

U6/miR-211 expression ratio as a purity parameter for HEK293 cell-derived exosomes

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Small extracellular vesicles (sEVs) including exosomes are produced by all cell types and can be isolated from biological fluids and cell culture supernatants. The separation of exosomes with high purity from protein-rich media remains challenging. Besides contaminating proteins, small microvesicles (MVs) and apoptotic bodies are usually co-isolated with exosomes. The optimization of exosome separation and purification depends on reliable methods for the determination of the purity of the preparation, but no standard measurement has been defined so far. We tried to advance purity assessment. sEVs were isolated from HEK293 cell culture supernatants by various combinations of centrifugation, precipitation and size exclusion chromatography. sEVs with a diameter within the size range of 30–150 nm, typical for exosomes, were obtained with all tested isolation methods as shown by electron microscopy. To estimate the levels of protein contamination, flow cytometric analysis of the obtained vesicles was used. Based on the controlled preferential loading and enrichment of miR-211 into exosomes, a novel approach for the estimation of the fraction of HEK293 derived exosomes as opposed to MVs and apoptotic bodies in sEV mixtures was developed. This novel approach represents a simple qRT-PCR-based approach to improve the precise characterization of sEV isolates that is necessary for the usage of exosomes as carriers for therapeutic nucleic acids. Compared to the precipitation and size exclusion chromatography, the differential ultracentrifugation turned out to give sEVs with fairly intact shape and the highest purity according to the novel qRT-PCR-based approach, as well as to other established methods for purification assessment.

Keywords: exosomes, small extracellular vesicles, ultracentrifugation, size exclusion chromatography

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Abbreviations: sEVs, small extracellular vesicles; MVs, microvesicles; MVB, multivesicular body; UC, ultracentrifugation; CFSE, carboxyfluorescein succinimidyl ester; PEG, polyethylene glycol; qRT-PCR, real-time quantitative reverse transcription PCR; SEC, size exclusion chromatography; TEM, transmission electron microscopy; lodi UC, iodixanol cushion centrifugation

INTRODUCTION

For the communication between cells information carriers are released, which can be single molecules such as interleukins, cytokines and hormones, or can come in the shape of vesicles constituting complex packages surrounded by membranes (van Niel *et al.*, 2006). Such vesicles are produced by different mechanisms. The disintegration of whole cells after activation of programmed cell death generates apoptotic bodies, which contain portions of the intracellular contents that may include nuclear material and mitochondria (Jiang *et al.*, 2017). Microvesicles/ectosomes with a diameter of about 100–1000 nm are created by an outward budding from the plasma membrane (Théry *et al.*, 2009; Tricarico *et al.*, 2017), while exosomes with a size of 30–150 nm are of endosomal origin (Raposo & Stoorvogel, 2013). Exosomes contain RNA, DNA, lipids and proteins. The transferred cargo is meaningful in cell-to-cell communication. The membrane of the vesicles protects this cargo from degradation and enables the delivery to specific target cells based on the interaction with defined surface receptors.

Exosomes are created by an inward budding of the membrane of an endosomal compartment, the multivesicular body (MVB). When MVBs fuse with the plasma membrane, exosomes are released into the extracellular space by exocytosis (Kalra *et al.*, 2012). Importantly, specific miRNAs are preferentially packed into exosomes (Kosaka *et al.*, 2013) and various mechanisms were suggested for the sorting process (Shurtleff *et al.*, 2017; Villarroya-Beltri *et al.*, 2013; Koppers-Lalic *et al.*, 2014).

The composition of exosomes is characteristic for the cells from which they originated, especially the pattern of miRNAs constitutes an individual signature that is characteristic for the producer cell. The composition of the exosomal cargo not only reflects the cell type but also its activation and metabolic state. Therefore, exosomes represent an important source of precise biomarkers and allow for a non – invasive method of early diagnosis or prognosis for many diseases (Toiyama *et al.*, 2018; Kalhuri & LeBleu, 2020; Weston *et al.*, 2019).

Exosomes constitute the body's natural vesicular transport system mediating for instance therapeutic effects of mesenchymal stem cells (Kot *et al.*, 2019), and can even reach the central nervous system by crossing the blood–brain barrier (Batrakova & Kim, 2015; Qu *et al.*, 2018). With these attributes exosomes open exciting opportunities to be used as drug carriers, especially for therapeutic nucleic acids (El Andaloussi *et al.*, 2013; Wahlgren *et al.*, 2012). For the employment of exosomes as drug delivery vehicles highly purified vesicle preparations are of crucial importance. However, the complex

composition of media from which exosomes are usually isolated - blood plasma, urine, tissue, or cell culture supernatants - keeps their purification challenging. Many commonly used methods are not suitable to entirely separate the vesicles from extra-vesicular proteins. Furthermore, the size and density of exosomes overlap with other kinds of extracellular vesicles, with which they also share some molecular markers. Although certain surface markers are enriched in exosomes, most of them are not exosome specific and cannot unambiguously determine the vesicle origin (Ferguson & Nguyen, 2016).

