Exosome isolation from cell culture by ultracentrifugation: optimized protocols

Introduction

Exosomes are a subtype of extracellular vesicles (EVs) with unique properties and biogenesis pathways. Exosome vesicles (30–150 nm in diameter) are generated by the inward budding of endosomal membranes, while microvesicles (MVEs) (100–1,000 nm in diameter) are formed by the outward budding of the plasma membrane (Figure 1) [1-3].

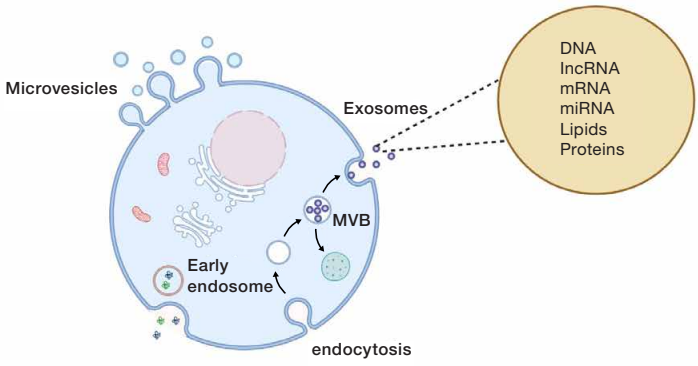

Figure 1. The exosome biogenesis pathway [1]. MVB: multivesicular body. Figure used under terms of the Creative Commons Attribution 4.0 International Public License.

The characteristics and cargo of EVs are influenced by the type and state of the cells involved in their generation. Evidence indicates that exosomes can transport genetic information from origin cells to recipient cells. Surface receptors on exosomes enable them to be targeted and captured by recipient cells. This allows them to modulate signaling pathways. These unique properties of exosomes draw the attention of many researchers for their use as disease biomarkers and drug delivery vehicles [1-3].

Exosomes can be isolated from different types of body fluids, such as semen, urine, breast milk, saliva, amniotic fluid, sputum, and cerebrospinal fluid. Also, many cultured cells can secrete exosomes when cultivated in T-flasks and cell culture media [4-17]. Due to the presence of lipoproteins and other EVs in body fluids, providing pure exosomes for therapeutic applications can be a challenge. Therefore, cell culture conditioned medium (CCM) has become one of the main sources of exosome isolation [4-17].

Because of its simplicity and proven efficacy, the ultracentrifugation technique is the preferred method for the purification of exosomes. The primary purpose here is to provide a workflow to isolate exosomes from CCM.

Protocols

Protocol 1. Differential ultracentrifugation technique for isolation of exosomes from human mesenchymal stem cells (hMSCs)

To prepare the conditioned medium, cryopreserved bone marrow–derived mesenchymal stem cells (BMSCs) and adipose tissue–derived mesenchymal stem cells (ADSCs) were revived and expanded in Gibco™ DMEM, low glucose (DMEM-LG) with 10% fetal bovine serum (FBS) and 2 mM Gibco™ L-glutamine, 100 U/mL of penicillin, and 100 U/mL of streptomycin [4]. When the cells reached 70% confluence, they were washed three times with phosphate-buffered saline (PBS) and cultured in a serum-free medium. After culturing the hMSCs for 48 hours, a serum-free medium was pooled together for exosome isolation [4].

1. Spin the conditioned medium at 300 x *g* for 10 min at 4°C [4].
2. Transfer the supernatant to a new centrifuge tube and spin it at 2,000 x *g* for 10 min at 4°C [4].
3. Transfer the supernatant to a new centrifuge tube and spin it at 10,000 x *g* for 30 min at 4°C [4].
4. Transfer the supernatant to a sterile ultracentrifuge tube that was rinsed with PBS and centrifuge it at 100,000 x *g* for 90 min at 4°C [4].
5. Resuspend the pellets with sterile PBS and repeat the centrifugation at 100,000 x *g* for another 90 min at 4°C [4].
6. To store isolated exosomes, resuspend the pellets in 500 µL of sterile PBS and store at –80°C [4].

