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The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles

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Background: Extracellular vesicles (EV), the collective term for vesicles released from cells, consist of vesicle species ranging in size from 30 nm to 5 μm in diameter. These vesicles are most commonly isolated by differential centrifugations, which pellets particles based on their differential movement through the liquid medium in which they are immersed. Multiple parameters, including the utilization of different rotor types, can influence the yield and purity of isolated vesicles; however, the understanding of how these factors affect is limited.

Materials and methods: Here, we compare the influence of multiple centrifugation parameters, including the use of swinging bucket and fixed angle rotors, as well as different centrifugation times, for the isolation of the smallest EVs, "exosomes." In particular, we determine the yields of exosomal RNA and protein, as well as the nature of the isolated vesicles and possible protein contamination with methods such as electron microscopy, western blot and flow cytometry.

Results: Our results show that application of a specific g-force or rotation speed by itself does not predict the ability of pelleting exosomes, and that prolonged centrifugation times can achieve greater yields of exosomal RNA and protein, whereas very long centrifugation times result in excessive protein concentrations in the exosome pellet.

Conclusion: In conclusion, rotor type, g-force and centrifugation times significantly influence exosome yield during centrifugation-based isolation procedures, and current commonly recommended isolation protocols may not be fully optimized for yield and purity of exosomes.

Keywords: *exosomes*; *extracellular vesicles*; *rotor*; *isolation protocol*; *ultracentrifugation.*

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Cells release several types of vesicles, which are collectively called extracellular vesicles (EV). In general, the term includes exosomes (30–100 nm), microvesicles (100–1000 nm) and apoptotic bodies (50–5,000 nm) (1–4). Apoptotic bodies are shed from dying cells and are usually collected with a low-speed centrifugation ($\sim$2,000 $\times$ g), while microvesicles are shed from the plasma membrane of viable cells and collected at 10,000–20,000 $\times$ g. On the contrary, exosomes are of endocytic origin and are released when multivesicular bodies fuse with the plasma membrane and release their intraluminal vesicles as exosomes to the extracellular milieu. These vesicles are collected with an ultracentrifugation above 100,000 $\times$ g. EVs harbour both proteins and different species of RNAs within their lipid shell, which can be transferred between cells (5,6) as part of intercellular communication in health and disease (7–9). The loading of proteins and RNA into exosomes has been indicated to be an at least partially specific event, with the exosomal cargo differing from the exosome-producing cell as well as between exosomes isolated from diffrent cell types and different cell conditions (10–13). Collectively, these traits have made exosomes, and EVs in general, attractive as possible biomarkers in different diseases, and as carriers of therapeutic molecules in the treatment of disease (14–16).

The most widely applied method for isolating EVs, in particular exosomes, is the isolation by differential centrifugations (17,18). This method involves a number of centrifugations, which sequentially increase in speed and

time, and thus pellet sequentially smaller particles. The pellets are discarded from each run and subsequent centrifugations are performed on the supernatant from the prior ones until the last centrifugal run, which aims to pellet exosomes. However, several factors can be altered in a differential centrifugation protocol, such as the duration of centrifugation, relative centrifugal force (RCF) and temperature. Any discrepancies in isolation protocols can inevitably lead to inconsistencies in isolated material, and may explain different results reported by different laboratories.

Apart from these commonly exercised variations of protocols, different rotors can be utilized. As the geometrical dissimilarities and performance of different types of rotors fundamentally differ, so does their pelleting efficiency. The two types of rotors that are commonly used in EV research are the swinging bucket (SW) rotors and the fixed angle (FA) rotors. These differ substantially in function, as the SW rotor stands out horizontally from the rotational axis during rotation, while the FA rotor is held at a constant angle during the whole centrifugation period. A direct consequence of this is that SW rotors generally have a longer sedimentation path length than FA rotors, which can result in a lower pelleting efficiency. These properties however grant the SW rotor better resolution, making it more suitable for the separation of particles with similar sedimentation coefficients, while the FA rotors are better suited for the separation of particles with more distinct sedimentation coefficients.

Although the theory of ultracentrifugation, including prediction of a rotors sedimentation capacity, has been thoroughly studied and well documented in the past (19–21), it has never been evaluated in real practice in the EV field. This issue therefore needs to be addressed to facilitate the development of standard isolation protocols, and the results would add to recent publications regarding this topic (18,22–25).

Therefore, the aim of the current study was firstly to determine the influence that different rotors, such as FA or SW rotors, may have on exosomal yield and purity. Secondly, we aimed to determine whether the commonly applied duration of centrifugation (70 minutes) is sufficient to pellet exosomes, and if variation in length of centrifugation is affecting exosomal yield and purity.

Materials and methods

Cells

The human mast cell line, HMC-1, were used to isolate exosomes and were kept at 37°C, with 5% CO_2 (Dr. Joseph Butterfield, Mayo Clinic, Rochester, MN, USA, and kindly provided by Gunnar Nilsson, Karolinska Institute, Stockholm, Sweden). This cell line has previously been shown to produce significant quantities of exosomes, and furthermore, these exosomes has been well characterized by electron microscopy, flow cytometry, Bioanalyzer and microarray analyses (26,27). Cells were grown in IMDM (HyClone laboratories, Inc., UT, USA) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1.2 U/ml alpha-thioglycerol (all supplemented products were from Sigma-Aldrich, St Louis, MO, USA). The FBS was depleted of exosomes by ultracentrifugation at $118{,}000 \times g_{avg}$ (Type 45 Ti rotor (FA), 38,800 rpm (revolutions per minute), k-factor 178.6, Beckman Coulter, Brea, CA, USA) for 18 hours. The pellet was discarded and the supernatant was filtered through 0.2 µm filters (Sarstedt, Numbrecht, Germany) before the FBS was used in cell cultures. Cells were seeded at a concentration of 0.5×10^6 cells/ml, and exosomes were harvested either three or four days later, after which cells were re-seeded.

