The development of methods for production, selection and characterisation of microcapsules containing animal cells

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Declaration

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Publications and presentations associated with this work

Publications

Cole, H.E., Demont, A., Marison, I.W. The Application of Dielectric Spectroscopy and Biocalorimetry for the Monitoring of Biomass in Immobilized Mammalian Cell Cultures. Processes 2015; 3: 384–405

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Abstract :

"The development of methods for production, selection and characterisation of microcapsules containing animal cells" – Aurelie Demont

Microcapsules for high cell density culture of mammalian cells have found an increasing interest over the past decades, however the poor stability of the microcapsules and the lack of characterisation methods led to few quantitative results. The aim of this project was therefore (1) to select the main capsule properties required for microencapsulated perfusion cultures and to select, develop and optimise methods to characterise these properties. (2) To investigate the possibility to use currently available polymers to develop and characterize a new retention system based on microencapsulation to enable the implementation of high cell density perfusion culture of mammalian cells in STR bioreactors. Microcapsules made of a range of polymer were developed, used to grow cells and compared to the well-characterized alginate-PLL microcapsules In this work the potential of microencapsulation to reach high cell densities with suspension-adapted cells was demonstrated since cell densities of 4 x 10^7cell/$mL_{capsule}$ and 1.1x10^8 cell/$mL_{capsule}$ were reached in perfusion cultures with PLL- and PLO-containing microcapsules. Due to polyelectrolyte microcapsule sensitivity to media composition or colonisation, microcapsules made with covalent membranes such as PGA or genipin were investigated, however neither of those options were shown to be suitable for encapsulated cultures. The possibility to use alginate-based microcapsules as a support to culture an adherent cell line, was investigated and the results showed that CHO-K1 were unable to grow in alginate beads whereas in PLL-containing microcapsules, the cells grew to a density of 8x 10^5 cells/$mL_{culture}$ in 7 days in alginate-PLL microcapsules with a growth rate of 0.016h^{-1}. Despite the shown potential of microencapsulation to grow mammalian cells and to reach high cell densities in encapsulated cultures, this work also showed that there is still a need to develop more stable microcapsules that can withstand the forces involved in culture conditions

1. Introduction

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Abstract

Nowadays, the majority of recombinant proteins are produced using mammalian cells. Due to mammalian cell sensitivity to shear stress and low-productivity, there is a need for high cell density systems which allow the production of high concentrations of recombinant protein. Entrapping the cells within semi-permeable membranes through encapsulation would enable cell growth in a protected environment, reducing the shear stresses encountered in the bioreactor and would allow perfusion cultures to be performed since a simple filter mesh should retain the microcapsules within the bioreactor. Microencapsulation of mammalian cells has been used for years and is well-documented in the cell transplantation field, however a lack of information can be observed concerning microencapsulation for high cell density culture for large-scale production of cell-derived molecules in the biotechnology industry. Many procedures have been developed to produce microcapsules, however most are descriptions of the production process and lack details of characterization of the microcapsule properties. As a result there is no consensus concerning the properties that the different microcapsules should have and many results have proven difficult to reproduce and compare. There is therefore a need to standardize the characterisation of microcapsules, thereby reducing potential lab-to-lab variations before investigating the possibility to use currently available polymers to develop and characterize a new retention system based on microencapsulation to enable the implementation of high cell density perfusion culture of mammalian cells in STR bioreactor.

1.1. General overview

1.1.1. Thesis aim, importance, strategy and scope

The work undertaken through this PhD project is funded by the Irish Research Council Employment Based Programme, in collaboration with NIBRT.

1.1.1.1. Particular focus of the present thesis

Nowadays, over 300 biopharmaceutical products are on the market, over 200 of these being recombinant proteins (Baeshen et al., 2014; Overton, 2014). According to Baeshen et al. (2014), the sales of these therapeutic proteins exceeded USD 100 billion in 2014, monoclonal antibodies having the major share of the market with more than USD 18 billion, followed by hormones (> USD 11 billion) and growth factors (> USD 10 billion) (Baeshen et al., 2014). Proteins, unlike many pharmaceuticals are complex molecules due to their structure and function and are therefore mainly synthesised through biological processes. From 2004 to 2013, 56% of the biopharmaceuticals approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) were produced by mammalian cells, 24% from *E.coli*, 13 % from *S. cerevisiae*, 3% from transgenic animals and plants and 4% from insect cells (Baeshen et al., 2014).

The production of recombinant proteins using animal cells is an increasingly developing area of bioprocessing in the pharmaceutical industry (Desimone et al., 2011; Harcum, 2005) as it is necessary to generate therapeutic proteins, which possess significant post-translational modifications, a proper folding and assembly and in vivo immunotolerance (Harcum, 2005; Wurm, 2004; Zhu, 2012). Unlike microbial cells, mammalian cells have the ability to glycosylate, fold, assemble and undertake post-translational modifications in a similar way than to human cells (Wurm, 2004).

Due to the ability of yeast to perform some post-translational modifications like those performed in higher eukaryotes, research has been undertaken to humanize the glycosylation pathway in yeast strains in order to create homogenous glycans on the target protein in order to produce low-priced proteins by combining the advantages of unicellular growth and the ability to perform eukaryotic post-translational modifications (Çelik and Çalık, 2012). According to Çelik and Calik (2012), additional

research is however necessary to master the quality control mechanism in the endoplasmic reticulum. Mammalian cells therefore currently remain the main means of production for recombinant proteins.

Over 70% of recombinant proteins are nowadays produced by immortalised Chinese hamster ovary (CHO) cells (Butler and Spearman, 2014; Wurm, 2004) due to the capacity to adapt and grow in suspension which is ideal for large scale culture, the capacity to grow in serum-free and chemically defined media ensuring reproducibility between batches and the capacity to produce post-translational modifications which are similar to those produced by humans, compatible and bioactive in humans. Furthermore, few human viruses are able to propagate in CHO cells. In addition, several gene amplification systems are well established in CHO cells, which take advantage of the genome instability to allow gene amplification and ultimately higher production yields of proteins (Lai et al. 2013). Other cells lines, such as those derived from mouse myeloma (N50, Sp2/0), baby hamster kidney (BHK), human embryo kidney (HEK293) and human retinal cells, have gained approval for recombinant protein production (Butler and Spearman, 2014; Wurm, 2004).

The use of animal cells for the production of recombinant proteins however, has some limitations such as: the time required to create stable, high producing cell lines secreting the protein to high levels, at high productivity and correctly assembled (Wurm, 2004; Zhu, 2012); the time taken to develop processes which allow cells to grow and secrete the recombinant proteins, using carefully controlled conditions, while avoiding the lysis of cells through shear forces present in the reactors; avoidance of medium and product (metabolite) limitations; and avoidance of product degradation (Harcum, 2005; Zhu, 2012); the low productivity of animal cells and the high production costs (Wurm, 2004).

In order to overcome these difficulties, there is the requirement for high productivity, high cell density systems which are carefully controlled and which allow the production of high concentrations of recombinant protein (Zhu, 2012). Nowadays, industries are still mainly using simple batch and fed-batch cultures to produce recombinant proteins at large- scale as the ideal cell retention device is non-existent and retention systems for perfusion cultures are still associated with uncertainty and risks in manufacturing (Clincke et al., 2013a, 2013b; Voisard et al., 2003; Xie and Zhou,

2005). Existing systems to perform a perfusion culture require the use of complicated technical devices mainly based on size and density separation, to retain the cells in the culture during media circulation (Clincke et al., 2013a, 2013b; Kompala and Ozturk, 2005; Voisard et al., 2003; Woodside et al., 1998). The separation systems based on size are usually based on filtration such as cross-flow filtration, hollow-fiber modules or spin filters however, the main drawback is the tendency to clog and foul (Clincke et al., 2013a, 2013b; Voisard et al., 2003; Woodside et al., 1998). The retention techniques based on density are based on the difference of density between cells and culture media, however this difference is rather small and gravitational settling velocities are low, making separation difficult. Techniques available today, such as gravity settlers, centrifuges, centrifugal bioreactors, hydrocyclones or acoustic settlers have been designed to enhance separation efficiency (Voisard et al., 2003; Woodside et al., 1998). These techniques have therefore a better potential for long-term operation according to Voisard *et al*. (2003), since they do not use a physical barrier (Voisard et al., 2003). It has been reported by Voisard *et al.* (2003) that companies are looking for a perfusion system, which is a reasonable investment, has reasonable running costs, which is available from several manufacturers and which can combine a high perfusion capacity with a high retention efficiency. The new system should be capable of operation over a complete process run without maintenance and should be robust in order to minimize the risks of failure. The system should also be easy to clean and to sterilize and avoid pumping of cells if internal (Voisard et al., 2003).

Entrapping the cells within semi-permeable membranes through encapsulation would enable cell growth in a protected environment, reducing the shear stresses encountered in the bioreactor and would allow simple perfusion cultures to be performed since a simple filter mesh should retain the microcapsules within the bioreactor. Furthermore, several studies showed that cell viability and protein production are increased within microcapsules (Breguet et al., 2007; Uludag et al., 2000a). Numerous polymers have been investigated to encapsulate cells, mainly for transplantation purposes, giving the microcapsules a range of properties, which can be tailored depending on the application (Uludag et al., 2000a).

The aim of this thesis is therefore (1) to select the main capsule properties required for microencapsulated perfusion cultures, to select, develop and optimise methods to

characterise these properties in order to create a characterization method toolbox that can be used to standardize the characterisation of microcapsules, thereby reducing potential lab-to-lab variation. (2) To investigate the possibility to use currently available polymers to develop and characterize a new retention system based on microencapsulation to enable the implementation of high cell density perfusion culture of mammalian cells in STR bioreactors, keeping in mind the characteristics that an ideal retention system should have from an industrial manufacturing point of view.

1.2. Cell microencapsulation

Encapsulation can be defined as a process, which involves the complete envelopment of pre-selected core material(s) within a defined porous or impermeable membrane using various techniques (Marison et al., 2004; Whelehan and Marison, 2011a). The main aims of encapsulation are the immobilization, protection, stabilization and control of release of the entrapped compound (Chan et al., 2009). Indeed, the entrapment of various materials such as flavours, living cells and pharmaceutical compounds within beads for different purposes is of great importance in the pharmaceutical, chemical and food industries, as well as in agriculture, biotechnology or medicine (Marison et al., 2004; Pruesse et al., 2008). An ambitious challenge is the entrapment of viable cells within semi-permeable membranes, which allow the transport of molecules essential for cell survival such as nutrients, waste and therapeutic products, while the transport of molecules larger than a defined critical size such as cells, antibodies or cytotoxic cells is prevented (Pajić-Lijaković et al., 2007; Uludag et al., 2000a). Nowadays, cell encapsulation is being pursued for diverse applications as shown in Table 1.1.

Table 1.1 : Applications of cell encapsulation technology

Application	Reference
Cell transplantation	(Briššová et al., 1998; de Vos et al., 2006; Haque et al., 2005; Hillberg et al., 2013; Ma et al., 2012; Orive et al., 2006; Uludag et al., 2000a)
High cell density culture for large-scale production of cell-derived molecules in biotechnology industry	(Breguet et al., 2007; Bugarski et al., 1992; Jarvis Jr. et al., 1986; Pajić-Lijaković et al., 2007; Uludag et al., 2000a)
Clonal selection of desired cell phenotypes	(Pueyo et al., 1995; Uludag et al., 2000a)
In vitro culture of cells dependent on close cell-cell contact	(Guo et al., 1989; Loty et al., 1998; Takabatake et al., 1991; Uludag et al., 2000a)
In vivo cell culture	(Hollingshead et al., 1995; McMahon et al., 1990; Okada et al., 1996, 1995; Uludag et al., 2000a)
Reproductive technology	(Jöchle, 1993; Nebel et al., 1993; Uludag et al., 2000a; Watson, 1993)
Cytotoxicity testing	(Goguen and Kedersha, 1993; Uludag et al., 2000a)

Bugarski et al. (1992) reported the possibility to encapsulate hybridoma cells for recombinant protein production, however except for Breguet *et al.* (2005, 2007) and Gugerli *et al.* (2003), there is very little literature concerning microencapsulated high cell density cultures as microencapsulated cells are mainly reported in articles about cell transplantation. Polyelectrolyte microcapsules have been reported to have a limited membrane stability (Breguet et al., 2007, 2005; Edwards-Lévy and Lévy, 1999; Gugerli, 2003), therefore, in order to perform microencapsulated high cell density culture for cell-derived molecule production, an encapsulation method that enables the stabilization of the polyelectrolytic membrane has to be found, together with the investigation of stronger polymers or polyelectrolytes and encapsulation methods enabling covalent interactions in the membrane formation.

1.2.1. Requirements for cell encapsulation

In order to use encapsulated cells for any of the above applications (Table 1.1), there is a need to isolate the desired cell population from the outside environment. The cells will therefore be subject to different immobilization conditions, due to their isolation through a membrane made of various materials. The final microcapsule properties will have to suit the needs of a specific application and will then be different from one field to another. It was however reported by Verica *et al.* (2009) that a few characteristics are generally considered as favourable for carriers; (1) it is better if the support is

renewable and has a high surface area-to volume ratio, (2) the biological or catalytic activity of the encapsulated material must not be negatively affected by immobilization, (3) the carrier should have a good mechanical, chemical and biological stability and not be easily damaged by enzymes, solvent, pressure changes or shear forces (Verica et al., 2009). Amongst the different capsule characteristics (Figure 1.1), a good balance will hence have to be maintained in order to support cell survival (Mahou, 2011; Uludag et al., 2000a). The mass transfer properties are critical since the essential molecules for cell survival must diffuse into the microcapsules while the metabolic end- products must diffuse to the outside of the membrane. The upper limit of capsule permeability is termed the molecular weight cut-off (MWCO) and will depend on the application of the encapsulated cells. For transplantation, the MWCO will have to restrict the ingress of antibodies and cytotoxic molecules into the hydrogel, while for cell cultures, the MWCO depends on whether it is desired that the produced biomolecules permeate through the capsule membranes (Lewińska et al., 2002; Mahou, 2011; Rosiński et al., 2005; Uludag et al., 2000a). Microcapsules also require enough mechanical resistance to avoid breakage and to withstand the various forces during the process. When microspheres are destined to be used in transplantation, their resistance was shown to be dependent on the site of implantation (Thanos et al., 2007), while for use in culture they will have to withstand the shear stresses due to agitation and aeration of the culture. Resistance should therefore be constant as a function of time. Besides permeability and mechanical resistance, it is also important to consider the availability of an extracellular matrix, especially for anchorage- dependent cells. It has been reported by Uludag *et al*. (2000) that the extracellular matrix enables the expression of the differentiated cell functions and the organization of the cell mass within the microcapsules for optimal viability (Uludag et al., 2000a). Microcapsule biocompatibility is critical in cell encapsulation. Biocompatibility is defined as “the ability to be in contact with a living system without producing an adverse effect.”(Vert et al., 2012), it is therefore important for the polymer not to induce the death of the encapsulated cells. If the encapsulated cells are intended for transplantation, the host response and graft survival will be determined by the biocompatibility of the biomaterial retaining the cells, the physicochemical nature of the biomaterial is thus important to enable capsule biocompatibility (Orive et al., 2006, 2003; Uludag et al., 2000a).

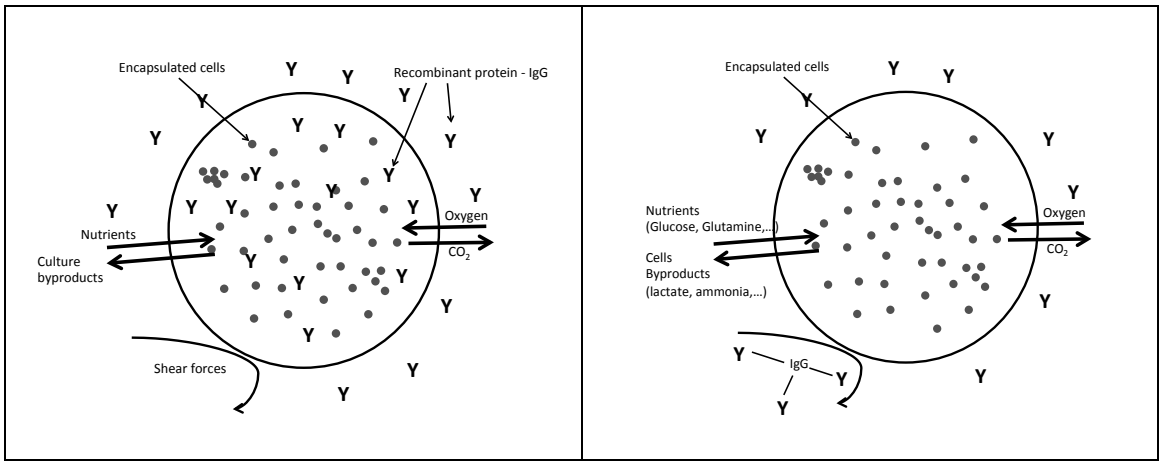

Figure 1.1 : Microcapsule requirements for use in recombinant protein production (left) or cell implant action (right)

Numerous methods have been reported in the literature in order to measure microcapsule permeability or microcapsule mechanical resistance. For permeability, several methods have been reported, the protein standards method measures the diffusion of a known protein or molecule such as BSA or vitamin B12 (Lewińska et al., 2002; Uludag et al., 2000a) while the dextran or pullulan standard method (Briššová et al., 1998; Rosiński et al., 2005; Uludag et al., 2000a) measures the ability of dextran or pullulan molecules to permeate through the capsule membrane. The protein method and the dextran method are complementary as the dextran and pullulan show the ingress of neutral molecules while the protein method shows the ingress of charged molecules that can interfere with the microcapsule membrane. None of the methods however, allow a rapid and simple visualization of the ingress of molecules into the microcapsules and both methods are dependent on a filtration step that could damage the microcapsules. The measurement of the penetration of FITC-Dextran into microcapsules was reported by Chen et al. (2009) and measured by confocal microscopy. This method allows simple visualization of the ingress of the molecules into the microcapsules, however it does not take into account the behaviour of charged molecules and does not quantify the amount of a molecule penetrating the microcapsules.

Different methods, some more qualitative and some more quantitative, have been reported in the literature to analyse the mechanical resistance of the microcapsules. These methods can be divided in two classes, the breakage methods that analyses the behaviour of microcapsules when subject to mechanical shear forces (Bartkowiak and Hunkeler, 2000; Liu et al., 1996; Lu et al., 1992; Uludag et al., 2000b) and the osmotic

methods that are based on the swelling of microcapsules when exposed to a change in media composition and osmotic pressure (Gåserød et al., 1999; Martins dos Santos et al., 1997; Ma et al., 2013; B. Thu et al., 1996; Van Raamsdonk and Chang, 2001). Breakage methods to measure the resistance of microcapsules include the texture analyser method which consists in compressing the microcapsules with a mobile probe at a defined speed using a texture analyser TA.XTPlus (Stable Micro Systems, UK) (Rosiñski et al., 2008) and the bead agitation method (Ma et al., 2013). The method based on osmotic changes is well documented and very straightforward to perform. It consists in exposing microcapsules to water, causing the microcapsules to swell and break. This method is reliable to compare microcapsules prepared from the same materials and is only applicable to physical hydrogels; in the case of chemically cross-linked hydrogels, changing the osmotic conditions will not induce the microcapsules to break (Gåserød et al., 1999; Martins dos Santos et al., 1997; Ma et al., 2013; B. Thu et al., 1996; Van Raamsdonk and Chang, 2001). Since all of the methods are based on different principles, it is very difficult to compare results analysed with several of them.

In order to compare different microencapsulation techniques and polymers, and the evolution of the microcapsules over the duration of a culture, different parameters of the microcapsules have to be analysed. The most important of these parameters can be divided into different subclasses including size and size distribution, transport properties (i.e. MWCO), mechanical resistance, stability and cell viability and cell dispersion in the capsules (Rosiński et al., 2005). Unfortunately, there is a lack of adequate and complete documentation of the desired requirements to characterize microcapsules. There is therefore a need to create a toolbox that standardizes the main characterization methods to enable a direct comparison between different experiments with different polymeric systems, to reduce lab-to-lab variations thereby allowing high reproducibility and comparison of results produced by different groups

1.3. Choice of polymer

Currently in microencapsulation, the materials of choice are natural or synthetic polymer systems, which can offer a large variety of properties that can either be chemical, structural or physical. A good knowledge of the different polymers properties is mandatory to understand how to design, develop and optimize microcapsules (Jones and McClements, 2010; Matalanis et al., 2011). The two main

classes of polymers used in cell encapsulation are proteins and polysaccharides and the specific characteristics are summarized in Table 1.2.

Table 1.2 : Summary of important protein and polysaccharide molecular properties in the assembly of biopolymer particles reproduced from (Jones and McClements, 2010).

Molecular property	Protein	Polysaccharide
Molecular conformation	Globular: non polar residues within interior; linear: restricted secondary structure	Linear or random coil
Electrical characteristics Hydrophobic characteristics	Positive: $-NH_3^+$; Negative: $-CO_2^-$; PI : if PI > 7, positively charged at pH 7 Functionality: Surface (effective) compared with internal (ineffective)	Positive: $-NH_3^+$; Negative: $-CO_2^-$, $-SO_4^-$ Interaction with non- polar residues; protein conjugation
Physical interactions Chemical reactivity	Van der Waals, electrostatic, hydrophobic and hydrogen bonding Disulfide interchanges, dehydration, phenolic oxidation, Maillard and transglutaminase reactions	Van der Waals, electrostatic, hydrogen bonding Conjugation, esterification, etherification, radicalization; depolymerisation

1.3.1. Microencapsulation by polyelectrolyte complexation

Polyelectrolytes are polymers containing a charged group in a monomer-repeating unit and can be split into two types, polycations or polyanions according to the charge (Huebner and Buccholz, 2002; Lacík, 2004; Uludag et al., 2000a). Polyelectrolytes have been used for diverse applications such as flocculents in water purification, protein precipitation and membrane technology (Huebner and Buccholz, 2002). The simplest way to produce a physical membrane barrier is by forming complexes when oppositely charged molecules interact with each other in solution via coulombic forces (Lacík, 2004; Uludag et al., 2000a). Polyelectrolyte complexes fit to the group of the physically crosslinked gels, in which the gel is held together through hydrogen bonding, hydrophobic and divalent ion cross-linking (Huebner and Buccholz, 2002; Jones and McClements, 2010; Lacík, 2004; Uludag et al., 2000a). In cell microencapsulation, charged polymers, due to their solubility in water, offer the possibility to develop an

aqueous encapsulation system compatible with cellular media (Uludag et al., 2000a). Both natural and synthetic polymers have been used for this purpose. Natural polymers are usually more cell-compatible however their harvest in large quantities results in a product with a heterogeneous composition (Uludag et al., 2000a). It is important to notice that purification systems for natural polymers have been described, resulting in rather homogeneous preparations (Uludag et al., 2000a). Synthetic polymers can be easily produced in large quantities, nevertheless it is critical that such polymers are cell compatible (Uludag et al., 2000a). Despite the fact that there are a large number of polyanion-polycation combinations, only a few of them can be used in cell microencapsulation (Huebner and Buccholz, 2002). When used as a core material, polycations interact with negatively charged phosphate groups of the cytoplasmic membrane, leading to membrane rupture and cell death, moreover they form stable complexes with DNA and proteins (Huebner and Buccholz, 2002). When the polycation is used in the membrane, these effects are not observed (Huebner and Buccholz, 2002). Due to the polycation toxicity for the cells, it is therefore strongly recommended to use a polyanion as core polymer. The most suitable polyanions to produce droplets from a cell suspension are polysaccharide derivates, with a pH-dependant high charge, a molar mass in the range of 10^6 Da and a cyclic backbone, such as cellulose sulphate and alginate (Huebner and Buccholz, 2002). For this thesis, the focus will be on microcapsules using alginate as polyanion.

1.3.1.1. Alginate

Alginate is the most widely used material for cell microencapsulation due to the property to rapidly form gels under very mild conditions as well as its reported *in vivo* biocompatibility (Santos et al., 2010; Strand et al., 2004). Alginate is used in the form of a salt of alginic acid, a polyuronic acid extracted from brown seaweeds and some bacteria, mostly derived from the genus *Pseudomonas* (Huebner and Buccholz, 2002; Santos et al., 2010; Strand et al., 2004). It is an unbranched anionic polysaccharide composed of linear block copolymers of 1->4 linked ß-D-mannuronic acid (M) and α-L-guluronic acids (G) forming regions of M-blocks and G-blocks, and of alternating MG-blocks structures (Figure 1.1) (Santos et al., 2010; Strand et al., 2004; Verica et al., 2009). The composition and sequential arrangement of the residues depends on the source of the alginate, and it is the structures comprised of homopolymeric and

heteropolymeric regions along the chains that create hydrogels when associated with divalent cations (Santos et al., 2010; Strand et al., 2004).

