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REVIEW

Systematic review of factors influencing extracellular vesicle yield from cell cultures

Johann Mar Gudbergsson · Kasper Bendix Johnsen · Martin Najbjerg Skov · Meg Duroux

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Abstract The potential therapeutic utility of extracellular vesicles (EVs) has spawned an interest into a scalable production, where the quantity and purity of EV samples is sufficient for clinical applications. EVs can be isolated using several different protocols; however, these isolation protocols and the subsequent methods of quantifying the resulting EV yield have not been sufficiently standardized. Therefore, the possibility of comparing different studies with respect to these parameters is limited. In this review, we have presented factors that might influence the yield and function of EVs from cell culture supernatants. The methods of isolation, downstream quantification, and culture conditions of the EV producing cells have been discussed. In order to examine the inter-study coherency of EV yields, 259 studies were initially screened, and 46 studies were included for extensive downstream analysis of EV yields where information pertaining to the isolation protocols and quantification methods was obtained from each study. Several other factors influencing yield were compared, such as cell type producing EVs, cell confluence level, and cell stimulation. In conclusion, various factors may impact the resulting EV yield, including technical aspects such as EV isolation and quantification procedures, and biological aspects such as cell type and culture conditions. The reflections presented in this review might aid in future standardization of the workflow in EV research.

Keywords EV · Exosome · Microvesicle · Isolation · Quantification · Stimulation

Abbreviations

EV	Extracellular vesicle
MVB	Multivesicular body
UC	Ultracentrifugation
SEC	Size-exclusion chromatography
UC-W	Ultracentrifugation with washing step
UC-G	Ultracentrifugation with gradient separation
FCS	Fetal calf serum
CM	Conditioned medium
BCA	Bicinchoninic acid
NTA	Nanoparticle tracking analysis
FA	Fixed angle
SW	Swinging bucket
DLS	Dynamic light scattering
TRPS	Tunable resistive pulse sensing

J. M. Gudbergsson · M. N. Skov · M. Duroux (✉)
Laboratory of Cancer Biology, Institute of Health Science and Technology, Aalborg University, Fredrik Bajers Vej 3B, 9220 Aalborg Ø, Denmark
e-mail: megd@hst.aau.dk

K. B. Johnsen
Laboratory of Neurobiology, Institute of Health Science and Technology, Aalborg University, Fredrik Bajers Vej 3B, 9220 Aalborg Ø, Denmark

Introduction

During the last decade, research on extracellular vesicles (EVs) has grown exponentially. EVs are a heterogeneous population of vesicles secreted by all cells in a size range of 30–2000 nm. One of the main subpopulations of EVs is the endosome-derived exosome (Akers et al. 2013). Exosomes are formed by inward budding of the endosomal membrane, hereby generating multivesicular bodies (MVBs), which fuse with the plasma membrane to release the exosomes into the extracellular space (Kowal et al. 2014). They are also distinguished from other EVs by their size distribution, where exosomes range from 30 to 120 nm (Vlassov et al. 2012), whereas microvesicles are reported in the size range of 50–2000 nm (Akers et al. 2013), and are formed by outward budding of the plasma membrane (Kowal et al. 2014). Cells secrete EVs to function in paracrine and autocrine signaling (Sharma et al. 2013), and they can be found in all body fluids and cell culture supernatants (Colombo et al. 2014). Vesicles contain vast amounts of different cargo material, including functional RNA (Valadi et al. 2007), DNA (Balaj et al. 2011) and proteins (Skog et al. 2008). By harvesting body fluids or cell culture supernatants, EVs can be isolated with a wide range of different methods, such as ultracentrifugation (UC) (Van Deun et al. 2014), ultrafiltration (Heinemann et al. 2014), commercial reagents (Schageman et al. 2013), immuno-affinity capture (Tauro et al. 2012) and size-exclusion chromatography (SEC) (Böing et al. 2014).

The different types of EVs are defined by their biogenesis; however, many studies tend to refer to EVs as exosomes only defined by their size (Gould and Raposo 2013). The term "extracellular vesicle" is becoming more popular, because it does not discriminate between the different subsets of vesicles isolated (Witwer et al. 2013). The distinction between the different types of vesicles is not feasible with current isolation methods, although expression of exosome-enriched proteins like CD9, CD63, CD81 and TSG101 can be identified (Lötvall et al. 2014). These exosome-associated markers cannot be assigned solely to exosomes, but rather as being abundantly present in the isolated EV fraction compared to the cell and larger microvesicle fractions (Witwer et al. 2013).

EVs have been extensively studied as biomarkers (Properzi et al. 2013) and mediators of cellular signaling (Sharma et al. 2013), infections (Hosseini et al. 2013), and pathophysiology (Record et al. 2014). Recently, a significant amount of effort has also been injected into the study of EV therapeutics (Johnsen et al. 2014). With the growing interest of EVs in therapeutics and diagnostics, there is now emphasis placed on a clinically scalable production of EVs together with a need of sample purity for clinical use (Yeo et al. 2013; Johnsen et al. 2014). EV yields have long been a subject of debate, since isolation and quantification methods are not technically adequate to give a precise yield, although progress is being made by refinement of the respective techniques (Witwer et al. 2013). In addition to the quantity perspective, the purity of EV samples is a concern in experiments since this might affect the study outcome (Webber and Clayton 2013).

