Video Article

Enrichment of Native Lipoprotein Particles with microRNA and Subsequent Determination of Their Absolute/Relative microRNA Content and Their Cellular Transfer Rate

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Abstract

Lipoprotein particles are predominately transporters of lipids and cholesterol in the bloodstream. Furthermore, they contain small amounts of strands of noncoding microRNA (miRNA). In general, miRNA alters the protein expression profile due to interactions with messenger-RNA (mRNA). Thus, knowledge of the relative and absolute miRNA content of lipoprotein particles is essential to estimate the biological effect of cellular particle uptake. Here, a quantitative real-time polymerase chain reaction (qPCR)-based protocol is presented to determine the absolute miRNA content of lipoprotein particles—exemplified shown for native and miRNA-enriched lipoprotein particles. The relative miRNA content is quantified using multiwell microfluidic array cards. Furthermore, this protocol allows scientists to estimate the cellular miRNA and, thus, the lipoprotein particle uptake rate. A significant increase of the cellular miRNA level is observable when using high-density lipoprotein (HDL) particles artificially loaded with miRNA, whereas incubation with native HDL particles yields no significant effect due to their rather low miRNA content. In contrast, the cellular uptake of low-density lipoprotein (LDL) particles—neither with native miRNA nor artificially loaded with it—did not alter the cellular miRNA level.

Video Link

The video component of this article can be found at <https://www.jove.com/video/59573/>

Introduction

Lipoprotein particles are composed of a monolayer of amphiphilic lipids and cholesterol enclosing a core of cholesteryl esters and triglyceride fats. The whole particle is stabilized by membrane-embedded apolipoproteins, which define the particle's biological functionality. Lipoprotein particles can be distinguished according to their respective increasing density and, thus, decreasing size, namely as very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL, and HDL particles. Despite the transportation of water-insoluble components in the bloodstream, it has been demonstrated that HDL particles carry noncoding strands of miRNA^{1,2}. Micro-RNAs are a class of short (usually two dozen nucleotides) RNA strands, which degrade intracellularly complementary mRNA strands and, thereby, alter the expression profile of certain proteins^{3,4,5,6}. Furthermore, alterations of the miRNA profile have been found in a variety of diseases and, thus, the profile is applicable as a biomarker for diagnosis and prognosis. The extracellular transport of miRNAs between cells via lipoprotein particles may serve as an additional mechanism for intercellular mRNA level modulation. To quantitatively estimate the biological effect, knowledge regarding the absolute and relative miRNA content of lipoprotein particles is needed.

Quantitative real-time PCR is a suitable and relatively quick method to gain this information. Hence, the relative quantification (RQ) value can be calculated, and relative differences between different samples and lipoprotein fractions are estimable. Multiwell microfluidic array cards are a fast and easy-to-use method to determine the relative presence (equates to the RQ value) of miRNAs in a sample. Multiwell microfluidic array cards consist of 96 or 384 individual reaction chambers for individual qPCR reactions embedded in a microfluidic device. Each chamber contains the required hydrolysis probe and specific qPCR primers for one individual miRNA. The advantages are a short handling time due to standardization, a simple workflow, and a reduced number of pipetting steps. Moreover, the required sample volume is minimized. In contrast to the relative quantification, the absolute miRNA content requires a comparison of qPCR sample results with standard curves of known absolute numbers of miRNA strands. It should be noted that, due to their relatively low miRNA content, standard and, moreover, even single-molecule sensitive imaging techniques are not feasible—the artificial enrichment of lipoprotein particles with miRNA is inevitable to study cellular lipoprotein particle interaction and miRNA transfer. Regarding this, delipidation of the HDL particle followed with subsequent relipidation⁷ allows the incorporation of and, thus, enrichment with miRNA strands. Similar enrichment of LDL particles with miRNA is not feasible due to the hydrophobicity of the apoB-100 protein, which is the main constituent of the LDL particle. However, by addition of the polar solvent dimethyl sulfoxide (DMSO), which is able to penetrate lipid membranes, LDL particles can be artificially loaded with miRNA strands as well.

High-speed atomic force microscopy (HS-AFM) is a powerful tool for the characterization of biological specimens offering subnanometer spatial and subsecond temporal resolution⁸. Hence, it is a well-suited technique for the quality control of modified lipoprotein particles as native/reconstituted/labeled lipoprotein particles can be imaged under a near-physiological environment.

Here, a qPCR-based protocol is presented step-by-step to determine the absolute/relative miRNA content of lipoprotein particles and cell samples, which allows an estimation of the cellular lipoprotein particle uptake rate. Moreover, a method for the enrichment of lipoprotein particles with miRNA is demonstrated. This method may be adapted for the general manipulation of lipoprotein content and, hence, demonstrates the applicability of lipoprotein particles as targets for drug delivery.

Protocol

Blood donations have been approved by the Ethics Committee, Medical University of Vienna (EK-Nr. 511/2007, EK-Nr. 1414/2016). Nomenclature is according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)⁹ guidelines.

1. Lipoprotein particle isolation from human blood

1. Precool an ultracentrifuge to 4 °C. Draw blood from healthy volunteers after overnight fasting.
NOTE: Typically required are three donors, donating 80 mL each, and blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant.
2. Centrifuge at 2,000 x *g* for 20 min at 4 °C and harvest plasma (upper phase); avoid shear forces. Determine the total volume *V* and adjust, if necessary, to a multiple of the centrifugation tube volume using phosphate-buffered saline (PBS). Measure the mass of 1 mL 3x and calculate the average density ρ .
3. Calculate the required amount of potassium bromide (KBr) for density adjustment with the following equation¹⁰ (for desired density in grams/milliliter, use $A = 1.019$, and for the specific volume of KBr, use $v = 0.364$ mL/g). Add KBr to the plasma and stir gently to avoid shear forces until KBr is totally dissolved.

