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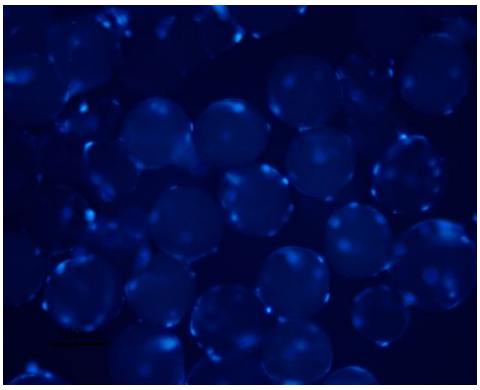

Expansion of multipotent mesenchymal stromal cells on gelatin coated alginate microcarriers

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Thesis to obtain the Master of Science Degree in

Bioengineering and Nanosystems

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Resumo

As células estaminais mesenquimais humanas (MSC) são consideradas como promissoras em aplicações no âmbito da medicina regenerativa, tais como terapias celulares ou engenharia de tecidos. MSC têm sido alvo de intenso estudo devido à sua capacidade de diferenciação, propriedades imunomodulatórias e habilidade para suportar a hematopoiese. MSC têm sido cultivadas tradicionalmente numa configuração 2-D como monocamada em frascos de cultura celular. As MSC têm sido também cultivadas em bioreactores utilizando microesferas onde as células aderem, ou utilizando a cultura em agregados que promove a interação célula a célula. Uma abordagem alternativa é a encapsulação em hidrogéis que protegem as células do ambiente exterior e possibilitam o cultivo em 3D. Este trabalho teve como objectivo estabelecer uma comparação entre a cultura em microcápsulas (encapsulação) e a cultura em microesferas. Foi observado que para microesferas de alginato ou gelatina/alginato, MSC encapsuladas não proliferaram e mantiveram uma forma esférica. Por outro lado, foi demonstrado que utilizando microesferas de alginato revestidas por uma camada de gelatina resultou na adesão e expansão de MSC em condições estáticas e dinâmicas. Após o crescimento de MSC em spinner flask, o seu imunofenótipo foi positivo para os marcadores CD73 e CD90, e negativo para CD31, CD80 e HLA-DR. MSC também demonstraram o potencial para diferenciação em osteoblastos, adipócitos e condrócitos.

Palavras-chave: bioreactores, CultiSpher-S, glutaraldeído, magnetite.

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. This project envisioned to obtain a global comparison between cell encapsulation and microcarrier configuration. It was observed that in either alginate microspheres or gelatin/alginate microspheres, encapsulated MSC did not proliferate and remained with spherical shape. On the other hand, 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.

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List of Acronyms

ASC - Adipose-derived stem cells

BM - Bone marrow

DMEM - Dulbecco's Modified Eagle Medium

ECM - Extracellular matrix

FBS - Fetal bovine serum

LCST - Lower critical solution temperature

MSC - Multipotent mesenchymal stromal cells

RGD - Arg-Gly-Asp

SMSC - Synovium-derived mesenchymal stromal cells

UCM - Umbilical cord matrix

Chapter 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 microenvironment 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. Study strategies for MSC encapsulation in alginate microspheres;
2. To develop an alginate microcarrier coated with an extracellular matrix protein (gelatin);
3. To evaluate the adhesion and detachment of MSC on the new microcarriers;
4. To investigate the expansion of MSC on the same microcarriers in static and dynamic conditions.

1.3 Thesis outline

This document is organized in 4 chapters. In chapter 1 is given a global description about MSC and their characteristics and are reviewed the recent studies regarding MSC encapsulation and MSC cultivation on microcarriers. In chapter 2 are described the materials and methods used in the experimental work. In chapter 3 are presented the results and discussion from two different strategies to cultivate MSC: cell encapsulation and microcarriers. In the first, MSC were encapsulated in alginate and alginate/gelatin microspheres. Second, MSC were cultivated on gelatin coated microcarriers in static conditions and CultiSpher-S (a commercial type of microcarrier made of gelatin) was used as control. Finally, MSC were cultivated on gelatin coated microcarriers in two different types of spinner flasks. In chapter 4 are summarized the conclusions and future work.

1.4 State of the art

1.4.1 Background

Stem cells are responsible for cells and tissues in the body to continually regenerate themselves. This is a result from the fact that stem cells are undifferentiated cells which have the capacity to self-renew and to differentiate into mature cells. MSC are classified as adult stem cells and they are often commited with a specific cell lineage. Examples of adult stem cells include MSC, hematopoietic stem cells and neural stem cells.

1.4.2 Multipotent mesenchymal stromal cells

MSC have been first isolated from bone marrow by Friedenstein and colleagues in the 1960s (Friedenstein et al., 1966). 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 (Figure 1.1).

Properties of MSC

It was demonstrated that MSC can interact with cells from the immune system, such as dendritic cells, naive and effector T cells an Natural Killer cells by inhibiting the anti-inflammatory pathways (Aggarwal and Pittenger, 2005). In addition, it was observed that T lymphocytes do not respond to MSC, thus MSC have the potential to be used in allogenic transplatation (Le Blanc et al., 2003). In addition, it is proposed that MSC can be activated in response to injury and they can be recruited to the local where the tissue was damaged (Caplan and Correa, 2011). MSC also have shown the potential to support the expansion of hemotopeitic stem cells by secretion of cytokines and growth factors (da Silva et al., 2010).

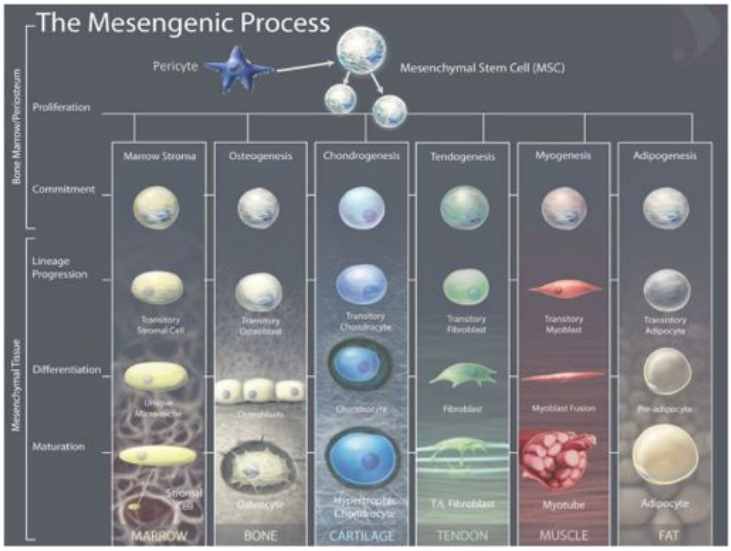


Figure 1.1 The Mesengenic Process (Caplan and Correa, 2011).

Sources of MSC and isolation procedures

They can be obtained from different sources such as bone marrow, adipose tissue, umbilical cord matrix, placenta and synovium.

Bone marrow. Bone marrow-derived MSC can be isolated from bone marrow (BM) aspirates. The low density BM mononuclear cells (MNC) are separated by a Ficoll density gradient. That fraction is then collected and after washing steps with culture medium, it is plated in tissue culture flasks. Since MSC are an adherent-dependent cell type, they will adhere to plastic and start to proliferate (dos Santos et al., 2010).

Adipose tissue. Adipose-derived stem cells (ASC) can be isolated from lipoaspirate tissue. This material which is usually discarded, represents an abundant and readily available source (Gimble et al., 2007). The standard procedure to isolate ASC is based on the digestion of the extracellular matrix by collagenase. After filtration and centrifuge it is obtained a pellet. This is the stromal vascular fraction (SVF). Next, the SVF can be resuspended in culture medium and plated in tissue culture flasks or alternatively, the SVF can be further purified by magnetic beads to remove specific cell lineages, such as $CD45^+$ cells or $CD31^+$ cells depending on the application (Locke et al., 2009).

Umbilical cord matrix. MSC have been successful isolated from the umbilical cord matrix (UCM) and expanded in xeno-free conditions (Simões et al., 2013). It was demonstrated that MSC from UCM can highly proliferate and have similar immunophenotype compared to MSC from bone marrow and adipose tissue.

Placenta. Like the umbilical cord, but in contrast with bone marrow, this source has the advantage the biological material can be obtained in abundance, while representing no risk for the donor. It has been demonstrated that the MSC obtained from placenta have similar features as the ones from bone marrow (Barlow et al., 2008).

Synovium. Synovium-derived mesenchymal stromal cells (SMSC) have demonstrate mesedormal differentiation, however among the MSC obtained from different sources, SMSC have shown superior capacity for chondrogenesis (Sakaguchi et al., 2005). SMSC have shown potential to be expanded *ex vivo* in xeno-free conditions (Santhagunam et al., 2013).

Characterization of MSCs

The International Society for Cell Therapy (ISCT) proposed the following criteria to define MSC (Dominici et al., 2006):

1. adherence to plastic in standard culture conditions;
2. in vitro differentiation in osteoblasts, adipocytes, chondrocytes;
3. MSCs are known to be positive for the markers CD73, CD90, and CD105, whereas negative for CD45, CD34, CD14 (or CD11b), CD79 (or CD19) and HLA-DR surface molecules.

In addition, several research groups have been used different surface markers panels to characterise MSC (Table1.1).

Table 1.1 Characterisation of MSC based on putative surface markers.

Positive markers	Negative markers
CD13, CD29, CD44, CD49a,b,c,e,f, CD51, CD54, CD58, CD63, CD71, CD73, CD90, CD102, CD105, CD106, CDw119, CD120a,b, CD123, CD124, CD126, CD127, CD140a, CD140b, CD166, p75LNGFR, STRO-1, SB-10, SH3, SH4, TGF-βIIR, SSEA-3	CD3, CD4, CD6, CD9, CD10, CD11a, CD14, CD15, CD18, CD21, CD25, CD31, CD34, CD36, CD38, CD45, CD49d, CD50, CD80, CD86, CD95, CD117, CD133, SSEA-1

Clinical therapies using MSCs

MSC have intrinsic therapeutic potential. MSC have been reported in the clinic to promote haematopoietic engraftment and to prevent or treat graft-versus-host disease after bone marrow transplant, restoring the immunomodulatory MSC properties.

At the moment, on Clinical-Trials.gov (http://clinicaltrials.gov) there are 194 studies (recruiting) for search term: mesenchymal stem cells and 41 studies for search term mesenchymal stromal cells (accessed on 08/06/2014).

One terminated study for stroke treatment involved CellBead® technology (CellMed AG, a subsidiary of BTG plc.), an implantable cell therapeutic system based on a genetically modified MSC cell line (MSC-TERT) encapsulated in alginate. At the moment, no study results were posted (clinical trial number: NCT01298830).

1.4.3 Methodologies for MSCs expansion

Expansion in 2D tissue culture flasks

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 O_2 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 (Zhao et al., 2005). 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 (Duggal et al., 2008). As a result, it is becoming increasingly accepted that 3D culture methods provide a cellular environment more consistent with that in vivo. In Table 1.2 are presentend the major advangens and disadvantages from different methodologies for MSC expansion.

Table 1.2 Advantages and disadvantages from different 3D methodologies for MSC expansion.