EV yields can be influenced by many factors; from how the cells are cultured to the choice and performance of isolation procedure and quantification methodology (Théry et al. 1999; Cvjetkovic et al. 2014; Franquesa et al. 2014). In this study, we focus on the smaller EVs, which includes exosomes and other membrane-derived vesicles that can be isolated with traditional "exosome" isolation protocols (Théry et al. 2006). We seek to give an overview of major factors that influence EV yields from cell culture supernatants, and elucidate the inter-study coherency in reporting EV yield. The major influences with respect to EV yields will be categorized and compared to the yields displayed in the studies analyzed.

Methodology

Literature search and reviewing was performed as displayed in Fig. 1. Briefly, PubMed searches were performed with the search terms "(*exosome? OR* "*extracellular vesicle?*" *OR microvesicle?*) *AND* (*supernatant OR* "*cell culture?*" *OR uptake*) *NOT review*" and "(*exosome? OR* "*extracellular vesicle?*" *OR microvesicle?*) *AND* (*yield OR isolation OR purification OR concentration OR uptake*) *NOT review*", from which 298 studies were included after pre-filtration based on title and abstracts. All 298 studies were assessed for the inclusion criteria and categorized based on EV isolation method(s). UC was further subcategorized into three different variants: regular ultracentrifugation, ultracentrifugation with

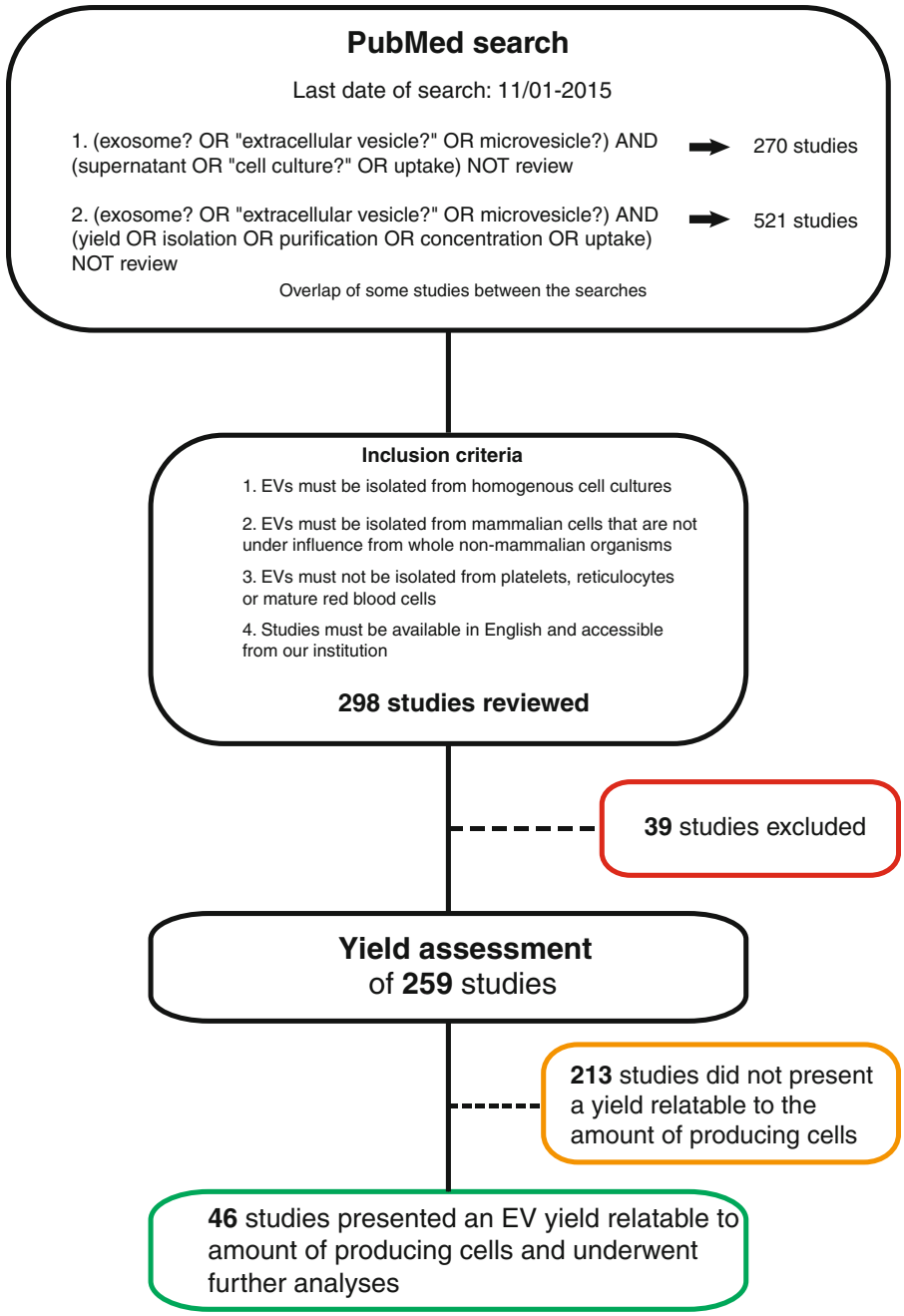

Fig. 1 The workflow for study analysis, including inclusion criteria and deliminations. A total of 259 studies met the initial inclusion criteria, whereas only 46 of these met the yield criteria

