Expansion of multipotent mesenchymal stromal cells on gelatin coated alginate microcarriers

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Abstract

Human multipotent mesenchymal stromal cells (MSC) are considered promising candidates for cellular therapies and for tissue engineering in regenerative medicine applications. They have been focus of intense research because of their multi-lineage differentiation capacity, immunomodulatory properties and ability to support of haematopoiesis. MSC have been traditionally cultivated in 2-D configuration as monolayer in tissue culture flasks. MSC have also been cultivated in bioreactors either with microcarriers where cells grow attached or in aggregates promoting cell-to-cell interaction. An alternative approach is encapsulation in hydrogels protecting cells from the external environment while offering 3-D support for cell cultivation. In this project, it was demonstrated that using gelatin coated alginate microcarriers resulted in MSC adhesion and expansion and static and dynamic conditions. After expansion, MSC immunophenotype was positive for CD73 and CD90 while negative for CD31, CD80 and HLA-DR, and the multilineage differentiative potential was maintained since MSC were able to differentiate in osteoblasts, adipocytes and chondrocytes.

Keywords: bioreactors, CultiSpher-S, glutaraldehyde, magnetite.

1. Introduction

1.1. Motivation

The motivation of this work results from the therapeutic potential of human multipotent mesenchymal stromal cells (MSC). These cells have gathered attention as building blocks for tissue engineering and have been seen as promising candidates for cellular therapies. In vivo MSC are usually present in a unique micro-environments known as the cellular niche. They can be obtained from various sources such as bone marrow, adipose tissue, cartilage and umbilical cord matrix. However, the number of cells that can be obtained from available donors is very low because MSC are very rare and their number decline with donor age. For clinical applications where the number of MSC required for one dose is 1 to 2 million MSC/kg body weight, in order to meet the clinical relevant doses, new scale-up methodologies need to be developed. Different types of bioreactors have been studied to obtain large numbers of MSC. Since MSC are an adherent-dependent cell type, microcarriers can used to provide surface area for cell growth. An alternative approach is encapsulation in hydrogels protecting cells from the external environment while offering 3-D support for cell cultivation.

1.2. Aim of studies

This research project aims at the evaluation of new strategies for the ex vivo expansion of MSC. Therefore, this project will explore the properties of alginate towards a final goal: the development of robust protocol for culture of MSC. This project presents an opportunity to learn the basic cell culture techniques and to combine materials for the development of new expansion procedures under a sterile environment.

Therefore, the specific objectives were:

1. To develop an alginate microcarrier coated with an extracellular matrix protein (gelatin);

2. To evaluate the adhesion and detachment of MSC on the new microcarriers;

3. To investigate the expansion of MSC on the same microcarriers in static and dynamic conditions.

1.3. State of the art

MSC are classified as adult stem cells and they are often committed with a specific cell lineage. Examples of adult stem cells include MSC, hematopoietic stem cells and neural stem cells. MSC have the ability to proliferate and give origin to specific mesenchymal tissues including bone, cartilage, muscle,
bone marrow stroma, fat, and other connective tissues. They can be obtained from different sources such as bone marrow [1], adipose tissue [2], umbilical cord matrix [3], placenta [4] and synovium [5].

MSC isolated from bone marrow and other tissues have been routinely cultivated as monolayers in tissue culture flasks (2D). The tissue culture flasks are easy to handle, however they have several limitations. These include the fact that the oxygen transport might be diffusion limited. Moreover, the pH and O2 tension are not controlled. In addition, the 2D cultivation is time and labour consuming when large number of cells are need for cellular therapies, since this methodology requires extensive inoculation, medium changes, replating the cells and harvesting. The application of three-dimensional (3D) cell culture techniques is receiving increased interest with evidence showing significant differences between the cellular phenotype and biological response of cells cultured in monolayer and 3D cell culture. The 3D methods facilitate greater cell-to-cell contacts and interactions of cells with the extracellular matrix (ECM), allowing cells to adapt to their native morphology, which may influence signalling activity.

