

Exosome enrichment by ultracentrifugation and size exclusion chromatography

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1. ABSTRACT

Exosomes are a subset of extracellular vesicles (EVs) that have important roles in intercellular communication. They contain and carry bioactive molecules within their membranes which are delivered to target cells. Reproducible isolation and enrichment of these exosomes will aid in evaluation of cellular communication. We present an approach that involved the pre-processing of plasma, combined with ultracentrifugation (UC) and size exclusion chromatography (SEC) to isolate EVs and subsequently enrich exosomes. Four variations of this approach (denoted methods I to IV) were compared. Coupling an ultracentrifugation method with size exclusion chromatography (Method II) provided the

best yield by nanoparticle tracking analyses (NTA), the presence of the exosomal markers CD63, Flotillin-1 and TSG-101 (immunoblotting) and showed exosome morphology using transmission electron microscopy (TEM). This method provides an efficient way to enrich the exosomes from blood (plasma), which could be potentially employed for clinical diagnostic assessment and therapeutic intervention.

2. INTRODUCTION

Over the last two decades, extracellular vesicles (EVs) have been studied intensely due to their functional roles in intercellular communication, such

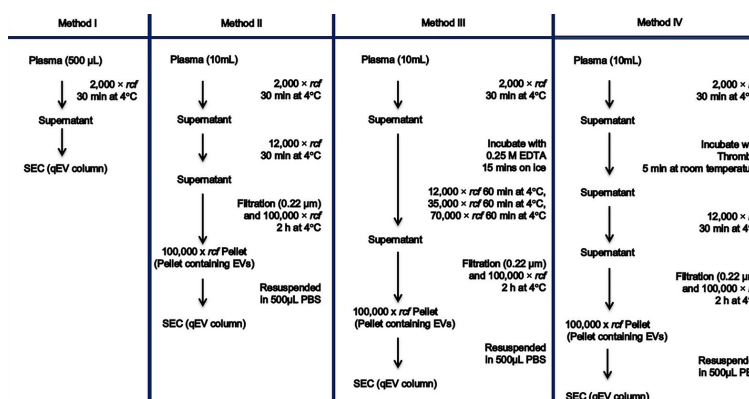


Figure 1. Comparison of the four exosome enrichment methods. Method I: qEV column to enrich exosomes from bovine plasma using size exclusion chromatography (SEC) only. Method II: ultracentrifugation (UC) to isolate bovine plasma extracellular vesicles (EVs) followed by the enrichment of exosomes through SEC. Method III: ethylenediaminetetraacetic acid (EDTA) pre-treated bovine plasma processed by UC and followed by SEC. Method IV: thrombin pre-treated bovine plasma processed by UC and followed by SEC.

as the shuttling of nucleic acids between cells and regulation of information carried to target cells (1). EVs constitute a group of vesicles including microvesicles, ectosomes, exosomes, shedding vesicles and microparticles (2).

Exosomes, are secreted vesicles of endocytic origin in the size range of 30–120 nm (3, 4) that encapsulate bioactive molecules (e.g. proteins, nucleic acids and lipids) and are released into body fluids including blood by different cell types (e.g. tumor cells, placental cells and immune cells) (5–7). Numerous studies have demonstrated the dynamic cell-cell communication mediated by exosomes (8–10). The uptake of exosomes by target cells may dynamically modulate cellular activities of recipient cells. The discovery of exosome mediated intercellular communication has spurred further research interest on exosomes for diagnostic and prognostic assessment of disease states as well as their potential for therapeutic intervention, such as targeted drug delivery systems (11–13).

Several methodologies exist to isolate and analyse EVs and the definition of an EV varies across different research groups (14). The current methods for exosome or EV isolation include ultracentrifugation or commercial vesicle precipitation kits (15). A major concern in the clinical utilization of exosomes is that the separation of exosomes from EVs and other non-vesicular entities (e.g. apoptotic bodies), cannot be fully achieved by these commonly used isolation techniques (15, 16). The composition of recovered EVs also varies vastly according to the isolation protocol being used. Ultracentrifugation alone, cannot achieve absolute separation of exosomes due to co-sedimentation of vesicles and other macromolecules (14, 16). Ultracentrifugation followed by differential gradient centrifugation, which separates vesicles based on density (e.g. Optiprep™ or sucrose cushions) is the current gold standard

for exosome enrichment from EVs (17, 18). This approach, is time consuming, costly and exosome yields are highly variable (due to user variability). Moreover, the successive washing and pelleting steps result in a reduction in exosome yield.

