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Original article

A method for the isolation and enrichment of purified bovine milk exosomes

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ABSTRACT

Exosomes are nanovesicles that play important roles in intercellular communication as they carry information to target cells. Isolation of high purity exosomes will aid in studying the exosomal cargo and quantity as well as how cell-specific messages are carried. We describe a new method incorporating size exclusion chromatography (SEC) to enrich milk-derived exosomes from extracellular vesicles (EVs). This involved the initial isolation of EVs from bovine milk via milk processing and ultracentrifugation; followed by a new method to enrich exosomes using SEC. This method was compared to buoyant density gradient centrifugation, a widely used method of enrichment. Exosomes were characterised by particle concentration and size (nanoparticle tracking analysis, NTA), morphology (transmission electron microscopy, TEM), presence of exosomal markers (immunoblotting) and protein concentration (bicinchoninic acid assay, BCA). Proteomic profiles of exosomal fractions were analyzed by mass spectrometry using Information Dependant Acquisition. Milk exosomal fractions were shown to contain exosomal markers flotillin-1 (FLOT-1) and tumor susceptibility gene-101 (TSG-101). The new method produced a higher yield of exosomes compared to buoyant density gradient centrifugation. Pooled exosomal fractions exhibited intact morphology by TEM. The use of SEC confirmed the fractionation of exosomes based on size while minimizing the interference with proteins. Tetraspanins CD9 and CD81 were observed via mass spectrometry in exosomal fractions. This new and efficient method confirmed the signatures for exosomes derived from unpasteurized bovine milk. Purification of exosomes is a foundational technique in the study of biomarkers for pathological conditions and effective drug delivery systems.

1. Introduction

Exosomes, a subtype of extracellular vesicles (EVs), are currently being studied in different biological fluids such as plasma, saliva and milk [1]. These studies aim to understand the roles of exosomes in the mechanisms underlying many diseases and identify potential candidate biomarkers for early detection of disease. Improving the purity of the exosomes isolated is essential for the integrity of biomarker and miRNA analysis and furthering the use of exosomes as drug delivery systems [2]. A number of laboratories are trying to isolate exosomes reproducibly, using diverse published techniques [3–8]. However, the purity of the exosomes isolated is highly variable due to the presence of contaminating particles, vesicles and molecules such as proteins and/or nucleic acids and other cellular components [7,9,10]. Minimizing contamination in the isolation of exosomes is vital in providing reliable information upon which to base new paradigms. Hence, there is a need for an efficient and robust method by which enriched populations of exosomes can be obtained. The enriched exosomes need to be well characterized and validated prior to subsequent studies [11,12].

There are several commercial exosomal isolation methods available that utilise exosome precipitation (*e.g.* ExoQuick precipitation [13]), ultrafiltration, or immunoaffinity capture based techniques. Exosome precipitation commercial kits have limitations that include the co-precipitation of other non-exosomal contaminants such as proteins and macromolecules, and cannot discriminate between exosomes and other EVs [14]. Buoyant density gradient centrifugation is a method that generates higher purity exosomes than the commercial kits, however it is time consuming, labour intensive and limited in the yields obtained [6]. Furthermore, most of these methods have been tested primarily on plasma samples.

Exosomes, derived from a range of bodily fluids, including milk, are being used to identify cows with poor fertility, and issues related to the health of the transition cow [15]. In dairy cows, the transition to lactation has been linked to physiological and metabolic stress as well as sub-optimal immune function [15,16]. During this transition period there is a high rate of infection and susceptibility to inflammatory

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disorders, such as mastitis and endometritis. These disorders result in a decrease in milk production and reproductive function [17–19]. Milk exosomes may hold promise for early diagnosis of these problems and other reproductive disorders in mammals. Bovine milk is easy to obtain in large quantities, can remain fresh for long periods of time, is stable under long-term storage conditions and is a good source of exosomes [4].

The objective of this study was to develop an efficient and robust method for the enrichment of exosomes derived from bovine milk. This method is based on a combination of recent approaches for the initial preparation of milk for extracellular vesicle isolation [4,8,20] and the incorporation of size exclusion chromatography (SEC) for enrichment of exosomes. SEC columns are packed with porous polymeric beads, and have been used previously for the separation of EVs derived from biological fluids such as plasma and urine [7,21]. Moreover, SEC has better separation (based on size) compared to buoyant density gradient centrifugation (based on density) and helps to eliminate contaminants with more confidence [22].

2. Methods and materials

2.1. Milk collection

Two litres of fresh unpasteurised milk was collected from a Holstein Friesian dairy herd located at Gatton, The University of Queensland. The collected milk was aliquoted (15 mL) and stored at −80 °C.