The assessment of the purity of exosome preparations brings about additional challenges. Due to their small size and heterogeneity, the analysis of exosomes is not trivial (Haraszti *et al.*, 2016; Kluszczyńska *et al.*, 2019). To visualize such small particles, transmission electron microscopy or atomic force microscopy are used, while quantification is possible by the Nanoparticle Tracking Analysis, Dynamic Light Scattering or Tunable Resistive Pulse Sensing (Kluszczyńska *et al.*, 2019). Exosomes can be examined on conventional flow cytometers when attached to latex beads or directly on high sensitivity flow cytometers (nanoscale flow cytometry). Western Blotting is very useful for the detection of exosome marker proteins but omits vesicle to vesicle variations and gives only an average data for heterogenic populations. Proteomic analysis based on mass spectroscopy has almost no limitation in protein detection but is very sensitive to contamination by co-isolated proteins (Kluszczyńska *et al.*, 2019). So far, no standard measurement of the purity of exosome preparations has been established. Webber and Clayton presented an approach that appears to provide a good purity measure, based simply on measuring the particle to protein ratio (Webber & Clayton, 2013). However, this approach also does not differentiate between exosomes and other kinds of vesicles in the same size range. All commonly used methods for exosome separation result in mixed populations of various vesicles together with non-vesicular components. Therefore, a mutual optimization process for purification techniques and the development of purity assessment methods has to take place.

In our approach to more precisely estimate the purity of exosome preparations, first a combined analysis of total exosomal RNA with the measurement of total protein content or of the level of specific proteins was used. The amount of extra-vesicular RNAs was visualized by RNase digestion experiments. The CD63 marker in bead - assisted flow cytometry was evaluated as a measure for exosome purity. Furthermore, to distinguish exosomes from MVs and apoptotic bodies, the ratio of miR-211 to U6 RNA was successfully utilized.

MATERIALS AND METHODS

Cell culture and sEVs harvesting

HEK293K cells were cultured in exosome depleted (ultra-centrifuged for 150 min at 100 000×g) Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO₂. Cells were passaged every 3rd day. Supernatants for sEV separation were collected after 48 h from, at that time point, confluent cell cultures. Cell culture medium was centrifuged for 4 min at 400×g to remove cell debris and for 30 min at 10 000×g to pellet the MVs. The resulting conditioned medium was subjected to ultracentrifugation for 150 min

at 100 000×g, the pellet was resuspended in PBS and the centrifugation was repeated. The pellet was resuspended in PBS to give the 2×UC prepared sEV suspension. The total protein yield of vesicle suspensions after each purification step was measured by standard Bradford assays (Thermo Fisher Scientific Polska, Warsaw, Poland).

OptiPrep™, a non-ionic iodixanol-based medium (60% iodixanol) with a density of 1.320 g/mL, was diluted to 40% with PBS and used to form a cushion. Conditioned medium was overlaid, and centrifugation was carried out for 150 minutes at 100 000×g. Several fractions were taken from the interphase region, and the protein concentration was determined by standard Bradford assays. Vesicle containing fractions were diluted with PBS, and the sEVs were pelleted at 100 000×g. Polyethylene glycol (PEG) precipitation was performed by mixing the conditioned medium with equal volume of 16% PEG 6000/8000 in PBS and incubating overnight at 4°C while mixing. Samples after the overnight precipitation were centrifuged at 1500×g for 60 min at 4°C. Pellets were resuspended in PBS and subjected to size exclusion chromatography (SEC) for which sEVs were separated on 3 ml columns filled with Sephadex G-150. A maximum of 200 µl was applied to each column, 200 µl fractions were collected. To detect sEVs labeled with CFSE, the fractions were placed on a black-wall 96-well plate and the fluorescence was measured using a FLUOStar Omega plate reader (BMG-Labtech, Germany) at a wavelength of 485/520 nm.

Transmission electron microscopy (TEM)

The sEV suspension was mixed with paraformaldehyde to 2% final concentration and applied onto formvar/carbon coated copper grids. The grids were washed twice with PBS, incubated with 1% glutaraldehyde and stained with 2% uranyl acetate. Dried samples were visualized during the same day on a Thermo Scientific Talos F200X electron microscope, at 200kV accelerating voltage.

