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Research Article

Evaluation of Small Extracellular Vesicles Isolation Methods From Human Serum for Downstream miRNA Profiling

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ABSTRACT

Exosomes are a type of extracellular vesicles that carry distinct profiles of biomolecules such as lipids, proteins, DNAs, and RNAs. Despite many years of research, there is still a lack of standardized methods to isolate exosomes from clinical samples for their downstream applications. Thus, this study compared three different methods, which are differential ultracentrifugation (DUC), polyethylene glycol-based precipitation (PEG), and a combination of both (PEG+UC) to isolate exosomes from human serum. The isolated exosomes were evaluated by their size distribution, recovered particle concentration, particle-to-protein ratio, exosomal marker expression, and miRNA recovery. Our results indicated that all three methods successfully isolated exosomes, however, with varying yield and purity. In particular, PEG+UC produced exosomes of both high yield and high purity, DUC produced exosomes of both low yield and low purity, whereas PEG produced exosomes of high yield but low purity. Using miR-30d-5p and let-7i-5p as selected targets, our qPCR results indicated significant differences in terms of exosomal miRNA recovery between all three methods. Overall, the PEG+UC method appeared to be a less labor-intensive alternative that can isolate exosomes of both high yield and high purity from human serum without compromising the yield of miRNAs.

Key words: Commercial kits, exosome, isolation, polyethylene glycol, purification, ultracentrifugation

INTRODUCTION

The cellular release of extracellular vesicles (EVs) has been the subject of numerous investigations since the 1980s, but only recently has the importance of these entities been appreciated as avenues for cell communication (Couch *et al*., 2021). EVs are a primitive, evolutionarily conserved mode of communication, functioning even as a means of communication from a tick to its host (Zhou *et al*., 2018). They are divided into three subpopulations: exosomes (30 nm to 150 nm), microvesicles (100 nm to 1 μm), and apoptotic bodies (>1 μm), which differ in size, composition, and biogenesis pathway (Théry *et al*., 2018). Exosomes, also referred to as small EVs, are lipid bilayer-enclosed structures released through the inward shedding of the plasma membrane via exocytosis. Microvesicles, also referred to as large EVs, are released through the outward shedding of the plasma membrane. Apoptotic bodies are released as byproducts of cell apoptosis (Doyle & Wang, 2019). Since the early 2000s, there has been a growing interest in exosomes to understand their roles as disease biomarkers (Fiandaca *et al*., 2015; Bellin *et al*., 2019; Zhang *et al*., 2019; Makler & Asghar, 2020; Martellucci *et al*., 2020; Tan *et al*., 2021). They carry various bioactive molecules, including lipids, proteins, DNA, RNA, and microRNA (miRNA), making them key players in intercellular communication (Valadi *et al*., 2007; Frydrychowicz *et al*., 2015; Zhang *et al*., 2019). Furthermore, the lipid bilayer structures of exosomes also offer a stable environment for these bioactive molecules, making them more suitable candidates for disease biomarkers when compared to cell-free proteins and RNAs (Cheng *et al*., 2014; Boukouris & Mathivanan, 2015).

In the past years, various methods based on the vesicles' density, size, or surface protein expressions have been developed for exosome isolation. Despite these advancements, many challenges such as purity, reproducibility, and scalability persist, which hinder the use of exosomes in clinical applications. Currently, differential ultracentrifugation (DUC) remains the gold standard for isolating exosomes (Li *et al*., 2019). This method utilizes multiple centrifugation steps to first remove cell debris,

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then the larger EVs such as microvesicles. A final step, involving high-speed ultracentrifugation, is used for pelleting exosomes (Livshts *et al*., 2015; Martins *et al*., 2023). Some of the advantages of DUC are that it does not require additional reagents other than the centrifuge tubes and ultracentrifuge, which makes it a more cost-effective method. Furthermore, it can prevent samples from being contaminated by other reagents as no other reagent was added during the isolation process (Yang *et al*., 2020). However, DUC is time-consuming, arduous to implement, and results in poor yield, which is not ideal in clinical settings (Li *et al*., 2019). Other factors such as the types of rotors used, centrifugation speed and duration as well as sample viscosity can also affect the yield of exosome pellet (Livshts *et al*., 2015).