2.2. Extracellular vesicle isolation

EVs were isolated from milk by differential centrifugation. Briefly, milk aliquots (15 mL) were added to 25 mL PBS (Gibco, Life Technologies Australia), centrifuged at 3000 *rcf* for 15 min at 4 °C to remove milk fat globules, cellular debris and somatic cells. The supernatant was divided into two groups, of a total of 20 mL each (*i.e.* equivalent of 7.5 mL milk each) and these were used for subsequent isolation and enrichment processes. An equal volume of 0.25 M EDTA (Sigma Aldrich, Castle Hill, New South Wales, Australia; pH 7) was added to the samples and incubated for 15 min on ice to precipitate casein and exosomes coated with casein as described by Kusuma et al. [20]. The 50 mL tubes were centrifuged at 12,000 *rcf* for 60 min at 4 °C. The supernatants were transferred to OptiSeal tubes (Beckman Coulter, Gladesville, Australia), and subjected to successive ultracentrifugation steps at 35,000 *rcf* for 60 min, and then at 70,000 *rcf* for 60 min at 4 °C (Beckman, Type 70.1 Ti Fixed angle ultracentrifuge rotor). The supernatant was filtered through 0.22 µm syringe filters and centrifuged at 100,000 *rcf* for 120 min at 4 °C to pellet the extracellular vesicles. The extracellular vesicles were resuspended in 600 µL PBS (Gibco, Life Technologies Australia) as shown in Fig. 1. Thereafter we evaluated the new method of exosome enrichment (Method B) and compared it with the most widely used current method (Fig. 2), employing buoyant density gradient centrifugation (Method A).

2.3. Exosome harvesting and enrichment

2.3.1. Method A

Briefly, EVs in 500 µL of PBS, from the previous step, was loaded onto a discontinuous iodixanol gradient (OptiPrep™ gradient, Sigma-Aldrich) in ultracentrifuge tubes [23]. The tubes were centrifuged at 100,000 *rcf* for 20 h at 4 °C (Beckman, Sw41Ti, Swinging-bucket ultracentrifuge rotor). Twelve individual fractions were obtained. Each fraction was transferred to separate ultracentrifuge tubes, diluted with PBS (Gibco, Life Technologies Australia) and ultracentrifuged again at 100,000 *rcf* for 2 h at 4 °C, to wash the exosomes. The pellets were resuspended in 100 µL PBS and used for further analysis. This procedure is described in the flowchart in Fig. 2.

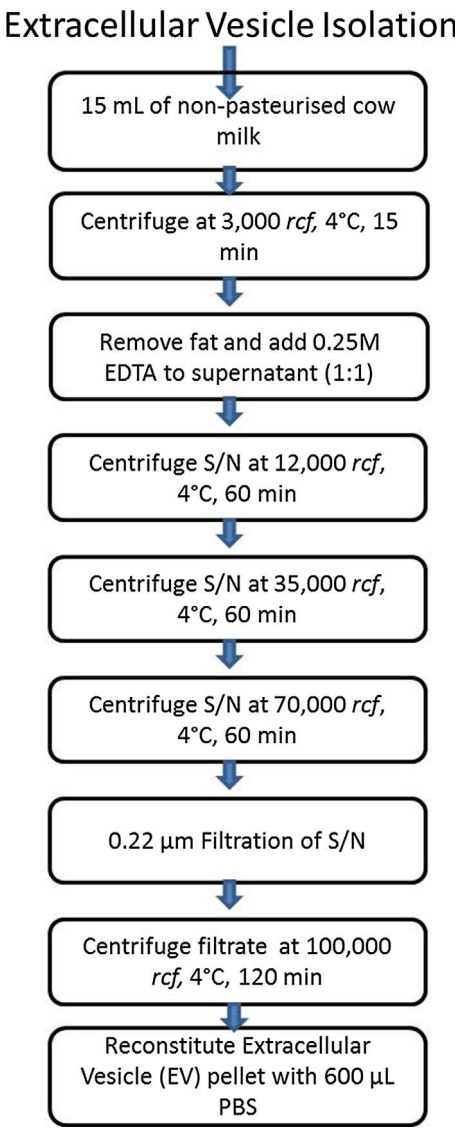

Fig. 1. Flowchart for the isolation of extracellular vesicles (EVs) from milk. EVs were isolated from milk by differential ultracentrifugation. Unpasteurised milk was centrifuged at 3000 *rcf* followed by 0.25 M EDTA treatment (1:1; v/v) and supernatants (S/N) subsequently centrifuged at 12,000, 35,000, 70,000 and 100,000 *rcf* respectively. The pellet obtained after the sequential centrifugation process contains EVs. After reconstitution in PBS, the EV suspension was used for exosome enrichment.

2.3.2. Method B

Briefly, EVs in 500 µL of PBS were loaded on a qEV column (Izon Science Ltd, New Zealand) and eluted with further PBS in 500 µL fractions to a total of 16 fractions, as per manufacturer's instructions (Fig. 2, Method B). The individual fractions were then used for further analysis. This procedure is described in detail in Fig. 2.