Isolation of exosomes

Cultures of HMC-1 cells grown as described above were centrifuged at $300 \times g$ for 10 minutes to pellet the cells. Supernatant was collected and transferred to ultracentrifuge tubes (Polyallomer Quick-Seal centrifuge tubes 25×89 mm, Beckman Coulter) using a syringe and a needle. Samples were then centrifuged in a Beckman Coulter ultracentrifuge (Optima L-90 K or Optima XE-90 Ultracentrifuge, Beckman Coulter) for 20 minutes at 4°C at $16{,}500 \times g_{avg}$ (Type 70 Ti (FA); k-factor: 950.6, 15,000 rpm, Beckman Coulter), in order to pellet apoptotic bodies, microvesicles and cell debris. Supernatant was then carefully collected and the pellet discarded. For the rotor comparison experiments, supernatant was transferred to new ultracentrifuge tubes through 0.2 µm filters (Sarstedt). Samples were then centrifuged either for 70 minutes with a Type 70 Ti rotor at 4°C at $118{,}000 \times g_{avg}$ (40,000 rpm, k-factor: 133.7), 70

Table I. Rotor settings

Isolation setting	Rotor	RCF (average)	RPM	Time (minutes)	k-factor
FA 70 minutes	Type 70 Ti (Fixed angle)	118,000	40,000	70	133.7
SW 70 minutes	SW 32 Ti (Swinging bucket)	118,000	31,000	70	217.6
SW 114 minutes	SW 32 Ti (Swinging bucket)	118,000	31,000	114	217.6

The three different centrifugation settings used for the comparison of rotors. The FA 70 minutes setting is used as a reference to which the SW 70 minutes setting (which is not compensated for the change in rotor) and the SW 114 minutes setting (which is compensated by prolonged centrifugation duration) were compared to.

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minutes at 4°C with a SW 32 Ti rotor at 118,000 × g_{avg} (31,000 rpm, k-factor: 217.6) or 114 minutes at 4°C with a SW 32 Ti rotor at 118,000 × g_{avg} (31,000 rpm, k-factor: 217.6) (see Table I). Centrifugation for 114 minutes with the SW rotor at 31,000 rpm corresponds to a 70 minutes centrifugation at 40,000 rpm with the FA rotor (Fig. 1, equation 5). These calculations are based on the k-factors, or clearing factors, of the rotors at their given rpm's. The k-factor takes into account the velocity and the dimensions of the rotors (Fig. 1, equation 1 and 2). The sedimentation coefficient is a value that is attributed to a particle that describes its migration through a medium based on Stokes law (Fig. 1, equation 3). The rotor conversion is possible only when the sedimentation coefficient of the desired particle is the same (Fig. 1, equation 4 and 5). Conditioned cell culture media from one large cell culture was used and split into the three settings employed to assure a paired analysis.

$$k = \frac{2{,}53 * 10^5 * \ln(r_{max}/r_{min})}{(RPM/1000)^2} \quad (1)$$

$$k_{adj} = k\left(\frac{maximum\ RPM}{actual\ RPM}\right)^2 \quad (2)$$

$$s = \frac{m}{6\pi\eta r} \quad (3)$$

$$T = \frac{K}{S} \quad (4)$$

$$\frac{T_1}{K_1} = \frac{T_2}{K_2} \quad (5)$$

Fig. 1. Equations used for conversion of a centrifugal run between different rotors. Equation 1 is used to calculate the k-factor (clearance factor) for a rotor. If a rotor is run below its maximum velocity, equation 2 should be applied to calculate the rotor's k-factor. Equation 3 explains the sedimentation coefficient, which describes a particles movement through a liquid medium and is based on Stokes law, and is best applied to spherical particles. Shown in equation 4 is the relation between the k-factor, time of centrifugation, and sedimentation coefficient, which is expressed here in Svedberg units. Equation 5 shows how equation 4 can be used to convert a centrifugal run with one rotor so that an equivalent run can be applied with another rotor. However, this demands that S remains constant. These conversions can easily be performed with the help of online tools, such as the one provided at the Beckman Coulter web page (www.beckmancoulter.com, March 17th, 2014) RPM: revolutions per minute; R_{max}/R_{min}: maximum/minimum distance from the rotational axis; s; the sedimentation coefficient; m: the mass of the particle; η: the viscosity of the medium; r: the radius of the particle; T: centrifugation time (in hours); K: k-factor; S: Svedberg unit.

For the centrifugation duration experiments, only the FA rotor was used. For these experiments, no filters were used to fully determine how the yield and purity was affected. All samples were centrifuged for either 70 minutes, 155 minutes, 4 hours, 11 hours or 37 hours with a Type 70 Ti rotor at 4°C at 118,000 × g_{avg} (40,000 rpm, k-factor: 133.7). Centrifugations were performed in three different sets, either for 70 minutes, 155 minutes, 4 hours, 11 hours and 37 hours or 70 minutes, 4 hours, 11 hours and 37 hours or for 70 minutes, 155 minutes and 11 hours. Values within each set are paired, as the same culture was used for each round of isolations. The pellets from both rotor and duration experiments were re-suspended and collected in either lysis buffer (miRCURY RNA isolation kit, Exiqon, Vedbaek, Denmark) or PBS, depending on the downstream experiment. The re-suspended samples were frozen in −80°C if not immediately used.

RNA isolation and quantification

Isolated exosomes were re-suspended in 350 µl of lysis buffer (with 1% 2-mercaptoethanol added; miRCURY RNA isolation kit, Exiqon) before 200 µl of 95% ethanol was added. The samples were vortexed and transferred to spin columns and centrifuged at 14,000 × g for one minute. The flow-through was discarded and 400 µl Wash Buffer was added to the spin columns which were centrifuged at 14,000 × g for one minute, whereby the flow-through was again discarded. This step was performed three times in total. A centrifugation at 14,000 × g for two minutes followed to dry the spin column. Fifty microliters of elution buffer was added and centrifuged for two minutes at 200 × g, followed by one minute at 14,000 × g. The flow-through was collected and either used right away or kept at −80°C. The RNA quantification and profiling was performed using a nano chip on a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Protein quantification

Isolated exosomes were re-suspended in PBS, mixed with 20 mM Tris-HCL 1% SDS and then sonicated for five minutes, three times, with vortexing in between. Protein contents were measured using a BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Twenty-five microliters of BSA standard or 25 µl of sample were transferred to a 96 well plate to which 200 µl working reagent was added (working reagent; 50:1 ratio of assay reagents A and B). The plate was incubated for 30 minutes at 37°C, before being analysed with a SpectraMax 384 Plus spectrophotometer at 562 nm and the SoftMax Pro software (Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA, USA).

RNase treatment

Samples were divided into two aliquots of equal volume, one of which was designated for RNase treatment and one for control. PBS was added to a final volume of 95 µl

to which five µl RNase A (final concentration 0.5 µg/µl; Thermo Fischer Scientific, Waltham, MA, USA) or RNase- and DNase-free H_2O was added to the treated and untreated samples, respectively. Treated samples were incubated in 37°C for 20 minutes while untreated control was kept on ice. RNA isolation as described above was then performed for all samples, with the exception that 2% 2-mercaptoethanol was used instead of 1% in the lysis buffer. To control that the RNase reaction worked, RNA isolated from cells was also treated.