Flow cytometry

Aldehyde/sulfate latex beads were incubated with sEVs for 20 min at room temperature (RT) with gentle stirring, then free binding sites were blocked by incubation with 1 mg/ml of bovine serum albumin (BSA) for 20 min. After spinning down for 2 min at 4000×g at RT, the beads were resuspended in 100 mM glycine in PBS and incubated for another 20 min. The beads were washed twice in FACS buffer (1% BSA in PBS with 0.1% sodium azide) and incubated with proper antibodies conjugated with PE (phycoerythrin) or FITC (fluorescein isothiocyanate) for 1 h at 4°C. After two more washing steps, the beads were analyzed on a Becton-Dickinson Calibur flow cytometer.

Determination of the sEV recovery rate

For the labeling with fluorescent dye, sEVs resuspended in PBS were incubated with CFSE (carboxyfluorescein succinimidyl ester) at a concentration of 5 µM for 10 min at 37°C, diluted with PBS and pelleted by ultracentrifugation.

Western Blotting

A whole cell extract from the HEK-293 cell line was prepared by lysing about 3×10⁶ cells in 0.3 mL RIPA Buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4; 0.1% Triton X-100, 2 mM EDTA, 0.1% SDS) that contained

a cocktail of protease inhibitors (Sigma Aldrich) on ice for 30 min. The lysate was centrifuged at $14000\times g$ for 15 min at 4°C and the supernatant was collected. The protein concentration was measured by the Bradford assay. For the measurement of total protein concentration at various stages of sEV isolation, the vesicle suspension was used in Bradford assays without prior lysis. For Western blotting of vesicle suspensions, sEVs were heated in Laemmli sample buffer to 95°C for 10 min without prior lysis. $40\ \mu\text{g}$ of protein per sample were separated on 8% or 10% SDS-polyacrylamide gels under denaturing conditions. After the electrophoresis, the proteins were transferred onto nitrocellulose membranes (Immobilon-P PVDF $0.45\ \mu\text{m}$, Millipore), which were blocked in TBS-Tween solution ($1\times\ \text{TBS}+1\%$ Tween-20) containing 5% BSA for 1.5 h at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies at a concentration of $0.8\ \mu\text{g}/\text{ml}$ in blocking buffer (anti-human Alix, sc-271975, GAPDH sc-25778, TSG-101 sc-7964, Calnexin sc-23954, all Santa Cruz Biotechnology, Dallas, Texas, USA). The following day, membranes were washed in TBS-Tween buffer and incubated with the secondary antibodies labeled with horseradish peroxidase for 1.5 h at RT ($0.04\ \mu\text{g}/\text{ml}$ in blocking buffer: goat-anti mouse IgG-HRP: 926-80010,

LI-COR). After washing the membranes, bands were detected using enhanced chemiluminescence (ECL) (Westar Supernova, CYANAGEN, XLS3 FS) and a G-Box system for analysis and documentation (Syngene).

RNase digestion of sEV isolates

To remove extra-vesicular RNA, the isolated sEVs were incubated for 10 min with RNase A (Thermo Fisher Scientific Polska, Warsaw, Poland) at a concentration of $10\ \mu\text{g}/\text{ml}$ in PBS at 37°C .

miRNA extraction and real-time quantitative reverse transcription PCR (qRT-PCR)

The miRcury isolation kit from Qiagen or the Genematrix Universal RNA/miRNA purification Kit from EurX were used to extract miRNA from vesicles according to the manufacturer's protocols. RNA concentration was determined using a Nanodrop spectrophotometer at a wavelength of 260 nm. MiRNA and U6 levels were determined by real-time quantitative RT-PCR using TaqMan[®] MicroRNA Assays (Applied Biosystems, Carlsbad, CA, USA) with pre-formulated primers and probe sets designed to detect and quantify mature miRNAs and U6 as the endogenous control. Reactions