2.4. Nanoparticle tracking analysis

The nanoparticle tracking analysis was conducted using a Malvern NanoSight™ NS500 instrument (NanoSight™, NTA 3.0 Nanoparticle Tracking and Analysis Release Version Build 0064; Amesbury, United Kingdom) as per manufacturer's instructions as we have described [23]. Briefly samples of each fraction (1–12; Method A) and (1–16; Method B) were characterized (including 3 technical replicates for each fraction) to determine the particle concentration (particles/mL)

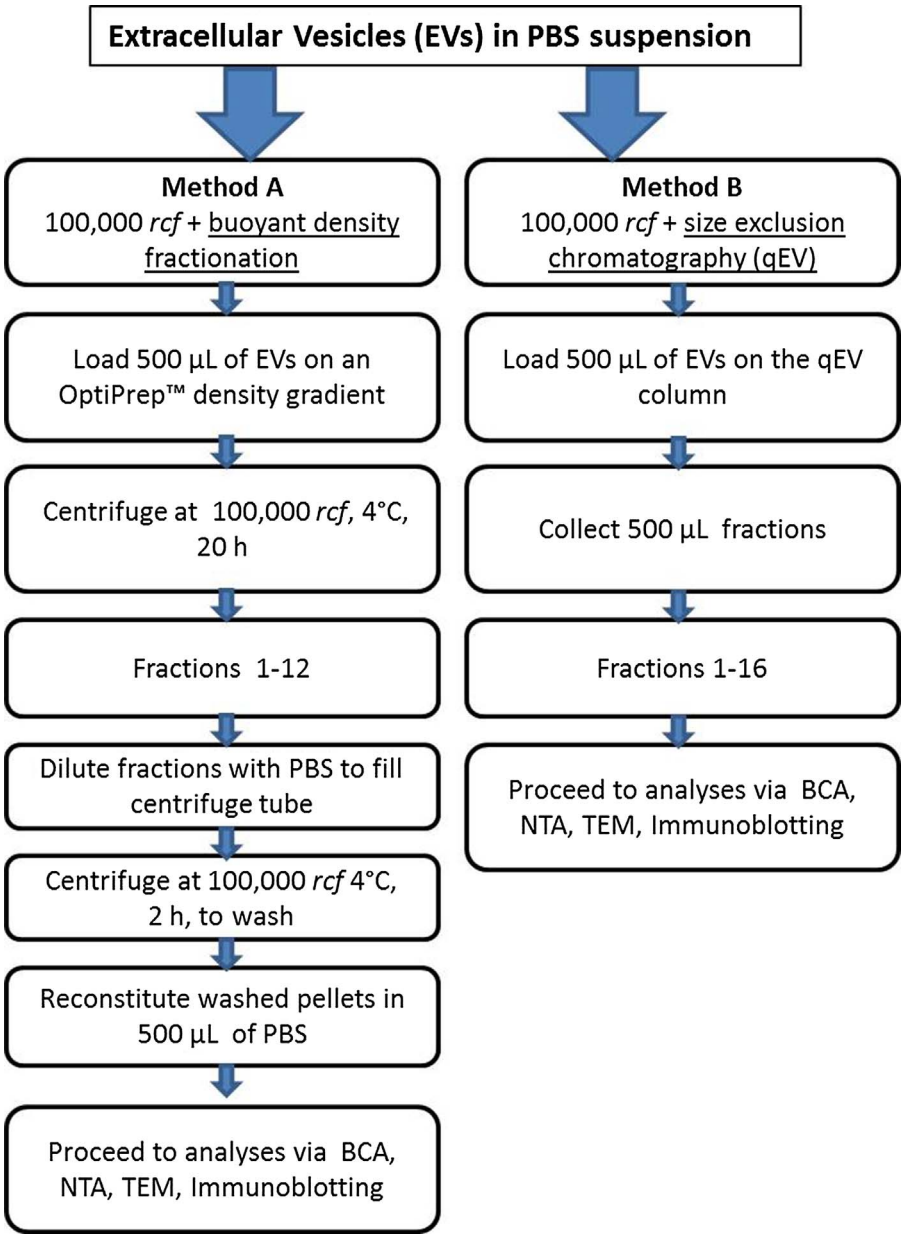

Fig. 2. Flowchart for the comparison of two exosome enrichment methods; buoyant density gradient centrifugation (Method A) and size exclusion chromatography (Method B). (A) 500 µL of EV suspension was loaded on top of the OptiPrep™ gradient and ultracentrifuged for 20 h to obtain 12 fractions as described in the flowchart. (B) 500 µL of EV suspension was introduced on top of a SEC column (qEV column) and processed via size exclusion chromatography to obtain 16 fractions as described in the flowchart.

2.5. BCA protein quantitation

Protein concentration was quantified using a Bicinchoninic acid (BCA) reagent kit (Sigma Aldrich, Castle Hill, New South Wales, Australia). All 12 fractions of Method A and 16 fractions of Method B were quantified against a bovine serum albumin (BSA) standard (0–2,000 µg/mL) (Sigma Aldrich, Castle Hill, New South Wales, Australia) to determine protein concentration.

2.6. Immunoblotting

Exosomal proteins (10 µg) were incubated for 10 min at 70 °C in reducing agent (NuPAGE Sample Reducing Agent, Life Technologies Australia, Mulgrave, Victoria, Australia) and loading buffer (NuPAGE LDS sample buffer, Life Technologies Australia Pty Ltd). Reduced proteins were electrophoresed (SDS-PAGE) on NuPAGE Novex 4–12% Bis-Tris Gels, and transferred to a polyvinyl difluoride (PVDF; Bio-Rad Laboratories, Australia) membrane using the Trans-Blot Turbo system with pre-packed transfer packs and the 7-min protocol (Bio-Rad Laboratories, Australia). Membranes were blocked for 1 h in blocking solution and probed overnight with primary rabbit polyclonal antibody anti-FLOT-1 (ab13493 Abcam, Boston, MA, USA) and, anti-TSG-101 (sc-7964; Santa Cruz Biotechnology, California, USA) at 4 °C, followed by secondary anti-rabbit IgG (1:1000; A0545, Sigma–Aldrich) and secondary donkey anti-goat IgG-HRP (1:1000; sc-2020, Santa Cruz Biotechnology, California, USA). SuperSignal West Dura-Extended Duration Substrate (Thermo Fisher Scientific Life Technologies, Australia) was used for development of the blot, and the signal visualized on X-ray film (Agfa, Mortsel, Belgium) and developed using a Konica Minolta SRX-101A processor (Konica Minolta medical and graphic INC, Japan).