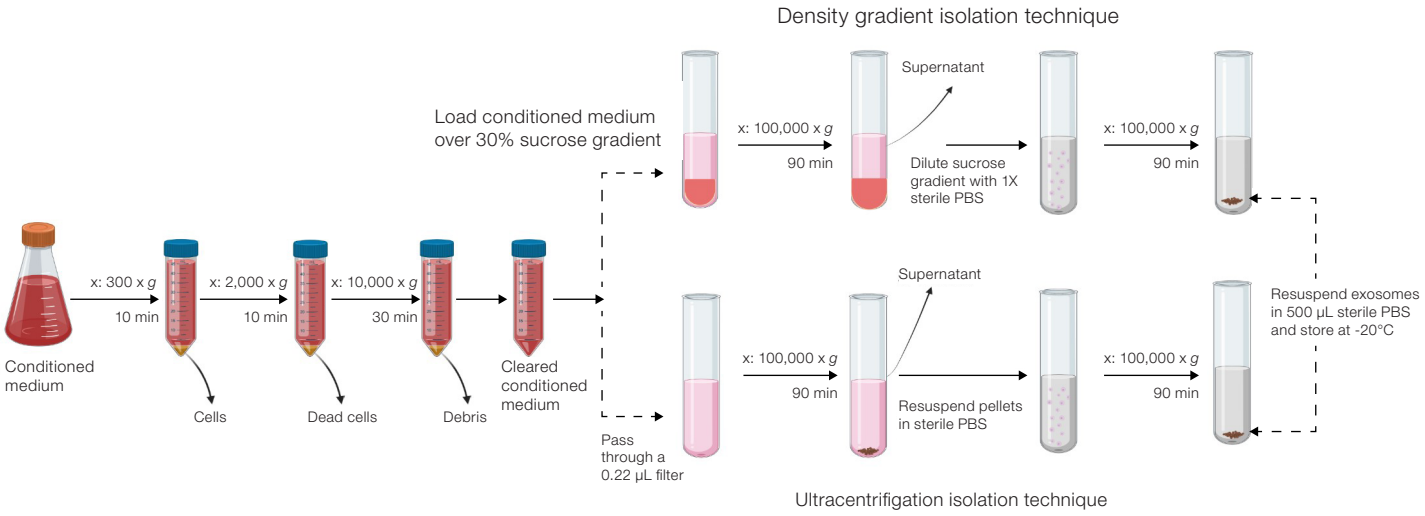

Figure 2. Workflow for exosome isolation from MSC culture medium by differential ultracentrifugation and one-step sucrose density gradient ultracentrifugation [4]. Courtesy of Dr. Mohanty's lab, Stem Cell Facility DBT–Center of Excellence for Stem Cell Research, All India Institute of Medical Sciences, New Delhi, India (www.mohantylab.in).

Protocol 2. One-step density gradient technique for isolation of exosomes from hMSCs

Even though the differential ultracentrifugation technique is the most commonly used procedure for exosome isolation, other particles like lipoproteins and proteins are also isolated with the exosomes during ultracentrifugation. Therefore, to produce exosomes with higher purity, researchers in Dr. Sujata Mohanty's laboratory combined differential centrifugation with one-step sucrose density gradient ultracentrifugation [4]. In this procedure, the isolation starts with the removal of debris and dead cells from the conditioned medium, which is then added to a 30% sucrose solution. During the one-step sucrose density gradient ultracentrifugation, denser proteins and lipoproteins are separated from exosomes because of the similarity of the densities of exosomes (1.12–1.18 g/mL) and the 30% sucrose gradient (1.15–1.19 g/mL) [4,5,10].

1. Centrifuge the conditioned medium at 300 x *g* for 10 min at 4°C [4].
2. Transfer the supernatant to another tube and centrifuge it at 2,000 x *g* for 10 min at 4°C [4].
3. Transfer the supernatant to a new tube and centrifuge it at 10,000 x *g* for 30 min at 4°C [4].
4. Prepare 4 mL of a 30% sucrose solution and add it to the ultracentrifuge tube [4].
5. Slowly and gently load about 30 mL conditioned medium from step 3 to the top of the sucrose solution. Be careful to not mix these two distinct phases [4].
6. Spin the tube an ultracentrifuge at 100,000 x *g* for 90 min at 4°C using a swinging bucket rotor [4].
7. Pipet off the supernatant and transfer the exosome-containing sucrose to another ultracentrifuge tube [4].
8. Dilute 5 mL of the exosome-containing sucrose with 5 mL of sterile PBS [4].
9. Ultracentrifuge the mixture at 100,000 x *g* for 90 min at 4°C [4].
10. Aspirate the supernatant and collect exosome pellets by resuspending them in 1 mL of PBS.
11. Exosome pellets can be stored at –80°C or processed for further analysis [4].

Figure 2 illustrates both the differential ultracentrifugation technique and the improvised sucrose gradient technique.