For these concerns, newer approaches such as size exclusion chromatography, immunocapturing-based methods, and the use of chemical precipitating reagents have been developed to mitigate the disadvantages of DUC. Nonetheless, these methods still come with their advantages and drawbacks. For instance, the widely adopted PEG-precipitation offers a rapid, cost-effective, and highly scalable approach that is more suitable for clinical settings (Alvarez *et al*., 2012; Helwa *et al*., 2017; Patel *et al*., 2019). However, this method is also known to produce exosomes with low purity due to the co-isolation of circulating lipids and proteins (Alvarez *et al*., 2012; Patel *et al*., 2019). Moreover, the residual precipitation agents found in the exosome pellet could potentially interfere with downstream analysis that requires high purity of exosomes such as mass spectrometry, limiting further exosome analysis with multiomics-based approaches (Gámez-Valero *et al*., 2016).

Therefore, the two-step exosome isolation methods have been widely experimented with to enhance the separation of exosomes from other EV subpopulations. When compared to using one-step exosome isolation, the two-step isolation methods were able to isolate exosomes of higher purity (Rider *et al*., 2016; Guerreiro *et al*., 2018). For instance, the combination of PEG-precipitation method with an ultracentrifugation step (PEG+UC) was reported to remove a majority of the impurities co-precipitated with exosomes in tissue culture supernatants (Rider *et al*., 2016; Ludwig *et al*., 2018). As different biofluids require different approaches, the efficiency of using PEG+UC to isolate exosomes from serum samples is yet to be confirmed. Considering the rapid development of exosome isolation methods, the absence of a standardized exosome isolation protocol from human serum samples greatly hinders their use as disease biomarkers. Therefore, it is imperative to standardize a method to maximize the yield and purity of exosomes (Gardiner *et al*., 2016). Using serum from healthy subjects, this study aimed to investigate the efficiency of PEG+UC, in comparison with DUC or PEG method alone in isolating exosomes from clinical samples. The ultrastructure, size distribution, particle concentration, total protein content, and marker expressions of isolated EVs were evaluated. Furthermore, the expression of selected miRNAs in exosomes was determined using qPCR. Our findings present a comprehensive comparison of DUC, PEG, and a combination of both methods (PEG+UC) for isolating exosomes, particularly for downstream biomarker discovery studies that involve miRNA profiling. This study provides a guideline for researchers in selecting the best method for exosome isolation, which paves the way for the future establishment and standardization of exosome isolation workflows for clinical applications.

MATERIALS AND METHODS

Sample collection and processing

This study was approved by the University of Nottingham Malaysia Science and Engineering Research Ethics Committee (ID: YFP100120). Written consent from all subjects had been collected before blood collection. The peripheral blood of six healthy volunteers was first collected in EDTA tubes. The sample was centrifuged at 1,500 × *g* for 10 min at 4°C, then the plasma layer was aliquoted and stored immediately at -80°C until further use.

Exosome isolation

To ensure homogeneity across comparisons, plasma from each volunteer was subjected to all three different isolation methods. Figure 1 provides a summary of the main steps involved in each isolation method used in this study.

Isolation method 1: Differential ultracentrifugation (DUC)

Human plasma was treated with thrombin and incubated for 5 min at 25°C. Treated serum was diluted with 1x phosphate-buffered saline (PBS). All centrifugal steps were performed at 4°C and supernatants were collected. Firstly, the samples were centrifuged at 300 × *g* for 5 min, followed by 2,000 × *g* for 10 min, 5,000 × *g* for 20 min, then 10,000 × *g* for 30 min. Next, the supernatants were ultracentrifuged at 110,000 × *g* for 70 min using the Beckman Coulter SW40Ti rotor. The resulting pellets were resuspended in PBS and centrifuged again at 110,000 × *g* for 70 min. Lastly, the exosome pellets were resuspended in appropriate buffers for downstream applications.

Isolation method 2: Polyethylene glycol-based (PEG) precipitation

The miRCURY Serum/Plasma exosome isolation kit (Qiagen, Germany) was used for exosome precipitation according to the manufacturer's instructions. Firstly, thrombin was added to plasma and incubated for 5 min at 25°C. The samples were centrifuged at 10,000 × *g* for 5 min and supernatant were collected. Precipitation buffer was added to the treated serum and left overnight at 4°C. The samples were centrifuged at 500 × *g* for 5 min at 20°C to collect exosomes pellet. Lastly, the pelleted exosomes were resuspended in appropriate buffers for downstream applications.

Isolation method 3: PEG precipitation followed by ultracentrifugation (PEG+UC)

The PEG-isolated exosomes pellet was resuspended in PBS before the ultracentrifugation step at 110,000 × *g* for 70 min at 4°C. The pellet was collected and resuspended in appropriate buffers.

