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A Simple Benchtop Filtration Method to Isolate Small Extracellular Vesicles from Human Mesenchymal Stem Cells

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Abstract

The ultracentrifugation-based process is considered the common method for small extracellular vesicles (sEVs) isolation. However, the yield from this isolation method is relatively lower, and these methods are inefficient in separating sEV subtypes. This study demonstrates a simple benchtop filtration method to isolate human umbilical cord-derived MSC small extracellular vesicles (hUC-MSC-sEVs), successfully separated by ultrafiltration from the conditioned medium of hUC-MSCs. The size distribution, protein concentration, exosomal markers (CD9, CD81, TSG101), and morphology of the isolated hUC-MSC-sEVs were characterized with nanoparticle tracking analysis, BCA protein assay, western blot, and transmission electron microscope, respectively. The isolated hUC-MSC-sEVs' size was 30-200 nm, with a particle concentration of 7.75×10^{10} particles/mL and a protein concentration of 80 µg/mL. Positive bands for exosomal markers CD9, CD81, and TSG101 were observed. This study showed that hUC-MSC-sEVs were successfully isolated from hUC-MSCs conditioned medium, and characterization showed that the isolated product fulfilled the criteria mentioned by Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV 2018).

Introduction

According to MISEV 2018, sEVs are non-replicating lipid bilayer particles with no functional nucleus present, with a size of 30-200 nm[1]. MSC-derived sEVs contain important signaling molecules that play important roles in tissue regeneration, such as microRNA, cytokines, or proteins. They have increasingly become a research

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"hotspot" in regenerative medicine and cell-free therapy. Many studies have shown that MSC-derived sEVs are as effective as MSCs in treating different conditions, such as immunomodulation[2,3,4,5], enhancing osteogenesis[6], diabetes mellitus[7,8], or vascular regeneration[9,10]. As early phase trials progress, three main key issues in relation to the clinical translation of MSCs-EVs have been highlighted: the yield of the EVs, the purity of the EVs (free from cell debris and other biological contaminants such as protein and cytokines), and the integrity of the phospholipid bilayer membrane of the EVs after isolation.

Various methods have been developed to isolate sEVs, exploiting the density, shape, size, and surface protein of the sEVs[11]. The two most common methods in sEVs isolations are ultracentrifugation-based and ultrafiltration-based techniques.

Ultracentrifugation-based methods are considered gold standard methods in sEVs isolation. Two types of ultracentrifugation techniques that are usually employed are differential ultracentrifugation and density gradient ultracentrifugation. However, ultracentrifugation methods often result in low yield and require expensive equipment for high-speed ultracentrifuge (100,000-200,000 × *g*)[11]. Furthermore, ultracentrifugation techniques alone are inefficient in separating EV subtypes (sEVs and large EVs), resulting in an impure sediment layer[11]. Lastly, density gradient ultracentrifugation could be also time-consuming and require additional precaution steps such as sucrose buffer addition to inhibit the gradient damage during acceleration and deceleration steps[12]. Hence, ultracentrifugation usually leads to a relatively low yield and is not capable of discriminating between different populations of EVs[13], which limits its application for large-scale EV preparation[11].

The second method of EV isolation is via ultrafiltration, which is based on size filtration. Ultrafiltration is relatively time- and cost-effective compared to ultracentrifugation, as it does not involve expensive equipment or long processing times[14]. Hence, ultrafiltration appears to be a more effective isolation technique than both aforementioned ultracentrifugation methods. The isolated products can be more specific based on pore sizes and higher yield[15]. However, the additional force incurred during the filtration process may result in the deformation or eruption of the EVs[16].

The current paper proposed a cost- and time-effective benchtop protocol for isolating MSC-derived sEVs for downstream analysis and therapeutic purposes. The method described in this paper combined a simple filtration method with bench top centrifugation to isolate high-yield and good-quality EVs from hUC-MSCs for downstream analysis, including particle size analysis, biomarker assay, and electron microscopic imaging.

Protocol

NOTE: See the **Table of Materials** for details about all materials, equipment, and software used in this protocol.

1. Human umbilical cord mesenchymal stem cells and culture

1. Culture the hUC-MSCs at a seeding density of 5 × 10^3/ cm^2 in DMEM, supplemented with 8% Human Platelet Lysate and 1% Pen-Strep. Incubate the cells at 37 °C in 5% CO_2. Replace the cell culture medium every 3 days to ensure proper cell growth.
2. Expand the cells to passage 5 in T175 flasks for sEV isolation.

