Studies on the influence of lipoproteins on extracellular vesicle isolation and analysis

Doctoral theses

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Introduction

Extracellular vesicles (EVs) are cell-secreted submicron structures enclosed by a lipid bilayer. The scientific interest in them rapidly grew over the last years. Due to recent changes in terminology recommendations, in this work we used a nomenclature based on the used EV isolation method. We performed size based separation of different EV subpopulations, thus the two main groups of EVs we studied were i) medium-sized EVs (mEVs, approx. 200-1000 nm) and ii) small EVs (sEVs, diameter < 200 nm). We mostly used human blood plasma for EV analysis and isolation. In that case the 1st crucial step is the removal of platelets by centrifugation. Unfortunately with this process we lost most of the EVs larger than 1000 nm. Therefore our study was restricted to mEVs and sEVs.

Upon EV isolation and analysis we followed the recommendations of the International Society for Extracellular Vesicles (ISEV) published in 2014.

Studies performed on EVs circulating in blood plasma revealed the importance of preanalytical factors many years ago. One has to carefully consider several different experimental parameters, from the choice of the anticoagulant through the right protocol for platelet removal up the storage of plasma samples. Many researchers raised the need for detailed studies on the relation of blood collection and food intake regarding EV analysis. However, so far no detailed studies were performed aiming to clarify these issues.

We must not forget that in blood plasma there is another big population submicron particles, lipoproteins. In contrast to EVs, these are not supported by a phospholipid bilayer, their hydrophobic core - where their lipid cargo is stored - is protected by a lipid monolayer. Their most important physiological function is to supply nutritients to the tissues, therefore it is not a surprise that their plasma levels show correlation with the metabolic state of the patients.

High density lipoprotein (HDL) and low density lipoprotein (LDL) are important parameters of cardiovascular risk estimation. After food intake (postprandial state) the gut secretes chylomicrons into the bloodstream which are responsible for transport of dietary lipids. In parallel, the liver decreases the secretion of LDL particles.

The major protein components of lipoprotein particles are the so-called apolipoproteins, which have special functions e.g. maintenance of the structural integrity of the lipoprotein particle and mediation of the interactions with lipoprotein receptors. The apolipoprotein composition of different lipoprotein particles changes dynamically, exchange/transfer of apolipoproteins between lipoprotein particles is possible and happens quite often.

Co-isolation of HDL with blood plasma derived sEVs is already reported. It is also known, that lipoprotein depletion of the blood plasma (e.g. lipoprotein apheresis) might result in substantial loss of EVs.

Current methods relying on particle enumeration are unfortunately not capable of distinguishing between EVs and other corpuscular structures like lipoproteins and protein aggregates. Thus, these methods that do not characterise surface markers of EVs, are often mistaken in the evaluation of both EV concentration and size distribution from biofluids. Regarding the information we mentioned above, we decided that there's an urgent need for detailed studies on the effect of food intake and dietary lipoproteins on EV analysis.

Technical challenges and the increasing role of blood plasma derived EVs in biomarker research also implicated the demand for such investigations.

Aims:

Our work initially focused on the effect of dietary chylomicrons. We decided to investigate whether they influence EV analysis and isolation or not.

We tried to give an answer to the following questions:

1. Do dietary chylomicrons disturb EV analysis? Compared to food intake, in what time period should we consider their effect?
2. Do dietary lipoproteins co-isolate with EVs? Does it only happen in the case of blood plasma or is it true for platelet concentrates as well?
3. Can co-isolated lipoproteins be separated from EVs
    - with size-exclusion chromatography or density gradient centrifugation in the case of mEVs?
    - with density gradient ultracentrifugation in the case of sEVs?
4. Co-isolated lipoproteins represent LDL aggregates or rather chylomicron remnants?
5. Is it possible to detect LDL aggregates in the EV size range with commonly used EV analysis methods?
6. It is simple co-isolation of LDL aggregates and EVs or a possible interaction between EVs and lipoproteins?

Materials and methods

Blood samples and platelet concentrates

We conducted studies both on human plasma samples and on supernatants of human platelet concentrates. For blood plasma studies we performed venepuncture of the cubital vein of 14 healthy adults both after 10 hours fasting and four hours after ingesting a standardised fat-containing meal. The human platelet concentrate was provided by the Hungarian National Blood Transfusion Service. In all experiments we strictly followed the ethical guidelines of the 1975 Helsinki Declaration and the experiments were approved by the Ethical Committee of the Semmelweis University, Budapest. All subjects have signed an informed consent.

