APPLICATION NOTE No. 476

Fast and Efficient Isolation of Exosomes from Stem Cells Using a Combination of Single-Use Bioreactors, High-Speed- and Ultracentrifugation

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Abstract

Cells release diverse types of membrane vesicles in the extracellular environment. Those are called extracellular vesicles (EVs) and comprise exosomes and microvesicles. Exosomes are relatively small extracellular membrane vesicles (30–150 nm) and known as an important mode of cell-to-cell communication by transferring biomolecules, such as nucleic acids, proteins, enzymes, and lipids, between cells. In addition, they can be used as biomarkers for various diseases and are also investigated as natural drug delivery vehicles system for next-generation therapeutic agents.
Here, we describe a fast and easy isolation process of exosomes from adipose-derived stem cells by a combination of high-speed and ultracentrifugation. The cells were cultured in BioBLU® 0.3c Single-Use Bioreactors and controlled by the DASbox® Mini Bioreactor System. The DASbox Mini Bioreactor System allowed high quantity stem cell culture and therefore high production of exosomes. Subsequent clearance of the conditioned culture medium was carried out by the Centrifuge CR22N, a high-speed centrifuge able to reach a maximum speed of 32,300 × g. Here, the fixed-angle Rotor R15A was used due to its high capacity of 10 × 50 mL and 10 × 15 mL conical tubes at once. In order to concentrate the exosomes further, the Ultracentrifuge CP100NX with a top speed of 803,000 × g was used in concert with the swing-bucket Rotor P32ST, which is able to hold up to 6 × 40 mL tubes. Furthermore, the use of a sucrose cushion during the ultracentrifugation step allowed a rapid isolation of pure and homogeneous exosomes compared to a more heterogenous exosome population without sucrose.
The high-volume capacities of the utilized rotors reduced the number of repetitive centrifugation steps and therefore the exosome isolation time to less than 4 hours, which is quicker than other isolation methods, e.g. precipitation or filtration.

Introduction

Cell signaling or cell communication is the ability of cells to receive, process, and transmit signals from or to their environment. It is a necessary process for the appropriate development and function of tissues [1]. This cell-to-cell communication is achieved by direct cell interactions or by the secretion of soluble factors. Most eukaryotic cells

Figure 1: In this study, the exosome production and isolation workflow was enabled by combining Eppendorf bioprocess and centrifuge solutions. As part of the Eppendorf bioprocess controller portfolio enabling small, bench, and large scale approaches, the DASbox Mini Bioreactor System is designed for parallel bioreactor operation on small scale. Furthermore, with their comprehensive rotor portfolio, the High-Speed Centrifuge CR22N and Ultracentrifuge CP100NX offer high volume capacities of up to 6 L and centrifugation speeds of up to 803,000 × g.

To learn more about the possibilities of the DASbox Mini Bioreactor System and the Eppendorf centrifuge solutions, please visit:
https://www.eppendorf.com/dasbox/
https://www.eppendorf.com/exosomes/

release membrane-derived vesicles, also called extracellular vesicles (EVs) that can have an impact on both neighboring and distant cells [2–4]. EVs, with sizes ranging from 30 to 1,000 nm, are released by numerous cell types such as blood cells, dendritic cells, endothelial and epithelial cells, nervous cells, tumor cells, as well as embryonic and adult stem cells in the extracellular space, both in physiological and pathological conditions [5]. EVs have also been identified in body fluids such as serum, saliva, amniotic fluid, synovial fluid, breast milk, and urine [6,7]. EVs are spherical cytosol fragments surrounded by lipid-bilayer membranes and hydrophilic proteins, similar to cell plasma membranes, and composed of various bioactive molecules, including RNAs, DNAs, proteins, mRNA, microRNA, and lipids [7,8].