Results

A nanoparticle tracking analysis (NTA) confirmed that both methods of isolation were able to obtain exosomes between 30 and 120 nm, which is within the expected range [4]. Sucrose gradient isolation has been shown to yield a higher concentration of exosomes when compared with differential ultracentrifugation [4]. The concentration of exosomes isolated from BMSCs increased from 2 x 10^9 per mL using the ultracentrifugation technique to 7 x 10^9 per mL using the sucrose gradient ultracentrifugation technique. For the same comparison for ADSCs, the concentration of exosomes increased from 3.06 x 10^9 per mL to 5.50 x 10^9 per mL (Figure 3).

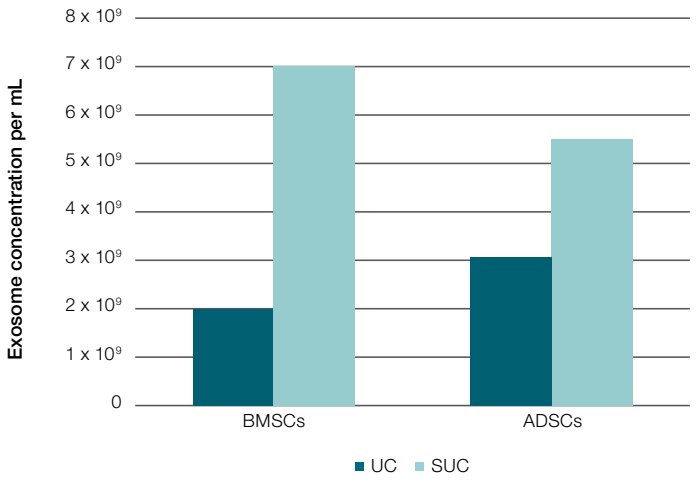

Figure 3. The concentration of exosomes after isolation with sucrose gradient ultracentrifugation (SUC) compared with ultracentrifugation (UC) [4]. Figure used under terms of the Creative Commons Attribution 4.0 International Public License.

Summary

Differential ultracentrifugation is a commonly used method for isolating exosome vesicles from biological fluids. The workflow for isolation of exosomes with ultracentrifugation starts with different steps of centrifugation that remove components such as large vesicles, debris, and cells; these steps are then followed by exosome precipitation with differential ultracentrifugation. Due to the presence of other types of extracellular vesicles and lipoproteins, differential ultracentrifugation should be combined with another technique in order to isolate pure exosomes. The combination of sucrose gradient separation followed by ultracentrifugation at 100,000 x *g* has been proven to produce high-quality exosomes with a high yield. Existing research protocols and instruments for the isolation of exosomes from different cell lines are shown in Table 1.

Table 1. Protocols for exosome isolation and purification reported in the literature.

Sample origin	RCF	Technique	Duration of ultracentrifugation	Rotor	Instrument	Ref.
Human mesenchymal stem cells (hMSCs)	100,000 x *g*	Differential ultracentrifugation	90 min	Thermo Scientific™ swinging bucket rotor	Thermo Scientific™ Sorvall™ WX 90+ Ultracentrifuge	4
		Density gradient ultracentrifugation	90 min	Thermo Scientific swinging bucket rotor	Sorvall WX 90+ Ultracentrifuge	4
Human tongue squamous cell carcinoma cell line (Tca8113)	100,000 x *g*	Differential ultracentrifugation	70 min	Swinging bucket rotor	Thermo Scientific™ Sorvall™ Ultra Pro 80 Ultracentrifuge	5
		Density gradient ultracentrifugation	70 min	Thermo Scientific™ SureSpin™ 630 Swinging Bucket Rotor	Sorvall Ultra Pro 80 Ultracentrifuge	5
HCT116 p53 +/+ human colon carcinoma cells	100,000 x *g*	Differential ultracentrifugation	90 min	Thermo Scientific™ Fiberlite™ F50L-8 x 39 Fixed-Angle Rotor	Sorvall WX 90+ Ultracentrifuge	6
Cervical cancer cell lines (CaSki, SiHa, and HeLa cells)	100,000 x *g*	Differential ultracentrifugation	120 min	SureSpin 630 Swinging Bucket Rotor	Sorvall WX 90+ Ultracentrifuge	7
Mesenchymal stem cells (MSCs)	110,000 x *g*	Differential ultracentrifugation	90 min	Thermo Scientific™ Sorvall™ AH-629 Swinging Bucket Rotor	Sorvall WX 90+ Ultracentrifuge	8

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