$$m_{\text{KBr}}/g = \frac{V/\text{mL} \times (A - \rho/\text{g/mL})}{1 - v/\text{mL/g} \times \left(\frac{A + \rho/\text{g/mL}}{2} \right)}$$

4. Measure the density as described in step 1.2 and adjust again, if necessary, by adding more KBr. Fill and seal centrifuge tubes suitable for ultracentrifugation with plasma. Avoid any air bubbles; otherwise, the tube may collapse. Place the tubes in the rotor according to the manufacturer's instructions and centrifuge at 214,000 x *g* for 20 h at 4 °C.
5. Open the tubes according to the manufacturer's instructions and discard the upper phase containing VLDL and IDL. Determine the total volume *V* and adjust, if necessary, to a multiple of the centrifugation tube volume using PBS.
6. Determine the density ρ of the bottom fraction. Calculate the required amount of KBr for density adjustment (use $A = 1.063$). Stir gently to avoid shear forces until KBr is dissolved. Repeat step 1.4.
7. Remove and collect the upper phase containing LDL particles. Store the LDL particle solution under an inert gas atmosphere at 4 °C. Determine the total volume *V* and adjust, if necessary, to a multiple of the centrifugation tube volume using PBS. Determine the density ρ of the bottom fraction as described in step 1.2.
8. Calculate the required amount of KBr for density adjustment (use $A = 1.220$) and add it. Stir gently to avoid shear forces until KBr is dissolved. Repeat step 1.4.
9. Remove and collect the upper phase containing HDL particles. Determine its total volume *V* and adjust, if necessary, to a multiple of the centrifugation tube volume using PBS. Determine the density ρ . A second centrifugation step of the upper phase at 214,000 x *g* for 20 h at 4 °C is recommended to remove albumin. If required, repeat step 1.8. Remove and collect the upper phase containing HDL particles.
10. Prepare and precool at least 20 L of dialysis buffer (0.9% NaCl, 0.1% EDTA [pH 7.4]) to 4 °C. Prewet dialysis tubes (molecular weight cut-off: 12–14 kDa) and add the LDL and HDL particle solution according to the manufacturer's instructions. Dialyze against 5 L of dialysis buffer at 4 °C and change the buffer after 1, 2, and 4 h.
11. After 24 h, recover the lipoprotein particle solutions from the dialysis tubes and determine the protein concentration using the Bradford assay¹¹ or another appropriate one. Store the HDL and LDL particle solutions under inert gas atmosphere at 4 °C.

2. Synthetic miRNA aliquots

NOTE: When handling RNA oligonucleotides, work RNase-free. Work only with fresh, disposable plastic consumables and always wear gloves, which should be changed frequently. Use only nuclease-free solutions. Always work on ice.

1. Spin down the vial acquired from the manufacturer at maximum force to form a pellet of the lyophilized synthetic miRNA. Add an appropriate volume of 10 mM Tris(hydroxymethyl) aminomethane (TRIS) buffer, pH 7.5, for a final concentration of 10 μM (stock concentration) miRNA.
2. Gently pipette up and down a couple of times for resuspension. Prepare aliquots of 100 μL each in sterile tubes. Store them at -20 °C if not used immediately. Avoid repeated thawing and freezing.

3. Reconstitution of HDL particles

1. Delipidation

1. Prepare buffer A (150 mM NaCl, 0.01% EDTA, 10 mM Tris/HCl [pH 8.0]). Precool the centrifuge to -10 °C. Precool 100 mL of a mixture of ethanol:diethyl ether (3:2) at -20 °C.

CAUTION: Wear appropriate personal protective equipment and work in a fume hood while handling diethyl ether as it is extremely flammable and harmful to the skin.

- Mix a volume corresponding to 5 mg of HDL particles with 50 mL of the precooled ethanol:diethyl ether (3:2) mixture and incubate for 2 h at -20 °C. Centrifuge at 2,500 x g for 10 min at -10 °C.
- Discard the supernatant, resuspend the pellet in 50 mL of precooled ethanol:diethyl ether mixture by vortexing, and incubate a second time for 2 h at -20 °C. Centrifuge again at 2,500 x g for 10 min at -10 °C.

NOTE: If desired, lyophilize the supernatant for an analysis of the miRNA content in the lipid fraction of the HDL particles.

- Dry the pellet under N₂ gas flow and resuspend it in 250 µL of buffer A (see step 3.1.1). Determine the protein concentration using the Bradford protein assay or another appropriate one and dilute to a final concentration of 1 mg of protein/250 µL of buffer A.

NOTE: The protocol can be paused here. Store the solution overnight at 4 °C under inert gas atmosphere.

2. Reconstitution

- Prepare a phosphatidylcholine (PC) stock solution using a mixture of chloroform:methanol (2:1) at a concentration of 5.6 mg of PC/mL. Similarly, prepare stock solutions of cholesteryl oleate (5 mg of CO/mL) and cholesterol (5 mg of C/mL). Store all solutions at -20 °C.
- In a clean glass tube, mix 500 µL of PC, 100 µL of CO, and 13.5 µL of C. These volumes correspond to an approximate molar ratio of 100 PC:22 CO:4.8 C. Dry the mixture under N₂ gas flow while rotating the tube to yield a homogeneous surface layer.
NOTE: The protocol can be paused here. Store the glass vial (if desired, stockpiling is possible) under inert gas atmosphere at -20 °C.
- Prepare a fresh 30 mM spermine solution in buffer A. Mix one aliquot (100 µL, 10 µM) of synthetic miRNA (see step 2.2) with 100 µL of spermine solution and incubate for 30 min at 30 °C.
NOTE: For negative control experiments, replace the miRNA and/or spermine solution with the same volume of buffer A.
- Dissolve a PC/CO/C master mix aliquot in the mixture from step 3.2.3.
- Prepare a solution of 30 mg/mL of sodium deoxycholate in buffer A and add 50 µL to the solution from step 3.2.4. Stir at 4 °C for 2 h.
- Add 250 µL of the delipidated HDL solution from step 3.1.4. This volume corresponds to an approximate molar ratio of 100 PC:22 CO:4.8 C:1 delipidated HDL proteins. Stir at 4 °C overnight.