EVs are a heterogeneous group of vesicles, comprising exosomes (30–150 nm) and microvesicles (MVs, 150 to 1000 nm) [4,7,9]. Exosomes originate from multivesicular bodies (MVBs), where they accumulate after being formed by inward-budding of the endosomal membrane. In contrast, MVs are formed through direct outward budding of the plasma membrane [10]. Exosomes were first described in 1983 as a process of "reverse endocytosis" and due to their endosomal origin, exosomes are enriched in late endosome components such as CD63, CD9, and CD81 [11,12]. In recent years, exosomes were linked to immune responses [13], central nervous system-related diseases [14], tumor genesis and cancer [15,16], the spread of viruses [17], and neurological disorders [18]. Because of this correlation, their utility as biomarkers in diagnosis [19,20] and support in the treatment of various pathologies has been researched extensively [21].

Many challenges remain in the field of exosome research, one of them being the low yield and the lack of an established biomanufacturing platform to efficiently produce exosomes from cells. Established methods of cell culture are therefore required and have been previously described by using BioBLU Single-Use Bioreactors [22–24]. In addition, research applications, contaminations from surrounding proteins, lipoproteins, and nucleic acid could lead to false positive results and error-prone interpretations, therefore

Figure 2: **(A)** The DASbox Mini Bioreactor Control System was used to culture exosome-producing human adipose-derived stem cells (hADSC) in **(B)** BioBLU 0.3c Single-Use Bioreactors with a single pitched-blade impeller. Growth parameters were monitored and controlled by the DASware® control software. Isolation was carried out **(C)** with the High-Speed Centrifuge CR22N equipped with the fixed-angle Rotor R15A able to simultaneously hold 10 × 50 and 10 × 15 mL conical tubes, as well as **(D)** the Ultracentrifuge CP100NX with the swing-bucket Rotor P32ST, which is able to hold up to 6 × 40 mL tubes at once.

leading to discrepancies between the scientific results of different research groups [25].

With the increasing interest in research applications of exosomes, reproducible methods of purification are becoming more and more important, the five most common ones being centrifugation, chromatography, ultrafiltration, precipitation, and immunoaffinity capture [26–32]. Centrifugation is nowadays the gold standard [33,34].

Therefore, in this application note, exosome isolation was carried out by the High-Speed Centrifuge CR22N and the Ultracentrifuge CP100NX after culturing human adipose-derived stem cells (hADSC) in suspension on microcarriers in the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors (Figure 2). This workflow enabled high-yields of viable cells and led to high-quality exosomes in high quantities (Figure 3).

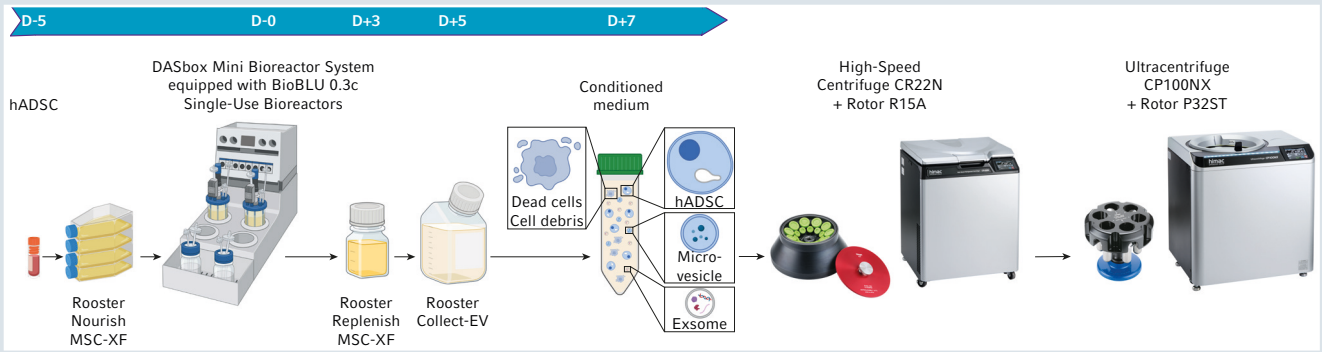

Figure 3: Schematic representation of cell expansion and exosome production in the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors, as well as exosome isolation using a combination of the High-Speed Centrifuge CR22N with the fixed-angle Rotor R15A and the Ultracentrifuge CP100NX with Rotor P32ST. Created with BioRender.com

