A centrifugal ultrafiltration-based method for enrichment of microvesicles

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Abstract/Introduction

Microvesicles encompass a wide range of small particles variably secreted by many cell types under both normal and pathological conditions. Exosomes, a unique subset, are distinguished by their size (30-100 nm), cellular content, and origin. Free exosomes are generated by release from endosomal-derived multi-vesicular bodies (MVBs) during fusion with the plasma membrane. Most significantly, exosomes carry mRNA, miRNA, and proteins derived from their cells of origin. The release of microvesicles has demonstrated biological relevance; these particles act as mediators of intercellular communication both within the local microenvironment as well as systemically. Exosomes have been linked to a range of physiological processes including: cell proliferation, cancer metastasis, immunomodulatory activity, and propagation of infectious agents such as retroviruses. Given these implications, and their presence in clinical samples (plasma, urine, saliva), exosomes represent a burgeoning target for biomarker discovery with prognostic/diagnostic implications.

Critical to understanding the physiological significance of these particles is development of techniques for reliable isolation of pure fractions. The current gold-standard for exosome isolation is multi-step differential ultracentrifugation. However, this method requires specific, costly instrumentation, is lengthy, and labor intensive. Other methods include immunoaffinity based isolation by magnetic beads or precipitation using commercial solutions. Here we present a rapid ultrafiltration-based approach for microvesicle isolation from biological samples. Since it is spin-based and dependent on size exclusion, the method has broad applications with regards to sample volume and/or type. Optimization was aided by a mid infrared (MIR)-based spectroscopy platform that permits simultaneous monitoring of protein quantitation and analysis of total lipid content during exosome fractionation. Given the ultrafiltration device's capacity for buffer exchange and sample concentration, purified fractions can be easily formatted to meet the requirements of any downstream analysis platform. To demonstrate application, fractions were assayed by flow cytometry, western blotting, ELISAs, and electron microscopy.

Methods

Cell Culture: MDA-MB-231 (ATCC[®] HTB-26[™]) cells were maintained under standard conditions [EmbryoMax[®] DMEM] (Dulbecco's Modified Essential Medium, #SLM-021-B) + 10% FBS). At near 100% confluency, media was removed, cells were washed with PBS (2X), and serum starved (DMEM, no FBS). After 48 hours, the culture supernatant (supe) was harvested.



Protein concentration: Total protein concentration and relative lipid content was determined using a Direct Detect[®] spectrometer (#DDHW000-10-WW).

Immunodetection: Protein samples were separated by SDS-PAGE and transferred to Immobilon[®]-P membrane (#IPVH08130) using a semidry system. In all cases, samples were prepared under reducing conditions. Blots were processed by SNAP i.d.[®] 2.0 Protein detection system (#**SNAP2BASE**) using the following antibodies: Flotillin-1, HSP90α, HSP70, Annexin A2, and Alix (all Cell Signaling Technology). Proteins were detected by chemiluminescence after 5 minutes incubation with Luminata[™] Forte western HRP Substrate (#WBLVF0500).

ELISA: Assay were performed using the ExoELISA[™] kits for CD63 and CD9 (System Biosciences) according to the user manual Briefly, lysed exosomes were immobilized on a microtiter plate. Following blocking, wells were incubated with 1Y Ab (anti-CD9 or CD63) followed by HRP-conjugated 2Y Ab (goat anti-rabbit). Accumulation of a colorimetric substrate (TMB) is read at 450 nm to determine exosome number.