NOTE: Many flasks are needed to harvest a high yield of sEVs (the yield increases with the number of cells).

2. Small extracellular vesicle isolation from hUC-MSCs

1. Replace the culture medium with fresh phenol-red free DMEM supplemented with 1% Pen-Strep [Conditioned medium (CM)] when the culture reaches 70%-80% confluency at passage 5.
   CAUTION: Use basal media only; avoid FBS and human platelet lysate supplement to avoid contamination by external sEVs.
2. After 24 h, centrifuge the CM at 200 × *g* for 5 min at 4 °C to remove cell debris. Collect and filter the supernatant through a 0.22 µm filter to remove particles larger than 220 nm.
3. Fill the centrifugal filter unit with 30 mL of phosphate-buffered saline (PBS) filtered through a 0.2 µm filter. Centrifuge the centrifugal filter unit at 3,500 × *g* for 5 min at 4 °C.
4. Fill the filtered CM into the centrifugal filter unit (each unit's maximum volume is 70 mL). Centrifuge the CM at 3,500 × *g* at 4 °C.
   NOTE: The duration of centrifugation should be monitored from time to time until the solution has reached the surface of the filter.
5. Discard the solution in the filtrate solution cup. Reverse centrifuge the sample filter cup with concentrate collection cup at 1,000 x g at 4 °C for 2 min.
6. Transfer the concentrated CM back to the centrifugal filter unit. Add 30 mL of filtered PBS into the centrifugal filter unit and centrifuge the unit at 3,500 × g at 4 °C.
7. Discard the solution in the filtrate solution cup. Install a concentrate collection cup on the filter unit and reverse-centrifuge at 1,000 × *g* for 2 min at 4 °C to obtain the purified sEVs.
8. Filter the sEVs through a 0.22 µm syringe filter.
9. Transfer the samples to new tubes and store them at -80 °C for further analysis.

3. Characterization of hUC-MSC-sEVs

1. Nanoparticle tracking analysis
   1. Dilute the isolated hUC-MSC-sEVs in filtered PBS to 20-100 particles/frame.
   2. Introduce 1 mL of the diluted sample into the NTA chamber using 1 mL disposable syringes.
   3. Set the measurement settings accordingly: adjust the **camera level** to **level 14**, determine the **detection threshold** to include as many particles as possible with the restriction that **10-100 red crosses** are counted, and the **blue cross count** is limited to **five**.
   4. For each measurement, record five 1 min videos and analyze them using the NanoSight Software with a detection threshold of five.
   5. Take measurements in triplicate for each sample.
2. Western blotting analysis
   1. Lyse the hUC-MSC-sEVs with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer, incubate for 30 min at 4 °C, and centrifuge at 200 × *g* for 5 min at 4 °C. Collect the supernatant.
   2. Quantify the protein using a Bicinchoninic Acid (BCA) Protein Assay Kit. Assemble the gel electrophoresis set accordingly and perform gel

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electrophoresis at 90 V until the protein reaches the stacking gel's end. Change the voltage to 200 V to separate the proteins until the end of the resolving gel.

3. Perform semidry transfer to transfer the proteins from the gel to a polyvinylidene difluoride (PVDF) membrane at 15 V for 1 h.

4. Block the PVDF membrane with 3% bovine serum albumin (BSA)/Tris-buffered saline-0.1% v/v Tween 20 (TBS-T) for 1 h on a shaker at room temperature. Incubate the PVDF membrane with primary antibodies as follows: mouse anti-CD 9 monoclonal antibody (1:500), mouse anti-CD 81 monoclonal antibody (1:500), mouse anti-GRP 94 monoclonal mouse anti-TSG 101 monoclonal antibody (1:500) at 4 °C overnight with constant shaking.

5. The next day, wash the PVDF membrane 5 x 5 min with TBS-T and further incubate with a secondary antibody: horseradish peroxidase-conjugated mouse IgG kappa binding protein (m-IgGκ BP-HRP) (1:5,000) for 1 h with agitation at room temperature.