washing step (UC-W) and ultracentrifugation with gradient separation (UC-G). Studies reporting a usable yield were included in an evaluation scheme, which gathered information such as EV donor cell type, amount of cells or cell confluency, type of cell manipulation (if any), fetal calf serum (FCS) depletion parameters, time and amount of conditioned medium (CM) produced, EV isolation protocols, method of quantification, and EV concentration. Not all studies provided all information.

Thirty-nine studies were excluded after pre-filtration, because they did not fulfill the initial search

criteria, which only became evident after the studies were analyzed. A total of 259 studies met the initial search criteria, and 46 of these studies displayed yields that were relatable in some way to the amount of cells producing the EVs. The yields from all 46 studies were normalized to protein amount (expressed in μg) or number of particles per 10^6 cells. A total of 52 yield counts (derived from 35 studies) were displayed as protein amounts and 34 yield counts (derived from 16 studies) were displayed as number of particles. Five studies displayed both protein and particle amounts, which were included in both categories. In cases where the yield was specified in a range (e.g. 1–3 μg), the mean value was used for further analyses.

Only yields displayed in protein amounts were included in EV yield analyses. Particle yields were not included due to the fact that 25 of the 34 particle yield counts were obtained with UC and UC-W. Less than four yield counts remained in each of the remaining isolation categories, impeding statistical comparison. Only yields from ultracentrifugation and commercial reagents were used in the comparison of isolation methods, since the other isolation categories contained less than five yield counts per category. Two studies were excluded from yield analyses since their major yields could be regarded as outliers (Epple et al. 2012; Brownlee et al. 2014).

Statistics

All statistical analyses were performed using GraphPad Prism 6. Comparison of yield populations was carried out using unpaired *t* test with Welch's correction, due to the difference in standard deviation between the groups. Statistical analyses were displayed as box plots with min/max.

Results

Reporting of EV yield

Study distribution between methods of quantification

Analysis of all 259 studies revealed inconsistencies in the way EV yield is generally reported. Some studies quantified EVs based on detection of a specific protein or total amount of RNA present in the EV fraction, but most studies used a more general quantification method in the form of total amount of protein or particle count (Fig. 2). More than 50 % of all studies quantified EVs in total amount of protein and 18 % utilized NTA, whereas the other methods accounted for less than 6 % each. Notably, 21 % of the studies did not quantify EVs in any way.

Study distribution between isolation methods

Several isolation methods have emerged with the growing interest in EV research, including ultracentrifugation, gradient separation, ultrafiltration, immuno-affinity capture, size-exclusion and commercial reagents (which includes ExoQuick, Total Exosome Isolation Reagent, PureExo and ExoSpin) (Momen-Heravi et al. 2013). The most widely used methods are based on ultracentrifugation, often accompanied by additional steps such as gradient separation and/or different washing steps. This was also reflected in our study cohort, which showed that 90 % of all isolations were performed with ultracentrifugation protocols (Fig. 3). The respective variations in the ultracentrifugation protocols were equally distributed, with approximately 1/3 in each category. The second most used isolation method was commercial reagents, which encompassed 11 % of the total, while other methods accounted for less than 3 % each.

Technical factors influencing the yield of EVs

Many procedures used in the isolation and quantification of the EV fraction are extensive, costly, time consuming and lack standardization. Since a golden

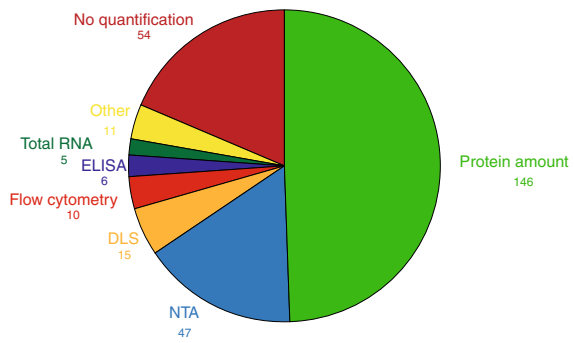

Fig. 2 Distribution of studies based on method of quantification. Studies reporting their quantification based on more than one method were counted in all respective categories. *Numbers* denote the amount of studies using the particular technique (total number of studies: 259)

Isolation method	% of total (259)
UC total (n = 234)	90 %
Commercial reagents (n = 29)	11 %
Immuno-affinity (n = 8)	3 %
Ultrafiltration (n = 5)	2 %
Liquid chromatography (n = 2)	< 1 %
Salt-out (n = 1)	< 1 %

UC variation	% of total (259)	% within UC (234)
UC (n = 73)	28 %	31 %
UC-W (n = 88)	34 %	38 %
UC-G (n = 80)	31 %	34 %

Fig. 3 Distribution of studies based on method of isolation. Studies isolating with more than one method were counted in all respective categories

standard methodology does not exist, the inter-study reproducibility of EV analysis (including yield) becomes limited; several different methods are applied with numerous different parameters, affecting the comparability between studies. In this section, EV isolation procedures and quantification methods will be discussed with regards to their influence on EV yield.