2.7. Proteomic profiling using information dependent acquisition (IDA) mass spectrometry

Exosomal fractions determined by our immunoblotting results which confirmed manufacturer's instruction were pooled. The pooled exosomal fractions (Method A: fractions 6–8; Method B: fractions 7–10) were analyzed by mass spectrometry. DTT/bicarbonate (10 µL of 20 mM) was added to each sample and incubated for 1 h at 60 °C.

Iodoacetamide (10 µL of 1 M in 100 mM bicarbonate) was added to each tube and incubated for 1 h at 37 °C in the dark. Parallel incubations were performed using BSA as a quality control for reduction/alkylation and digestion reactions. Samples were digested with Trypsin Gold (1 µg) for 18 h at 37 °C. Post digestion, formic acid (100 µL, 0.1%) was added. Peptides were desalted using stage tips; a 3-mm piece of an Empore C18 (Octadecyl) SPE Extraction Disk was excised and placed in a gel loader tip and POROS slurry was added to form a microcolumn. Trifluoroacetic acid (1 vol, 0.1% in water) was added to the sample and loaded onto the microcolumn. The microcolumn was washed with trifluoroacetic acid (20 µL, 0.1% in water). Peptides were eluted from the microcolumn by three washes of acetonitrile (20 µL × 3, 0.1% formic acid). Eluates were pooled and samples were evaporated at room temperature in a vacuum evaporator for 45 min. Samples were reconstituted in 30 µL formic acid.

The digested protein samples were analyzed using the TripleTOF® 5600 mass spectrometer (ABSciex, Redwood City, California, USA) and Eksigent 1D+ NanoLC system to obtain initial high mass accuracy. MS/MS data were screened to identify the peptides present in the samples. A 10 µL aliquot of digested material was injected onto the column and separated with a linear gradient of 5 to 10% Buffer B for 2 min (Buffer A: 0.1% Formic acid/water; Buffer B: acetonitrile/0.1% formic acid), 10 to 40% Buffer B (58 min), 40 to 50% Buffer B (10 min), 50 to 95% (10 min) with a flow rate of 500 nL/min. The column was flushed at 95% buffer B for 15 min and re-equilibrated with 5% Buffer B for 6 min. The in-depth proteomic analysis was conducted using Information Dependant Acquisition (IDA) on the TripleTOF® 5600 System interfaced with a nanospray source. Results were analyzed using ProteinPilot™ (ABSciex. Redwood City, California, USA).

2.8. Transmission electron microscopy (TEM)

Exosomal fractions determined by our immunoblotting results which confirmed manufacturer's instruction were pooled and based on the presence of exosomal markers in Immunoblots. 5 µL of the pooled exosomal fractions (Method A: fractions 6–8; Method B: fractions 7–10) were analyzed by TEM. Samples were placed on formvar coated copper grids for 2 min, washed briefly in ultrapure water and negatively stained with 1% uranyl acetate. The samples were then viewed using the JEOL 1010 transmission electron microscope operated at 80 kV, and images were captured with an Olympus Soft Imaging Veleta digital camera. Individual fractions 6–8 from Method A and 7–10 from Method B were also analyzed by TEM (Supplemental figure).

2.9. Statistical analyses

The number of exosomes from the bovine milk for each method are presented as number of particles per mL (mean ± SD, n = 3). Exosomal yield for each method was evaluated by Student's T tests (two-tailed) using a commercially-available software package (Prism 6, GraphPad Inc, La Jolla, CA 92037 USA). Significance was defined as $p < 0.05$.

3. Results

3.1. Nanoparticle tracking analysis

The yield of exosomes obtained after Method A was approximately $7 \times 10^{10} \pm 1.87 \times 10^{10}$ particles/mL (n = 3). In comparison Method B yielded approximately $3 \times 10^{12} \pm 4.42 \times 10^{11}$ particles/mL (n = 3) (Fig. 3). The pattern of particle number across the SEC fractions showed low particle numbers in fraction 1–6 (void volume) and an increase in particle number in exosomal fraction 7 through to fraction 9 and dropping slightly at fraction 10, with numbers declining from fraction 11 onwards.

