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Benchtop isolation and characterization of functional exosomes by sequential filtration

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ABSTRACT

Early and minimally invasive detection of malignant events or other pathologies is of utmost importance in the pursuit of improved patient care and outcomes. Recent evidence indicates that exosomes and extracellular vesicles in serum and body fluids can contain nucleic acid, protein, and other biomarkers. Accordingly, there is great interest in applying these clinically as prognostic, predictive, pharmacodynamic, and early detection indicators. Nevertheless, existing exosome isolation methods can be time-consuming, require specialized equipment, and/or present other inefficiencies regarding purity, reproducibility and assay cost. We have developed a straightforward, three-step protocol for exosome isolation of cell culture supernatants or large volumes of biofluid based on sequential steps of dead-end pre-filtration, tangential flow filtration (TFF), and low-pressure track-etched membrane filtration that we introduce here. Our approach yields exosome preparations of high purity and defined size distribution and facilitates depletion of free protein and other low-molecular-weight species, extracellular vesicles larger than 100 nm, and cell debris. Samples of exosomes prepared using the approach were verified morphologically by nanoparticle tracking analysis and electron microscopy, and mass spectrometry analyses confirmed the presence of previously reported exosome-associated proteins. In addition to being easy-to-implement, sequential filtration yields exosomes of high purity and, importantly, functional integrity as a result of the relatively low-magnitude manipulation forces employed during isolation. This answers an unmet need for preparation of minimally manipulated exosomes for investigations into exosome function and basic biology. Further, the strategy is amenable to translation for clinical exosome isolations because of its speed, automatability, scalability, and specificity for isolating exosomes from complex biological samples.

1. Introduction

The early detection of cancer enables more immediate intervention and could drastically reduce cancer-associated mortality and morbidity. Nevertheless, few established screening methods fulfill the World Health Organization guidelines regarding specificity, mortality reduction, and cost effectiveness [1,2]. Recent approaches for the early detection of cancer have focused on identifying protein biomarkers, metabolic cancer-associated events, and circulating tumor cells [3]. However, the low relative abundance of protein markers in relation to the total protein content in human plasma makes detection of these markers difficult and somewhat unspecific [4]. Analyses of metabolic cancer-associated events are, while potentially promising, yet to be translated into widespread clinical utility. Circulating tumor cells are particularly difficult to identify and separate from blood samples owing to their paucity and high rate of anoikis-associated cell death and attrition [5].

Considerable recent interest in exosomes and microvesicles has revealed these extracellular entities to be abundantly represented in serum and biofluids and to contain nucleic acid, protein, and

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other tumor biomarkers. Accordingly, there is also an emphasis on understanding their significance in tumorigenesis and metastasis [6,7].

Exosomes are defined as small, lipid-bilayer-enclosed vesicles with a diameter of approximately 40–100 nm that are released into the extracellular milieu as a result of the fusion of intracellular multivesicular bodies (MVB) with the plasma membrane. Thus, typical exosome-associated proteins (e.g. CD63, CD81 and Rab proteins) are also characteristic of endosome trafficking [8]. Microvesicles represent a distinct class of extracellular vesicles that originate via external budding of the plasma membrane. They are more heterogeneous in size (100–1000 nm), and can potentially contain a broader array of cell surface proteins [9,10].

Exosomes and other extracellular vesicles have been reported to play important roles in physiological cell communication and tissue homeostasis events [8–10]. They facilitate the transfer of nucleic acids locally between neighboring cells, but their presence in body fluids, such as blood [11], urine [12], or breast milk [13] also enables epigenetic reprogramming of target cells at distant sites [14]. These particular exosome capabilities have also been found to play a crucial role in tumor growth and metastasis [15]. Their supportive role in formation of premetastatic niches suggests an early abundance of exosomes in body fluids such as blood and urine before the occurrence of tumor cell extravasation and dissemination [6]. Thus, the clinical detection of such tumorigenesis-supporting exosomes would be an ideal method for the early detection or therapeutic targeting of cancer [16,17]. Such an approach, however, requires standardized and reliable methods for isolating patient-derived exosomes. Currently, exosomes are frequently enriched from biofluids by protocols involving steps of centrifugation and ultracentrifugation, sometimes employing additional density gradient ultracentrifugation [18]. These procedures, however, require dedicated, expensive equipment and have been shown to yield highly aggregated exosomes, accumulated protein and protein aggregates, and other non-exosome contaminants [19]. These obstacles can confound downstream functional assays and hinder robust translation into clinical diagnostic utility. Other protocols involving antibody-coated beads are only suitable for composition analysis and not for functional studies, since modifications of the exosome surface during the isolation process are difficult to avoid [20].