In this study, we compared the (Method I) direct use of size exclusion chromatography (SEC) for the isolation of exosomes with (Method II) using ultracentrifugation (UC) coupled to the enrichment of exosomes using size exclusion chromatography (SEC) and (Methods III and IV) the pre-processing of plasma prior to UC for the isolation of EVs coupled with SEC. The exosomes are characterised by nanoparticle tracking analysis (NTA) for particle number, bicinchoninic acid assay (BCA) for protein concentration, immunoblotting (Western blot) for presence of exosomal markers and transmission electron microscopy (TEM) for morphology based on the requirement described by Witwer *et al* and Lotvall *et al* (14, 15).

3. MATERIALS AND METHODS

3.1. Animal samples (blood plasma)

Blood from dairy cows was obtained and pooled. The procedures for plasma collection were managed by DairyNZ with the approval by the Ruakura Animal Ethics Committee (AEC 13927). The plasma collected was pooled. 10mL of the pooled plasma was utilized for each exosome enrichment method II, III and IV (Figure 1), except for method I, which utilized 500µL of pooled plasma, as per manufacturer's instruction.

3.2. Plasma processing

3.2.1. Method I: Plasma preparation for size exclusion chromatography (plasma + SEC)

The pooled plasma (500µL) was centrifuged at 2,000 × rcf for 30 min at 4 °C to remove debris.

The enrichment of exosomes continued using size exclusion chromatography (see section 3.3.).

3.2.2. Method II: Plasma preparation for extracellular vesicles isolation by ultracentrifugation (plasma + UC + SEC)

Extracellular vesicles were isolated from 10mL pooled plasma by successive differential centrifugation steps at $2,000 \times rcf$ for 30 min and $12,000 \times rcf$ for 30 min at 4°C . The supernatant was filtered through a $0.22\text{-}\mu\text{m}$ filter (Corning Costar) and ultracentrifuged at $100,000 \times rcf$ for 2 h at 4°C (Beckman, Type 70.1 Ti, Fixed angle ultracentrifuge rotor). The pellet was reconstituted in 500 μL phosphate-buffered saline (PBS; Gibco) and the enrichment of exosome continued using size exclusion chromatography (see section 3.3.).

3.2.3. Method III: EDTA treated plasma preparation for extracellular vesicles isolation by ultracentrifugation (EDTA treated plasma + UC + SEC)

The pooled plasma (10mL) was centrifuged at $2,000 \times rcf$ for 15 min at 4°C to remove debris. An equal volume of 0.25M ethylenediaminetetraacetic acid (EDTA; Sigma–Aldrich) at pH 7 was added to the samples and incubated for 15 min on ice. The sample was further centrifuged at $12,000 \times rcf$ for 60 min, $35,000 \times rcf$ for 60 min and then $70,000 \times rcf$ for 60 min at 4°C (Beckman, Type 70.1 Ti Fixed angle ultracentrifuge rotor) the pellet was discarded at each step. The supernatant was filtered through a $0.22\text{-}\mu\text{m}$ filter (Corning Costar) and centrifuged at $100,000 \times rcf$ for 2 h at 4°C (Beckman, Type 70.1 Ti, Fixed angle ultracentrifuge rotor). The pellet was reconstituted in 500 μL PBS and the enrichment of exosome continued using size exclusion chromatography (see section 3.3.).

