

Expansion of Human Bone Marrow-Derived Mesenchymal Stem Cells in BioBLU® 0.3c Single-Use Bioreactors

Vincent Dufey¹, Aurélie Tacheny¹, Muriel Art¹, Ulrike Becken^{2*}, Françoise De Longueville¹

¹ Eppendorf Application Technologies S.A., Namur, Belgium

² Eppendorf AG Bioprocess Center, Juelich, Germany

* corresponding author: becken.u@eppendorf.com

Abstract

The use of adult stem cells holds great promise for new cell-based therapies and drug discovery. For their routine application, large numbers of cells have to be produced with consistently high quality. Two-dimensional cultivation systems, such as T-flasks, are widely used, but they are limited in terms of control and scalability. Stem cell expansion in rigid-wall, stirred-tank bioreactors, however, facilitates the precise control of critical process parameters like pH and dissolved oxygen, and allows a more straightforward scale-up to larger process dimensions. We tested the suitability of Eppendorf BioBLU 0.3c Single-Use Vessels controlled by a DASbox® Mini Bioreactor System for the expansion of human bone marrow-derived mesenchymal stem cells on microcarriers,

and obtained 1×10^8 cells in a working volume of 250 mL. The cells were able to differentiate into osteocytes and chondrocytes, respectively, demonstrating that their expansion in stirred-tank bioreactors did not affect multipotency.

BioBLU Single-Use Vessels with maximum working volumes of 250 mL and 3.75 L were previously used for the expansion of human induced pluripotent stem cells as cell-only aggregates [1] and of adipose-derived mesenchymal stem cells on microcarriers [2]. These results, and our current data, suggest that Eppendorf BioBLU Single-Use Vessels are widely applicable for the expansion of different stem cell types at various scales.

Introduction

Although the term *stem cells* had already appeared in the scientific literature by the mid-1800s, the number of research studies on these cells only increased at the beginning of the twenty-first century. Stem cells are unspecialized cells which have the ability to self-renew, and the capacity to differentiate into specialized cells. According to their origin, they are classified into embryonic stem cell (ESCs), derived from an embryo, and adult stem cells (ASCs), found in adult tissues. Mesenchymal stem cells (MSCs) are adult stem cells,

which can originate from a large variety of tissues such as bone marrow, adipose tissue, placenta, muscle or umbilical cord. MSCs are defined as multipotent, meaning they are capable of differentiating into more than one, but not all, cell types. In contrast, ESCs are pluripotent, meaning they can generate all cell types. MSCs are attractive candidates for therapeutic applications, especially in the field of regenerative medicine [3]. Despite their limited differentiation potential, MSCs offer great advantages

compared to ESCs, as they do not pose ethical issues, they can be isolated from various sources, and they reduce the risk of rejection reactions. The doses of human MSCs (hMSCs) needed for clinical trials are estimated at between one and 200 million cells per patient, depending on the disease being tackled [4]. One of the most important challenges in providing hMSCs for curative use is the production of large quantities of cells in a robust manner. Indeed, whatever the tissue source, the number of hMSCs extracted is very low, and not sufficient for clinical use; hence the hMSCs have to be expanded following isolation. Besides providing the needed cell quantities, hMSC

production must also comply with the manufacturing process regulations required of a fully controlled production system. hMSC expansion in stirred-tank bioreactors can be monitored and is scalable, and hence can fulfill these requirements from experimental quantities to production. An Eppendorf DASbox Mini Bioreactor System equipped with BioBLU 0.3 Single-Used Vessels has already been successfully used for the expansion of pluripotent stem cells in suspension [1]. In the study presented here, we used the same bioreactor system to culture adherent multipotent stem cells on microcarriers, and reached a clinically relevant number of cells.

Material and Methods

Initial cell culture in T-flasks

We obtained human bone marrow-derived mesenchymal stem cells (hMSCs-BM), cryopreserved after the second passage, from Lonza®, Switzerland. To reach the cell quantity needed for this study, we first expanded hMSCs-BM in Eppendorf Cell Culture Flasks T-175 for four passages. hMSCs-BM were cultured in MSCGM™ Mesenchymal Stem Cell Growth BulletKit™ Medium (Lonza), including both the basal medium and the necessary supplements. At passage six, we harvested the cells to be used for three-dimensional microcarrier culture in a stirred-tank bioreactor.