3. Dialysis

- Precool at least 15 L of PBS at 4 °C. Add 50 g of adsorbent beads to 800 mL of double distilled water (ddH₂O) and stir for 1 min. Wait 15 min, decant the supernatant, and repeat the procedure with PBS.
- Prewet dialysis cassettes (molecular weight cut-off: 20 kDa) or appropriate dialysis tubes and add the solution from step 3.2.6, using a syringe according to the manufacturer's instructions.
- Add the adsorbent beads from step 3.3.1 to 3 L of PBS and dialyze at 4 °C. Change the buffer and the beads after 1 h and 2 h.
- After 24 h, recover the reconstituted HDL (rHDL) particle solution and determine the protein concentration using the Bradford assay. Store the rHDL particle solution under inert gas atmosphere at 4 °C.

4. Labeling of LDL particles

- Prepare 10x LDL buffer (1.5 M NaCl, 3 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA, pH 7.4]) and store it at room temperature.
- Prepare a fresh 30 mM spermine solution in RNase-free water. Mix an aliquot (100 µL, 10 µM) of synthetic miRNA (see step 2.2) with 100 µL of spermine solution and incubate for 30 min at 30 °C.
NOTE: For negative control experiments, replace the miRNA and/or the spermine solution with the same volume of 1x LDL buffer.
- Add 100 µL of DMSO to the miRNA/spermine solution from step 4.2 and dilute it further with 1.2 mL of 1x LDL buffer.
- Dilute the LDL particle solution to a final concentration of approximately 4 mg/mL with PBS and mix 450 µL with 50 µL of 10x LDL buffer. Incubate it for 10 min on ice.
- Combine the LDL particle solution from the previous step and the miRNA/spermine/DMSO solution (from step 4.3) and incubate it for 2 h at 40 °C.
- Perform dialysis similar as described in section 3.3 and store the labeled LDL particle solution accordingly.

5. Quality control of reconstituted/labeled lipoprotein particles

NOTE: For quality control, the diameter and general shape of lipoprotein particles can be determined using, for instance, AFM or electron microscopy (EM). Here, HS-AFM is used to measure the size distribution of native/reconstituted/labeled lipoprotein particles.

- Dilute the HDL/LDL particle solution in PBS (1:100–1:1,000) and incubate it on freshly cleaved mica for 5 min. For cleaving the mica¹², press adhesive tape against the substrate and remove the upper mica layers by pulling the tape off.
NOTE: Depending on the particular AFM instrument, the observation area, and the initial concentration of particles, the dilution factor has to be adjusted to observe individual particles.
- After incubation, rinse the sample with PBS and perform HS-AFM imaging in PBS and in the tapping mode with cantilevers having spring constants of $k_{cant} < 0.2$ N/m. It is recommended to use scan sizes $< 1 \mu m^2$ and to keep the imaging forces as low as possible.
- Determine the height of the imaged particles with respect to the mica surface with suitable software.
 - Load the data into Gwyddion (freeware), detect the particles via grain analysis (**Mark grains by threshold**) and set the threshold above the substrate background. Flatten the image (**Remove polynomial background**) choosing the **Exclude masked region** option.
 - Export the height values of the detected particles (**Distribution of various grain characteristics**) and repeat these steps for all recorded images. Perform statistical analysis by either creating histograms or calculating probability density functions of the obtained particle heights.

4. Repeat steps 5.1–5.3 with native as well as reconstituted/labeled lipoprotein particles and compare the obtained results to verify particle quality. If observing debris and/or conglomerates, discard the sample.

6. Cell culture

1. Grow adherent cells according to an established protocol (e.g., IdIA7-SRBI¹³) until reaching confluency.
NOTE: Several independent chambers depending on the number of experiments (recommended are two chambers per experimental setting) and negative control experiments (recommended are two chambers without the addition of lipoprotein particles) are needed. Additionally, two chambers are required for the determination of the cell number.
2. Gently wash the cells 3x with prewarmed Hank's balanced salt solution (HBSS) to remove cell debris and cover the cell layer with an appropriate volume of serum-free growth medium. Add an appropriate volume of lipoprotein particle solution to reach a final concentration of 50 µg/mL lipoprotein particles. Incubate at 37 °C and 5% CO₂ for 16 h.
NOTE: Depending on the experimental design and cell line, the incubation time has to be adapted to achieve a sufficient (i.e., measurable) increase in the cellular miRNA level.
3. Gently wash the cells 3x with prewarmed (37 °C) HBSS to remove cell debris/lipoprotein particles and cover the cell layer with an appropriate volume of serum-free medium.
4. Determine the cell density using an appropriate method (e.g., hemocytometer, automated cell counter) in at least two independent chambers to calculate the number of cells in the sample volume from step 6.3. This number is used for normalization.

7. miRNA extraction from cell and lipoprotein particle samples

NOTE: The extraction of miRNA from cells is performed using the miRNA extraction kit with the following modifications.