3.2.4. Method IV: Thrombin treated plasma preparation for extracellular vesicles isolation by ultracentrifugation (thrombin treated plasma + UC + SEC)

10 mL pooled plasma was centrifuged at $2,000 \times rcf$ for 30 min and $12,000 \times rcf$ for 30 min at 4°C . Bovine thrombin (T7153, Sigma–Aldrich) was added to the supernatant (1:100) and incubated for 5 min at room temperature. The sample was centrifuged $12,000 \times rcf$ for 30 min at 4°C , filtered through a $0.22\text{-}\mu\text{m}$ filter (Corning Costar) and centrifuged at $100,000 \times rcf$ for 2 h at 4°C (Beckman, Type 70.1. Ti, Fixed angle ultracentrifuge rotor). The pellet was reconstituted in 500 μL PBS and the enrichment of exosome continued using size exclusion chromatography (see section 3.3.).

3.3. Exosome enrichment using size exclusion chromatography (SEC)

The processed plasma (method I) and EVs obtained from method II, III and IV were loaded on

top of qEV size exclusion columns (Izon Science, Christchurch, New Zealand) followed by elution with PBS. 500 μL fractions were collected in separate tubes (a total of 16 tubes), as per manufacturer's instructions. The 16 fractions were concentrated using a vacuum concentrator (Eppendorf Concentrator Plus) for 1.5 h at room temperature and stored at -80°C until further analyses.

3.4. Nanoparticle tracking analysis (NTA)

NTA measurements were performed using a NanoSight NS500 instrument (NanoSight NTA 3.0. Nanoparticle Tracking and Analysis Release Version Build 0064). This was used to determine the concentrations of particles.

3.5. Protein quantification

Protein concentration of each exosomal fractions (method I–IV) was determined by a bicinchoninic acid (BCA) assay using the bicinchoninic acid reagent kit from Sigma–Aldrich and bovine serum albumin standards (19).

3.6. Immunoblotting (Western blot)

10 μg of exosomes (as determined by BCA) were incubated for 10 min at 70°C in reducing agent (NuPAGE Sample Reducing Agent, Life Technologies Australia Pty Ltd) and loading buffer (NuPAGE LDS sample buffer, Life Technologies Australia Pty Ltd). The reduced proteins were electrophoresed on NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies Australia Pty Ltd). The gel was transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories Pty Ltd, Australia) using the Trans-Blot Turbo system. Membranes were blocked for 1 hour in 2% BSA blocking solution and probed overnight with primary antibody; anti-Flot-1 (ab13493, Abcam), anti-CD63 (ab213092, Abcam) and, anti-TSG101 (sc7964, Santa Cruz Biotechnology) at 4°C , followed by secondary antibody; anti-rabbit IgG (A0545, Sigma–Aldrich), anti-mouse IgG (18765, Sigma–Aldrich) and anti-goat (sc-2020, Santa Cruz Biotechnology). The targeted proteins were visualized with SuperSignal West Dura-Extended Duration Substrate (Thermo Fisher Scientific) on X-ray films using Konica SRX101A processor (Konica Minolta medical and graphic INC, Japan).

3.7. Transmission electron microscopy (TEM)

5 μL of enriched exosomes were added onto formvar coated copper grids for 2 min, then briefly washed in ultrapure water and negatively stained with 1% uranyl acetate. The samples were then visualised using the JEOL 1010 transmission electron microscope operated at 80kV, and images were captured using an Olympus Soft Imaging Veleta digital camera.