Preparation of microcarriers

We prepared Cytodex® type 1 (GE Healthcare® Bio-Sciences, Sweden) and Cytodex type 3 microcarriers (Sigma-Aldrich®, USA) according to the manufacturer's instructions. This included four steps: the microcarrier hydration, a washing with PBS, the microcarrier sterilization by autoclaving, and a final rinsing in the culture medium.

Culture of hMSCs-BM on microcarriers in BioBLU 0.3c Single-Use Vessels

We processed the experiments in parallel in a 4-fold Eppendorf DASbox Mini Bioreactor System for cell culture (Figure 1), equipped with Eppendorf BioBLU 0.3c Single-Use Vessels. We inoculated the cultures with hMSCs-BM cultured in T-flasks, combined with Cytodex (type 1 or 3) microcarriers. To ensure a similar cell-to-bead ratio (10 cells/bead), we used 140 mg of Cytodex type 1 and 200 mg of Cytodex type 3 microcarriers. The initial number of cells per bioreactor was 6×10^6 cells, which corresponds

to a cell density of 9,700 cells/cm² for Cytodex type 1 and 11,000 cells/cm² for Cytodex type 3. We cultured hMSCs-BM in MSCG Mesenchymal Stem Cell Growth BulletKit Medium containing both the basal medium and the necessary supplements, in an initial working volume of 100 mL. To promote the initial cell adhesion, we did not agitate the culture for 24 hours. After this attachment period, we manually adjusted the culture volume to 200 mL, and set the agitation speed to 60 rpm for the entire proliferation phase. The cells were cultured at 37°C. The pH of the growth medium was controlled at 7.6 by automatic addition of CO₂ in the vessel headspace. We set the dissolved oxygen (DO) level to 40 %. The DO setpoint was maintained by delivering gas (N₂, air, and O₂) into the medium with the gas flow set at 0.1 sL/h. To maintain the culture until its maximum yield, we exchanged 50 % of the culture medium after 6 and 8 days of culture. After 9 days, up to 70 % of the medium was replaced almost daily. During those refreshment steps, agitation was stopped to let the microcarriers sediment. The cells were cultivated for 20 days on Cytodex type 1 and for

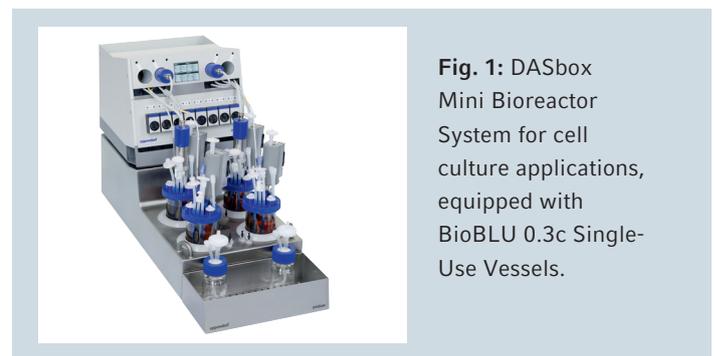


Fig. 1: DASbox Mini Bioreactor System for cell culture applications, equipped with BioBLU 0.3c Single-Use Vessels.

27 days on Cytodex type 3 microcarriers.

Analysis of cell viability

During the proliferation phase, we regularly analyzed the cell viability on the microcarriers by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) coloration. To do so, we supplemented 400 μL of cell/microcarrier suspension with 40 μL of 5 % MTT solution (Sigma-Aldrich), and incubated the suspension at 37°C for 45 minutes. MTT produces a yellowish solution that is converted to dark blue, water-insoluble MTT formazan by the mitochondrial dehydrogenases of living cells. The dark blue crystals precipitate inside the cells, and can be seen by light microscopy.

Cell counting and metabolite measurement

To evaluate cell growth, we counted the cells at days 6, 9, 13, 15, 16, and 20. At each of these points in time, we collected independent samples of 3 mL of the cell/microcarrier suspensions. Cells were detached from microcarriers by addition of 0.25 % Trypsin-EDTA, and the cell number was determined using the CASY® Cell Counter and Analyzer, model TT (Omni Life Science®, USA).