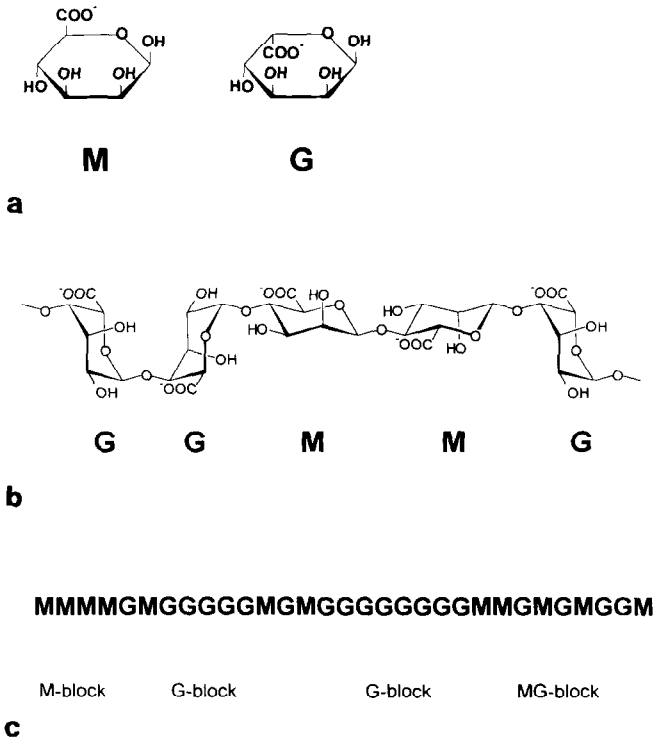

Figure 1.2 : a, the monomers of alginate, Haworth conformation. M : ß-D-mannuronate and G : α-L-guluronate. b, the alginate chain, chair conformation. c, symbolic representation of the alginate chain. (B. Thu et al., 1996)

The composition and sequence of alginate blocks is of great importance for the properties of the gel matrices as it conditions features such as mechanical stability, capsule diameter, permeability and degradation rate (Santos et al., 2010). Gels produced from alginate with a high content of G-blocks are more stable and have a greater permeability compared to gels produced from alginates with a high content of M-blocks (Santos et al., 2010). It was reported that the length of the G-blocks is the principal factor responsible for cross-linking with divalent cations while M residues are reported to provide the elasticity of the gel (Santos et al., 2010). The relative rigidity of the polymer chains is reported to increase as follows: MG<MM<<GG (Santos et al., 2010). Alginate containing a high G content with long G-blocks will thus produce stiff, open and static networks, while alginates containing a high number of M-blocks will produce more entangled networks due to their relatively long elastic segments (Santos et al., 2010).

The affinity of alginates toward the different divalent cations has been shown to decrease in the following order: Ba > Sr > Ca > Pb > Cu > Ni > Cd > Zn > Co > Mn (Santos

et al., 2010), yet, only a few of these can be used to make capsules for therapeutic applications , the most commonly used being Ca^{2+} and Ba^{2+} (Mørch et al., 2006). Due to its physiological and biocompatible properties, calcium provides excellent viability for the encapsulated cells and has therefore been commonly used as the cross-linking ion in cell microencapsulation (Huebner and Buccholz, 2002; Mørch et al., 2006; Santos et al., 2010). In physiological solutions though, due to a constant exchange between Ca^{2+} ions and other non- gelling ions such as monovalent cations and Mg^{2+}, and the affinity of Ca^{2+} ions for chelating agents such as phosphate, EDTA and citrate, calcium alginate beads show a tendency to suffer from osmotic swelling, leading to an increase in the membrane pore size, destabilization and finally rupture of the matrix (Mørch et al., 2006; Santos et al., 2010).

A possibility to solve the stability of calcium-alginate beads would be to replace calcium ions with ions presenting higher affinity towards alginate. Morch *et al* (2006) studied the replacement of calcium ions by strontium or barium ions and showed that for high-G alginate gels, the dimensional stability increased when using Ba^{2+} ions instead of Ca^{2+} ions. Moreover, the size of alginate beads decreased when barium was used and the permeability to immunoglobulin G was reduced. This study showed that strontium gels have characteristics lying between those of calcium and barium (Mørch et al., 2006). For high-M alginate, the behaviour of beads cross-linked with strontium and barium ions showed the opposite behaviour to the high-G alginate beads; the beads were larger than those made of calcium-alginate and had a higher swelling tendency, resulting in an increase of permeability (Mørch et al., 2006). The investigation also revealed that different block structures in the alginate bind the ions to a different extent, Ca^{2+} was thus found to bind to G- and MG-blocks, Ba^{2+} to G- and M-blocks and Sr^{2+} to G-blocks only (Mørch et al., 2006). Despite the advantages of replacing Ca^{2+} ions by Ba^{2+} ions, Ba^{2+} has drawbacks such as inhibiting potassium channels in cell membranes at concentrations greater than 5-10 mM and at cross-linking times greater than 15 minutes. In addition, using Ba^{2+} may result in inhomogeneous microcapsules as a consequence of the gradient occurring during the binding process (Mørch et al., 2006; Santos et al., 2010).

Alginate is a single stranded polymer and is susceptible to a variety of depolymerisation processes. It is reported by Draget *et al*. that acid and alkaline

degradation mechanisms and oxidation with free radicals can cleave the glycosidic linkages (Draget et al., 1997). The degradation of alginate as a function of pH is described to be at its minimum around neutral pH and increases at pH above or below this value. The increased instability at <pH 5 is due to a proton-catalysed hydrolysis while the degradation at >pH 5 is caused by a ß-alkoxy-elimination (Figure 1.2)(Draget et al., 1997). Draget et al reported that contamination of alginate by reducing agents like polyphenols from brown algae can create an oxidative-reductive depolymerisation reaction provoking degradation of the alginate by free radicals (Draget et al., 1997). It also has to be noticed that autoclaving is not recommended for sterilization of alginate solutions since the depolymerisation reactions are reported to increase with temperature (Draget et al., 1997).

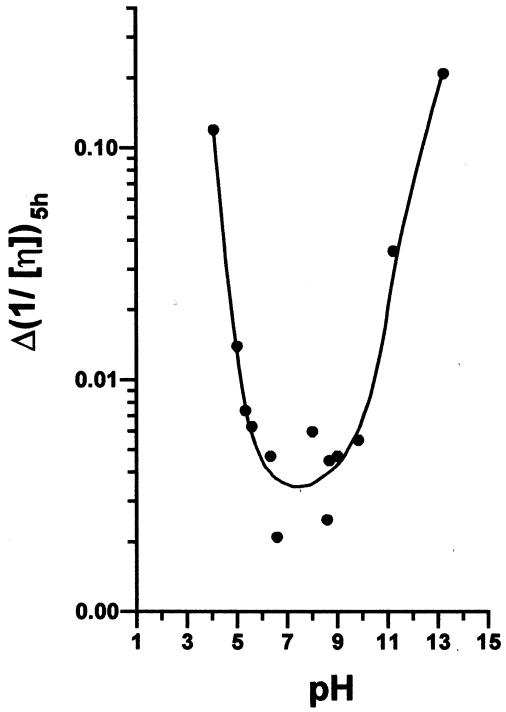

Figure 1.3 : Reported degradation of alginate isolated form *Laminaria digitata* measured as the change in intrinsic viscosity after 5h at different pH and at 68°C (Draget et al., 1997)

The coating of Ca-alginate beads after gellification with an additional layer of polycation such as poly-L-lysine (PLL), poly-L-ornithine (PLO) or chitosan is usually used to increase the mechanical stability and to induce further restriction in microcapsule permeability. This new positively charged membrane can however provoke immunogenic reactions and it is therefore necessary to block the positive charges with an additional alginate coating (Santos et al., 2010).

1.3.1.2. Alginate based microcapsules

After the formation of an ionically cross-linked alginate core, the beads are usually coated with a polycation layer in order to increase mechanical stability, to induce further permeability restrictions and to stabilize the gel against osmotic swelling. This semi- permeable membrane will allow the bi-directional diffusion of nutrients, oxygen and metabolic waste products and protect the cells from mechanical stress and the host immune system in case of microcapsule transplantation (Huang et al., 2012). Alginate and polycations form complexes due to electrostatic forces between opposite charges (Santos et al., 2010; Strand et al., 2004; B. Thu et al., 1996). The new positively charged membrane was however, reported to have immunogenic effects due to positive charges; it is then necessary to block the positive charges by an additional alginate coating. It has been reported that the surface of the microcapsule is formed from a single mixed layer formed by a complex of polycation and alginate as the polycation penetrates the alginate core to create this overlapped membrane of polyelectrolyte and alginate (Santos et al., 2010; Strand et al., 2003). The choice of polycation is a key issue in alginate-polycation microcapsule formation. Different polycations exist such as Poly-L-lysine, Poly-L-ornithine, chitosan, oligo-chitosan, lactose modified chitosan; however this thesis will focus only on Poly-L-lysine and Poly-L-ornithine..

1.3.1.2.1. Alginate-poly-l-lysine-alginate microcapsules

The complexation between polyanionic alginate and polycationic poly-L-lysine (PLL) has been the first process utilized for cell microencapsulation and has been widely used and studied for encapsulating a variety of cell phenotypes, especially pancreatic islets for diabetes treatment (Lim, 1982; Lim and Sun, 1980; Schneider et al., 2001; Beate Thu et al., 1996; B. Thu et al., 1996; Uludag et al., 2000a). Poly-L-lysine is a synthetic polymer made of a repetition of molecules of the amino acid lysine. Poly-L-lysine is positively charged at physiological pH and therefore able to bind to alginate, as the isoelectric point is 9.6 (Figure 1.3).

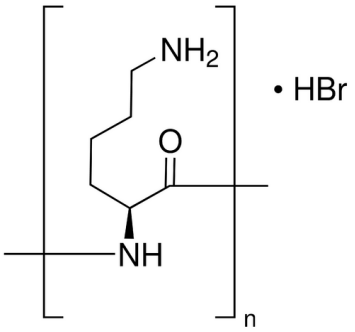

Figure 1.4 : Poly-L-lysine hydrobromide structure

As reported by Uludag *et al*. (Uludag et al., 2000a), alginate-PLL microcapsules provide an interesting perspective into the development of encapsulated cells since :

1. A large volumes of published data from independent laboratories is accessible
2. Alginate-PLL was the first system developed to explore the challenges of encapsulation technology
3. Most findings with this system are relevant to encapsulation systems pursued for other applications.

The common method was initially developed by Lim and improved by Sun to encapsulate pancreatic islets (Lim, 1983, 1982; Lim and Sun, 1980). The technique is based on the immobilization of cells in alginate beads, which are then washed and transferred to a precipitation bath containing a PLL solution. Due to the negative charges of the alginate, the membrane is formed with the positively charged PLL that binds to the alginate beads. An additional coating with a low concentration alginate solution is undertaken to counterbalance the remaining positive charges of the PLL at the surface in order to avoid cell or protein binding at the surface of the capsule. The inner alginate core of the microcapsules can then be dissolved with a chelating agent such as EDTA or sodium citrate, to obtain a liquid core capsules (Huebner and Buccholz, 2002; Lim and Sun, 1980; Beate Thu et al., 1996; B. Thu et al., 1996; Uludag et al., 2000a).

The stability of the PLL layer is mainly governed by the composition of the alginate since high M alginate ensures a more rapid binding of the PLL during the coating step and a much thicker PLL layer (Huebner and Buccholz, 2002). Since the reaction between alginate and PLL is very fast, the diffusion of PLL into the alginate gel is the rate-limiting step of the reaction. The porosity of the gel and the relative molecular weight of the polycation determines the free diffusion of PLL (Huebner and Buccholz,

2002). Different parameters are known to influence the alginate-PLL membrane, a decrease in alginate concentration, increase of the incubation time and concentration of PLL will lead to an increase in the membrane thickness, while an increase of pH and molecular weight of PLL will lead to a decrease of the membrane thickness (Gugerli, 2003). The PLL layer can then be improved through an increased concentration of PLL and by using a lower molecular weight PLL. A certain minimum polymer length is however necessary to obtain stable capsules (Huebner and Buccholz, 2002). The strength of the membrane is related to its thickness, however thick membranes are more fragile. Thinner membranes lead to more elastic but weaker capsules.

Because only a small fraction of the core-alginate can diffuse through the membrane, the capsules are predisposed to osmotic swelling under physiological conditions when the cross-linking divalent cations of the beads are exchanged with monovalent cations (Huebner and Buccholz, 2002). In order to reduce capsule swelling to improve mechanical stability of microcapsules made with alginate-PLL complex, the use of intermediate to high G content alginate is preferred, as the binding with calcium occurs on the G block of the alginate (Santos et al., 2010). It is however known that PLL binds better with high M content alginate, a compromise between the amount of G blocks and the amount of M blocks is important in order to get good capsule stability without prejudicing membrane thickness. Strengthening the PLL layer by allowing more polycations to bind to the capsule surface, exchanging a fraction of the calcium ions with barium ions with a higher affinity toward alginate, alternating PLL-alginate layer or decreasing the capsule diameter have also been investigated in order to improve capsule stability (Huebner and Buccholz, 2002).

Alginate-PLL microcapsules were mainly investigated for transplantation purposes, however reports were made about the possibility to use them to produce recombinant proteins (Breguet et al., 2007; Bugarski et al., 1992; Gugerli, 2003). Breguet et al. (2007) investigated alginate-PLL microcapsule stability in culture conditions and showed the benefits and limitations of this method. They reported that microcapsules proved to be unsuitable for long-term bioreactor culture due to the alginate entrapped into the microcapsule core and a destabilisation of the polyelectrolyte membrane. They also reported that a 3-fold increase of specific productivity of the target product was observed in microencapsulated culture (Breguet et al., 2007). Due to the severe

limitations of alginate-PLL in culture conditions, it was suggested by Breguet et al. (2007) to replace the alginate-PLL system by polyelectrolytes with higher affinities for one another.

1.3.1.2.2. Alginate-poly-L-ornithine-alginate microcapsules

Alginate-PLL microcapsules have been widely studied in cell microencapsulation, however the fragility and low resistance against swelling make them difficult to handle. This lack of stability of alginate-PLL microcapsules has stimulated the study of novel polycations such as poly-L-ornithine.

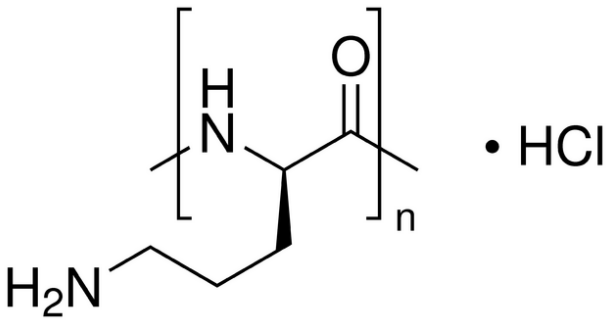

Figure 1.5 : Poly-L-ornithine hydrochloride structure

Like poly-L-lysine, poly-L-ornithine (PLO) is a synthetic polymer. The amino acid comprising PLO presents one methyl group less in the backbone than PLL (Figure 1.4), resulting in shorter monomers. It is suggested that this could possibly permit a more efficient binding to alginate and enable coatings of increased thickness and strength, with a tighter permeability and apparently more stable complex than in the case of PLL in alginate-PLL capsules (Lacík, 2004; Santos et al., 2010).

Poly-L-ornithine is reported to bind mainly to high-M alginates (Santos et al., 2010) and to produce capsules with reduced swelling (Darrabie et al., 2005; Hillberg et al., 2013). It is however, still unclear whether PLO coatings can improve capsule strength and capsule biocompatibility. De Castro *et al.* have reported that no important differences were detected between the classical PLL microcapsule and the PLO microcapsule while Ponce *et al*. (2006) concluded that PLL coatings resulted in more stable and less immunogenic beads than PLO coated beads (De Castro et al., 2005; Ponce et al., 2006). Darrabie *et al*. (2005) however, reported that alginate beads coated with a PLO membrane had a reduced pore size compared to beads coated with PLL in addition, the PLO coating nearly eliminated bead swelling while significantly increasing the

mechanical strength of the alginate microcapsules (Darrabie et al., 2005). They also suggested that the reduction in bead swelling could result in lower shear stress thereby increasing the biocompatibility and the cell viability (Darrabie et al., 2005). This difference in stability between PLL and PLO can be explained, according to Darrabie *et al. (2005),* by the shorter structure of PLO compared to PLL, allowing PLO to bind more efficiently to the alginate membrane, increasing the membrane thickness and strength. Darrabie *et al.* (2005) reported that the immunological effects of PLO on cell viability are still unclear, however they suggest that it may be a better alternative to PLL-coated microcapsules (Darrabie et al., 2005). The study of Tam *et al*. (2011) confirmed the suggestion made by Darrabie *et al.* (2005) that using PLO instead of PLL results in less immune cell adhesion after 2 days in mice, moreover PLO microcapsules were also characterized by a greater hydrophilicity and superior resistance to swelling and damage under osmotic stress (Tam et al., 2011).

Thanos et al. (2006) investigated the stability of alginate-PLO microcapsules when implanted in different transplant sites. They showed that identifying appropriate and stable transplant sites was critical since the stability of the microcapsules varies according to host environment (Thanos et al., 2007). Luca et al. (2001) and Opara et al (2010) both described the possibility to encapsulate cells into alginate-PLO microcapsules for transplant purposes. Luca et al. (2001) showed that Sertoli cells could be successfully co-encapsulated with rat islets within alginate-PLO microcapsules, while Opara et al. (2010) showed that islets could be co-encapsulated with angiogenic proteins in multilayer alginate-PLO microcapsules to be transplanted in an omentum pouch. These capsules were reported to remain stable for up to 30 days (Opara et al., 2010).

1.3.2. Microencapsulation by chemically cross-linked polymers

The stability of alginate-based microcapsules is dependent on the ionic links between calcium ions and alginate and on the polyelectrolyte complex force between alginate and the polycations. As calcium ions in the core are exchanged with non-gelling ions in physiological and culture media, the microcapsules can be destabilized due to the resulting osmotic swelling. Improving the capsule stability is essential for applications

involving cell culture. This can be done by covalent stabilization of the alginate-based microcapsules with different possible cross-linkers.

1.3.2.1. PGA

Propylene glycol alginate or PGA is a derivative of alginate produced by esterification with propylene oxide. A method to produce stable membranes involving covalent bonds around alginate beads was developed by Levy and Edwards-Levy (1996). The method involves the production of beads made of alginate, PGA and protein such as BSA or HSA by drop-wise addition of the mixture into a $CaCl_2$ solution. A membrane is then formed around the beads using a transacylation reaction between the ester functions of the PGA and the amino groups of the protein. The transacylation reaction starts by alkalization of the bead suspension and results in the formation of a membrane made of protein linked to alginate by amide bonds (Hurteaux et al., 2005; Levy and Edwards-Levy, 1996). It has been reported that the membrane made of covalently linked polymers is stable in various conditions and mechanically resistant, however as protein is involved in the membrane the membrane can be degraded by proteases (Hurteaux et al., 2005; Levy and Edwards-Levy, 1996). Different studies such as the one of Breguet *et al*. (2005) showed that PGA-microcapsules can be used in cell microencapsulation with no negative impact of the transacylation process on the cells (Breguet et al., 2005).

The mechanical properties and stability of this type of microcapsules was investigated by Edward-Lévy's research group together with the microcapsule resistance to steam sterilization and lyophilisation (Callewaert et al., 2007; Edwards-Lévy and Lévy, 1999) and the possibility to encapsulate peptides (Callewaert et al., 2009; Hurteaux et al., 2005). There is very few reports in the literature which mention the possibility to encapsulate cells in such microcapsules; Breguet et al., (2005) showed that by combining the PGA-system with a synthetic polymer such as PLL, cells could be cultivated for 1 month in repeated batch mode, the initial mechanical resistance of this new type of capsule being 5-fold higher than of conventional PLL-alginate microcapsules. This result was then further confirmed (Marison et al., 2004). Junkat cells were also successfully cultured in flask by Munin et al. (2006) in PGA-containing microcapsules.

1.3.2.2. Genipin

Genipin (Figure 1.5) is a molecule derived from geniposide that is extracted from the fruit of *Genipa americana* and *Gardenia jasminoides*. The fruit of Gardenia containing geniposide was used in oriental traditional medicine for the treatment of inflammation, jaundice, headache, fever, hepatic disorders and hypertension, since geniposide present in the fruit is enzymatically hydrolyzed to genipin by intestinal bacteria when administered orally, with genipin being the compound that exhibits pharmacological activities (Butler et al., 2003; Muzzarelli, 2009).

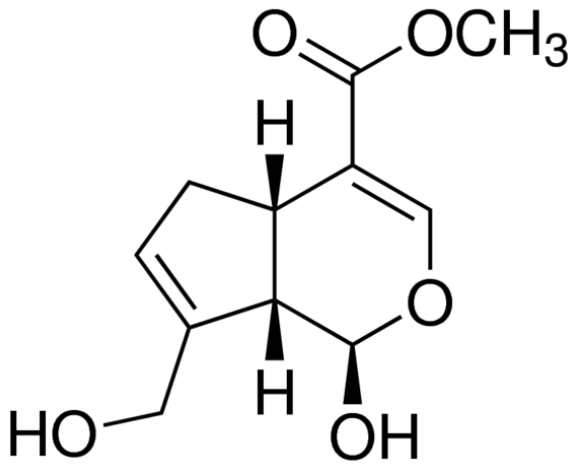

Figure 1.6 : Chemical formula for genipin

Genipin is difficult to extract from Gardenia fruit directly, moreover its concentration is rather low, between 0.005-0.01% (Butler et al., 2003; Muzzarelli, 2009). Since geniposide is present in the range of 3.06-4.12%, the common manufacturing procedure is to isolate genipin from Gardenia fruit through a microbiological process involving *Penicillium nigricans* that produces ß-glucosidase that hydrolyses the geniposide into the aglycone genipin (Butler et al., 2003; Muzzarelli, 2009).

Due to the capacity to covalently cross-link molecules containing residues with primary amine groups and the low cytotoxicity compared to other cross-linkers such as glutaraldehyde, genipin has been used to substitute chemical cross-linkers to crosslink biomaterials (Butler et al., 2003; Chen et al., 2006; Hillberg et al., 2013; Muzzarelli, 2009).

Different studies have been made which investigate the possibility to use genipin in microencapsulation to cross-link microspheres made of chitosan, gelatin or alginate-

polyelectrolytes. Chitosan microsphere cross-linking was investigated by Yuan *et al*. (2007) and they showed that the degree of cross-linking of the microsphere increased with cross-linking time or genipin concentration while the swelling ratio decreased with increasing cross-linking time or genipin concentration (Yuan et al., 2007). Chen *et al*. (2006) investigated the possibility to use alginate-chitosan microcapsules cross-linked with genipin to produce capsules for cell encapsulation and other delivery applications. They showed that the cross-linking conditions have an influence on the cross-linked membrane, especially the temperature of the reaction and the reaction time whereas the concentration had little impact. They also reported that the cross-linking of genipin with the primary amide generates fluorescence allowing an easy evaluation of the cross-linking reaction and the membrane distribution in the microcapsules (Chen et al., 2006). Hillberg *et al*. (2013) investigated the possibility to improve PLO-alginate microcapsule biocompatibility and to increase, by cross-linking, the PLO layer using genipin (Hillberg et al., 2013). His research confirmed the possiblity to grow islets within PLO-microcapsules cross-linked with genipin over a month and demonstrated an improvement in microcapsule biocompatibility when cultured in cell culture media (Hillberg et al., 2013).

Paul et al. (2011) showed that the membrane of alginate-chitosan microcapsules cross-linked with genipin offers immune protection to the entrapped cells, the growth rate of the ASC cells being similar to that in alginate-PLL microcapsules. Genipin-containing microcapsules also showed an improvement in stability compared to alginate-PLL microcapsules when cultured in cell culture media (Paul et al., 2011).