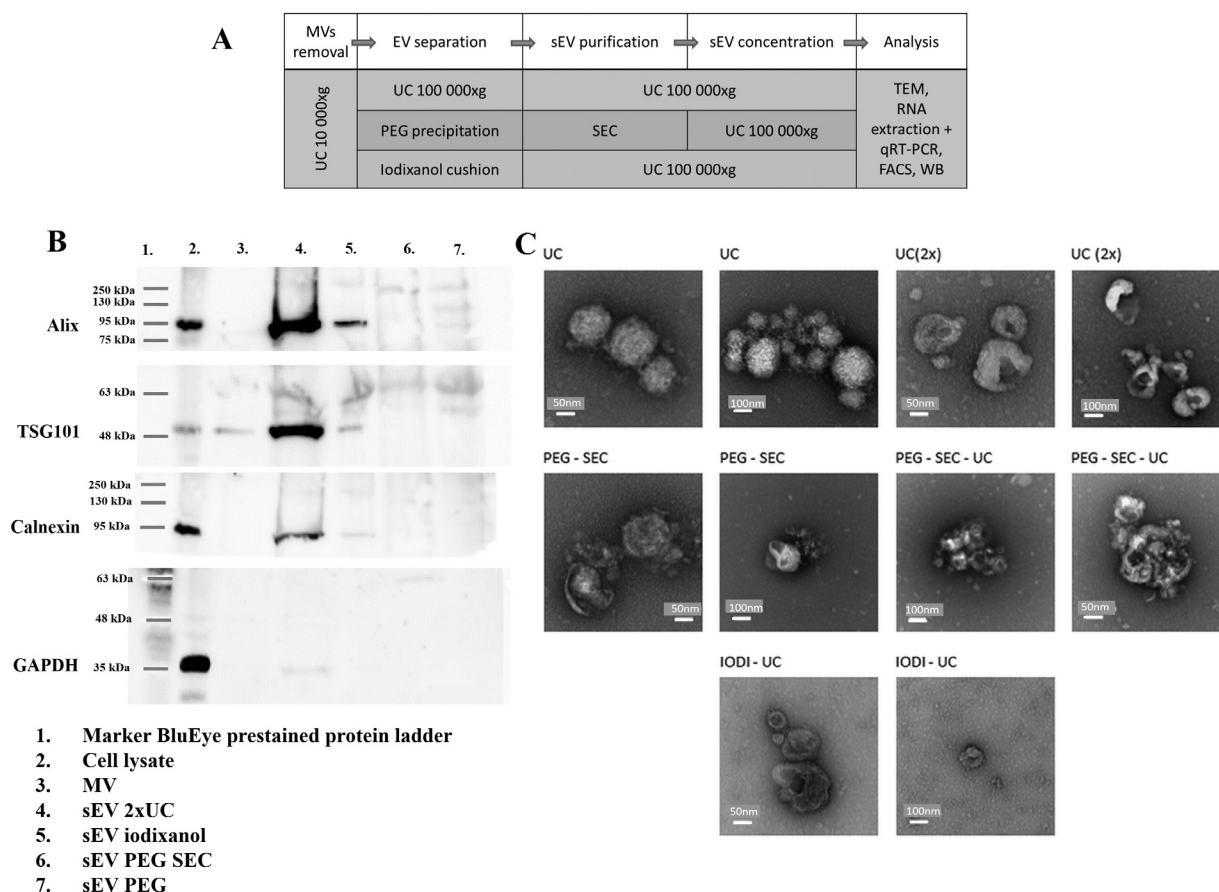


Figure 1. Separation of sEVs from cell culture medium.

(A) Three different approaches were used to separate sEVs. The first one (2 \times UC) consisted of two consecutive ultracentrifugation steps after the removal of larger vesicles. In the second approach, the sEVs were precipitated with 8% PEG 6000/8000, purified by SEC and concentrated by UC. The third one consisted of a centrifugation onto a iodixanol cushion, followed by an UC. (B) Western blots showing stainings for the exosome markers Alix and TSG101, as well as the cellular proteins Calnexin and GAPDH in lysates from HEK293T cells, microvesicles (MV) and exosomes after 2 \times UC, iodixanol cushion, PEG precipitation and PEG-SEC purification. (C) Vesicles in the range between 50 and 120 nm were obtained by all separation methods and were visualized by transmission electron microscopy.