Automated laboratory analysis

Triglicerides, total cholesterol, LDL cholesterol, apoA1 and apoB100 were measured from fasting and postprandial serum samples as well. The measurements were performed in the Central Laboratory of Semmelweis University, Budapest with the kind help of Tamás Géza Szabó, MD.

Processing of blood samples and platelet concentrates

For EV analysis from blood plasma we chose ACD-A blood collection tubes because our group has previously proved that in this kind of tube the *in vitro* platelet activation and EV release is minimal. During the processing of blood samples we followed the guidelines of the International Society for Thrombosis and Haemostasis. The platelet depleted plasma samples (platelet-free plasma, PFP) were aliquoted, snap frozen in liquid nitrogen and preserved at -80°C until further analysis.

In the case of platelet concentrates we pelleted the platelets from the concentrates in the presence of aggregation/activation inhibitors. Afterwards we performed the same two-step centrifugation of the supernatant we did in the case of blood samples in order to remove any residual platelets.

EV isolation from blood plasma samples and supernatant of platelet concentrates

Fisrt of all, to reduce the viscosity of the sample we diluted the plasma/supernatant with an aqueous buffer in 1:1 ratio. Afterwards we performed gravity-driven filtration through a 800 nm pore size syringe filter (Sartorius). Next we pelleted the mEVs at 20,500 g for 60 minutes using a benchtop micro centrifuge. Before pelleting the sEVs from the supernatant we performed an additional gravity-driven filtration through a 200 nm pore size filter. Then the filtrate was ultacentrifuged at 100,000 g for 70 minutes in order to pellet the sEVs (OptimaMAX-XP benchtop ultracentrifuge, Beckman Coulter Inc;). Both mEV and sEV pellets were resuspended in buffer, re-centrifuged as a washing step, then aliquots in 50 μL buffer were snap frozen is liquid nitrogen and kept at -80°C until analysis.

EV purification with density gradient ultracentrifugation

We layered 1-1 mL of 40, 20, 10 and 5 m/m% Iodixanol (Optiprep™, Sigma-Aldrich) solutions over each other from bottom to top in a 4,5 mL polyallomer ultracentrifuge tube to form a discontinuous

gradient. Onto the top 0.5 mL of resuspended mEV/sEV was carefully applied. Afterwards ultracentrifugation was performed at a 100,000 g for 20 hours at 4°C in a swing bucket rotor. At the end of the centrifugation 500 µL fractions were collected from top to bottom (total nr of 9). Each mEV fraction was diluted to 2 mL and re-pelleted at 20,500g for 60 minutes. Each sEV fraction was diluted to 8,6 mL with buffer and re-pelleted at a 100,000g for 3 hours at 4°C. The pelleted fractions were resuspended in buffer, snap frozen in liquid nitrogen and stored at -80°C until further analysis. Samples for Western blotting were resuspended in protein lysis buffer complemented with protease inhibitor cocktail.

EV purification with size-exclusion chromatography (SEC)

Size exclusion chromatography is usually used for separation of particles with distinct diameter. In our experiments we run fasting and postprandial PFP samples and mEV samples isolated from platelet concentrates as well on a ready-made chromatography column (qEV™ designed for EV isolation by IZON Sciences Ltd.) following the manufacturer's instructions.

Flow cytometry

Measurements of PFP samples and isolated EVs were carried out on a BD FACSCalibur flow cytometer applying protocols previously described by our workgroup. For mEV measurements an EV gate was set up using a mixture of silica and polystyrene fluorescent calibration beads. EV surface markers were detected with the following reagents (1 µL reagent/10 µL PFP, in a total volume of 60 µLs): annexin-V-PE (AX, BD Biosciences), anti-CD41a-APC (BD Biosciences, anti-CD41a HIP8 clone), anti-CD9-FITC (Sigma-Aldrich, MEM-61 clone), anti-CD63-PE (Sigma–Aldrich, MEM-259 clone). In all cases the background signal of the antibody/stain in buffer was higher than the signal coming from unstained EV samples, we used buffer staining controls as negative controls. Lipoproteins in the samples were detected by a goat polyclonal anti-human-apoB100/48-FITC conjugated antibody (Mybiosource). The antibody was diluted 10 times then centrifuged at 20,000 g for 60 minutes to remove aggregates. Only the aggregate-free supernatant was used for sample staining (40 µL 10x diluted antibody/10 µL PFP, in a total volume of 60 µLs). We performed apoCII and apoE detection in our samples as well (4 µL antibody/ 10 µL PFP). All immunolabelings were performed at room temperature for 30 minutes, protected from light. Afterwards samples were diluted with 1 mL buffer and the mEVs were re-pelleted to remove the unbound antibodies. The analysis of sEVs due to technical challenges was performed on the surface of 4 µm diameter sulphate/aldehyde latex beads (Life Technolgies). After bead-binding we performed the staining and the analysis of the EV-covered beads. Data analysis was carried out using the FlowJo 10.0.8 software.

