APPLICATION NOTE No. 440 | 2021

The DASbox® Mini Bioreactor System as a Tool for Process Development And Stem-Cell Derived Exosome Production in Standardized Culture Conditions

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Abstract

Exosomes are the smallest particles of the extracellular vesicles (EVs), secreted by different cells of the body and considered as important players in cell-to-cell communication. Their biological functions rely in the transfer of cargo molecules including membrane and cytosolic proteins, lipids, nucleic acids, and metabolites. Exosomes, and more specifically stem cell-derived exosomes, are now of great interest as cell-free therapeutic tools due to their strong diagnostic and therapeutical potential in various diseases models including skin, nervous system, heart, liver, and kidney. However, to explore the use of exosomes in the different biomedical areas, large amounts of high-quality exosomes need to be produced. To achieve this challenge, the culture of cells in the controlled environment offered by stirred-tank bioreactors can facilitate the standardized expansion of high yield of viable cells and enhance the reproducible production of extracellular vesicles. In this study, we tested the suitability of BioBLU® 0.3c Single-Use Bioreactors, controlled by a DASbox® Mini Bioreactor System, in the production of exosomes secreted by human adipose-derived stem cells. After a successful cell expansion phase (34.7 fold induction after 5 days of expansion), the presence of exosomes in the conditioned media has been demonstrated. Following collection and isolation, those EVs have been characterized through different classical read-outs. These results illustrate the great potential of the DASbox Mini Bioreactor System used in combination with BioBLU 0.3c Single-Use Bioreactors to determine optimal culture parameters required for scaling-up from R&D and process development to manufacture.

Introduction

Intercellular communication is essential to maintain homeostasis and cellular response in tissues and organs of multicellular organism. Cells exchange informative signals by direct cell-cell contact or by sensing circulating endogenous bioactive compounds carried by extracellular vesicles (EVs) released by other cells (2). EVs carry a cargo of various cell-specific molecules including membrane- and cytosolic proteins, lipids, nucleic acids, and metabolites and can be broadly classified, based on their mechanism of release and size in exosomes (less than 150 nm in diameter), microvesicles/microparticles/ectosomes (100-1000 nm) and apoptotic bodies (> 1000 nm) (3). Exosomes are generated intracellularly inside multivesicular endosomes or multivesicular bodies that fuse with the plasma membrane to be released later into the extracellular space. Consequently, exosomes play significant roles in a wide range of biological processes in health and diseases such as cellular homeostasis, coagulation, antigen presentation, angiogenesis, inflammation, apoptosis and intercellular signaling (2). Their biological function relies on the transfer of their bioactive cargos. As exosomes can be detected in most body fluids, great interest has been observed for this nanovesicles as disease biomarkers, therapeutic targets or even drug-carriers. Furthermore, their low immunogenicity and ideal biocompatibility as well as their ability to pass biological barriers make them good candidates for cell-free therapeutic approaches (4).

The use of exosomes as a cell-free therapeutic tool requires the production of clinically relevant amounts of high-quality vesicles. However, as every multistep process, the exosome production workflow offers many opportunities of variability, questions, and challenges (Figure 1). An efficient manufacture process must be scalable and be in accordance with appropriate regulation requirements. This process has also to be controlled and standardized from cell culture to final product characterization to ensure reproducibility.

Exosome content being cell type-specific, the upstream process begins with the selection of cells used as exosome source. Adipose-derived stem cells (ADSCs), which are mesenchymal stem cells derived from stromal-vascular fragments of adipose tissue, are now considered as one of the most promising cell types for cell-based therapies in regenerative medicine, not only of their self-renewal and expansion potential, multipotency and low immunogenicity but also for the secretion of a wide array of bioactive materials involved in tissue renovation and tissue substitution (5-6). Among these bioactive material, ADSC-derived exosomes have attracted