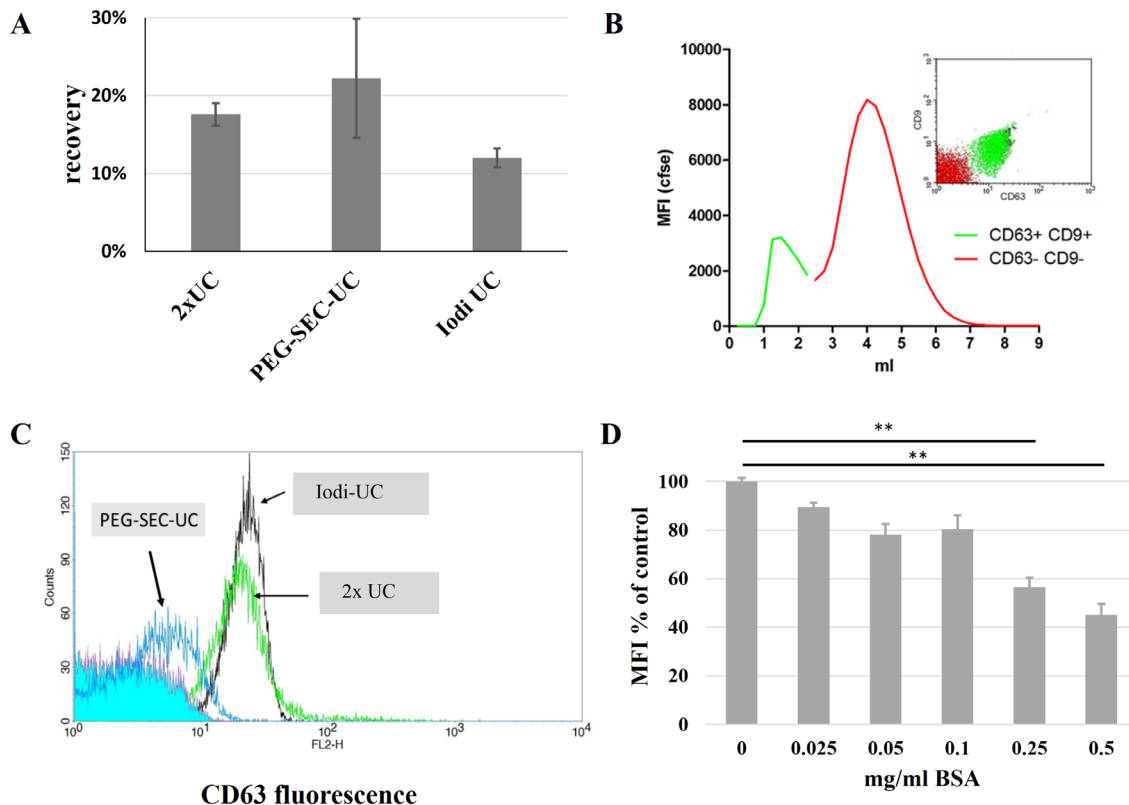


Figure 2. One title for whole figure

(A) Recovery of fluorescently labelled sEVs. CFSE-labelled sEVs were suspended in sEV-depleted medium. Then the medium was divided into three parts and sEVs were separated according to one of the three protocols used in this study. The fluorescence of the resulting vesicle suspension was measured at a fluorescence plate reader and related to the input. (B) Size-exclusion chromatography (SEC) reproducibly resulted in an early peak (green) that contained exosomes (corresponding to the green population in the inserted flow cytometer dotplot) and a CD9/CD63 negative fraction of contaminating proteins (depicted in red). (C) A flow cytometry histogram showing an overlay of the CD63 staining of sEVs isolated with different methods as indicated. (D) sEVs were isolated using the 2xUC protocol. Increasing concentrations of admixed BSA diminished the mean fluorescence intensity (MFI) of CD63 staining. The percentage values relative to a sample without added BSA are shown (mean and S.D.). For the statistical analysis, ANOVA and HSD were used, $**P \leq 0.01$.

for U6 RNA, hsa-miR-128-3p, hsa-miR-155-3p, hsa-miR-211-5p, hsa-miR-494-3p, and hsa-miR-181b-3p were realized.

Statistical analyses

Experiments were at least repeated twice ($n=3$). The differences between two independent groups of data were calculated with the use of the parametric Student's *t*-test. To compare the means from three or more groups the one-way ANOVA test (indicating an overall statistically significant difference in the group means) with post-hoc Tukey honestly significant difference (HSD – was run to confirm where the differences occurred between groups) was used. The homogeneity of variances was verified by Levene's test and Brown-Forsythe test. All statistical analyses were done using Statistica ver. 8.0 software (StatSoft Inc., Tulsa, OK, USA). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

We focused on three principles to isolate sEVs – first by ultracentrifugation (UC), where all particles in a similar sedimentation range are pelleted or concentrated at the interphase between the conditioned medium and a high-density phase, here an iodixanol cushion. The pellet/interphase is usually enriched in exosomes but can

also contain MVs, protein aggregates, parts of damaged membranes or small apoptotic bodies. The second principle of sEV separation was based on precipitation with PEG (Weng *et al.*, 2016), and the third on size exclusion chromatography. These three purification principles were combined into three major purification schemas (Fig. 1A). The first one consisted of two rounds of ultracentrifugation (2xUC), the second one combined PEG precipitation, size exclusion chromatography (SEC) and UC, while centrifugation onto an iodixanol cushion followed by UC was used in the third approach (Iodi-UC). The presence of proteins characteristic for exosomes was shown by Western blotting (Fig. 1B). Calnexin, an endoplasmic reticulum protein, should not be detected in exosomes (however, a small contamination is visible in the 2xUC preparation). Further analysis using electron microscopy showed vesicles in the size range of 40–120 nm from all preparations (Fig. 1C). Repeated ultracentrifugation seemed to damage the vesicles, while precipitation using PEG increased aggregation.