Particle enumeration with tunable resistive pulse sensing (TRPS)

A device based on the Coulter counter's principles was used to determine the particle size and concentration in our samples (EVs and lipoproteins). The device is available in the market under the name qNaNo and it is manufactured by IZON Scineces Ltd. New Zealand. The method is called *Tunable Resistive Pulse Sensing (TRPS).* The setup is constructed by a nanopore with a given size and two electrolyte chambers on the two sides of the nanopore. To determine exact size and concentration of our particles we used nanopores with different sizes to cover different size-ranges (100, 150, 200, 300, 400 and 800 nm pore size pores were used). For calibration of the instrument calibration beads with a known size and concentration were used provided by the manufacturer. In all cases we registered at least 500 events or continued the measurement for 5 minutes.

EV isolation from cell culture conditioned media

As EV source we chose the 5/4E8 Th1 hibridoma cell line. The cells were incubated in serum free conditioned media for 24 hours which was subsequently followed by EV isolation. Fist cells were pelleted at 300 g for 10 minutes, afterwards the supernatant was filtered through a 5 μm filter only usimg the force of gravity. Next large EVs were pelletd at 2,000 g for 20 minutes. The resulting supernatant was filtered through an 800 nm filter, then mEVs were pelleted at 12,500 g.

Transmission electron microscopy (TEM)

The TEM analysis was done in collaboration with the Institute of Experimental Medicine of the Hungarian Academy of Sciences by Prof. Ágnes Kittel. In our experiments we used two different approaches. In the first case we used a protocol previously set up by our workgroup, where we analysed EVs in a fixed pellet. After fixation and a washing step the samples were dehydrated with an increasing series concentration of ethanol solutions. In 50% ethanol samples were contrasted with 1% uranyl-acetate for 30 minutes then they were embedded in Taab 812 polymer. Ultrathin sections were made and they were analysed by a Hitachi 7100 Transmission electron microscope equipped by a 2000 x 2000 megapixel CCD camera (Veleta, Olympus). The other approach was chosen because the specific structure of lipoproteins (lipid monolayer around a lipophilic core) cannot be visualised using the embedding technique. EV and lipoprotein samples in suspension were mixed with equal volumes of 1% OsO_4 solution. After fixation, drops of samples were dried onto TEM grids for 10 minutes at room temperature. Next the grids were washed 3 times for 5 minutes in distilled water. The grids were dried and we performed TEM analysis with the microscope setup mentioned above.

Western blot

The relatively large molecular weight of the analysed apoB protein required modifications in the electrophoresis protocol as well, the separation was performed in 5 m/m% agarose gels (Sigma-Aldrich). Standardisation of sample loading onto the gels exceptionally was not based on protein content, we rather decided to load equal volumes of density-gradient fraction lysates. The electrophoresis was performed at

a 100 V constant voltage for 5 hours, with constant cooling of the system and one buffer-exchange after 2.5 hours. After electrophoretic blotting of the samples to PVDF membranes, and blocking with BSA-Tween-buffer, apoB was detected with a polyclonal anti-apoB100/48 antibody produced in rabbit (Novus, 1:1000 dilution, overnight at 4°C). The presence of EVs in our samples was verified by a monoclonal anti-human CD63 antibody produced in rabbit (Santa-Cruz, 1:1000 dilution, overnight at 4°C). In both cases we used an anti-rabbit HRP-conjugated antibody produced in goat (abcam, 1:30,000 dilution, 40 minutes incubation time, room temperature). The resulting chemiluminescent signal was detected using the Pierce ECL Western Blotting Substrate kit (Thermo Fisher Scientific).