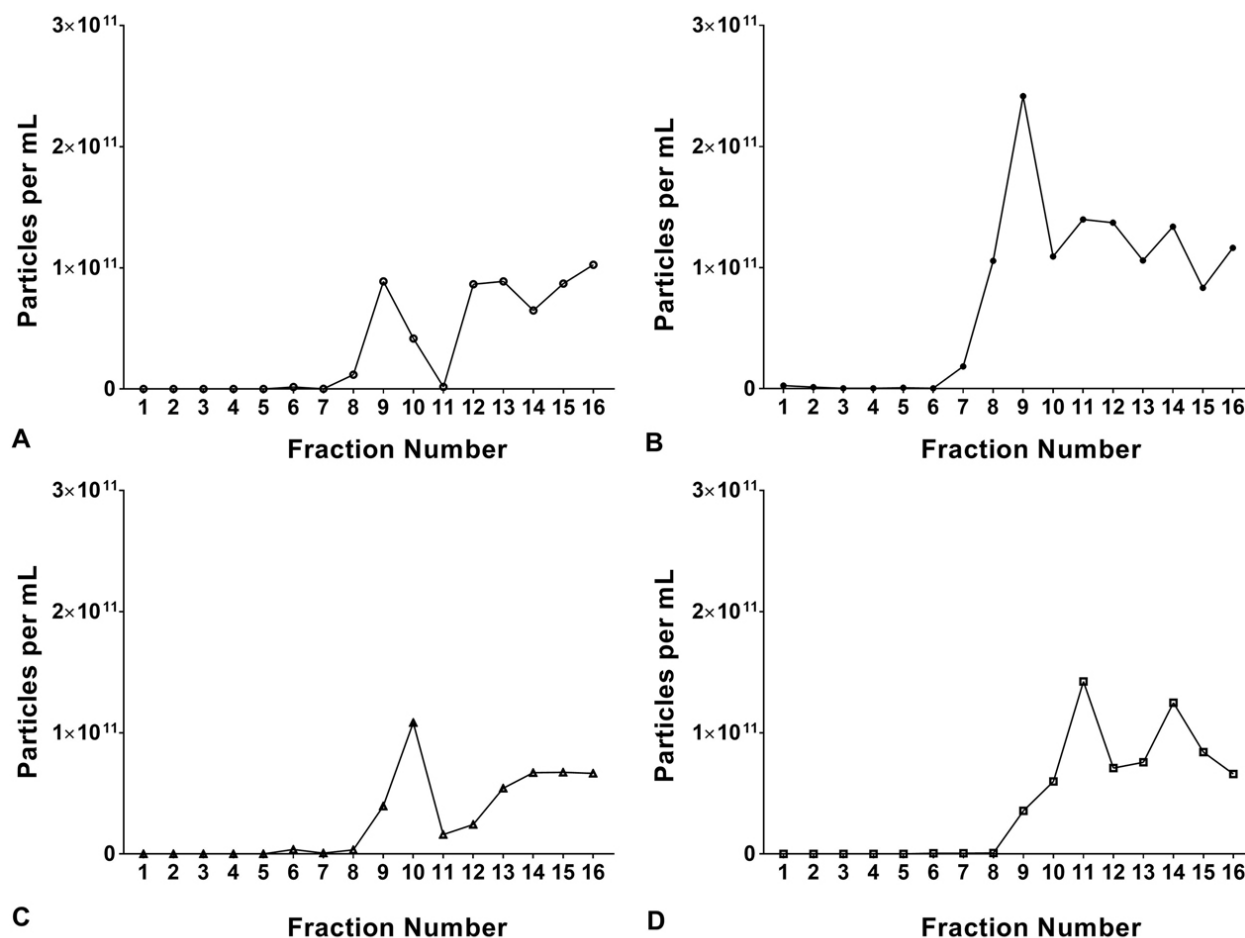


Figure 2. Concentration (particles per mL) of fractions 1–16 following the isolation and enrichment of bovine plasma exosomes using (A) methods I (○) size exclusion chromatography only, (B) method II (●) coupling ultracentrifugation (UC) followed by size exclusion chromatography (SEC), (C) method III (△) ethylenediaminetetraacetic acid (EDTA) pre-treated bovine plasma processed by UC and followed by SEC and (D) method IV (□) thrombin pre-treated bovine plasma processed by UC and followed by SEC.

4. RESULTS

4.1. Nanoparticle tracking analysis (NTA)

Fractions 1–16 from each enrichment method (I–IV) were analysed using a Nanosight NS-300 system to determine particle density and size range. Particle numbers were negligible in fraction 1 to 6 for all the methods (method I–IV, Figure 2A–D), corresponding with the column void volume as described in the manufacturer's instructions. Fractions 7–16 for each method showed variable number of exosomes (method I–IV, Figure 2A–D). Method II had the highest yield of 8.25×10^{11} particles per mL (Table 1).