6. Again, wash the PVDF membrane with TBS-T five times, and visualize the membrane in a charge-coupled (CCD) imager using a chemiluminescence detection reagent. Analyze the expression level of the proteins using ImageJ software. First, open the image file in ImageJ (**File | Open…**). Enhance the quality of the image by adjusting the brightness and contrast (**Image | Adjust | Brightness/Contrast**). Adjust the image until the blots are clearly visible, click on the **Apply** button, and then save the images in TIFF format (**File | Save As | TIFF…**).

3. Transmission electron microscope

    1. Dilute one part of the hUC-MSC-sEVs sample with four parts of filtered PBS to a total volume of 10 µL and incubate on a carbon-coated copper grid for 15 min.

    2. Remove excess sample using a laboratory wipe and allow it to air-dry for 3 min.

    3. Incubate 10 µL of 1% phosphotungstic acid (PTA) solution to stain the sample for 3 min.

    4. Remove excess 1% PTA solution using a laboratory wipe and allow it to air-dry for 3 min.

    5. Use the sample for transmission electron microscope imaging.

    NOTE: Refer to **Figure 1** for the summarized schematic experiment steps.

Representative Results

Figure 2 shows that hUC-MSC-sEVs have a particle size mode at 53 nm, while other significant peaks of particle size were 96 and 115 nm. The concentration of hUC-MSC-sEVs measured by NTA was 7.75×10^{10} particles/mL. The protein concentration of hUC-MSC-sEVs measured with the BCA assay was approximately 80 µg/mL.

In western blotting analysis, hUC-MSC-sEVs demonstrated positive bands for exosomal markers CD9, CD81, and TSG101, but were negative for GRP94 (**Figure 3**). GRP94 is an endoplasmic reticulum marker commonly used as a negative marker for sEVs. The isolated hUC-MSC-sEVs were visualized under TEM to determine their size and morphology.

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hUC-MSCs-sEVs sized ~100 nm and showed a cup-like bilayer membrane structure (**Figure 4**).

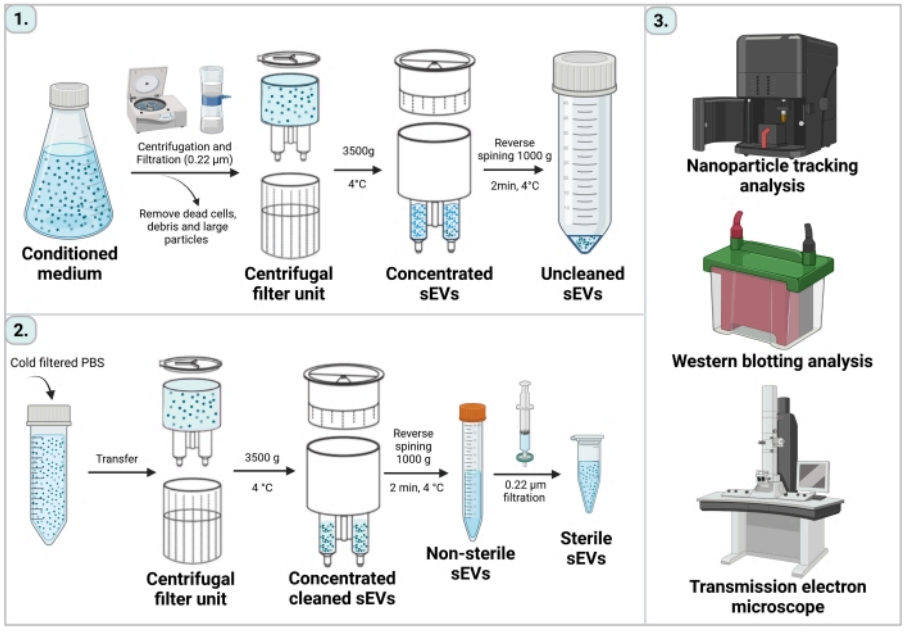

Figure 1: Working schematic of the isolation, cleaning, and characterization of sEVs. The conditioned medium was collected from MSC culture and filtered through a 0.22 µm filter to remove large particles. The filtered medium was transferred to a centrifugal filter unit to concentrate the sEVs and reverse-centrifuged to collect the concentrated sEVs. The concentrated sEVs were then resuspended with cold, filtered PBS, and the steps were repeated with a centrifugal filter unit for the washing steps. Lastly, the sEVs were sterilized by 0.22 µm filtration before being characterized using nanoparticle tracking analysis, western blotting analysis, and transmission electron microscopy. Please click here to view a larger version of this figure.