1.3.1 Culture in microcarriers

Human bone marrow (BM) mesenchymal stem cells (MSC) have been cultivated in low-serum containing medium (2% of fetal bovine serum, FBS) in CultiSpher-S (a gelatin cross linked microcarrier) in spinner flasks [6]. The authors showed that FBS improved the cell adhesion resulting in a reduced lag phase. For clinical applications, MSC should be cultivated in xenofree medium. This would eliminate the potential risk of immune response against xenogeneic antibodies, such as animal proteins, bacteria or virus that can derive from the FBS [7] [8]. Therefore, the potential to expand human BM MSC and adipose-derived stem cells (ASC) in xenofree conditions have been assessed [9]. Cells were cultivated in MesenPRO RS/ StemPro MSC SFM XenoFree medium in spinner flasks. More recently, this research group has developed the same approach for a 1L-scale controlled stirred-tank bioreactor [10].

Alginate has also been modified to work as a microcarrier. Alginate has been a polymer of choice for a wide number of biomedical applications such as drug delivery, wound dressings, cell culture and tissue engineering [11]. Alginites are constituted by (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers, sequential G units, and regions of organized M and G units. Gelling of aqueous alginate solutions is due the presence of divalent cations, such as Ca2+. The first example of an ECM protein coated alginate microcarrier showed the expansion of human chang liver (CCL-13) and mouse fibroblast (L929) cell lines in static conditions [12]. Both cell lines showed rapid proliferation in collagen-coated Ba2+-alginate microcarriers. Another study has demonstrated the proliferation of CHO-K1 and PA317 cells on calcium-alginate microcarriers with cross-linked gelatin in spinner flasks [13]. The gelatin cross-linking was achieved by immersion of the calcium alginate microcarriers in a 0.4% glutaraldehyde solution for 30 min. This step promoted the covalent bound between gelatin and alginate the while increasing the mechanical strength of the microcarriers.

MSC have been cultivated in RGD-modified alginate microcarriers in spinner flasks [14]. The authors demonstrated that at a given RGD peptide density, the increase in the microcarrier diameter resulted in the increase of cell adhesion by factor of three. On the other hand, the increase in the microcarrier diameter resulted in a decrease of the growth rate by a factor of four. In addition, when the differentiation in the osteogenic lineage was induced, it was showed that by increasing the RGD peptide density, the cellular secretion of osteogenic differentiation markers (osteocalcin and osteopontin) was increased.

2. Materials and Methods

For cell culture, sodium alginate 1.8% (w/v) was prepared by dissolving alginate powder (Sigma) in Milli-Q water under constant agitation at 45°C. To avoid contamination, the alginate solution was sterilized via sterile filtration using a 22 μm syringe filter (Milipore).

2.1. Alginate microcarriers preparation

Sodium alginate solution 1.8% (w/v) was pumped at 5 ml/h (Harvard Apparatus 22, Southnatick, MA, USA) trought a needle of 101.6 μm. Alginate microspheres were obtained by applied voltage (10kV) between the needle and a copper plate which was inside a CaCl2 300mM solution. The cross-linking of alginate within the microspheres was achieved under constant stirring (80 rpm) after 15 min. To avoid swelling the alginate microcarriers kept in CaCl2 300mM solution and stored at 4°C.

2.2. Gelatin coating of alginate microcarriers

Alginate microcarriers were incubated in gelatin 1% (w/v) solution for 2h with cycles of 2 min at 750 rpm and 10 min with non-agitation at 37°C. The supernatant was discarded and a aqueous solution of gluteraldehyde 0.4% (v/v) was added to promote covalent crosslinking of gelatin. The crosslinking time was 30min at 750 rpm, 22°C continuously. The gluteraldehyde solution was removed and the cross-
linked gelatin coated alginate microcarriers were incubated in a glycine solution (100 mg/mL) for 1 hour at room temperature with adequate agitation. Quenched with glycine solution was removed and the microcarriers were washed twice with PBS. The microcarriers were observed in the optical microscope for diameter distribution analysis.

