What Are We Looking At? Extracellular Vesicles, Lipoproteins, or Both?

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Unawareness or neglecting that extracellular vesicles (EVs) and lipoproteins coisolate when using a traditional 1-step physical-based purification method to isolate one of these entities from plasma is a major issue. This issue ultimately leads to wrong conclusions about the composition and biological functions of these bionanoparticles. Here, I highlight the biophysical similarities between EVs and lipoproteins and their different abundances in plasma that result in the unintentional coisolation of these 2 different entities. Concerns related to nonquantitative measures of mass spectrometry (MS) and quantitative polymerase chain reaction (qPCR)–based data and the dynamics of certain apolipoproteins are also stressed because these facets, like the coisolation issue, may challenge our understanding of what we are actually looking at when we are studying apparently purified EV or lipoprotein samples.

EVs and lipoproteins are biological nanoparticles that can be found in the circulatory system. Among the many biological functions, including cardiovascular, of EVs and lipoproteins is their proposed ability to deliver biological cargo (beyond lipids in the case of lipoproteins) from one cell to a distant recipient cell. Thus, these biological vehicles may be key mediators of intercellular communication. As such, EVs and lipoproteins have the potential to serve as disease biomarkers and to deliver therapeutic compounds to specific biological targets. However, a proper isolation and characterization of EVs and lipoproteins is crucial and often a prerequisite for being able to investigate the biological properties of these specific entities and to exploit and realize their full clinical potential.

EVs are cell-derived nanoscale vesicles that carry nucleic acids, proteins, and other biomolecular compounds from their cells of origin. EVs constitute a heterogeneous population of vesicles secreted via different mechanisms—to all kinds of body fluids. The young EV research field has received increased attention during the last decade. EV classification efforts are impeded by the lack of unique antigens representative for a general EV class and EV subpopulations. Nevertheless, the EV community has reached some kind of consensus in regard to classifying a subset of EV subpopulations as exosomes, microvesicles, and apoptotic bodies:[1] Exosomes—the size range from 30 to 100 nm—originate from multivesicular bodies and are the most abundant subpopulation of EVs. Microvesicles—the size range 100 to 1000 nm—are budded from the plasma membrane. That said, recent studies have shown a considerable overlap in size of these 2 types of EVs. Other less-abundant and larger EVs include apoptotic bodies. Exosomes and microvesicles will be referred to as EVs from here on. The density of EVs is commonly reported to be in the range from 1.08 to 1.21 g/mL (Online Table I), and the EV particle concentration for healthy individuals has been suggested to range between 10^7 and 10^9 EVs/mL plasma.[2] However, the estimated EV concentrations should be taken with caution because the methods used to enumerate EVs experience limited sensitivity and specificity.[2] Moreover, there are also issues related to retaining all EVs during the purification steps.

The far more abundant plasma lipoproteins (≈10^{16} lipoproteins/mL plasma; Online Table I) are submicron assemblies (except for the largest chylomicrons) of lipids and apolipoproteins that are secreted from the intestine and liver (with and without lipids) and then mature in circulation. Although lipids and proteins populate the surfaces of both EVs and lipoproteins, a distinguishing feature is the presence of an aqueous core in EVs, whereas the core of lipoproteins is comprised of lipids. Hence, the plasma lipoproteins play an important role in the transport of lipids. The plasma lipoprotein family includes high-density lipoproteins (HDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), very low-density lipoproteins (VLDL), and chylomicrons. They are classified according to their mass density (Online Table I). In addition, specific apolipoproteins are associated with specific species of lipoproteins. The apolipoprotein ApoA-I is mostly associated with HDL and accounts for almost 70% of the mass of HDL protein content. ApoB-100 is associated with LDL, IDL, and VLDL, whereas apoB-48 is uniquely associated with chylomicrons.

Lipoprotein Contamination in EV Samples

The observed coisolation of HDL with EVs when using a density-gradient ultracentrifugation step for EV purification[3] is in line with the Figure. Even though the density of LDL is slightly lower than that of EVs, the difference is likely not sufficient to overcome the limits of resolution of the applied density gradient and a huge excess of LDL compared with EVs (10^{15} LDL particles/mL versus ≈10^7–10^9 EVs/mL,[2] according to the estimated calculation shown in Online Table I), thus leading to a significant spillover of LDL to EV fractions in addition to HDL contamination.