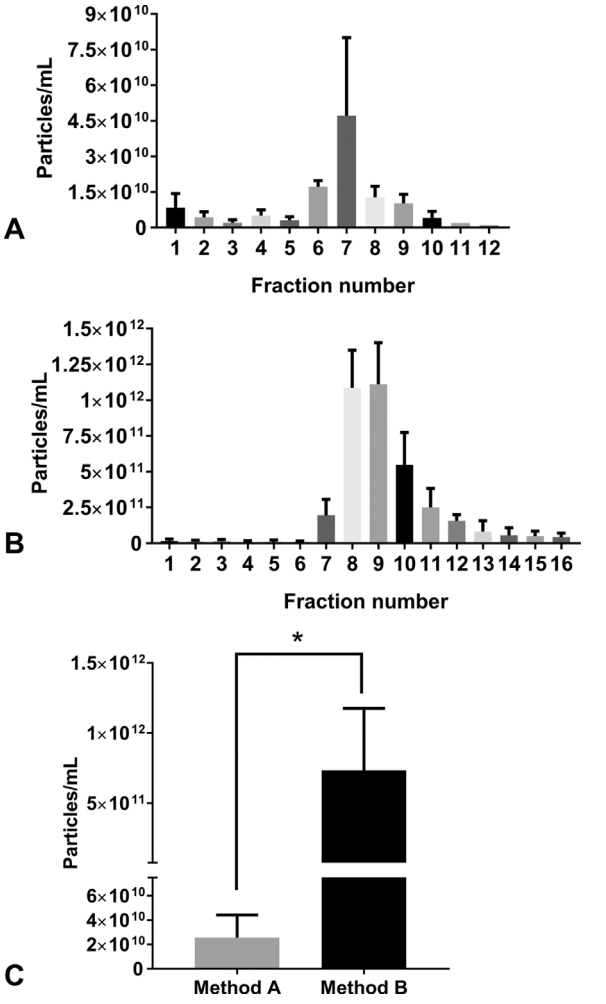

Fig. 3. A comparison of particle concentration between buoyant density gradient centrifugation (Method A) and size exclusion chromatography (Method B). Nanoparticle tracking analysis (NTA) was utilized to determine the particle concentration (particles/mL) of exosomes obtained after isolation and enrichment using; (A) Buoyant density gradient centrifugation enrichment of 12 fractions (n = 3 experimental replicates) and; (B) SEC enrichment of 16 fractions (n = 3 experimental replicates). (C) Comparison of exosome yields obtained from Method A and Method B. Each fraction was evaluated in technical triplicate (n = 3) on NTA. Data are presented as mean ± SD and $p < 0.05$ defined as significant.

3.2. Protein concentration

Method A fraction 7 had the highest protein concentration determined by BCA assay. In comparison, protein concentration in Method B showed an increasing trend with increasing fraction number (Fig. 4).

3.3. Immunoblotting

SDS-PAGE showed the presence of exosomal markers in fraction 7 for Method A (with faint detection in fractions adjacent to fraction 7) (Fig. 5). FLOT-1(49 kDa) and TSG-101(47 kDa) were clearly observed in the exosomal fractions 7–10. Coomassie staining of SDS-PAGE for Method B revealed a distinct protein profile for exosomal fractions 7–10 compared to non exosomal fractions 11–16. Moreover, exosomal markers TSG-101 (47 kDa) and FLOT-1 (49 kDa) were detected in fractions 7–10 in Method B (Fig. 6B).

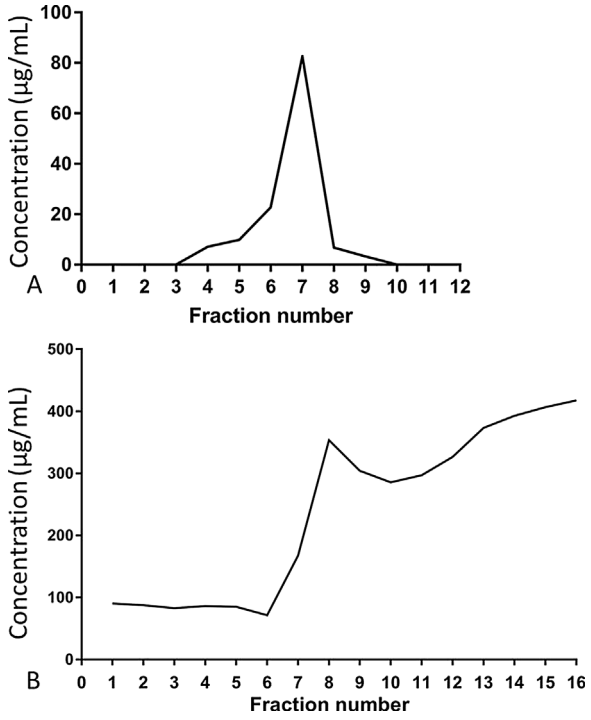

Fig. 4. A comparison of protein concentration between buoyant density gradient centrifugation (Method A) and size exclusion chromatography (Method B). A representative graph of protein concentration (µg/mL) of all fractions obtained after isolation and enrichment following (A) buoyant density gradient centrifugation, fractions 1–12 and (B) SEC, fractions 1–16 as determined by BCA assay.

3.4. Proteomic analyses by mass spectrometry

Using Protein Pilot™, tetraspanin proteins CD9 and CD81 were detected in pooled exosomal fractions from both Methods A and B, but not in non-exosomal fractions. Method B detected more peptides (3 peptides of CD9 and 2 peptides of CD81) with greater than 95% confidence, compared to Method A (1 peptide each) (see Table 1 for peptide information).