1.3.3. Available polymer reviews

Alginate-PLL microcapsules have been widely characterized for both cell implants and recombinant protein production. Alginate-PLO microcapsules and genipin-containing microcapsules however, have been mainly characterised for cell transplantation (Darrabie et al., 2005; De Castro et al., 2005; Luca et al., 2001; Thanos et al., 2007) and there are no known reports that have shown the effect of bioreactor shear stress and the long- term effects of medium changes on microcapsules made of this polymers. When microcapsules are intended to be used in bioreactor cultures, they must be capable of long-term stability in cell culture media or in physiological environments particularly since these media are usually rich in ions, which can destabilize the

integrity of the alginate gels or the polyelectrolyte interactions involved in the microcapsules integrity (Breguet et al., 2007; Gugerli, 2003). Some studies suggested that poly-L-ornithine (PLO) may have some advantages for microencapsulation such as a reduction of swelling and an increase in mechanical strength compared to the traditional use of poly-L-lysine (PLL) for coating alginate microcapsules (Darrabie et al., 2005), although other studies reported that replacing PLL by PLO did not result in a significant improvement (De Castro et al., 2005). Genipin-containing microcapsules were reported to improve microcapsule stability and biocompatibility when genipin is used to cross-link microspheres made of chitosan, gelatin or poly-L-ornithine. No studies have been found which describe stabilising alginate-PLL microcapsules using genipin, despite the fact that alginate-PLL microcapsules are the most widely characterised microcapsules for both cell implants and recombinant protein production. PGA-containing microcapsules showed promising results in Breguet et al. (2005), Marison et al. (2004) and Munin et al. (2006) however, there are very few other reports of this type of capsule since. Moreover, as with the other types of capsules, there is a lack of data concerning stability under culture conditions, the effect of long-term medium change and long-term culture stability of PGA-containing microcapsules. Many procedures have been developed to produce microcapsules however, most are descriptions of the production process and lack details of characterization of the microcapsule properties. As a result there is no consensus concerning the properties of microcapsules and many results may prove difficult to reproduce.

1.4. Cell microencapsulation methods

Successful application and performance of microcapsules for cell encapsulation requires a methodology capable of producing mono-dispersed, homogeneous and spherical beads of small size and narrow size distribution under mild conditions with no negative effect on cell viability. Moreover, the method must be relatively easy to set-up and simple to operate, with a high efficiency and a high production rate, the apparatus must have the possibility to be sterilized and the production time must be short such as not to harm the cells.

Techniques such as dripping and jet break-up reach all or most of the criteria required for an application in medical and biotechnological processes (Whelehan and Marison,

2011a, 2011b) and are therefore the main methods used to encapsulate cells. Bacteria, yeast, plant cells or mammalian cells have been widely encapsulated for different applications (Ahmed and Youssef, 2012; Bučko et al., 2005; Nedović et al., 2001; Xie and Wang, 2007) using dripping and jet break-up methods. Several dripping and jet break-up methods can be described, however, the main focus will be on the vibrating jet technology as it will be used to produce the microcapsules for this work.

The vibrating-jet technique, also called vibrating nozzle or prilling (Del Gaudio et al., 2005; Whelehan and Marison, 2011a) is one of the most widely used techniques to produce microspheres (Senuma et al., 2000; Whelehan and Marison, 2011a). The technology is based on the principle that the application of a vibration frequency with defined amplitude to the extruded laminar jet will break it into equally sized droplets (Heinzen et al., 2004, 2002; Whelehan and Marison, 2011a). Different methods can be use to apply the sinusoidal force such as vibrating the nozzle (vibrating nozzle technique), periodic changes of the nozzle/orifice diameter during extrusion or pulsating the polymer in a chamber before passing through the nozzle (vibrating chamber technique) (Heinzen et al., 2004; Stark, 2001; Whelehan and Marison, 2011a; Wyss-Peters, 2005). The choice of the technique applying the vibrational force is dependent on the system it is being applied to however, these different methods to produce a sinusoidal force to the laminar jet will be termed as "vibrating-jet techniques" in this chapter (Heinzen et al., 2004; Whelehan and Marison, 2011a).

The nozzle diameter, the polymer flow rate and the vibrating frequency are the main parameters influencing microcapsule diameter. When droplets are formed by the break-up of a jet by the vibration method, they are often dispersed by a system based on electrostatic repulsion forces (Brandenberger et al., 1999; Heinzen et al., 2004). As reported by Brandenberger *et al.* (1999), viscoelastic fluid jets break-up into monodisperse droplets however, coalescence between droplets occurs due to irregularities in the filaments contraction after break-up, resulting in beads of double or triple volume (Brandenberger et al., 1999). Having the droplets fall through an electrostatic field after the break-up will charge them causing repoulsion, therefore the droplets do not hit each other during the flight and are distributed over a large surface of the gelation bath, thereby resulting in monodisperse beads (Brandenberger et al., 1999; Heinzen et al., 2004). The electrode potential is, according to

Brandenberger *et al.,* in the range of 400-1500V depending on the droplet diameter, the jet velocity and the geometrical set-up (Brandenberger et al., 1999). Figure 1.7 describes an encapsulation device based on the vibrating jet technology.

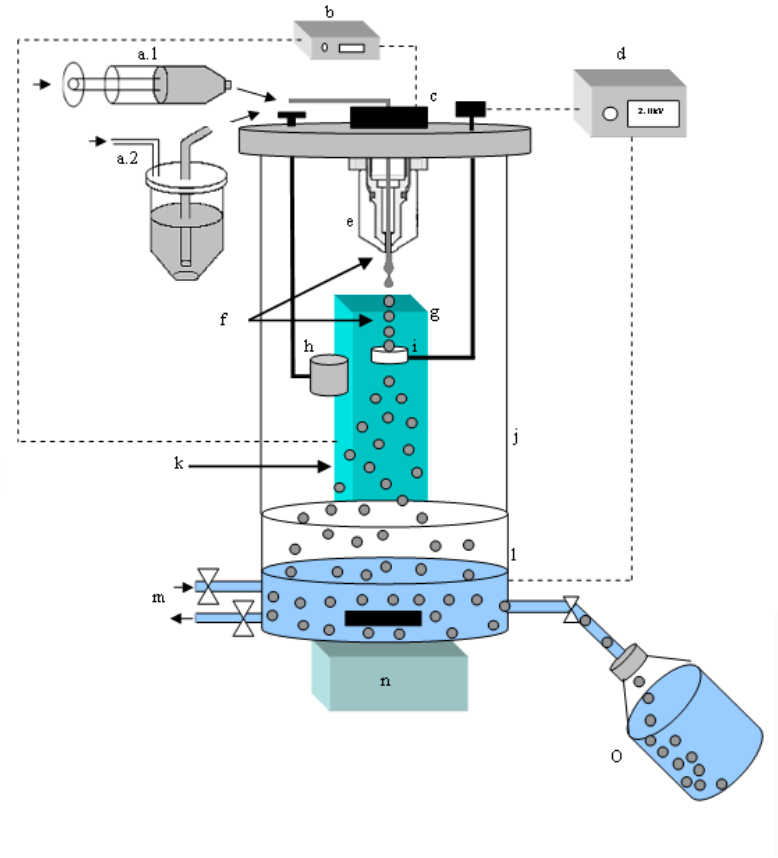

Figure 1.7 Representation of an encapsulation device based on the laminar jet break-up principle by vibrational frequencies with: (a) the product delivery mechanism which can be either a syringe pump (1) or a pressure regulation system (2), (b) a vibrational frequency control system, (c) a vibrating (pulsating) chamber, (d) an electrostatic charge generator, (e) a vibrating (single) nozzle, (f) a break-up of the liquid (optimal break-up of extruded jet resulting in a single bead chain), (g) a stroboscopic light, (h) a bypass system, (i) a electrode, (j) a reaction vessel casing, (k) dispersed droplets due to the negative charge applied, (l) a gelling bath, (m) the removal of gelling material for continuous operation, (n) a magnetic stirrer and (o) the product collector. Reproduced from (Whelehan and Marison, 2011a) with permission

As the maximum flow rate per nozzle is limited in vibration technology, scale-up has to be done by increasing the number of nozzles (Figure 1.8). It has however, to be taking into account that each nozzle of the multinozzle unit must show similar production conditions, meaning that the flow rate, the frequency and the amplitude must be identical for each nozzle. Brandenberger and Widmer (Brandenberger and Widmer, 1998) increased microsphere output by adding more nozzles to the nozzle plate of their encapsulator and obtained a vibrating-jet monocentric multi-nozzle device (Brandenberger and Widmer, 1998; Whelehan and Marison, 2011a). Brandenberger and Widmer overcame the problem of keeping the flow rate constant of the multi-nozzle system by pumping the polymer through a concentric split, in this way they

were able to obtain a relative flow difference of less than 2.0% between all the nozzles of the device (Brandenberger and Widmer, 1998; Whelehan and Marison, 2011a). The disturbance resulting in the jet break-up is transmitted in a vibrating chamber through which the polymer is flowing. Indeed, subjecting all liquid jets with the same sinusoidal force by vibrating each nozzle would be challenging from an engineering point of view. This method of creating the perturbation by a pulsating chamber allows a good size distribution of the droplets and any difference in droplets size is due to small difference in the diameters of the nozzles (Brandenberger and Widmer, 1998; Whelehan and Marison, 2011a).

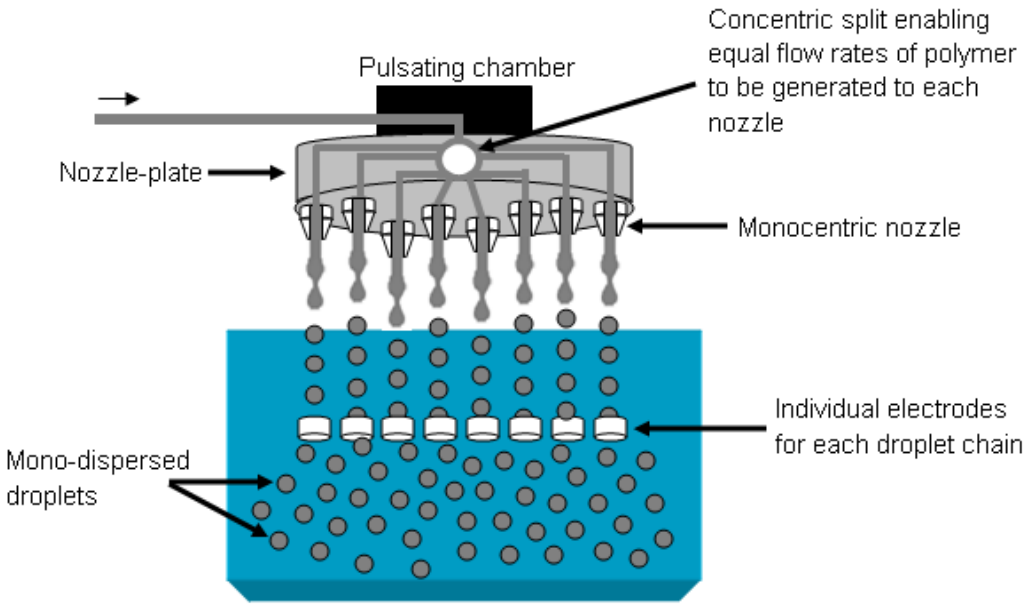

Figure 1.8 Multinozzle device such as that supplied by EncapBioSystems. Reproduced from (Whelehan and Marison, 2011a) with permission

More information about dripping and jet break up techniques can be found in Demont A. and Marison I.W., 2015, Microencapsulation by Dripping and Jet Break-Up in: Munmaya Mishra (ed), Handbook of Encapsulation and Controlled Release, CRC Press Taylor and Francis Group, Bocca Raton, NY

1.5. Structure of the thesis

1.5.1. Objectives of the thesis

Microencapsulation of mammalian cells has been used for many years and is well documented in the cell transplantation field however, a lack of information can be observed concerning microencapsulation for high cell density culture for large-scale production of cell-derived molecules in biotechnology industry. Many procedures have been developed to produce microcapsules however, most are descriptions of the production process and lack details of characterization of the microcapsules properties. As a result there is no consensus in the properties of the different microcapsules and many results have proven difficult to reproduce and compare. This shows the importance of quantitative microcapsule characterisation (de Vos et al., 2009; Tam et al., 2011).

The aim of this thesis was first to set-up a characterization methods toolbox to standardize the characterization of microcapsule key parameters. The production of stable polyelectrolyte microcapsules for cell encapsulation and high cell density cultures was then investigated using different polyelectrolytes (poly-l-ornithine and poly-l-lysine) to understand the potential and limitations of this system to grow adherent and suspension mammalian cells in bioreactors. Finally, the last goal was to investigate whether a solution or alternative could be found to overcome polyelectrolyte microcapsule limitations to enable the production of robust microcapsules for high cell density perfusion cultures in bioreactors.

1.5.2. Outline of the thesis

This thesis contains 7 chapters and has been structured in the following way:

- **Chapter 1 – Introduction:**

  A brief outline of the thesis aims and focus is described in the present chapter. Additionally a general overview about cell microencapsulation, the potential, promise and challenges as well as the main requirements and the microencapsulation techniques for cell encapsulation are also being presented in this first chapter.
- **Chapter 2 – Material and methods : Microcapsule characterizations toolbox**:

The aim of this chapter is to regroup the different methods used in the work and to obtain a good overview of the capsule key parameters characterization techniques by creating a toolbox that standardizes the main methods to allow a direct and quantitative comparison of the experiments.

- **Chapter 3 - Characterisation and stabilisation of alginate-PLL microcapsules**

  The objective of this study is to study the widely used alginate poly-L-lysine microcapsules as a reference for cell encapsulated cultures. The capsules are characterised with the methods developed in Chapter 3 under batch and perfusion culture conditions to avoid lab-to-lab variation and allow reproducibility.

- **Chapter 4 - Development, characterization of alginate-PLO microcapsules for cell culture proposes**

  The aim of this chapter was to investigate the possibility to produce microcapsules suitable for perfusion culture using PLO to coat alginate beads and to compare them with alginate PLL microcapsules. The capsules are characterised with the methods developed in chapter 3 under batch and perfusion culture conditions to avoid lab-to-lab variation and allow reproducibility.

- **Chapter 5 – Stabilisation of polyelectrolyte microcapsules using different cross-linking methods**

  The present investigation aims to investigate the possibility to stabilise alginate-PLL microcapsules using either the crosslinking reaction with genipin or the transacylation reaction with the PGA-containing system to see whether the produced microcapsules are suitable for perfusion cultures in bioreactors from a stability and a cell perspective. The capsules are characterised with the methods developed in chapter 3 under batch and perfusion culture conditions to avoid lab-to-lab variation and allow reproducibility

- **Chapter 6 – An understanding of potential and limitations of alginate-based microcapsules to culture anchorage dependent cells**:

  In this work, the possibility of growing adherent cells in alginate based microcapsules is investigated and a comparison between encapsulated cultures and non-encapsulated cultures to grow adherent cells is undertaken.

- **Chapter 7 - Conclusion:**

  This chapter reviews the conclusions from chapters 3-6 and gives an outline of additional applications of the present results. Potential problems and solutions are discussed before an overall conclusion of the work is made.

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2. Materials and methods

Abstract

In order to compare different microencapsulation techniques and polymers, and the evolution of microcapsules over the duration of a culture, different parameters have to be compared and characterised. The purpose of this chapter is to define those parameters, to develop or adapt existing characterization methods and to combine them to create a characterization method toolbox that can be used to standardize the characterisation of microcapsules. This chapter is therefore divided in two parts, the first part aims to describe the method in order to have a usable “toolbox” while the second part shows in detail the development and optimisation of the molecular weight cut- off and mechanical resistance determination methods.

2.1. Introduction

In order to compare different microencapsulation techniques and polymers, and the evolution of microcapsules over the duration of a culture, different parameters of the microcapsules have to be analysed. The most important of these parameters can be divided into different subclasses including size and size distribution, transport properties (i.e. MWCO), mechanical resistance, stability cell viability and cell dispersion in the capsules (Rosiński et al., 2005). Unfortunately, there is a lack of adequate and complete documentation for the quantitative characterisation of microcapsules. This chapter presents an overview of the techniques for the quantitative characterization of the key parameters by creating a toolbox that standardizes the main characterization methods to enable a direct comparison between different experiments with different polymeric system, to reduce lab-to-lab variation thereby allowing high reproducibility and comparison of results produced by different groups. Particular attention is addressed to the molecular weight cut- off and mechanical resistance since they are key parameters for successful application of microcapsules.

2.2. Part I : Methods description

2.2.1. Encapsulation methods

2.2.1.1. Polyelectrolyte microcapsule formation

The encapsulation method used was a modification of the technique originally developed by Lim and Sun (Lim, 1983, 1982; Lim and Sun, 1980) undertaken under completely sterile conditions using a vibrating nozzle encapsulation device (Encapsulator Biotech, EncapsBioSystem Inc, Greifensee, Switzerland or Inotech IE-50R, Inotech, Greifensee, Switzerland) as described elsewhere (Serp et al., 2000). Alginate (Kelton LV, FMC biopolymers, UK; Protanal CR, FMC biopolymers, UK; Manucol DH, FMC biopolymers, UK; Büchi Alginate, Büchi, Switzerland) beads were first formed by extrusion of 300mL pre-sterilised (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland) sodium alginate solution (1.5% w/v) through the encapsulator nozzle into an aqueous solution of $CaCl_2$ 110mM (Sigma, St-Louis, USA). After 5 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules incubated in the polyelectrolyte solution (0.05% w/v poly-L-lysine 30-

70kDa or 0.15%w/v poly-L-ornithine (Sigma, Saint Louis, Missouri, USA)) for 30 min at room temperature under agitation. The microcapsules were then washed with saline buffer followed by incubation in 1L 0.03% sodium alginate for 10min under gentle agitation. After washing with saline buffer, the solid alginate core of the microcapsules was liquefied by incubation for 5min in 0.05M sodium citrate (Sigma, Saint Louis, Missouri, USA). After core liquefaction with citrate, the microcapsules were washed with saline buffer followed by a wash with cell culture medium before being transferred to cell culture medium to be inoculated. When cells were encapsulated, the cells ($0.7 \cdot 10^6$ $_v$cells/$mL_{alginate}$) were mixed with the 1.5% alginate solution and the bead formation was made according to the method above.

2.2.1.2. Genipin microcapsules formation

Alginate (Manucol DH, FMC biopolymers, UK) beads were first formed by extrusion of 100mL pre-sterilised (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland) sodium alginate solution (1.5% w/v) through the encapsulator nozzle into an aqueous solution of $CaCl_2$ 110mM (Sigma, St-Louis, USA). After 5 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules incubated in the polyelectrolyte solution (0.05% w/v poly-L-lysine 30-70kDa (Sigma, Saint Louis, Missouri, USA)) for 30 min at room temperature under agitation. The microcapsules were then washed with saline buffer followed by incubation in 3L 0.03% sodium alginate for 10min under gentle agitation. After washing with MOPS buffer solution (8.5g/L NaCl, 2.09g/L MOPS, pH7), the microcapsules were incubated in 300mL genipin solution (1mg/mL genipin in MOPS buffer solution) for 0 to 6 hours at 37°C under agitation. The solid alginate core of the microcapsules was then liquefied by incubation for 5min in 0.05M sodium citrate (Sigma, Saint Louis, Missouri, USA). After core liquefaction with citrate, the microcapsules were washed with MOPS buffer twice followed by a wash with cell culture medium before being transferred to cell culture medium to be inoculated. When cells were encapsulated, the cells ($0.7 \cdot 10^6$ $_v$cells/$mL_{alginate}$) were homogenised in the alginate solution and the bead formation was made according to the method above.

2.2.1.3. PGA microcapsules formation

Propylene-glycol alginate (PGA) (Kelcoloid S, FMC biopolymers, UK) was dissolved in 20mM MOPS buffer (4.18g/L MOPS, 8.5g/L NaCl, pH7) to make a 5% stock solution.

The pH of the solution was constantly adjusted to pH7 as it tends to drop below 4 during PGA dissolution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland). This solution must be prepared fresh, as PGA is not stable in aqueous solution above pH 4.

Sodium alginate (Manucol DH, FMC biopolymers, UK) was dissolved in 10mM MOPS buffer (2.09g/L MOPS, 8.5g/L NaCl, pH7) to make a 2% stock solution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland).

BSA (Sigma, Saint Louis, Missouri, USA) was dissolved in 10mM MOPS buffer (2.09g/L MOPS, 8.5g/L NaCl, pH7) to make a 40% stock solution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland).

PEG 6000 (Sigma, Saint Louis, Missouri, USA) was dissolved in 10mM MOPS buffer (2.09g/L MOPS, 8.5g/L NaCl, pH7) to make a 50% stock solution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland).

Beads were first formed by extrusion of a solution composed of 1% alginate, 1,8% PGA, 4% BSA, 1% PEG through the encapsulator nozzle (300µm) into a 4% BSA (Sigma, Saint Louis, Missouri, USA), 5% $CaCl_2$ dihydrate (Sigma, Saint Louis, Missouri, USA), 0.2% Tween 20 (Sigma, Saint Louis, Missouri, USA) aqueous solution. After 10 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules incubated in the polyelectrolyte solution (0.05% w/v poly-L-lysine 30-70kDa (Sigma, Saint Louis, Missouri, USA) 4% BSA (Sigma, Saint Louis, Missouri, USA), 5% $CaCl_2$ dihydrate (Sigma, Saint Louis, Missouri, USA)) for 30 min at room temperature under agitation. The microcapsules were then transacylated by raising the pH to 11 by addition of 2M NaOH. The reaction was stopped after 10 seconds by neutralization with 2M MOPS, the beads were then agitated for 10min before being washed with 10mM MOPS buffer. The solid core of the microcapsules was then liquefied by incubation for 5min in 0.05M sodium citrate (Sigma, Saint Louis, Missouri, USA). After core liquefaction with citrate, the microcapsules were washed with MOPS buffer twice followed by a wash with cell culture medium before being transferred to

cell culture medium. When cells were encapsulated, the cells ($0.7 \cdot 10^6$ $_v$cells/$mL_{alginate}$) were mixed with the solution containing alginate, BSA and PEG and the bead formation made according to the method described above.

2.2.2. Cell culture and culture analysis methods

2.2.2.1. CHO-DP12 cell culture

The cells used were CHO-DP12, a *dhfr*$^-$ cell line that has been adapted to serum-free cell culture media and suspension culture and which have been stably co-transfected with the IgG1-*dhfr*$^-$ gene to produce an IgG1, a 150kDa protein. Cells from a working bank, stored at -196°C, were rapidly thawed at 37°C and used to inoculate a shake flask (125ml, Corning Inc, Corning, NY, USA) containing 10mL of a protein-free culture medium (EX-CELL CHO DHFR$^-$, Sigma Aldrich, Saint Louis, Missouri, USA). After 6 hours incubation at 37°C, under a humidified atmosphere of air containing 5% CO_2, cells were counted using a Neubauer Haemocytometer. A new shake flask (125mL, Corning Inc, Corning, NY, USA) containing 20mL of a protein- free culture media was inoculated to a viable cell density of $0.3 \cdot 10^6$ cells/mL and incubated in a CO_2 incubator (Galaxy 170S, New Brunswick Scientific, Eppendorf, Germany) at 37°C at 100rpm, under a humidified atmosphere of air containing 5% CO_2. The cells were passaged every 3 to 4 days in a new shake flask (125ml, Corning, Inc) containing 25mL of protein- free culture media with an initial viable cell density of $0.3 \cdot 10^6$ cells/mL. The inoculum for the reactor was cultured in a 1L shake flask containing 400mL of protein free culture medium with an initial viable cell density of $0.3 \cdot 10^6$ cell/mL. The cells were then harvested and re-suspended in sodium alginate (1.5%) at a viable cell density of $0.75 \cdot 10^6$ cells/$mL_{alginate}$ prior to being encapsulated.

EX-CELL CHO DHFR$^-$ medium (Sigma Aldrich, Saint Louis, Missouri, USA) was supplemented with 4mM L-glutamine (Sigma Aldrich, Saint Louis, Missouri, USA), 10mL/L penicillin-streptomycin (Life Technologies, Carlsbad, California, USA) and 2.2mL/L Na-phenol red (Sigma Aldrich, Saint Louis, Missouri, USA). For encapsulated perfusion and fed-batch cultures in bioreactors with PLL containing microcapsules, the medium was also supplemented with 1.31mmol/L $CaCl_2$.

2.2.2.2. CHO-K1 cell culture

Cells from a working bank, stored at -196°C, were rapidly thawed at 37°C and used to inoculate a T25 (Corning Inc, Corning, NY, USA) containing 5mL of a protein-free culture medium (Dulbeco's modified Eagle's medium nutient mixture F-12 HAM (DMEM-F12), Sigma Aldrich, Saint Louis, Missouri, USA). After 24 hours incubation at 37°C, under a humidified atmosphere of air containing 5% CO_2, the medium was removed, the layer of cell was rinced with sterile PBS buffer and 5mL of fresh medium was added to the flask. The cells were observed under a microscope to determine confluency and split once they reached 70-80% confluence.