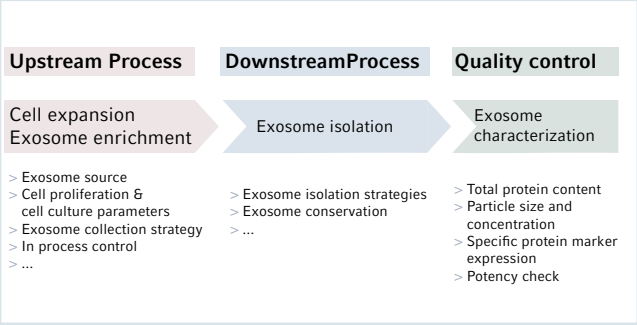

Fig. 1: The exosome production workflow

the attention of experts for their therapeutic potential in skin wound healing (7), neurological and cardiovascular protection (8, 9), tumor inhibition, and kidney preservation (10).

As a next step, those selected cells should be expanded in high quantity. Culture conditions used during the ex vivo cell expansion phase will largely impact the yield and the bioactivity of released exosomes. Indeed, several parameters such as cell density, cell passage number, but also culture support have been described to play an important role on exosome composition and functions (11-15). Very interestingly, 3D culture systems, such as stirred-tank bioreactors, seems to be beneficial for exosome yield and quality (14-15). As a valuable advantage, and by contrast with conventional 2D culture systems, bioreactors combine scalability with the maintenance of a more stable and physiological environment.

In the present study, we use an DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors as a system to culture human ADSCs in suspension on microcarriers to produce exosomes. This fully controlled system allows an expansion of high yield of viable cells and optimizes a production of high-quality extracellular vesicles in standardized culture conditions. The DASbox Mini Bioreactor System is a perfect tool to support the process development from benchtop to production scale, optimizing culture conditions before scaling-up.

Material and Methods

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

Lonza® human Adipose Derived Stem Cells (hADSC) (Lonza, PT-5006,) at passage 3 (P3) have been initially expanded on T75 CellBIND flasks (Corning®, 3290) in the presence of RoosterNourish_MSC-XF media (RoosterBio®, KT-016).

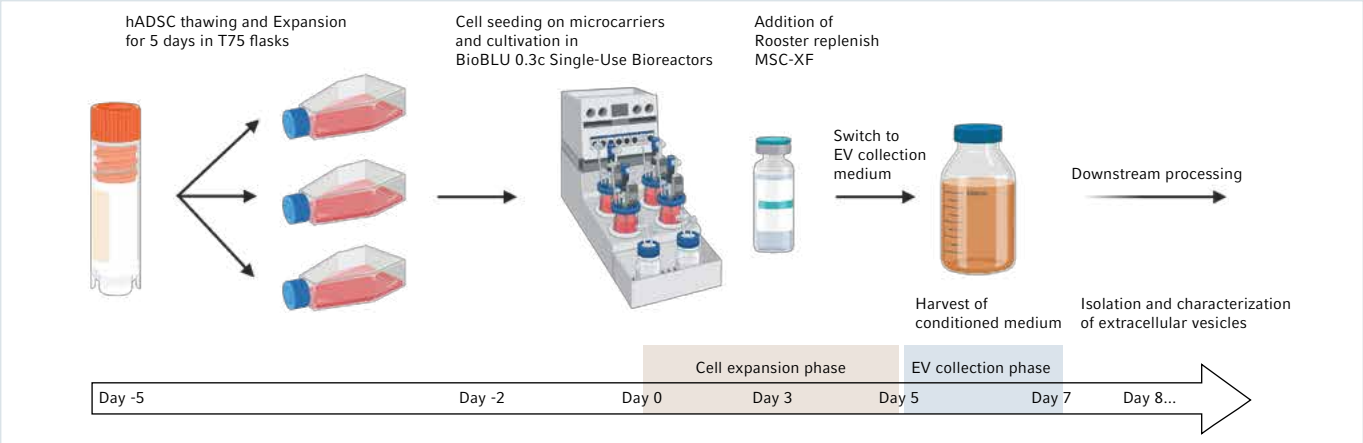

Fig. 2: Schematic representation of cell expansion and exosome production in BioBLU 0.3c Single-Use Bioreactors controlled by a DASbox Mini Bioreactor System. Created with: biorender.com