Material and Methods

Culture of hADSC on microcarriers in BioBLU 0.3c Single-Use Bioreactor

Human adipose-derived stem cells (hADSC, Lonza, PT-5006) at passage 3 (P3) were expanded on T75 BioCoat® Collagen I-coated culture flasks (Corning®, 10175430) in the presence of RoosterNourish-MSC-XF medium (RoosterBio®, KT-016). After 5 days, cells were trypsinized using 0.025% Trypsin-EDTA (Lonza, CC-5012) and Trypsin neutralizing solution (Lonza, CC-5002). Cells were counted using the Vi-CELL XR automated cell counting device (Beckman Coulter®, 731050). A total amount of 6×10^6 hADSC combined with 3.4 g of Synthemax® II low-density microcarriers (Corning, CLS3781), were cultured in suspension in a volume of 250 mL of RoosterNourish MSC-XF medium using DASbox Mini Bioreactor System. This system was equipped with two BioBLU 0.3c Single-Use Bioreactors. To promote the initial cell adhesion to the beads, cells and microcarriers were not agitated for the first 4 hours. Later, the agitation speed was set to 80 rpm for the remaining process. The cells were cultivated at 37°C with the level of dissolved oxygen (DO) set to 40%. Growth medium was maintained at pH 7.2 by the automatic addition of CO_2 in the vessel headspace and NaOH (1 M). On day 3, 5 mL of RoosterReplenish-MSC-XF (RoosterBio, SU-023) were added to the suspension providing additional growth factors to the cells. On day 5 of culture, each BioBLU 0.3c Single-Use Bioreactor was transferred under a cell culture hood, where medium was discarded, beads were washed using 100 mL of phosphate-buffered saline (PBS), and 250 mL of RoosterCollect-EV medium (RoosterBio, M2001) were added. Then, the BioBLU 0.3c Single-Use Bioreactors were returned to the DASbox Mini Bioreactor System for 48 hours (EV collection phase). Cell proliferation was evaluated at different time points by staining the cells with the fluorescent molecule Invitrogen Calcein AM (Thermo Fisher Scientific®, C3099) and visualized under the Thermo Scientific Invitrogen Evos FL Auto 2 microscope (Thermo Fisher Scientific, AMAFD2000). On day 7, the cell culture-conditioned medium (CCM) was collected for subsequent centrifugation steps. Cells were counted via the Vi-CELL XR.

Isolation of exosomes by high-speed and ultracentrifugation

To isolate exosomes from the culture supernatant, CCM was collected and distributed in 50TC (50 mL) and 15TC (15 mL) tubes and centrifuged at 500 × g for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged a second time at 2,000 × g for 10 min at 4°C. The supernatant was then transferred again into a new tube for a final centrifugation step at 20,000 × g for 20 min at 4°C. A small aliquot of the supernatant was kept at -80°C for potential further analysis. All centrifugation steps up to this point were performed using the high-speed centrifuge CR22N equipped with the fixed-angle rotor R15A. Next, in order to compare the impact of different centrifugation methods on the purity and integrity of the exosomes, either a direct ultracentrifugation run or a run with a 30% sucrose cushion was performed [35]. These ultracentrifugation runs were performed using the Ultracentrifuge CP100NX equipped with the swing-bucket Rotor P32ST. For sucrose-based isolation, the CCM was transferred into 40PET tubes containing 4 mL of 30% sucrose solution (prepared in PBS) by slowly overlaying using a 27 gauge needle and syringe. For the direct ultracentrifugation method, the CCM was immediately transferred into 40PET tubes. PET was used because this material is highly transparent and therefore allows to see pellet after centrifugation. Next, the tubes were centrifuged at 100,000 × g for 90 min at 4°C. The supernatant was discarded while 4 mL remained at the bottom of the tube. In order to balance the tubes again, additional CCM or PBS (depending on the initial volume of CCM) was slowly added on top, followed by another centrifugation step at 100,000 × g for 90 min at 4°C to collect and concentrate exosomes as a pellet. The maximum volume of supernatant was discarded while not disrupting the pellet. The pellet potentially containing the exosomes was resuspended in 600 µL of PBS and stored in aliquots of 200 µL at -80°C for further use. Non-conditioned medium (RoosterCollect-EV medium) was used simultaneously with the CCM as a negative control.