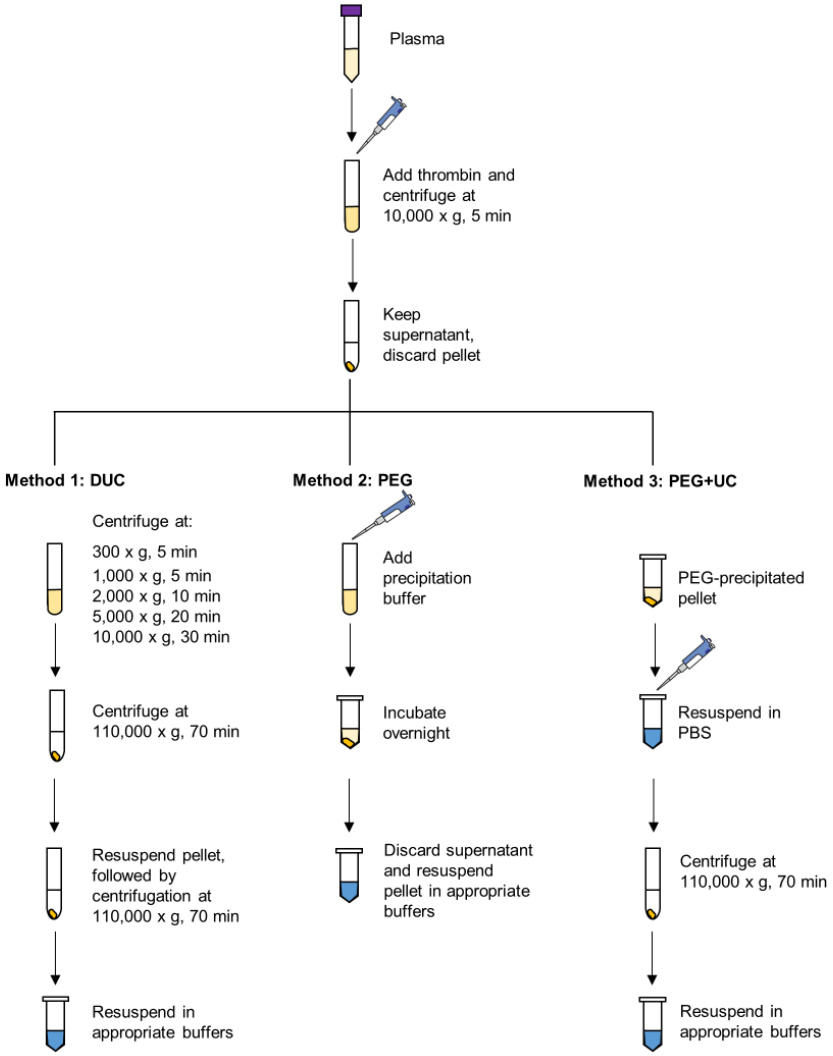

Fig. 1. Schematic representation of exosome isolation methods. Isolation method 1: Differential ultracentrifugation (DUC) only. Isolation method 2: Precipitation using polyethylene glycol-based commercial exosome isolation kit (PEG) only. Isolation method 3: Combination of PEG and ultracentrifugation (PEG+UC).

Exosome characterisation

Characterization method 1: Transmission electron microscopy (TEM)

The exosome pellet was resuspended in 1.5 mL of fixative containing 2.5% glutaraldehyde. 50 µL of the sample was dropped onto a cooper mesh grid of the electron microscope. The sample was set to dry at room temperature for 5 min before being negatively stained with 1% ammonium molybdate for 10 sec. The excess staining solution was wiped away using filter paper. Finally, the sample was viewed using a Tecnai G2 Spirit transmission electron microscope (FEI Co., Hillsboro, OR) operating at 100 kV.

Characterization method 2: *Nanoparticle tracking analysis (NTA)*

The particle number and mean size of isolated EVs were measured on the NanoSight NS300 and analyzed using the NTA 3.4 software (Malvern Panalytical, UK). Briefly, isolated EVs were diluted using deionized water. Each sample was measured at 5 x 60 sec, a constant temperature of 27°C, and a viscosity of 0.849-0.850 cP. The detection threshold of the camera was set to 20, slider shutter to 1300, slider gain to 295, FPS to 25.0, and number of frames to 1498.