After 5 days, cells were trypsinized (0.25 % Trypsin-EDTA, Lonza, CC-5012) and counted using the Vi-CELL® automated cell counting device (Beckman Coulter, USA). A total amount of 6×10^6 hADSC (2.4×10^4 cells/ml) combined with 3.4 g of Synthemax® II low density microcarriers (Corning, 3781), representing a cell-to-bead ratio of 3.7 cells/bead, were cultured in suspension in a volume of 250 ml RoosterNourish_MSC-XF media using the DASbox Mini Bioreactor System. This System was equipped with 4 BioBLU 0.3c Single-Use Bioreactors. To promote the initial cell adhesion, the suspension of cells and microcarriers was not agitated for the first 4 hours. Later, the agitation speed was set to 100 rpm. The cells were cultivated at 37°C and the dissolved oxygen (DO) level was set to 40%. The pH of the growth medium was controlled at 7.2 by automatic addition of CO_2 in the vessel headspace and NaOH (1N) as summarized in Table 1. Three days later, 5 ml of RoosterReplenish™-MSC-XF (RoosterBio, SU-023) were added to the suspension to provide additional growth factors to the culture, as recommended by culture medium's supplier.

Table 1: Overview of process parameters for the cultivation of hADSC in BioBLU 0.3c Single-Use Bioreactors.

Microcarriers	Synthemax II low density (3.4 g)
Inoculation cell density	2.4 x 10^4 cells/mL
Working volume	250 mL
Cell/carrier ratio	3.7 cells/carrier
Dissolved oxygen	40 %
pH	7.2
Temperature	37 °C
Gas flow	0.1 sL/h
Stirring speed	(no aggitation during the first four hours) 100 rpm

Following a 5-day proliferation (Cell expansion phase), the medium was replaced, after rinsing with PBS, by the Extracellular vesicles (EV) collection media (RoosterCollect™-EV media, RoosterBio, M2001) used in combination with the EV Boost reagent at low concentration (Roosterbio, S2001), for a period of 48 hours (EV collection phase). Cell proliferation has been evaluated through cell counting performed at different time points during the cultivation using the Vi-CELL automated cell counting device. Cell viability was determined, by staining the cells with the fluorescent molecule Calcein AM (Invitrogen™, C3099) and visualized under the Evos™ FL Auto 2 microscope (Invitrogen). The lactate and glucose concentrations were monitored at different timepoints with the YSI 2900 Biochemistry analyzer (YSI). At the end of the incubation, the cell culture conditioned media (CCM) was collected, centrifuged at 3000 x g for 15 minutes at room temperature to remove cell debris, filtrated on Sartolab® P20 (0.22 µm) (Sartorius® Stedim Biotech GmbH, 18053) and stored at – 80°C for further analysis (Figure 2).

The present study is based on two independent cell culture experiments.

A negative control was also run for each experiment consisting in RoosterCollect-EV media combined with the EV Boost reagent. This non-conditioned media followed the same procedure as the CCM for the isolation and characterization of exosomes.

Isolation of extracellular vesicles by size exclusion chromatography

Exosome enrichment of the filtrated CCM was obtained by concentrating the samples by centrifugation at 3000 × g and 4°C with the Centriplus® YM 10 (Amicon®, 4421). A volume of 1 ml of the concentrated sample was then loaded onto the size exclusion chromatography columns (HansaBioMed Pure-EVs, HBM-PEV) and the exosomes were eluted with PBS without divalent cations, in approximately 22 consecutive fractions of 500 µl. Protein concentration of each fraction was analyzed by Nanodrop 2000 (Thermo Scientific™, ND-2000).