Using fluorescently labelled sEVs, that were added into cell conditioned medium before application of the separation/purification procedures, the recovery rate for the three different purification methods was determined (Fig. 2A). This analysis revealed that 18% ($\pm 1\%$), 22% ($\pm 8\%$), and 12% ($\pm 1\%$) of the sEVs were recovered during the isolation by 2xUC, PEG-SEC-UC, and Iodi-UC, respectively. To demonstrate the separation po-

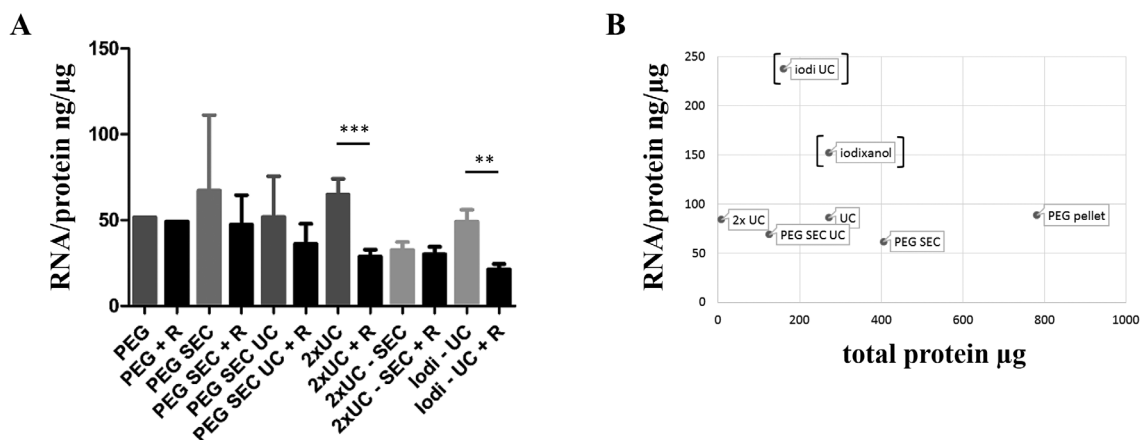


Figure 3. Estimation of RNA contamination and total yield.

(A) Yield of total RNA from vesicle preparations with (+R) or without RNase A digestion. The same volume of HEK 293 cell culture medium served as the starting material for each approach (mean and S.D.). ANOVA and HSD were used, $**P \leq 0.01$; $***P \leq 0.001$. (B) The ratio between total RNA and total protein plotted against the total protein yield. The dots for RNA/protein after iodixanol cushion centrifugation are put into brackets as they do not show the actual values – the RNA measurement is influenced by the iodixanol.

tential of SEC, the obtained fractions were combined with latex beads. Antibody staining demonstrated that CD9⁺/CD63⁺ vesicles could be separated from protein impurities (artificially colored in Fig. 2B). A disadvantage of SEC is the production of relatively diluted samples, which need to be concentrated for certain further applications by a final ultracentrifugation step.

Next, flow cytometry was used to assess the protein contamination. When sEVs isolated according to various schemas were tested, 2×UC and Iodi-UC isolated sEVs showed similar mean fluorescence intensities, while PEG-SEC-UC isolated sEVs presented with markedly reduced staining indicated higher protein contamination (Fig. 2C). To more precisely examine the influence of contaminating protein on the fluorescence intensity of CD63 staining, BSA was mixed with 2×UC purified sEVs before the adsorption to latex beads. Increasing concentrations of BSA could progressively reduce the intensity of CD63 staining, probably due to partial inhibition of the adsorption of sEVs to latex beads (Fig. 2D).

As free RNA in the solution is not pelleted by UC, the ratio between total RNA and the total protein of the vesicle preparation should provide a rough estimation of the total yield. However, complexes of extra-vesicular RNA with proteins were found in diverse sEV containing liquids that could be pelleted and contributed to the total RNA yield (Jeppesen *et al.*, 2019). To assess the amount of co-isolated extravesicular RNA, vesicle preparations were digested with RNase A before RNA isolation. As shown in Fig. 3A, the amount of total RNA was reduced up to 50% upon incubation with RNase A. When sEVs were isolated by 2×UC, a significant reduction in the RNA/protein ratio was observed upon RNase digestion (compare '2×UC' with '2×UC+R' in Fig. 2A). However, when SEC was added as another purification step before RNase digestion, no difference in samples treated with RNase A versus untreated control was found, demonstrating removal of extra-vesicular RNA by SEC. Similarly, specific miRNAs could be clearly separated between EVs and protein/HDL containing fractions (extra-vesicular) by SEC in another study (van Eijndhoven *et al.*, 2016).