Characterization method 3: Protein assay

Exosomes were lysed on ice in radio-immunoprecipitation assay (RIPA) buffer (Abcam, UK). A total of 25 µL diluted exosome lysate was loaded into each well in a 96-well plate, then added in 200 µL of working solution from Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). The plate was incubated at 37°C for 30 min and measured at 562 nm absorbance.

Characterization method 4: Western blot

An equal volume (7.5 µL) of extracted proteins was separated by a 12% SDS-PAGE gel at 80 V and transferred onto PVDF membranes. The membranes were blocked with 3% milk or 5% bovine serum albumin (BSA) for 45 min at room temperature. Next, the membranes were incubated against CD9, Calnexin, Albumin (Cell Signalling Technology, Danver, MA), and TSG101 (Thermo Fisher Scientific, Waltham, MA). The membranes were washed in 0.1% TBST and incubated for 1 hr in secondary antibodies (Cell Signalling Technology, Danver, MA). Signals were detected using Clarity™ Western ECL kit (Bio-Rad, Hercules, CA). Finally, the images were captured and analyzed using the Image Lab software (Bio-Rad, Hercules, CA).

Quantitative PCR

Total RNA from exosomes was extracted using the miRNeasy Micro kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration and purity of isolated RNAs were measured using Nanodrop One (Thermo Scientific, Waltham, MA). Immediately after total RNA extraction, exosomal RNA was reverse transcribed into cDNA using the miRCURY RT kit (Qiagen, Germany). The cDNA templates were diluted at 1:10 for the qPCR run using the miRCURY LNA SYBR Green PCR Kit (Qiagen, Germany). The PCR cycling protocol was set according to the manufacturer's protocols.

Statistical analysis

GraphPad Prism software (version 10.1.0; GraphPad Software, San Diego, CA) was used to perform all statistical analyses. The significant differences between all isolation methods were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Data were presented as mean±SD. Significant differences were considered with $p<0.05$.

RESULTS

Ultrastructure of exosomes

The microscopic images presented a heterogeneous population of both round and spherical particles ranging from 40 to 160 nm, consistent with the reported morphology and size of exosomes. Overall, no major changes in terms of vesicle ultrastructure were observed across different isolation methods. However, some contaminating aggregates were found in EVs isolated using DUC and PEG (shown by black arrows). PEG+UC exhibited individual vesicles with the least background interference and contaminating particles (Figure 2a).

Size distribution analysis

The NTA results demonstrated that mean particle size differed significantly across isolation methods ($p<0.05$). PEG isolated EVs of the largest mean size (mean = 157.8 ± 3.9 nm), followed by DUC (mean = 113.0 ± 12.1 nm) and PEG+UC (mean = 84.8 ± 8.1 nm). PEG+UC had a significantly lower mean particle size as compared to PEG ($p<0.05$) (Figure 2b). Further analysis showed that only 54.7% of PEG-isolated particles fell within 30-150 nm when compared to 86.4% in DUC, and 96.7% in PEG+UC-isolated samples (Figure 2c).

Particle concentration and total protein analysis

The particle concentration and total protein amount in the isolated EVs were determined by NTA and BCA assay. The NTA results reported that particle concentration differed significantly across isolated methods ($p<0.05$). PEG yielded the highest number of particles (concentration range = $1.68 \times 10^{12} \pm 1.79 \times 10^{11}$ particles/mL), followed by PEG+UC (concentration range = $8.75 \times 10^{11} \pm 1.12 \times 10^{11}$ particles/mL) and DUC (concentration range = $6.94 \times 10^{10} \pm 6.92 \times 10^{9}$ particles/mL) (Figure 2d). Similarly, PEG samples contained the highest protein amount (total protein = 30.4 ± 12.2 mg; $p<0.01$). The subsequent UC step in PEG+UC significantly reduced the total protein by 9-fold (total protein = 3.7 ± 2.2 mg; $p<0.01$). Meanwhile, the DUC recovered the least amount of protein (total protein= 0.9 ± 0.1 mg; $p<0.01$) (Figure 2e). Further analysis of particle to protein ratio revealed that PEG+UC had the highest EVs purity ($5.88 \times 10^{7} \pm 6.65 \times 10^{6}$), followed by DUC ($1.88 \times 10^{7} \pm 2.73 \times 10^{5}$) and PEG ($1.38 \times 10^{7} \pm 1.42 \times 10^{6}$) (Figure 2f).