Western blot analysis

Immunoblotting was used to detect the presence of the exosomal marker CD63 in a set of chromatography fractions. Briefly, after concentration of fractionated samples by UltraCel YM-10 (Microcon®, 42407), the extracellular vesicles presented were lysed by RIPA Lysis and Extraction Buffer (50% v/v) (Thermo Fisher Scientific, 89900) containing a protease inhibitor cocktail (cOmplete, EDTA-free, protease inhibitor cocktail, Roche, 11873580001). Lysate samples were separated on NuPAGE 4-12 % Bis-Tris protein gels (Thermo Fisher Scientific, NP0321BOX) in non-reducing conditions and transferred onto Low-Fluorescence PVDF transfer membrane (Thermo Fisher Scientific, 22860). The membranes were blocked with the Intercept (TBS) blocking buffer (Li-Cor,927-60001) for 1 hour at room temperature and probed with the primary anti-CD63 antibody (rabbit IgG) (SBI, EXOAB-CD63A-1) diluted 1:1,000 in Intercept (TBS) antibody diluent (Li-Cor,927-65001) overnight at 4°C. Following incubation, the membranes were incubated with the WesternSure® HRP goat secondary anti-rabbit IgG antibody diluted 1:20,000 in the Intercept (TBS) antibody diluent for 1 hour at room temperature. Visualization of the bands was performed using the WesternSure PREMIUM chemiluminescent substrate (Li-Cor,926-95000) and blots were scanned on C-DiGit® blot scanner (Li-Cor, 3600-00).

CD63 ELISA for exosomes detection

After concentration of the collected fractions, the presence of exosomes was determined using the ExoELISA-ULTRA Complete Kit _CD63 Detection (EXEL-ULTRA-CD63-1, SBI) following manufacturer's instructions. Briefly, 50 µl of each fraction was added to the plate and incubated at 37°C for 1 hour. After 3 washes, the CD63 primary antibody was added and incubated for 1 hour at RT. Before the addition of the secondary antibody, 3 washes were also performed and the plate with the secondary antibody was incubated for 1 hour at RT. After the final 3 washes, the plate was incubated for 15 min at RT with the ELISA substrate before the addition of the stop buffer. Optical densities were recorded at 450 nm.

Size distribution analysis

The size distribution profile of EVs was estimated either by Dynamic Light Scattering (DLS) or by Centrifugal Liquid Sedimentation (CLS). The measurements were realized by the SIAM (Synthesis, Irradiation and Analysis of Materials) Platform and the Pharmacy Department of the University of Namur.

In DLS, the particle size distribution (PSD) of extracellular vesicles was measured by a Zetasizer® Nano ZS (Malvern Instruments, UK) equipped with a laser of $\lambda = 633$ nm wavelength. A concentrated sample of CCM was analyzed in transparent cuvettes of 10 mL. Liposomes were selected as model System (measurement protocol) (Refractive index= 1.45, viscosity= 1.2, temperature =20°C), as these lipid vesicles share some physical characteristic with exosomes, such as composition, size and density (1).

The PSD of extracellular vesicles was also measured with the CLS technique. In this case, measures were performed with a Disc Centrifuge CPS 24,000 (CPS Instruments Inc., USA) working at 22,000 rpm. A certified PVC standard (particle size: 0.263 µm) is used before each test to ensure the accuracy of the measured sizes. The laser of the disc centrifuge, to measure the extinction coefficient, has a wavelength of $\lambda = 405$ nm. A sucrose gradient was prepared using pure water at 2% and 8% concentrations. The volume of the injected sample was 1 mL. The particles were considered as spherical in shape with a density of 1.2 mg/mL with refractive index and absorption coefficient of 1.45 and 0.001, respectively.

Results

Robust and stable proliferation of hADSC in the BioBLU 0.3c Single-Use Bioreactor

After an initial expansion of hADSC on T-flasks to ensure an appropriate number of cells for the culture in the bioreactor, cells were seeded at a density of 2.4×10^4 cells/ml (6×10^6 cells/vessel) using Synthemax II low density microcarriers as matrix support. Cell counting and viability staining at different time points during the expansion phase showed a fast proliferation rate and a homogeneous distribution of the cells on the microcarriers (Figure 3A & B). At the end of

the expansion phase (at day 5), the cell number increased 34.7 fold, corresponding to a cell density of 8.3×10^5 cells/ml (2.08×10^8 cells/vessel). This cell amount corresponds approximatively to more than 115 confluent T-75 flasks (average density of 20.000 to 25.000 cells per cm^2) generated in less then one week of culture.