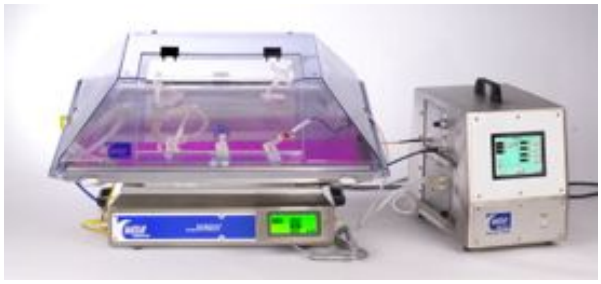
Methodology	Advantages	Disadvantages
microcarriers	Provide high surface area	Need for cell detachment from microcarriers
spheroid suspension culture	No need for materials to cells adhere	Difficulty in control cell agglomeration
encapsulation	Protection from shear stress and prevent cells from aggegration	Limitation in diffusion

Expansion in bioreactors

Bioreactors can overcome many limitations of 2D cell culture, such as limitations on nutrients and metabolites transport, better oxygen diffusion and control of several parameters such as pH, temperature and dissolved oxygen concentration (DO_2). Some examples of bioreactors are shown in Figure 1.2. Since MSC are an adherent-dependent cell type, the most common strategy has been the expansion of MSC in microcarriers. Since microcarriers have a high surface area to volume ratio, they can provide a large surface area, thus overcoming the limitations of 2D tissue culture flasks.

(a) StemSpan™ Spinner Flask (STEMCELL Technologies Inc).

(b) BIOSTAT®B twin version with stirred glass vessel and with single-use vessel (Sartorius).



(c) Wave bioreactor (GE Healthcare Life Sciences).

(d) Rotary bioreactor (Z®RP Bioreactor (Zellwerk GmbH).

Figure 1.2 Different bioreactors suitable for mammalian cell culture.

Cell encapsulation

Mammalian cell encapsulation has received much attention in regenerative medicine. For example, cell encapsulation has been used as a scaffold to maintain the normal cellular physiology after cell transplantation. Another application has been the use of microcapsules as a platform to guide the differentiation of stem cells (Wang et al., 2009).

Alginate. 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 (Lee and Mooney, 2012). Alginates are constituted by (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers. Therefore, the alginate molecule is classified as a block copolymer composed by regions of sequential M units, regions of 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 Ca^{2+}, that cooperatively bind between the G blocks of adjacent alginate chains, creating ionic interchain bridges.

(a) Chemical structure of sodium alginate. (b) Ionic cross-linking (egg-box model).

Figure 1.3 Chemical structure of sodium alginate and schematic of ionic cross-linking with Ca^{2+} ions (Lee and Yuk, 2007).

Alginates hydrogels have many satisfactory properties necessary in biomaterials, such biocompatibility, low immunogenicity and tunable permeability. For *in vivo* applications, alginate is previously oxidized to a desired degree which enables the degradation in an aqueous environment. However, alginate has the limitation of being unable to interact with a class of mammalian cells that are known as anchorage-dependent cells (Rowley and Mooney, 2002). These cells, including MSC, require cell anchorage in order to carry many of the cellular functions such as migration, proliferation and differentiation. MSC have the ability to recognize several proteins, including fibronectin, gelatin, collagen, which interact with integrins present in the cell membrane (Gronthos et al., 2001).

Several authors have studied modified alginates with peptide sequence to encapsulate mammalian cells. MSC have been encapsulated in alginate containing the Gly-Arg-Gly-Asp-Tyr (GRGDY) peptide to investigate their viability and proliferation (Markusen et al., 2006). The peptide was covalently linked to alginate by carbodiiamide chemistry (Rowley et al., 1999). Markusen *et al.* demonstrated that MSCs maintained viability ($>$80%) within the alginate-GRGDY beads and that GRGDY promoted cell attachment. Moreover, the authors found that MSC did not proliferate within the alginate-GRGDY beads during the 2 weeks of culture. In another study MSC were encapsulated in RGD-modified alginate (Bidarra et al., 2010). The Arg-Gly-Asp (RGD) peptide sequence has been known as a synthetic extracellular matrix to promote cell adhesion, proliferation and migration. Bidarra *et al.* demonstrated that RGD-alginate microspheres promoted MSC adhesion, maintained their metabolic activity and supported their osteogenic differentiation. Besides alginate, other natural and synthetic polymers have been used to encapsulate MSC. In Table 1.3 are summarized a few studies that aimed at differentiation and expansion of encapsulated MSC.

Table 1.3 Studies involved encapsulated MSC to evaluate their differentiation and proliferation capacity.

Materials	Aim of study	Reference
PEG-peptide hydrogels	multilineage differentiation	(Anderson et al., 2011)
Collagen-agarose	culture and delivery MSC for tissue engineering	(Batorsky et al., 2005)
Silk ionomers	differentiation into osteogenic and adipogenic lineages	(Calabrese and Kaplan, 2012)
Alginate	effect of shear stress on osteoblastic differentiation	(Yeatts et al., 2012)

Culture in microcarriers

The recent studies of MSC culture using microcarriers are resumed in Table 1.4. The microcarrier type column corresponds to the commercial name. CultiSpher-S is made of gelatin cross linked and Cytodex 3 has a cross-linked dextran matrix coated by denatured collagen.

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 in spinner flasks (Eibes et al., 2010). The authors showed that FBS improved the cell adhesion resulting in a reduced lag phase.

MSC from placenta have cultivated on CultiSpher-S microcarriers in wave bioreactor-like (Timmins et al., 2012).

Recently, one research group has shown the expansion of MSC on microcarriers (type) in a 5 L stirred-tank bioreactor (2.5 L working volume) (Rafiq et al., 2013).

For clinical applications, MSC should be cultivated in xenofree medium. This would eliminate the potential risk of immune response against of xenogeneic antibodies, such as animal proteins, bacteria or virus that can derive from the FBS (Spees et al., 2004) (Sundin et al., 2007). Therefore, the potential to expand human BM MSC and adipose-derived stem cells (ASC) in xenofree conditions have been assessed (dos Santos et al., 2011). 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 (dos Santos et al., 2014).

Alginate has also been modified to work as a microcarrier. The first example of a ECM protein coated alginate microcarrier showed the expansion of human chang liver (CCL-13) and mouse fibroblast (L929) cell lines in static conditions (Gröhn et al., 1997). Both cell lines showed rapidly proliferation in collagen-coated Ba^{2+}-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 (Kwon and Peng, 2002). 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.

In another study, MSC have been cultivated in RGD-modified alginate microcarriers in spinner flasks (Schmidt et al., 2011). 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.

1.4.4 Methodologies for harvesting MSC from microcarriers

After *ex vivo* expansion, MSC are harvested from the bioreactor or tissue culture flask. In this step, MSC need to be detached from the surface were they have been cultivated, either from the microcarriers

Table 1.4 Expansion of MSCs in microcarrier cultures.

Species	Sources	Microcarrier type	Reactor type	Culture medium	Expansion	Reference
Human	Bone marrow	CultiSpher-S	Spinner flask	MesenPRO RS® containing 2% of FBS	4.2×10^5 cells/mL (8.4±0.8 fold)	(Eibes et al., 2010)
Human	Bone marrow and adipose tissue	CultiSpher-S and polystyrene beads	Spinner flask	MesenPRO RS®/StemPro® MSC SFM	1.4×10^5 and 2.2×10^5 cells/cm^2	(dos Santos et al., 2011)
Human	Placenta	CultiSpher-S	Wave bioreactor-like	Serum-containing medium		(Timmins et al., 2012)
Human	Fetal bone marrow	Cytodex 3 (GE Healthcare)	1-L bioreactor	D10 medium	6.0×10^5 cells/mL (12- to 16 fold expansion)	(Goh et al., 2013)
Human	Bone marrow	Non-porous Plastic P-102L microcarriers (Solohill Engineering Inc., USA)	5 l bioreactor (2.5 l working volume)	D10 medium	1.7×10^5 cells/ml (6 fold expansion)	(Rafiq et al., 2013)
Human	Bone marrow	CultiSpher-S	Spinner flask	DMEM with 10% FBS	3.2×10^4 cells/mL (8 fold expansion)	(Yuan et al., 2012)
Human	Synovium	Non-porous plastic micro-carriers (Solohill Engineering)	Spinner flask	Xeno-free medium (StemPro;Invitrogen)	8.8×10^5 cells/mL (34 fold expansion)	(Santhagunam et al., 2013)
Human	Bone marrow and adipose tissue	Non-porous plastic micro-carriers (Solohill Engineering)	1-L bioreactor	StemPro® MSC SFM	1.1×10^8 and 4.5×10^7 cells/mL	(dos Santos et al., 2014)
Mouse	Bone marrow	Alginate microcarriers coupled with RGD peptide	Spinner flask	DMEM with 10% FBS		(Schmidt et al., 2011)

or polystyrene flask. The common methodologies for MSC harvesting as in general for any achorage-dependent mammalian cell require the use of proteases. The most common is trypsin however to meet the requirements for clinical therapies, such as non-animal origin, other commercial products have been available including StemPro® Accutase® (Life Technologies™), TrypZean® (a non-animal recombinant trypsin solution, Sigma-Aldrich) and TrypLE™ Select CTS™ (Life Technologies™).

However, for clinical trials where high number of doses are required, the use of these products represents a significant cost. Therefore, the development of enzyme free harvesting materials would be advantageous. To address this problem, the majority of studies have been on the development of temperature-responsive polymers or thermoresponsive polymers. The interest behind thermoresponsive polymers derives from the fact that some polymers are able to change their conformation in solution from expanded coil conformation to a compact globuli according to their upper or lower critical solution temperature. For example, the thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAAm) has a lower critical solution temperature (LCST) of 32°C. When the temperature is above 32°C, PNIPAAm adopts a compact globuli conformation. When the temperature is below 32°C, the PNIPAAm chains start to swell to adopt a expanded coil conformation. This mechanism is illustrated in Figure 1.4. At 37°C, the PNIPAAm chains grafted on the surface are collapsed and the endothelial cells attached have elongated shape. At 20°C, PNIPAAm adopted the expanded coil conformation and the cells after detachment have round shape. Recently one research group have successfully grafted PNIPAAm on the surface of two commercially available microcarriers (dextran-based Sephadex® and vinyl acetate-based VA-

OH(Biosynth®) (Çakmak et al., 2013), but results evaluating cell harvesting have not been presented yet.

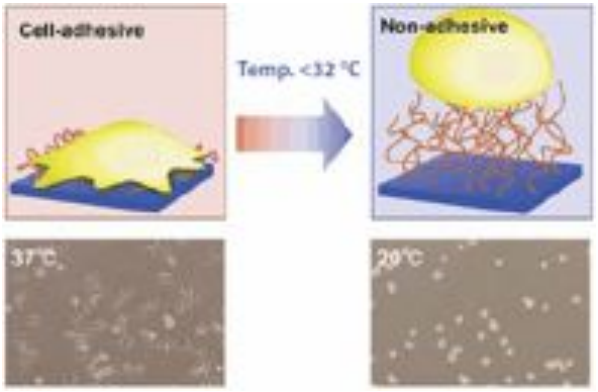


Figure 1.4 Schematic representation cell detachment based on the LCST of PNIPAAm (Masamichi et al., 2010).

An alternative system for expansion of anchorage dependent cell in designed for free enzyme harvesting is use of alginate microcarriers as referred earlier (Schmidt et al., 2011). Since the alginate structure can be broken in the presence of the chelating agents such as EDTA or citric acid, alginate could be a suitable material for the microcarrier core. To promote cell adhesion, different ECM proteins could be attached at the microcarrier surface, including fibronectin, gelatin and collagen. Therefore, one strategy would be the development of alginate microcarriers coated with ECM proteins and sensitive to chelating agents, not requiring the use of proteases.