Western blot

Samples were mixed with 20 mM TrisHCL 1% SDS and then sonicated for five minutes, three times, with vortexing in between. Volumes corresponding to 25 µg of protein from isolates were separated on a 10% polyacrylamide gel. Samples were then transferred onto a nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA, USA) which was then blocked with 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories) in Tris-buffer saline (TBS) for two hours. Membrane was then incubated with primary antibodies against calnexin (1:1000; clone H-70; Santa Cruz Biotechnology, Santa Cruz, CA, USA), TSG101 (1:1000; clone 4A10; Abcam, Cambridge, UK) and CD81 (1:800; clone H-121; Santa Cruz Biotechnology) dissolved in 0.25% Blotting Grade Blocker Non-Fat Dry Milk in TBS-Tween (TBST) overnight at 4°C, after which the membrane was washed with TBST for 10 minutes, three times. Secondary antibodies [for calnexin and CD81: (1:10 000) ECL anti-rabbit IgG horseradish peroxidase-linked $F(ab')_2$ fragment (donkey-anti-rabbit); for TSG101: (1:2000) ECL anti-mouse IgG horseradish peroxidase-linked $F(ab')_2$ fragment (sheep-anti-mouse); GE Healthcare, Buckinghamshire, UK] were diluted in 0.25% Blotting Grade Blocker Non-Fat Dry Milk in TBST and incubated for 1.5 hours. Membranes were analysed with ECL Prime Western Blotting Detection (GE Healthcare) and a VersaDoc 4000 MP (Bio-Rad Laboratories).

Flow cytometry

Samples were incubated with CD63-coated magnetic beads (15 µg exosomes/70,000 beads/antibody; Exosome Isolation CD63, Lot OK527, Life Technologies AS, Oslo, Norway) over night with gentle agitation. The bead-sample complexes were washed with 1% exosome-depleted FBS in PBS and then incubated with human IgG (Sigma-Aldrich) for 15 minutes at 4°C. The bead-sample complexes were again washed and then incubated with PE-labelled antibodies against CD9, CD63 and CD81 or Isotype Control (BD Bioscience, Erembodegem, Belgium) for 40 minutes with gentle agitation at room temperature. Another wash step was performed before running the samples in a FACSAria (BD Bioscience) and analysing the data using FlowJo Software (Tri Star, Ashland, OR, USA).

Electron microscopy

Vesicle samples used for electron microscopy were dissolved in PBS and a volume corresponding to 15 µg of protein was loaded onto formvar carbon-coated grids (Ted Pella Inc., Redding, CA, USA). Samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde and contrasted in 2% uranyl acetate. Samples were examined in a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Jena, Germany). Vesicles were measured with the iTEM® software (Olympus-SiS, Münster, Germany).

Statistics

Statistical significance was evaluated using either repeated measures ANOVA or one-way ANOVA with a Tukey's multiple comparison test and a confidence interval of 95% for all samples. To estimate correlation, a Spearman-ranked correlation test was performed.

Results and discussion

The effect of rotor type on exosomal yield

When reviewing the field of exosomes, a basic protocol with a 70 minutes centrifugation at approximately 100,000 $\times$ g is most often applied (22,24,28). It is however rarely stated in the methods section which type of rotor has been used nor is it explained in which exact position of the rotor the specified g-force is applied. Since different rotors have different sedimentation path lengths, and the g-force varies with the distance from the rotational axis, the centrifugal run should be converted to fit the particular rotor used. To determine the outcome of exosome isolation when different rotors are used, three different centrifugation settings were performed (Table I). Firstly, a 70 minutes centrifugation with a FA rotor was used as a reference point to which the other two were compared (Table I, FA 70 minutes). Secondly, a 70 minutes centrifugation with the SW rotor was used, which had therefore not been adjusted for the use of another rotor (Table I, SW 70 minutes). The third and last settings used the SW rotor, but had a longer centrifugation time (114 minutes) that took the dimensions and performance of the SW rotor into account (Table I, SW 114 minutes). Thus, the third protocol was adjusted to be equivalent to the FA reference, based on the different k-factors that these two rotors have (equations in Fig. 1).

The RNA and the protein yields were measured to determine the difference in exosome isolation between the rotors. The FA 70 minutes setting gave an average RNA yield of 21.7 ± 4.7 ng/10^6 cells (Fig. 2A). The unadjusted protocol with the SW rotor (SW 70 minutes) yielded an average of 13.9 ± 2.6 ng/10^6 cells and the adjusted protocol (SW 114 minutes) averaged at 25.4 ± 4.7 ng/10^6 cells. This suggests that an unadjusted protocol for a rotor with a lower k-factor does not pellet exosomes with the same efficiency. Therefore, we can conclude that different

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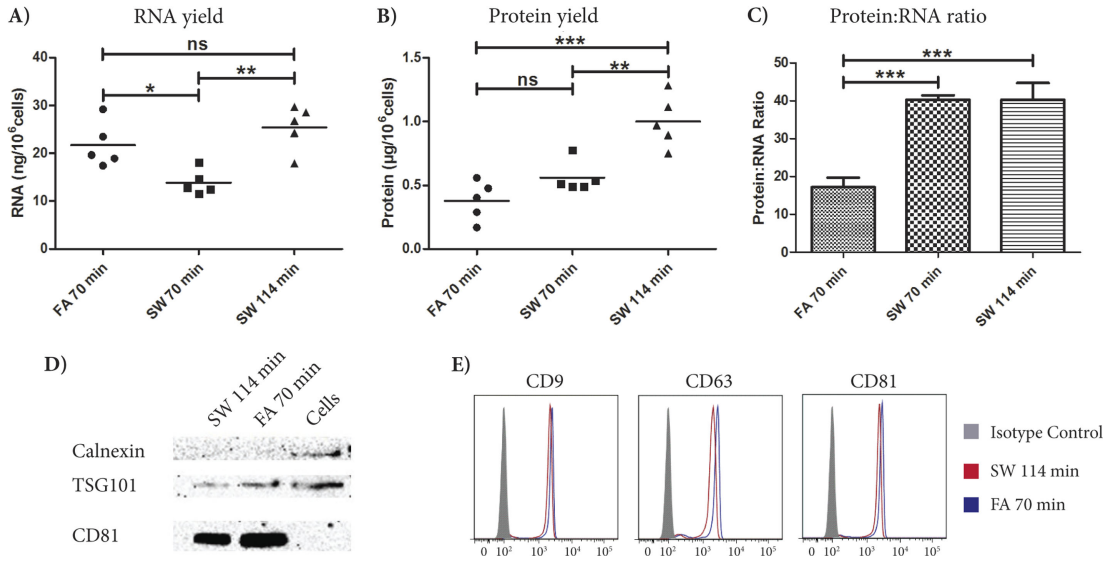