3.5. Transmission electron microscopy (TEM)

TEM confirmed the intact donut/cup shaped spherical morphology of exosomal vesicles that are concave in the middle, by both buoyant density gradient centrifugation (Method A) and size exclusion chromatography (Method B) (Fig. 7). The vesicles observed ranged in size from 30 to 200 nm. Exosomes were also observed in the individual fractions 6–8 (Method A) and 7–10 (Method B), (Supplemental Figure). Although comparable morphology was observed in both methods, observed abundance, within a field of view, was lower in samples from Method A compared to Method B.

4. Discussion

Extracellular vesicle (EV) pellets obtained after ultracentrifugation contain exosomes as well as other vesicles, macromolecules and protein aggregates. Our study evaluated the isolation of EVs using a series of ultracentrifugation steps (Fig. 1) and thereafter the enrichment of exosomes from the isolated EVs. We compared two methods for exosome enrichment from bovine milk: buoyant density gradient centrifugation (Method A) and; size exclusion chromatography (SEC) utilizing a commercially available column (Method B) (Fig. 2). The new method (Method B) described in our study differs from other milk exosome isolation methods that use only SEC without an initial EV isolation step [24]. In our method (Method B) we first remove soluble proteins, fats, casein and other contaminants, by differential centrifugation during the EV isolation step. The SEC method is cost-effective, as the columns can be regenerated (by washing them with NaOH and PBS) and reused. SEC of plasma has proven to be time-efficient, and demonstrates a narrow elution profile of exosomes [7,9,25]. Using our new method of exosome enrichment (as described in this paper) a sample can be processed in approximately 30 mins (including washing and regeneration of the column). Moreover, multiple columns (2 to 4) can be run in tandem by a single operator to increase workflow productivity. In comparison, exosome enrichment via buoyant density gradient centrifugation (Method A) requires a 16 or 20 h centrifugation step and multiple wash steps (at least one) to remove the sucrose and/or OptiPrep™ (See Fig. 2, Method A). Therefore, processing 6 samples in tandem using Method A would take approximately 4.5 h per sample. In

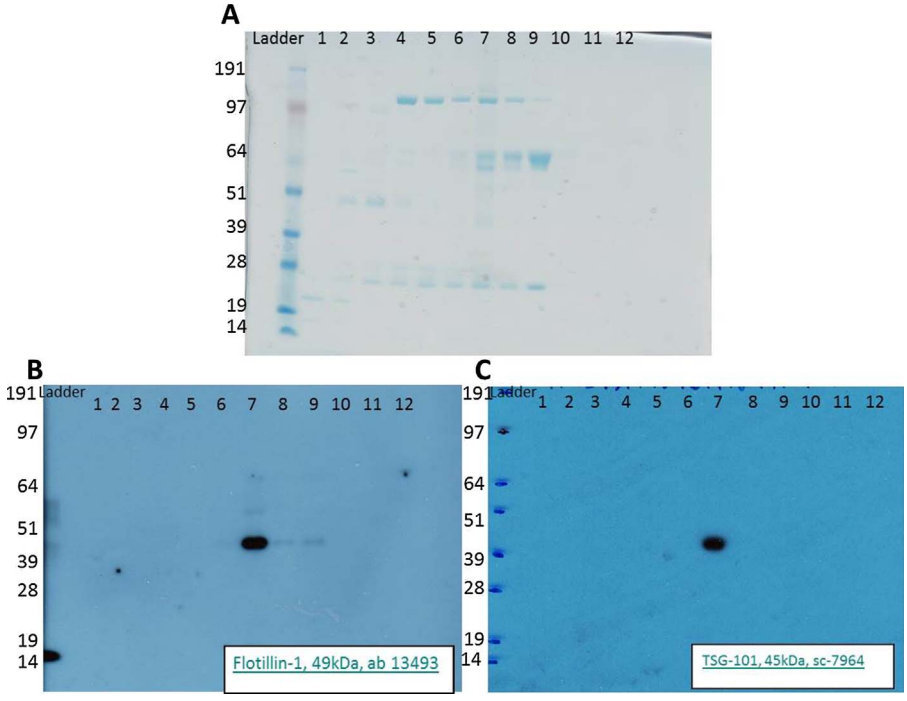

Fig. 5. Immunoblotting for the presence of exosomal markers, using Method A. SDS-PAGE (A) showing differences in protein profiles between all 12 fractions. Immunoblots indicating the presence of TSG-101(B) and FLOT-1(C) in fractions (6–8) containing the exosomes.