To passage the cells, the medium was gently removed from the T-flask and the cell layer was gently washed with sterile PBS. The PBS is then removed and 2mL of trypsin added in order to detached the cells from the flask. The flask was then incubated for 5min at 37°C. Once the cells were detached, 3mL of media was added to inactivate the trypsin and the cells split 1:10 into a new T25 T-flask (Corning Inc, Corning, NY, USA) and incubated at 37°C, under a humidified atmosphere of air containing 5% CO_2.

The inoculum for the flasks culture was cultured in several T75 (Corning Inc, Corning, NY, USA) containing 15mL of medium. The cells were then harvested according to the same procedure as for passaging the cells before being centrifuged and re-suspended in sodium alginate (1.5%) at a viable cell density of $2 \cdot 10^5$ cells/$mL_{alginate}$ prior to being encapsulated.

DMEM-F12 medium (Sigma Aldrich, Saint Louis, Missouri, USA) was supplemented with 10mL/L penicillin-streptomycin (Life Technologies, Carlsbad, California, USA).

2.2.2.3. Shake flask encapsulated cultures and stability tests

Encapsulated cells were grown in shake flask (Corning Inc, Corning, NY, USA), with a working volume of 100mL and a microcapsule volume representing 25% of the medium volume. The culture was then incubated at 37°C, under a humidified atmosphere of air containing 5% CO_2 and an agitation of 100rpm. For perfusion cultures, the microcapsules were allowed to settle before a known volume of medium was aseptically removed with a sterile pipette and replaced with the same volume of fresh medium. Perfusion was performed once daily.

2.2.2.4. Bioreactor encapsulated cultures

Encapsulated cells were grown in a stirred tank bioreactor (Minifors, Infors, Bottmingen, Switzerland), with a working volume of 1.2L for PLO and PGA microencapsulated cultures and in a Mettler- Toledo RC1 (Mettler-Toledo, Columbus, OH, USA) 2L biocalorimeter, with a working volume of 1.4L for PLL microcapsules. The bioreactor was equipped with a pH and temperature control with air sparging. The bioreactor was operated in perfusion mode at a temperature of 37°C, agitation of 100 rpm with a sparged airflow of 0.01 vvm and a headspace airflow of 0.2 vvm and 0.1 vvm CO_2. The pH was maintained at 7.2 by addition of NaOH and CO_2 to the headspace. For perfusion cultures, the volume was maintained constant by the use of a feed system, which added and removed medium at the same rate. A 200µm nylon mesh was installed on the medium outlet port to avoid capsule removal in the feed outlet. A microcapsule volume representing 25% of the medium volume was used for all bioreactor cultures.

2.2.2.5. Perfusion feeding strategy

Prior to perfusion, sampling of the cultures was performed. Based on the predetermined cellular growth rates, specific metabolite consumption and production rates, the concentration of glucose and glutamine required by the cells to maintain the growth rate was calculated. The required amount of anhydrous glucose (Sigma Aldrich, Saint Louis, Missouri, USA) and L-glutamine (Sigma Aldrich, Saint Louis, Missouri, USA) powder were then weighed and added to fresh medium. The medium was then sterile filtered (0.2µm Steritop filters, Merck Millipore, Ireland) before being added to the culture.

2.2.2.6. Cell viability

The concentration of cells was determined microscopically using the Trypan blue exclusion method to count cells with a Neubauer haemocytometer. Encapsulated cells were liberated by mixing a precise volume of microcapsules with a precise volume of 50mM sodium citrate before extruding them gently through a 27 gauge needle (Becton-Dickinson & Co, New Jersey, USA) for PLO microcapsules and 30 gauge needle (Becton-Dickinson & Co, New Jersey, USA) for alginate microbeads, PLL microbeads, PLL microcapsules and PGA micrcapsules in order to break the capsules and dissociate cell clusters.

In order to be able to visualise the cells within the microcapsules, cells were stained with calcein (viable cells) (Sigma, Saint Louis, Missouri, USA) and propidium iodide (dead cells) (Sigma, Saint Louis, Missouri, USA), and incubated at 37°C for two hours followed by confocal imaging. The microcapsules were analysed with a multiphoton confocal microscope (Zeiss LSM 710 and Confocor 3, Zeiss Microscopy, Jena, Germany).

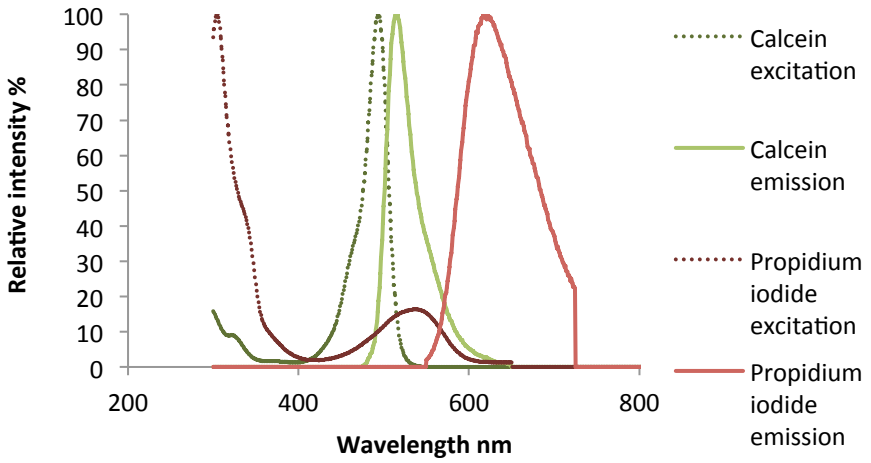

Figure 2.1 : Excitation and emission wavelength of propidium iodide and calcein used for fluorescent cell viability determination with confocal microscopy

The excitation wavelength was set at 488 nm, since this wavelength can excite both dyes, and an emission between 500 and 600 nm for calcein and between 600 and 700nm for propidium iodide (Figure 2.1). The gain was adapted from 500 to 1000 for each emission wavelength; the pinhole was set at 33.5 and the averaging was set at 4. Moreover, in order to perform a z-stack imaging to see the cell distribution within the microcapsules, the top and bottom position of the microcapsules was defined, such that the width of each slice of image taken was known.

2.2.2.7. Metabolites and IgG analysis

Glucose and lactate concentration were determined by HPLC analysis (Agilent Instruments 1200, Agilent Technologies Ltd Cork, Ireland) using a Supelcogel^{TM}C-610H column (Supelco, Bellefonte, Pennsylvannia, USA), H_2SO_4 0.01M as mobile phase at a constant rate of 0.5mL/min for 32min and a RI detector (Agilent Instruments 1200 series, Agilent Technologies Ltd. Cork, Ireland).

L-Glutamine and ammonia were measured enzymatically using a standard method (L-glutamine-Ammonia assay kit, Megazyme, Ireland).

The secreted IgG1 protein was determined by HPLC analysis (Agilent Instruments 1200, Agilent technologies Ltd, Cork, Ireland) at 25°C using a protein A column (POROS Prepacked Protein A Affinity Colum, Life Technologies, Thermo Fisher Scientific, USA). Two mobile phases were used at a constant rate of 2ml/min for 5 min according to the elution program described below (Table 2.1). The recombinant protein was detected using a UV/Vis detector set to 280nm.

Table 2.1 : Elution program for IgG quantification by HPLC

Time	% Mobile phase A	% Mobile phase B
0 - 2.5 minutes	100	0
2.5 – 3.5 minutes	0	100
3.5 – 5 minutes	100	0

Mobile phase A: 20mM sodium phosphate, 500mM NaCl, pH7, Mobile phase B: 50mM sodium phosphate, 500mM NaCl, pH2

The protein A column must be regenerated at the beginning and at the end of the sequence and after every 20 samples. This was done by injecting 3 x 100µl of 2M guanidine HCl (Sigma Aldrich, Saint Louis, Missouri, USA) and eluting using mobile phase B at 2ml/min. The column regeneration was followed by 3 blank analysis consisting of injecting mobile phase A.

2.2.3. Microcapsule characterisation methods

The optimisation and development of the characterisation methods described in this section are fully detailed in the next section: characterisation methods optimisation and development.

2.2.3.1. Microcapsule size and size distribution

The shape and dimension of beads and microcapsules are of great importance as some properties such as permeability, mechanical resistance and chemical stability are correlated with the size and the spherical aspect of the capsules. It is therefore

necessary to have a reliable method to measure the size and to examine the general aspect of the microcapsules.

The general aspect of the microcapsules can be observed using an optical microscope connected to a digital video camera with software for image analysis (Leica application suite v3.4.0, Leica, Switzerland)

The diameters of thirty capsules were measured using a 3-point measurement method (Leica application suite v3.4.0, Leica, Switzerland) in order to assess statistically significant average microcapsule diameter and size dispersity, according to the method described by Breguet et al (2007). The values are given in µm ± the standard error of the mean.

The membrane thickness of PLL and PLO microcapsules was observed using confocal microscopy. The PLL and PLO were labelled by adding 1.2 mg of FITC (Sigma Aldrich, Saint Louis, Missouri, USA) to a 1.2 L water solution containing 600 mg of PLL or PLO brought to pH 8-9 by drop-wise addition of NaOH 2M. The solution was agitated for one hour before the pH was adjusted to 7 by addition of acetic acid, MOPS 10mM and $CaCl_2$ 100mM (Sigma Aldrich, Saint Louis, Missouri, USA) enabling an average label yield of $7.5 \cdot 10^{-4}$ moles FITC/mole of lysine monomer and $6.79 \cdot 10^{-4}$ moles FITC/mole of ornithine monomer (Figure 2.2).

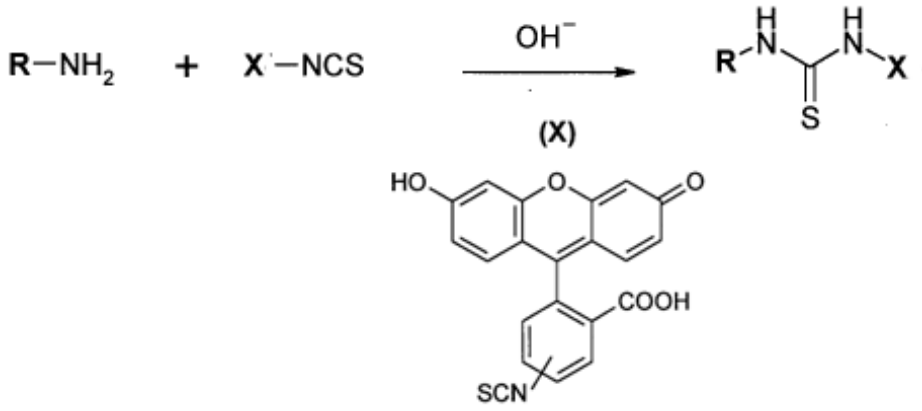

Figure 2.2: Reaction schemes for the labelling of the poly-L-lysine (R-NH_2) with the fluoresceine isothiocyanate X (Lamprecht et al., 2000).

The freshly labelled PLL-FITC and PLO-FITC solutions were then filtered through a sterile 0.22µm Steritop filter (Merk Millipore, Ireland) in order to remove the precipitated unfixed FITC and to sterilise the solution. The microcapsules were analysed using a multiphoton confocal microscope (Zeiss LSM 710 and Confocor 3,

Zeiss Microscopy, Jena, Germany). The excitation wavelength was set at 488nm and the emission wavelength at between 500 and 600 nm. The gain needed to be adapted from 500 to 900 in order to have the maximal intensity without being saturated for the first sample and then kept constant throughout the culture enabling the detection of an intensity decrease in the membrane that would show a leakage in PLL from the membrane. The pinhole was set at 33.5 and the averaging is set to 4. The membrane width was defined as the distance between each side of the peak at 50% of the peak height. A minimum of 10 measurements were performed and the values are given in µm ± the standard error of the mean.

The membrane thickness of PLL-genipin microcapsules was observed usinga multiphoton confocal microscope (Zeiss LSM 710 and Confocor 3, Zeiss Microscopy, Jena, Germany). The excitation wavelength was set at 543nm and the emission wavelength at between 500 and 700 nm. The gain needed to be adapted from 500 to 900 in order to have the maximal intensity without being saturated for the first sample and then kept constant throughout the culture enabling the detection of an intensity decrease in the membrane. The pinhole was set at 33.5 and the averaging is set to 4. The membrane width was defined as the distance between each side of the peak at 50% of the peak height. A minimum of 10 measurements were performed and the values are given in µm ± the standard error of the mean.

2.2.3.2. Mechanical resistance

The mechanical resistance of microcapsules was measured using a texture analyser (TA.XT plus texture analyser, Stable Micro System, UK) which featured a mobile probe driven at a set speed of 0.299mm/s, the area of the probe was $3.17 \cdot 10^7$ µm^2 and the initial distance of the probe from the plate was $4.7 \cdot 10^3$ µm.

Mechanical resistance is defined as the force required for each microcapsule to reach 50% deformation. In order to calculate it, the number of microcapsules under the probe (n_{caps}) has to be calculated (1):

$$\frac{P_a}{C_a} = n_{caps} \quad (1)$$

Where P_a is the probe surface area and C_a is the average microcapsule area at its widest part. Next, the distance the probe has moved to reach 50% deformation ($D_{50\%}$) must be calculated (2):

$$D_{50\%} = D_i - r_c \quad (2)$$

Where D_i is the probe distance from the plate before it starts moving and r_c is the microcapsule radius. When this distance is determined it is possible to calculate the force at the 50% deformation point ($F_{50\%}$). This is done by using the raw data from the texture analyser and locating the $D_{50\%}$ value, $F_{50\%}$ is the force at $D_{50\%}$.

However, this value is the force that is exerted on the total number of microcapsules under the probe. To determine the force per capsule (F_c) the force $F_{50\%}$ has to be divided by the number of microcapsules under the probe n_{caps} (3) :

$$F_c = \frac{F_{50\%}}{n_{caps}} \quad (3)$$

If the microcapsules burst under the probe, the mechanical resistance is defined as the force required for each capsule to burst. In order to calculate it, the distance travelled by the prove when the microcapsules burst (D_b) has to be determined from the raw data and its related force (F_b) will be used. The bursting force is the force required for the total amount of microcapsules under the probe to burst, to find the force per capsule (F_c), the force F_b has to be divided by the number of microcapsules under the probe n_{caps} (4)

$$F_c = \frac{F_b}{n_{caps}} \quad (4)$$

2.2.3.3. Molecular weight cut-off (MWCO)

To 900µl of a solution of different MW 0.05% FITC-labelled-molecule dextrans (10 kDa, 40 kDa, 70 kDa, 150 kDa, 250 kDa, 500 kDa, Sigma Aldrich, Saint Louis, Missouri, USA) and IgG (Sigma Aldrich, Saint Louis, Missouri, USA) were added 300µl of microcapsules. The solution was incubated at room temperature with continuous stirring for 2 hours and a sample taken and analysed by confocal microscopy to confirm the presence or absence of dextran in the microcapsules. The microcapsules were analysed using a multiphoton confocal microscope (Zeiss LSM 710 and Confocor 3, Zeiss Microscopy,

Jena, Germany). The excitation wavelength was set to 488 nm and the emission wavelength to between 500 and 600 nm. The gain was adapted from 500 to 800 between samples in order to get the best possible picture quality, the pinhole was set up to 33.5 and the averaging was set up to a value of 4.

2.3. Part II: Characterisation methods optimisation and development

2.3.1. Molecular weight cut off (MWCO) method development

2.3.1.1. Overview

Bioencapsulation has potential application in many fields, such as biotechnology, pharmacy, food, cosmetics and medicine. Depending on the field of application, the permeability of the capsule membrane is extremely important (Dautzenberg et al., 1999). In the case of bioartificial organs and cell encapsulation, the essential nutrients need to diffuse freely into the microcapsules and toxic metabolites must diffuse freely fro the microcapsules to ensure survival of the cells (Uludag et al., 2000). Moreover the recombinant proteins synthesized by the cells should be able to leave the capsules while antibodies and other components of the immune system should not enter the capsules in the case where microcapsules are used as bioartifical organs (Briščová et al., 1996). In bioprocessing, however, allowing the produced molecules to stay in the capsules or to exodiffuse will make the downstream processing easier. In the literature, different methods are described to measure the permeability of the membrane. The protein standards method measures the diffusion of a known protein or molecule such as BSA or vitamin B12. It is a simple way to obtain a study of the diffusion properties of the capsule membrane. To measure the MWCO, standards of different defined proteins may also be used (Lewińska et al., 2002; Uludag et al., 2000). The dextran or pullulan standard method measures the ability of dextran of pullulan molecules to permeate through the capsule membrane. Different known molecular weight pullulans or dextrans can be mixed with microcapsules. Small molecular mass molecules are able to permeate the microcapsule membrane, consequently the concentration in supernatant decreases, while molecules with a molecular mass higher than the cut-off are not able to diffuse through the membrane and the concentration remains constant. After a pre-determined amount of time, samples of the supernatant are removed and analysed by size exclusion chromatography (Briščová et al., 1998, 1996; Rosiński et al., 2005; Uludag et al., 2000). It is important to consider that dextran and pullulan are neutral polysaccharides and that they therefore interact differently

with the microcapsule membrane compared with proteins. However, the molecular weight is less important than the diffusional Stokes radius (size of particle in solution) to allow or exclude the diffusion of molecules in or out of the microcapsules (Wang et al., 1997).

The protein method and the dextran method are complementary as the dextran and pullulan show the ingress of neutral molecules when the protein method shows the ingress of charged molecules that can interfere with the microcapsule membrane. None of the methods however, allow a rapid and simple visualization of the ingress of molecules into the microcapsules and both methods are dependent on a filtration step that could damage the microcapsules. The method developed here, using labelled dextran and proteins will allow a quick and simple visualization of the ingress of molecules into the capsules using confocal microscopy and a simple quantification by spectrophotometry and confocal microscopy (Chen et al., 2009).

2.3.1.2. Molecular weight cut off determination method

2.3.1.2.1. Method development

The spectrum of absorbance of FITC was measured with a 0.1% FITC Dextran 70kDa solution labelled with 0.003-0.020 moles FITC per mole glucose (Sigma) in PBS buffer to determine if an absorbance can be measured and at which wavelength. In order to determine the volume of solution in each microtitre (96-well plates), 3 standard curves from 0% to 1% were prepared and the absorbance measured with 100µl, 200µl and 300µl of solution in each well. Following these first steps, 3 standards curves from 0% to 1% FITC-Dextran were prepared for each molecular weight of labelled dextran (4kDa, 10kDa, 70kDa, 150kDa, 250kDa, 500kDa) in order to determined the Limit of Detection, the Limit of Quantification and the linear range of the method. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as follows:

$$LOD = \frac{3.3 \cdot \sigma_{intercept}}{\bar{s}} \quad (5)$$

$$LOQ = \frac{10 \cdot \sigma_{intercept}}{\bar{s}} \quad (6)$$

with $\sigma_{intercept}$ the standard deviation of the intercepts (y) and $\bar{s}$ the average of the slopes.

The linearity was first evaluated by visual inspection of the plot of the signal. The linear range was then estimated and the linear relationship was confirmed with two statistical methods:

(1) A regression line was determined by the method of the least squares and the coefficient of determination r^2 was calculated; (2) The linearity was also investigated using an F-test

2.3.1.2.2. Microcapsule preparation

Microcapsules were prepared according to the method described in section 2.2.1.1

2.3.1.2.3. Diffusion kinetics

Microcapsules (1mL) were continuously agitated with 3ml of 0.05% FITC-Dextran. At defined time intervals, a sample of supernatant was taken, the absorbance measured at 450nm and the sample returned to the solution.

2.3.1.2.4. Molecular weight cut-off measurement

To 900µl of a solution of 0.05% FITC-labelled-dextran of 10kDa, 150kDa and 500kDa (Sigma Aldrich, USA) molecular weight were added 300µl of capsules. The solution was mixed for a few seconds to homogenize the capsules in the dextran solution and a sample removed and filtered though a 0.45µm nylon mesh and the absorbance at 450nm determined. This volume represents the initial concentration. The solution was then incubated at room temperature with continuous stirring for for 2 hours and a sample taken and filtered. Part of the sample (200µl) was transferred to a 96 well microtitre plate and the absorbance measured with a VersaMax plate reader set to 450nm. The absorbance measured was then compared with the absorbance of the initial concentration. To confirm the presence or absence of dextran in the microcapsules, a confocal analysis was also performed. Confocal analysis was also performed when the capsules were too weak to use the filtration process.

2.3.1.2.5. Confocal analysis of the molecular weight cut off of the capsules

The microcapsules were analysed using a multiphoton confocal microscope (Zeiss LSM 710 and ConfoColor 3). The excitation wavelength was set to 488 nm and the emission wavelength to between 500 and 600 nm. The gain was adapted from 500 to 800

between samples in order to get the best possible picture quality and avoid saturation, the pinhole was set up to 33.5 and the averaging was set up to a value of 4.

2.3.1.3. Results and discussion

The absorbance spectrum of FITC was measured using a Thermo Scientific Evolution 60S UV-Vis spectrophotometer with a 0.1% FITC Dextran 70kDa solution labelled with 0.003-0.020 moles FITC per mole glucose (Sigma) to determine if an absorbance can be measured and at which wavelength. The results (Figure 2.3) show that FITC-labelled dextran had an absorbance between 400nm and 500nm, with a maximum between 440nm and 490nm. The absorbance for preparation of standard curves and sample analysis was chosen to be 450nm.

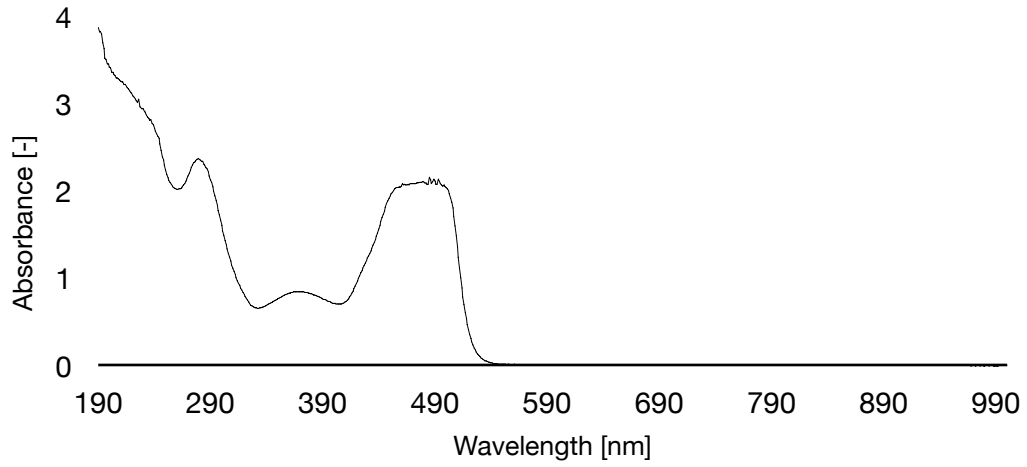

Figure 2.3 : FITC- labelled dextran spectrum from 190nm to 1000nm

In order to determine the volume of solution in each well, 3 standard curves from 0% to 1% were prepared and the absorbance measured with 100µl, 200µl and 300µl of solution in each well. Both 200µl and 300µl of solution showed good linearity between 0.5% and 0.005% of FITC dextran, while 100µl showed a poor correlation. In order to use as few micro capsules as possible, the chosen volume of solution was 200µl.

The linear range, the limit of detection (LOD) and the limit of quantification (LOQ) were determined for each molecular weight of FITC dextran. By observation of the curves (Figure 2.4), the linear range was determined to be between 0.005% and 0.2% of FITC-dextran for each molecular weight of dextran. A linear regression was determined using the least squares method and the coefficient of determination r^2 calculated for

each sample of each molecular weight dextran. The linearity was also investigated using an F-test and gave a cumulative probability P of 99.9% linearity.

The limit of detection LOD and the limit of quantification LOQ were calculated in the previously determined linear range, namely 0.005% and 0.2% of FITC-dextran. The LOD were between 0.001 and 0.003 of absorbance while the LOQ were between 0.003 and 0.01 of absorbance (Figure 2.4). The lowest concentration of FITC-dextran that could be measured using this method is 0.005% FITC-dextran, as the highest LOQ will have to be the lowest concentration limit.