In Fig. 3B, the yield in total protein and the ratio of total RNA to total protein are depicted. While the RNA/protein ratio gives a rough estimation of sEV

purity, the total amount of protein is related to the yield. In case of double ultracentrifugation ('2×UC') and PEG precipitation ('PEG pellet'), followed by SEC ('PEG-SEC') and UC ('PEG-SEC-UC'), each additional purification step reduced the total yield, while the RNA/protein ratio did not markedly increase. In case of the iodixanol cushion centrifugation ('Iodi-UC'), the RNA/protein ratio improved, however, RNA measurement based on absorbance at 260 nm is erroneous as iodixanol shows some absorption at this wavelength. Thus, the points in Fig. 3B do not reflect actual values for RNA yield and were put into brackets for that reason. In contrast, the RNA concentration in Fig. 3A was measured after an additional purification step by ultracentrifugation, therefore these values give correct RNA levels and RNA/protein ratios. In conclusion, the sole determination of the total RNA/protein ratio lacks the required precision and specificity for a satisfactory estimation of vesicle purity. When we compared our methods for sEV isolation and purification with published data, our yields were in a similar range as obtained by others. Patel *et al.* compared four commercially available kits for sEV isolation and purification with the differential ultracentrifugation (Patel *et al.*, 2019). They found no major differences in quality or quantity of the RNA isolated from sEVs. The yield of total protein showed some differences; however, it seems that – similar to our study – impurities might have affected the apparent yield. A similar study comparing various sEV isolation techniques, including ultracentrifugation, precipitation, columns or filter systems, found PEG precipitation to be a favorable method for RNA isolation, however, blood plasma was used in this study (Andreu *et al.*, 2016). A few publications focus on the methodological assessment of purification versus purity. Buschmann *et al.* compared different ways of sEV isolation from the serum of healthy donors and sepsis patients: precipitation, SEC, membrane affinity and sedimentation – using commercially available kits; the extracted RNA was analyzed by next generation sequencing (NGS). They demonstrated that the method of isolation is crucial and can result in different exosome composition and cargo. Precipitation methods gave bigger yield of total RNA, while SEC methods decreased the RNA reads (Buschmann *et al.*, 2018). Webber and Clayton presented an approach that appears to provide a good

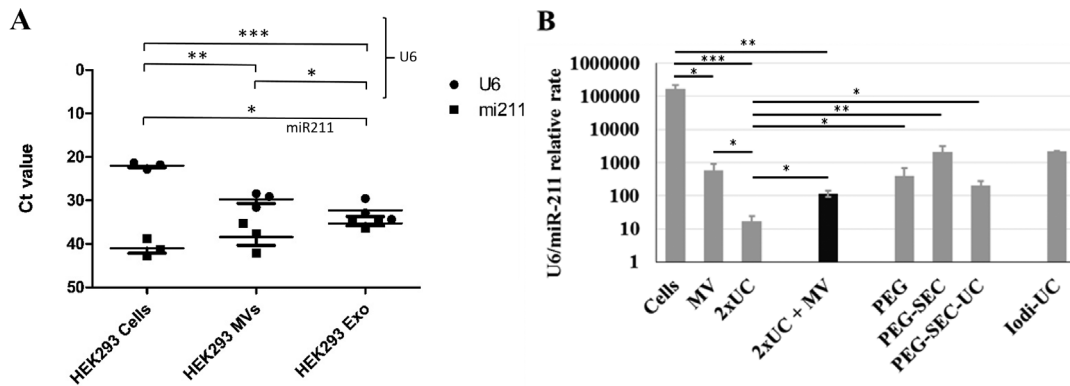


Figure 4. The miR-211 and U6 RNA levels and their ratio determined by qRT-PCR in cells and vesicle preparations.