Size distribution analysis

The size distribution profile of EVs was measured by Dynamic Light Scattering (DLS). The measurements were performed by the Platform and the Pharmacy Department of the University of Namur. In DLS, the particle size distribution (PSD) of EVs was measured by a Zetasizer Ultra (Malvern Panalytical, ZSU5700) equipped with a laser of $\lambda = 633$ nm

wavelength. A Zetasizer Low Volume Disposable Sizing Cell Kit (Malvern Panalytical, ZSU1002) was used to analyze the exosome pellets in transparent cuvettes. Liposomes were selected as the model system (refractive index: 1.45, viscosity: 1.2 PI, temperature: 20°C), as these lipid vesicles share some physical characteristics with exosomes, such as composition, size, and density [36]. Thus, the particles were considered spherical with a density of 1.2 mg/mL and a refractive index and absorption coefficient of 1.45 and 0.001, respectively.

Electron microscopy
The measurements were performed by the GIGA Neurosciences Laboratory for Cell and Tissue Biology (University of Liège). Briefly, exosome suspension solution was loaded on transmission electron microscope (TEM) grids and incubated for 1 hour. Exosomes were stained for 10 minutes with filtered 2.5% uranyl acetate solution that was applied to the surface of the TEM grid by using a syringe. Excess uranyl acetate solution was removed from the grid by filter paper. The grid was quickly washed with water to remove the excess staining solution. A Jeol transmission electron microscope (Jeol, JEM-1400) at 80 kV was used to analyze the exosome appearance.

Exosomes quantification by CD63 ELISA
Exosome quantity was measured using the ExoELISA-ULTRA Complete Kit CD63 Detection (System Biosciences, EXEL-ULTRA-CD63-1) following the manufacturer's instructions. Briefly, 50 µL of each sample were added to a 96-well plate and incubated at 37°C for 1 hour. The plate was washed three times for 5 min while shaking using the Eppendorf ThermoMixer® C. CD63 primary antibody was diluted in blocking solution (1:100). Then 50 µL of antibody solution were added to each well and incubated for 1 hour at room temperature (RT) while shaking. The primary antibody was discarded, followed by three washing steps of 5 min. The secondary antibody was diluted in blocking solution (1:5000), 50 µL were added to each well and incubated for 1 hour at RT while shaking. After the final three washing steps, the plate was incubated for 15 min at RT with 50 µL of TMB (3,3',5,5'-Tetramethylbenzidin) ELISA substrate. The same amount of stoping buffer was added and the plate was analyzed immediately using a spectrophotometric microplate reader xMark (Bio-Rad Laboratories, 1681150) at 450 nm to provide a fixed endpoint for the assay.

Results and Discussion

Proliferation and culture of hADSC in the BioBLU 0.3c Single-Use Bioreactor
After an initial expansion of hADSC in T75 culture flasks for 5 days (Figure 4, D-2/D-0) to ensure an appropriate confluence of 85-90%, cells were trypsinized (5.3×10^5 cells/mL, viability: 96.7%) and seeded on Low Concentration Synthemax® II Microcarriers (Corning) with a cell-to-bead ratio of 3.7 cells/bead. Microscopy image following calcein staining on day 1 of the expansion phase in BioBLU 0.3c Single-Use Bioreactors showed a successful attachment of the cells to the beads (Figure 4, D+1).
On day 6, proliferation of the cells and a homogeneous distribution on the microcarriers was observed (Figure 4, D+6). After 5 days of bioreactor culture, the EV collection was initiated by exchanging the RoosterNourish-MSC-XF medium for 250 mL of RoosterCollect-EV medium for the next two days. This low-particulate medium is used in bioprocess scales for clean EV collection when using human mesenchymal stem cells (hMSCs). Cell viability at the end of the culture periods (D+7) reached 97% and cell densities of 1.96×10^6 cells/mL. The close monitoring of various culture parameters, such as temperature, pH, and DO levels by DASbox Mini Bioreactor System enabled a culture system ideal for yielding standardized high numbers of viable cells throughout the whole process. This is crucial to ensure reproducible exosome quantity and quality during cell culture.