2.3. Mechanical stability gelatin coated alginate microcarriers and test to EDTA
To understand how the gelatin coating can improve the mechanical strength of alginate microcarriers, different gelatin concentrations were used for cross-linking. The microcarriers were submitted to agitation at 750 rpm at 37°C for 21min using a Thermomixer®confort (Eppendorf AG). After visual inspection, the microcarriers were used to evaluate the if EDTA could be used to harvest the cells (instead of using proteases) by chelation of Ca²⁺ ions from the alginate structure.

In another experiment, new gelatin coated alginate microcarriers were added to StemSpan™ Spinner Flasks (30mL) with IMDM to observe if the microcarriers would break due to shear stress or if they would swell in the presence of presence of phosphate and bicarbonate buffers. The impeller rotational speed was increased every day from 30 to 80rpm and after 10 days. A sample of microcarriers was collected every day and observed under the microscope for size and shape. The dimensions of the impeller are 2.4cm (width) and 2.7cm (diameter).

2.4. Expansion of MSC on gelatin coated alginate microcarriers in static conditions
To investigate cell adhesion and proliferation on the gelatin coated microcarriers, MSCs from the same donor at passage 7 were seeded on a 24-well plate Ultra low Attachment (Costar®) at 5x10³ cells/cm² in the presence of DMEM/ 10% FBS/1% penicillin-streptomycin and cultured for 10 days at 37°C and 5% CO₂. Before cell seeding, the alginate micr0carriers coated with gelatin were washed twice with PBS and incubated in pre-warmed culture medium for 1 hour. The culture medium was changed every three days and the number of viability cells were counted every two days by Trypan Blue exclusion method. Cultispher-S microcarriers (Sigma) were used as positive control and non coated alginate microcarriers were used as negative control.

2.5. Expansion in spinner flask
Cultispher-S microcarriers (chosen as the control) were sterilized by autoclaving and equilibrated in pre-warmed (37°C) culture media. Before inoculation of the spinner flask, gelatin coated alginate microcarriers and Cultispher-S microcarriers were suspended in 10 mL of pre-warmed culture medium and added to StemSpan™ Spinner Flasks. Next, MSCs were inoculated at 5x10⁵ cells per mL in 1/2 of the final medium volume (15 mL). Cells were incubated at 37°C and 5% CO₂ with intermittent stirring for a total period of 24h (15 min. at 25-30 rpm, followed by 60 min. statically). After this period, the volume was brought to 30 mL (final volume). During time in culture, 25% of the culture medium was changed every two days. This was procedure was done by allowing the microcarriers to settle down in the bottom of the flask and the adequate volume of supernatant was removed. Then, the volume was brought to 30 mL by adding fresh medium to the spinner flask. In another experiment, Belco spinner flasks were used to obtain two replicates for gelatin coated microcarriers. MSCs were seeded at 5x10³ cells per mL in 40 mL (initial and final volume).

2.6. Immunophenotypic analysis
At the end of expansion in spinner flasks, MSCs were evaluated for immunophenotypical analysis. For each surface marker, 1 x 10⁵ cells were added to each FACS tube. Next, 5 µL of the respective monoclonal antibody was added and the mixture was placed in the dark for 15 min. Then, 2mL of PBS was added and the mixture was centrifuged at 1000 RPM for 5 min. Finally the supernatant was discarded and 500 µL of paraformaldehyde 2% was added. The analysis of MSC surface markers was done using the FlowJo software.

2.7. Mesodermal differentiation
For the osteogenic and adipogenic differentiation assays, cells were plated on 24-well plate (Falcon BD Biosciences®) at 3x10³ cells/cm² in the presence of DMEM with 10% FBS MSC qualified and 1% Penicillin-Streptomycin and incubated at 37°C and 5% CO₂. The medium was changed every 3 to 4 days until cells reach 80% confluence. Then, the culture media was replaced by the respective differentiation media, either osteogenic or adipogenic, and cells were cultured during 14-15 days with the medium changed every 3 to 4 days. For chondrogenic differentiation, the cells were plated on a 24-well Ultra Low Attachment plate (Costar®). A pellet of 2x10⁵ was resuspended and droplets of this suspension were plated on the surface of each well. The cells were placed in the incubator for 1h30 min to allow the droplets to dry. Then, the chondrogenic differentiation culture medium was added and changed every 3 to 4 days.