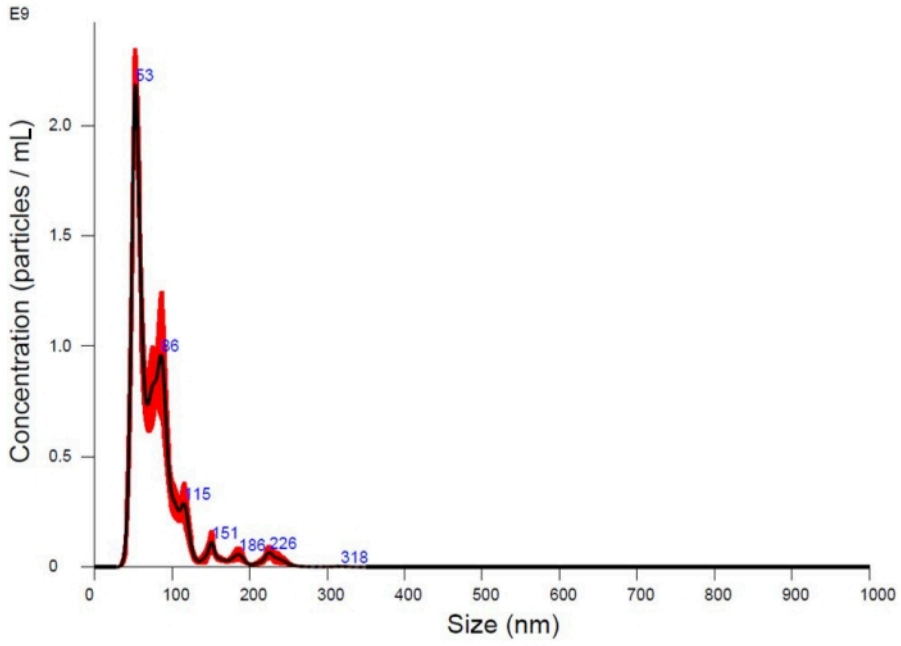

Figure 2: Nanoparticle Tracking Analysis of isolated hUC-MSC-sEVs. The hUC-MSC-sEVs were 100-1,000x diluted with clarified PBS and measured using Nanosight NS300 equipped with a CMOS camera, a 20x objective lens, a blue laser module (488 nm), and NTA software. The figure is a representation of NTA in triplicate. Abbreviations: hUC-MSC-sEVs = human umbilical cord-derived MSC small extracellular vesicles; NTA = nanoparticle tracking analysis; CMOS = complementary metal-oxide semiconductor. Please click here to view a larger version of this figure.