Exosome isolation from the conditioned medium using serial centrifugation steps
Medium from the two BioBLU 0.3c Single-Use Bioreactors (500 mL) were pooled for the following steps. The medium was then redistributed into 15TC and 50TC conical tubes and hereafter, three centrifugation steps using the High-Speed

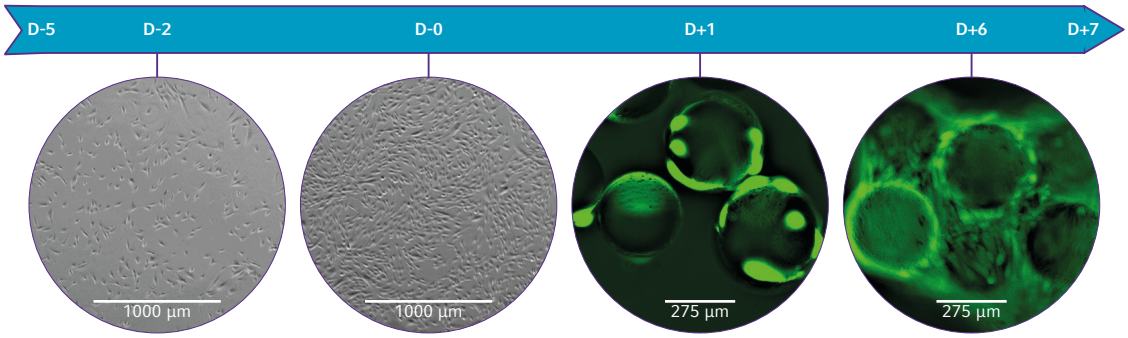

Figure 4: Cell culture in T75 flask (D-5 to D-0) and on beads using the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactor (D-0 to D+7). Calcein staining was used to follow cell growth on beads.

Centrifuge CR22N with the fixed-angle Rotor R15A were performed (Figure 5A). These three centrifugation steps were used to remove living cells and beads (500 × g), cell debris, dead cells, apoptotic bodies (2,000 × g), and microvesicles (20,000 × g). After each centrifugation step, the supernatant was transferred to new tubes. During this process 1 mL of medium was left inside the tube to prevent taking up cells, cell debris, apoptotic bodies, or MV pellets while transferring. After the last centrifugation step, approximately 470 mL of cleared medium were available for ultracentrifugation. Using the Ultracentrifuge CP100NX and swing-bucket Rotor P32ST, only two runs were required for the total volume of two BioBLU 0.3c Single-Use Bioreactors (Figure 5B/C), thereby reducing working steps and time during exosome collection.

Exosomes are usually collected from crude medium centrifuged firstly at 100,000 × g, followed by a second step at the same speed but using a sucrose gradient to collect only homogeneous, pure, and intact exosomes. In order to save time, the sucrose gradient step was replaced by a technique consisting of 4 mL of a 30% sucrose cushion at the bottom of the tube (Figure 5C). Using the sucrose