We used the supernatants collected during cell counting to quantify glucose and lactate concentrations, using the Glucose RTU™ Kit (Biomérieux®, France) and L-Lactate Assay Kit (Abcam®, United Kingdom), respectively.

hMSC-BM differentiation—osteogenic lineage

For osteogenic lineage differentiation, we detached hMSCs-BM from microcarriers by trypsin treatment, and seeded them into BD BioCoat™ Fibronectin Cellware, 24-well plates

(Corning®, USA) at a density of 7×10^3 cells/well. One day after seeding, we replaced the expansion medium with hMSC Osteogenic Differentiation BulletKit Medium (Lonza). This serum-containing induction medium is designed to induce osteogenic differentiation of hMSCs into mature, functionally active osteoblasts. We exchanged the medium 3, 7, 10, 14, and 18 days after the first induction. At days 14 and 21 we assessed the bone cell mineralization using the OsteoImage™ Mineralization Assay (Lonza). Moreover, at day 21 we stained calcium deposits with an anthraquinone dye (Alizarin Red S Staining Kit, ScienCell, USA).

hMSC-BM differentiation—chondrogenic lineage

For chondrogenic lineage differentiation we detached hMSCs-BM from microcarriers by trypsin treatment, and seeded them into BD BioCoat Fibronectin Cellware, 24-well plates at a density of 8×10^4 cells/well. Three days after seeding, we replaced the expansion medium with hMSC Chondrogenic Differentiation BulletKit Medium (Lonza). This is a serum-containing medium designed to induce chondrogenic differentiation of hMSCs into mature, functionally active chondrocytes through the addition of TGF- β 3. We exchanged the medium 2, 4, 7, 9, and 11 days after the first induction. At day 14, we detected proteoglycans secreted by chondrocytes by Alcian Blue staining (Sigma-Aldrich).

Results

hMSC-BM expansion on microcarriers in BioBLU 0.3c Single-Use Vessels

In order to investigate the suitability of BioBLU 0.3c Single-Use Vessels for the scalable production of multipotent stem cells, we cultured hMSCs-BM in parallel on two microcarriers types, Cytodex type 1 and Cytodex type 3. Cytodex 1 microcarriers are based on a dextran matrix covered with positively charged groups, while a layer of denatured collagen is covalently bound on the Cytodex 3 dextran surface.

The quantity of MSCs in the bone marrow is very low, and

hMSCs-BM have to be strongly expanded to obtain clinically relevant cell numbers. One possibility to expand cells in a microcarrier culture is the use of a technique called *bead-to-bead transfer* or *colonization*. By adding fresh microcarriers into the existing culture, cells can switch from one carrier to another, and start to grow on the empty beads [4]. This allowed us to avoid subculturing techniques traditionally used with adherent cells, and was successfully employed for the culture of cell lines such as Vero cells [5], as well as for hMSC expansion [6]. We used MTT coloration to visually monitor cell proliferation, and to detect the appropriate

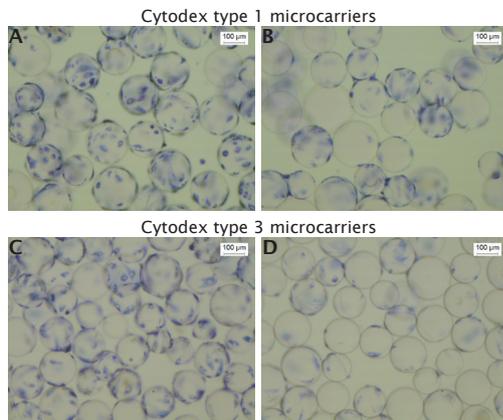


Fig. 2: MTT-coloration of hMSCs-BM. Upper panel: Cells were cultured on Cytodex type 1 microcarriers and stained before (A) and one day after (B) the addition of fresh beads. Lower panel: Cells were cultivated on Cytodex type 3 microcarriers and stained before (C) and one day after (D) the addition of fresh beads.

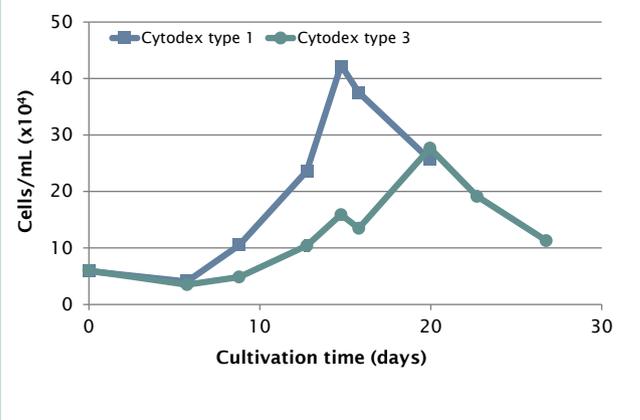


Fig 3: hMSC-BM growth profiles in BioBLU 0.3c Single-Use Vessel with Cytodex type 1 and Cytodex type 3 microcarriers. Mean values of two experiments carried out in parallel are shown.

moment to add fresh microcarriers. When stained cells were visible on all beads (Figure 2A and 2C) we added fresh microcarriers to offer additional growth surface. Figure 2B and Figure 2D demonstrate that the colonization already started the day after carrier addition.