For the osteogenic differentiation staining, cells were washed with PBS and stained with 2.5% (w/w) silver nitrate (Sigma®) for 30 min at room temperature. Then, cells were washed three times with distilled water and observed under the microscope. For chondrogenic differentiation, cells were washed once with PBS and fixed with PFA 2% so-
lution for 30 min at room temperature. Then, cells were washed with PBS and stained with Alcian Blue 1% solution (Sigma®) prepared in 0.1N HCl for 30 min. The excess was removed by washing three times with PBS and observed under the microscope. For adipogenic differentiation, cells were washed with PBS and fixed with PFA 2% solution for 30 minutes at room temperature. Then, cells were washed once in distilled water and incubated with Oil Red-O solution 0.3% (Sigma®) for 1 hour at room temperature. Cells were washed twice with distilled water and observed under the microscope.

2.8. Metabolite analyses
To evaluate the nutrient consumption by MSCs and their metabolic production at different time points of culture, the supernatant was collected and the concentrations of glucose, glutamine, glutamate, lactate, ammonia and potassium were determined automatic using a BioProfile® 400 Analyzer (Nova® Biomedical, Waltham, MA).

3. Results and discussion
3.1. Alginate microcarriers
The microcarriers produced include a alginate core and a gelatin shell. The core of the microcarriers were manufacture by forming beads of controlled diameter crosslinking using Ca²⁺. The gelatin shell coating was stabilized with glutaraldehyde and serves two purposes: one is to bring mechanical stability, the second purpose is to provide a surface for cell adhesion. Additionally, it was tested the hypothesis of by using a lower amount of gelatin, potentially forming a more open gelatin shell that this would allow EDTA to diffuse across the gelatin layer, chelating the Ca²⁺ in the alginate core. Therefore, by controlling the gelatin layer thickness, cells could be detached by using EDTA in low concentration, avoiding the use of proteases.

First, alginate microcarriers were obtained by applied voltage as described in 2.1. Next, the alginate microcarriers were coated with gelatin with different concentrations (Table 1). Alginate microcarriers and gelatin coated alginate microcarriers were observed under the microscope and the diameter was determined using the Image Measurement Utility from MATLAB. It was observed that increasing the gelatin concentration of the solution used for alginate core coating leads to an increase in microcarrier diameter. Microcarriers are named according with concentration of the gelatin solution used. Therefore, for example "gelatin 1% coated alginate microcarriers", means that a solution of 1%(w/v) gelatin was used in the coating step.

To evaluate the mechanical strength, the different gelatin coated microcarriers were submitted to agitation at 750 rpm at 37°C for 21min. It was observed that for all the gelatin concentrations present in Table 1, the microcarriers were still intact. The same microcarriers were then used to evaluated if EDTA could break their spherical structure. It was observed that regardless the EDTA concentration used (35, 50 and 100mM), the alginate microcarriers coated with a gelatin concentration above 0.1% (w/v) were stable, indicating that EDTA could not diffuse across the gelatin shell layer. It was noticed that in the absence of the gelatin layer, the alginate microcarriers were easily dissolved by EDTA (35 mM). Therefore, the use of gelatin 0.1% (w/v) solution for coating the alginate microcarriers provides a boundary condition where microcarriers integrity is EDTA concentration dependent (all experiments performed with 21 min exposition of the same microcarriers density to solutions of EDTA at different concentrations).

Next, the stability of gelatin 0.1% coated alginate microcarriers to shear stress at the maximum of 80 rpm was evaluated. Gelatin 0.1% coated alginate microcarriers were added to a StemSpan™ Spinner Flask and non coated alginate microcarriers were used as a control. After 10 days of agitation, gelatine 0.1% coated alginate microcarriers and non-coated alginate microcarriers remained intact. Besides the mechanical strength, other important parameter is the microcarrier swell. It was observed that gelatine 0.1% coated alginate microcarriers and non-coated alginate microcarriers had an increase in microcarrier size throughout agitation period.