Various chemical agents have also been proposed to facilitate exosome isolation using reduced centrifugal force, thus obviating the requirement for ultracentrifugation. However, the ability of available reagents to distinguish exosomes from microvesicles or protein aggregates remains uncertain [9]. Microfluidic approaches based on affinity capture or novel filter materials have been reported as well [21,22]. These are promising for some applications, but, as currently implemented, sample throughput is limited to 0.06–0.96 ml per hour. A method to harvest exosomes from cell culture supernatants has been proposed by Lamparski *et al.* [19]. This protocol employs tangential flow filtration (TFF) and ultracentrifugation on a deuterium-sucrose cushion to produce clinical-grade therapeutic exosomes and it has been implemented in clinical trials [23,24]. Although this method is a vast improvement over previous techniques, it still requires access to an ultracentrifuge. Such ultracentrifugation steps are incompatible with future high-throughput automation of the exosome isolation and characterization process and, consequently, development of clinically implementable exosome diagnostics or therapies.

The aim of our work was to improve on the currently available methods by demonstrating a simplified, clinically applicable method of robust and specific isolation of exosomes from biofluids. In these proof-of-principle studies, cell culture supernatant is used as a readily obtainable biofluid with properties similar to urine or diluted plasma.

Our proposed method enables the efficient and simplified purification of functional exosomes, and may open new paths towards the early detection of malignant events. We believe that cancer exosomes hold a unique fingerprint of the original and progressing neoplasm [17,25], and that analysis of circulating exosomes and the discrimination between physiological and cancer-derived exosomes could prove to be highly valuable in future early-stage cancer diagnostics.

2. Materials and methods

2.1. Exosome-depletion from cell culture media

Fetal bovine serum (FBS)-containing mammalian culture medium was depleted of exosomes and free protein aggregates by tangential flow filtration at 4 °C (Fig. 1) using a 500-kDa modified polyethersulfone (mPES) hollow fiber filter MidiKros module and KrosFlo Research II TFF System (Spectrum Laboratories, Rancho Dominguez, CA, USA). The maximum transmembrane pressure was held below 1.5 pounds per square inch (PSI) in order to prevent driving of small elastic vesicles through the filter membrane and into the permeate. The resultant permeated medium was sterile filtered in normal (dead-end) filtration mode through a 0.1-μm polyethersulfone (PES) filter membrane (Membrane Code VEPP, Millipore, Billerica, MA, USA) in a biological culture hood and stored at 4 °C.

2.2. Exosome production

Equal numbers of MDA231 breast cancer cells were seeded into six T175 flasks (1.29×10^6 cells per flask). After two days of culture (~70% confluence) the medium was carefully removed, the cells were rinsed twice with phosphate-buffered saline (PBS), and exosome-depleted medium (containing 0.2% FBS) was added. The supernatant was collected at 48 h, and exosome isolation was initiated immediately thereafter (Fig. 2).

2.3. Exosome isolation by sequential filtration

2.3.1. Dead-end (normal) filtration (Step 1)

The supernatant (150 ml) was depleted of floating cells and cell debris by dead-end filtration at 22 °C. We used a 0.1-μm Millipore Express (PES) membrane Stericup Filter Unit with low protein-binding properties. The mesh-like filter quickly eliminated large and rigid media components; exosomes and larger flexible particles (such as microvesicles) were able to pass through the filter, even if their diameter was larger than 100 nm. Fifty milliliters of PBS was added to the filter to wash out residual exosomes for a higher yield.

2.3.2. Tangential flow filtration (Step 2)

Filtrate from Step 1 was transferred into a conical bottle under sterile conditions and subjected to tangential flow filtration at 4 °C (Fig. 1). A Spectrum Laboratories KrosFlo Research II TFF pump fitted with a 500-kDa MWCO mPES hollow fiber MidiKros filter module was used. The exosome-containing sample was continuously aspirated from the conical bottle, pumped through the hollow fiber system, and recirculated into the conical bottle. Small molecules, including free protein, were driven through the hollow fiber pores, subsequently eluted as permeate, and eventually discarded. Molecules too large to pass through the pores, such as exosomes and microvesicles, were kept in circulation as retentate, and thus concentrated in the conical bottle to a final volume of 50 ml.

Pressure monitoring and adjustable clamps were used to stably maintain a very low transmembrane pressure of between 1.5 and 2.5 PSI in order to minimize the loss of small exosomes