Mass spectrometry (MS)

The mass spectrometry was performed with the kind help of Prof. Károly Vékey, Dr. László Drahos and Dr. Lilla Turiák in the MS Proteomic Workgroup of the Hungarian Academy of Sciences. For the MS analysis mEV samples isolated from fasting and postprandial PFP were resuspended in 20 µl distilled water. Disruption of the EVs was performed by several freeze-thaw cycles. Proteomic digestion was done as described previously. Peptides were separated on a 25 cm Acclaim Pepmap RSLC nano HPLC column with a Dionex Ultimate 3000 NaNo HPLC system. The digested and separated peptides were analysed on a Bruker Maxis II Q-TOF mass spectrometer. The results were evaluated using the ProteinScape 3.0 softwer with Mascot search engine.

Data analysis and statistics

Microsoft Excel was used to collect and organise our results. Statistical analysis was performed with the GraphPad Prism v.6 software. Paired t-test was used for the comparison of fasting and postprandial samples if the distribution of the data points followed the Gaussian distribution. In other cases, Wilcoxon matched-pairs signed rank test was used. Normality was tested with D'Agostino-Pearson normality test. For the comparison of more than two groups one-way ANOVA was used with Dunett's post-hoc correction (* $p< 0.05$; ** $p< 0.01$; *** $p<0.001$). The error bars represent the standard error of the mean (SEM). Figure editing was performed with the Adobe Photoshop CS4 software, illustrations were drawn in Microsoft Powerpoint.

Results

Event number increases in human plasma after a fat containing meal, detected by flow cytometry

After a 12 hours fasting period venous blood was taken from 3 healthy subjects. Next, all individuals ingested a standardised fat-containing meal. Blood collection was performed 15 minutes, 30 minutes, 90 minutes, 3 hours and 6 hours after the food intake. PFP was made from the blood samples and it was analysed by flow cytometry. We found that 90 minutes after food intake there was a significant increase in the event number detectable in the size range of mEVs (***$p<0.001$). The increase in the event number was detectable even 6 hours after the food intake. In our further experiments we decided to perform postprandial blood sampling 4 hours after food intake. The same increase in the particle concentration in plasma was detectable with TRPS as well, without any difference between the size of the particles in fasting and postprandial samples.

Particles that appear upon food intake carry apoB on their surface

Fasting and postprandial (4h) PFP samples were analysed by flow cytometry. The apoB labelling of the samples resulted in an increased signal in the postprandial cases, compared to both the signal given by the EV markers and the fasting apoB signal. When we summarised the results obtained from the 12 subjects, we found that events that carried EV markers and were sensitive to detergent lysis showed a significant decrease while the increase in the apoB signal was also significant. Thus, we proved that chylomicrons that appear after food intake are responsible for the increased particle concentration of these samples.

We also verified the presence of lipoprotein particles on the top of ultracentrifuged postprandial PFP samples (dark, round, homogenous structures forming rouleau-like aggregates) with TEM analysis.

The mEV samples isolated from fasting and postprandial plasma samples contain significant amount of lipoproteins

In the case of isolated mEV samples we could also detect an increase in particle concentration both with flow cytometry and TRPS without further significant increase in the detected apoB signal. In contrast, the EV marker annexin showed a significant decrease in the isolated mEV samples similar to that observed in the case of PFP samples. Just like in the case of PFP samples, isolated mEVs did not differ in size before and after food intake measured by TRPS. The presence of lipoproteins (and other contaminating plasma proteins) in the samples was verified with MS analysis as well both in fasting and in postprandial samples. In both cases the apoB protein was among the 1st 10 hits.

Because of the large apoB positivity detected in fasting samples, we decided to use fasting PFP samples for our further experiments.

In isolated mEV samples, previously we could not detect lipoproteins with TEM when we used the approach that analyses pelleted and embedded EVs. However, when we introduced the approach that

studied samples after direct osmification on a TEM grid, in the exact same sample we could detect the presence of lipoproteins.

Lipoproteins cannot be separated from isolated mEV samples nor with size exclusion chromatography (SEC), nor with density gradient ultracentrifugation

Fasting and postprandial (4h) PFP samples were processed by SEC. To our surprise, particle concentration in postprandial samples were increased compared to fasting samples, measured by TRPS. This led us to the conclusion that SEC might not be able to completely remove lipoproteins that appear upon food intake from EV samples. We performed SEC purification of mEV isoltates isolated from platelet concentrate supernatant. Flow cytometric analysis of these SEC fractions revealed that in those fractions where mEVs leave the column, we always find apoB carrying particles as well.