1. **Cell samples**
 1. Precool the centrifuge to 4 °C. Add 350 µL of lysis reagent each to two chambers containing cells. As a negative control experiment, use a chamber without cells.
CAUTION: Wear appropriate personal protective equipment and work in a fume hood while handling lysis reagent as it contains phenol and thiocyanate.
 2. Wait for 3–5 min (depending on the cell line) for cell detachment. If required, check for cell detachment with brightfield microscopy. Pool the contents of the two chambers in a 1.5 mL tube.
 3. Using a 20 G needle and a 5 mL syringe, homogenize/disrupt the sample 5x–10x by aspiration and incubate for 5 min. Add 140 µL of chloroform (CHCl₃), shake vigorously for 15 s, and incubate for 3 min.
 4. Centrifuge at 12,000 x g for 15 min at 4 °C. Afterward, stop cooling the centrifuge.
 5. Transfer the upper aqueous phase to a new 1.5 mL tube; avoid phase mixing/contamination as the interphase contains DNA and the lower phase contains proteins. Add 1.5x the volume of 100% ethanol and mix thoroughly by pipetting.
 6. Place a spin column in a 2 mL collection tube and add 700 µL of the mixture from step 7.1.5. Centrifuge at 8,000 x g for 15 s at room temperature. Discard the flow-through and repeat this step with the residual sample volume.
 7. Discard the flow through and add 700 µL of the first wash buffer to the spin column. Centrifuge it at 8,000 x g for 15 s at room temperature.
 8. Discard the flow through and add 500 µL of the second wash buffer to the spin column. Centrifuge it at 8,000 x g for 15 s at room temperature. Repeat this whole step a second time with 2 min of centrifugation time.
 9. Place the spin column into a new 2 mL collection tube and centrifuge it at full speed for 1 min to dry the membrane.
 10. Place the spin column into a 1.5 mL collection tube. Add 30 µL of RNase-free water at the center of the membrane for elution and centrifuge it at 8,000 x g for 1 min. Repeat this whole step a second time with the first flow through as elution solution. The reverse transcription step is done immediately after the extraction; otherwise, store the extracted miRNA samples at -20 °C.
2. **Lipoprotein particles**
 1. Set the volume of the sample with the lowest protein concentration to 100 µL (= maximum sample volume). Calculate the sample volumes of the other samples according to this normalization, inversely to their concentration. For negative control experiments, use 100 µL of RNase-free water.
NOTE: Normalization is not required but simplifies the direct comparison of results during the qPCR step and analysis.
 2. Precool the centrifuge to 4 °C. Add 700 µL of lysis reagent to the sample volume of step 7.2.1.
 3. Perform miRNA extraction according to steps 7.1.3–7.1.10.

8. Reverse transcription

NOTE: The reverse transcription of miRNA is performed using a reverse transcription kit with the following modifications.

1. Thaw the kit reagents and the reverse transcription primers on ice. Prepare the following master mix in a reaction tube on ice: 45.7 µL of RNase-free H₂O, 16.5 µL of 10x reverse transcription buffer, 11 µL of reverse transcription enzyme, 2.1 µL of RNase inhibitor, and 1.7 µL of dNTP mix. Mix gently, do not vortex.
NOTE: The scale depends on the sample quantity; here, it is calculated for 10 reactions.
2. Label 0.2 mL tubes accordingly and mix 7 µL of the master mix from step 8.1 with 5 µL of the extracted sample from step 7.1.10 and 3 µL of reverse transcription primer. Mix gently, centrifuge shortly, and store the mixture on ice.
3. In order to use the same cell number for each cell line, reduce the sample volume of the cell line with a higher overall cell number; use this as the residual volume to reach the total sample volume of 5 µL of RNase-free water.

NOTE: The lipoprotein particle samples are already normalized in step 7.2.1. For standard curve preparation, dilute an aliquot of miRNA sequentially in RNase-free water and calculate the number of strands per sample volume. Required are at least five data points within the range of the resulting sample quantification cycle (c_q) values. Usually, total dilution factors ranging from 10^2 to 10^6 are suitable.

4. Place tubes into the thermocycler machine and start the following program: 30 min at 16 °C (annealing step), 30 min at 42 °C (reverse transcription), 5 min at 85 °C (melting step), and pause at 4 °C (storage). Perform the qPCR step immediately after reverse transcription; otherwise, store the complementary DNA (cDNA) synthesized from the miRNA samples at -20 °C.

9. qPCR

1. Perform a qPCR of the cDNA (reverse transcribed from miRNA) using a commercially available assay (see the **Table of Materials**) with the following modifications.
2. Thaw all reagents (supermix, RNase-free H₂O), cDNA samples (from step 8.4), and the primers on ice. Prepare the following master mix in a reaction tube on ice: 75 µL of supermix, 47.5 µL of RNase-free H₂O, 7.56 µL of primer. Mix gently, do not vortex.
NOTE: The scale depends on the sample quantity; here, it is calculated for 10 reactions.
3. Label 0.2 mL tubes accordingly and add 2 µL of the cDNA sample to 13 µL of the master mix and mix gently. In general, measure each sample 2x.
NOTE: At least two negative control samples are required additionally—use RNase-free water as a sample. If the calibration curve is not determined in the same run as the sample, one sample from the calibration curve measurement is also required. It is used to calibrate each individual run to the same reaction efficiency.
4. Place the tubes in the PCR machine and start the following program: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C, and 60 s at 60 °C. Repeat the last two steps of the program up to 50x.
5. For an analysis of the c_q values with the PCR machine's software package, activate **DynamicTube Normalization** (for the compensation of different background levels using the second derivative of each sample trace) and **Noise Slope Correction** (normalization to the noise level).
NOTE: The c_q value of the negative control should be several cycles higher than the highest sample c_q value.
 1. For a calibration curve analysis, determine the threshold for the calculation of the c_q for each miRNA from the standard curves of each miRNA individually, using the **Auto-find threshold** function of the software package, and keep it constant for each specific miRNA.
 2. For sample analysis, if necessary, compensate for different reaction efficiencies of the sample run with the data point from the calibration curve sample. The software calculates the c_q values of the samples from the threshold level of the respective calibration curve measurement.