4.2. Protein quantification

The protein concentration were determined for all fractions (method I–IV) using BCA assay (Figure 3A–D). An increase in protein concentration

was observed from fraction 9 through to fraction 16 across all the methods. This trend was similar across methods II–IV, while method I showed a greater increase in protein concentration from fraction 14–16.

4.3. Immunoblotting (Western blot)

Flotillin-1 was consistently identified in the fractions 12–16 (method I–IV, Figure 4A–D). Further immunoblotting analyses for exosomal markers CD63 and TSG101 were carried out for method II. Both CD63 (Figure 5A) and TSG101 (Figure 5B) were detected in the fractions 11–16.

4.4. Transmission electron microscope (TEM) analyses

The enriched exosomes (method II) displayed a cup-shaped morphology by TEM (Figure 6).

Table 1. Particle yields (method I-IV) obtained from nanoparticle tracking analysis (NTA)

Methods for exosome enrichment	Particle yields obtained from NTA (particles per mL)
Method I: plasma + size exclusion chromatography	4.73×10^{11}
Method II: plasma + ultracentrifugation + size exclusion chromatography	8.25×10^{11}
Method III: EDTA treated plasma + ultracentrifugation + size exclusion chromatography	4.04×10^{11}
Method IV: thrombin treated plasma + ultracentrifugation + size exclusion chromatography	6.24×10^{11}