Chapter 2

Materials and methods

2.1 Alginate viscosity

Sodium alginate with different concentrations [1.1 - 2.2% (w/v)] was prepared by dissolving alginate powder (Sigma) in Milli-Q water under constant agitation at 45°C. After enough time to allow the released of the air bubbles trapped in alginate, the viscosity of the different solutions at room temperature was measures using a rotational viscometer (Brookfield Engineering Laboratories, Inc.).

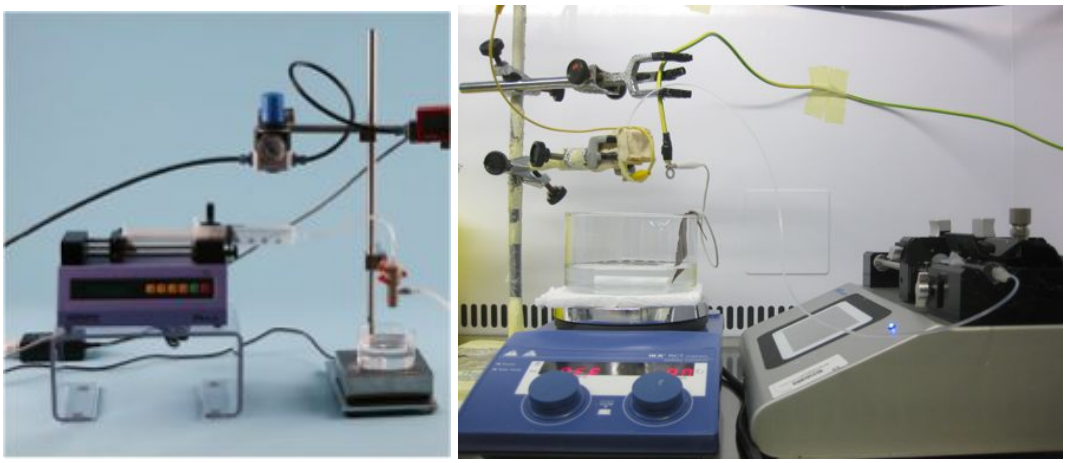
2.2 MSC encapsulation in alginate microspheres

For MSC encapsulation, 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 filtration using a 22 μm syringe filter (Milipore). MSC at passage 8 (male donor, 67 years old) were cultivated on tissue culture flasks with Dulbecco's Modified Essential Medium (DMEM, Gibco), 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. MSC were incubated at 37°C and 5% CO_2 in a humidified atmosphere and the medium was changed every 3 to 4 days until cells reach confluence (80-90%). After MSC harvesting, the pellet obtained is mixed in sodium alginate 1.8% (w/v) to achieve a concentration of 1×10^6 cells/mL alginate. MSC were encapsulated by extrusion of the alginate solution under a coaxial nitrogen-flow (using a Var J1 encapsulation unit (Nisco, Switzerland) at a speed of 10 mL/h (Harvard Apparatus 22, Southnatick, MA, USA). The cross-linking of alginate within the microspheres was achieved under constant stirring in an isotonic 50mM $CaCl_2$ solution for 15 min. The microspheres were washed twice in DMEM with 10% FBS to remove the excess of calcium and the microspheres were transferred to a 24-well plate Ultra low Attachment (Costar®) and incubated for 10 days at 37°C and 5% CO_2.

2.3 Alginate microcarriers

2.3.1 Alginate microcarriers preparation

First, sodium alginate (Sigma) was dissolved in mili-Q water at a final alginate concentration of 1.8% (w/v) and the solution was filtered using a 22 μm filter. Then, the alginate solution 1.8% (w/v) was pumped at 5 ml/h (Harvard Apparatus 22, Southnatick, MA, USA) trough 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 $CaCl_2$ 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 were kept in $CaCl_2$ 300mM solution and stored at 4°C .



(a) Var J1 encapsulation unit (Nisco, Switzerland).

(b) Alginate microcarriers preparation using applied voltage.

Figure 2.1 Different settings used to obtain alginate beads.

2.3.2 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 aquous solution of glutaraldehyde 0.4% (v/v) was added to promote covalent crosslinking of gelatin. The crosslinking time was 30min at 750 rpm, 22°C continua sly. 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.

2.3.3 Mechanical stability gelatin coated alginate microcarriers and test to EDTA

To understand how the gelatin coating can improve the mechanical strength of alginate mirocarriers, 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 microscopic inspection, the microcarriers were used to evaluate the if EDTA could be used to harvest the cells (instead of using proteases) by chelation of Ca^{2+} 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).

2.3.4 Magnetite loaded alginate microcarriers

One interesting functionality of alginate microcarriers is that they can be designed to respond to a magnetic field. To achieve this, magnetite (Fe_3O_4) was mixed with sodium alginate and the protocol described earlier for alginate microcarriers preparation was followed using the same conditions. Then the microcarries were observed under the microscope and a qualitative test for the microcarriers movement was done in the presence of a neodymium magnet.

2.4 Proliferative capacity of MSC on 12-well plate

In this experiment, BM MSC were obtained from a male donor with 67 years old and were seeded in the 12-well plate at passage 7. To evaluate the expansion potential, MSC were seeded at a initial concentration of 1,000 cells/cm^2 on a 12-well plate in which each well has 4 cm^2 of surface area. The cell culture kinetics was obtained by harvesting the cells at day 3, 5, 7, 9, 11 and 13.

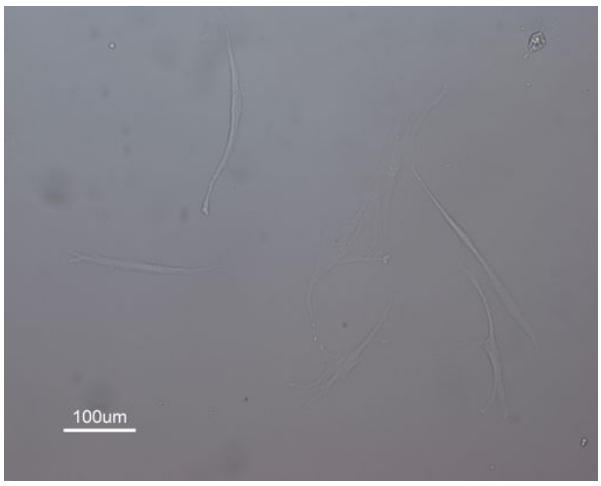


Figure 2.2 MSC on 12-well plate at day 3.

2.5 Expansion of MSC on gelatin coated alginate microcarriers

2.5.1 Expansion 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 5×10^3 cells/cm^2 in the presence of DMEM/ 10% FBS/1% penicillin-streptomycin and cultured for 10 days at 37°C and 5% CO_2. Before cell seeding, the alginate microcarriers 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 viable 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.

2.5.2 Expansion in spinner flask

MSC from the same donor at passage 6 were seeded at 3000 cells/cm^2 in two T-175 flasks in DMEM with 10% FBS and with 1% penicillin-streptomycin and were expanded during 12 days at 37°C and 5% CO_2. Cells were harvest with trypsin at 0.05% and viable cell were counted by Trypan blue.

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 50×10^3 cells per mL in 1/2 of the final medium volume (15 mL). Cells were incubated at 37°C and 5% CO_2 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, Bellco spinner flasks were used to obtain two replicates for gelatin coated microcarriers. MSC were seeded at 50×10^3 cells per mL in 40 mL (initial and final volume).

2.5.3 Monitoring of cell culture in spinner flask

To study the kinetics of MSCs in spinner flasks, samples (0.5 mL) of homogeneously mixed culture media were collected (in duplicates) to FACS tubes once a day during the expansion period. Microcarriers were washed twice with 1 mL of PBS and 1 mL of trypsin 0.05% was added to enzymatically dissociate MSCs from the microcarriers. The FACS tubes were agitated at 650 rpm for 7 min. The number of viable cell was calculated by Trypan Blue exclusion method. In order to follow the metabolic profiles during the culture, supernatant samples of 1.2 mL were collected (before and after changing the medium) and centrifuged at 1500 rpm for 10 min. The supernatant samples were kept at -20°C for posterior metabolite analyses.

(a) StemSpan™ Spinner Flask. (b) Bellco Spinner Flasks.

Figure 2.3 Spinner flasks.

2.5.4 Kinetic analysis

To compare the results obtained in the stirred microcarriers cultures with the control plates, a first order kinetic model for cell expansion and death was used. The balance for viable cells (X_v) can be written as

$$\frac{dX_v}{dt} = \mu \cdot X_v - k_d \cdot X_v = \mu_{app} \cdot X_v \tag{2.1}$$

where μ and k_d are the growth and death rates, respectively, and μ_{app} is the apparent growth rate. The calculated values were based on the initial and final cell densities. The maximum growth rates μ_{max} were also estimated, using the same model applied to the slope of the curves during the exponential phase.

The specific growth rate can be written as:

$$\mu = \frac{(Ln(Cx(t)/Cx(0)))}{\Delta t} \tag{2.2}$$

where μ is the specific growth rate (h^{-1}), Cx(t) and Cx(0) are the cell numbers at the end and start of exponential growth phase respectively and t is time (h).

Doubling time:

$$t_d = \frac{Ln2}{\mu} \tag{2.3}$$

Where td is the doubling time (h) and μ is the specific growth rate (h^{-1}).

Population doubling:

$$Population\ doubling = 3.32\ \times (\log(\frac{Cx(t)}{Cx(0)})) \tag{2.4}$$

Where Cx(t) and Cx(0) are the cell numbers at the end and start of exponential growth phase respectively and t is time (h).

Fold increase:

$$Fold\ increase = \frac{Cx(f)}{Cx(0)} \tag{2.5}$$

Where Cx(f) is the final cell number at the end of passage and Cx(0) is the initial cell number.

Reynolds number:

$$Re = \frac{ND^2\rho}{\mu} \tag{2.6}$$

Where Re is the Reynolds number (dimensionless), N is the impeller speed (s $^{-1}$), D is the impeller diameter (m), ρ is the fluid density (kg/m^3) and μ is the fluid viscosity (kg/ms^{-1}).

2.5.5 Metabolite analyses

To evaluate the nutrient consumption by MSCs and their metabolite 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).

The specific metabolite consumption rate, q_{Met}, was given by the following equation:

$$q_{\text{Met}} = \frac{\Delta Met}{\Delta t X_{\text{v}}} \tag{2.7}$$

Where ΔMet is the difference in the concentration of the metabolite at the start and end of each time point and X_{av} is the average of viable cells at that respective time point.

The apparent yield of lactate from glucose, $Y'_{\text{lactate/glucose}}$, was given by the following equation:

$$Y'_{\text{lactate/glucose}} = \frac{q_{\text{lactate}}}{q_{\text{glucose}}} \tag{2.8}$$

Therefore, $Y'_{\text{lactate/glucose}}$ is the ration between glucose consumption (q_{glucose}) and lactate production (q_{lactate}) over a specific time period.

2.6 Characterization of MSC after expansion

2.6.1 Dapi staining

DAPI staining was used to visualize the nuclei of MSCs by fluorescence microscopy (Olympus U-RFLT50). At day 1, samples of microcarriers (0.5 mL) were collected from the spinners flasks and were transferred to 24-well plate. Samples were washed with 0.5 mL of PBS and fixed with 0.4 mL of paraformaldehyde 4% for 20 min at room temperature. Samples were washed twice with PBS and then cells were incubated with 0.4 mL of DAPI (4, 6-diamidino-2-phenylindole, 1.5 mg/mL in PBS) for 5 min at room temperature (protected from light) and washed twice with PBS.