10. Calculation of miRNA content

1. **Calibration curve**
 1. Calculate, from the initial number of miRNA strands per aliquot (100 µL of 10 µM miRNA, molecular weight from datasheet) and the subsequent serial dilution steps, the number of miRNA strands in the sample volume (the 5 µL sample volume from step 8.3).
NOTE: Assuming a reverse transcription efficiency of 1, this number is equal to the number of cDNA strands.
 2. Calculate the number of cDNA strands in the sample volume of 2 µL from step 9.3. Consider thereby the additional dilution factor of 7.5 (the 2 µL sample volume from step 9.4 from the 15 µL sample volume from step 8.4).
 3. Plot the c_q value from step 9.5.1 against the number of strands $n_{strands}$ calculated in step 10.1.2 in a base-10 semilogarithmic plot, and fit the data points with the following regression curve (M = the slope of the linear regression curve, B = offset).
$$c_q = -M \times \log(n_{strands}) + B$$

Confirm that the correlation coefficient (R^2) for the line is >0.99.
2. **Lipoprotein particles**
 1. Calculate the number of miRNA strands per sample volume using the measured sample c_q value (step 9.5.2) and the following equation (M and B are the calibration curve parameters of the specific miRNA).
$$n_{strands} = 10^{\frac{c_q - B}{M}}$$
 2. Calculate the corresponding number of lipoprotein particles in the sample volume, starting from the volume in step 7.2.1 (100 µL), its known concentration, and the subsequent dilution steps (100 µL → 30 µL sample volume in step 7.1.10 → 5 µL [dilution 1:6] sample in the 15 µL total volume in step 8.4 → 2 µL [dilution 1:7.5] in step 9.4). Assume a molecular weight of 250 kDa for HDL particles and 3 MDa for LDL particles and a 100% recovery of miRNA during the miRNA extraction step (ignoring any lipid contribution to the molecular weight yields a slight overestimation of the number of miRNA strands per lipoprotein particle).
 3. Divide the number of miRNA strands from step 10.2.1 by the number of particles calculated in the previous step to yield the number of miRNA strands per lipoprotein particle.
3. **Cells**
 1. Calculate the number of miRNA strands per sample volume according to step 10.2.1.
 2. Calculate the corresponding number of cells in the sample volume according to step 10.2.2, starting with the initial cell number concentration measured in step 6.4.
 3. Divide the number of miRNA strands from 10.3.1 by the number of cells calculated in the previous step to yield the number of miRNA strands per cell.
 4. Calculate the uptake rate of lipoprotein particles by dividing the total amount of miRNA strands from the previous step after correction for the background miRNA level of the cells obtained from a negative control experiment by the miRNA/particle ratio (step 10.2.3) and the incubation time (16 h, see step 6.2).

11. Multiwell microfluidic arrays

1. miRNA extraction

1. Perform miRNA extraction as described in step 7.

2. Reverse transcription

1. Thaw the reverse transcription primers, the reverse transcription kit components, and MgCl_2 (25 mM) on ice. For eight samples, mix 8 μL of reverse transcription primers (10x), 2.25 μL of dNTPs with dTTP (100 mM), 16.88 μL of reverse transcriptase (50 U/ μL), 9.00 μL of 10x reverse transcription buffer, 10.12 μL of MgCl_2 , 1.12 μL of RNase inhibitor (20 U/ μL), and 1 μL of nuclease-free water.
2. Mix gently and centrifuge briefly. Add 4.3 μL of reverse transcription reaction mix to 3.5 μL of extracted miRNA in a tube and mix, spin down, and incubate on ice for 5 min. Place the tubes in a thermocycler machine and start the following program: 16 °C for 2 min, 42 °C for 1 min, and 50 °C for 1 s repeated 40x in total, and then, as stop reaction, 85 °C for 5 min and hold at 4 °C until stopped.

3. Preamplification

1. Thaw the primers on ice and invert and centrifuge briefly. Mix the preamplification master mix (2x) by swirling the bottle. Prepare the preamplification reaction mix according to the following instruction for eight samples: 112.5 μL of preamplification master mix (2x), 22.5 μL of preamplification primers, and 67.5 μL of nuclease-free water. Invert and centrifuge briefly.
2. Mix 2.5 μL of the reverse transcription reaction product from step 11.2.2 with 22.5 μL of preamplification reaction mix from the previous step and invert and centrifuge briefly. Incubate the samples on ice for 5 min.
3. Place the tubes in a thermocycler machine and incubate at the following settings: enzyme activation at 95 °C for 10 min, annealing at 55 °C for 2 min, extending at 72 °C for 2 min, repeated 12x: denaturing at 95 °C for 15 s and annealing/extending at 60 °C for 4 min, enzyme inactivation at 99.9 °C for 10 min, and 4 °C on hold.
4. Spin down, add 7.5 μL of 1x TE (pH 8.0) and 67.5 μL of nuclease-free water, invert, and centrifuge briefly. The samples can be stored at -20 °C for up to 1 week.

4. qPCR

1. Mix the master mix by swirling the bottle. Prepare the PCR reaction mix for one card: 450 μL of master mix, 441 μL of nuclease-free water, and 9 μL of the preamplification sample from step 11.3.4. Invert and centrifuge briefly.
2. Load each fill reservoir of the multiwell microfluidic array card with 100 μL of prepared PCR reaction mix according to the manufacturer's instructions and centrifuge 2x for 1 min at 3,000 x g. The acceleration during the two consecutive centrifugation steps is important for properly filling the card. Seal the card according to the manufacturer's instructions.
3. Use a PCR system with the following program: enzyme activation at 95 °C for 10 min and then, repeated 40x: denaturing at 95 °C for 15 s and annealing/extending at 60 °C for 1 min.
4. Import the results file from the PCR system and calculate the RQ values using the system's software package with the following analysis settings: a maximum allowable c_q value of 40.0, maximum c_q values in calculations included, and outliers among replicates excluded. Activate the Benjamini–Hochberg false discovery rate as option for p -value adjustment (correction of the occurrence of false positives¹⁴) and global normalization as normalization method (using a median threshold value for all samples¹⁵).