On the contrary, when isolating the bioparticles according to size, it becomes clear that size-exclusion chromatography (SEC)–based purification of EVs will lead to the coisolation

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Nonstandard Abbreviations and Acronyms

EV	extracellular vesicle
HDL	high-density lipoproteins
LDL	low-density lipoproteins
SEC	size-exclusion chromatography
VLDL	very low-density lipoproteins

of chylomicrons and likely also VLDL with EVs (Figure). To reduce the amount of these 2 lipoprotein species in SEC-purified EV samples, blood samples from fasting donors should be collected. The VLDL is known to increase after food intake,[4] and levels of chylomicrons/apoB-48 have been reported to increase ≈2-fold (from ≈5 µg, corresponding to ≈10^{13} chylomicron particles/mL [Online Table I], to ≈10 µg [apoB-48]/mL) 3 to 4 hours postprandial.[5]

Even EV isolation and purification including differential ultracentrifugation followed by a qEV SEC purification step has shown the presence of LDL in plasma EV preparations.[6] This latter result is likely an outcome of several orders of magnitude higher concentration of LDL particles than EVs in human plasma that may challenge the resolution of the purification methods. In addition, these 2 purification methods do partly (ultracentrifugation; below) or fully (SEC) rely on particle size and thus, do not separate based on density only. Hence, a clear separation according to the coordinate system shown in the Figure is not obtained.

Ultracentrifugation is also prone to lipoprotein contamination because the sedimentation rate of spherical objects at constant acceleration is proportional to the difference between the density of the particle and the density of the medium and to the square of the particle radius. Thus, if we assume that the density of HDL and EVs is similar, the sedimentation rate of 100 nm EVs is ≈100× faster than 10-nm–sized HDL. The final EV product, after the classical 100 000 g centrifugation for the sedimentation of ≈100-nm–sized exosomal EVs, eventually contains a significant amount of HDL particles if not the vast majority because of the much higher concentration of HDL compared with EVs in plasma. Similar arguments hold for LDL contamination of EV samples purified by ultracentrifugation. It is worth noting that a work based on 259 studies showed that >80% of the studies used only ultracentrifugation for EV purification.[7]

EV Contamination in Lipoprotein Samples

The reverse contamination of lipoproteins with EVs could also be an issue. However, the EV contamination in purified lipoprotein samples should be small because of the much lower particle concentration of EVs compared with the concentrations of the different types of lipoproteins in plasma (Online Table I). As mentioned above, the coisolation of EVs with lipoproteins is likely occurring when a density gradient is applied for purification. This is particularly important considering that density-gradient methods are commonly used for isolating lipoproteins. Interestingly, proteomic analysis of purified VLDL and LDL has demonstrated the presence of proteins known to be associated with EVs, for example, CD14, protein S100-A8, human leucocyte antigen class I molecules, and LDL-receptor.[8] A comprehensive database containing the proteomic profile of HDL based on 17 different and independent MS studies[9,10] contains 21 (minimum 1 hit per 17 studies) of the 28 proteins that have been reported in EVs derived from platelets.[11] It should be noted that platelet-derived EVs are one of the most abundant EVs in blood. Four of the 17 independent studies reported the same 8 proteins, including platelet factor 4 and platelet glycoprotein 1b beta chain. The presence of the membrane-spanning protein CD9 antigen in 5 of the 17 studies indicates that some of these HDL samples were likely contaminated with EVs (CD9 is often found on the surface of EVs). Although the concentration of plasma lipoproteins is much higher than EVs, the spillover of even a small portion of EVs into the purified lipoprotein preparation can significantly alter the biomolecular composition data and final conclusions about lipoproteins. Thus, it is important to be critical of the results that the sensitive MS proteomics and qPCR-based RNA analyses provide before we derive conclusive statements about lipoproteins and EVs, especially in the case where the protein and RNA signatures are not put on an absolute scale in terms of concentration. Furthermore, we should wonder why certain highly hydrophilic proteins and RNAs are suggested to be associated to lipoproteins. The presence of miRNA in HDL isolated by KBr-density–gradient ultracentrifugation without further downstream purification steps has been reported.[12] Concerns related to the presence of EV contaminants in these HDL samples have been rightly raised,[3] and the proposed EV contamination is supported by the presence of CD9 in 3 different MS studies of HDL samples purified by KBr-density–gradient ultracentrifugation.[10] Because the aqueous core of EVs are proposed to carry miRNA, the amount of miRNA associated with HDL is uncertain. However, it should be mentioned that the study by Vickers et