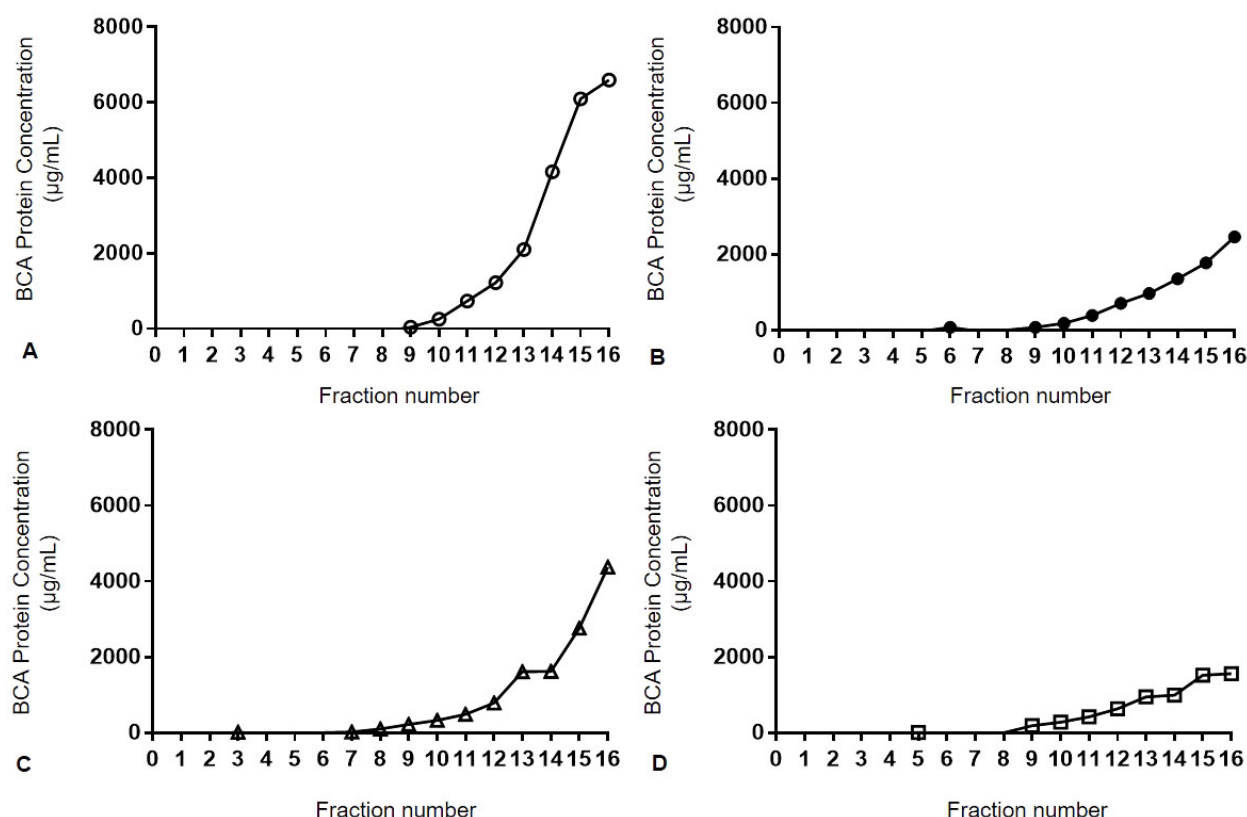


Figure 3. Protein concentration (µg/ml) of the 16 fractions by bicinchoninic acid (BCA) assay following the isolation and enrichment of bovine plasma exosomes using (A) methods I (○) size exclusion chromatography only, (B) method II (●) coupling ultracentrifugation (UC) followed by size exclusion chromatography (SEC), (C) method III (Δ) ethylenediaminetetraacetic acid (EDTA) pre-treated bovine plasma processed by UC and followed by SEC and (D) method IV (□) thrombin pre-treated bovine plasma processed by UC and followed by SEC.

5. DISCUSSION

Exosomes are specific subset of extracellular vesicles that originate from the endosomal membrane cell compartment. They encapsulate selectively sorted bioactive molecules (e.g. proteins, lipids, and nucleic acids), and are released into the extracellular environment through fusion of multivesicular bodies with the cell plasma membrane. Exosomes can be transported through body fluids (e.g. blood) and

mediate communication between neighbouring and/or distant cells by delivering encapsulated bioactive molecules to recipient cells. Owing to their vital role in cell-to-cell communication, exosomes are known to be involved in physiological and pathological processes such as tumor development (20), pregnancy (21) and immunomodulation (22). Exosomes have the potential to be used in diagnostic and prognostic tests for disease and to be used in therapeutic interventions. A challenge for clinical application is the inability to efficiently and