Fig. 2. Comparison between fixed angle (FA) rotor and swinging bucket (SW) rotor on exosome isolation. To equally deplete larger extracellular vesicles in all samples, one sample was centrifuged at 16,500 × g in a FA rotor and filtered through 0.2 µm filters and then divided into three samples. These three samples were then centrifuged for either 70 minutes in a FA rotor (FA 70 min), 70 minutes in a SW rotor (SW 70 min) or for 114 minutes in a SW rotor (SW 114 min). The SW 114 minutes centrifugation is calculated to be equal to the FA 70 min centrifugation in terms of pelleting efficiency. (A) The exosomal yield presented as nanogram (ng) RNA/10^6 cells for the three different centrifugation settings. (B) The exosomal yield presented as microgram (µg) protein/10^6 cells for the three centrifugation settings. (C) The protein to RNA ration is calculated based on the measurements from A and B ((µg protein/10^6 cells)/(µg RNA/10^6 cells)). (D) Detection of the vesicular markers TSG101 and CD81 as well as the endoplasmatic reticulum marker calnexin with western blot in isolates from cells, FA 70 minutes and SW 114 minutes. (E) Detection of CD9, CD63 and CD81 by flow cytometry using CD63-coated beads with isolates from FA 70 minutes (blue) and SW 114 minutes (red). Repeated measures ANOVA followed by Tukey's post hoc test were used to determine significant differences. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns; non-significant. Protein:RNA ratio data is presented as mean ± SEM.

centrifugation times (alternatively, different centrifugal speeds, see Fig. 1) are needed for different types of rotors to pellet exosomes with the same efficiency.

For the protein yield, the FA 70 minutes setting resulted in an exosome pellet with an average of 0.38 ± 0.15 µg protein/10^6 cells, while the unadjusted and adjusted protocol with the SW rotor averaged at 0.56 ± 0.12 and 1.00 ± 0.20 µg/10^6 cells, respectively (Fig. 2B). When comparing protein yields, it is evident that there are other differences between the rotors than just the difference in k-factor. The reason the protein yield does not follow the mathematical prediction, nor the trend for the RNA yield, can only be speculated upon. Factors that could affect this include the position of the pellet in both of the centrifugation tubes. In the FA rotor, the pellet is positioned in the lower outer side of the tube and in the SW rotor the pellet is positioned at the very bottom of the tube, which may result in an elevated protein value. It is also possible that the characteristics or geometry at the bottom of the centrifugation tube influence the retention of loosely pelleted proteins.

When a protein to RNA ratio was calculated, both of the protocols using the SW rotor showed ratios at around 40, while the FA protocol had a ratio of approximately 20, which indicates that the SW rotor is pelleting more protein in relation to RNA than the FA rotor (Fig. 2C).

To determine the presence of vesicles in the sample, as well as the nature of these vesicles, the samples were analysed with Western blot and flow cytometry for the presence of proteins previously found in exosomes. For Western blot, samples from both the FA rotor and the SW rotor were probed for the commonly used exosomal markers TSG101 and CD81, as well as calnexin, a marker for the endoplasmic reticulum. Both the FA and SW rotor sample were positive for CD81 and TSG101 (Fig. 2D). Furthermore, both samples were negative for calnexin (Fig. 2D), which indicates that the samples contain exosomes with little or no contamination of vesicles from the endoplasmic reticulum. Additionally, the presence of the tetraspanins, that is, CD9, CD63 and CD81, were evaluated with flow cytometry, for which both the samples from the FA and SW rotor were positive (Fig. 2E). Thus, western blot and flow cytometry together show the presence of vesicles in the samples. Furthermore, it also shows that both rotors are isolating similar types of vesicles, when it comes to the expression of CD9, CD63, CD81 and TSG101.

In conclusion, both rotor types isolate vesicles with similar features, however, the protein to RNA ratio argues that the choice of rotor has an impact on the isolated material, including RNA-containing exosomes,

and therefore needs to be handled and reported in a more standardized and diligent manner. Thus, we suggest that only describing the g-force (RCF) in a protocol, and leaving out which rotor was used for an experiment, is insufficient for accurate replication of any exosomal isolation procedure. The k-factor for different rotors must also be accounted for, as the k-factor for a rotor is describing its pelleting efficiency.

In Table II, the k-factor for some of the commonly used rotors within the exosome field are shown, which highlights that 70 minutes with one rotor is not equivalent to 70 minutes with another rotor, when it comes to pelleting capacity. This could explain some of the variation seen in exosomal yield between different studies, as 70 minutes with the TLA-100.3 rotor would most likely result in a substantially higher yield per volume than 70 minutes with a SW 32 Ti rotor, for example. Including the k-factor for the rotor used under the centrifugal conditions as a standard in describing methods would allow for improved accuracy and adaptation of such protocols.