Because the cell proliferation rate is different depending on the microcarrier type used, we followed each culture independently. In the culture performed on Cytodex type 1 we added fresh microcarriers at days 6 and 10, while additional beads were added at days 6 and 15 into the culture using Cytodex type 3 carriers.

We cultured hMSCs-BM for 20 days on Cytodex type 1 microcarriers and for 27 days on Cytodex type 3 microcarriers. During the expansion we counted the cells at different time points. As shown in Figure 3, we obtained the best proliferation rate on Cytodex type 1 microcarriers. The cell number increased 17.5 fold to a maximum cell density of 1×10^8 cells/bioreactor at day 14, corresponding to 4×10^5 cells/mL. On Cytodex type 3 microcarriers, 20 days of culture were needed to reach a maximum cell number of 7×10^7 cells per bioreactor, which is 11.5-fold higher than the initial seeded quantity and corresponds to 2.5×10^5 cells/mL.

Close glucose monitoring revealed a rapid nutrient consumption of the hMSC-BM culture on Cytodex type 1 microcarriers (Figure 4A) and on Cytodex type 3 microcarriers (not shown). Two days after cell seeding, the

glucose concentration had decreased by 40 %. Despite the addition of 100 mL of fresh medium at day 2 the glucose concentration continued to drop. To maintain the culture until its maximum yield, we regularly exchanged the medium as described in the Material and Methods section. As a result of glucose consumption and feeding, the glucose concentration varied in a characteristic pattern, but never dropped below 0.1 g/L. As by-products of their glucose metabolism, the cells produce metabolites, such as lactate and ammonia, which accumulate in the medium. High lactate concentrations can affect hMSCs growth as well as cell morphology. We monitored the lactate concentration throughout the entire cultivation period and maintained it below 25 nM to ensure that a concentration of 35.4 nM, which was found to inhibit growth [7], was never reached (Figure 4B).

Multipotency analysis of hMSC-BM cultured in BioBLU 0.3c Single-Use Vessels

To confirm that hMSCs-BM cultured on microcarriers in BioBLU 0.3c Single-Use Vessels retained their differentiation capacity, we harvested cells from the microcarriers and used them for *in vitro* osteogenic and chondrogenic differentiation assays. Since the highest cell density was obtained on Cytodex type 1 microcarriers, only data generated from cells expanded on this support are shown. However, we obtained

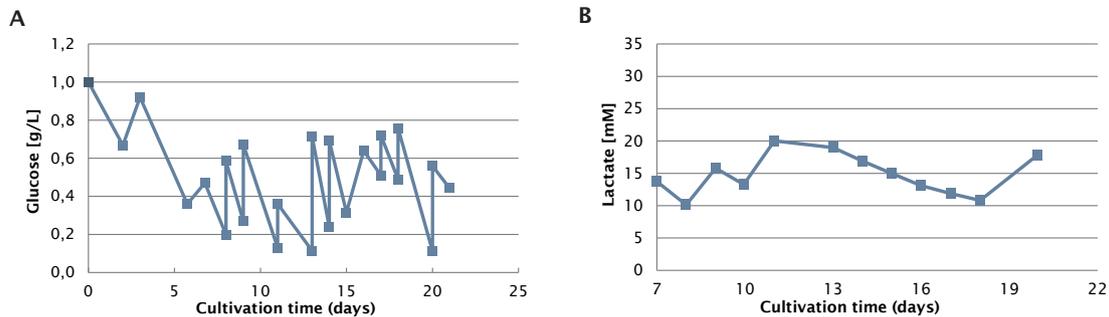


Figure 4: Metabolic profiles of the hMSC-BM culture on Cytodex type 1 microcarriers.

A: Glucose consumption profile of the hMSC-BM culture on Cytodex type 1 microcarriers. A representative result is shown.

B: Lactate production in the hMSC-BM culture on Cytodex type 1 microcarriers. A representative result is shown.

equivalent results with cells cultured on Cytodex type 3 microcarriers.