Isolation procedure

The choice of isolation method has an impact on purity and yield of the EV sample. Generally, the less extensive EV isolation procedures generate higher yields than the more labor-intense procedures, but the resulting sample purity might be compromised (Webber and Clayton 2013). Due to the low purity of samples isolated with UC, a washing step is often added (UC-W), which theoretically should reduce the presence of contaminants (Théry et al. 2006). To investigate this theory, the coherency between the inter-study yields was assessed as a function of EV isolation procedures. The analyzed studies were categorized based on the method of isolation and the average yields were compared (Fig. 4). UC-G was found to recover the least amount of protein compared to other ultracentrifugation protocols and commercial reagents. The difference in protein yield between UC and UC-W was found to be minimal, questioning the importance of a washing step. Supporting this finding, UC-G presented EV samples with the lowest yield, but greatest purity (Van Deun et al. 2014). EpCAM affinity capture was found to isolate more exosomes compared to UC and UC-G, defined by the higher expression of the exosome-enriched proteins Alix, TSG101 and HSP70 (Tauro et al. 2012). The purity of the EV samples impacts the yield, as co-purified protein aggregates in less pure samples was shown to artificially increase yields when quantifying EVs with

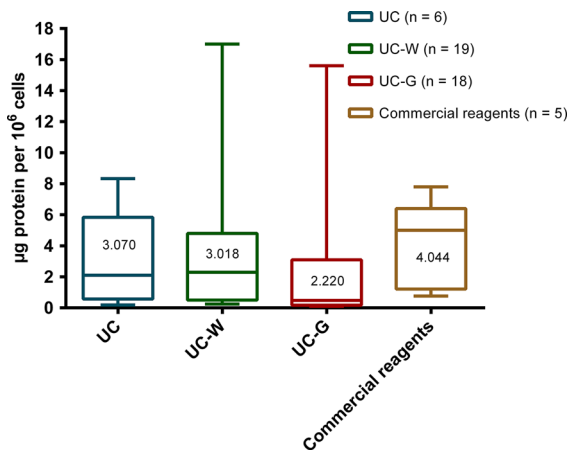

Fig. 4 Comparison of EV protein yields from different isolation procedures. Mean yield is plotted in the *boxes*, and *bars* show the maximum and minimum yield. Commercial reagents displayed the highest yields and UC-G the lowest. UC and UC-W displayed similar yields

the most frequently used methods, such as the bicinchoninic acid (BCA) assay or nanoparticle tracking analysis (NTA) (György et al. 2011). This suggests that the higher EV yield obtained with UC could be an artefact due to the presence of contaminants, and that the actual EV amount in a specific sample more likely corresponds to that obtained from UC-G. This observation was supported by another study, showing that UC-G gave a threefold increase in the particle/protein ratio compared to UC-W (Webber and Clayton 2013). It was found that the difference in yield and particle/protein ratio between UC and UC-W was minimal, indicating that the washing step was inefficient with respect to clearing protein contaminants (Webber and Clayton 2013).

Not only the choice of isolation procedure impacts the EV yield, but also the detailed parameters (see (Taylor and Shah 2015) for a detailed discussion on how the method of isolation impacts downstream

analysis of EVs). As an example, the rotor type used for ultracentrifugation and the time of ultracentrifugation play important roles in the efficiency of pelleting EVs. Fixed angle (FA) and swinging bucket (SW) rotors need different centrifugation times to achieve the same sedimentation efficiency, where SW rotors sediment twofold more protein, as indicated by a doubling of the protein/RNA ratio in the pellet (Cvjetkovic et al. 2014). In addition, ultracentrifugation times of 70 min resulted in insufficient recovery of EVs, whereas 4 h or more, resulted in excessive co-pelleting of proteins (Cvjetkovic et al. 2014).

The EV yield is highly dependent on the choice of isolation methodology, where UC and commercial reagents display the highest yields. Still, the sample purity must also be taken into account, which favors the utilization of UC-G. Since UC-G is highly time-consuming, other novel methods might be more time-effective such as SEC (Böing et al. 2014) or liquid chromatography-based systems (Chen et al. 2011; Nordin et al. 2015). Immuno-affinity capture isolation, which is antibody dependent, might also give high purity samples and be ideal to map the function of different subtypes of EVs (Tauro et al. 2012), but for generalized EV studies this method falls short due to the high specificity towards certain proteins.