The effect on exosomal yield by prolonged centrifugation periods

The second aim of this study was to determine the influence of longer centrifugation periods on yield, specifically asking whether 70 minutes of high speed centrifugation is sufficient to pellet all exosomes present in the conditioned media, or if a longer centrifugation period allows for greater quantities of material to be acquired. This was investigated by a series of centrifugation durations, where the RNA and protein yields were estimated and compared. Both the RNA (Fig. 3A) and the protein (Fig. 3B) yields followed similar trends, with longer centrifugations resulting in higher quantities. The relationship between yields and centrifugation periods also showed significant correlation with a correlation coefficient of 0.8980 and 0.9036 for the RNA and protein yields, respectively ($p < 0.05$). This shows that only a fraction of materials is normally pelleted and that extending the centrifugation duration hugely increases yields. To visualize the increase of RNA and protein yields in relation to each other, a protein to RNA ratio was calculated, which revealed a larger amount of proteins being acquired in relation to RNA with centrifugations longer than 155 minutes (Fig. 3C). This is especially evident with the 11 and 37 hours centrifugations, and less so with the shorter centrifugation periods tested. The unequal increase in yield for RNA and protein was further seen when values were re-calculated as a percentage of the 70 minutes centrifugation (with the 70 minutes centrifugation set as 100%), which revealed that yields increased by approximately 10,000% for the protein but only 800% for RNA when prolonging centrifugation duration to 37 hours. To further determine how the RNA and protein yields related to each other, the yields of RNA and proteins were plotted on a time scale. It was demonstrated that the largest incline for the RNA graph was seen during the first 155 minutes and it levelled off after 660 minutes (11 hours), while the protein incline was stable up to 2,220 minutes (37 hours) (Fig. 3D). The incline of the curve reveals the rate of acquisition for the different time points, but since the incline of longer centrifugations are affected by material isolated at earlier time points, the information on rate of pelleting between different time points in a centrifugation is masked. Therefore, by subtracting the pelleted material of earlier centrifugations, as well as the time it took to pellet it, from latter ones, the rate of pelleting at different centrifugation intervals can be visualized. It was then demonstrated that the rate of RNA pelleting is as high up to 4 hours of centrifugation as it is during a regular 70 minutes centrifugation, after which it is reduced (Fig. 3E). By contrast, a gradual increase could be seen in the rate of protein acquisition up to 11 hours, after which it drops (Fig. 3F). This further suggests that RNA-poor and protein-rich vesicles/particles and/or soluble proteins are enriched in the pellet with extended centrifugation, but that the yield of RNA-containing structures, such as exosomes, still improves well beyond 70 minutes of centrifugation. It could thus be argued that the first 4 hours of centrifugation are efficient in pelleting RNA containing structures, but beyond 4 hours of centrifugation, proteins accumulate to a greater extent. The very uniform rate in pelleting of RNA during the first hours of centrifugation suggests that the RNA collected during this period likely is of the same origin as that collected during

Table II. Rotor types and their properties

Rotor	g_{avg}	RPM	Migration path length	k-factor	Equivalent centrifugation duration (minutes)
Type 70 Ti	118,000	40,045	52.4	133.4	70
Type 45 Ti	118,000	38,837	67.9	178.3	93
SW 41 Ti	118,000	30,913	85.7	217.5	114
SW 32 Ti	118,000	30,998	85.7	217.6	114
TLA-100.3	118,000	52,724	20.8	51.3	27

Some common rotors and their characteristics, adjusted to a centrifugal force of 118,000 × g at the average distance from the rotational axis. Seventy minutes with the Type 70 Ti rotor is set as a reference and the equivalent centrifugation time (calculated with the equations in Fig. 1) for the other rotors are presented in the last column to illustrate the differences.

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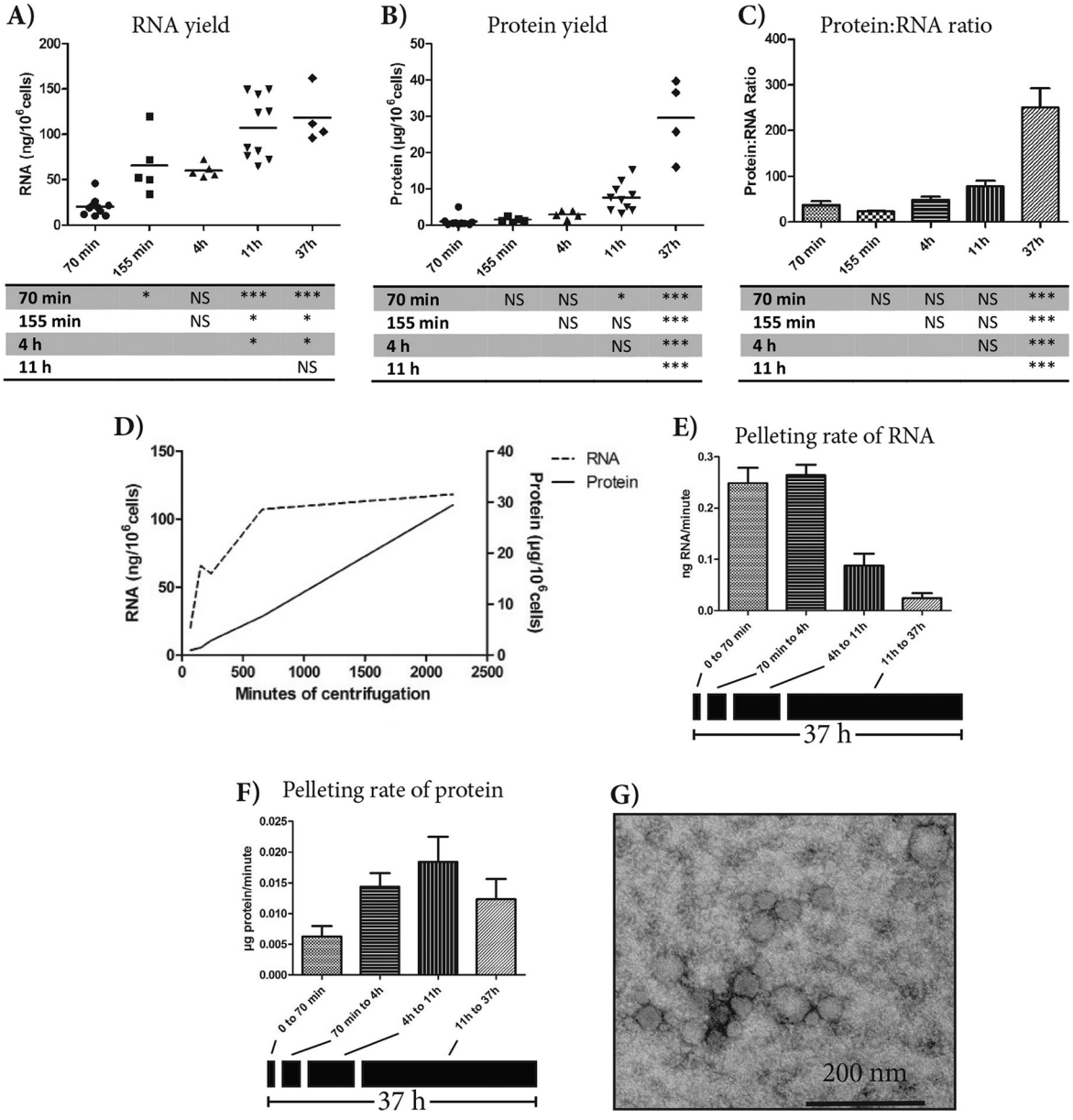

	70 min	155 min	4h	11h	37h
70 min	*	NS	***	***	
155 min		NS	*	*	
4 h			*	*	
11 h				NS	

	70 min	155 min	4h	11h	37h
70 min	NS	NS	*	***	
155 min		NS	NS	***	
4 h			NS	***	
11 h				***	