Method for the enrichment of exosome from bovine plasma

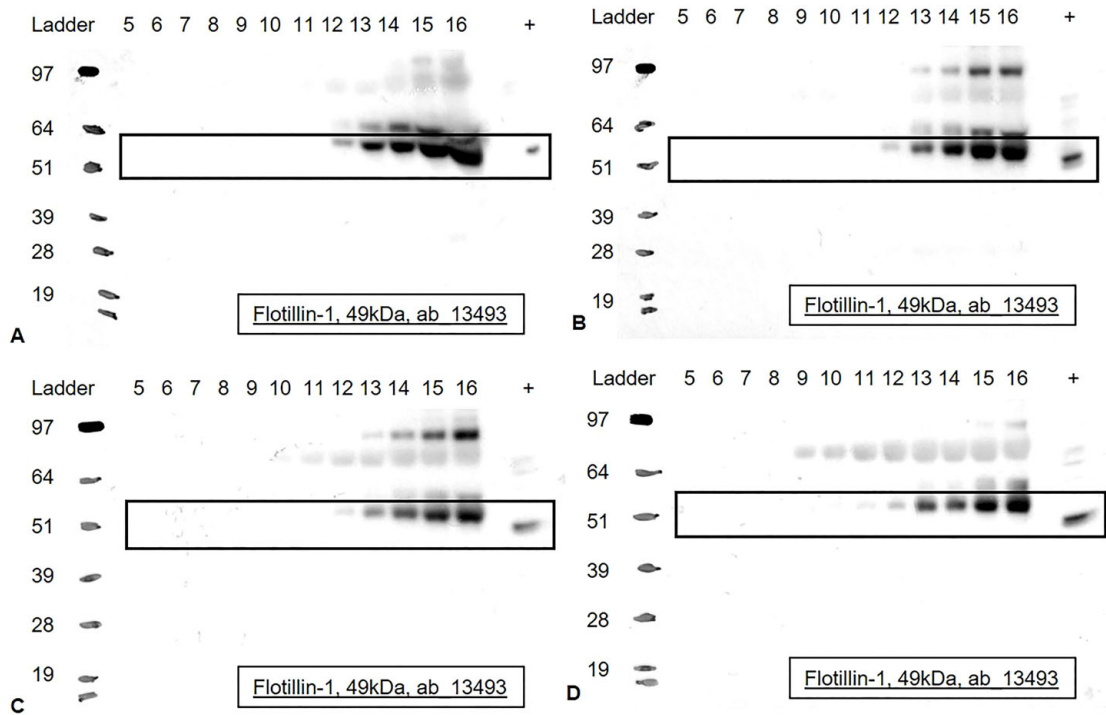


Figure 4. Immunoblots for exosomal marker Flotillin-1 (49kDa) for SEC fractions 5–16 for methods (I-IV); each lane depicts a separate fraction. “+” represents a positive control. Each gel includes a protein size ladder. (A) method I: size exclusion chromatography only (SEC), (B) method II: ultracentrifugation (UC) followed by size exclusion chromatography (SEC), (C) method III: ethylenediaminetetraacetic acid (EDTA) pre-treated bovine plasma processed by UC and followed by SEC. (D) method IV: thrombin pre-treated bovine plasma processed by UC and followed by SEC.

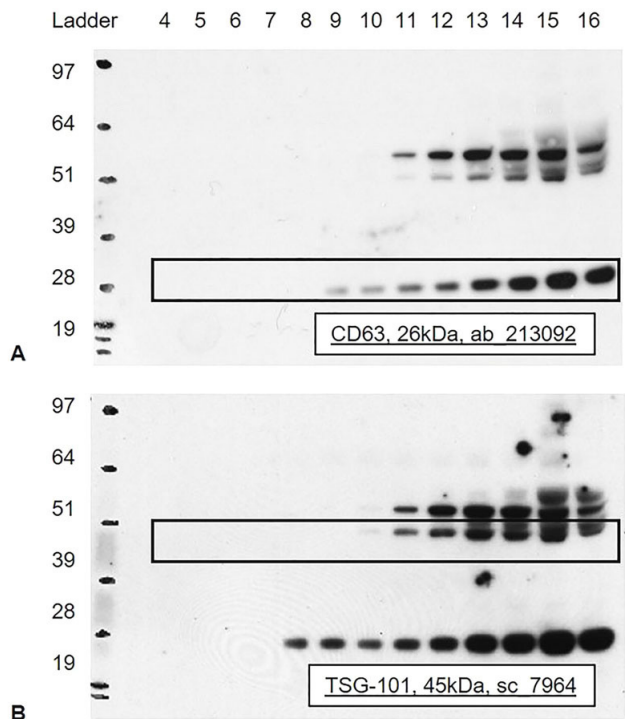


Figure 5. Immunoblots for exosomal markers (A) CD63 (26kDa) and (B) TSG-101 (45kDa) on fractions 4–16 obtained after size exclusion chromatography following Method II. Exosomal fractions 11–16 display a bands specific (in size) for both markers. Each gel includes a protein size ladder.

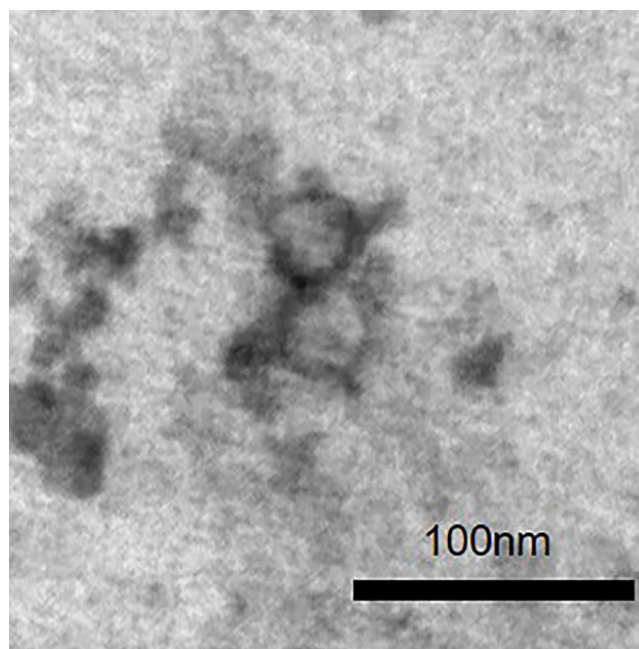


Figure 6. Representative transmission electron microscopy of exosome morphology. Scale bar 100 nm.

reproducibly isolate exosomes, due in part to a lack of a well-defined rigorous isolation protocol.