2.6.2 Immunophenotypic analysis

At the end of expansion in spinner flasks, MSCs were evaluated for immunophenotypical analysis. For each surface marker, 1×10^5 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.

Table 2.1 Panel of mouse anti-human monoclonal antibodies used for the analysis of MSC surface markers.

Surface markers	Commercial brand	Conjugated fluorophore	Isotype
CD31	BioLegend®	PE	IgG1
CD73	BD Biosciences©	PE	IgG1
CD80	BioLegend®	PE	IgG1
CD90	BioLegend®	PE	IgG1
CD105	BioLegend®	PE	IgG1
HLA-DR	BD Biosciences©	PE	IgG1
IgG1	BioLegend®	PE	-

2.6.3 Mesodermal differentiation

For the osteogenic and adipogenic differentiation assays, cells were plated on 24-well plate (Falcon BD Biosciences® at 3×10^3 cells/cm^2 in the presence of DMEM with 10% FBS MSC qualified and 1% Penicillin-Streptomycin and incubated at 37°C and 5% CO_2. 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 2×10^5 was ressupended 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 chondrogenesis differentiation culture medium was added and changed every 3 to 4 days.

Osteogenic Differentiation

The 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.

Chondrogenic differentiation

The cells were washed once with PBS and fixed with PFA 2% solution 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.

Adipogenic differentiation - Oil Red-O solution

The 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.

Chapter 3

Results and discussion

3.1 Cell encapsulation

After the preparation of alginate solutions with different concentration, the viscosity at room temperature was measured (Figure A.1). It was observed, that by increasing the alginate concentration, the viscosity at room temperature increased. Alginate 1.8% (w/v) was selected for cell encapsulation because at room temperature, the viscosity of this solution allowed the formation of intact alginate microspheres with controlled diameter and it was possible to homogenise the cells in the solution better than higher alginate concentrations. After encapsulation, MSC were cultivated for 12 days. At different time points, the number of cells was estimated by Trypan Blue (direct method), Alamar Blue® and WST-1 (indirect methods) (Figure 3.1). It was observed that MSC did not proliferate in alginate microspheres and maintained a round shape (Figure 3.2). MSC were also encapsulated in alginate/gelatin 1.8%/1% (w/w) microspheres to investigate if gelatin promoted cell adhesion. However, MSC remained with spherical shape throughout the culture time as expected (Markusen et al., 2006).

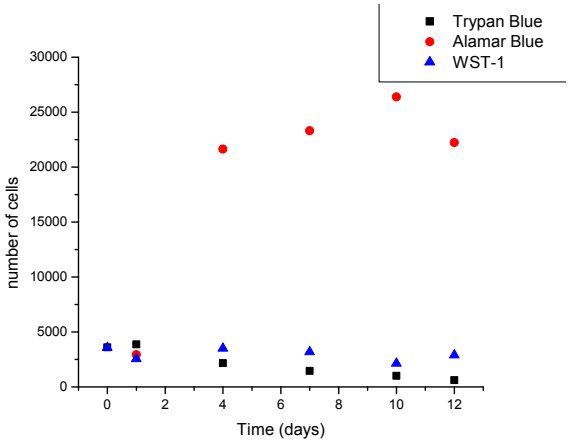
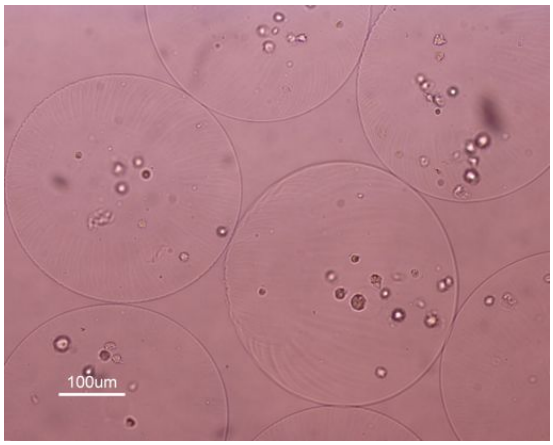
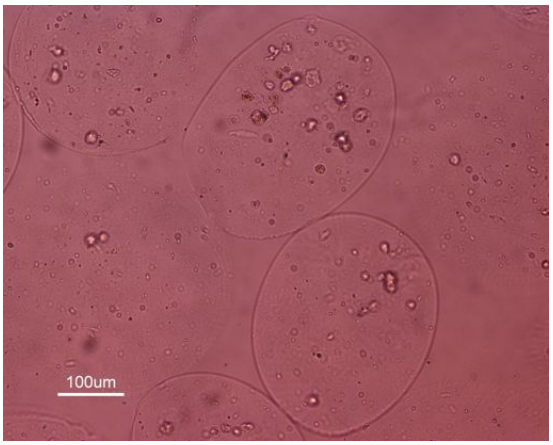


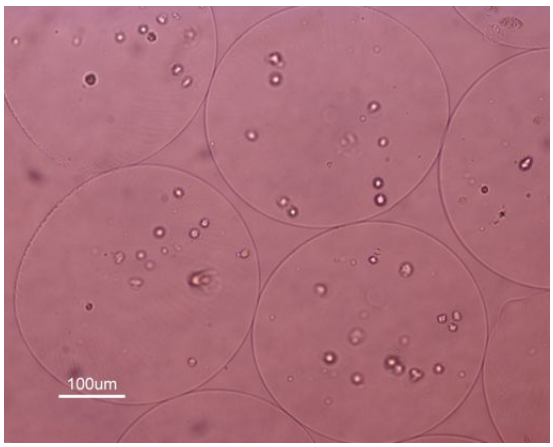
Figure 3.1 Different methods used to evaluate cell proliferation of MSC encapsulated in alginate microspheres.



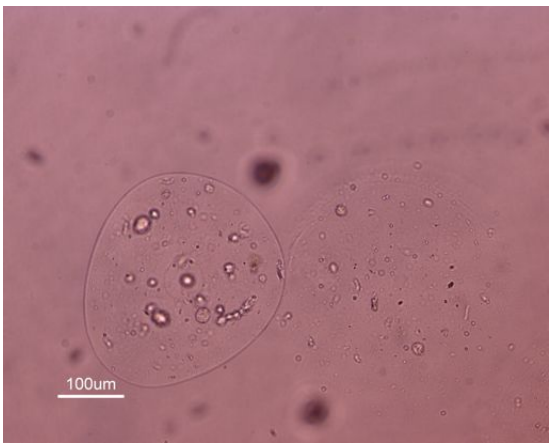
(a) MSC encapsulated in alginate 1.8% (w/v) microspheres at day 0.



(b) MSC encapsulated in alginate/gelatin 1.8%/1% (w/w) microspheres at day 0.



(c) MSC encapsulated in alginate 1.8% (w/v) microspheres at day 3.



(d) MSC encapsulated in alginate/gelatin 1.8%/1% (w/w) microspheres at day 3.

Figure 3.2 MSC encapsulated in alginate 1.8% (w/v) microspheres and in alginate/gelatin 1.8%/1% (w/w) microspheres.

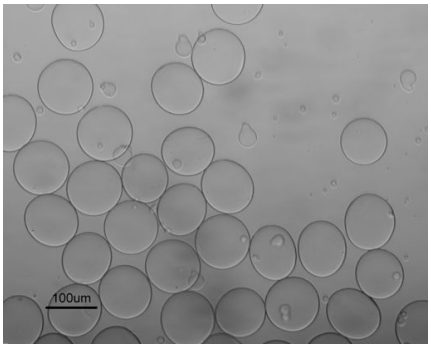
3.2 Alginate microcarriers

The microcarriers produced include a alginate core and a gelatin shell . The core of the microcarriers were manufactured by forming beads of controlled diameter crosslinking using Ca^{2+}. 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 adesion. 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 Ca^{2+} 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.

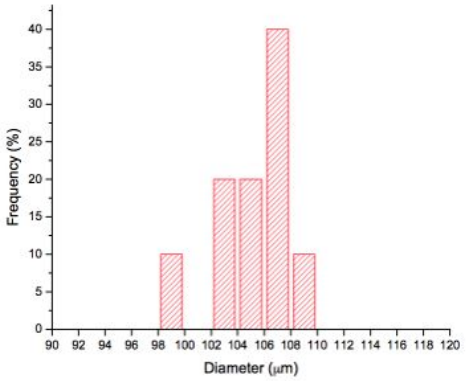
First, alginate microcarriers were obtained by applied voltage as described in 2.3.1. Next, the alginate microcarriers were coated with gelatin with different concentrations (Table 3.1). Alginate microcarriers and gelatin coated alginate microcarriers were observed under the microscope and the diameter was determined using the Image Measurement Utility from MATLAB. (Figure 3.3). 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) gelatine 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 using a Thermomixer® confort (Eppendorf AG). It was observed that for all the gelatin concentrations present in Table 3.1, 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).

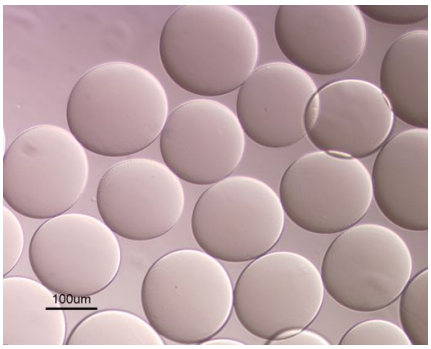
Next, the stability of gelatin 0.1% coated alginate microcarriers to shear stress at the maximum of 80 rpm was evaluated. Gelatin 0.1% coated alginate microcarriers were added to a StemSpan™ Spinner Flask and non coated alginate microcarriers were used as a control. After 10 days of agitation, gelatine 0.1% coated alginate microcarriers and non-coated alginate microcarriers remained intact (Figure 3.4). 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.



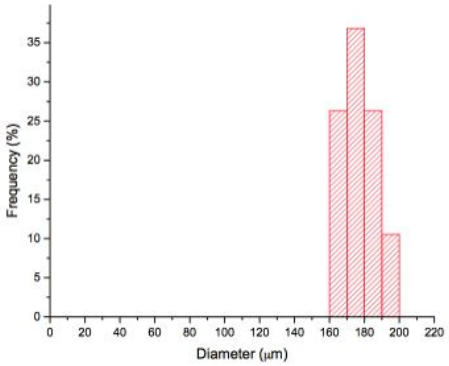
(a) Alginate microcarriers.



(b) Histogram of alginate microcarriers (average diameter 105±3μm).



(c) Gelatin 1% coated microcarriers.

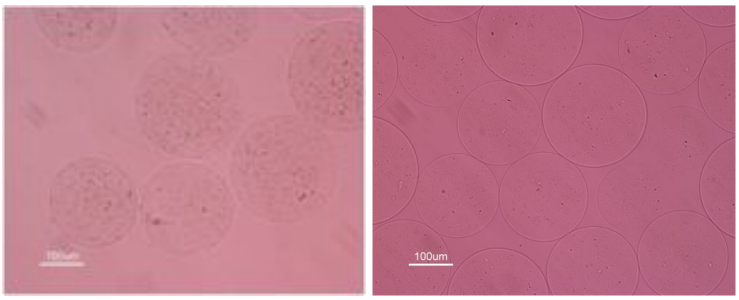


(d) Histogram of gelatin 1% coated microcarriers (average diameter 178±10μm).

Figure 3.3 Microcarriers and size distribution.

Table 3.1 Assay to evaluate the ”response” of gelatin coated alginate microcarriers to EDTA.