Method of quantification

After EV isolation, the obtained fraction is generally characterized with respect to the size distribution and quantities. Particle quantification and determination of size distribution can be performed in several ways, including colorimetric protein assays (e.g. BCA or Bradford), dynamic-light scattering (DLS), NTA and tunable resistive pulse sensing (TRPS) (Fig. 2). In this section, the most widely used quantification methods (protein assays, DLS and NTA) will be discussed, along with the potential of the novel application of TRPS in EV research as this method is gaining it's foothold in the field.

Colorimetric protein assays Colorimetric protein assays are widely used to estimate EV yield, both alone and in supplement to particle analysis. The most used assays are the Bradford and BCA assays, as they are commercially available and rapidly give an estimate of protein quantity in a sample. The Bradford assay relies on the unspecific binding of Coomassie Brilliant Blue to proteins (Bradford 1976), whereas BCA is dependent on the reduction of Cu^{2+} to Cu^{1+} in the presence of proteins under alkaline conditions (Smith et al. 1985). However, both methods have biochemical drawbacks when estimating a protein concentration in a heterogeneous molecular mixture such as the EV fraction. For example, many compounds interfere with color formation in BCA, such as phospholipids and reducing sugars (Kessler and Fanestil 1986; Sapan et al. 1999). Also, the Bradford assay was shown to underestimate protein amount by 80 % (compared to quantitative amino acid analysis) in outer membrane vesicle samples (Rossi et al. 2014). In general, for colorimetric protein assays, the use of homogenous standards is a major problem when working with heterogeneous samples like EVs (Sapan et al. 1999). Since the color formation is dependent on the composition of proteins (Wiechelman et al. 1988; Fountoulakis et al. 1992) and the degree of interfering factors (Kessler and Fanestil 1986; Kirazov et al. 1993), this technique falls short when estimating EV quantities. It might be possible to compensate for the inaccurate estimation of protein amount by adding a color yield factor (a ratio between protein amount obtained by quantitative amino acid analysis and the respective colorimetric technique) or a protein-lipid ratio (Rossi et al. 2014; Osteikoetxea et al. 2015). Although estimation of protein amount alone might not be the ideal method to depict EV yields, most studies that were analyzed displayed yields in this manner. In general, the protein yields from the studies were surprisingly coherent; the average yields from the analyzed studies displayed similar outcomes seen in multiple EV isolation comparison studies with regards to the yield distribution as a function of isolation procedure (Fig. 4) (Tauro et al. 2012; Van Deun et al. 2014; Zhu et al. 2014).

Dynamic light scattering DLS is based on intensity fluctuations of light scattered from particles undergoing Brownian motion in a solution. The intensity of the scattered light is recorded, and converted to a hydrodynamic diameter by applying the Stokes–Einstein equation (Karow et al. 2014). This is sufficient to estimate the particle size distribution of a monodispersed sample, whereas for polydispersed samples, additional mathematical functions need to be applied (Frisken 2001; Karow et al. 2014; Hassan et al.

2015). Due to the fact that polydispersity is accounted for mathematically and not practically, limitations arise since sample characteristics, including the degree of polydispersity, may interfere with correct measurement (Karow et al. 2014; Hassan et al. 2015). For example, since the light scattering intensity from large particles is higher than that of small particles, the presence of small amounts of large particles in a sample might obscure the signal from smaller particles, resulting in an artificial increase in measured size distribution (Filipe et al. 2010; Karow et al. 2014). DLS can separate binary populations if the mean diameter peaks are not too close and the concentration ratio between these is appropriate, but for highly polydispersed samples, like EVs, the method falls short (Filipe et al. 2010; Sokolova et al. 2011). The estimation of particle concentration might also be faulty, because the size distribution profile measured with DLS favors the larger particles present in a sample. Thus, size and yield estimation of EV samples acquired with DLS must be carefully considered.

Nanoparticle tracking analysis The most widely used method for EV quantification and single particle analysis is NTA. Similar to DLS, NTA relies on the light scattering properties of particles in a solution by illuminating these with laser light. Instead of recording intensity fluctuations of scattered light, a video is being captured of the particle's Brownian motion. Subsequently, the video data are analyzed by tracking the Brownian motion of individual particles to estimate the hydrodynamic diameter using the Stokes–Einstein equation (Gardiner et al. 2013). NTA was shown to be the most useful single particle analysis tool, since it covers most of the EV size spectrum and gives both size distribution and concentration of the analyzed particles. Parameters that enables calculation of the refractive index of the particles are also measured (Gardiner et al. 2014). In contrast to DLS, NTA is able to distinguish differently sized populations of nanoparticles, which makes this technique applicable for polydispersed samples like EVs (Filipe et al. 2010; Sokolova et al. 2011). However, similarly to DLS, the presence of larger particles might hinder the detection of smaller particles due to saturation of the camera (van der Pol et al. 2014a). This results in an underestimation of smaller particles and an overestimation of larger particles, setting the lower size limit of detection considerably higher than the smallest EVs (van der Pol et al. 2014a). Also, protein aggregates can be tracked and size-estimated in the same way as EVs, which is a bias in the resulting yield measured with the NTA (Filipe et al. 2010). By only including tracked particles within the refractive index range of EVs [RI: 1.37–1.39 (Gardiner et al. 2014; van der Pol et al. 2014b)], the protein aggregates [RI: 1.59–1.64 (McMeekin et al. 1964)], lipoproteins [RI: 1.45–1.60 (Mills et al. 2000)] and other potential contaminants could be excluded from the data analysis and give a more correct EV yield. In addition, several technical aspects need to be considered when using NTA to estimate an EV concentration, including the type of NTA instrument, setup of the instrument, video analysis parameters, standardization procedure and sample preparation (please refer Gardiner et al. 2013 for a detailed discussion on the use of NTA).