Exosome markers expression

To assess the purity of isolated EVs, a western blot was performed to identify selected exosomal protein markers. The results demonstrated successful isolation of exosomes using all three methods as indicated by the presence of exosomes positive markers, TSG101 and CD9 (Figure 2g). PEG demonstrated the strongest expression of CD9 and TSG101. However, at the same time, it also co-isolated the highest abundance of microvesicles as shown by the strong intensity of Calnexin. DUC demonstrated minimal contamination of microvesicles but also presented faint expression of CD9 and TSG101. PEG+UC, on the other hand, demonstrated strong CD9 and TSG101 expressions but were absent in Calnexin. Albumin was present in abundance in the EVs isolated using PEG, followed by PEG+UC and DUC. The overall results suggested that PEG+UC was efficient in recovering a higher yield of exosomes with minimal contamination of microvesicles.

Exosomal miRNA expression

The yield and purity of total RNAs isolated from exosomes are presented in Table 1. Overall, no significant differences were observed between the purity and concentration of isolated RNAs from exosomes across different methods.

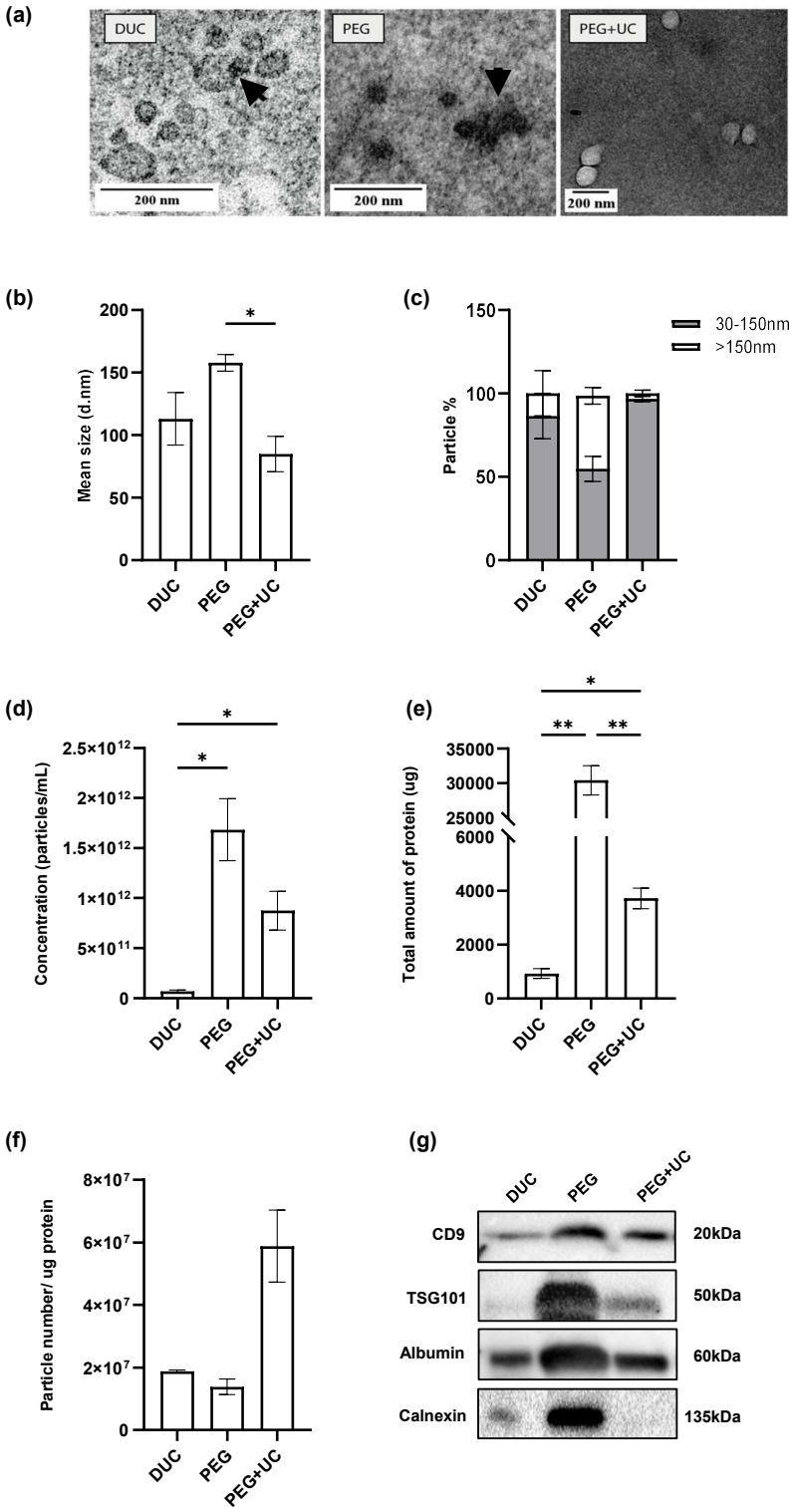

Fig. 2. Exosome characterization. (a) TEM images of exosomes isolated using the DUC (left), PEG (middle), and PEG+UC method (right). Vesicle aggregations were shown by the black arrows in DUC and PEG. (b) The mean particle size of isolated particles measured by NTA. PEG isolated the particles of the largest mean size, followed by PEG+UC and DUC. (c) Particle distribution (represents by particle %) of isolated vesicles. PEG+UC had the highest number of particles within the exosome size range (30-150nm), followed by DUC and PEG. (d) Total particle concentration measured by NTA. PEG isolated the highest number of particles, followed by PEG+UC and DUC. (e) The total amount of protein in EV samples was measured by protein assay. PEG samples contained the highest amount of protein, followed by PEG+UC and DUC. (f) EVs purity analysis