### Table 1: Assay to evaluate the "response" of gelatin coated alginate microcarriers to EDTA.

<table>
<thead>
<tr>
<th>Gelatin % (w/v)</th>
<th>EDTA [mM]</th>
<th>Break</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35, 50, 100</td>
<td>No</td>
</tr>
<tr>
<td>0.75</td>
<td>35, 50, 100</td>
<td>No</td>
</tr>
<tr>
<td>0.5</td>
<td>35, 50, 100</td>
<td>No</td>
</tr>
<tr>
<td>0.25</td>
<td>35, 50, 100</td>
<td>No</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>100%</td>
</tr>
<tr>
<td>0.1</td>
<td>50</td>
<td>80-90%</td>
</tr>
<tr>
<td>0.1</td>
<td>35</td>
<td>20-30%</td>
</tr>
<tr>
<td>0</td>
<td>35</td>
<td>100%</td>
</tr>
</tbody>
</table>

3.2. Expansion of MSCs on gelatin coated microcarriers in static conditions
It was evaluated the cell adhesion and prolifera- tion of MSC on the gelatin coated microcarriers in the presence of DMEM/ 10% FBS/1% penicillin-streptomycin. MSC were seeded on a 24-well plate Ultra low Attachment (Costar®) at 5×10³ cells/cm². At day 1, the initial cell adhesion efficiency was calculated as the number of cells adherent on microcarriers divided by the initial number of cells at day 0. The cell adhesion efficiency were 13.8 ±1.7 % and 12.8 ±4.5 %, for gelatin 1% coated alginate microcarriers and for CultiSpher-S microcar-
riers (control), respectively (Figure 1b). The maximum number of cells was achieved at day 10 for both cultures. For gelatin 1% coated alginate microcarriers this corresponded to $4.4 \pm 0.5 \times 10^4$ cells (21$\pm$2.3-fold) and for CultiSpher-S microcarriers to $3.0 \pm 0.3 \times 10^4$ cells (15.5$\pm$1.5-fold) (Figures 1a and 1c).

The next step was to evaluate the proliferative capacity of MSCs on gelatin coated alginate microcarriers in dynamic conditions and to compare it with the static cultures. The initial cell adhesion efficiency for gelatin 1% coated alginate microcarriers was 22%. CultiSpher-S microcarriers were used as control, however the microcarriers were completely destroyed by the impeller due to insufficient space between the impeller and the bottom of the flask. After day 4, the cell culture enters an exponential phase reaching a maximum cell number of $3.9 \pm 0.2 \times 10^6$ cells at day 9 (8.8-fold) (Figures 2a and 2b). The specific growth rate for this culture was 0.034h$^{-1}$. After day 10, the number of cells started to decrease. This could be due to surface limitation or could be associated with lack of nutrients and (or) accumulation of metabolites. To study this question, in the next experiment at different time points of culture, the supernatant was collected and the concentrations of nutrients and metabolites were determined to evaluate the nutrient consumption by MSC and their metabolite production.

![Graph](image1.png)

**Figure 1:** Expansion of MSC on gelatin coated alginate microcarriers in 24-well plate.

3.3. **Expansion of MSCs on gelatin coated microcarriers in dynamic conditions**

The next step was to evaluate the proliferative capacity of MSCs on gelatin coated alginate microcarriers under stirred conditions and to compare it with the static cultures. The initial cell adhesion

![Graph](image2.png)

**Figure 2:** Expansion of MSC on gelatin coated alginate microcarriers in StemSpan Spinner Flask.

After expansion, the immunphenotype of MSC was analyzed. It was observed that MSC were negative for CD 31, CD80 and HLA-DR. The expression the surface markers CD73 and CD90 was above 90%. The expression of CD105 was only 7% (Figure 3). From the literature, CD105 is indicated as being very sensitive to the action trypsin, so usually
has a lower value compared with CD73 and CD90. However, in this experiment a mistake could have been during the labelling with the antibodies.

![FACS histograms](image)

Figure 3: Analysis of MSC surface markers after expansion in spinner flask.

After expansion, the mesodermal differentiation potential was evaluated by replating MSC in differentiation culture medium as described in 2.7. It was observed that MSC were able to differentiate in osteoblasts, adipocytes and chondrocytes indicating that the multilineage differentiative potential was maintained (Figure 4).