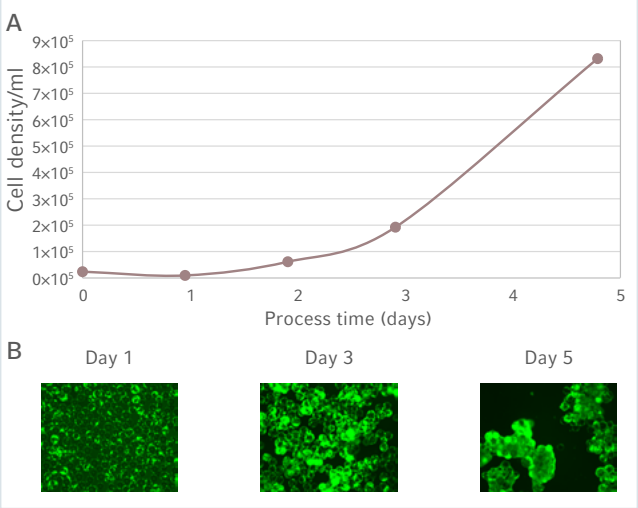

Fig. 3: (A) Representative cell proliferation curve of hADSC at passage 3 (P3) in the BioBLU 0.3c Single-Use Bioreactor controlled by a DASbox Mini Bioreactor System during a 5 day- expansion phase. Results are representative of two independent culture replicates. (B) Cell growth and viability of hADSC on Corning Synthemax II microcarriers was monitored on day 1, 3 and 5 by cell staining with a green, fluorescent dye for cell viability (Calcein-AM). The images show representative areas at different days.

Once the cell expansion phase has reached the expected number of cells, the EV collection phase is initiated by switching the expansion medium for the EV collection medium supplemented with the EV boost reagent for the next 2 days. During this period, cell proliferation remains stable and the particle concentration in the conditioned medium is enhanced in the expected size range for EVs. Cell viability at the end of the culture (Day 7) was monitored by Calcein-AM staining (data not shown).

Glucose monitoring reveals a stable consumption of this important nutrient during the whole culture process (Figure 4). The decrease in glucose concentration follows the characteristic pattern of the cell proliferation until day 5 where the expansion medium was switched to the EV collection medium as indicated in the graphic. Glucose content in the EV collection medium was lower than in the expansion medium. In the next 2 days its concentration decreases progressively.

As well as glucose, lactate monitoring (a by-product of the glucose metabolism) indicates a time-increase in its concentration for the first 5 days until the switch of the media and for the next 2 days during the collection phase. A fine control of these parameters was essential to maintain healthy cells and improve extracellular vesicles production during the EV collection phase.

The close monitoring of different culture parameters such as temperature, pH and oxygen ensured by the DASbox Mini Bioreactor System allows a fully controlled culture system favourable to generate standardized, high yield of viable cells through the entire process. This is particularly important to ensure reproducible exosome quantity and quality at the end of the process.

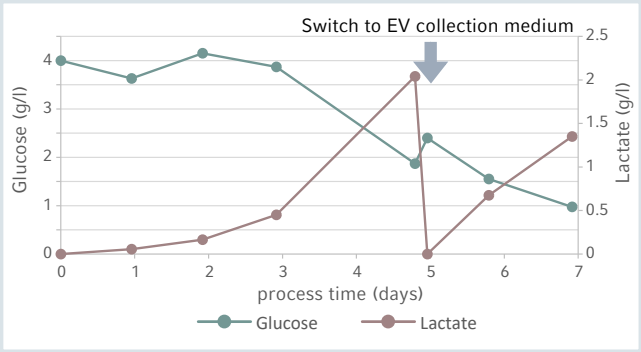

Fig. 4: Glucose consumption and lactate production profile of hADSC cultured for 7 days in the BioBIU 0.3c Single-Use Bioreactor controlled by a DASbox Mini Bioreactor System. Results are representative of two independent culture replicates.