Tunable resistive pulse sensing Another single particle analysis technique is TRPS, which relies on the Coulter principle to estimate particle sizes (Maas et al. 2014). In TRPS, conducting medium containing nanoparticles passes through a nano-sized pore in a non-conductive membrane. The pore can be stretched (tuned) to fit a polydispersed sample of a given size range. By applying a voltage across the pore, an electrical current is established which is constantly measured. When nanoparticles pass through the pore, an increase in resistance occurs (called a resistive pulse), and the magnitude of this increase (blockade height) can be correlated to the diameter of the passing nanoparticle (Coumans et al. 2014). Currently, the qNano (Izon Science) is the most widely used TRPS system in the EV field and commercial tunable nanopores suited to measure polydispersed EV samples are available. The smallest tunable nanopores (NP100) can measure particles in a size range of 50–70 to 200 nm (Maas et al. 2014, 2015). However, this does not cover the full size spectrum of EVs, which stretches down to 30 nm (Vlassov et al. 2012). It has been suggested that the variability in the lower detection limit could be due to pore-to-pore variability (Coumans et al. 2014). If a sample contains particles with larger diameters than the pore, clogging can occur (Maas et al. 2015). The qNano sample flow is controlled by a variable pressure module, allowing for a more sensitive concentration estimation

(Willmott et al. 2010). Since the pressure controls the rate of pulse measurements, the concentration estimate is proportionally affected, and therefore a calibration with particles of known concentrations is needed to determine the concentration of an unknown sample (Willmott et al. 2010). Several technical aspects need consideration when using TRPS in EV analysis, which include pore geometry, steric factors and Brownian motion of the EVs, particle trajectory and ionic distribution of the fluid (Weatherall and Willmott 2015).

In general, single particle analyzers are much more effective than colorimetric protein assays or bulk particle analysis (DLS) in determining true EV quantities. Although TRPS has been shown to be suitable to estimate polydispersed particle samples similar to other methods (van der Pol et al. 2014a; Maas et al. 2015), the wide size span of EVs and current pore sizes hinder detection of the smallest EVs and result in frequent clogging. Currently, NTA must be considered the most adequate technique to estimate EV size and concentration since most of the EV size spectrum is covered, although standardization of sample measurements needs to be done.

Biological factors influencing yield

As emphasized above, technical aspects of EV isolation and quantification play important roles in the outcome of yield. Although the current techniques are still somewhat in their infancy with regards to EV isolation and analysis, knowledge of the biological factors that encompass the machinery of EV formation and secretion set further limits to elucidate the rate and amount of EV secretion (yield). In this section, some of the major biological factors that might influence EV yield will be presented and assessed.

Cell type

Determination of the most efficient EV-producing cell type has yet to be established. Few studies investigating the therapeutic potential of EVs have commented on this issue (Chen et al. 2011; Yeo et al. 2013). Immature dendritic cells were shown to produce limited amounts of EVs (Alvarez-Erviti et al. 2011; Tian et al. 2014), whereas mesenchymal stem cells were shown to secrete vast amounts, relevant for the production of EV therapeutics on a clincial scale (Chen et al. 2011; Yeo et al. 2013). Due to the fact that inhibition of Rab27a and Rab27b partially stalls EV secretion (Zheng et al. 2013), cells overexpressing these proteins might be effective EV producers. Interestingly, various cancer cells were shown to overexpress Rab27a and Rab27b, including breast cancer cells (Wang et al. 2008), hepatocellular carcinoma cells (Dong et al. 2012), glioma cells (Wu et al. 2013) and pancreas cancer cells (Wang et al. 2014). The presence of invadopodia, which are docking sites for Rab27a-positive MVBs, also enhanced secretion of EVs in cancer cells (Hoshino et al. 2013). This evidence suggests that cancer cells potentially release more EVs compared to non-cancer cells. To investigate whether this was reflected in the studies analyzed here, a comparison between yields from cancer cells and non-cancer cells was done (Fig. 5). Comparison of 33 EV yields from cancer cells (from 19 studies) and 21 EV yields from non-cancer cells (from 17 studies) showed no significant difference in EV yields ($p = 0.62$). Many factors could have contributed to this outcome, such as the small study cohort and the inter-study variability in isolation and quantification procedures. However, this might also indicate a more complex relationship between cell type and EV yield, and that a categorization of cancer cells versus non-cancer cells is simply too general.