MSC were also expanded on gelatin 1% coated alginate microcarriers in Bellco® Spinner Flasks (Figure 5). At day 1, the cell adhesion efficiencies were 43.9 ±2.2 % and 48.9 ±4.2 % for spinners A and B, respectively. In Spinner A, the maximum cell number was 3.4± 0.2×10⁶ cells at day 7 (3.8-fold). At day 8, the cell number on spinner A decreased although on day 9 this value was much higher which can be attributed to experimental error on sample collection. At day 8, new gelatin 1% coated alginate microcarriers (280 cm²) were added to spinner B to promote more surface area during the exponential phase for MSC to attach. In spinner B, the maximum cell number was 2.9±0.3×10⁶ cells at day 8 (2.9-fold).

### 3.3.1 Metabolism of MSCs

In order to understand the impact on the growth rate of mammalian cells of different parameters such as oxygen tension or feeding regime, it is important to analyse the cellular metabolism. It has been described that the growth of MSC can be inhibited by metabolites at certain concentration. One study showed that the inhibitory concentration for human MSC of lactate and ammonia were 35.4mM and 2.4 mM, respectively [15]. The inhibitory effect from

![Osteogenesis](image)

(a) Osteogenesis.

![Adipogenesis](image)

(b) Adipogenesis

![Chondrogenesis](image)

(c) Chondrogenesis

Figure 4: Differentiation of MSC after expansion in StemSpan™ Spinner Flask.
the excess of lactate results from the decrease in the pH and the change in the osmolarity. Unprotonated ammonia (NH₃) can diffuse across the cell membrane and change the intracellular pH. Throughout the culture, 25% of the medium was changed every two days. It was observed that for both spinners, the concentration of glucose decreased rapidly and at the end of day 6 and 8, the values for glucose were close to zero (Figure 6a). The consumption of glutamine was higher in the initial stage between day 0 and day 4. The lactate concentration increased reaching a maximum at day 8. The values obtained for lactate concentration are lower than the inhibitory growth values reported in the literature. It appears that the decrease in cell number at day 9 could be related with limitation in glucose concentration. Regarding the yield of lactate from glucose, this value was approximately 2 until day 8. This shows that MSC used the glycolysis pathway to produce ATP since 1 mol of glucose generates 2 mol of lactate. Therefore, the glycolysis pathway was efficient.

Figure 5: Expansion of MSC on gelatin coated alginate microcarriers in Belco® Spinner Flask.

Figure 6: Concentration profiles of nutrients and metabolites during the expansion of MSC in Belco® Spinner Flask.
microcarriers could be made with controlled diameter. Gelatin was used as a model protein to coated the alginate microcarriers providing mechanical strength and an anchorage surface for MSC. Alginate microcarriers were also coated with gelatin in low concentration % (w/v) to enable the detachment of MSC using EDTA. It was observed that MSC did not adhere on gelatin 0.1% coated alginate microcarriers. MSC were able to adhere and proliferate on gelatin 1% coated alginate microcarriers in static (24-well plate) and dynamic conditions (spinner flasks). Comparing the three systems, MSC achieved the higher fold increase on the 24-well plate. The higher cell adhesion was obtained in the Belco® Spinner Flask. Although MSC adhered and proliferated on gelatin 1% coated alginate microcarriers, cells entered in death phase at day 9. After the metabolites analysis, it was observed that this could be related with limitation in glucose concentration. To test this hypothesis, we could perform another experiment with the same parameters but using two different feedings, such as 25% and 50%. After expansion in StemSpan® Spinner Flask, the immunophenotype and differentiation potential of expanded MSC was evaluated. MSC immunophenotype was positive for CD73 and CD90 while negative for CD31, CD80 and HLA-DR, and MSC were able to differentiate in osteoblasts, adipocytes and chondrocytes.

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References

Figure 7: Metabolic analysis of the expansion of MSC in Belco® Spinner Flask.

4. Conclusions
In this work, it was demonstrated that alginate microcarriers could be made with controlled di-
stem cells from the umbilical cord matrix: Successful isolation and ex vivo expansion using serum-/xeno-free culture media,” *Biotechnology journal*, 2013.