Representative Results

A general scheme of lipoprotein particle isolation

Figure 1 shows the general scheme of lipoprotein particle isolation starting from whole blood, using sequential flotation ultracentrifugation¹⁶. If desired, other lipoprotein particle fractions like VLDL and IDL particles can be harvested during this protocol. The fixed-angle titanium rotor in combination with polypropylene quick-seal tubes is suitable to withstand the centrifugation forces. To avoid tube collapse, it is important to avoid air bubbles in the tube. Centrifugation is carried out at 4 °C to minimize protein degradation. Usually starting from plasma (60–80 mL per donor) of pooled blood donations of three volunteers, a yield of LDL and HDL particle solution volumes of 3 mL each with concentrations in the range of 1–3 mg/mL can be expected. The whole procedure, starting from blood donation, took around 7 days.

Figure 1: Flowchart of lipoprotein isolation. Centrifuge blood from healthy volunteers in vacuum container tubes and collect plasma (upper phase). After the adjustment of its density to $\rho = 1.019$ g/mL using KBr, centrifuge the solution at 214,000 $\times$ g for 20 h at 4 °C. After the adjustment of the density of the bottom fraction to $\rho = 1.063$ g/mL using KBr, centrifuge the solution again at 214,000 $\times$ g for 20 h at 4 °C. Store the upper fraction containing LDL particles temporary at 4 °C. After the adjustment of the density of the bottom fraction to $\rho = 1.220$ g/mL using KBr, centrifuge the solution twice at 214,000 $\times$ g for 20 h at 4 °C. Collect the upper fraction containing HDL particles, dialyze both the HDL and LDL particle solutions and exchange the buffer after 1, 2, and 4 h. After 24 h, determine the protein concentration and store the samples under inert gas at 4 °C. [Please click here to view a larger version of this figure.](#)

Reconstitution of HDL particles

The reconstitution of HDL particles was performed according to a protocol previously published by Jonas⁷. The first step was the delipidation of HDL particles as shown in **Figure 2A**, followed by the second step of relipidation (i.e., reconstitution) as shown in **Figure 2B**, using lipid PC, CO, and C in addition to a mixture of miRNA and spermine. We chose human mature miR-223 and miR-155 because miR-223 shows a high abundance and miR-155 is rare in lipoprotein particles¹⁷. Usually, both steps are performed on two sequential days. During reconstitution, other lipophilic and/or amphiphilic components could be added as desired. The complete evaporation of ethanol/diethyl ether and methanol/chloroform solvent of PC, CO, and C was critical. The last step—as shown in **Figure 2C**—was the dialysis procedure to separate reconstituted HDL particles (rHDL) from free lipids/miRNA/detergent. This took an additional 1-2 days. The addition of absorbent beads to the dialysis solution kept the density gradient along the dialysis membrane constant. A yield of 50% of rHDL particles can be expected.

Figure 2: Flowchart of HDL particle reconstitution. (A) Delipidation: Mix the HDL particle solution with precooled ethanol/diethyl ether and incubate at -20 °C for 2 h. After discarding the supernatant, resuspend the pellet and repeat the procedure. Dry the pellet with N₂ gas and resuspend it in buffer A. After the determination of the concentration, store the delipidated HDL under inert gas atmosphere at 4 °C. (B) Reconstitution: After mixing phosphatidyl-choline (PC), cholesteryl oleate (CO), and cholesterol (C), evaporate the solvent using N₂ gas while rotating the tube. Incubate the miRNA aliquot with spermine solution for 30 min at 30 °C, add sodium deoxycholate and resuspend the dried lipid film. Stir the sample for 2 h at 4 °C, add the delipidated HDL solution, and stir the sample again, this time overnight at 4 °C under inert gas atmosphere. (C) Dialysis: Transfer the solution from panel B containing reconstituted HDL (rHDL) particles to a dialysis membrane chamber (molecular weight cut-off: 20 kDa) and dialyze against PBS and absorbent beads at 4 °C. Exchange the buffer and the beads after 1 h and 2 h. Recover the rHDL particle solution after 24 h, determine the concentration, and store the sample under inert gas atmosphere at 4 °C. [Please click here to view a larger version of this figure.](#)

Labeling of LDL particles

The labeling of LDL particles with miRNA (Figure 3) as demonstrated for HDL particles was not feasible due to the hydrophobicity of the apoB-100 protein, which is the main constituent of the LDL particle. DMSO was used for the penetration of the lipid monolayer of the LDL particle and, thus, mediated the miRNA association. The whole procedure took 1-2 days with a yield close to 100%.

Figure 3: Flowchart of LDL particle labeling. Incubate the miRNA aliquot with spermine solution for 30 min at 30 °C and add DMSO and LDL buffer. Incubate LDL sample with LDL buffer for 10 min on ice and add miRNA/spermine/DMSO mixture. After incubation at 40 °C for 2 h, transfer the solution into a dialysis membrane chamber (molecular weight cut-off: 20 kDa) and dialyze against PBS and absorbent beads at 4 °C. Exchange buffer and beads after 1 & 2 h. Recover labeled LDL particle solution after 24 h, determine the concentration and store under inert gas atmosphere at 4 °C. [Please click here to view a larger version of this figure.](#)

Quality control of lipoprotein particles

HS-AFM can be used to examine the size and shape of native and reconstituted/labeled lipoprotein particles on mica. Just before use, mica has to be freshly cleaved (use adhesive tape to remove the upper layer[s]) in order to provide a clean and flat surface. When incubating HDL/LDL particles on mica, the dilution factor (and/or the incubation time) needs to be adjusted to observe individual particles. Clusters do not allow a determination of particle dimensions. HDL particles are mobile on mica. When using conventional AFM instead of HS-AFM, the immobilization protocol needs to be adapted accordingly (buffer, surface coating) to reduce the lateral particle mobility. While scanning the sample, the imaging force has to be kept low (tapping mode) to avoid any deformation of the particles, which will consequently affect the measured values. For data analysis, particles were detected via a threshold algorithm (e.g., in Gwyddion: **Grains > Mark by threshold**) and their height was determined with respect to the mica surface. Measuring the particles' height is the most precise way to determine particle sizes, as the apparent lateral dimensions are broadened by the tip shape (see exemplary images in **Figure 4**). Probability density functions (*pdfs*) of particle heights were calculated for statistical evaluation and comparison of size distributions of the various lipoprotein particles. A comparison of native and miRNA-labeled LDL particles as shown in **Figure 4** makes it possible to verify the principal similarity between labeled and nonlabeled (i.e., native) lipoprotein particles (labeled LDL particles without the addition of miRNA/spermine mixtures are shown as a control for the labeling procedure itself). The whole procedure took approximately 1 day.