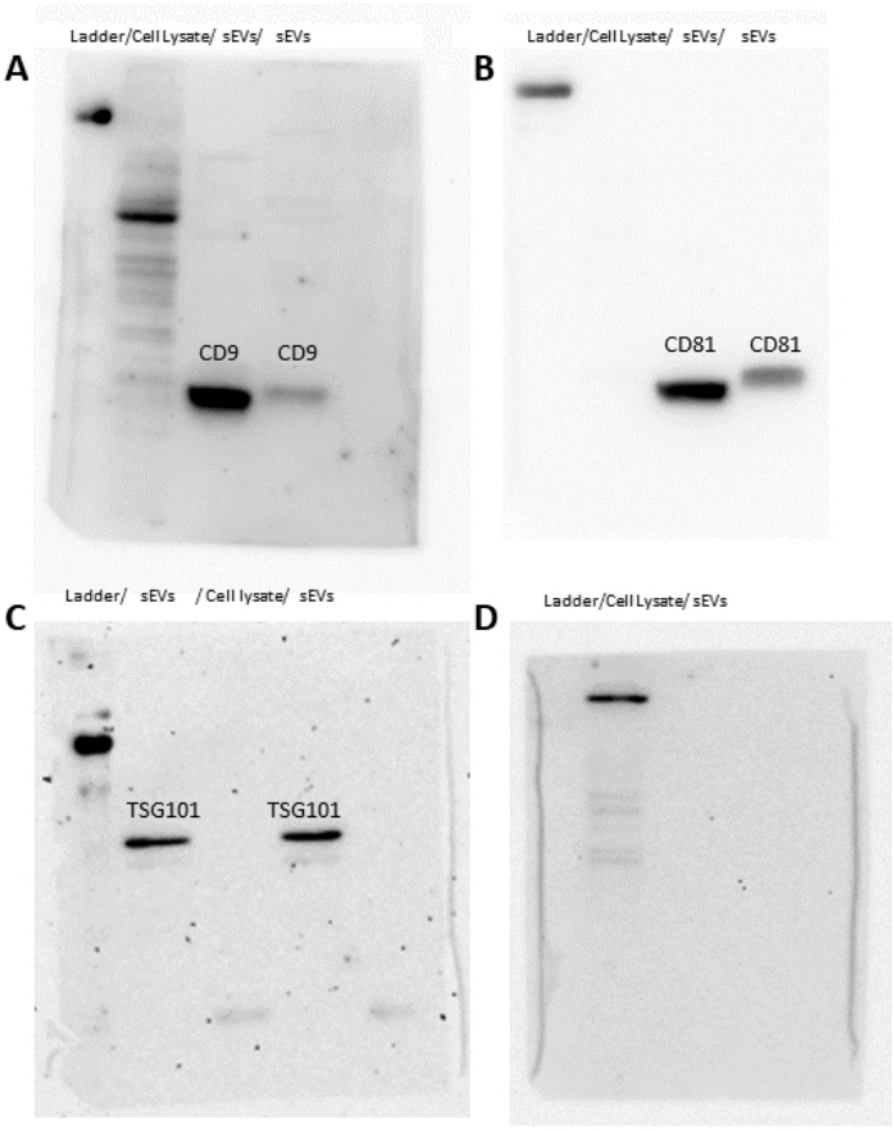

Figure 3: The biomarker expression of hUC-MSC-sEVs. (**A**) Western blot analysis of CD9 exosomal positive marker. (**B**) Western blot analysis of CD81 exosomal positive marker. (**C**) Western blot analysis of TSG101 exosomal positive marker. (**D**) Western blot analysis of GRP94 negative marker. All four markers were compared with cell lysate as a control. From left to right, Ladder, Cell lysate, hUC-MSC-sEVs (**A,B**); Ladder, hUC-MSC-sEVs, Cell lysate, hUC-MSC-sEVs, Cell lysate (**C**); Ladder, Cell lysate, hUC-MSC-sEVs (**D**). The cell lysate pairs are from two replicates, and these images are representative images from experiments performed in triplicate. Abbreviation: hUC-MSC-sEVs = human umbilical cord-derived MSC small extracellular vesicles. Please click here to view a larger version of this figure.