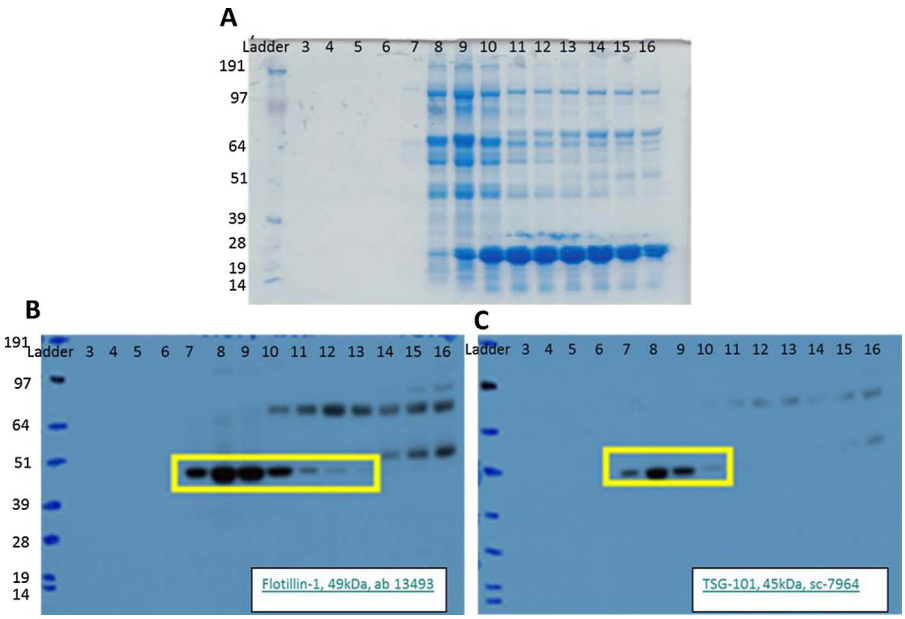

Fig. 6. Immunoblotting for the presence of exosomal markers, using Method B. SDS-PAGE (A) showing differences in protein profiles between all 16 fractions. Immunoblotsindicating the presence of TSG-101 (B) and FLOT-1 (C) in fractions (7–10) containing the exosomes.

addition, the washing steps result in incomplete recovery of exosomes, reducing yield [26]. This poor recovery helps explain why the new method presented in this paper, obtained significantly higher particle yield (approx. 3×10^{12} particles/mL, $p < 0.05$) of approximately two orders of magnitude higher than the benchmark method (approx. 7×10^{10} particles/mL), using NTA. SEC, unlike buoyant density gradient centrifugation, also removes the need to make gradients, which is labour intensive and subject to significant user variability [27].

Many definitions for EVs exist in the literature. EVs include (but are not limited to) microvesicles, ectosomes and shedding vesicles [28]. Microvesicles are EVs that are secreted into the extracellular space, and on average tend to be larger in size (*i.e.* 100–1000 nm) compared to exosomes (30–120 nm) [29]. The size overlap (especially in the range of 100 nm to 120 nm) between the two vesicle types makes it difficult to separate the two groups using existing techniques. In our study SEC fraction 7 most likely contained the larger nanovesicles and some small microvesicles. Smaller microvesicles of about 100 nm may have been eluted in fraction 7, together with the larger exosomes and the extent of microvesicle contamination was difficult to determine. SEC fractions 8 and 9 contained the majority of nanoparticles (mainly exosomes), while fraction 10 contained the smaller exosomes (Method B). This suggests that when these four fractions were pooled, an enriched exosomal preparation was produced that was relatively free from contaminants such as soluble proteins, but may still contain a low concentration of smaller microvesicles and possibly other types of vesicles whose sizes overlap in this range. Buoyant density gradient centrifugation also has the same issue of contaminating microvesicles present in exosomal fractions 6–8, due to some of these vesicles possessing similar densities.

A peak in protein concentration (Fig. 4B) overlapped with particle concentration (particles/mL) (Fig. 3) for the exosomal fractions 7–10, while the later fractions had a continual increase in protein concentration that corresponded with elution of residual and/or aggregated proteins. As proteins and their aggregates are smaller than exosomes, they were retained longer on the Izon™ nano-beads compared to the larger particles that eluted earlier in the process [5,30]. This increasing trend in protein concentration in the later fractions (11–16, Method B) did not correspond with increased particle densities, since the majority of exosomes had eluted earlier in the chromatography process.

TEM on pooled fractions 7–10 (Method B) clearly showed that most of the exosomal structures had been preserved and they corresponded to classical exosome morphology (*i.e.* spherical morphology) of approximately 30–120 nm in diameter [31]. These results are similar to those from a study by Gamez-Valero et al. described that SEC minimally alters the morphologic characteristics of isolated vesicles [32]. In our study both methods (Methods A and B) had some larger vesicles, over 120 nm diameter, by TEM. These vesicles could be the larger EVs that may not necessarily be exosomes (*e.g.* microvesicles), which have co-eluted in the SEC in fraction 7 (Method B).

The exosomal markers FLOT-1 and TSG-101 (protein markers that meet ISEV guidelines) were clearly observed by immunoblotting to be enriched in the exosomal fractions 7–10 (Method B), as expected. Aggregated proteins were eluted after fraction 10, confirming that the SEC enrichment method has a narrow elution profile for exosomes. However, FLOT-1 and TSG-101 may also be present in the contaminating small microvesicles that co-elute in fraction 7 (as these markers may also be present in membranes of other vesicles, and not not specific to exosomes). FLOT-1 and TSG-101 were not detectable by mass spectrometry-IDA (in both methods), possibly due to being in low abundance. Comparative analyses by mass spectrometry of the exosomal versus non-exosomal fractions detected CD9 and CD81 in the exosomal fractions (6–8, Method A and 7–10, Method B) only, making these potentially useful indicators (markers) for milk exosomes.

As we demonstrated in this study, there are many overlapping

Table 1
Peptides unique to Tetraspanins CD9 and CD81 detected in exosomal fractions from Methods A and B. More peptides of CD9 and CD81 were detected in Method B compared to Method A.