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Method Validation

Relative exosome quantitation via microbead-based flow cytometry

Determine relative efficiency of exosome recovery during process optimization





Capture – PureProteome[™] SA beads + biotinvlated Ab (CD9, CD63, CD81) Detection possible via two methods

 Direct – Exosomes pre-labeled with Bodipy[®] FL Dye (Life Technologies) Indirect – PE conjugated Ab (anti-CD63, CD9, CD81, EpCAM)

Acquire samples on a guava easyCyte[™] 8HT flow cytometer

Establish a standard curve using sample titration (MFI signal), Interpolate samples (Relative)

Method Optimization







Given the differences in spin duration needed to achieve sample concentration, identifying the spin speed (A) and MWCO format (B) most compatible with high recovery was critical. Yield was greatest at 4000g (Maximum velocity). MWCOs > 10 kDa showed significant exosome loss; given their size, loss is most likely due to high deformability. (C) The process was monitored using a Direct Detect[®] spectrometer. MIR-based quantification relies on relative Amide I band (1600-1690 cm⁻¹) signal strength. Both graphs show the presence of a distinct peak at 1590 cm⁻¹ which obscures the ability to accurately determine protein concentration. The responsible molecule was eliminated by buffer exchange without affecting exosome yield.

Serum Albumin

FBS contains high amounts of albumin; overly abundant proteins will greatly limit detection of the far rarer target species (as illustrated below).



To address this, albumin removal was attempted via vacuum filter-based size using Biomax[®] 500 membrane (500 kDa MWCO, #PBVK04710). Gel analysis of the wash fractions (F1-6) and final concentrated sample are shown. While membrane filtration resulted in albumin depletion, there was also loss of other protein species (compared to standard UF concentration). Significant exosome loss was confirmed by the bead capture assay.



Electron Microscopy – confirmation of microvesicles

Centrifugal Ultrafiltration-purified microvesicle fractions were whole mounted, stained [methylcellulose and reverse staining] (Uranyl Acetate)], and visualized by electron microscopy. Measurement of several microvesicles indicates a range of diameters between 66 to 120 nanometers. Several multivesicles (up to 485 nm) were also observed within the preparations. TEM preparation and visualization was performed at Core Electron Microscopy Facility, UMass Med, Worcester, MA.



Comparative Analysis – Ultrafiltration vs. Ultracentrifugation + Precipitation





(A) Ultrafiltration (UF) demonstrated similar recovery to ultracentrifugation (UC) when assayed for spiked-in labeled exosomes; both outperformed a precipitation method (PPT). (B) Both showed similar yield of total exosomes as determined by CD9-Alexa647 staining of bead-captured exosomes. (C) These findings were consistent with results from two independent ELISA assays.

Due to their endosomal origin, all exosomes contain membrane transport and fusion proteins (GTPases, Annexin A2, Flotillin-1), tetraspannins (CD9, CD63, CD81, CD82), heat shock proteins (Hsp70, Hsp90α), proteins involved in multivesicular body biogenesis (Alix, TSG101). This signature provides an additional parameter for assessing the relative purity, and yield, of fractions. Four exosome-specific markers were detected in the ultrafiltration retentate by immunoblotting using the SNAP i.d.[®] 2.0 protein detection system.

Summary

Size-selective ultrafiltration provides a relative fast, easy, and effective alternative method for exosome enrichment from serum-free cell culture media



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Supernatant was harvested from MDA cells following 48 HR serum starvation. An aliquot was processed via ultrafiltration; the resulting fraction was labeled with Bodipy[®] FL Dye. Labeled microvesicles (@ 20% load – 10 mL starting supe) were spiked into a second enrichment (40 mL) performed by either ultrafiltration (UF), ultracentrifugation (UC), or precipitation [PPT -ExoQuick-TC[™] Solution (System Biosciences)]. Recovered fractions were assayed for microvesicle recovery (CD63 Bead capture/flow cytometry), total content (ELISAs), and Western blotting (UF and UC only).





The following fractions (in each case, 1 µg total protein) were assayed: (1) Starting culture, (2) UF filtrate 1st spin, (3) UF filtrate 2nd spin, (4) UF retentate, (5) UC supernatant, and (6) UC pellet

- Fractions purified using Amicon[®] Ultra filters demonstrated similar characteristics (exosome yield and protein cargo) as the differential ultracentrifugation method
- Bead-based flow cytometry offers a platform for relative exosome quantitation
- The presence of high levels of albumin presents a challenge to working with cultures grown under standard conditions (+ serum)