is determined by the particles-to-protein ratio. PEG+UC resulted in the highest purity of exosome samples, followed by DUC and PEG. (g) Western blot analysis. CD9 and TSG101 were used as positive markers to indicate the presence of exosomes, and calnexin and albumin were used to indicate the presence of microvesicles and albumin, respectively. DUC, differential ultracentrifugation; PEG, polyethylene-glycol precipitation; PEG+UC, PEG precipitation followed by UC; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis. * p<0.05, ** p< 0.01. All data shown are presented as mean±SD (n=3-5).

Table 1. Purity and concentration of isolated RNAs from exosomes measured with Nanodrop

	A260/280	A260/230	Concentration (ng/μL)
DUC	1.384±0.05	0.43±0.32	49.8±12.6
PEG	1.367±0.06	0.26±0.17	47.01±30.7
PEG+UC	1.396±0.07	0.58±0.49	54.35±31.1

To investigate the feasibility of using isolated EVs for miRNA research, the levels of miR-30d-5p and let-7i-5p were measured using qPCR. The results demonstrated that all three methods have successfully recovered miRNAs from exosomes (Figure 3). Further analysis showed that PEG had the highest expression for miR-30d-5p (Cq value = 24.23±1.35) and let-7i-5p (Cq value = 25.70±1.11). The ultracentrifugation step in PEG+UC significantly reduced both miR-30d-5p (Cq value = 27.39±1.76; $p<0.05$) and let-7i-5p expression (Cq value = 27.50±1.37; $p<0.05$). Conversely, DUC recovered significantly lesser amount of both miR-30d-5p (Cq value = 29.79±1.67; $p<0.05$) and let-7i-5p (Cq value = 29.09±1.05; $p<0.01$) as compared to PEG+UC. By previous results, it can be concluded that while PEG recovered a higher abundance of miRNAs, it is likely due to the co-precipitation of microvesicles.

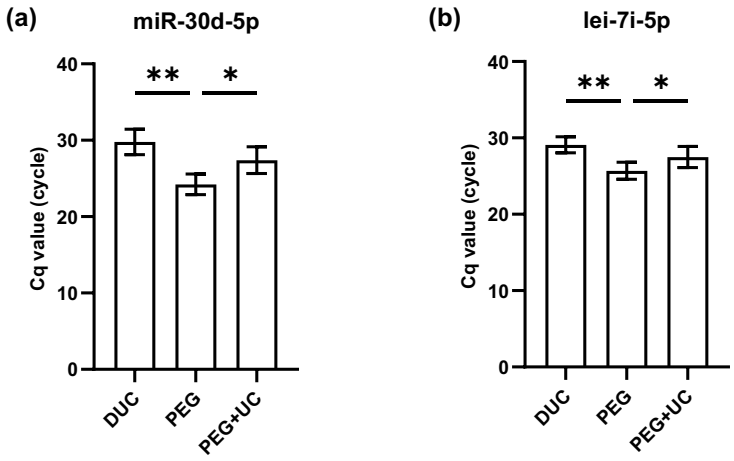

Fig. 3. Quantitation of exosomal miRNA using qPCR. (a) miR-30d-5p. (b) let-7i-5p. The results demonstrated all three isolation methods successfully recovered miRNAs from exosomes, with PEG reporting the highest expression of both miR-30d-5p and let-7i-5p. Data presented as mean±SD (n=6). *$p<0.05$, ** $p< 0.01$.