	70 min	155 min	4h	11h	37h
70 min	NS	NS	NS	***	
155 min		NS	NS	***	
4 h			NS	***	
11 h				***	

Fig. 3. Impact of centrifugation duration on exosomal RNA and protein yield. Conditioned media were centrifuged with a Type 70 Ti fixed angle (FA) rotor for 70 minutes, 155 minutes, 4 hours, 11 hours or 37 hours. The yield was determined by measuring RNA and proteins. (A) The exosomal yield presented as nanogram (ng) RNA/10^6 cells for the different centrifugation durations. (B) The yield presented as microgram (µg) protein/10^6 cells for the different centrifugation durations. The correlation between centrifugation duration and yield was also calculated giving a significant correlation coefficient of 0.8980 and 0.9036 for RNA and protein, respectively. (C) The RNA and protein yields from A and B were used to calculate a protein to RNA ratio ((µg protein/10^6 cells)/(µg RNA/10^6 cells)) to determine if increased centrifugation duration affected the purity of exosomes by also pelleting soluble proteins. (D) The increase of RNA (left Y-axis) and protein (right Y-axis) over time in minutes (X-axis). E and F) To determine the gain in yield made by prolonging the centrifugation time, a pelleting rate for different centrifugation time intervals for RNA (ng/minute) (E) and for protein (µg/minute) (F) was calculated based on 5 experiments where all samples were paired. G) Electron micrographs of samples from a 4 hours centrifugation performed on the supernatant from a regular 70 minutes centrifugation shows the presence of vesicles in the size range of 30–100 nm. The scale bar represents 200 nm. Data for C, E and F are presented as mean ± SEM. One-way ANOVA followed by Tukey's post-hoc test were used to determine significant differences. *$p < 0.05$, ***$p < 0.001$, NS; non-significant.

the first 70 minutes, which is probably a vesicular one. The pelleting of proteins is however more gradual, and extends beyond 4 hours, in all probability representing proteins of both vesicular origin as well as a heterogeneous population of soluble proteins. From a sedimentation coefficient viewpoint, it can be suggested that the RNA and proteins gathered the first hours, when the rate of RNA pelleting is constant, are from material with the same sedimentation

coefficient, again most likely vesicles such as exosomes. On the contrary, the material, which is pelleted after these hours, has a significant contribution of particles with lower sedimentation coefficients, such as soluble proteins, and/or vesicles with a lower sedimentation velocity. Furthermore, electron microscopy showed that 30–100 nm exosome-like vesicles could, with a 4 hours centrifugation, be isolated from the supernatant remaining after a 70 minutes centrifugation at 118,000 × g (Fig. 3G). This suggests that vesicles do remain in the supernatant after a 70 minutes centrifugation and could thus be the factor that contributes to the increase in RNA and protein yields seen during the longer centrifugations. Considering all data, this strongly argues that a 70 minutes centrifugation period with the settings employed here is insufficient to isolate exosomes, which can be of particular importance when working with rare clinical samples or small volumes of cell culture media.

Characterization of vesicles isolated during longer centrifugation durations

To evaluate if the increase in protein to RNA ratio was due to co-isolation of soluble proteins and if other subpopulation of vesicles with different size and protein composition were pelleted during the longer centrifugation times, Western blot, flow cytometry and electron microscopy were performed. Electron microscopy confirmed the presence of vesicular structures in all samples (data not shown). All vesicles detected in all samples, except in the 37 hours samples where too few vesicles were present, were measured manually with the iTEM® software and presented as graphs (Fig. 4A). The median size was similar in all samples (~53 nm, Fig. 4A). However, larger (>101 nm) and smaller (20–50 nm) vesicles were more abundant than intermediately sized vesicles (51–100 nm) during longer centrifugations (Fig. 4B). The velocity of a sedimenting particle is dependent on its size and density (assuming a spherical particle), which would suggest that the larger vesicles pelleted with longer centrifugations are more likely to be less dense, while the smaller vesicles probably owe their slower sedimentation partly, or wholly, to their smaller size. Previously published studies have suggested that both the length of a centrifugation and the size of the vesicles affect how vesicles migrate in a sucrose gradient (29,30), which would support this argument.

Western blot demonstrated that the 70 and 155 minutes samples were both positive for TSG101 and CD81 while the 4 hours centrifugation was positive only for CD81 but with weaker band intensity (Fig. 4C). The 11 and 37 hours samples were only weakly positive for TSG101 and CD81 when a larger quantity (100 µg instead of 25 µg) was loaded per well. All isolates were negative for calnexin, which suggests that longer centrifugations do not lead to contamination of vesicles from the endoplasmic reticulum (Fig. 4C). Flow cytometry revealed that all samples were positive for all three tetraspanins, but with a decrease in signal intensity with longer centrifugations (Fig. 4D). As CD81 was detected with flow cytometry in all samples, but only in the 70 minute, 155 minute and 4 hour samples with Western blot, it indicates that a weaker, or even non-existing signal, does in all probability not reflect an absence of vesicles, but rather a very high non-vesicular protein content which drowns out the signal. The fact that CD81 and TSG101 were detected when larger amount of samples was loaded supports this argument.