We tried density gradient ultracentrifugation, which is considered as a gold standard method of choice for the purification of EV samples. We detected most of the mEVs in the 6^{th} fraction of the gradient. Most of the apoB carrying particles floated in the top three fractions, while some apoB signal was always detectable in the mEV containing 6^{th} fraction. This was confirmed both with flow cytometry and Western blotting. Surprisingly, the band that was detected by the anti-apoB antibody was characterised by the molecular weight of 550 kDa instead of the expected 250 kDa.

The sEV samples isolated from blood plasma or from platelet concentrate supernatant also contain lipoproteins

sEVs were isolated from fasting PFP and platelet concentrate supernatant with differential ultracentrifugation and were analysed by flow cytometry after conjugation onto the surface of latex beads. In both cases, we detected significant apoB signal in our samples, accompanied by less prominent, but solid apoCII, and in the case of PFP samples, light apoE positivity. The presence of EVs in the sample was verified by detection of the CD9 and CD63 tetraspannins.

Lipoproteins cannot be completely removed from sEV samples with density gradient ultracentrifugation either

In the case of sEV samples we also tried density gradient ultracentrifugation for the purification of lipoprotein containing samples isolated by simple differential ultracentrifugation. The results were comparable with those we had in the case of mEVs. Most of the apoB carrying lipoproteins floated onto the top three fractions of the gradient, but in all cases sEV containing fractions had detectable apoB signal as well (FR7-8). We obtained similar results with sEVs isolated from platelet concentrates. The relatively large molecular weight of the detected apoB band by Western blotting led us to the conclusion that the major contaminants in EV samples derived from fasting PFP or platelet concentrates are not chylomicrons but mostly LDLs.

LDL is capable of forming aggregates in the size range of EVs, thus it is detectable with flow cytometry and TRPS during analysis of EVs derived from biofluids

We conjugated commercially available LDL onto the surface of latex beads and performed flow cytometry just like we did in the case of sEV samples. As expected, we detected apoB and apoCII, but not CD9 and CD63. TRPS analysis of this commercially available LDL showed that it was capable of forming relatively large aggregates, up to a diameter of 2-300 nm.

We tried the differential detergent lysis previously introduced by our workgroup for distinguishing EVs from protein aggregates (0.1% Triton-X 100). We wanted to find out whether this method was suitable for the differentiation between lipoproteins and EVs as well. Unfortunately the apoB signal detected in PFP samples and the apoB signal obtained from the commercially available LDL were both sensitive to detergent treatment.

LDL does not only co-isolates with EVs, but shows *in vitro* association with them

mEVs were isolated from cell culture conditioned media during serum- and lipoprotein free conditions. These EVs were visualised by TEM with fixation in suspension and direct analysi on a TEM grid. In parallel, half of the EVs were mixed with human LDL in 2 mg/mL concentration and after 30 minute incubation at room temperature these samlpes were suspected to TEM analysis as well. As a control, we analysed LDL alone as well. According to our result, LDL is capable of forming EV-sized aggregates, furthermore shows *in vitro* association with EVs, extensively covering their surfaces.

Conclusions

Our results shed light on a previously unexpected fact, that chylomicrons, LDL and aggregates of the latter might be similar to circulating EVs in their biophysical properties, thus raising difficulties upon their isolation and detection.

We have shown that LDL co-isolates with EVs isolated from blood plasma and platelet concentrate supernatants. Furthermore, with the most commonly used EV isolation and purification methods LDL cannot be completely separated from EVs.

The differential detergent lysis previously introduced by our group for the distinguishing of EVs and protein complexes is unfortunately not suitable for differentiating between EVs and lipoproteins.

The plasma concentration of LDL on human blood is approximately 10^{14} particles/mL, whereas the amount of EVs is estimated to fall around 10^{12}/mL, or according to less optimistic estimations, rather falls around $10^{7\text{-}9}$/mL. Keeping that in mind, even if only a very small proportion of LDL is aggregated or co-isolated with EVs, it might still outnumber them, misleading techniques based on particle enumeration (TRPS, NTA), resulting in overestimation of EV numbers.

Regarding our results, we must consider the possible presence of EV-lipoprotein complexes. Because of that we must have a fresh look on functional studies conducted with EVs or lipoproteins isolated from blood. It easily might be the case that the effects reported by these studies are rather combinatorial, since they did not consider the effects of contaminating co-isolated EVs or lipoproteins, and the differences in the triggered biological responses.

Another important aspect we must consider, is the extensively grooving field of biomarker research based on circulating EVs. If the surface of an EV is partially covered by lipoproteins, it might hinder potential molecules which could be otherwise good candidates for biomarker detection.

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Publications related to the doctoral thesis

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