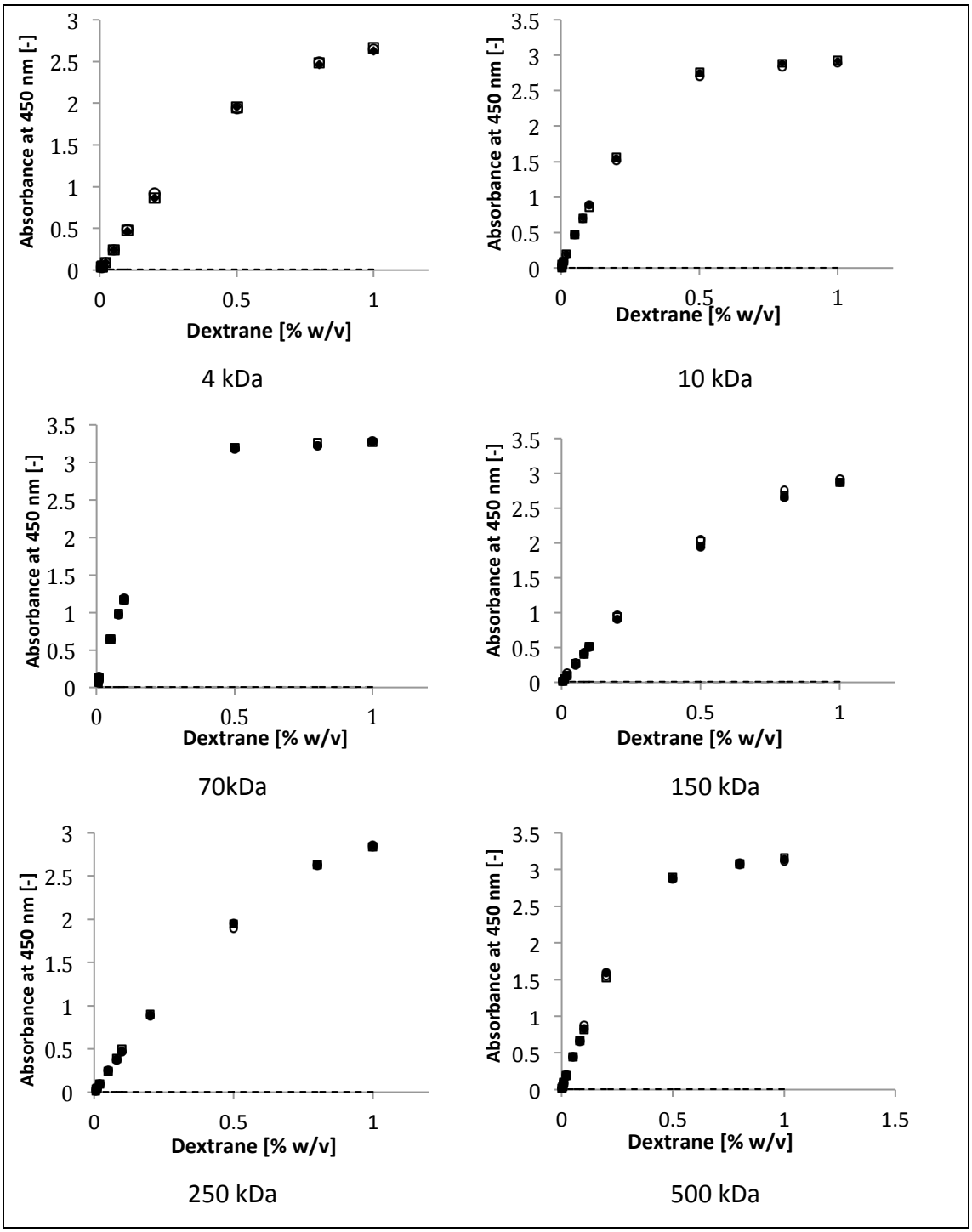

Figure 2.4 : 3 standard curves (full and empty spheres, full squares), linear range (0.2% to 0.005%), LOD (grey dashed line) and LOQ (black dashed line) for each molecular weight of FITC dextran : 4kDa (top left), 10kDa (top right), 70 kDa (middle left), 150kDa (middle right), 250kDa (bottom left) and 500kDa (bottom right)

In order to test the method, the molecular weight cut- off of alginate-poly-l-lysine-alginate microcapsules was determined. To determine the required time of reaction a kinetic study was performed with a wide range of molecular weight dextrans (Figure 2.5). If C/C_0 is equal to or greater than 1, the dextran does not enter the capsules, if C/C_0 is between 1 and 0.75 dextran diffuses into the microcapsules with difficulty and if C/C_0 is equal 0.75, the dextran diffuses freely into the microcapsules. The results

(Figure 2.5) show that 10kDa diffuses freely into the microcapsules, the 150kDa dextran diffuses slowly and the 500kDa does not enter the microcapsules.

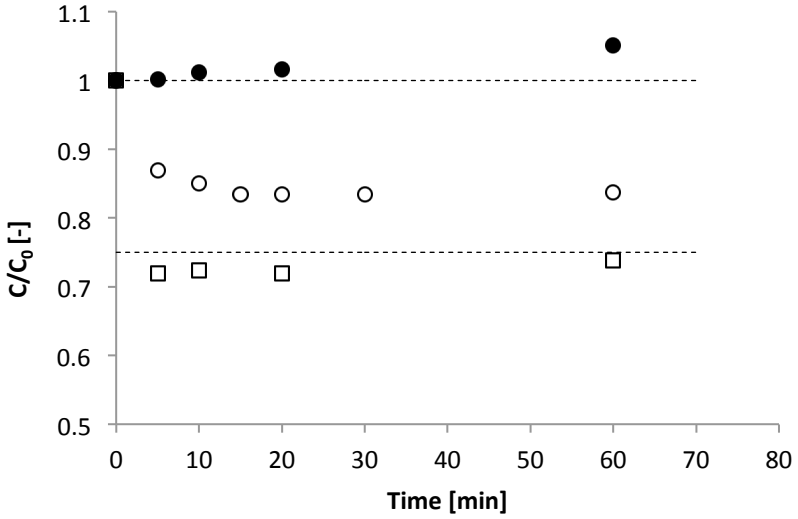

Figure 2.5 : Kinetics of Dextran ingress into the microcapsules for different molecular weight dextran. 500kDa (full spheres) does not enter the microcapsules, 150kDa dextran (empty spheres) ingress is limited and 10kDa dextran (empty squares) diffuses into the capsules rapidly.

The kinetics (Figure 2.5) show that the reaction ends after one hour, therefore, in order to test the method, a time of 2 hours incubation was chosen. 300µl of microcapsules were added in 900µl of FITC-dextran, the solution was agitated for 2 hours and 200µl of liquid was then transferred in a 96 well plate. The measured absorbance was compared with the initial concentration from a sample previously taken in order to determine the presence or absence of dextran into the capsules. The concentration of dextrans in the microcapsules obtained for 3 different molecular weight dextran (10kDa, 150kDa and 500kDa) are summarized in the Table 2.2.

Table 2.2 : Ingress of FITC-dextran into microcapsules obtained after 1 hour of incubation with the capsules for a molecular weight of dextran of 10kDa, 150kDa and 500kDa

Dextran Molecular weight	10 kDa	150 kDa	500 kDa
Initial dextran concentration in the surrounding medium [%]	0.038	0.043	0.04
Dextran concentration after 1 hour in the surrounding medium [%]	0.028	0.036	0.04
% ingress	100%	64%	0%

The FITC-labelling experiments (Table 2.2) and the kinetic experiments (Figure 2.5) showed that the 10kDa dextran diffuses into the capsules freely, the 150kDa dextran diffuses in a limited way and the 500kDa dextran does not enter the microcapsules. These results were confirmed by confocal microscopy analysis (Figure 2.6) that show that the FITC was located on the outside of the capsules for the 500kDa dextran, that the majority of the dextran was on the outside of the microcapsules for the 150kDa dextran while for the 10kDa, the amount of dextran inside the microcapsule was equal to the amount of dextran on the outside of the microcapsules.

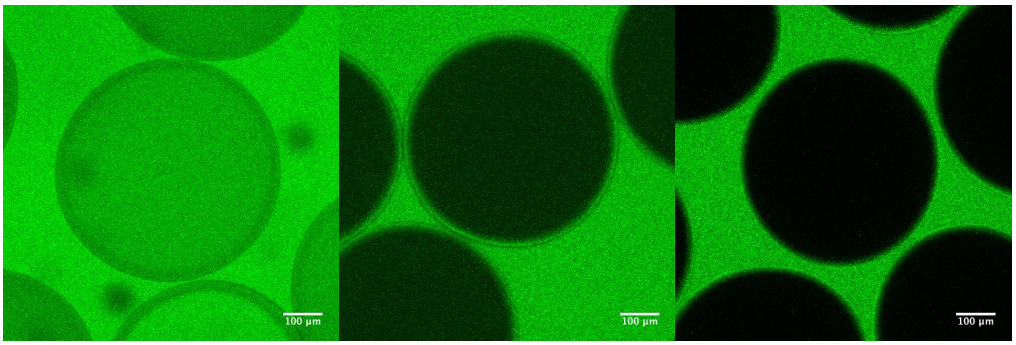

Figure 2.6 : Confocal microscopy images of microcapsules after 2 hours incubation in FITC dextran of different molecular weight : 10kDa (left), 150kDa (middle), 500kDa (right)

The absorbance method requires careful washing of the microcapsules with MOPS or PBS buffer before performing the analysis in order to remove any interfering substances from the microcapsule environment, such as phenol red present in cell culture media that could interfere with the absorbance measurements. The preparation of the samples also needs to be performed very carefully when the

microcapsules become fragile, as the microcapsules may burst or become damaged during the filtration step resulting in erroneous results that will be interpreted as if the dextran entered the microcapsule. A confocal analysis should therefore be performed in parallel to the absorbance method.

Confocal analysis can also be used in order to determine the MWCO of the microcapsules by comparing the difference in intensity between the interior and exterior of the microcapsules. In order to analyse the differences of intensity, the ratio I_i/I_o is calculated. If the measured molecule diffuses freely in the capsule, the intensity inside of the microcapsule is the same as the intensity outside of the capsules; the ratio I_i/I_o will therefore be 1. If the molecule does not diffuse into the microcapsule, the ratio I_i/I_o will be 0 and if the molecule diffuses into the microcapsules in a limited manner, the ratio I_i/I_o will be between 0 and 1. In order to reduce signal noise, a moving point average was performed on the intensity data (Figure 2.7). The intensity inside the microcapsules was then compared with the intensity outside the microcapsules and the MWCO profile was expressed with a graph of MW of the molecule versus (1- I_i/I_o) (Figure 2.7).

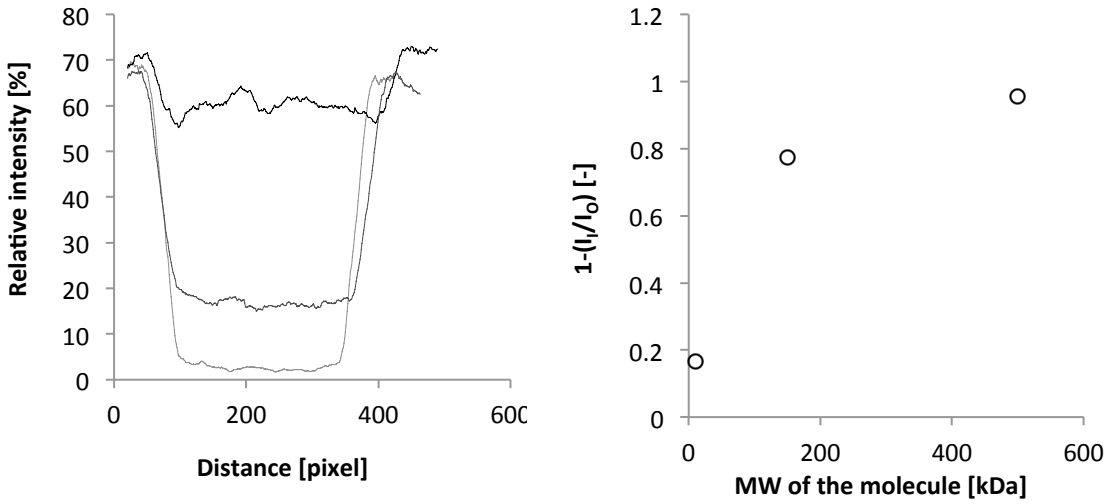

Figure 2.7 : Intensity profiles of different molecular weight dextran (light grey line 500kDa, black line 10 kDa and dark grey line 150kDa) after moving point average noise reduction (on the left) and MWCO profile calculated from the intensity profiles (on the right)

The intensity method does not involve a filtration step since microcapsules are directly analysed under the confocal microscope; the method is therefore more adapted for weak and fragile microcapsules. Moreover, the preparation of the samples does not need to be as rigorous as for the absorbance method as coloured materials e.g. medium will have no influence on the fluorescence intensity. However, this method

has some limitations such as when the microcapsules are packed with cells, the shadow of cells will mask what goes on inside the microcapsule and no intensity will be detected.

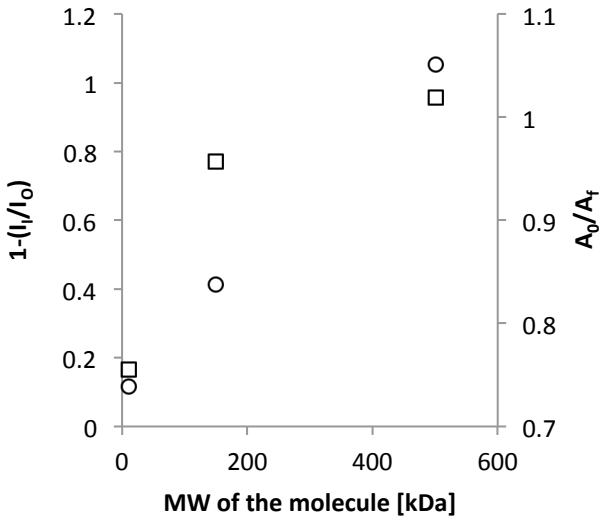

Figure 2.8: Comparison of the MWCO profile of microcapsules measured using the absorbance method (empty sphere on the right axis) and with the intensity method (empty square on the left axis)

When the absorbance and confocal intensity methods are compared, the confocal intensity measurement is more precise especially when the dextran diffuses freely inside the microcapsule. The absorbance method gives a ratio of 0.75 for the 10kDa dextran (Figure 2.8), which means that the dextran should diffuse freely inside the microcapsules. However, the confocal images (Figure 2.6) and the intensity measurements (Figure 2.8) show that the intensity of dextran inside the microcapsule is slightly lower than the intensity outside the microcapsules, which shows that the dextran diffuses easily but not freely into capsules.

2.3.1.4. Conclusion

The aim of this work was to develop a method to determine the MWCO of microcapsules used for cell encapsulation. The developed method uses FITC-labelled dextran with the ingress of labelled molecules into microcapsules using absorbance (spectrophotometry) and confocal microscopy. Confocal microscopy is more precise and provides more accurate results particularly for weak and fragile microcapsules. Although sample preparation is more straightforward, once the microcapsules begin to be significantly colonised, the confocal method is not suitable, only the absorbance method is able to measure the ingress of dextran into microcapsules. It is also

important to realise that dextrans are polysaccharides and that they interact differently with the microcapsule membrane compared with proteins. However, the type of molecule is less important than the diffusional Stokes radius (size of particle in solution) to allow or exclude the diffusion of molecules in or out of the microcapsules. For example, the molecular weight of IgG is 150 kDa and the Stoke radius is 5.29 nm, which corresponds to a dextran with a molecular weight of between 40kDa and 70 kDa. It would therefore be important to test the ingress of a labelled-protein such as IgG into the microcapsules to confirm that the Stoke radius is enough to allow or exclude the diffusion of a molecule into or out of microcapsule.

2.3.2. Microcapsule mechanical properties and stability

2.3.2.1. Overview

Microencapsulation can have many applications in many different fields. Depending on the field the microcapsules are used, characterization of the mechanical resistance and stability of the microcapsules is very important. If the microcapsules are used to protect cells for culture in bioreactors, they will be subject to shear stresses due to agitation and aeration. The microcapsules therefore need to be resistant enough to avoid breakage and the resistance should be constant with respect to time. Different methods, some more qualitative and some more quantitative, have been reported in the literature to analyse the mechanical resistance of the microcapsules. These methods can be divided in two classes, the breakage methods that analyses the behaviour of microcapsules when subject to mechanical shear forces (Bartkowiak and Hunkeler, 2000; Liu et al., 1996; Lu et al., 1992; Uludag et al., 2000) and the osmotic methods that are based on the swelling of microcapsules when exposed to a change in media composition and osmotic pressure (Gåserød et al., 1999; Martins dos Santos et al., 1997; Ma et al., 2013; Thu et al., 1996; Van Raamsdonk and Chang, 2001). The method based on osmotic changes is well documented and very straightforward to perform. It consists in exposing microcapsules to water, causing the microcapsules to swell and break. This method is reliable to compare microcapsules prepared from the same materials and is only applicable to physical hydrogels; in the case of chemically cross-linked hydrogels, changing the osmotic conditions will not induce the microcapsules to break.

Another very common method to measure the resistance of microcapsules is the evaluation of microcapsules mechanical resistance using a Texture analyser TA.XTPlus (Stable Micro Systems, UK) (Rosiñski et al., 2008). The texture analyser consists of a mobile probe with a defined compression speed, which compresses the microcapsules. By measuring the force corresponding to the rupture of the microcapsule or at a given degree of compression, it is possible to obtain information about different batches of microcapsules. Two methods are commonly

used to measure the resistance of microcapsules. A number (20-30) of microcapsules can be individually compressed and the mechanical resistance values presented as the mean force necessary to break one capsule. A layer of microcapsules can also be compressed at the same time. This method assumes that the capsules are arranged in a hexagonal plan and that the capsules have a relatively narrow size distribution. The total force applied divided by the number of microcapsules beneath the piston gives the mechanical resistance per microcapsule. For both compression methods, the mechanical resistance will be given in gram per capsule instead of the Young modulus since changes of the contact area between the microcapsule and the piston are difficult to measure as a function of degree of compression.

By using the Texture analyser method, some inconsistences appear when measuring the initial force on different batches of capsules made from the same polymer and with the same procedure. This study aims to understand if these inconsistencies were due to the capsules or to the texture analyser itself. The possible causes are the calibration, the movement of the probe, the surface tension due to water and variability in the capsules size and shape

In order to get consistent results with the Texture analyser method, parameters had to be optimized and the conditions of measurement thoroughly characterized.

2.3.2.2. Mechanical resistance measurement using a Texture analyser TA.XTPlus

2.3.2.2.1. Determination of the reproducibility of the method

The reproducibility between each measurement for the same sample and the reproducibility between each sample (error in the preparation) has been analysed. Three different monolayers of APA microcapsules were prepared on a microscope glass slide and 6 mechanical resistance measurements were taken on each slide. Each monolayer of microcapsule needs to be exempt of extracapsular water; the water was therefore removed by capillarity using absorbent paper. The reproducibility between each measurement for the same sample was analysed by comparing the results of the measurements taken on one glass slide when the

error in the preparation is analysed by comparing the results of the 3 different samples prepared on the 3 different glass slides.

2.3.2.2.2. Determination of the influence of the movement of the probe

In order to analyse the influence of the movement of the probe on measurements, the texture analyser was calibrated with a 2000g weight and 6 measurements were made using a glass slide as a sample. An analysis of the noise was then performed to see if a correlation between noise and movement of the probe could be found.

2.3.2.2.3. Determination of the influence of the surface tension

In order to determine the influence the surface tension due to water on mechanical resistance measurement, a drop of water was placed on a glass slide under the probe and measurements made. In between each measurement, the probe was dried with absorbent paper and the glass slide changed.

2.3.2.2.4. Determination of the influence of the water content in the sample

In order to measure the influence of the water content of the sample on the mechanical resistance measurements, a monolayer of APA microcapsules was prepared on a glass slide and the water removed using absorbent paper. The amount of water that was removed varied between the different glass slides in order to measure the mechanical resistance as a function of the amount of residual water associated with the microcapsules.

2.3.2.2.5. Determination of the influence of the calibration

In order to investigate the influence of the texture analyser calibration on measurements, 5 measurements were made using a glass slide as a sample. The analyser was calibrated with a 2000g weight between each measurement.

2.3.2.2.6. Determination of the burst force and mechanical resistance of microcapsules

The mechanical resistance of microcapsules was measured using a texture analyser (TA.XT plus texture analyser, Stable Micro System, UK) which featured a mobile probe driven at a set speed of 0.299mm/s, the area of the probe was $3.17 \cdot 10^7$ μm^2 and the initial distance of the probe from the plate was $4.7 \cdot 10^3$ μm.

Mechanical resistance is defined as the force required for each microcapsule to reach 50% deformation and can be calculated following the equations in section 2.2.3.2.

2.3.2.3. Results and discussion

In order to understand the errors observed in the mechanical resistance method using the texture analyser, the error in the preparation of the sample and the error in the measurement were analysed in order to test the reproducibility of the method. The influence of calibration, the movement of the probe and the influence of water were also tested. The reproducibility in the method and the influence of water were tested on APA microcapsules, the calibration and the movement of the probe were tested using a glass slide as a sample.

Among the different hypothesis, it is important to understand if a mechanical problem in the texture analyser was responsible for errors in the results. It is therefore important to analyse the movement of the probe to detect an eventual problem. The probe is supposed to move smoothly along the arm of the machine, however if it does not this can have an influence on the measurement of the mechanical resistance. In order to detect an eventual problem in the movement of the probe, a noise analysis was performed, the noise being defined as the ability of the arm to move smoothly. If the noise is regular and correlated with a time interval, it is possible that the movement is not smooth and that the probe measures irregularly. It is also important to compare the noise with the measured force to see if noise has an influence on the measurement.

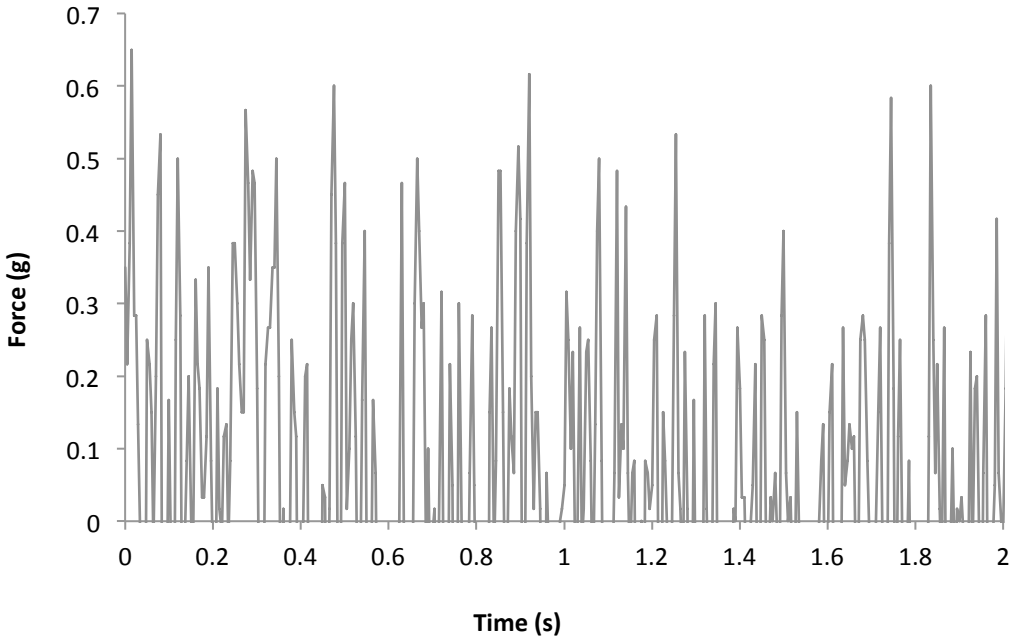

Figure 2.9: Illustration of the signal noise pattern for the two first seconds of the probe descent.

The signal noise analysis shown in Figure 2.9 shows that no regular pattern could be detected, suggesting that the probe movement is smooth and regular, consequently the signal noise was not correlated with the motor of the analyser probe. Moreover, the maximum force reached by the noise is 0.65 g when the forces measured for the capsules mechanical resistance are bigger than 10 g. There is negligible no influence of the noise, i.e. of the mechanical movement of the probe on the measurement of the mechanical resistance.

Since no mechanical problem was found, it was important to understand if a calibration problem could be causing the inconsistences in the method. In order to test the calibration of the machine, 5 measurements were taken on a glass slide, the machine being calibrated each time between the measurements. The result of this test shows that for small strength values, a relative error of 20% between the measurements is detected, due to the calibration between each measurement. This relative error was reduced to 2% at the highest measurements values. However it is not possible to produce microcapsules of this strength. The error produce by the calibration is probably due to the correction that the analyser needs to make to bring the value to the known 2000 g weight. This correction is larger than the measured value and creates errors between results determined with two different calibrations. The analyser cannot be calibrated every day, as the

calibration needs to remain the same during an experiment in order to obtain comparable values.