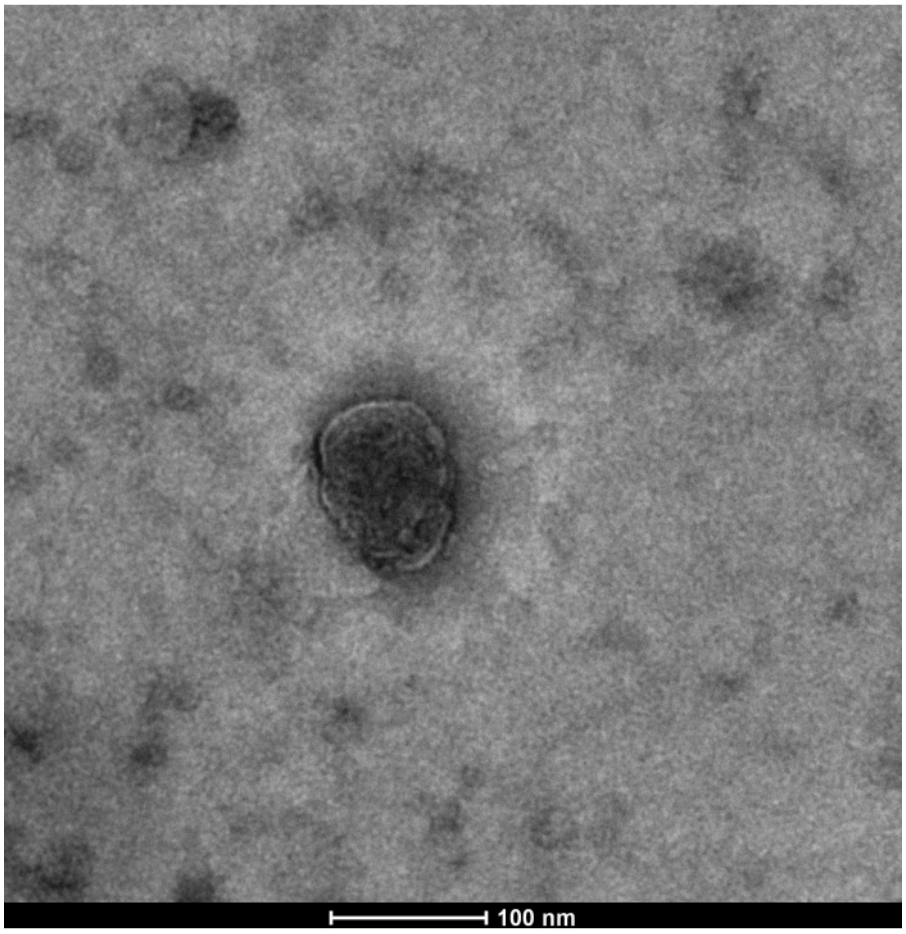

Figure 4: Morphology of single hUC-MSC-sEVs under transmission electron microscopy. The hUC-MSC-sEVs were incubated on a carbon-coated copper grid and stained with 1% phosphotungstic acid before being viewed under the transmission electron microscope. The hUC-MSC-sEVs, sized around 100 nm, showed a cup-like bilayer membrane structure. Scale bar = 100 nm. The image is a representative of three independent captures. Abbreviation: hUC-MSC-sEVs = human umbilical cord-derived MSC small extracellular vesicles. Please click here to view a larger version of this figure.

Discussion

EVs are one of the important subsets of the secretome in MSCs that play a crucial role during normal and pathological processes. However, sEVs, with a size range between 30 to 200 nm, have risen as a potential tool for cell-free therapy in the past decade. Various techniques were developed to isolate sEVs from MSCs. However, differential ultracentrifugation, ultrafiltration, polymer-based precipitation, immunoaffinity capture, and microfluidics-based precipitation possess different advantages and disadvantages[17]. None of these isolation techniques can achieve a high recovery rate and specificity. Thus, researchers must select the appropriate methods according

to their preferences based on a high recovery rate or high specificity.

Prior to the isolation of MSCs sEVs, the MSCs were assessed for positive surface markers (CD90, CD105, CD73) and negative markers (CD34, CD11b, CD19, CD45, and HLA-DR) by flow cytometry as described previously[18]. The technical advantage of this study of sEVs might provide a solution to the current situation. It only requires a basic benchtop set up in the laboratory to avoid using high-end equipment to isolate sEVs. The MSCs were starved with culture media in the described protocol without adding any serum. This is crucial to prevent any contamination of EVs originating from serum supplementation. However, considering that serum supplements are unavoidable in some cases, exosome-depleted serum supplements can be considered in formulating complete culture media. In addition, phenol red-free media could be used as a substitute for standard culture media as this common pH indicator could potentially cause issues with colorimetric measurements[15]. The protocol can be improved by using a 0.22 µm pump filter instead of a conventional syringe filter during the removal of particles larger than 220 nm. Precautions should be taken to improve the yield of the sEVs. In the last centrifugation of CM through a centrifugal filter unit, prolonged centrifugation should be avoided to ensure there is sufficient volume of solution to elute the sEVs from the filter. Overlooking this may reduce the yield of sEVs collected during the reverse-centrifugation step.

This protocol is limited to the laboratory scale and, as such, may not be suitable for large-scale isolation in industry. This is due to the high cost of the one-time use of a centrifugal filter unit, which is not feasible for large-scale isolation, which generally involves a volume of up to thousands of liters of CM. In summary, we provide a simple, benchtop-scale filtration protocol for isolating sEVs derived from MSCs. This protocol is also suitable for purifying isolated sEVs for further downstream analysis. Although this protocol is designed specifically for sEVs derived from MSCs, a part of the described protocol can be used to isolate sEVs from different sources, such as body fluid, cell lines, or other liquid-type sources.

Disclosures

The authors declare no conflicts of interest.

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