Figure 4: Flowchart and representative results of HS-AFM measurements. Dilute the HDL/LDL particle sample in PBS ($1:10^2$ - $1:10^3$) and incubate it on freshly cleaved mica for 5 min, followed by a careful rinse with PBS to remove free (not electrostatically adsorbed) particles. Perform HS-AFM imaging and check the particle density on the surface. Carry out the measurements in PBS at room temperature. The top image of this figure shows a too high particle density; the bottom image is suitable for analysis. The height of single particles was analyzed after thresholding and native (black curve) and reconstituted/labeled (red and green curve) particles were compared in a statistical evaluation. The scale bar = 100 nm. This figure has been modified from Axmann et al.¹⁹. [Please click here to view a larger version of this figure.](#)

miRNA extraction, reverse transcription, and qPCR

The extraction of miRNA from native/artificially enriched lipoprotein or cell samples was performed using a miRNA extraction kit as shown in **Figure 5A**. Hereby, an RNase-free environment was critical. This step took approximately 1 h. Reverse transcription of the extracted miRNA sample (**Figure 5B**) was performed using standard biochemical procedures as described by the manufacturer. This step took approximately 1.5 h. Finally, the amount of cDNA obtained during the last step was determined using qPCR (**Figure 5C**). A standard curve, which relates the obtained C_q values to the absolute miRNA strand number, yielded the absolute miRNA content of the initial sample. This took approximately 2.5 h.

Figure 5: Flowchart of miRNA extraction, reverse transcription, and qPCR. (A) miRNA extraction: Mix the sample with lysis reagent and lyse it via aspiration using a syringe. Incubate for 5 min and add CHCl_3 . Shake vigorously for 15 s and incubate for 3 min. After centrifugation at $1,200 \times g$ for 15 min at 4°C , collect the top phase and mix it with ethanol. Transfer the solution to a spin column (maximum volume $<700 \mu\text{L}$) and centrifuge it at $8,000 \times g$ for 15 s. Discard the eluent and repeat the last step with the rest of the solution. Add the first washing buffer and centrifuge at $8,000 \times g$ for 15 s. Discard the eluent, add the second washing buffer, and centrifuge at $8,000 \times g$ for 15 s. Repeat the last step with a centrifugation time of 2 min. Further dry the membrane via centrifugation at maximum speed for 1 min. Elute the miRNA with H_2O and centrifuge at $80,000 \times g$ for 1 min. Store the extracted miRNA sample at -20°C . (B) Reverse transcription: Thaw the 10x buffer, H_2O , dNTP mix, inhibitor, and enzyme on ice and prepare the master mix. Add the extracted miRNA from panel A to the master mix and the reverse transcription primer and perform reverse transcription using a thermocycler machine. Store the cDNA sample at -20°C . (C) qPCR: Thaw the supermix, H_2O , and primer on ice and prepare the master mix. Add the cDNA from panel B to the master mix and perform qPCR. Analyze the data to obtain c_q values and calculate the absolute miRNA content of the sample (see Figure 6 and the representative results for details). [Please click here to view a larger version of this figure.](#)

Absolute miRNA content and transfer rate

The absolute miRNA content of native and artificially enriched HDL and LDL particles was calculated from the c_q values of the samples and a standard curve of the respective miRNA as shown in Figure 6. Figure 6A shows data as calculated by the analysis software (with activated **DynamicTube normalization** [for the compensation of different background levels using the second derivative of each sample trace] and **Noise slope correction** [normalization to the noise level]). c_q values of standard curves were determined using the **Auto-find threshold** function of the software package on the normalized fluorescence signal measured by the qPCR machine. Hereby, the software maximized the R-value of the fit of the standard curve. The threshold level was kept constant for each specific miRNA sample analysis. Subsequently, the c_q values were plotted as a function of the number of miRNA strands, and a regression line was calculated. Sample c_q values were determined with the same threshold level as shown in Figure 6B; reaction efficiency differences between different qPCR runs were compensated automatically by the software using one additional calibration curve sample included in each run. Using the regression line equation, the unknown amount of miRNA in the sample could be calculated. The lipoprotein particle number was estimated from the initial protein concentration and its average molecular weight ($\text{MW}_{\text{HDL}} \sim 250 \text{ kDa}$). Thereby, no lipid contribution to the molecular weight was assumed—thus, the number of miRNA strands per lipoprotein particle was slightly overestimated. Moreover, a 100% recovery rate of miRNA during the miRNA extraction step was assumed. Furthermore, the miRNA content of the cells before and after the incubation with HDL particles was determined and the miRNA transfer rate was calculated as shown in Figure 6C.