The reproducibility in the preparation of the capsule monolayer and in the measurement is very important when analysing samples, as the different samples and the different measurements of one sample need to be comparable. In order to test the reproducibility in the measurements, six measurements were made on one monolayer of microcapsules. This test was repeated on three different samples to show that the measurements are consistent despite differences in sample preparation. It can be seen in Table 2.3 that the reproducibility between the different measurements for the same sample shows an error of between 13.9 and 22%. This error can be explained by the configuration of the monolayer of microcapsules, some monolayers being broader than others, thereby changing the displacement of the microcapsules when crushed by the probe. The water content of each sample can also vary between the different samples and within the monolayer, which can add to the measurements. In order to analyse the reproducibility in the sample preparation, the error on the average strength measurement of the 3 samples was calculated by propagation of error. A relative error of 15.1% was obtained. This error is mainly due to the error of the measurement and it would therefore be interesting to find a method to prepare the samples to reduce this error. Among the different hypothesis above, the water content in the sample is probably the most important error source in sample measurement and will therefore be analysed.

Table 2.3 : Reproducibility analysis of the different measurements made with the texture analyser and of the sample preparation

Reproducibility in the measurement	Sample	Average strength (g/capsule)	Standard error (g/capsule)	Relative standard error (%)
	1	0.145	0.022	15.0
	2	0.158	0.022	13.9
	3	0.147	0.033	22.6
		0.150	0.023	15.1
Reproducibility in the sample preparation		Average strength on the 3 samples preparation (g/capsule)	Standard error on the 3 samples preparation (g/capsule)	Relative error on the 3 samples preparation (%)

As the water content in the sample has an influence on capsule strength measurements, measurements with different amount of water in the samples were made. The water was removed from the sample by capillarity with an absorbent paper. In the sample with a high amount of water, no liquid was removed from the monolayer of microcapsules. For the medium amount of water, liquid was absorbed with a paper until no more water is visible on the slide and for the low amount of water; water was removed until the capsules are deformed in a hexagonal shape. Results show that with a high amount of water, an 8.2% relative error is measured, when for a medium amount of water a 15% error is measured. This is probably due to the fact that with a large amount of water, capsules float and can be easily displaced by the probe, resulting in few microcapsules under the probe when the probe touches the microcapsules. With a medium amount of water the microcapsules do not move, however the remaining water in the sample is not homogenously distributed in the microcapsule monolayer, with more water remaining in the centre of the monolayer compared with the edges. This difference in distribution will lead to a larger error in the measurements between different spots on the microcapsule monolayer. When the majority of the water was removed, a 5.4% relative measurement error was observed. This is due to the fact

that the water is homogeneously removed from the sample and that the microcapsules do not move on the slide when crushed by the probe. It can therefore be said that an optimal sample preparation for an optimal microcapsule strength measurement will have to define how the water in the sample must be removed in order to have consistent measurements.

2.3.2.4. Conclusion

Texture analyser method showed a lack of reproducibility in measurements of initial mechanical strength on different batches of microcapsules made from the same polymer solutions and following the same procedure. In order to get quantitative results, it was therefore essential to understand if the lack of reproducibility was due to actual variations in the microcapsule sample or to the way in which the texture analyser operates. Different causes such as the calibration, the movement of the probe, the surface tension of the samples due to water and variability in the microcapsules were suggested and different experiments were performed to test them.

By analysing the noise of the different analysis performed, no regular pattern was noticed suggesting that the analyser was not the problem and that the probe was moving smoothly and regularly. By taking different measurements of a sample, it was shown that the measurements were reproducible with a 15% relative error however, by reducing the amount of water in the microcapsule samples until the amount was low and constant, the relative error could be reduced to 5.4%. The error in the sample preparation was shown to be mainly due to the error between the different measurements and it can be concluded that there is no influence of the sample preparation on the inconsistent measurements. There is however an influence of the calibration on the measurements. The calibration refers a measured value to a known standard value therefore creating an error when different calibrations are performed between measurements.

In order to obtain reproducible measurements with the texture analyser it is recommended that a single calibration is used prior to the analysis of a complete batch of experimental samples rather than calibration before each sample. It is also important to reduce the amount of water in the sample as much as possible. By taking both these recommendations into account, the texture analyser allows

reliable measurement of the mechanical resistance of microcapsules with a 15% relative error. In other words, if there is more than 15% difference between two results they can be considered being significantly different.

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3. Characterisation and stabilisation of alginate poly-L-lysine microcapsules

Partially published in: Demont, A., Cole, H., Marison, I.W. An understanding of potential and limitations of alginate/PLL microcapsules as a cell retention system for perfusion cultures. J. Microencapsul. 2015 (in press)

Abstract

Microcapsules for high cell density culture of mammalian cells have found an increasing interest over the past decades, however the poor stability of the microcapsules and the lack of characterisation methods led to few quantitative results. Alginate-poly-L-lysine microcapsules have been studied here in detail in order to form a basis for comparison of capsules made from different polymers. Since the microcapsules can be easily retained in the bioreactor without the need for a cell separation device, high cell densities were achieved with a maximum of $4 \cdot 10^7$ cell/$mL_{capsules}$, corresponding to a colonisation of 5% of the internal capsule volume. However, measurement of microcapsule integrity and mechanical resistance showed that alginate-poly-L-lysine microcapsules are not suitable for perfusion cultures since they are very sensitive to media composition, mainly the presence of non-gelling ions that have a higher affinity for alginate than poly-L-lysine and Ca^{2+}, leading to the leakage of poly-L-lysine and Ca^{2+}, and to capsule rupture under perfusion conditions.

3.1. Introduction

The production of recombinant proteins using animal cells is an increasingly developing area of bioprocessing in the pharmaceutical industry (Desimone et al., 2011; Harcum, 2005). The use of animal cells is necessary to generate therapeutic proteins, which possess significant post-translational modifications, a proper folding and assembly and *in vivo* immunotolerance (Harcum, 2005; Wurm, 2004; Zhu, 2012).

The use of animal cells for the production of recombinant proteins however has some limitations such as the time required to create stable, high producing cell lines secreting the protein to high levels, at high productivity and correctly assembled (Wurm, 2004; Zhu, 2012); the time taken to develop processes which allow cells to grow and secrete the recombinant proteins, using carefully controlled conditions, while avoiding the lysis of cells through shear forces present in the reactors, avoidance of medium and product (metabolite) limitations, and avoidance of product degradation (Harcum, 2005; Zhu, 2012); the low productivity of animal cells and the high production costs (Wurm, 2004).

In order to overcome these limitations, there is the requirement for high productivity, high cell density systems which are carefully controlled and which allow the production of high concentrations of recombinant protein (Zhu, 2012). At the current time, the pharmaceutical industry uses simple batch and fed-batch processes to produce recombinant proteins at large scale since the ideal cell retention device is non-existent and retention systems for perfusion cultures are still associated with uncertainty and risks in the manufacturing process (Clincke et al., 2013a, 2013b; Voisard et al., 2003; Xie and Zhou, 2005). Microencapsulation may offer the possibility to be an efficient cell retention system, while protecting the cells from the shear stresses associated with the bioreactor. Microcapsules with a narrow size distribution (<3%) can be produced using the jet break-up technique. This technique has been extensively characterised for diverse polymers, including alginate, cellulose sulphate or gelatin (Brandenberger and Widmer, 1998; Brandenberger et al., 1999; Gugerli, 2003; Serp et al., 2000) and the results show that the presence of high diversity in molecular size and the presence of impurities might prevent the possibility to form a stable jet. The polymer used is therefore highly dependent on the method to produce the beads. Alginate microcapsules have the advantage that a relatively high alginate

concentration (up to 3% w/v) can be extruded with the jet break-up technique into mild encapsulation conditions (Whelehan and Marison, 2011). Moreover, this method allows the coating of the alginate bead core with different poly-cations such as poly-L-lysine to control membrane permeability, facilitating the downstream processing by either concentrating the product in the microcapsules or by releasing it in the medium (Breguet et al., 2007; B. Thu et al., 1996a, 1996b). Microcapsules have been widely used for diverse mammalian cell culture applications (Breguet et al., 2007; Guo et al., 1989; Hollingshead et al., 1995; Jarvis Jr. et al., 1986; Jöchle, 1993; Loty et al., 1998; McMahon et al., 1990; Nebel et al., 1993; Okada et al., 1996, 1995; Pajić-Lijaković et al., 2007; Pueyo et al., 1995; Santos et al., 2010; Serra et al., 2011; Takabatake et al., 1991; Uludag et al., 2000; Watson, 1993; Wilson and McDevitt, 2013), however there is little information concerning microencapsulated high cell density cultures performed in continuous stirred tank bioreactors (CSTR). The poor mechanical resistance of hydrogel-based microcapsules and the fact that it has not been established whether productivity and specific growth rate of such systems is comparable or superior to suspension cultures can be reasons for the lack of industrial applications of microencapsulation to large-scale mammalian cell culture (Duff, 1985; Gugerli, 2003). Since microcapsules are intended to be used in bioreactor cultures, the microcapsules must have long-term stability in cell culture media and physiological environments particularly since these media are usually rich in ions which can destabilize the integrity of the alginate gels or the polyelectrolyte interactions involved in the microcapsule integrity (Breguet et al., 2007; Gugerli, 2003).

In this work, the widely used alginate poly-L-lysine microcapsules have been studied as a reference for cell encapsulated cultures. The variation of burst force with time, the microcapsule stability in culture medium and integrity was evaluated for encapsulated batch, fed-batch and perfusion cultures. The kinetics and stoichiometric parameters of CHO-DP12 cells have been determined for suspension batch culture, encapsulated batch, fed-batch and perfusion cultures, together with the possibility to monitor cell growth using dielectric spectroscopy.

3.2. Material and Methods

Material and methods used in this chapter are described in section 2.2. Specific experiments set-up are described below.

3.2.1. Shake flask encapsulated cultures and stability tests

20mL of cell containing microcapsules or empty microcapsules were cultured in shake flask (Corning Inc, Corning, NY, USA), with a working volume of 100mL and a microcapsule volume representing 25% of the medium volume. The culture was then incubated at 37°C, under a humidified atmosphere of air containing 5% CO_2 and an agitation of 100rpm. For perfusion cultures, the microcapsules were allowed to settle before a known volume of medium was aseptically removed with a sterile pipette and replaced with the same volume of fresh medium. Perfusion was performed once daily. Each culture and stability test carried out in shake flask were carried out only once

3.2.2. Bioreactor encapsulated cultures

Encapsulated cells were grown in a Mettler Toledo RC1 2L biocalorimeter, with a working volume of 1.4L. The bioreactor was equipped with a pH-sensor (Metler Toledo, Delaware, USA), an inbuilt PT-100, a dieletric probe (Aber Instrument, UK) and an air sparger. The bioreactor was operated either in batch, fed batch or perfusion mode at a temperature of 37°C, agitation of 100 rpm with a sparged airflow of 0.01 vvm and a headspace airflow of 0.2 vvm and 0.1 vvm CO_2. The pH was maintained at 7.2 by addition of NaOH and CO_2 to the headspace. For perfusion cultures, the volume was maintained constant by the use of a feed system, which added and removed media at the same rate. A feed of $CaCl_2$ was applied in parallel to the NaOH pH control to maintain a 1:50 gelling/non-gelling ions ratio for perfusion and fed-batch culture. A 200µm nylon mesh was installed on the medium outlet port to avoid capsule removal in the feed out. A microcapsule volume of 25% of the working volume was used for all bioreactor cultures. For encapsulated perfusion and fed-batch cultures in the bioreactor, the media was also supplemented with 1.31mmol/L $CaCl_2$. Each culture carried out in the Mettler Toledo RC1 biocalorimeter were carried out only once.

3.2.3. Perfusion feeding strategy

Prior to perfusion, sampling of the cultures was performed. Based on the predetermined cellular growth rates, specific metabolite consumption and production rates, the concentration of glucose and glutamine required by the cells to maintain the growth rate was calculated. The adjustments in glucose and glutamine concentration in the fresh medium were made to meet the concentrations that would be required by an anticipated cell number which would be present in the culture on the following day based on the growth rate displayed by the cells. The required amount of anhydrous glucose (Sigma Aldrich, Saint Louis, Missouri, USA) and L-glutamine (Sigma Aldrich, Saint Louis, Missouri, USA) powder were then weighed and added to fresh medium. The medium was then sterile filtered (0.2µm Steritop filters, Merck Millipore, Ireland) before being added to the culture.

3.2.4. Encapsulation

The encapsulation method used was a modification of the technique originally developed by Lim and Sun (Lim and Sun, 1980; Lim, 1983, 1982;) undertaken under completely sterile conditions using a vibrating nozzle encapsulation device (Encapsulator Biotech, EncapsBio, Switzerland or Inotech IE-50R, Inotech, Switzerland) as described elswhere (Serp et al., 2000). Alginate (Manucol DH, FMC biopolymers, UK) beads were first formed by extrusion of 300mL pre-sterilised (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland) sodium alginate solution (1.5% w/v) containing cells ($0.7 \cdot 10^6$ $_v$cells/mL$_{alginate)}$ through the encapsulator nozzle into an aqueous solution of $CaCl_2$ 110mM (Sigma, St-Louis, USA). After 5 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules were washed with saline buffer (0.85% NaCl in 10mM MOPS, Sigma, St-Louis, USA) followed by incubation in 1.2L 0.05% (w/v) poly-L-lysine 30-70kDa (Sigma, St-Louis, USA) for 30 min at room temperature under agitation. The microcapsules were then re-washed with saline buffer followed by incubation in 1L 0.03% sodium alginate for 10min under agitation. After washing with saline buffer, the solid alginate core of the microcapsules was liquefied by incubation for 15min in 50mM sodium citrate (Sigma, St-Louis, USA). After core liquefaction with citrate, the microcapsules were washed with saline buffer followed by a wash with cell culture media before being transfer to cell culture media to be inoculated.

3.2.5. Microcapsule characterisation and culture analysis

Homogenous culture samples containing microcapsules were regularly removed from the bioreactor to determine intra/extra-capsular cell number, concentration of key culture metabolites, microcapsule size, mechanical resistance, molecular weight cut-off (MWCO) and membrane thickness using methods described in sections 2.2.2 and 2.2.3.

The microcapsules colonisation was estimated by first determining the amount of capsules within 1mL of capsules and then by calculating the amount of cell per capsule. The theoretical maximum cell concentration within the capsule was estimated afterwards by dividing the microcapsule volume by the volume of a cell. The microcapsule colonisation was then estimated by dividing the maximum theorical cell concentration within one capsule by the real cell concentration within one capsule. It was therefore assumed that both the cells and the microcapsules were capable of deformation to fill up the free spaces and were therefore not behaving like solid spheres.

3.2.6. Dielectric spectroscopy monitoring

The encapsulated culture was monitored by dielectric spectroscopy following the method described elsewhere (Cole et al., 2015)

3.3. Results - Part I: Determination of the working window

3.3.1. The influence of alginate on microcapsule stability

3.3.1.1. Alginate characterization

As previously reported by Santos et al (2010), PLL has a higher affinity for medium mannuronic alginates which ensure a more rapid binding of the PLL during the coating step and a much thicker PLL layer. The choice of the alginate is therefore an important parameter in microcapsule development in order to optimise the microcapsule stability. Several available alginates were therefore tested in order to choose the most suitable alginate to perform perfusion culture with PLL-containing microcapsules. The viscosity and the water content of each alginate were measured (Figure 3.1). Moreover, the monomer composition was compared through FTIR spectroscopy.

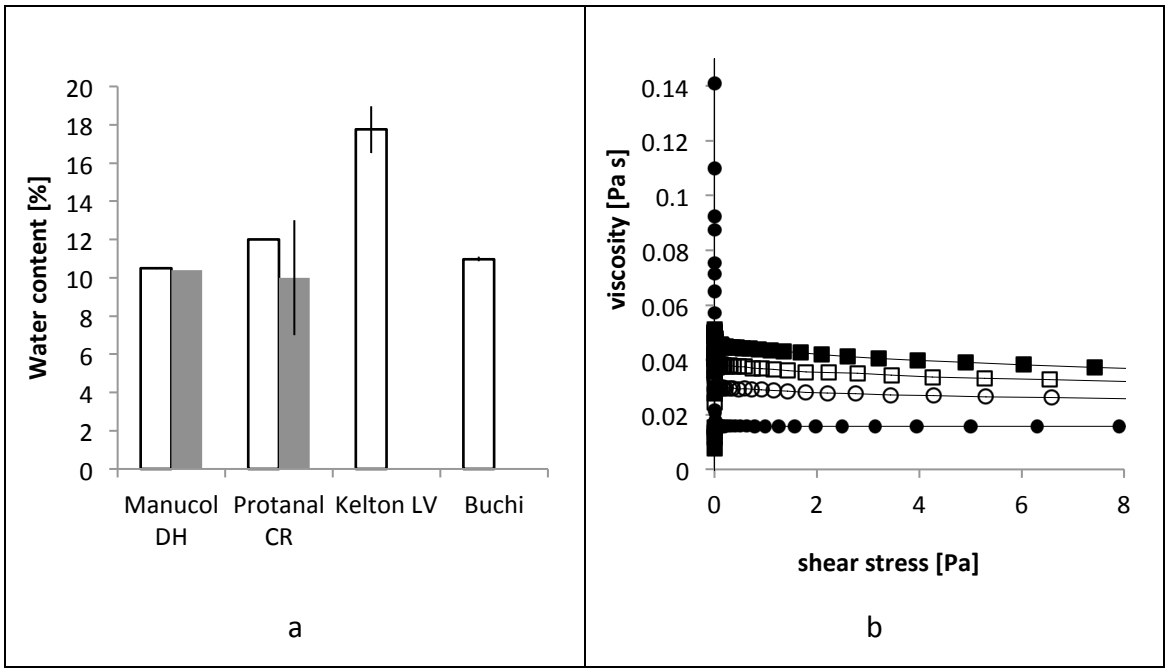

Figure 3.1: a) Water content of different alginates. The measured water content is represented by the empty bars and the advertised water content by the full bars. The measured alginate water content is given in % and the error bars represent the standard error of the mean (n=3). b) Viscosity of 1% (w/v) solutions of Kelton LV (full spheres), Protanal CR (empty spheres), Büchi (empty squares) and Manucol DH (full squares).

The water content of Manucol DH, Protanal CR and Buchi alginate give similar results, between 10 and 12% of water in the samples. The water content of the Kelton LV however is slightly higher, with a measurement of 18% water, which suggests that

Kelton LV might have been badly stored (Figure 3.1a). The viscosity of a 1% solution of each of the alginate was also measured and shows that the viscosity of the Kelton LV alginate was much lower than the viscosity of the other 3 alginates, which would confirm the storage hypothesis. The Manucol DH shows the highest viscosity, followed by Büchi and Protanal CR (Figure 3.1b).

3.3.1.2. Alginate stability in culture conditions

The 4 alginates characterized above have a high M/G ratio and should have a good affinity for PLL. The stability of PLL-microcapsules produced with these alginates in culture conditions should therefore be analysed. PLL-microcapsules were therefore produced by extruding each alginate through a 300µm nozzle before coating the beads with a 0.05%PLL solution and liquefying the core with a sodium citrate solution. Different parameters such as the size, the mechanical resistance and the swelling of the microcapsules were analysed along the microcapsules production and the stability test

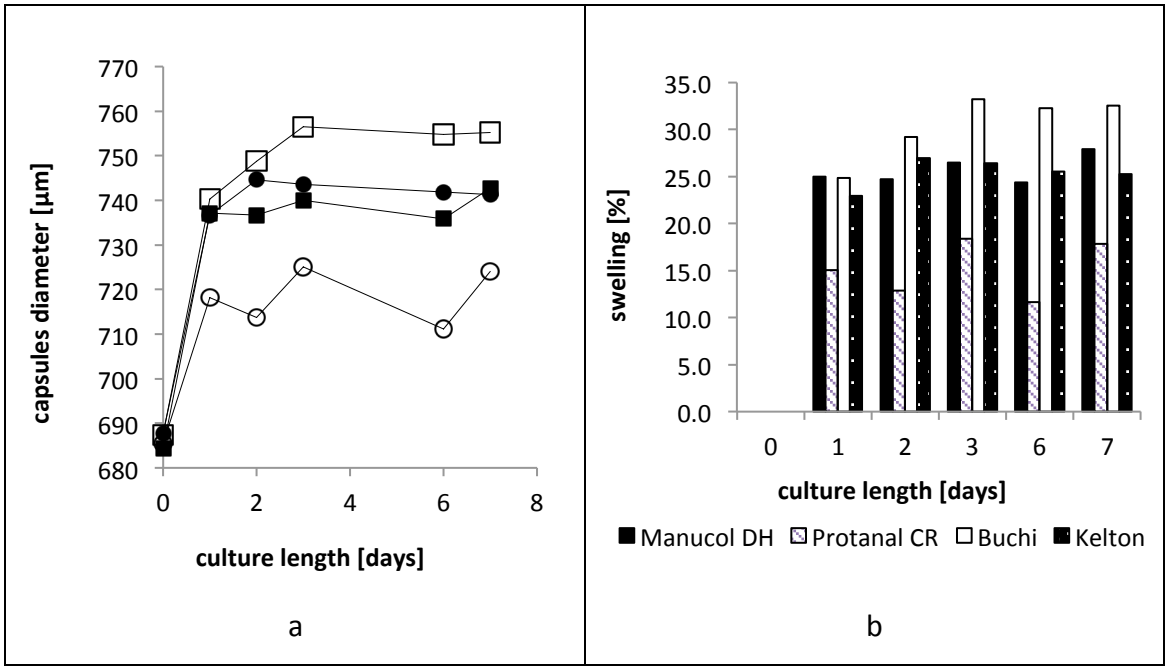

Figure 3.2: a) Microcapsules size as a function of time for microcapusles made of Manucol DH (full squares), Büchi (empty squares), Kelton LV (full spheres) and Protanal CR (empty spheres) alginate (n=30). The microbead diameter is given in µm and the error bars represent the standard error of the mean (n=30). b) Microcapsule swelling as a function of time for microcapsules made of Manucol DH (full bar), Büchi (empty bar), Kelton LV (full bar with white dots) and Protanal CR (empty dashed bar).

Figure 3.3a and b show that the microcapsules swell after being incubated in media. Those microcapsules produced using Manucol DH alginate and Protanal CR alginate

have less swelling while those produced with Buchi and Kelton LV alginate have the greatest swelling observed. This swelling suggests that PLL has a higher affinity for Manucol and Protanal alginate and therefore binds with a higher affinity to these alginates allowing a retention of the beads initial shape. This affinity can be observed through the shrinkage that occurs during the PLL coating step Figure 3.4b. During binding PLL attracts the alginate chains and extrudes water, provoking a reduction in bead size, which is more important for Protanal and Manucol than for Kelton and Buchi alginates. During the citrate step swelling is observed (Figure 3.4b) due to a higher presence of free calcium ions inside of the capsules than outside, provoking a flux of water into the capsules. Finally, when in medium (Figure 3.4b), the capsules keep swelling as more water enters the capsules to equilibrate the salt concentration, in addition calcium ions bound to alginate will be displaced by sodium ions. The initial bead sizes (Figure 3.4b) are bigger for Protanal, Manucol and Buchi than for Kelton alginate, due to higher intramolecular interactions making it harder to separate the beads. The mechanical resistance shown in Figure 3.4a appears to be higher for Manucol, Protanal and Buchi alginates than for Kelton LV alginate at the beginning of the culture however, after 7 days, Buchi alginate seems to have a lower mechanical resistance than the Kelton alginate.

The results obtained are interesting. Büchi and Kelton LV alginate have the more swelling, the less affinity for PLL and the lowest mechanical resistance. This could be explained by the fact that Kelton LV has a higher water content than the other 3 alginates and that the initial concentration used for Kelton LV alginate is therefore lower than that of the other 3. Kelton LV alginate is, therefore, not good for use anymore and should be discarded from the tests. Büchi alginate on the other hand has the same water content than Manucol DH and Protanal CR, however its stability is lower than that of Manucol DH and Protanal CR, while its swelling is greater.