DISCUSSION

Comparison of exosome isolation methods

This study reports an optimized method to isolate exosomes from limited clinical samples (human serum). The characteristics of exosomes and miRNA contents isolated using the PEG-precipitation followed by an ultracentrifugation step (PEG+UC) were compared to the gold standard DUC and PEG alone. Overall findings suggested that PEG+UC successfully isolated exosomes of both high yield and high purity from human serum. This protocol was adapted from Ludwig *et al*. (2018) and Rider *et al*. (2016), who previously used the PEG+UC method to isolate exosomes from cell culture supernatant. Here, we further validated that PEG+UC was able to reduce pellet contamination in serum samples, thereby resulting in high-purity exosomes for downstream analysis (Table 2).

Table 2. Summary of different isolation methods. Overall, PEG+UC resulted in exosomes of both high yield and high purity

Method	Mean particle size (nm)	Particle concentration (particle/mL)	Particle-to-protein ratio	Protein markers				miRNA recovery	EVs specificity (ranked)	EVs recovery (ranked)
				Exosomes		Microvesicles	Plasma protein			
				CD9	TSG101	Calnexin	Albumin			
DUC	113.0 ± 12.1	$6.94 \times 10^{10} \pm 6.92 \times 10^{9}$	$1.88 \times 10^{7} \pm 2.73 \times 10^{5}$	+	+	+	+	+	2	3
PEG	157.8 ± 3.9	$1.68 \times 10^{12} \pm 1.79 \times 10^{11}$	$1.38 \times 10^{7} \pm 1.42 \times 10^{6}$	+++	+++	++	++	+++	3	1
PEG+UC	84.8 ± 8.1	$8.75 \times 10^{11} \pm 1.12 \times 10^{11}$	$5.88 \times 10^{7} \pm 6.65 \times 10^{6}$	++	++	-	+	++	1	2

Generally, all three methods have successfully isolated exosomes. In terms of yield, PEG+UC resulted in a high abundance of exosomes. The western blot results demonstrated that PEG+UC reported strong expressions of CD9 and TSG101, which are commonly known as exosome-positive markers. In terms of purity, PEG+UC eliminated a vast majority of co-precipitated plasma proteins from PEG-precipitation. The western blot results demonstrated the absence of a marker for endoplasmic reticulum proteins found in microvesicles, Calnexin, as well as a reduction in plasma proteins represented by albumin. The electron microscopic images and size distribution analysis also showed that PEG+UC yielded a homogeneous population of intact vesicles. The results were further supported by the high particle-to-protein ratio, indicating high purity in PEG+UC-isolated samples.

Despite conventional DUC being recognized as the gold standard method for exosome isolation, our study demonstrated that using DUC alone had resulted in low exosome yield. As reported in previous studies, the multiple centrifugation steps in DUC have likely caused losses in exosome recovery, thereby resulting in the weak expression of CD9 and TSG101 in western blot (Nigro *et al*., 2021). These results were also supported by the low particle concentration as measured by NTA. Furthermore, the presence of Calnexin in the western blot indicated that the microvesicles had co-precipitated together with the exosomes during the ultracentrifugation process. As microvesicles belong to another subset of EVs that carry their genetic profiles and regulate distinct mechanisms of genetic transfer from exosomes, it is imperative to remove them from exosome analyses (Ståhl *et al*., 2019). Despite one way to circumvent the limitation of low exosome yield would be to increase the volume of starting materials this will not be feasible in clinical settings as samples are often limited and difficult to obtain (Alotaibi, 2023; Yedigaryan & Sampaolesi, 2023). In addition, the multiple centrifugations followed by repeated ultracentrifugation steps have also made it a tedious protocol. Therefore, DUC is not an ideal method when it comes to using clinical samples such as plasma or serum as starting material to isolate exosomes.