(A) The cycle threshold values (Ct values) derived from RT-PCR reactions are shown for U6 RNA and miR-211 isolated from cells, MVs and sEVs after 2xUC (three independent isolations – mean). ANOVA and HSD were used. (B) The 2^{4Ct} values derived from the Ct measurements of miR-211 and U6 RNA were used to calculate a relative ratio (RR) for cells, MVs and sEVs isolated by various separation steps. For the '2xUC + MV' data point (black column), equal amounts of protein from the MV fraction and the sEVs isolated by 2-fold ultra-centrifugation were mixed together before RNA isolation (mean and SD). ANOVA and HSD were used. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

purity measure, based simply on measuring the particle to protein ratio (Webber & Clayton, 2013). However, also this approach does not differentiate between exosomes and other kinds of vesicles in the same size range. Tian *et al.* compared five commercial exosome isolation kits with UC (Tian *et al.*, 2019). Although they obtained much higher particle numbers when using the kits, the purity of sEVs isolated by UC was superior. Purity was assessed by counting the isolated particles before and after treatment with a non-ionic detergent that was supposed to lyse the phospholipid bilayer of the sEVs.

The flow cytometry analysis of vesicles illustrates the degree of protein contamination but does not provide information about the fractions of exosomes, MVs and apoptotic bodies. We tried to utilize the differences between the levels of specific RNAs in cells versus exosomes to estimate exosome enrichment by various isolation methods. The rationale for this approach relates to the controlled packing of specific RNAs into exosomes resulting in levels that can be higher or lower than those in cells. We assumed that the RNA expression pattern in MVs that are budding from the plasma membrane or in apoptotic bodies would be similar to the pattern inside the cells. The RNA expression pattern determined in vesicle preparations could resemble more that of the cell or that of exosomes and in this way give an indication for the proportion of exosomes in the isolate. We tested various combinations of the levels of the U6 small nuclear RNA (U6 spliceosomal RNA) and miR-211, miRNA-128a, miRNA-155, miRNA-494, and miRNA-181b (not shown) isolated from cells, MVs and sEVs. The cycle threshold value (Ct) of qRT-PCR reactions, inversely corresponding to the levels of miR-211 and U6 RNA, showed the most significant differences between the three sources of RNA (Fig. 4A). Cells contain higher levels of U6 RNA than vesicles, but lower levels of miR-211. When calculating the relative ratio (RR) of miR-211 and U6 RNA using the $RR = 2^{4Ct}$ formula (Schmittgen & Livak, 2008), differences of more than five orders of magnitude were observed. A $RR > 10^6$ was calculated for cellular RNA, while in the MV fraction RR dropped to 300–600, and to a value below 10 for the sEV isolate (Fig. 4B). To demonstrate the robustness of the approach, equal amounts (μ g protein) of MVs and sEVs were mixed before RNA isolation. The resulting RR ranged exactly between the values for pure sEVs and

MVs (Fig. 4B, black column). Concerning the RR values for the other isolation methods (Iodi-UC and PEG-SEC-UC) we cannot exclude that the potential leakage visible in the TEM pictures might have influenced the miR-211 and U6 level determination.

For the generalization of the novel method to estimate the amount of contaminating MVs or apoptotic bodies, we suggest selecting specific RNA molecules, for example based on microarray data, which show big differences between cells and sEVs. Calculating a relative rate between the levels of two or more RNA species can be used to establish an exosome specific purity parameter. In other words, an increase in the specific pattern of defined RNA molecules during various purification steps indicates the enrichment of exosomes, as it seems unlikely that contaminating RNA or vesicles of different origin would show the same pattern as found for exosomes. In our study, increasing the number of isolation/purification steps shifted the RNA rate towards the exosome specific pattern. In HEK293 cells, miR-211 was one of the miRNAs more efficiently packed into exosomes in HEK293 cells than others. To the best of our knowledge, the presented work is the first study trying to utilize the controlled packing of specific miRNAs into exosomes to estimate the relative fraction of exosomes in sEV preparations.

CONCLUSION

In most studies that aim at exosome isolation, a mixed population of vesicles is obtained. Therefore, it is important to verify, analyze and characterize the obtained preparations. Different methods, even on exactly the same sample, result in enrichment of varying vesicle subpopulations. There is no standard method giving 100% pure exosomes to which any other method could be compared. Therefore, a mutual optimization process for purification techniques and the development of purity assessment methods has to take place. The quantitation of miR-211 and U6 RNA was sufficient to generate a purity parameter for exosomes in sEV preparations from HEK293 cells based on the selective loading of miRNAs into exosomes. This purity parameter can be combined with already established ones to improve purity assessment of exosome preparations. When comparing the three isolation/purification protocols used in our study,

the differential ultracentrifugation (2×UC) gave the purest sEV samples, and the yield was the lowest compared to the other methods. The purity assessment of the isolated sEVs is crucial for choosing the best method for further applications, e.g., when using sEVs as carriers for therapeutic agents.

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