Gelatin % (w/v)	EDTA [mM]	Break
1	35, 50, 100	No
0.75	35, 50, 100	No
0.5	35, 50, 100	No
0.25	35, 50, 100	No
0.1	100	100%
0.1	50	80-90%
0.1	35	20-30%
0	35	100%

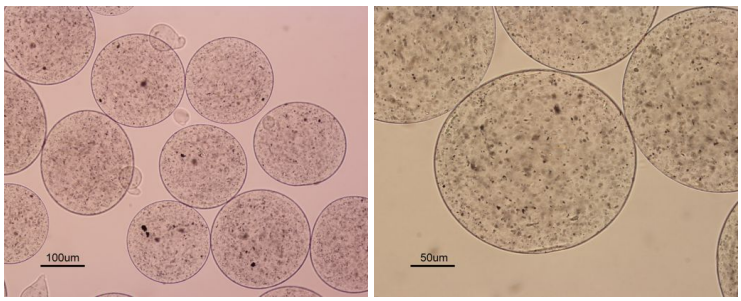


(a) Gelatine 0.1% coated alginate microcarriers.

(b) Alginate microcarriers.

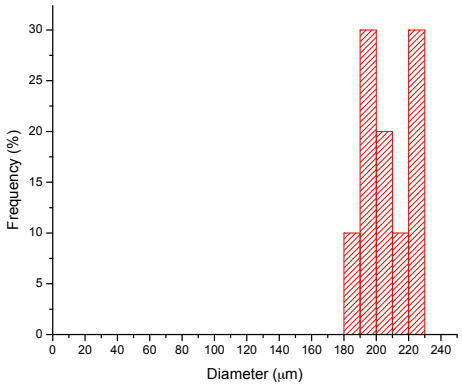
Figure 3.4 Microcarriers after agitation in StemSpan™ Spinner Flasks.

Alginate microcarriers containing magnetite could be used to easily change the culture medium without removing the cells by simple applying a magnetic field. In Figure 3.5 is showed the magnetite loaded microcarriers obtained from dilution 1:10 (w/w). After Ca^{2+} cross linking it was observed that only a few amount of magnetite was incorporated in the core of the microcarriers. The higher amount of magnetite was present in the $CaCl_2$ solution probably due to diffusion during the agitation period. The microcarriers had a average diameter of 208$\pm$13 μm.



(a) Magnetite loaded microcarriers (100x).

(b) Magnetite loaded microcarriers (200x).

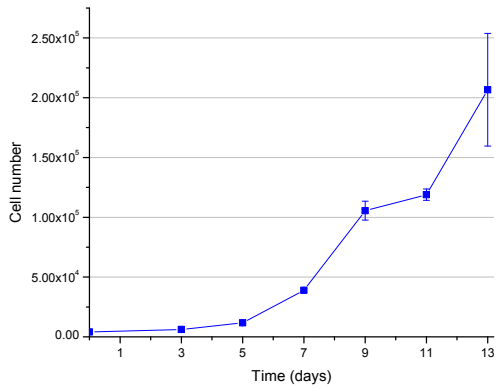


(c) Histogram of magnetite loaded microcarriers.

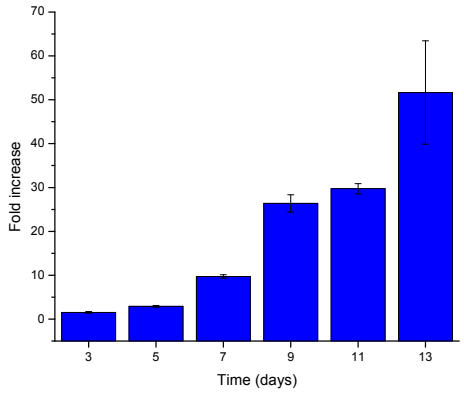
Figure 3.5 Magnetite loaded alginate microcarriers.

3.3 Proliferative capacity of MSC on 12-well plate

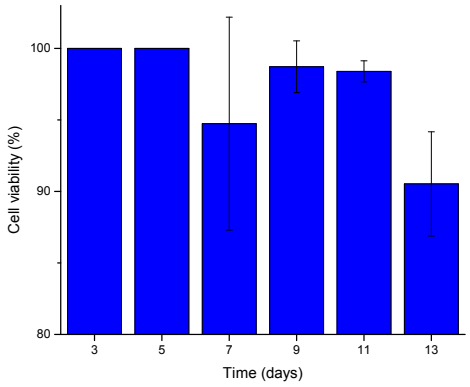
The cell number and the viability were determined, after harvesting MSC at the selected time points (day 3, 5, 7, 9, 11 and 13) followed by counting the cells using the Trypan Blue exclusion method. The MSC fold increase was calculated by dividing the number of cells harvested by the number of cells at day 1. In the first 3-4 days there was little expansion. This period is usually defined as lag phase where cells adapt themselves to growth conditions. After day 5, the cell culture entered in the exponential phase and the cells started to divide continuously. The maximum cell number was 2.1$\pm$0.5 x 10^5 cells after 13 days of culture. This corresponded to a fold increase in total cell number of 51.7$\pm$11.8 (n=2) when the culture reached about 90% confluence. In Figure 3.6a it is observed that cell number increased over time until day 13 and this stage the culture was sacrificed. It would be expected that for longer culture times, the cells will stop to growth, entering on a stationary phase followed by a death phase. Since MSC adhere to tissue culture plastic as monolayer, when cell culture reaches 100% confluence, the cells will have limited adherent surface for further grow.



(a) Cell number.



(b) Fold increase.



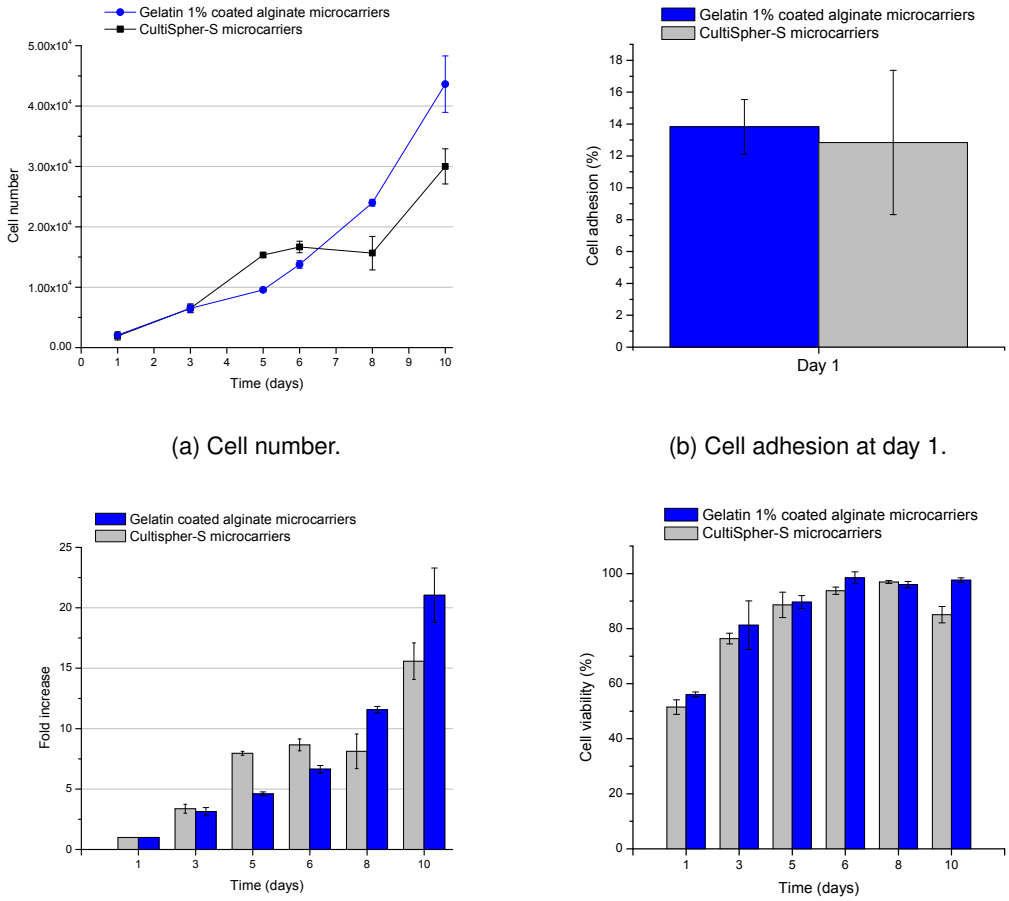
(c) Cell viability.

Figure 3.6 Expansion of MSC in 12-well plate.

3.4 Expansion of MSCs on gelatin coated microcarriers

3.4.1 Expansion in static conditions

It was evaluated the cell adhesion and proliferation 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 5×10^3 cells/cm^2. 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 $\pm$1.7 % and 12.8 $\pm$4.5 %, for gelatin 1% coated alginate microcarriers and for CultiSpher-S microcarriers (control), respectively (Figure 3.7b). The maximum number of cells was achieved at day 10 for both cultures. For gelatin 1% coated alginate microcarriers this corresponded to 4.4$\pm$0.5$\times10^4$ cells (21$\pm$2.3-fold) and for CultiSpher-S microcarriers to 3.0$\pm$0.3$\times10^4$ cells (15.5$\pm$1.5-fold) (Figures 3.7a and 3.7c). In both cultures, the cell viability at day 1 was relatively low (50-55%). At day 3, the cell viability increased and then it was maintained above 80-90% (Figure 3.7d). Throughout the culture time, either gelatin 1% coated alginate microcarriers and CultiSpher-S microcarriers showed a continued aggregation (Figure 3.8).



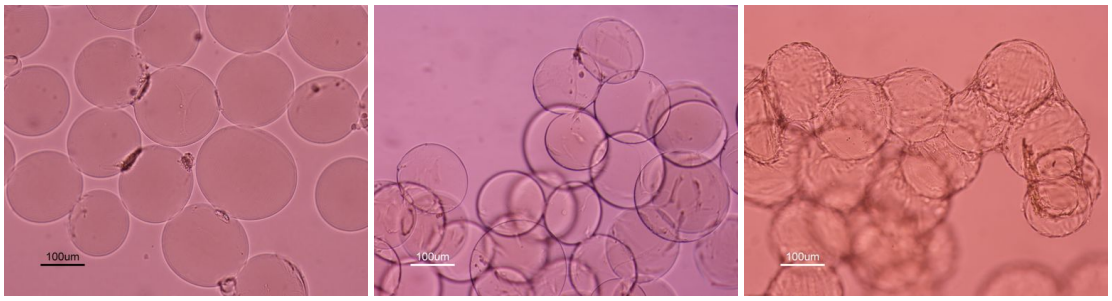
(a) Cell number.

(b) Cell adhesion at day 1.

(c) Fold increase.

(d) Cell viability.

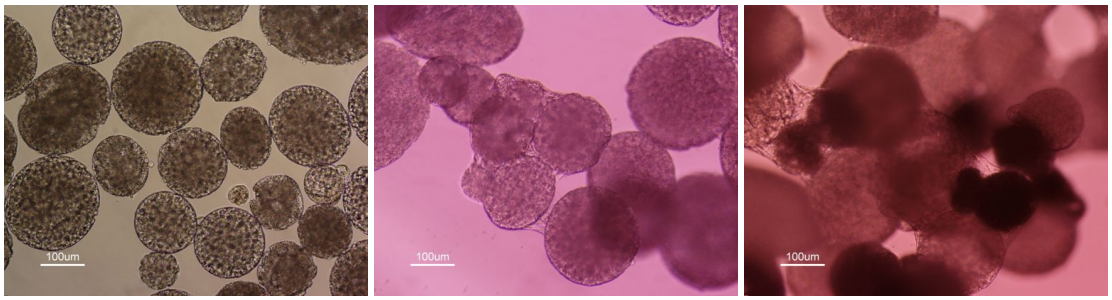
Figure 3.7 Expansion of MSC on gelatin coated alginate microcarriers in 24-well plate.