Osteogenic differentiation is divided into three stages: cell proliferation (from day 1 to 4), extracellular

matrix maturation (from day 5 to 14), and finally, matrix mineralization (from day 14 to 28). This last step is characterized by high expression of osteocalcin and osteopontin, followed by calcium and phosphate deposition [8]. Bone is composed of the organic protein collagen and the inorganic mineral hydroxyapatite. By specific staining of the hydroxyapatite portion, bone mineralization can be quantified during the differentiation process. As shown in figure 5A, bone mineralization was detectable already after 14 days of induction and was clearly increased at day 21. Hydroxyapatite was only detected on induced cells. After 21 days of osteogenic lineage induction, calcium deposits, indicating the matrix mineralization phase, were furthermore detected by Alizarin Red S staining on induced hMSCs-BM, while we observed no deposition in the not-induced negative control (Figure 5B).

Next we assessed chondrogenic differentiation, which is a complex, multi-stage process characterized by the production of cartilage-specific molecules such as type II collagen and proteoglycans. Detectable by Alcian Blue staining, proteoglycans are a good indicator of cartilage formation and are considered to be a marker of cell chondrogenesis. After 14 days of chondrogenic lineage induction, we detected strong cartilage proteoglycan synthesis, while the level stayed low in the negative control (Figure 6).

These results clearly demonstrate that the differentiation potential of hMSCs-BM is maintained after intensive expansion on microcarriers in BioBLU 0.3c Single-Use Vessels. We successfully differentiated cells into osteocytes and chondrocytes, indicating that multipotency is maintained when hMSCs are cultured in a stirred-tank bioreactor.

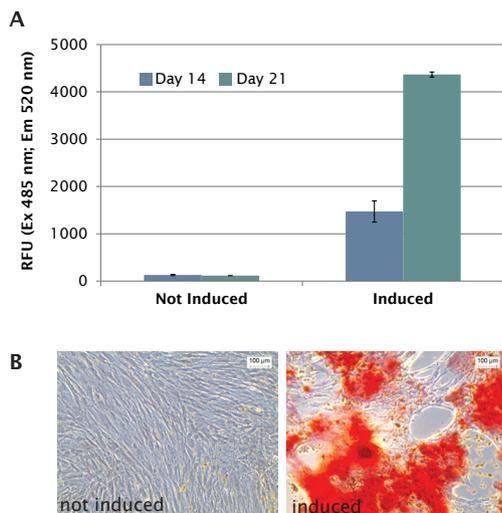


Fig. 5: Osteogenic lineage differentiation

A: OsteoImage Mineralization assay with hMSCs-BM cultured on Cytodex type 1 microcarriers after 14 and 21 days of osteogenic lineage induction. Means and standard deviations are shown (n=3 replicates). RFU: Relative fluorescence units. Ex: Excitation. Em: Emission.

B: Alizarin red S staining of hMSCs-BM cultured on Cytodex type 1 microcarriers. Negative control (left) and induced cells (right) on type 1 microcarriers. A representative result is shown.



Fig 6: Chondrogenic lineage differentiation
Alcian Blue staining of hMSCs-BM cultured on Cytodex type 1 microcarriers after 14 days of chondrogenic lineage induction.

Conclusion

In this application note we demonstrate the successful expansion of hMSCs-BM in an Eppendorf DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Vessels. We evaluated Cytodex type 1 and Cytodex type 3 microcarriers as growth surfaces for these adherent stem cells. We obtained the highest cell number on Cytodex type 1 microcarriers, with a 17.5-fold expansion, corresponding to a maximum cell density of 1×10^8 cells/batch at day 14. On Cytodex type 3 microcarriers we reached a maximum cell

number of 7×10^7 cells/bioreactor, which is 11.5-fold higher than the initial seeded quantity. In this study, we furthermore demonstrated that expansion in BioBLU 0.3c Single-Use Vessels did not affect the hMSC multipotency, as they conserved their ability to differentiate into osteocytes and chondrocytes. Taken together, these results demonstrate that the DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Vessels is suitable to expand multipotent stem cells in a safe and controlled manner.

Literature

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System for Cell Culture Applications , max. 5 sL/h gassing	
4-fold system for single-use vessels	76DX04CCSU
8-fold system for single-use vessels	76DX08CCSU
16-fold system for single-use vessels	76DX16CCSU
24-fold system for single-use vessels	76DX24CCSU
BioBLU® Single-Use Vessels	
BioBLU® 0.3c Single-Use Vessel, cell culture, 1 pitched-blade impeller, sterile, 4-pack	78903508
Eppendorf Cell Culture Flasks	
Eppendorf Cell Culture Flasks T-175, sterile, free of detectable pyrogens, RNase and DNase, DNA. Non-cytotoxic, TC treated, with filter cap, 48 flasks (12 bags × 4 flasks)	0030 712.129

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