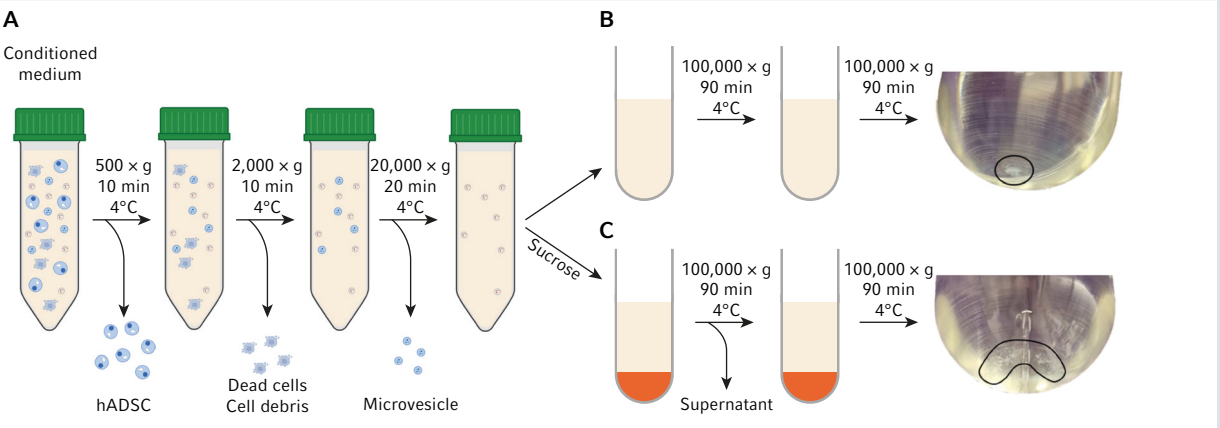

Figure 5: Combination of high-speed centrifugation to clear the medium **(A)** by using the High-Speed Centrifuge CR22N and fixed-angle Rotor R15A and **(B)** ultracentrifugation without sucrose cushion or **(C)** with sucrose cushion to pellet the exosomes using the Ultracentrifuge CP100NX and swing-bucket Rotor P32ST. Created with BioRender.com

cushion, an intact and homogeneous population of exosomes should be received without the labor-intensive need to create a glucose gradient within the collection tube.

In this application note, this sucrose cushion technique was compared to two consecutive centrifugation steps without sucrose (Figure 5B/C). After the two ultracentrifugation steps, the exosome pellet shape clearly differed from one technique to the other. Indeed, the centrifugation without a sucrose cushion generated a predominantly condensed pellet at the bottom of the tube (Figure 5B), while the sucrose cushion technique (Figure 5C) generated a more diffused and spread-out pellet. This can be explained by the fact that the exosomes traverse the sucrose cushion and are therefore pelleted more slowly to the bottom of the tube in a linear and straight direction, while no sucrose condensed the pellet to the same spot.

Exosomes characterization and quantification

After ultracentrifugation, exosome pellets were resuspended in PBS and characterized using DLS, electron microscopy (Figure 6A), and ELISA (Figure 6B). The DLS was used to determine the size distribution profile of small particles in the PBS suspension and therefore the homogeneity of the exosome population. As mentioned above, fresh non-conditioned xeno-free medium (RoosterCollect-EV) was used as a negative control at the same time. DLS measurements showed that the RoosterCollect-EV medium used for the collection of exosomes during the last 48 h of the experiment did not contain any exosomes. DLS measurements of exosomes isolates with or without sucrose cushion showed size distributions typical for exosomes with peaks at around 100 nm for both techniques (Figure 6A). Of note, the area under the curve exosome isolation techniques were different between both approaches. Indeed, the peak width of exosomes without sucrose cushion was larger with a tendency to be shifted to higher size detection. In comparison, the peak width of exosomes with sucrose cushion was smaller in comparison and more concentrated around the lower size. Therefore, the exosomes population without sucrose cushion is more heterogeneous with larger exosomes versus a more homogeneous population of intact exosomes with sucrose cushion. This data was confirmed by using the electron microscopy technique to visualize the exosome population (Figure 6A). This analysis confirmed our previous observations. No exosomes were detected in the RoosterCollect-EV medium (negative control). The population of exosomes without sucrose cushion was bigger and heterogeneous, while the exosomes in the sucrose cushion were smaller and more homogeneous with less debris detected (Figure 6A). The expression of exosomal biomarkers was analyzed by ELISA detection of CD63 (Figure 6B). Using this method, the resulting relative exosome abundance was comparable for both exosome isolation approaches. It also confirmed the absence of external exosomes in the RoosterCollect-EV negative control, where no expression of CD63 was detected in the sample. This data demonstrate exosome yields by using the High-Speed Centrifuge CR22N and Ultracentrifuge CP100NX for isolation. Furthermore, the use of a sucrose cushion during the ultracentrifugation step allowed for the collection of a rich and homogeneous exosome population.