(a) Gelatin 1% coated alginate microcarriers at day 1.

(b) Gelatin 1% coated alginate microcarriers at day 5.

(c) Gelatin 1% coated alginate microcarriers at day 10.



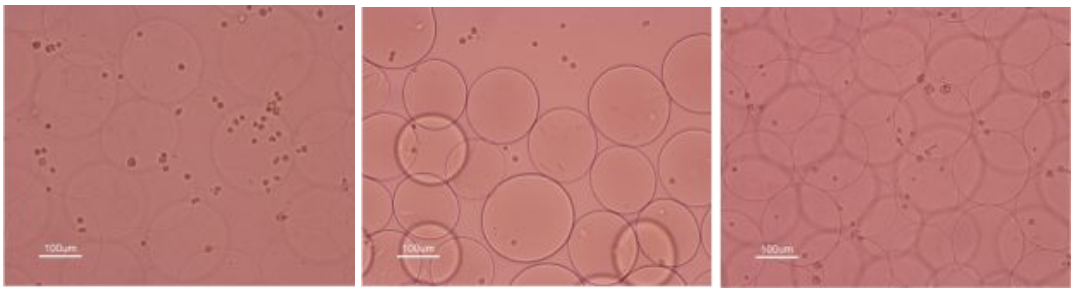
(d) CultiSpher-S microcarriers at day 1.

(e) CultiSpher-S microcarriers at day 5.

(f) CultiSpher-S microcarriers at day 10.

Figure 3.8 Bright field images of MSC on gelatin 1% coated alginate microcarriers and on CultiSpher-S microcarriers in static conditions.

In another experiment, it was evaluate if MSC would adhere and proliferate on 0.1% coated alginate microcarriers. Here, it was aimed to detach the cells using EDTA instead of trypsin. However, it was observed as in the control that MSC did not adhere on 0.1% gelatin coated microcarriers (Figure 3.9). This could be due to insufficient number of amino groups to react with glutaraldehyde and to create a tridimensional structure around the alginate bead.



(a) Gelatine 0.1% coated alginate microcarriers.

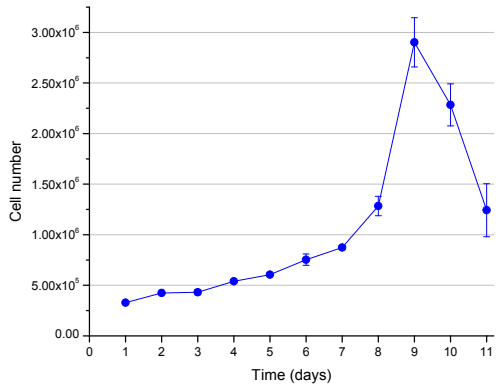
(b) Gelatine 1% coated alginate microcarriers.

(c) Alginate microcarriers.

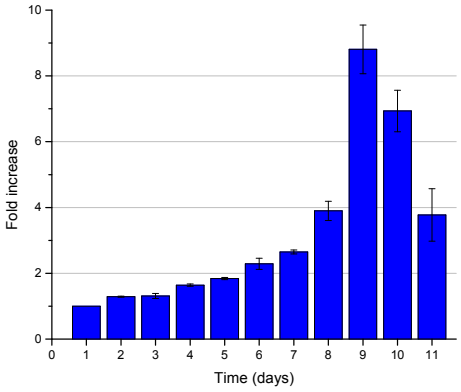
Figure 3.9 Bright field images of MSC at day 1.

3.4.2 Expansion in dynamic conditions

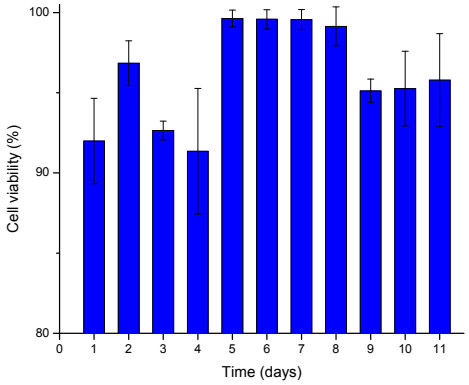
The next step was to evaluate the proliferative capacity of MSCs on gelatin coated alginate microcarriers under stirred conditions and to compare it with the static cultures. In Table 3.2 are summarized the values used for seeding (cells/mL) and for available surface area in static and dynamic conditions. 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 (Figure B.3). The initial cell adhesion efficiency for gelatin 1% coated alginate microcarriers was 22%. 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 3.10a and 3.10b). The specific growth rate for this culture was $0.034 h^{-1}$. After day 10, the number of viable cells started to decrease (Figure 3.10c). 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.



(a) Cell number.



(b) Fold increase.

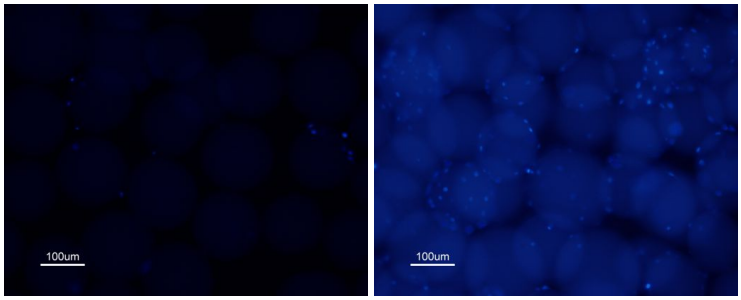


(c) Cell viability.

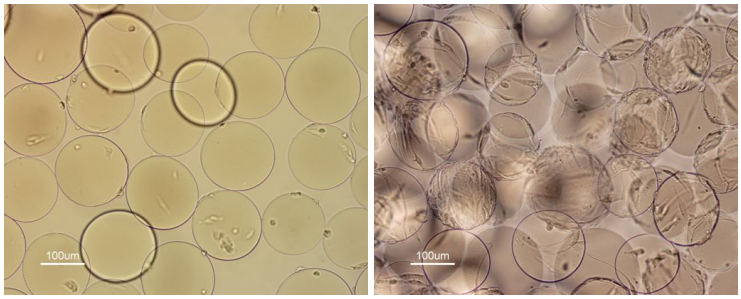
Figure 3.10 Expansion of MSC on gelatin coated alginate microcarriers in StemSpan™ Spinner Flask.

Table 3.2 Available surface area for MSC on microcarriers in static and dynamic conditions.

Culture type	**cells/mL**	**cm^2/mL**	**volume (mL)**
12-well plate (2D)	1×10^3	4	1
microcarriers in 24-well plate	15×10^3	3	1
microcarriers in StemSpan™ Spinner Flask	50×10^3	7.2	30
BellCo Spinner Flask	50×10^3	7.2	40



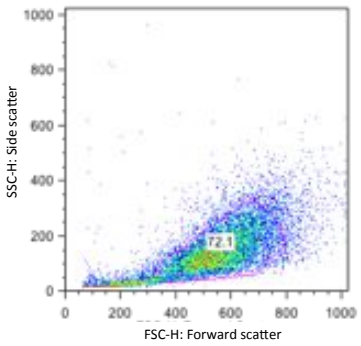
(a) DAPI staining at day 1. (b) DAPI at day 9.



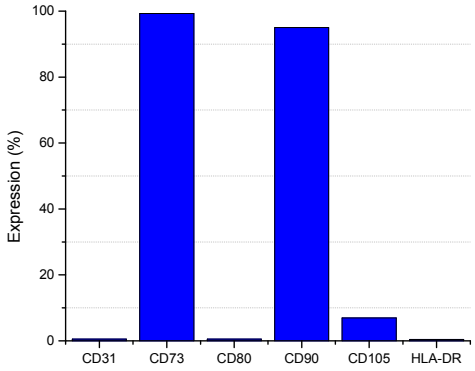
(c) Bright field image at day 1. (d) Bright field image at day 9.

Figure 3.11 DAPI staining and bright field images of MSC after expansion in StemSpan™ Spinner Flask.

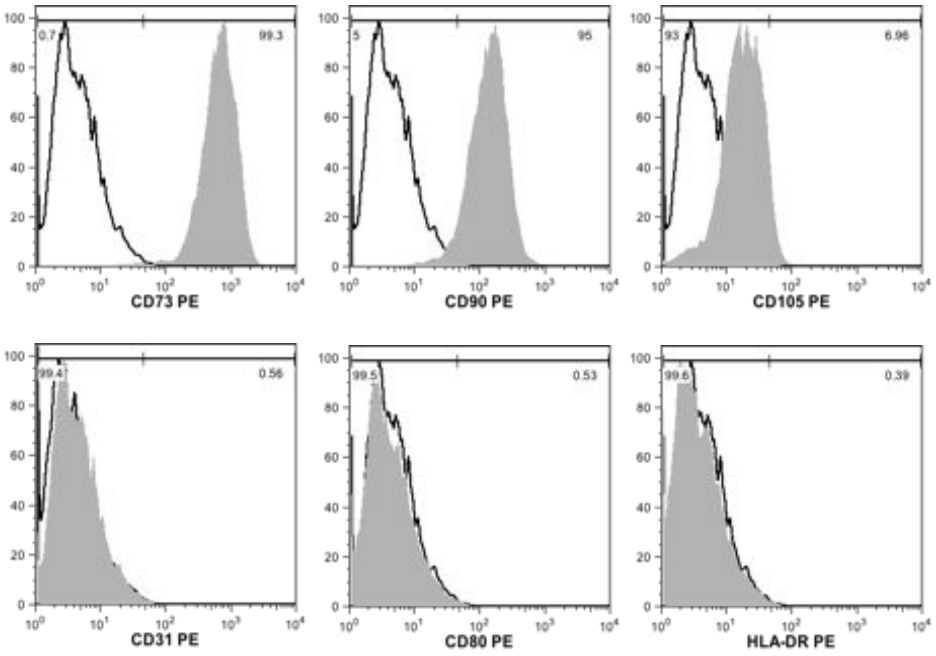
After expansion, the immunophenotype of MSC was analyzed. It was observed that MSC were negative for CD 31, CD80 and HLA-DR. The expression the surface markers CD73 and CD90 was above 90%. The expression of CD105 was only 7% (Figure 3.12). From the literature, CD105 is indicated as being very sensitive to the action trypsin, so usually has a lower value compared with CD73 and CD90.



(a) Dot plot with gate for MSC population highlighted.



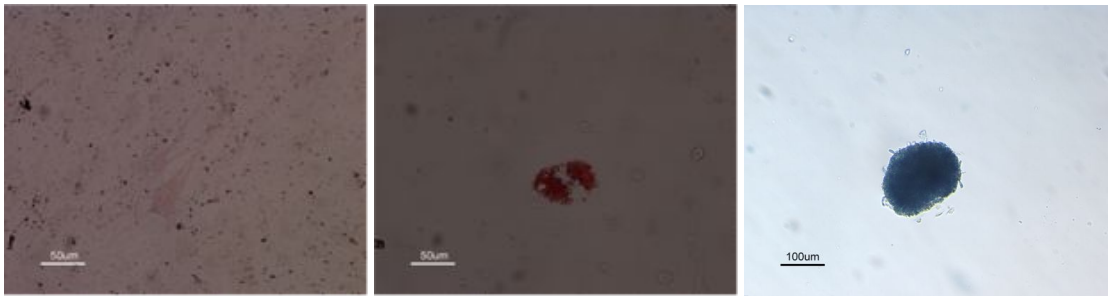
(b) Expression of MSC surface markers.