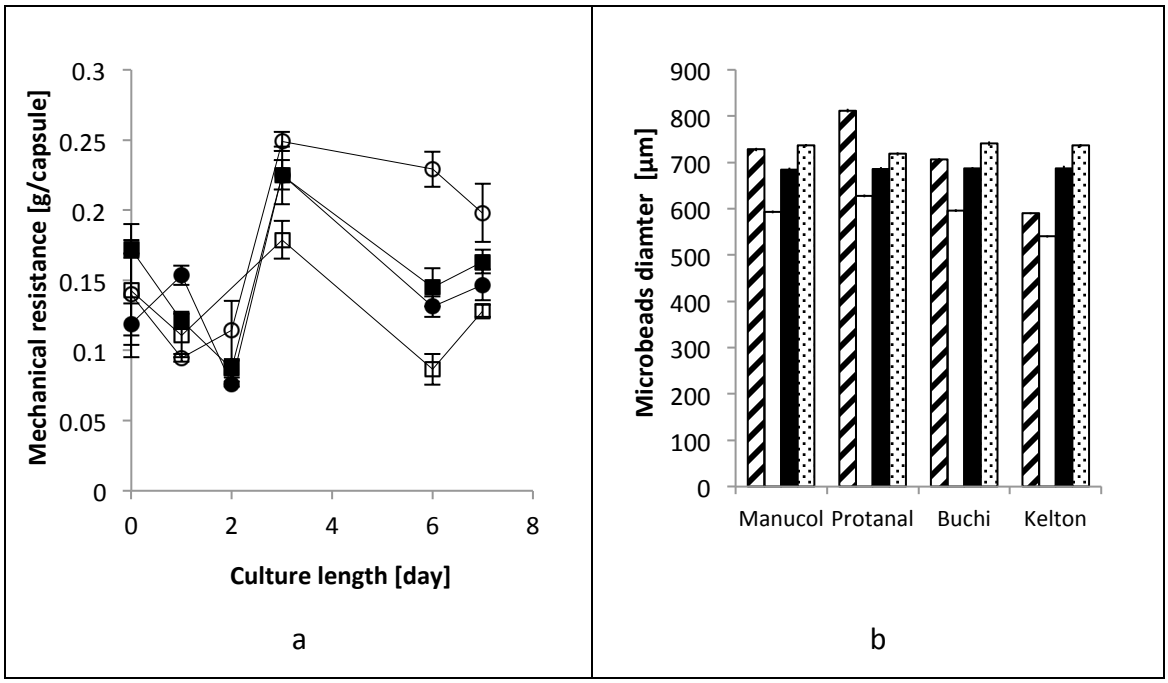

Figure 3.3 : a) Microcapsule mechanical resistance as a function of time for microcapusles made of Manucol DH (full squares), Büchi (empty squares), Kelton LV (full spheres) and Protanal CR (empty spheres) alginate. The burst force is given in g per microcapsule and the error bars represent the standard error of the mean (n=5). b) Microcapsule diameter along as a function of time for microcapsules made of different alginates. Samples were analysed after extrusion (dashed line bar), after PLL coating (empty bar), after citrate liquefaction (full bar) and once in cell culture media (empty bar with dots). The microbead diameter is given in µm and the error bars represent the standard error of the mean (n=30).

The results of the stabiliy test in culture condition of alginate-PLL microcapsules produced with different alginates showed that Manucol DH and Protanal CR have a good affinity for PLL and a good stablity under culture conditions while Buchi alginate has a good affinity for PLL however the stability is lower. Due to the low viscosity, high water content and low mechanical resistance, Kelton LV alginate was discarded since the quality appears to have been damaged during storage. In order to choose the most suitable alginate, alginates still need to be tested with respect to the influence on cell growth. It is indeed mandatory that the alginate that is selected has no negative effect on cell growth

3.3.1.3. Effect of the alginate on CHO-dp12 cell growth

In order to test the effect of the different alginates on cell growth, a suspension of cells in a 1.5% solution of Manucol DH and Protanal CR alginate was thus extruded in order to produced APA microcapsules for cell culture in batch mode. As Kelton LV and Büchi alginates did not meet the stability criteria, it was decided not to test them further. Different parameters were analysed during the culture and are reported in Table 3.2.

Table 3.1 : Kinetic parameters of suspension and encapsulated CHO cells cultures.

	Suspension	Protanal CR	Manucol DH
Culture length [h]	168	360	240
μ [h^{-1}]	0.0204	0.0159	0.0211
Max viable cell density [cell/mL]	5.9 x 10^6	8.65 x 10^6	6.89 x 10^6
Viability at max viable cell density [%]	92.7	75	92.4

The results reported in Table 3.2 show that the growth rate of the cells is similar for both suspension and encapsulated cultures in Manucol DH. The growth rate in Protanal CR is however lower. The cell viability in both suspension and encapsulated cultures in Manucol DH are once again similar with a value of 92%, while for Protanal CR, the viability only reaches 75% at the maximum viable cell density. Due to these two parameters, Manucol DH was therefore chosen to encapsulate the cells.

3.3.2. The impact of size on microcapsule stability

In order to compare the influence of microcapsules size on microcapsule stability, alginate-PLL microcapsules were produced using Manucol DH alginate beads of different size and the mechanical resistance compared using the method previously reported by Bartkowiak and Hunkeler (2000). A direct comparison of the microcapsule mechanical resistance based on the bursting force values is impossible as the differences in microcapsule diameter influence the size of the microcapsule contact surface (*S*) during the deformation, resulting in forces that do not reflect the microcapsule internal pressure during compression (Bartkowiak and Hunkeler, 2000). It was assumed by Bartkowiak and Hunkeler (2000) that at high deformation, the shape of the deformed microcapsule is similar to that of a flat cylinder with a surface *S*.

As there is no change in microcapsule total volume, the contact surface *S* can be expressed as a relationship between the initial radius of the microcapsule *r* and the percentage of deformation d_f. In order to have comparable results, it was decided that the percentage of deformation would be 50%. The contact surface *S* and the microcapsule internal pressure *P* can be calculated following the equation below (Bartkowiak and Hunkeler, 2000) where *S* is the contact surface in mm^2, *r* is the microcapsule initial radius in *mm*, d_f is the percentage of deformation, *P* is the internal pressure in *kPa* and *F* is the force in *N.*

$$S = \frac{2}{3}\pi \frac{r^2}{\left(1-\left(\frac{d_f}{100}\right)\right)} \text{ and } P = \frac{F}{S}$$

On the basis of the calculations summarized in Table 3.3, it can be concluded that the mechanical resistance of the microcapsules increases with a reduction of the microcapsule diameter.

Table 3.2 : Mechanical properties of different size microcapsules

Caps. rad. *r* [mm]	**Force *F* [N]**	**Mem. Thick. [mm]**	**Deformation d_f [%]**	**Surface *S* [mm^2]**	**Pressure *P* [kPa]**
0.342	$1.69\ 10^{-3}$	0.019	50	0.49	$3.45\ 10^{-3}$
0.238	$1.52\ 10^{-3}$	0.019	50	0.23	$6.41\ 10^{-3}$

3.3.3. The influence of the cell culture medium on microcapsules stability

Microcapsule stability has been reported to be dependent on the gelling/non-gelling cation ratio in cell culture media (Beate Thu et al., 1996; B. Thu et al., 1996). It is therefore important to understand how the microcapsules behave in different cell culture media and what the influence of medium composition on microcapsule stability is. Investigating whether the microcapsules can be stabilized by addition of calcium ions in the cell culture media is an important step, however stabilisation must not have a negative effect on cell viability due to calcium toxicity or gel density. The toxicity of calcium will therefore be investigated on suspension cultures while the influence of stabilisation will be analysed using encapsulated cultures.

Table 3.3 : Estimation of the salt composition of non- defined media (DHFR) from different salt concentrations found in some chemically defined mammalian cell culture media. The ratio of gelling/non gelling cations is estimated at 1:109.

Salt concentration [mmole/L]	MEM	DMEM	IMDM	RPMI 1640	F10	F12	McCoys5A	199	**Average**
$CaCl_2$	1.80	1.80	1.49	-	0.30	0.30	0.90	1.80	**1.20**
KCl	5.37	5.37	4.43	5.37	3.82	3.00	5.37	5.37	**4.76**
$MgSO_4$	0.81	0.81	0.81	0.41	0.62	-	0.81	0.81	**0.73**
NaCl	116.35	109.51	77.00	10.27	126.62	130.02	87.26	116.35	**96.67**
$NaHCO_3$	26.19	44.04	36.00	23.81	14.28	14.00	26.19	26.19	**26.34**
NaH_2PO_4	1.17	1.04	1.04	-	-	-	4.83	1.17	**1.85**

As DHFR medium is a chemically non-defined cell culture media, an estimation of its composition in gelling and non-gelling ions has been made from the known composition of different cell culture media supplied by Sigma Aldrich. This estimation is illustrated by Table 3.4.

Table 3.4 : Added calcium chloride (mmol/L) to DHFR medium

DHFR medium	
Gelling/non-gelling cations ratio	Added $CaCl_2$ concentration (mmol/L)
1:10	11.0
1:20	4.6
1:35	3.4
1:50	1.31
1:70	0.7
1:109	0.0

From the values in Table 3.4, the non-gelling cation concentration is estimated at 130 mM and the gelling cation at 1.2 mM, the corresponding divalent to monovalent cations ratio being 1:109. From these values, the EX-CELL CHO $DHFR^-$ media was supplemented with calcium chloride according to Table 3.5.

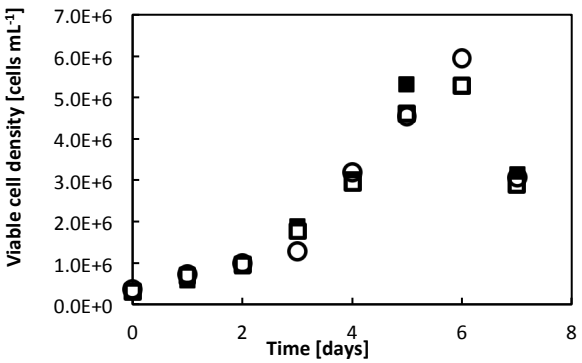

Figure 3.4: Comparison of the viable cell density evolution of Ca^{2+} supplemented suspension cultures. The VCD in the culture without calcium is represented by the empty spheres and has a μ of 0.49d^{-1}, the VCD in the culture with 4.6mmol/L $CaCl_2$ is represented by full squares and has a μ of 0.56d^{-1} and the VCD in cultures with 3.4mmol/L $CaCl_2$ is represented by the empty squares and has a μ of 0.54d^{-1}.

It can be seen in Figure 3.5 that suspension cells develop at the same rate with or without supplementation of the culture media with $CaCl_2$ and that cell growth is not affected by the addition of calcium to suspension cell culture medium, over the range of concentrations studied.

Table 3.5 : Growth rate (μ) comparison between the Ca^{2+} supplemented encapsulated cultures. The Ca^{2+} concentrations are expressed on a x-y form, x being the concentration of Ca^{2+} in the inoculation medium while y is the concentration of Ca^{2+} in the perfusion feed.

Added $CaCl_2$ concentration (mmol/L)	Growth rate, μ [day^{-1}]
0-0	0.7
0-4.6	0.59
0-3.4	0.56
0-1.31	0.65
0-0.7	0.6
4.6-4.6	0.54
3.4-3.4	0.52

Table 3.6 shows a comparison of the different growth rates obtained during encapsulated cultures with different calcium concentrations added in the feed and in the medium at the beginning of the culture. It can be seen than when calcium is added form the beginning of the culture the growth rate is slightly slower than when no calcium is added in the media at the beginning. Moreover it can also be observed that

the growth rate when calcium is added in the feed is slightly slower than the growth rate obtained in the flask with no calcium addition. It can, however be said that by comparing the different growth rates that μ is independent of the calcium concentration added.

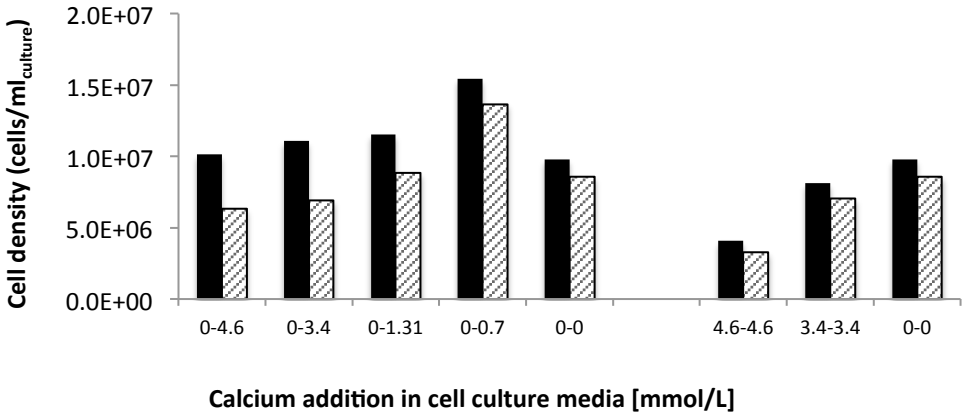

Figure 3.5 : Maximum cell density per mL culture obtained in different tested Ca^{2+} supplemented enapsulation culture. The total cell density is represented by the full bars while the viable cell density is represented by the dash-lined bar.

The highest cell densities were obtained in the flasks where 1.31mmol/L $CaCl_2$ and 0.7mmol/L $CaCl_2$ were added (Figure 3.6). This can be explained by the fact that the microcapsules in the flasks with no addition of calcium started to disintegrate and the cells were therefore washed out with the media change.

Figure 3.6 show that the addition of 0.7mmol/L calcium chloride gave the optimal growth rate, however the increase in strength is not significant when compared with the strength before the feed (Figure 3.7), therefore a concentration of 1.31mmol/L calcium chloride was chosen as a significant increase in growth rate and stabilisation can be observed.

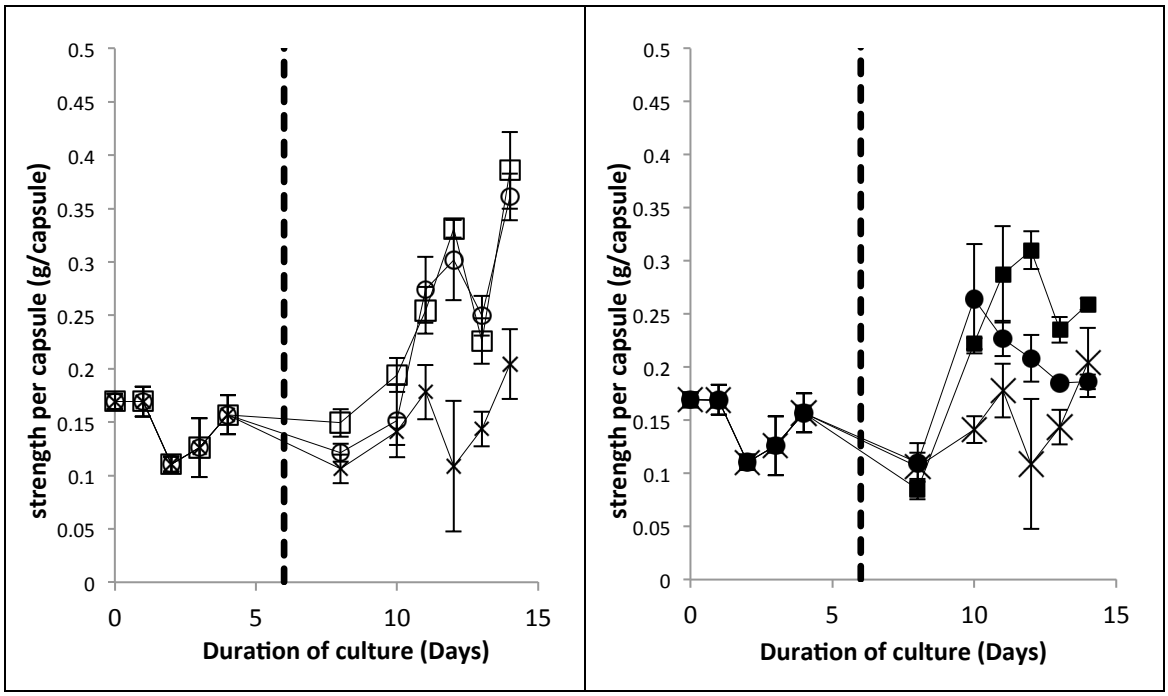

Figure 3.6 : Microcapsule mechanical resistance as a function of time depending on the monovalent to divalent ratio in the feed. The burst force is given in g per microcapsule and the error bars represent the standard error of the mean (n=5). The crosses represent the medium without calcium addition, on the left, the empty squares represent medium with an addition of calcium chloride of 4.6mmol/L, the empty spheres represent medium with an addition of calcium chloride of 3.4mmol/L, on the right full squares represent an addition of 1.31mmol/L and the full spheres an addition of 0.7mmol/L

3.3.4. Results summary

It has been shown in the previous sections that the most suitable alginate to produce alginate PLL microcapsules was Manucol DH and that the mechanical resistance of the microcapsules increased with reduced microcapsule diameter, suggesting that microcapsules produced with a 200µm nozzle will be stronger that microcapsules produced with a 300µm nozzle. The influence of cell culture media on microcapsule stability was investigated and the microcapsules stabilised with no negative effect on the encapsulated cells by the addition of 1.31 mmole/L of calcium chloride in the feed culture medium, which correspond to an estimated gelling:non-gelling ions ratio of 1:50. It can therefore be concluded that the most stable microcapsules to run a bioreactor culture would be produced extruding manucol DH alginate through a 200µm nozzle. The cell culture medium will then need to be supplemented with 1.31mmol/L of calcium chloride in order to have the optimal gelling/non-gelling ions ratio. Calcium chloride should be added from the beginning of the culture as it will reduce the microcapsules swelling and stabilise the microcapsules during the batch phase. An addition of calcium chloride will also have to be set-up in parallel of the

alkali inlet to compensate the Na^+ ions that are being fed into the reactor due to pH control and to maintain the ratio of 1:50 into the reactor throughout the culture.

3.4. Results - Part II: An understanding of potentials and limitations of alginate-PLL microcapsules as a cell retention system for perfusion cultures

3.4.1. Microcapsule characterisation in batch and perfusion cultures

In order to investigate the possibility of using alginate-PLL-alginate (APA) microcapsules as a retention method for perfusion cultures, encapsulated batch cultures were first set- up as a reference to see if cells can be cultured in microcapsules under the same conditions, cell density and growth kinetics as suspension cultures. The batch culture duration was 14 days and it was observed that the hydrogel structure was partially or totally damaged at the end of the culture. The microcapsule resistance (Figure 3.8) was shown to decrease from 0.2g capsule^{-1} to 0.03g capsule^{-1}, with the lowest values obtained within 5-14 days. A loss in mechanical resistance of 50% was observed within 2 days of inoculation and 70% within 5 days. Studies have shown that alginate is highly sensitive to the presence of monovalent ions, such as Na^{+} or K^{+}, during the gelling process using Ca^{2+} (Draget et al., 1998; Martinsen et al., 1989; Sobol et al., 2013; B. Thu et al., 1996a, 1996b). A constant displacement of the equilibrium between the medium and the microcapsules is expected which might be destructive for the microcapsules (Breguet et al., 2007). The displacement of Ca^{2+} by other non-gelling ions present in the medium together with a separation of PLL from the hydrogel would therefore be predicted to cause microcapsule destabilisation and lead to partial destruction of the hydrogel. Due to the poor stability, the alginate-PLL polyelectrolyte system was shown to be limited to a duration of less than 15 days for the cultivation of cells, under the conditions employed.

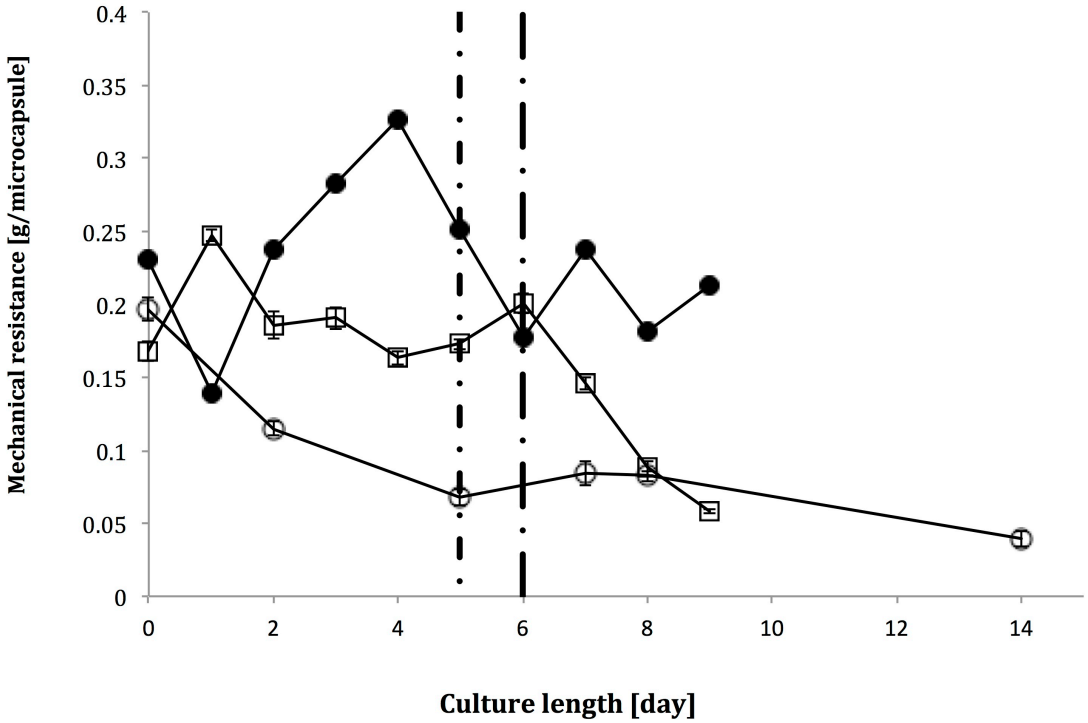

Figure 3.7 : Mechanical resistance of microcapsules as a function of time in encapsulated batch (empty spheres), fed-batch (filled spheres) and perfusion (empty squares) cultures. The burst force is given in g per microcapsule and the error bars represent the standard error of the mean (n=5). The feed started at day 5 for the encapsulated perfusion culture and at day 6 for the encapsulated fed-batch culture.

By finely regulating the calcium ions in the medium, the Ca^{2+}/Na^{+} ratio was maintained at an estimated ratio of 1:50 thereby stabilising the microcapsules during the batch phase of the perfusion culture. Once the feed started however, a continuous loss in mechanical resistance (Figure 3.8) was observed, the resistance decreasing from 0.2 g $microcapsule^{-1}$ to 0.05g $microcapsule^{-1}$. Moreover, a 25% loss in mechanical resistance was observed after 1 day of feed in which the medium was replaced 2.7 times. The hydrogel structure of the microcapsules was partially or totally damaged after the third day of medium feed leading to the release and wash out of the cells. Due to the low stability, the alginate-PLL polyelectrolyte system has been clearly shown to be unsuitable for perfusion culture operation however, stabilisation with calcium ions indicates that this system may be suitable for encapsulated batch culture and fed-batch cultures.

The stabilisation of the microcapsules with calcium ions enabled a fed-batch culture that lasted 9 days, with a batch phase of 6 days, without significant loss in microcapsule stability (Figure 3.8). The dilution due to the feed was negligible (1.01-fold) which should not affect the equilibrium between the microcapsules and the medium in terms of PLL and gelling ions. In order to supply nutrient feed over a longer

duration however, a higher dilution should be tested and the microcapsule stability would be expected to decrease by a maximum of 25% with a 2.7x dilution, as observed with the perfusion culture (Figure 3.8).

The microcapsule radius remained stable throughout the encapsulated batch, fed-batch and perfusion cultures (Figure 3.9). It is however interesting to notice that the initial radius of the microcapsules in the batch culture is 1.45- fold higher than for the perfusion culture and 1.3 fold higher than for the fed-batch culture. This is probably due to no Ca^{2+} ions being added to the medium to stabilise the microcapsules in the batch culture, which leads to a swelling of the microcapsules as they adapt to the culture conditions. The thickness of the PLL-membrane also remains stable at average of 16.88 ± 0.70µm during the culture.