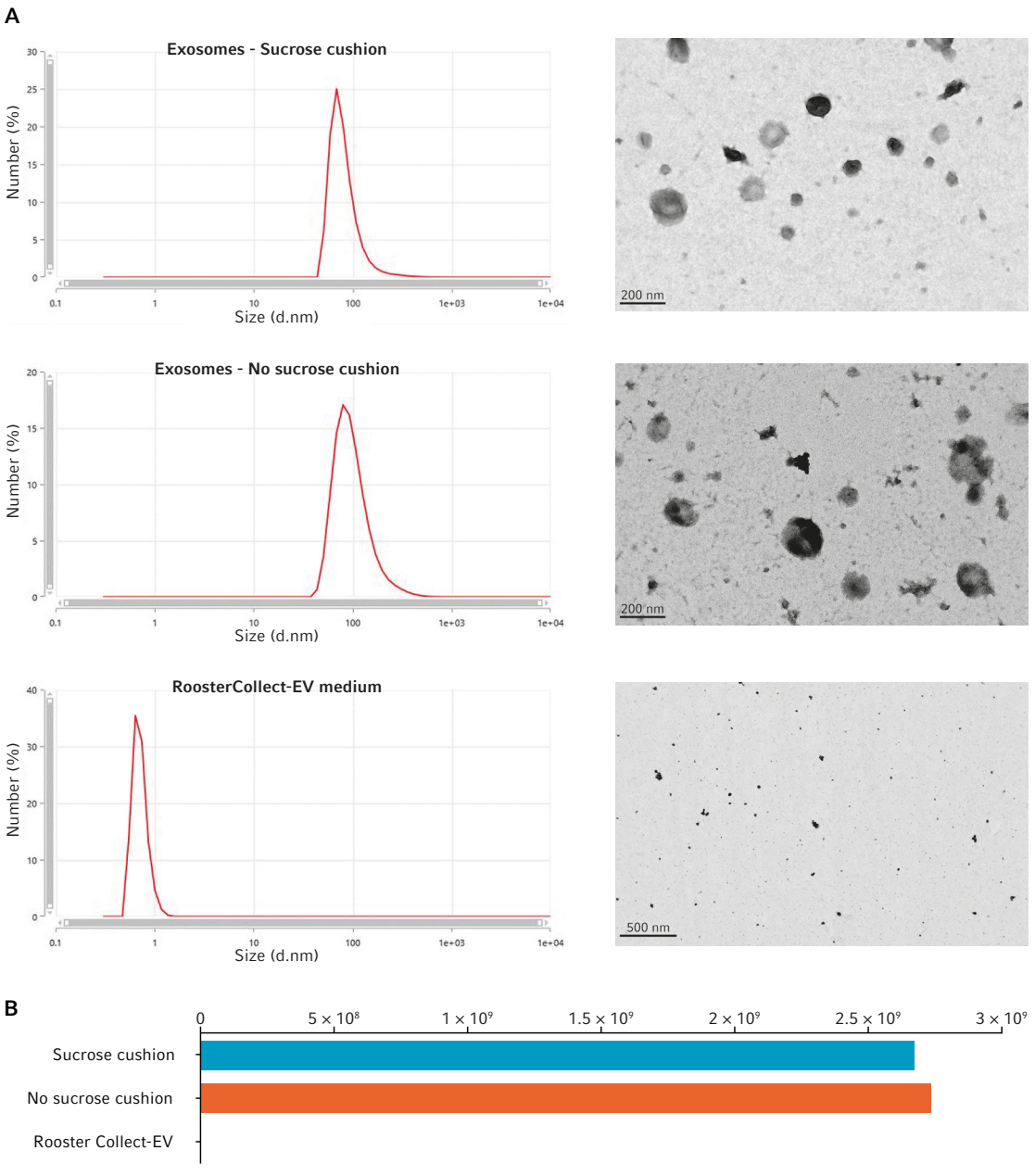

Figure 6: (A) DLS (left) analysis and electron microscopy (right) of exosome populations isolated with or without a sucrose cushion during the ultracentrifugation step, as well as Rooster Collect-EV medium as a negative control. **(B)** ELISA quantification of the exosome abundance from the samples shown in (A).