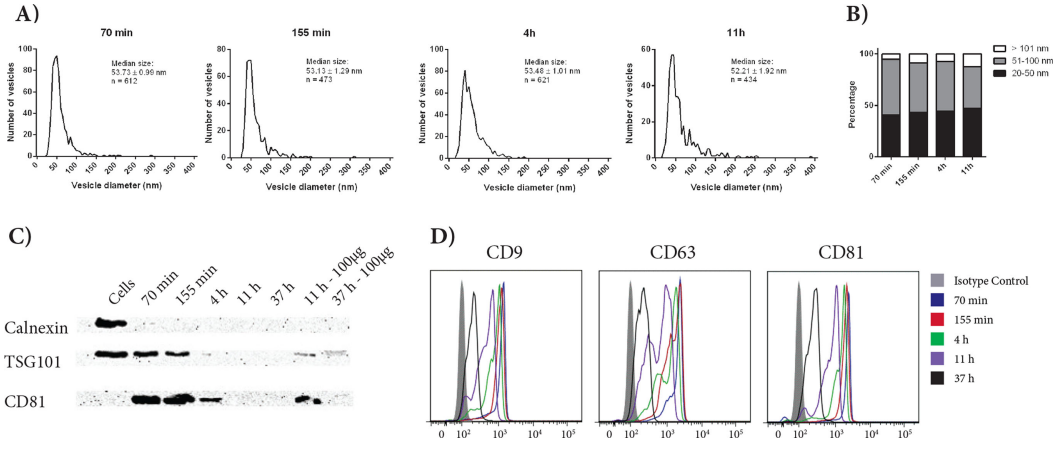

Fig. 4. Characterization of vesicles isolated with longer centrifugation durations. (A) Size distribution of vesicles from 70 minutes, 155 minutes, 4 hours and 11 hours estimated by electron microscopy and presented as graphs with number of vesicles on y-axis and vesicle diameter in nm on x-axis. (B) Size distribution as percentage of vesicles larger than 100 nm (white), between 100–151 nm (grey) and between 20–50 nm (black), based on the same data as in A. (C) Western blot for isolates from 70 minutes, 155 minutes, 4 hours, 11 hours and 37 hours centrifugations. HMC-1 cell lysates were used as positive control and 25 µg was loaded per well with one extra set of 11 and 37 hours samples loaded with 100 µg protein (right hand side). (D) Flow cytometry on isolates from 70 minutes (blue), 155 minutes (red), 4 hours (green), 11 hours (purple) and 37 hours (black) probed for CD9, CD63 and CD81 markers as well as isotype control (grey).

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Interestingly, doing a backwards calculation to come to the predicted sedimentation coefficient that the different centrifugation durations aim to pellet, one attains the approximate values of 114, 52, 33, 12 and 4 Svedberg Units (S) for the 70 minutes, 155 minutes, 4 hours, 11 hours and 37 hours centrifugations, respectively. Generally, the lower this value is the smaller and/or less dense particles are pelleted. Larger particles such as viruses will be pelleted at 50–100 S depending on species and ribosomal subunits are named after which S they are pelleted at. Some soluble proteins have a Svedberg unit of approximately 30 S and below, with albumin having that of approximately 4–5 S. This highlights the limitation of longer and/or faster centrifugation and puts the consequences of adjusting either of these factors into perspective. From the time points evaluated, the 155 minutes centrifugation gives an increase in yields without any apparent loss of quality, in terms of a higher protein to RNA ratio or a substantial loss of intensity in the Western blot and flow cytometry analyses. This does however not seem to pellet all the vesicles. Thus, longer centrifugations could also be a good option if these are followed by a purification process, such as a sucrose or iodixanol gradient, or alternatively purification by size exclusion through a Sepharose column.

RNase treatment and RNA profiles after increased centrifugation duration

To determine if the increased yield in RNA was protected inside vesicles or on the outside of the vesicles or even free, all samples were RNase treated. The RNA in all samples was protected from RNase A degradation, which indicates that it resides inside a protecting structure, such as a vesicle (Fig. 5A). Furthermore, the RNA profiles of the treated sample did not differ from those of the control samples, which strengthens the notion that the RNA is protected from RNase A degradation (data not shown).

As a measurement of the purity of isolated vesicles, the RNA profiles of the isolated material was determined and compared between the different centrifugation durations. This was performed to establish whether the increased yield in RNA seen in Fig. 3 was due to pelleting of more RNA-containing exosomes, or whether other RNA-

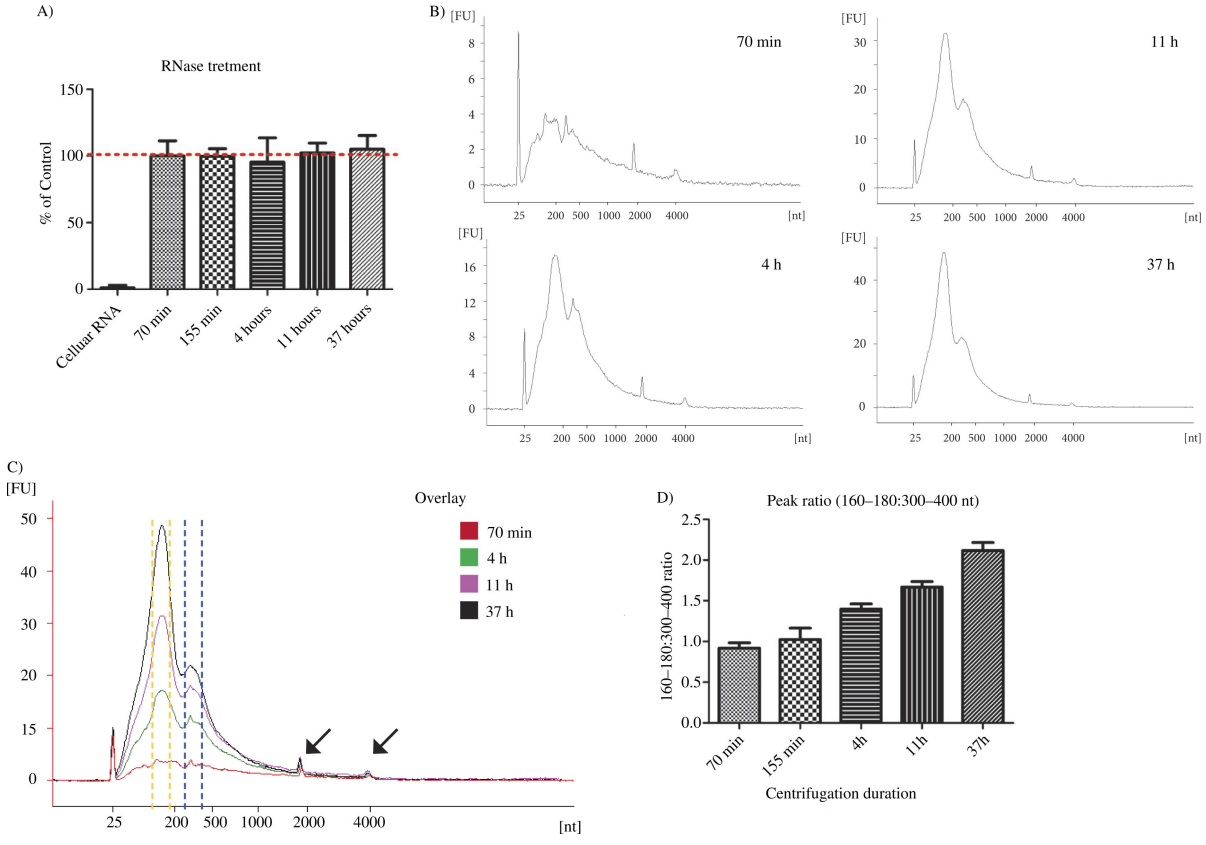

Fig. 5. Alterations in the RNA profile during longer centrifugation duration. (A) RNase treatment of samples. Treated samples are presented as a percentage of RNA yield in comparison to yield of untreated control, which was kept on ice (red line marks 100%). (B) The RNA profile and yield of samples isolated with 70 minutes, 4 hours, 11 hours or a 37 hours centrifugation were determined with a Bioanalyzer. One representative experiment is shown per centrifugation time. (C) Profiles from B are shown in an overlay. Black arrows: 18 and 28 S ribosomal peaks; yellow dashed lines: 160–180 nt interval; blue dashed lines: 300–400 nt interval. (D) Ratios of the highest peak between 160 and 180 nt (yellow dashed lines in C) and the highest peak between 300 and 400 nt (blue dashed lines in C).