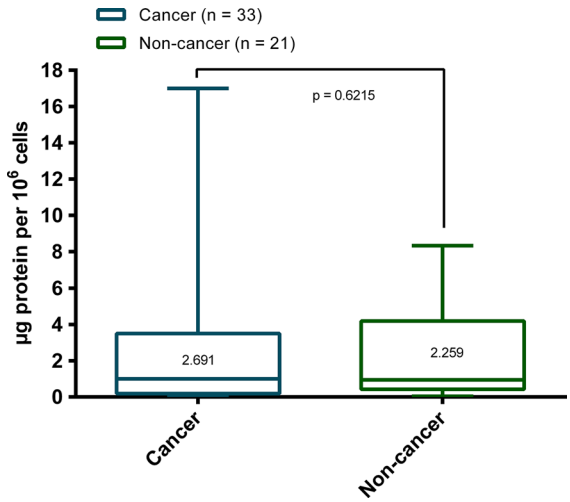

Fig. 5 Comparison of EV protein yields from cancer and non-cancer cells. Mean yield is plotted in the *boxes*, and *bars* show the maximum and minimum yield. No significant difference in average protein yields between the two categories was seen ($p = 0.62$)

Cell confluence

In our study cohort, EVs were produced and harvested from cell cultures with varying confluences (typically 60–90 %), which may have an effect on EV yield and the functional characteristics of the EVs. Confluent cell cultures have been shown to display a tenfold decrease in cholesterol metabolism compared to cells in the pre-confluent state (Gal et al. 1981). In relation to this, EVs from prostate cancer cells were highly enriched in cholesterol (Llorente et al. 2013), and therefore the EV production might be decreased in confluent cell cultures. Contact inhibition, which triggers confluent cells to enter quiescence, may decrease EV secretion and/or alter their characteristics compared to actively dividing cells (Steinman et al. 2003; Hayes et al. 2005). Confluence estimates vary between individual researchers, which prevents a feasible comparison of EV yields as a function of cell confluency. To be able to compare yields in this manner, it would be ideal to specify the amount of cells from which the EVs were isolated and the surface area and format of the culture dish. Therefore, cells should be quantified immediately after harvesting the CM. However, this might result in unnecessary cell-stress if the cells are not to be passaged at the time of CM harvest. A general standardization of this by estimating cell numbers at different cell confluences (e.g. 50–100 %, in 10 % increments) for any given cell line, could be an effective way to rapidly display the amount of cells producing the isolated EVs. Since the production time of CM varies, this could also aid in correcting for the difference between cell confluency at the start and at the time of harvesting CM. Due to the inconsistencies in reporting confluency of the producing cell culture (and the resulting EV yield), it has not been possible to investigate the effect of this factor on the production of EVs.

Cell stimulation

Exogenous stimulation of cells is known to alter their phenotype, including their secretome. Various stimulations have been assessed for their effects on EV production and composition; For example, Ca^{2+} ionophores (Savina et al. 2003), hypoxia (Svensson et al. 2011; King et al. 2012; Kucharzewska et al. 2013) and detachment of cells (Koumangoye et al. 2011) increased the EV release, whereas lipopolysaccharide stimulation reduced it (Théry et al. 1999).

Most in vitro culturing of cell monolayers requires the presence of serum in the culture medium to allow for the cells to adhere and grow, in which the most commonly used is FCS (Bryan et al. 2011). The FCS has obvious inherent effects on the cultured cells, which varies between cell types and their differentiation status (Lund et al. 2009; Bryan et al. 2011). Since FCS contains vast amounts of EVs, most studies perform an EV depletion protocol of FCS before using it for production of CM, or change to serum-free medium (Shelke et al. 2014). The health of cultured cells is reflected in their secretome, since cell stress was shown to induce phenotypic alterations that were reflected in the protein and RNA content of EVs (de Jong et al. 2012). In relation to this, 13 out of 46 of the studies displaying yields, CM was produced under serum-free conditions, when cells would normally be cultured with serum. This abrupt change in culture conditions induces alterations in the cell's metabolism and in some cases could force the cells to stop proliferating due to starvation (Pirkmajer and Chibalin 2011). As cellular stress can be reflected in the EV fraction, any functional characterization of EVs under such conditions must be affected and perhaps fallacious, unless the goal with the experiment is to characterize this particular aspect. 6 of the 13 studies that abruptly changed to serum-free conditions performed some kind of subsequent functional experiments with the EV fraction (Xie et al. 2010; Ji et al. 2013; Tauro et al. 2013; Inder et al. 2014; Klein-Scory et al. 2014; Lee et al. 2014). Although EV depletion of FCS might be the least of the evils, a change to EV-depleted medium during isolation might also alter the phenotypic profile of the cells, as FCS-derived EVs have been found to exert a positive effect on cell migration (Shelke et al. 2014). EV-depleted medium has also been shown to reduce cell proliferation in a number of cell lines, which could be restored by the addition of FCS-derived EVs to the EV-depleted culture medium (Eitan et al. 2015). A comparison of the protein yields from studies that used EV-depleted serum and studies that abruptly changed to serum-free conditions showed that studies using EV-depleted serum reported a significantly higher average yield ($p = 0.02$) (Fig. 6). However, the EV depletion efficiency is dependent on the time of depletion (with UC), which has been shown to be ~95 % for a depletion time of 18 h and only ~50 % for 1.5 h (Shelke et al. 2014). Some studies did not display

