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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.

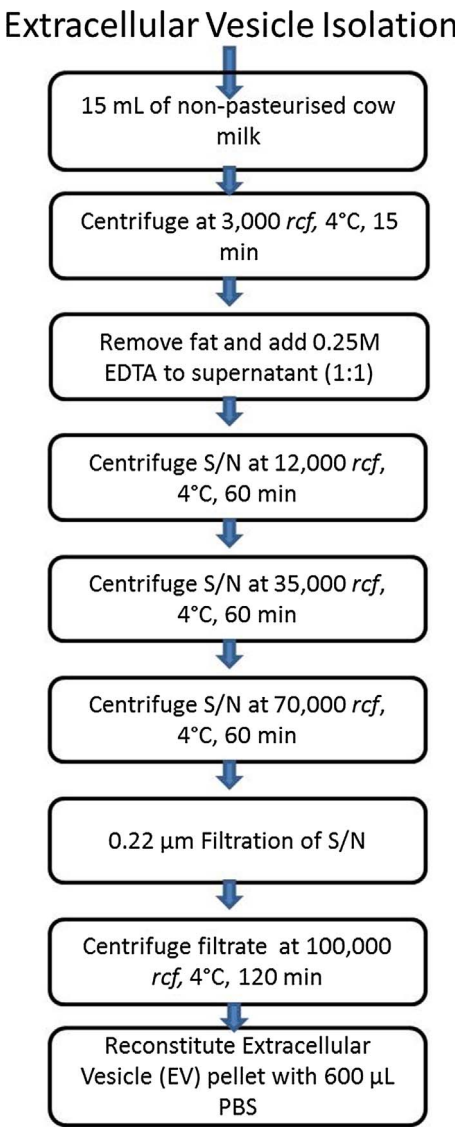


**Fig. 1.** Flowchart for the isolation of extracellular vesicles (EVs) from milk. EVs were isolated from milk by differential ultracentrifugation. Unpasteurised milk was centrifuged at 3000 *rcf* followed by 0.25 M EDTA treatment (1:1; v/v) and supernatants (S/N) subsequently centrifuged at 12,000, 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)