PEG, on the other hand, is a simple and rapid process commonly used for exosome isolation (Yakubovich *et al*., 2022). Our results demonstrated that using PEG alone to isolate serum exosomes has resulted in the co-isolation of small plasma proteins and microvesicles (Alvarez *et al*., 2012; Patel *et al*., 2019). Co-isolation of these microvesicles other than just exosomes could be the reason for the larger particle size distribution observed in PEG. Additionally, the high expression of selected miRNAs, miR-30d-5p and let-7i-5p, from PEG isolates may be attributed to co-precipitation of microvesicles present in the circulation as they were reported to contain their profile of genetic materials (Vickers *et al*., 2011; Pan *et al*., 2019; Zhang *et al*., 2020; Ma *et al*., 2022). The selection of these two miRNAs was based on unpublished next-generation sequencing (NGS) data, which indicated high expression levels in exosomes. However, it should be noted that these miRNAs were used for comparison of miRNA recovery from exosomes across different isolation methods, rather than focusing on the functionalities of each miRNA. Next, previous studies have reported the negative impact of PEG which interfered and obscured the results of protein-based analyses (Abramowicz *et al*., 2018; Ludwig *et al*., 2019). PEG also co-precipitated IgG, a highly soluble protein that is abundant in the circulation during exosome isolation (Crowley-Nowick *et al*., 1996). Hence, using PEG alone to isolate exosomes may contribute to misinterpretation and discrepancies between data, ultimately leading to irreproducible results. Therefore, despite being a rapid, highly reproducible, and scalable method, there are limitations to using PEG alone to isolate exosomes from fluids of high protein abundance.

Our findings suggested that the subsequent washing of PEG-precipitated EVs using PBS and re-concentrating by ultracentrifugation, PEG+UC, is a way to circumvent the limitations of using either DUC or PEG alone. PEG+UC prevents vesicle aggregation or being mechanically damaged as a result of repeated ultracentrifugation steps, yet can remove the majority of non-exosomal contaminants and PEG residue as demonstrated by TEM (Linares *et al*., 2015). As a result, the high-purity exosomes isolated using the PEG+UC method would contribute greatly to research that has a primary focus on exosomes, as most of the other contaminants such as microvesicles have been successfully eliminated.

Notably, in line with reported studies, our results also reported that none of the three isolation methods could remove albumin, the most abundant circulating protein in plasma or serum samples (Sódar *et al*., 2016). As one of the more commonly used methods to characterize exosomes is protein quantitation, the high abundance of albumin, which represents plasma proteins, might mask the detection of lower abundance exosomal proteins. However, since there are no current studies demonstrating that albumin is involved in miRNA transport, the focus of isolating exosomes from clinical samples should be on reproducibility and scalability, instead of discriminating all non-exosomal compounds as it is not achievable with all methods (Iftikhar & Carney, 2016). The results should not be a major concern if the contaminants would not interfere with miRNA interpretation (Andreu *et al*., 2016).

Limitations of study

This study had a few limitations. First of all, this current study selected only a handful of the many available exosome isolation methods for comparison. The size exclusion chromatography (SEC) was initially evaluated in this study. However, our results suggested that SEC produced inconsistent data and diluted end products which were not suitable for TEM analysis (data not shown). An extra step is needed to concentrate the filtrate, which would make the isolation process more tedious and not ideal in clinical settings. Hence, SEC was eventually excluded from this study. As the main objective of this study was to find out the best method for biomarkers research, other more complicated methods that require specialized equipment or materials were also excluded from this study. For example, the density gradient ultracentrifugation includes a prolonged ultracentrifugation step of approximately 16 hr, which could potentially damage the structure of exosomes and not be favorable to be upscaled in clinical settings (Li *et al*., 2019). The immunoaffinity captured-based techniques isolate exosomes by their specific markers, which are not suitable for biomarker research as the surface proteins of exosomes are constantly changing in different disease conditions (Khanabdali *et al*., 2024). Furthermore, despite the higher purity end products, these methods often resulted in lower yields in which the isolated exosomes might not be sufficient for downstream miRNA analysis.

Secondly, this study employed only one standard protocol (e.g., centrifugation speed & type of rotor used) for each comparison method. There are many protocols cited in the literature, our purpose was not to evaluate each protocol but focus

on comparing different exosome isolation methods. The exact composition of the commercial kit used was not fully known as these are proprietary products.

Thirdly, this study was designed for human serum as a starting material. The isolation of exosomes from other biofluids may cause variation in results. Our study reported a standardized exosome isolation method that can be used within a given group of clinical samples for biomarker research.

CONCLUSION

Taken together, the presented results in this study suggested that the PEG+UC method acts as a simpler and more efficient method to isolate exosomes from limited serum samples. This study also serves as a guide to provide insights to researchers on the efficiency, reproducibility, and scalability of three exosome isolation methods, namely the DUC, PEG, and PEG+UC, in terms of yield, purity, and miRNA recovery.

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ETHICAL STATEMENT

This ethic from this study was approved by the University of Nottingham Malaysia Science and Engineering Research Ethics Committee (ID: YFP100120).

CONFLICTS OF INTEREST

All authors declared no conflicts of interest.

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