Exosome isolation and characterization from EV conditioned medium

At the end of the collection phase, the hADSC-conditioned culture media was collected, filtered, and concentrated as indicated in the Material & Methods section.

Efficient exosome isolation from the concentrated hADSC-conditioned media was achieved by size-exclusion chromatography (SEC). This technique of isolation of exosomes was chosen as several published studies have shown that SEC retains the biophysical properties of exosomes and results in higher exosome yield (16). Once all the fractions were collected, a typical elution profile is observed based on the

determination of total protein concentration in each fraction (Figure 5A). As expected, most of proteins present in the sample eluted in the later fractions (from fraction 16 to 24).

Several tetraspanins, in particular CD9, CD63, CD81, CD82 or CD37 are specifically enriched in the membrane of exosomes. Consequently, they are often described and used as exosome biomarkers (17). This family of proteins with four transmembrane domains (TM4SF) are critically important for trapping both membrane and luminal proteins and are involved in a multitude of biological processes (18).

The expression of exosomal biomarkers in the fractionated samples of hADSC conditioned media was analyzed by ELISA detection of CD63 (Figure 5A). High relative content of CD63 was found in the fractions 16 to 20 with a peak in fraction 19, corresponding to a relative quantity of 9.1 $\times 10^{10}$ exosomes in this fraction. Non-conditioned media used as negative control, showed analogous profile in total protein determination, however, no expression of CD63 was detected in the sample (data not shown).

These results were confirmed by the detection of CD63 by western blot in a set of SEC fractions (Figure 5B). CD63 was detected in SEC fractions 13 to 20 with a gradual increase from fraction 13. The negative control, a non-fractionated non-conditioned sample, shows no expression of CD63, confirming the results obtained by ELISA. Exosomes isolated from a human mesenchymal stem cell line from SBI, were used as positive control and a non-fractionated sample, were also included in the test to confirm the presence of CD63+ exosomes in the whole sample.

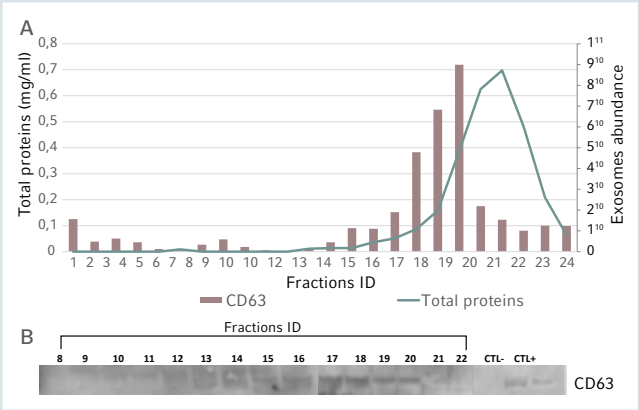

Fig. 5: Isolation and characterization of stem cell-derived-exosomes from hADSC- conditioned media. (A) Total protein profile of fractionated conditioned media (Nanodrop analysis) and CD63 determination by ELISA detection of SEC fractions. (B) CD63+_Western blot analysis of a set of fractions (from fraction 8 to 22). CTL (-): non-conditioned media, CTL (+): Exosomes isolated from a human mesenchymal stem cell line from SBI and NF: non-fractionated sample.