Figure 6: Flowchart of calculation of the absolute miRNA content and transfer rate. (A) Standard curve for miR-155: A miR-155 aliquot (100 μ L, 10 μ M) was serially diluted with RNA-free water as indicated. qPCR yielded c_q values for each serial dilution sample (measured twice) using the **Auto-find threshold** function of the software package. Negative control experiments (without the addition of miRNA) yielded c_q values of >35. Data points of c_q values as function of the number of miRNA strands per sample volume (calculated from the initial concentration and the serial dilutions) were fitted with the presented equation (red line, right image), yielding $M = -3.36$ and $B = 42.12$. The determined PCR efficiency was 0.98. The error bars were calculated from the results of experimental repetitions and were smaller than the diameter of the data point circle. (B) c_q values of native/artificially enriched HDL particles were determined with the same threshold level as determined in panel A and converted to the number of miRNA strands in the qPCR sample volume. The absolute ratio of miRNA of the original sample was calculated from the number (concentration) of HDL particles in the sample volume (3.2×10^{11} particles). (C) Cell samples (cell line IdIA7-SRBI) were incubated for 16 h with artificially enriched HDL particles (50 μ g/mL) and analyzed similarly. The determined c_q values were 22.5, 22.5, and 19.3 for cells only, for cells incubated with native HDL, or for cells incubated with rHDL particle solution (both 50 μ g/mL), respectively. These values were converted to the number of miRNA strands as done in panel B. The number of miRNA strands after incubation (7.3×10^6) were corrected by subtraction of the number of miRNA strands before incubation (8.6×10^5). The result was divided by the number of cells in the sample volume (3,100), the miRNA-particle-ratio (1.5×10^{-4}), and the incubation time period (16 h). This yielded the transfer rate of lipoprotein particles via miRNA uptake (240 HDL particle uptake events per cell and second). This figure has been modified from Axmann et al.¹⁹. [Please click here to view a larger version of this figure.](#)

Multiwell microfluidic array

Due to small yields of miRNA extraction, reverse transcription of the extracted miRNA was followed by a preamplification step. Finally, qPCR, as shown in **Figure 6**, was performed. For all steps, standard biochemical procedures were used as described by the manufacturer. Here, a part of the global miRNA profile on HDL particles of uremic patients recruited for a study on the influence of CRF on cholesterol efflux from macrophages¹⁸ is shown. In this study, the cholesterol acceptor capacity of HDL or serum in—besides others—17 young adult uremic patients (CKD stages 3-5) and 14 young adult hemodialysis patients without associated diseases and matched controls was measured. To analyze the data, default settings were used (maximum allowable CT value: 40.0, including maximum CT values in calculations and excluding outliers among replicates). *P*-values were adjusted using **Benjamini-Hochberg false discovery rate** (correction of the occurrence of false positives), and as normalization method, **Global Normalization** was selected, which finds the common assays among all samples to use its median C_T for normalization. In the representative results, some RQs of miRNAs isolated from HDLs of uremic patients are depicted (RQs of controls are 1). Obviously, miR-122 and miR-224 are highly expressed in the HDLs of uremic patients. This whole step took approximately 1 day.

Figure 7: Flowchart and representative results of the multiwell microfluidic array. After the miRNA extraction as shown in **Figure 5A**, mix the miRNA sample with reverse transcription primer and a master mix containing 10x buffer, H₂O, dNTP mix, inhibitor, MgCl₂, and enzyme. After incubation on ice for 5 min, perform the reverse transcription using a thermocycler machine. Add the preamplification master mix, incubate for 5 min on ice, and perform preamplification using a thermocycler machine. Add 0.1x TE (pH 8.0) and mix an aliquot with PCR master mix and H₂O. Pipette the PCR reaction mix into the fill port of the multiwell microfluidic array and spin twice at 3,000 x g for 1 min each. Perform qPCR using a PCR system and analyze the data to yield RQ values (here, the figure shows RQ values of HDL particles of uremia patients in comparison to a healthy control group¹⁸). [Please click here to view a larger version of this figure.](#)

Discussion

Here, the isolation of lipoprotein particle fractions from human blood and the determination of their individual miRNA content is described step-by-step. It is critical to work in an RNase-free environment while handling isolated and synthesized miRNA—particle-embedded miRNA is obviously sheltered from enzymatic degradation. As the miRNA/particle ratio of native lipoprotein particles is rather low, artificial enrichment with miRNA is required to study the whole particle uptake of cells. Thereby, the reconstitution of HDL particles as described previously⁷ is modified to incorporate miRNA strands. Additionally, the separation of the lipid and protein fraction during this procedure allows scientists to examine lipid- and protein-associated components of the lipoprotein particle¹⁹. In a similar way, the labeling procedure of LDL particles is adapted. Interestingly, the addition of spermine—a natural stabilizer of nucleotides—did not influence the miRNA/particle ratio. It should be noted that, in principle, the method allows the infolding of other substances than miRNA within a lipoprotein particle. Of course, there is a limit with regard to the physical size of the substance based on the overall size of HDL (diameter: 5-12 nm) and LDL particles (diameter: 18-25 nm).

Concerning the quality control of reconstituted/labeled lipoprotein particles, HS-AFM is an applicable method to characterize HDL/LDL particles at the single particle level. In comparison to EM, it allows for shorter preparation times and near-physiological conditions (wet, room temperature).

Due to its inherent sensitivity and amplification, qPCR is the method of choice to detect low miRNA concentrations. Alternatively, single-molecule sensitive fluorescence microscopy, which is able to detect even individual molecules, would not be suitable due to the low concentrations of, for instance, fluorescently labeled miRNA strands per particle. Thus, the ratio of miRNA strands per native lipoprotein particle has been found to be 10⁻⁸. Artificial enrichment increases the ratio by a factor of 10,000, which facilitates the estimation of the cellular lipoprotein uptake rate (no significant difference is detected using native lipoprotein particles¹⁹). The high sensitivity of qPCR makes it possible to determine this uptake rate by measuring the number of miRNA strands after the incubation time and the miRNA/particle ratio. It should be noted that the calculated value ignores cellular degradation and release of miRNA and, thus, represents at least a lower limit for the lipoprotein particle uptake ratio.

In the future, the method can be adapted to transfer pharmaceutical substances (notably also lipophilic ones) in cells and correlate their biological effect to the intracellular concentration (determined via the uptake rate of lipoprotein particles).

Disclosures

The authors have nothing to disclose.

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