STATISTICAL ANALYSIS

Lipid spots were quantified using densitometry with the ImageJ software and represented as the area under the curve.

LIMITATIONS

Saturated lipid species are not revealed by the revelation method used for HPTLCs described in this protocol.

TROUBLESHOOTING

Problem 1

The lipid mixture does not clear up during liposome extrusion.

Potential solution

Make sure that the extruder is properly assembled and that the pore membrane is well clamped in between the O-rings.

Problem 2

The solution with liposomes after extrusion is becoming turbid.

Potential solution

Avoid vortexing of the liposomes, as this well cause the liposomes to fuse / break down back into multilamellar sheets. Do not store the liposomes longer than 4 days and make sure the liposomes are cooled down before putting them at 4°C. Putting liposomes too quickly at 4°C can cause thermal shock and lead to liposome fusion. Make sure that the buffer is filter-sterilised before extrusion to avoid bacterial contamination.

Problem 3

The polydispersity index in the DLS measurement is >0.5.

Potential solution

If also the Z-average is much larger than expected, the liposomes have either fused or larger particles are contaminating the sample. Prepare new liposomes and ensure that the buffer is filter-sterilised to prevent salt aggregations and other contaminations. Handle the liposomes with care to prevent fusion. If the DLS output graph shows two peaks indicating two populations of particles, re-extrude the liposomes.

Problem 4

The attenuator in the DLS measurement is 5–6.

Potential solution

Dilute the sample until an attenuator of 8–9 is reached.

Problem 5

The attenuator in the DLS measurement is 10–11.

Potential solution

Remix the liposomes carefully by flicking or pipetting to homogenise sedimented liposomes in the solution and remeasure. If the attenuator stays low, this indicates that the sample is either too diluted or that the liposomes have fallen apart into smaller particles. A higher number of smaller particles is needed to get to the same intensity as larger particles. Prepare a new dilution with a lower dilution factor. If that does not improve the attenuator, prepare new liposomes.

Problem 6

The Z-average is too small. If the Z-average is smaller than the size of the pore membrane, the liposomes have fallen apart.

Potential solution

Prepare new liposomes.

Problem 7

The Z-average is too large (>200 nm). The liposomes have fused or dust particles or other contaminants are present in the sample.

Potential solution

Prepare a new dilution of the sample to measure the size in case of dust particle contamination. If that does not improve the Z-average, re-extrude the liposomes or prepare new liposomes.

Problem 8

There are spikes in the RALS curves.

Potential solution

These spikes are coming from dust particles on the outside of the cuvette that move into the window of the laser. Wipe the quartz cuvette free of dust with ethanol-soaked Kimtech wipes before starting the measurement.

Problem 9

Addition of ApoE does not lower the light intensity detected in RALS.

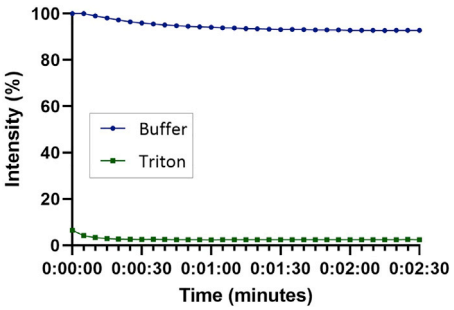

Figure 9. Destruction of liposomes with 0.1% Triton-X detected by right-angle light scattering

Addition of 0.1% Triton-X leads to a sharp decline of the light intensity due to the complete breakdown of the liposomes. y-axis: light intensity represented as a percentage relative to time 0 (t/t0 of corresponding liposomes with buffer alone).

Potential solution

If the liposomes have fallen apart, the size will not decrease and therefore neither the light intensity. Confirm that liposomes are of good quality with DLS. An additional check of the liposomes can be performed by adding 0.1% Triton-X to the quartz cuvette. This should directly dissolve all liposomes and will lead in an immediate decrease in light scattering intensity (see Figure 9). If the liposomes are passing all the tests, then it's likely that the protein is non-functional. Take a fresh aliquot or repurify the protein.

Problem 10

Antibodies could potentially not recognise lipidated forms of apolipoproteins, because the protein could have a different conformation in its lipid-bound form, hiding the epitope. Additionally, the epitope could be responsible for lipid binding and thus, is not recognised by the antibody. Apolipoproteins synthesised by different cell types or organs could have distinct post-translational modifications, preventing an efficient recognition by antibodies.

Potential solution

Use antibodies that are not recognising the lipid-binding domain of the apolipoproteins and test the antibody recognition efficiency before proceeding to immunoprecipitation.

Problem 11

Lipid spots are not visible (concentration too low, Figure 10A) or they do not separate properly, forming merged patterns (concentration too high, Figure 10B). A good outcome could be visualised in Figure 8.

Potential solution

A lipid spotting and migration test should be made before the final experiment. It will allow the correct dosage of the lipid extract to obtain a good separation and visualisation of the lipids. For low lipid concentrations, detection at 488 nm enhances the lipid spot intensity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anne-Claude Gavin (anne-claude.gavin@unige.ch).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article (Lindner et al.[1]) includes all datasets generated or analysed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101894.

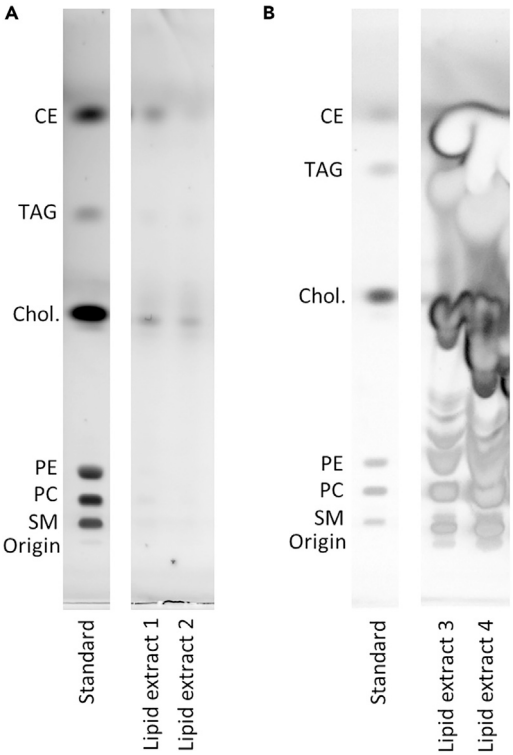

Figure 10. Lipid revelation problems depending on lipid concentration

(A and B) Representative fluorescent-inverted images of HPTLC plates show too low (A) or too high (B) lipid concentrations. Lipid standards: 1 μg SM, 1 μg PC, 1 μg PE, 0.1 μg cholesterol, 1 μg TAG, 0.1 μg CE. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Chol., cholesterol; CE, cholesteryl ester.

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AUTHOR CONTRIBUTIONS

A.-C.G. and K.B. designed the research; K.L. conducted the *in cellulo* lipoprotein particle formation assays and the lipid analysis of the particles; K.B. and L.C.v.E., with the help of V.R., conducted the *in vitro* lipoprotein particle formation assays; K.A. and F.H. developed protocols for the extraction of lipids; A.V.K. developed the ApoE purification protocols; K.L. and L.C.v.E wrote and all authors reviewed the manuscript.

DECLARATION OF INTERESTS

K.B., K.A., F.H., and A.V.K. are employees of AbbVie. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

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