(c) FACS histograms

Figure 3.12 Analysis of MSC surface markers after expansion in spinner flask.

After expansion, the mesodermal differentiation potential was evaluated by replating MSC in diferentiation culture medium as described in 2.6.3. It was observed that MSC were able to differentiate in osteoblasts, adipocytes and chondrocytes indicating that the multilineage differentiative potential was maintained (Figure 3.13).



(a) Osteogenesis. (b) Adipogenesis (c) Chondrogenesis

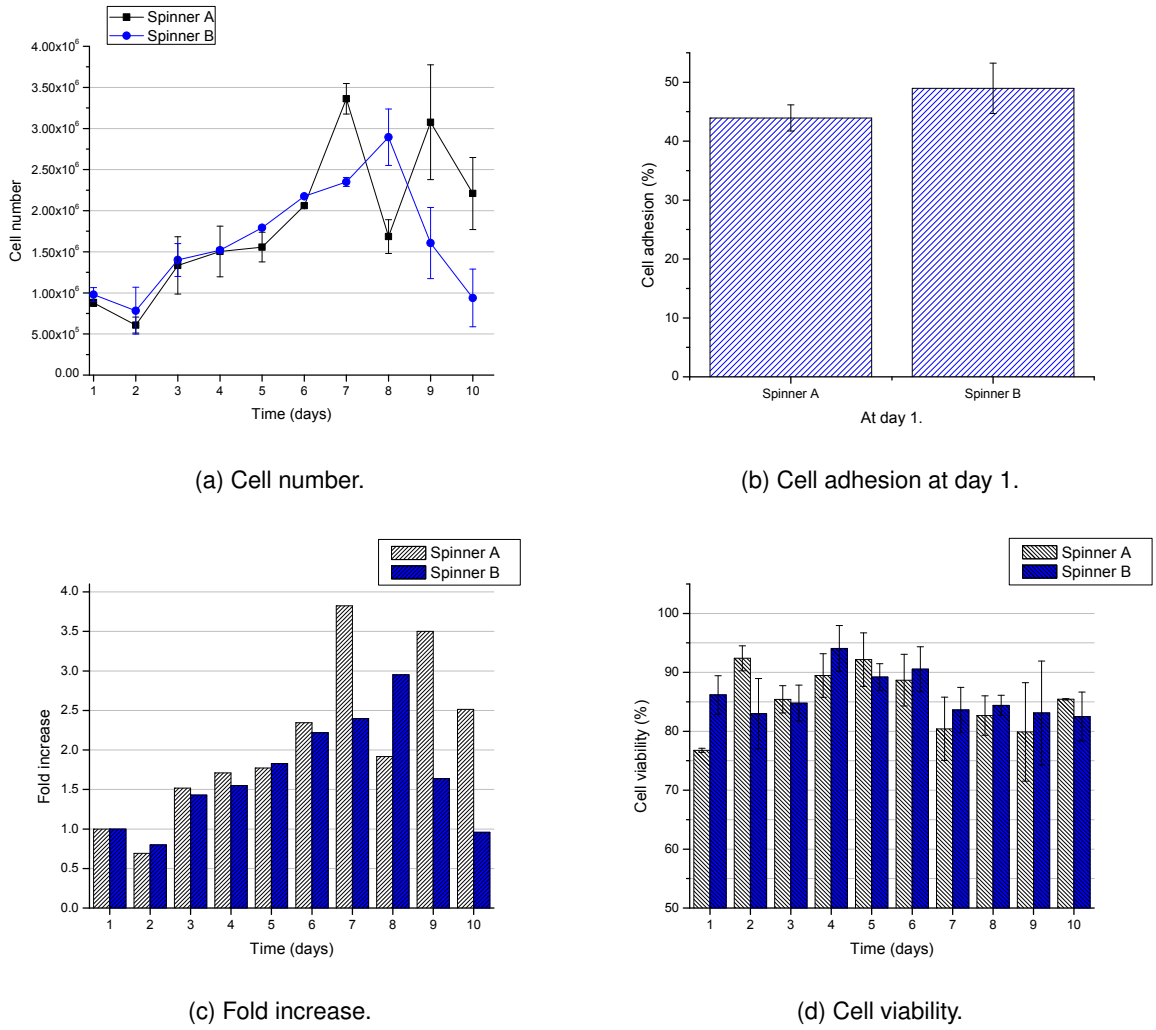
Figure 3.13 Differentiation of MSC after expansion in StemSpan™ Spinner Flask.

Table 3.3 Parameters of the microcarriers cultures.

Parameter	StemSpan™ Spinner Flask	Bellco® Spinner Flask
Microcarriers average diameter (D_{mc})	100 μm	100 μm
Vessel working volume (V_L)	30 mL	40 mL
Vessel diameter (D_t)	5 cm	5.5 cm
Impeller width (W_i)	2.4 cm	2.5 cm
Impeller diameter (D_i)	2.7 cm	5 cm
Culture medium density (ρ)	1.005 g·cm^{-3}	1.005 g·cm^{-3}
Dynamic viscosity at 37° C (μ)	0.0092 g·cm^{-2}·s^{-1}	0.0092 g·cm^{-2}·s^{-1}
Impeller rotational speed (N)	0.67s^{-1} ($\simeq$ 40 rpm)	0.5s^{-1} ($\simeq$ 30 rpm)
Reynolds number (Re)	617	1365
Dimensionless power number (N_P)	3.7	1.3
Power consumed (P)	15.45 μW	50.5 μW
Energy dissipation rate per unit mass (ε)	0.52 μW·g^{-1}	12.6 μW·g^{-1}

MSC were also expanded on gelatin 1% coated alginate microcarriers in Bellco® Spinner Flasks (spinner A and B). At day 1, the cell adhesion efficiencies were 43.9 $\pm$2.2 % and 48.9 $\pm$4.2 % for spinners A and B, respectively (Figure 3.14b). In Spinner A, the maximum cell number was 3.4$\pm$ 0.2$\times 10^6$ 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 cm^2) 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$\pm$0.3$\times 10^6$ cells at day 8 (2.9-fold). The cell viability for both spinners was between 76-94% (Figure 3.14d).

The parameters of the microcarriers in dynamics are summarized in Table 3.3.



(a) Cell number.

(b) Cell adhesion at day 1.

(c) Fold increase.

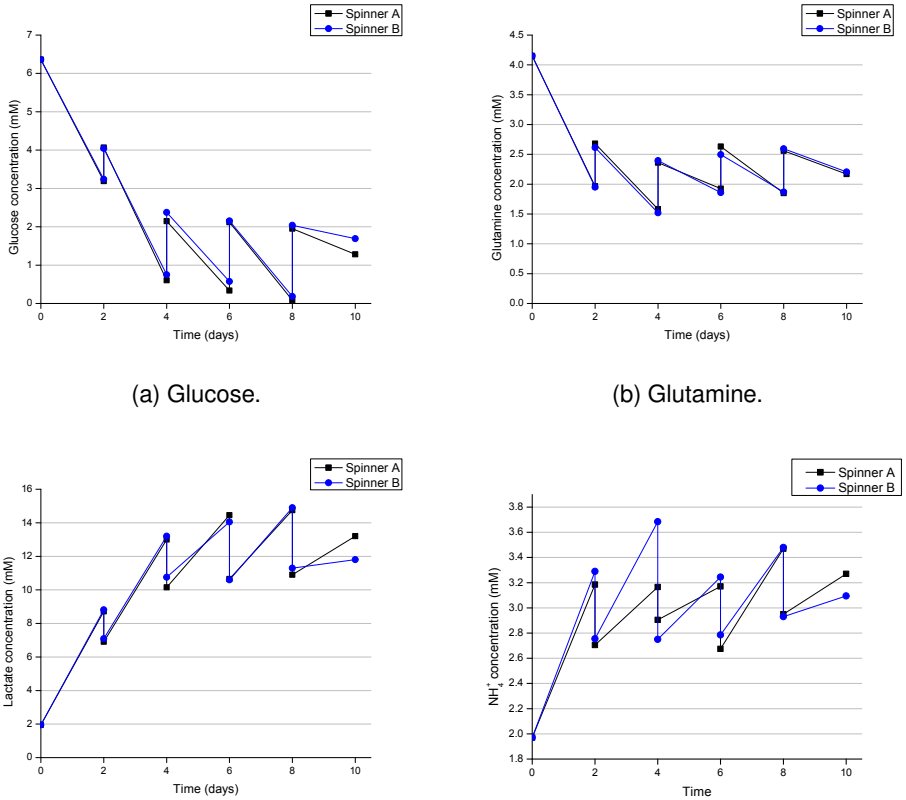
(d) Cell viability.

Figure 3.14 Expansion of MSC on gelatin coated alginate microcarriers in Bellco® Spinner Flask.

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 (Schop et al., 2009). The inhibitory effect from the excess of lactate results from the decrease in the pH and the change in the osmolarity. Unprotonated ammonia (NH_3) 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 3.15a). 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.



(a) Glucose.

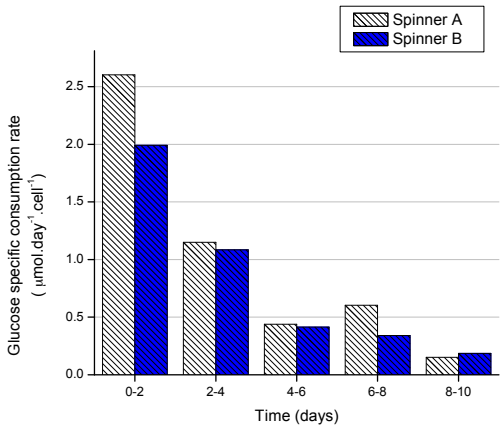
(b) Glutamine.

(c) Lactate.

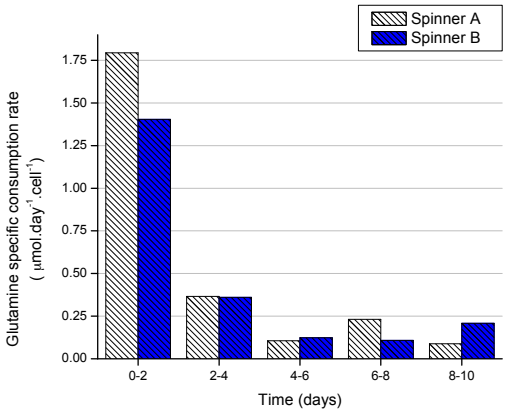
(d) Ammonia.

Figure 3.15 Concentration profiles of nutrients and metabolites during the expansion of MSC in Bellco® Spinner Flask.

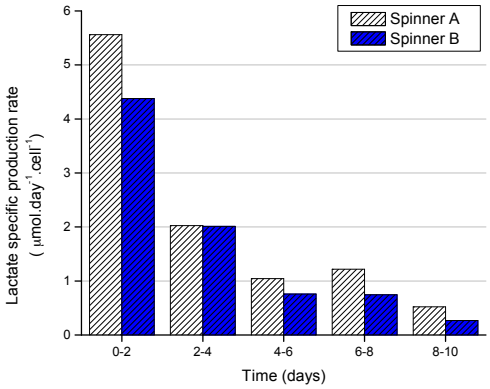
Regarding the yield of lactate from glucose, this value was approximately 2 until day 8. This shows that MSC used the glycolysis pathway to produce ATP since 1 mol of glucose generates 2 mol of lactate. Therefore, the glycolysis pathway was efficient.