Method A		Method B	
CD9	CD81	CD9	CD81
NLIDSLK	QFYDQALQQAIVDDDANNAK	NLIDSLK FYEDTYNK AIHIALDCCGLTGVPEQFLTDTCPPK	QFYDQALQQAIVDDDANNAK NSLCPSSGNVITNLFK

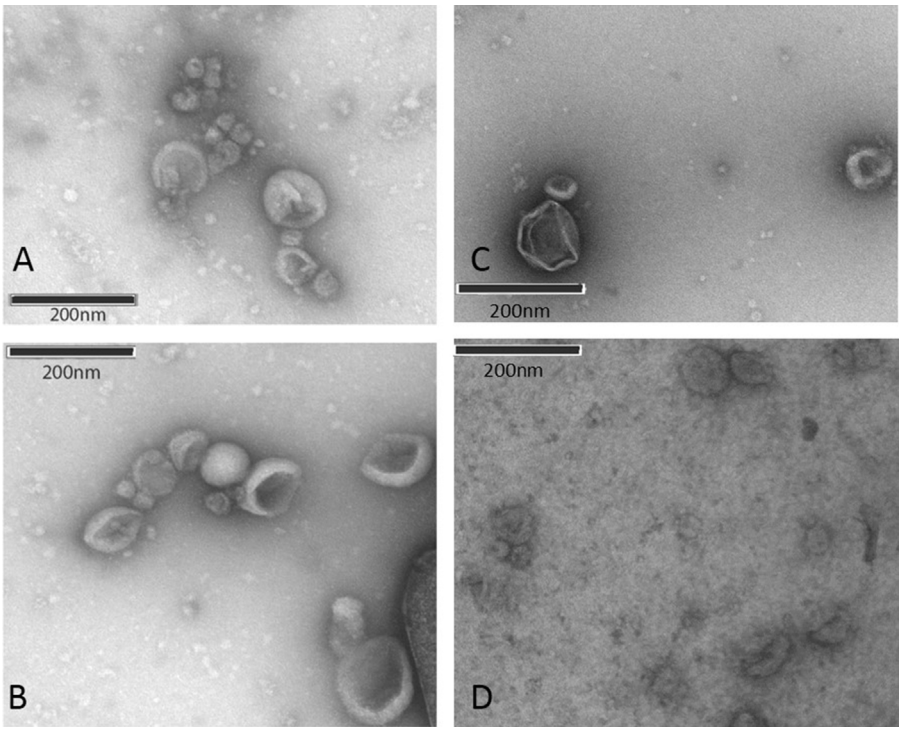

Fig. 7. Transmission electron microscopy displaying exosomal morphology and size. Representative electron micrographs of pooled exosome isolations from (A) and (B): (A) and (B) is to be followed by size exclusion chromatography. (C) and (D) is to be followed by buoyant density gradient centrifugation buoyant density gradient centrifugation (Method A); and (C) and (D): buoyant density gradient centrifugation (Method B). A and C show the presence of some relatively smaller particles, while B and D show relatively larger particles from each population. Scale bar 200 nm.

characteristics between the distinct populations of exosomes and other EVs [31] and so far none of the techniques available are able to sufficiently discriminate and isolate a highly purified population of exosomes [29,33–35]. This is one limitation of the SEC and buoyant density gradient enrichment methods. Careful consideration must be given to the defining characteristics for “exosomes” and “extracellular vesicle” populations as well as other vesicles, as this has the potential to affect downstream experimentation.

There are several benefits of using milk exosomes as a diagnostic tool. Under real life conditions where sample volumes may be limited (*i.e.* tissue biopsies), large volumes of milk can be obtained through a non-invasive procedure. Milk exosomes can be isolated with high yield and quality making milk exosomes an ideal candidate in early diagnostic tests. In addition, milk exosomes are an ideal candidate vehicle for delivering therapeutic agents and are tolerated across species, with bovine milk exosomes known to be taken up by human phagocytes [36] with no adverse immune or inflammatory responses observed [20]. The bi-lipid membrane and aqueous internal core of milk exosomes means they are able to carry both hydrophilic and lipophilic drugs [4]. Coupled with good physical and biological stability, this versatility makes milk exosomes a potential biocompatible and cost-effective vector for therapeutic delivery.

In conclusion, the new method we have described enables the isolation of exosomes with increased yield, reduced contamination and co-precipitation of other macromolecules. This method is also time efficient allowing for higher throughput and is less labour intensive than other widely used methods such as buoyant density gradient centrifugation. With increasing interest into the roles of exosomes in cellular communication (i.e. packaging and delivering biologically active cargo) we believe the reliability of the new method will enable researchers to further investigate the potential utility of exosomes in diagnostics and therapeutics. In the dairy cow, the isolation and evaluation of bovine milk exosomes could enable earlier identification of conditions such as mastitis and metabolic disorders which commonly occur in the transition period.

Authors’ contributions

YQK and KV performed the experiments, collected and interpreted data. HNP, FBA and MDM were responsible for the study concept and participated in the study design. HNP, YQK and FBA, provided initial help with analysis. KV and HNP collected samples. KV and YQK conceptualized and wrote the manuscript. HNP and MDM reviewed the manuscript prior to publication.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.repbio.2017.09.007.

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