Several techniques have been commonly used to characterize the physicochemical properties of exosomes such as size, shape, surface charge, density, and porosity (2). This includes techniques like Nanoparticle Tracking Analysis (NTA), Dynamic Light Scattering (DLS), Centrifugal Liquid Sedimentation (CLS) and flow cytometry among others (2-16). In the present study, the size of the vesicles isolated from the hADSC-conditioned media was determined first by the DLS technique. The range of EVs size in the sample showed a mono-modal distribution (only one population) of approximately 60 – 110 nm with a mean size of ~ 80 nm (Figure 6A). This data was confirmed by the CLS technique. In this case, the range of EVs size in the sample showed a bi-modal distribution (two nearby populations) of approximately 35 – 140 nm with a first peak at ~ 42 nm and a second peak at 57 nm (Figure 6B). The slightly different results obtained from both techniques could be explained by the better resolution presented by the CLS technique, due to the use of a

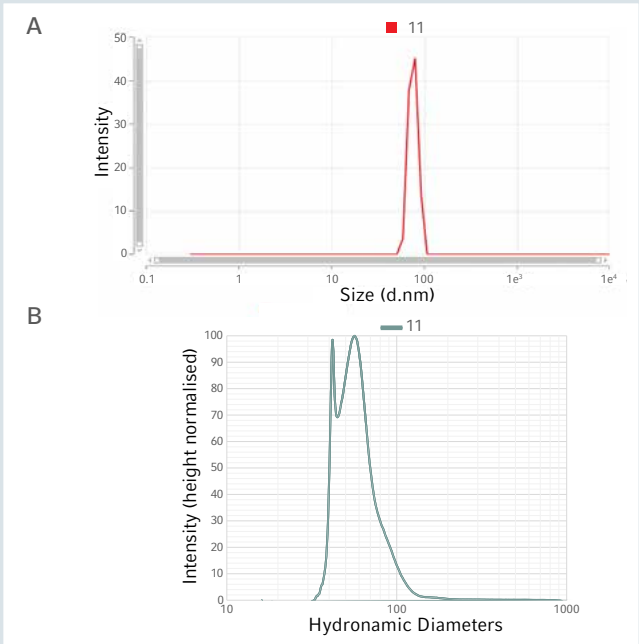

Fig. 6 : Particle size distribution of isolated hADSC-derived exosomes. Size of vesicle measured by (A) DLS and (B) CLS in hADSC conditioned media collected at the end of the collection phase (2 days).

gradient of sucrose (2 - 8%) and a lower wavelength of the laser compared to the DLS technique (16). However, in both cases, the range size of the vesicles presents in the hADSC conditions media sample correspond to the expected size of exosomes (less than 150 nm in diameter).

Conclusion

In comparison with conventional 2D culture platforms, bioreactors offer important advantages to optimize the upstream process of an exosome production workflow among which process monitoring, control and scalability opportunities. The DASbox Mini Bioreactor System, processing in parallel 4 bioreactors of 60 to 250 ml working volume, is of particular interest to identify the most appropriate cell culture parameters required for an optimal exosome collection. This system is your ideal partner into upstream process development from R&D to production. The present study illustrates the successful expansion of hADSC in an DASbox Mini Bioreactor System equipped with BioBLU 0.3 Single-Use Bioreactors. Indeed, following this procedure, a final cell density of 8.3×10^5 cells/ml has been obtained from an initial seed density of 2.4×10^4 cells/ml after only 5 days of culture expansion, representing a 34.7 fold-expansion. Following the cell expansion phase, the system ensures a fine control of the main bioprocess parameters during 2 additional incubation days in EV collection medium, ensuring optimal conditions for EV quality preservation. From a total of 88.41×10^6 hADSC, we have estimated the quantity of exosomes present in the conditioned media to 3.71×10^{11}, meaning almost 4200 particles per cell. Presenting the expected size range and positive for the CD-63, those EV could be considered as exosomes.

As demonstrated by the present work, the DASbox Mini Bioreactor System equipped with BioBLU 0.3 Single-Use Bioreactors is suitable for the successful collection of high quantity of hADSC-derived extracellular vesicles. To complete those data, efficient stem cell exosome production has also been demonstrated at large scale on the SciVario® twin bioreactor control system (19).

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Ordering information

Description	Order no.
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DASbox® GA4 Exhaust Analyzing Module*, O_2 1 – 50 %, CO_2 0 – 25 %, including I/O option and accessories for 4 vessels	76DXGA4X
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