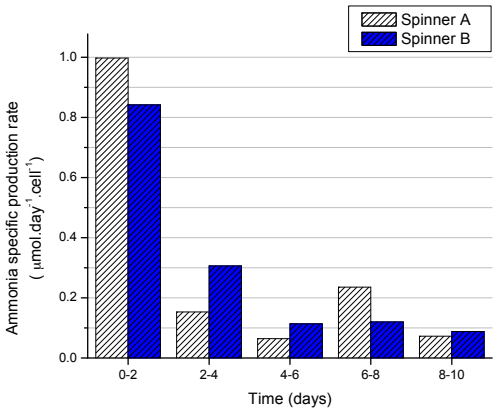
(a) Glucose specific consumption rate.



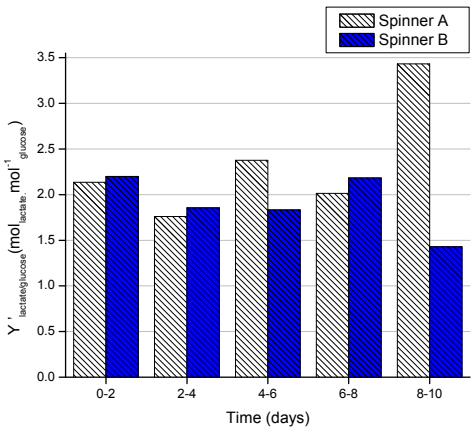
(b) Glutamine specific consumption rate.



(c) Lactate specific production rate.



(d) Ammonia specific production rate.



(e) Apparent yield of lactate from glucose.

Figure 3.16 Metabolic analysis of the expansion of MSC in Bellco® Spinner Flask.

Chapter 4

Conclusions

In this work, it was demonstrated that alginate 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 Bellco® Spinner Flask. Althought 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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Appendix A

MSC encapsulation

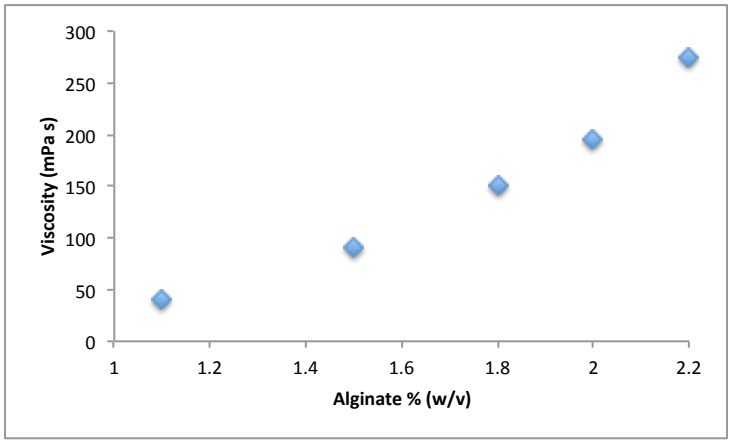


Figure A.1 Sodium alginate viscosity as function of the concentration (% w/v).

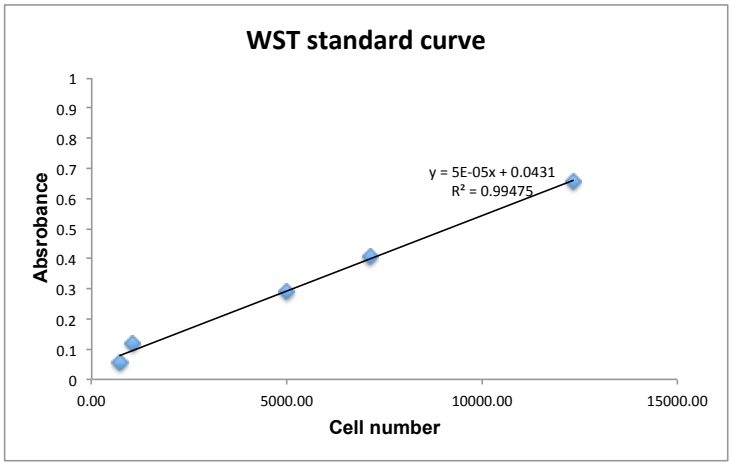


Figure A.2 Standard curve for WST absorbance at λ= 450nm. (reference at λ= 600nm.)

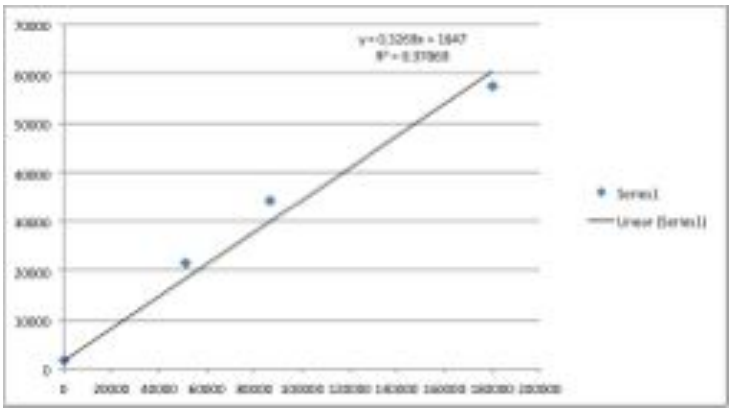


Figure A.3 Standard curve for Alamar Blue® absorbance at λ= 570nm. (reference at λ= 600nm.)

Appendix B

Microcarriers

In order to follow cell adhesion on microcarriers, it was aimed to design microcarriers containing a fluorophore. Rhodamine was chosen because it can be easily dissolved in sodium alginate. The protocol followed was described in chapter 2.

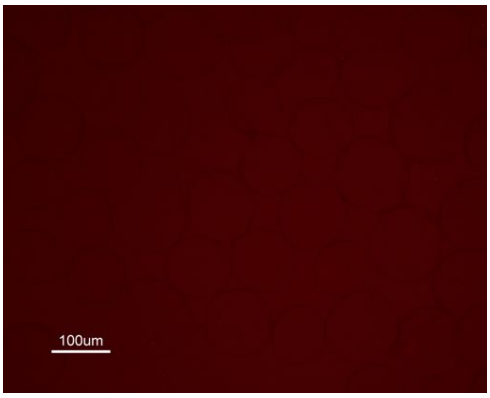
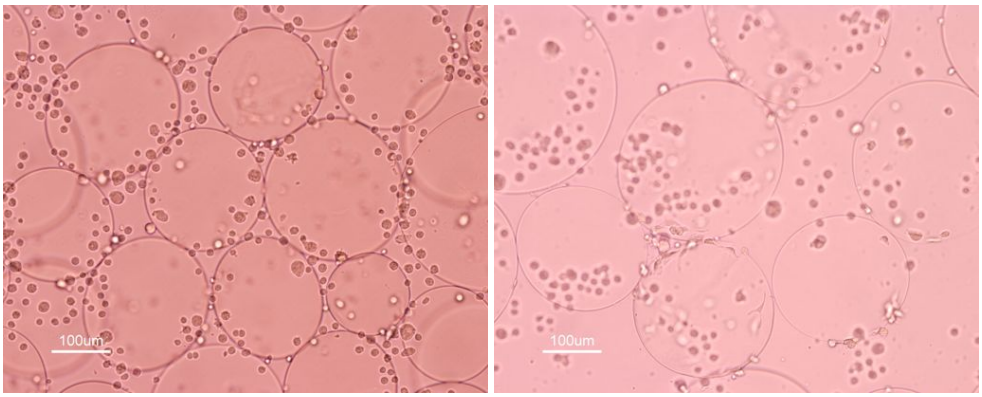


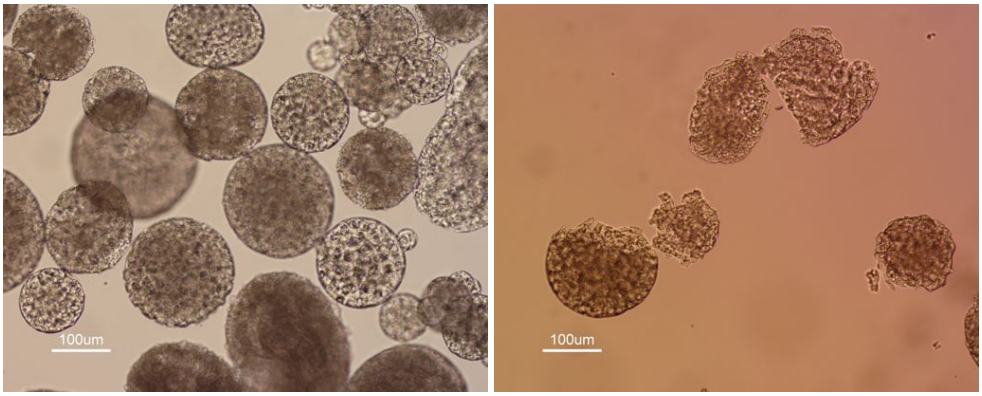
Figure B.1 Attempt to encapsulate rhodamine in alginate microcarriers.



(a) t=0. (b) t=1.5h.

Figure B.2 Gelatin 1% coated alginate microcarriers in 24-well plate.

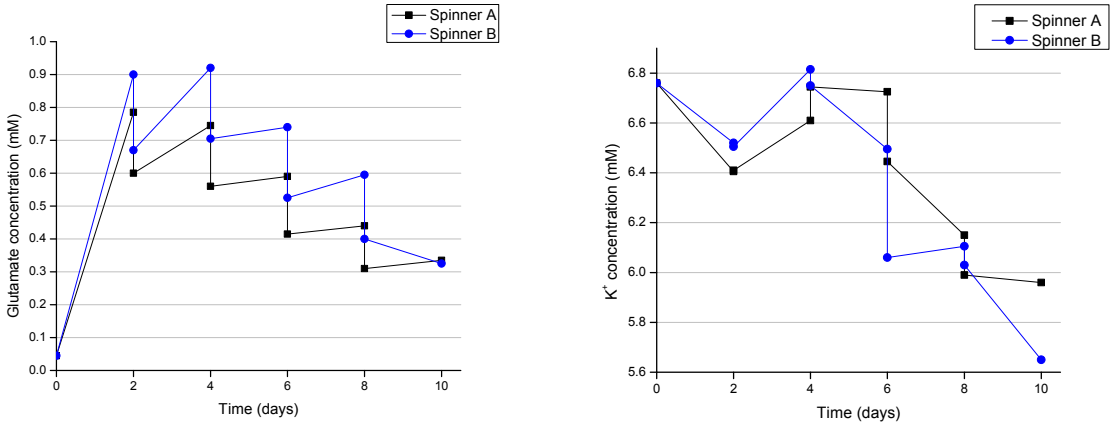
B.1 Expansion in dynamic conditions



(a) Cultispher S microcarriers at day 1. (b) Cultispher S microcarriers at day 3.

Figure B.3 Cultispher S microcarriers in StemSpan™ Spinner Flasks.

B.1.1 Metabolites analysis



(a) Glutamate. (b) Potassium.

Figure B.4 Concentration profiles of glutamate and potassium during the expansion of BM MSC in Bellco spinner flask.