There are many different techniques and commercial kits available for exosome isolation, and one has to take into account the additional workload, time, cost and most importantly reproducibility when considering which approach is suitable. No existing exosome isolation method has been accepted as universally appropriate, and the advantages and disadvantages of the most widespread methods have previously been discussed (23). The choice of approach for exosomes isolation is also dependent on the source matrix, due to the inherent differences in composition and complexity of body fluids (14). Furthermore, species differences may exist in the composition of body fluids, including blood plasma (24, 25) and milk (26). Therefore, careful consideration of EV/exosome isolation methods must be taken into account, as this may have the potential to affect downstream experimentation. Ultracentrifugation followed by differential gradient centrifugation using Optiprep™ or sucrose cushions, which separates vesicles based on density remains the gold standard for exosome enrichment from EVs (17, 18). The limitations of using this approach, however, is that it is time consuming, and expensive. Moreover, there exists tremendous user variation in the quantity and quality of exosomes isolated, making the reproducibility of exosome isolation for this method poor. Importantly there is a reduction in exosome particle yield after several washing and pelleting steps.

In this study, we evaluated four methods for the enrichment of exosomes. The best approach as determined by the requirements for exosome isolation as described by Witwer *et al* and Lotvall *et al* (14, 15) coupled ultracentrifugation (to first isolate the EVs) with the subsequent enrichment of exosomes by size exclusion chromatography (Method II). The approach provided the most consistent yield of blood plasma exosomes based upon the particle yield, presence of exosome markers and exosome morphology. The initial step for ultracentrifugation discussed by Thery *et al* (18), enables the removal of dead cells, some proteins and macromolecules and other contaminating debris. However, ultracentrifugation alone will not remove all soluble proteins (27). The direct isolation of exosomes through size exclusion chromatography (e.g. qEV column) have been demonstrated to separate exosomes from soluble contaminants with high efficiency (28, 29). However, the design of the qEV column only allows loading of a 500µL maximum sample size. The maximum capacity of the size exclusion chromatography column limits its scalability for high throughput applications. The use of an initial ultracentrifugation-based step for the enrichment of EVs also allows the ability to process a higher volume of the starting material, and therefore to achieve greater exosome yield. The subsequent use of the size exclusion chromatography column enables efficient removal of soluble proteins and other contaminants, hence enriching specifically for exosomes. This approach demonstrated decreased user variability and the time required for exosome enrichment as well as

improved yield and reproducibility. This approach may also be useful for the enrichment of exosomes from other types of body fluids such as milk, saliva and urine.

We further investigated the pre-treatment of the bovine plasma with anticoagulants to evaluate if the addition of an anticoagulant step would provide a better yield and/or purity of exosomes. To date, the choice of which anticoagulant is most effective for utility in EVs/exosomes study is unclear (30). In this study, bovine plasma was either treated with EDTA which chelate calcium (31, 32), an important co-factor for blood clot or thrombin which can facilitate the removal of fibrinogen (29) prior to EVs/exosomes isolation. No differences in yield were observed with the addition of an anticoagulant pre-treatment step. This may be due to the loss attributed to the additional ultracentrifugation steps required for the processing of the anticoagulant treated plasma.

Clinical exosome based applications especially for clinical diagnostics requires a time-efficient, reproducible and robust method. Our approach (method II) which couples ultracentrifugation with size exclusion chromatography enables the use of a greater starting volume for exosome isolation, is highly reproducible, time-efficient and can provide a greater yield. This method provides an efficient way to enrich the exosomes from blood (plasma), which could be employed in clinical diagnostic assessment and potentially therapeutic intervention.

6. ACKNOWLEDGMENTS

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