Conclusion

The DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors offers efficient upstream process optimization for the exosome production workflow: It allows parallel processing of 4 bioreactors of 100 to 250 mL working volume, as well as the precise control of critical process parameters such as temperature, pH, DO, and gas flow. Some of the biggest challenges during the exosome isolation process are preventing low purity and ensuring the integrity of the plasma membranes at the end of the process. Purification and isolation methods like precipitation or chromatography techniques generate either exosome aggregation or degradation due to the operation buffers. Ultracentrifugation, known as the gold standard, has the advantage of being able to separate the exosome from a large quantity of biomaterials at relatively low costs and without the need of additional chemical reagents which can contaminate the exosome preparation. Nevertheless, ultracentrifugation also has its disadvantages, such as labor intensity because of repetitive centrifugation steps for large volumes of medium, and dependence on separation efficiency by the rotor types (fixed-angle or swing-bucket) and their specific capacity. Another significant disadvantage is the presence of non-exosomal impurities in the exosome fraction. To increase the purity and integrity, exosomes are most often isolated in a sucrose gradient (2.0–0.25 M) and centrifuged at 210,000 × g for up to 16 hours. To improve the protocol and reduce time, ultracentrifugation was carried out by using the sucrose cushion technique, which reduced the centrifugation time to only 90 min per run.

The present work demonstrates, that a workflow consisting of the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors as well as high-speed and ultracentrifugation is a viable combination for the successful collection of intact and homogeneous hADSC-derived exosomes. Use of the High-Speed Centrifuge CR22N equipped with the fixed-angle Rotor R15A allows centrifugation of up to 650 mL in one step, considering its ability to simultaneously hold 10x15TC plus 10x50TC conical tubes at the same time. This unique feature allows clearance of 650 mL medium from cells, cell debris, microvesicles etc. in less than 1 hour, which save time and repetitive work. Similarly, with its possibility to accommodate up to 6x40 mL tubes at once, the swing-bucket Rotor P32ST (used in this work together with the Ultracentrifuge CP100NX) is able to hold a maximum volume of 240 mL medium.

These volume capacities enabled the clearance of the medium content of two BioBLU 0.3c Single-Use Bioreactors in only one centrifugation run by use of the High-Speed Centrifuge CR22N, and two runs to concentrate the exosomes with Ultracentrifuge CP100NX. With that, the whole process took less than 4 hours which showcase the potential of this workflow for efficient time management during process development.

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System, for cell culture applications, 4-fold system for single-use vessels	76DX04CCSU
BioBLU® 0.3c Single-Use Bioreactor, cell culture, open pipe, 1 pitched-blade impeller, no pH, sterile, 4 pieces	1386 100 000
DASware® control software, including PC, OS, and licenses, for 4-fold DASbox® Mini Bioreactor System	7860 016 7
Centrifuge CR22N	Inquire*
Centrifuge CP100NX	Inquire*
Rotor R15A , for CR22N, Max. speed 15,000 rpm, Max. RCF 32,200 × g	5721 221 007*
Rotor P32ST, for CP-NX series, Max. speed 32,000 rpm, Max. RCF 180,000 × g	5720 214 003*
Eppendorf ThermoMixer® C, basic device without thermoblock	5382 000 015
50TC Tube (100Pcs), 50 mL	5721 221 007
15TC Tube (100Pcs), 15 mL	5721 302 110
40PET Tube (50 Pcs), 40 mL	5720 411 148

*Inquire the part number for your country

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