containing structures may contribute to the increased RNA quantities. A similar RNA profile, as the 70 minutes centrifugation may suggest, that more of the same type of vesicles is isolated with prolonged centrifugations, whereas a dissimilar RNA profile may indicate that other types of vesicles, particles and/or protein aggregates containing RNA are contributing to the pellet with longer centrifugations. Analysis of RNA with a Bioanalyzer demonstrates that the RNA profiles are conserved despite extended centrifugation periods, which indicates that the gain made in RNA yield by longer centrifugations is likely to be from the same source, most likely exosomes (Fig. 5B). Since the ribosomal RNA peaks (black arrow, Fig. 5C) do not change markedly throughout the different centrifugation durations, it can be suggested that the gain in small RNA with longer centrifugations is not from microvesicles or apoptotic bodies as they have been shown to contain ribosomal RNA (27), but rather of exosomal origin. However, when examining the RNA profile more closely, it can be observed that one of the two prominent peaks (160–180 nt in size, see yellow dashed lines, Fig. 5C) increase to a greater extent with prolonged centrifugation than the other (300–400 nt in size, see blue dashed lines, Fig. 5C). The average ratio between the 160–180 and 300–400 nt peaks gradually increase from the 70 minutes to the 37 hours centrifugations (Fig. 5D), with a correlation coefficient of 0.9018 ($p < 0.05$). This suggests that RNA acquired after prolonged centrifugations may be harboured in two different types of vesicles and/or protein/RNA containing structures, with one of them contributing more to the 160–180 nt peak than the 300–400 nt peak.

Conclusion

For the first time, this study has determined the influence of rotor types on the isolation of exosomes using differential centrifugation, and has described the influence of prolonged centrifugation periods on exosomal RNA yield. Firstly, a FA and SW rotor need different centrifugation settings in order to pellet particles equally, and proper rotor conversion calculations should be performed with the k-factor reported in any publication using such isolation techniques.

Secondly, by extending the centrifugation time, significantly higher vesicle yields can be attained, which shows that a regular centrifugation protocol of 70 minutes is substantially insufficient for the isolation of exosomes. However, centrifugations that are too long, that is, beyond approximately 4 hours, should probably be avoided in studies of EVs, to reduce excessive contamination of soluble proteins to any vesicular material collected at earlier time points.

Conflict of interest and funding

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References

1. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009;9:581–93.
2. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics. 2010;73:1907–20.
3. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev. 2012;64:676–705.
4. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci. 2011;68:2667–88.
5. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.
6. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics. 2009;6:267–83.
7. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. 1996;183:1161–72.
8. Torregrosa Paredes P, Esser J, Admyre C, Nord M, Rahman QK, Lukic A, et al. Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. Allergy. 2012;67:911–9.
9. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2012;18:883–91.
10. Lässer C, Eldh M, Lötvall J. Isolation and characterization of RNA-containing exosomes. J Vis Exp. 2012;(59):e3037.
11. Hedlund M, Nagaeva O, Kargl D, Baranov V, Mincheva-Nilsson L. Thermal- and oxidative stress causes enhanced release of NKG2D ligand-bearing immunosuppressive exosomes in leukemia/lymphoma T and B cells. PLoS One. 2011;6:e16899.
12. Faure J, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, et al. Exosomes are released by cultured cortical neurones. Mol Cell Neurosci. 2006;31:642–8.
13. Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J Immunol. 2002;168:3235–41.
14. Liang B, Peng P, Chen S, Li L, Zhang M, Cao D, et al. Characterization and proteomic analysis of ovarian cancer-derived exosomes. J Proteomics. 2013;80C:171–82.
15. Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer. 2009;10:42–6.
16. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol. 2011;29:341–5.

17. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic Acids Res. 2012;40:D1241–4.
18. Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles. 2013;2:20389, doi: http://dx.doi.org/10.3402/jev.v2i0.20389
19. Bowen TJ, Rowe AJ. An introduction to ultracentrifugation. London: Wiley-Interscience; 1970. 1, ii–xviii, 171 p., 8 plates.
20. Scott DJD, Harding SE, Rowe AJ. Analytical ultracentrifugation: techniques and methods. Cambridge: Royal Society of Chemistry; 2005.
21. Stephenson FH. Calculations in molecular biology and biotechnology: a guide to mathematics in the laboratory. Amsterdam: Academic; 2003.
22. Momen-Heravi F, Balaj L, Alian S, Mantel PY, Halleck AE, Trachtenberg AJ, et al. Current methods for the isolation of extracellular vesicles. Biol Chem. 2013;394:1253–62.
23. Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al. Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. Front Physiol. 2012;3:162.
24. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles. 2013;2:20360, doi: http://dx.doi.org/10.3402/jev.v2i0.20360
25. Kalra H, Adda CG, Liem M, Ang CS, Mechler A, Simpson RJ, et al. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. Proteomics. 2013; 13:3354–64.
26. Ekström K, Valadi H, Sjöstrand M, Malmhäll C, Bossios A, Eldh M, et al. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. J Extracell Vesicles. 2012;1:18389, doi: http://dx.doi.org/10.3402/jev.v1i0.18389
27. Crescitelli R, Lässer C, Szabo TG, Kittel A, Eldh M, Dianzani I, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles. 2013;2:20677, doi: http://dx.doi.org/10.3402/jev.v2i0.20677
28. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol, 2006;Chapter 3:Unit 3.22.
29. Palma J, Yaddanapudi SC, Pigati L, Havens MA, Jeong S, Weiner GA, et al. MicroRNAs are exported from malignant cells in customized particles. Nucleic Acids Res. 2012;40: 9125–38.
30. Aalberts M, van Dissel-Emiliani FM, van Adrichem NP, van Wijnen M, Wauben MH, Stout TA, et al. Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans. Biol Reprod. 2012;86:82.