parameters for EV depletion protocols (4 yield counts), while others had depletion times below 2 h (4 yield counts). By excluding these yield counts, the statistical significance was lost ($p = 0.12$), but the results still indicate that some of the increase in EV yield can be explained by insufficient EV depletion of FCS or that starved cells secrete less EVs. In contradiction to this, a recently published study by Li et al. (2015) reported an increase in EV yield from N2a and SH-SY5Y cells that received serum-free medium during CM production compared to cells that received EV-depleted medium. Although this has not been thoroughly studied experimentally, it can safely be concluded that an abrupt change to serum-free medium does alter the cells and their secretomes (Fig. 6).

Taken together, stimulation of cells with exogenous compounds or alteration of their culture conditions does affect the resulting EV yield. The available literature and analyses of the studies suggest that an abrupt change to serum-free conditions might affect the EV yield and content. Therefore, care should be taken when analyzing and using EVs isolated under such conditions in functional studies. When using EV-depleted medium, the inherent effects of the FCS-derived vesicles needs to be considered when analyzing the effects of EVs.

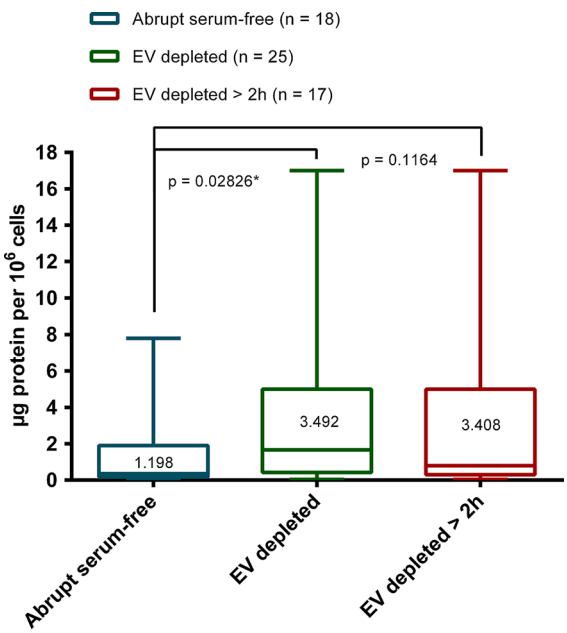

Fig. 6 Effects of serum conditions on EV protein yield. Mean yield is plotted in the *boxes*, and *bars* show the maximum and minimum yield. A significant difference was seen between EV yields obtained under abrupt serum-free conditions and those obtained with EV depleted FCS ($p = 0.03$). However, after excluding studies that did not display EV depletion parameters and studies that depleted for less than 2 h, the significance was lost ($p = 0.12$)

Conclusions

Considering the discussion of available literature and the subsequent analyses, it is evident that isolation and quantification of EVs are not simple and straightforward procedures. Numerous factors influence EV yield, ranging from cell culture conditions to the choice and performance of isolation and quantification methods. We identified and discussed a few major influencing factors, and analyzed 46 studies that presented yields with respect to these factors. The analyses revealed that 90 % of all analyzed studies utilized a UC-based protocol for EV isolation, and that UC-G isolated the least amounts of EVs, but presented with the purest samples. Technical parameters for the isolation of EVs also have an impact on the yields; for example, the time of ultracentrifugation influences the yield and purity of the isolated EV sample. In addition, some parameters for the quantification procedure may be a cause of uncertainty when estimating EV yields, including user-generated biases (handling) and limitations of the respective techniques. Although technical factors most likely encompass the majority of the limitations in EV yield estimation, biological factors also contribute to the variation and lack of reproducibility of EV yields. Factors such as cell type, cell confluency or density, stimulation of cells with exogenous compounds, and abrupt change in culture conditions during production of CM, might all potentially affect the functional characteristics of the EVs and the yield outcome. Even though cancer cells are candidates for being efficient EV producers, an examination of EV yields from the analyzed studies showed that cancer cells might not produce more EVs than non-cancer cells. In contrast, an abrupt change to serum-free medium presented a significant decrease in EV secretion, which could indicate that the stress experienced by the cells under such circumstances reduces the EV output. The presence of FCS EVs residing in the medium would also interfere with the measured EV quantities. In future research, more

attention should be drawn towards the analysis of EV yield and purity, which includes generating more effective isolation procedures. Fortunately, research into this issue is increasing with novel and promising EV isolation approaches being established, such as SEC and liquid chromatography. Measuring the quantities of EVs needs more specificity to enable exclusion of co-precipitated protein, such as differentiation of particles by their refractive index. Researchers working with EVs derived from cell cultures need to establish some form of stoichiometric relationship between the cells and the EV yield and quality for future comparison between studies.

Compliance with ethical standards

Conflict of interest The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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