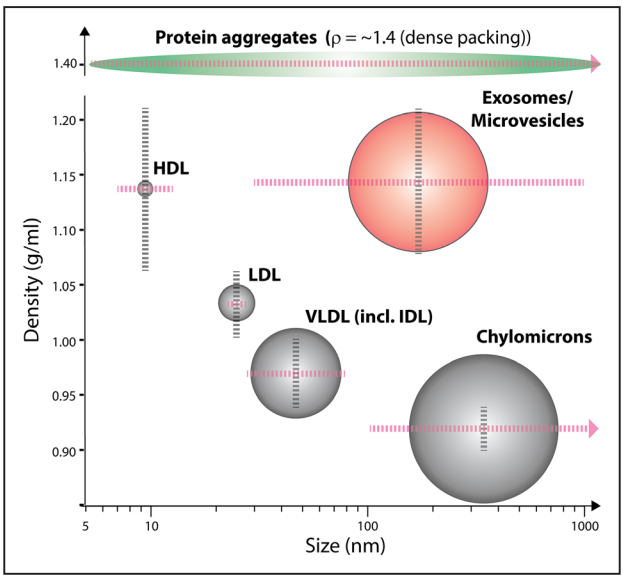

Figure. A graphical representation of the biophysical properties of extracellular vesicles (EVs) and lipoproteins (Online Table I). The grey, vertical dashed lines represent the density ranges for each of the species, whereas the pink, horizontal dashed lines represent their corresponding size ranges in log scale. In addition, protein aggregates are also added because these are also likely to be present in plasma samples. HDL indicates high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low-density lipoprotein.

al[13] also suggests that miRNA can be associated with HDL. This study was based on a thorough 3-step isolation of HDL.

SEC-based purifications are often used to isolate the small and most abundant lipoproteins, HDL and LDL. However, protein complexes/aggregates and the deformability of EVs may introduce contaminations to SEC-purified HDL samples. Three SEC columns connected in series followed by phospholipid binding resins[14] or density gradient followed by SEC[15] have been applied to avoid contaminated HDL samples for MS-based proteomic studies. The latter work observed a relative low number of different proteins (10–16) associated with HDL compared with the recent advances in proteomics that have extended the list of HDL-associated proteins to >90.[9,10] The low protein numbers reported in this work could be because of a better HDL-purification procedure compared with previous work.

Conclusions

I suggest at minimum a purification protocol based on size (only) followed by mass density-based purification or vice versa, when using physical-based methods for isolating EVs and lipoproteins. This 2-step procedure should according to the Figure lead to lipoprotein-free EV or EV-free lipoprotein samples or at least minimize the coisolation issues significantly. However, nothing comes for free; thus, a more thorough purification protocol might result in some loss of the particles of interest and molecules that are weakly associated to these bioparticles. Along these lines, it should be noted that, for example, density–gradient centrifugation introduces high shear forces and in some cases high-salt concentrations that may disrupt both HDL and EV protein interactions. Importantly, the coisolation issue is more pronounced in EV isolation compared with lipoprotein isolation because of the much higher abundance of lipoproteins compared with EVs in blood. I would also like to stress the importance of using quantitative proteomic and qPCR studies to evaluate the significance of the observed molecular findings. Along these lines, the distinction between lipoproteins and EVs based on a protein level is challenged because of the lack of unique EV biomarkers and the dynamics of certain apolipoproteins. The large nonexchangeable apoB variants associated with chylomicrons (apoB-48) and VLDL/LDL (apoB-100) are specific and, thus, can serve as valuable lipoprotein biomarkers. Of note, I would hesitate to use the exchangeable apoA-I as a biomarker for HDL.[2] Nonlipidated plasma apoA-I could potentially associate with EVs because of its dynamical properties and capability to bind to artificial vesicles. Our laboratory is currently investigating this interesting aspect. At the same time, the CD9, CD63, and CD81 proteins that share the membrane-spanning characteristics and are frequently observed at high abundance in EVs are likely EV but not lipoprotein specific.

I hope that this work will bring awareness about the challenges in isolating pure EV or lipoprotein samples and stress the need for caution when making conclusive statements on the molecular composition and biological functions of EVs and lipoproteins. Moreover, this work will hopefully also encourage and guide further research toward developing improved and novel bionanoparticle isolation and characterization methods and protocols that will ensure that we know what we are looking at when we are studying purified EV and lipoprotein plasma samples.

Disclosures

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