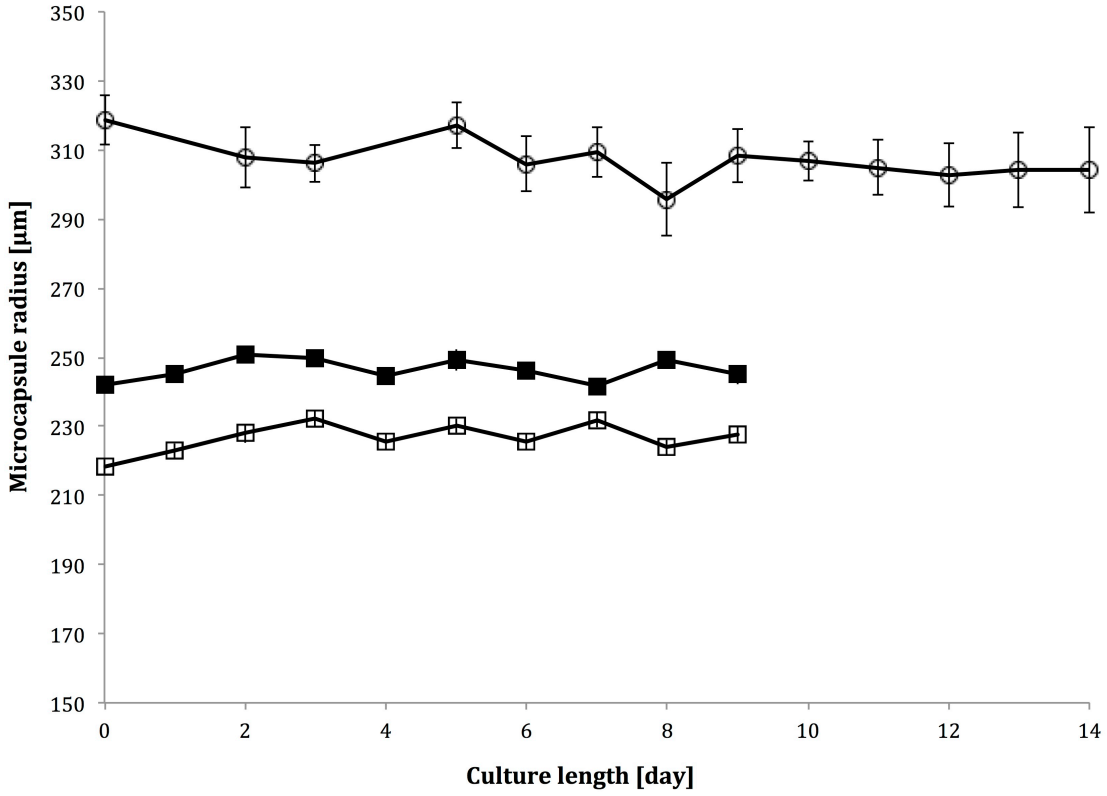

Figure 3.8 : Size of microcapsules as a function of time during encapsulated batch (empty spheres), fed-batch (full squares) and perfusion (empty squares) cultures. The size of the microcapsules is given in µm and the error bars represent the standard error of the mean (n=30).

Since microcapsules were used to culture CHO-DP12 cells, which produce a recombinant IgG, it is important to test the molecular weight cut-off (MWCO) of the microcapsule membrane in order to determine whether the IgG will be retained by the polyelectrolyte membrane or if it will diffuse to the outside. The MWCO determination

was performed using fluorescent-labelled dextran and fluorescent-labelled IgG, and both showed that the protein should diffuse through the microcapsule membrane in batch, fed-batch and perfusion cultures (Figure 3.9).

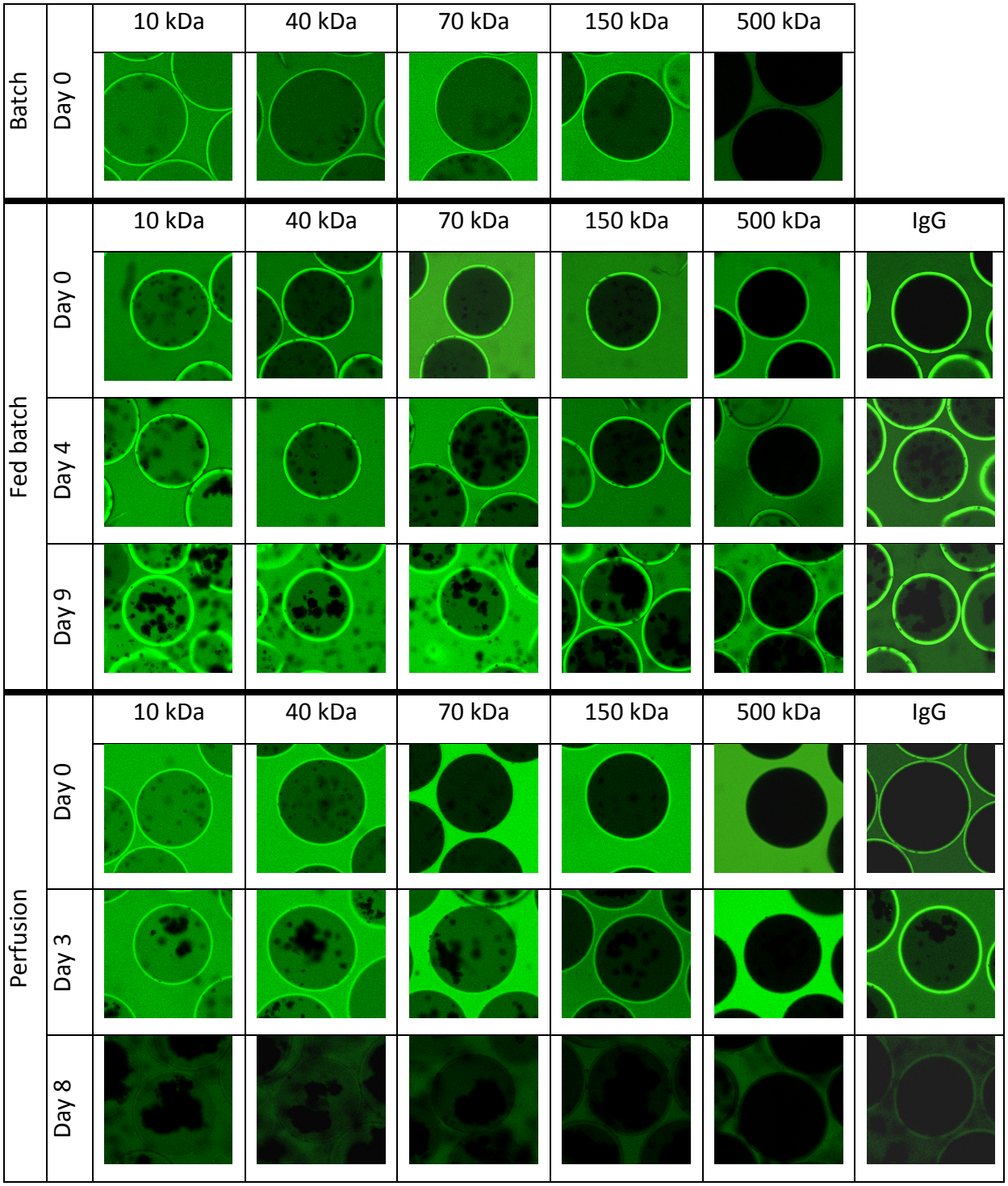

Figure 3.9 : Determination of the molecular weight cut off in the encapsulated bioreactor cultures

In this part of the work it was demonstrated that APA microcapsules can be stabilized by the addition of Ca^{2+} to the medium and medium feed to be used as a growing/retention system for cells in a fed-batch culture. Since the CHO cell line is suspension adapted, there is no technical advantage to growing them in

microcapsules. However, APA microcapsules were shown to be a successful retention and culture system for batch and fed-batch cultures.

3.4.2. Cell growth in suspension and encapsulated batch and perfusion cultures

The bioreactor was inoculated at an initial cell concentration of 1 x 10^5 cells $mL^{-1}_{culture}$ for the encapsulated culture and 2.4 x 10^5 cells $L^{-1}_{culture}$ for the batch suspension cultures. The batch suspension culture was performed under the same conditions as the encapsulated cultures. The encapsulated perfusion culture was grown in batch mode for 5 days before initiation of a medium feed of 1.7L d^{-1}. The encapsulated fed-batch was grown in batch mode for 6 days before initiation of the medium feed and had a starting volume of 1.4L. No lag phase was observed for the suspension batch, encapsulated fed-batch and encapsulated perfusion cultures, however a lag phase of 6 days was observed for the encapsulated batch culture. This lag phase can explain the lower cell concentrations reached in the encapsulated batch culture compared to the suspension batch culture as some nutrients were consumed by the cells during the lag phase (Figure 3.10).

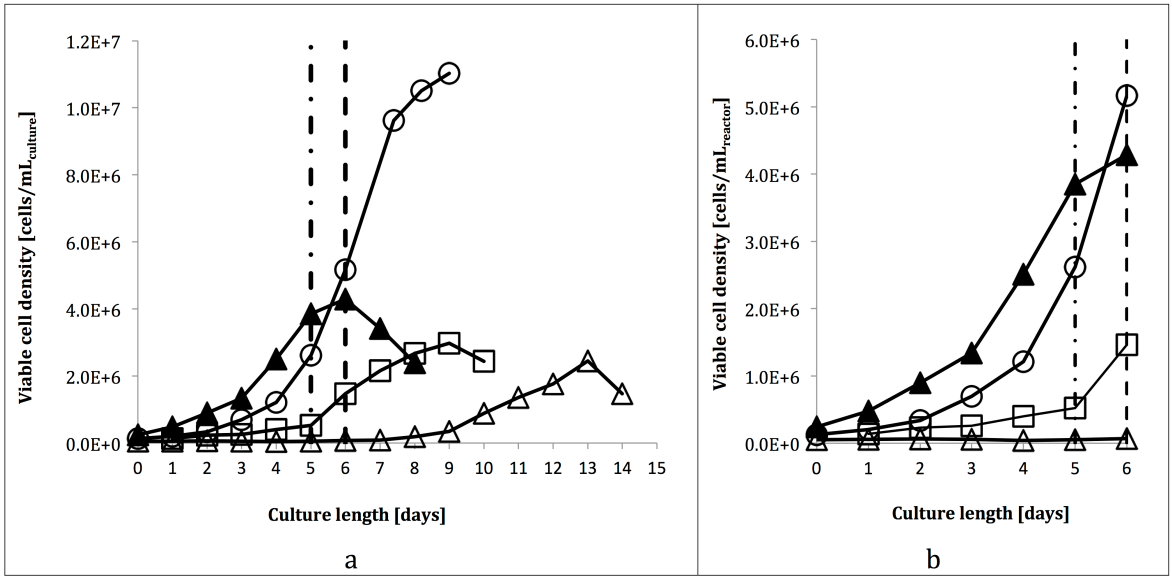

Figure 3.10 : (a) Growth of CHO cells in suspension batch culture (filled triangles), encapsulated batch (empty triangles), fed batch (empty squares) and perfusion (empty spheres) culture. (b) Growth of CHO cells in suspension batch culture (filled triangles), encapsulated batch (empty triangles), fed batch (empty squares) and perfusion (empty spheres) culture from day 1 to 6 of the culture. The culture was inoculated with 0.5 x 10^5 cells/mLculture for the encapsulated batch, 1.3 x 10^5 cells/mLculture for the encapsulated fed-batch and perfusion cultures and with 2.4 x 10^5 cells/mL for the suspension batch. The medium feed started at day 5 for the encapsulated perfusion and at day 6 for the encapsulated fed-batch.

The growth of the encapsulated cells in batch culture can be divided into 3 distinct phases: an exponential growth phase followed by a stationary phase and a decline phase. For the encapsulated perfusion, two growth phases can be observed, an exponential phase and a linear growth phase. The linear growth phase is however, not due to a decrease in cell growth and viability, but to a loss of cells into the feed due to microcapsule breakage since a viability of more than 90% was found at the end of the culture in the microcapsules. Under fed-batch conditions, 4 distinct phases can be observed, an exponential growth phase followed by a linear growth phase once the feed started, a stationary phase and finally a decline phase (Figure 3.10).

Table 3.6 : Kinetic and stoichiometric parameters of suspension and encapsulated CHO cells cultures

Value	Units	Susp. batch	Encapsulated batch	Encapsulated perfusion	Encapsulated fed-batch	**Perfusion with ATF by Clincke et al. (2013a)**	**Perfusion with TFF by Clincke et al. (2013a)**
Microcapsule radius	(μm)	-	308 ± 2	227±1	246±1	-	-
μ	(h^{-1})	0.023	0.024	0.028	0.022	0.01375	0.01167
Culture duration	(h)	192	336	216	216	307.2	1144.8
$X_{culture,\ max}$	(cell $mL^{-1}_{culture}$)	5.17×10^{6}	4.19×10^{6}	1.94×10^{7}	5.03×10^{6}	1.37×10^{8}	2.24×10^{8}
$X_{caps,\ max}$	(cell mL^{-1}_{caps})	-	1.21×10^{7}	3.67×10^{7}	1.26×10^{7}	-	-
Max cells	(cell $caps^{-1}$)	-	1.48×10^{3}	1.8×10^{3}	786	-	-
Colonisation	(%)	-	1.65	5.33	1.8	-	-
q_s	(mol $cell^{-1}$ h^{-1})	1.78×10^{-13}	2.01×10^{-13}	2.18×10^{-13}	3.95×10^{-13}	-	-
$q_{lactate}$	(mol $cell^{-1}$ h^{-1})	2.70×10^{-13}	2.46×10^{-13}	2.11×10^{-13}	4.05×10^{-12}	-	-
q_{NH3}	(mmol $cell^{-1}$ h^{-1})	2.34×10^{-11}	3.15×10^{-11}	6.63×10^{-11}	8.17×10^{-11}	-	-
q_p	(pg $cell^{-1}$ h^{-1})	0.43	1.65	2.11	0.89	-	-
C_{IgG}	($mg_{IgG}/mL_{culture}$)	0.085	0.13	0.11	0.22		
$Y_{X/S}$	(cell mol^{-1})	1.33×10^{11}	1.19×10^{11}	1.28×10^{11}	5.59×10^{10}	-	-
$Y_{lactate/S}$	(mol mol^{-1})	1.48	1.22	1.24	1.25	-	-

Symbols: $X_{culture,\ max}$ the maximum total cell density obtained per $mL_{culture}$; $X_{caps,max}$ the maximum total cell density obtained per $mL_{capsule}$; Max cells the maximum total amount of cells per capsule; μ the specific growth rate; q_s the specific consumption of glucose; $q_{lactate}$ the specific productivity of lactate; q_{NH3} the specific productivity of NH_3; q_p the specific productivity of IgG; $Y_{X/S}$ the yield of biomass with respect to glucose; and $Y_{lacatate/S}$ the yield of lactate with respect to glucose; C_{IgG}, the concentration of IgG in the bioreactor at the end of the culture. The yields for the perfusion culture were calculated for the batch phase only since the loss in cells due to capsule breakage made it impossible to estimate accurately.

The suspension cells grew to a maximum cell density ($X_{culture,max}$) of 5.17×10^{6} cells/$mL_{culture}$ and the encapsulated batch to a maximum cell density of 4.19×10^{6} cells/$mL_{culture}$ (1.21×10^{7} cells/$mL_{capsule}$) with similar growth rates, μ, (0.023 h^{-1} for the suspension batch and 0.0.024 h^{-1} for the encapsulated batch) (Table 3.7). This result shows that there is no negative effect of the immobilisation of the cells in alginate beads on cell growth. In perfusion culture, the intact microcapsules were totally retained within the bioreactor and became colonized with cells to a maximum concentration of 1.94×10^{7} cells/$mL_{culture}$ (3.67×10^{7} cells/$mL_{capsule}$) (Table 3.7). These values are considerably higher than those reported by Breguet et al (2007). In fed-batch culture, an exponential feed was implemented and a total dilution of 1.01-fold was reached in the bioreactor by the end of the culture. A maximum cell density of 5.03×10^{7} cells/$mL_{culture}$ (1.26×10^{7} cells/$mL_{capsule}$) was achieved before the cells

entered the stationary phase (Table 3.7). The choice to keep the dilution of the bioreactor volume low was made in order to keep the microcapsules as stable as possible, which led to an accumulation of metabolic by-products such as lactate (68mM) and ammonia (7.7mM) that could have become inhibitory for cell growth (Lao and Toth, 1997; Ozturk et al., 1992; Schneider et al., 1996). Indeed it was shown by Lao and Toth, (1997) and Ozturk et al., (1992) that lactate had a significant inhibitory effect on cell growth at concentrations above 50mM. The increase in cell density between encapsulated batch and fed-batch was not significant enough to show an advantage in culturing cells under fed-batch operation under these conditions. It is also interesting to notice that the encapsulated cultures have a higher growth rate (μ) than the perfusion culture performed by Clincke et al. (2013a) (Table 3.7), showing therefore that compared to other existing systems, encapsulation has no negative effect on cell growth.

The yield of lactate to glucose (Table 3.7), $Y_{lact/s}$, for the encapsulated culture (1.22 mol mol^{-1} in batch, 1.24 mol mol^{-1} in perfusion and 1.25 mol mol^{-1} in fed-batch) was 16% lower than for the suspension batch culture (1.48 mol mol^{-1}). This result indicates a more efficient utilization of glucose via respiration in encapsulated cultures compared with suspension cultures, which is in agreement with Breguet et al., (2007). A 3.8-fold higher IgG productivity, $q_{p,}$ (Table 3.7) was observed between suspension (q_p= 0.43 pg $cell^{-1}$ h^{-1}) and encapsulated batch cultures (q_p= 1.65 pg $cell^{-1}$ h^{-1}), which clearly indicates that microencapsulation significantly stimulated IgG production. This is further confirmed by the finale concentration of IgG in the culture, C_{IgG}, which shows that the finale concentration of IgG in the culture is bigger in encapsulated batch than suspension batch.

A low colonisation of the microcapsules may be observed in perfusion culture (5.3% of the theoretical maximum cell density). This low colonisation may be due to microcapsule instability, but may also be an indication that the cells were not able to grow freely within the core of the microcapsules as suggested by Breguet et al (2007). The similar value of the specific consumption rate of glucose, $q_{s,}$ and the specific productivity of lacate, $q_{lactate}$, between the encapsulated and suspension cultures however suggests that there was no significant diffusional limitation and that the low

colonisation was mainly due to cell leakage from broken or damaged capsules (Table 3.7).

Microcapsules were shown to enable cell culture in bioreactors, in batch, fed- batch and perfusion modes, with no significant influence of cell entrapment on cell growth. A more efficient consumption of glucose via respiration was observed in microencapsulated cultures compared to suspension cultures, together with a significant increase in productivity. The microcapsules were retained in the bioreactor using a simple gauze for a separation device and were shown to be capable of reaching high cell densities, comparable to those obtained with suspension perfusion cultures employing conventional cell separation devices such as spin-filters or tangential flow filtration systems (Clincke et al., 2013a, 2013b; Gugerli, 2003). The main advantage of this system is, however, that even higher cell densities may be obtained in bioreactors by simply increasing the volume of capsules in the bioreactor from the 25% used in this study to the maximum estimated reactor loading for a stirred tank reactor of 75%.

Through continuous on-line monitoring using dielectric spectroscopy, it was possible to follow the growth of the encapsulated cells in real time. The results (Figure 3.11) demonstrate that dielectric spectroscopy could be an efficient way to measure encapsulated biomass using a non-destructive method. The correlation between capacitance measurements and viable cell density for both encapsulated and suspension cultures were very good, showing that there was no influence of the microcapsules on the dielectric measurement.

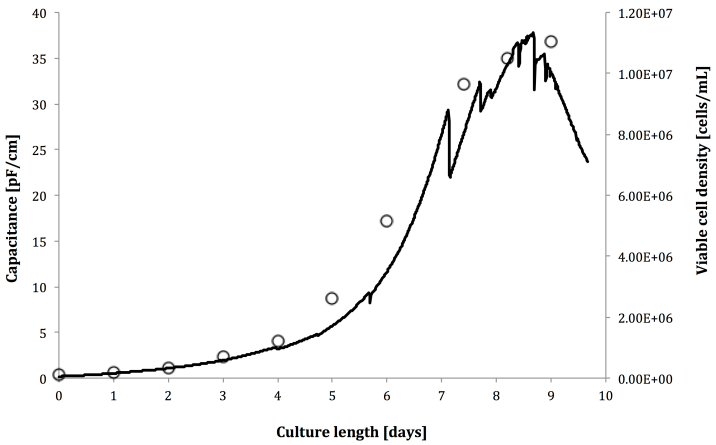

Figure 3.11 : Continuous on-line monitoring of viable cell density with dielectric spectroscopy in an encapsulated perfusion culture. The growth of the cells inside the microcapsules (empty spheres) can be followed online by capacitance measurements (solid line)

3.5. Discussion

In the present study, the possibility of using alginate-PLL-Ca^{2+} system to encapsulate cells in order to grow them in CSTR bioreactors has been evaluated. Due to the mild encapsulation process, the alginate-PLL-Ca^{2+} system was found to possess the required properties to allow cell encapsulation without damaging the cells, as previously shown by Breguet et al. (2007). The microcapsules however, do not possess the necessary stability to protect against the conditions prevailing in the bioreactor due to the dependence of stability on the medium ionic composition, bead size and type of alginate.

It was previously demonstrated that the stability of PLL-microcapsules is influenced by the alginate composition, since high mannuronic alginates ensured a rapid binding of PLL during the coating step resulting in a much thicker PLL layer (Huebner and Buccholz, 2002). The choice of alginate is therefore an important step in microcapsule development in order not to influence stability in a negative way. Manucol DH alginate, with a M/G ratio of 1.56 (Chan et al., 2011), was therefore chosen to be the most suitable alginate for alginate-PLL formation (Chan et al., 2011).

The influence of microcapsule size has previously been investigated by Strand *et al*. (2002). These authors showed that reducing the microcapsule size from 700µm to a diameter of 500µm resulted in increased capsule stability, further reducing the diameter to 200µm lead to a collapse of the capsules during the PLL coating step (Strand et al., 2002). Bartkowiak and Hunkeler (2000) also investigated the influence of microcapsules size on microcapsule stability and their results are in accordance with those reported by Strand et al. (2002). It was therefore decided to produce microcapsules using a 200µm nozzle, resulting in microcapsules with an external diameter of 500µm to provide the optimum strength and stability. It was moreover demonstrated by Breguet et al., (2007) that decreasing the microcapsule size had no significant influence on the cell growth rate and on the biomass yield $Y_{X/S}$.

The binding of PLL to the extruded alginate bead is governed by the affinity of PLL for alginate and by the density of negative charges on the surface (Strand et al., 2002; B. Thu et al., 1996a, 1996b). Thus Thu *et al.* (1996a, 1996b) suggested that inhomogeneous beads will have better PLL binding than homogenous beads, due to a

higher charge density on the surface of the beads. By reducing the bead size, the surface area to volume ratio increases resulting in more alginate surface, the result being a more dense alginate-PLL network and increased stability (Strand et al., 2002; B. Thu et al., 1996a, 1996b).

Commercially available alginates have been shown to be highly sensitive to the presence of monovalent ions, such as Na^+ or K^+, during the gelling process using Ca^{2+} (Draget et al., 1998; Martinsen et al., 1989; B. Thu et al., 1996a, 1996b). Draget *et al*. (1998) showed that the addition of KCl and NaCl in the buffer solution results in decreased alginate gel strength, with the effect more pronounced for NaCl than for KCl (Draget et al., 1998). Microcapsule stability was therefore expected to be dependent on the presence of non-gelling ions, such as Na^+, in the culture medium as they compete with Ca^{2+} and PLL for the alginate, resulting in a destabilization of the microcapsule core. This displacement of Ca^{2+} by other non-gelling ions present in the medium feed during perfusion operation would therefore be predicted to influence capsule stability provoking a loss in capsule strength and rupture of the capsules. Consequently by regulating the gelling/non-gelling ion ratio during the perfusion culture would be expected to solve the microcapsule stability problem by maintaining the equilibrium between sorption/desorption of Ca^{2+} even when significant amounts of NaOH are added to control the pH. Although it was possible to improve the stability of the microcapsules during the batch phase of the perfusion, the loss of PLL from the alginate complex resulted in loss of stability. Such desorption of PLL has been reported by Gåserød et al. (1998, 1999) where a loss of 20-40% PLL occurred when microcapsules were incubated in a saline solution (Gåserød et al., 1998, 1999). Consequently, it is not feasible to carry out perfusion cultures for any significant duration since, while Ca^{2+} desorption can be overcome by supplying Ca^{2+} to maintain a constant monovalent: divalent ratio, it is not possible to overcome the desorption of PLL. As a result alginate-PLL microcapsules may be suitable for batch and single-cycle fed-batch cultures where there is no net PLL removal from the culture. However, there is little reason to culture suspension adapted cell lines as encapsulated cultures under batch or fed-batch conditions, since the results clearly show that similar results are obtained for both suspension and encapsulated cultures. This does open the possibility of using the encapsulation system developed here for the cultivation of adherent cell lines or those which are particularly sensitive to the shear forces found in bioreactors

(Castilho et al., 2008). In the perfusion cultures supplemented with Ca^{2+}, the maximum cell density attained was $1.94 \cdot 10^7$ cells/$mL_{culture}$, whereas the maximum theoretical cell density that could be attained, assuming complete capsule colonisation and stability was estimated to be $1.34 \cdot 10^8$cells/$mL_{culture}$ for a culture in which the microcapsules represent only 25% of the culture volume. Consequently, the cell density attained represents only 5.33% capsule colonisation. A number of literature reports concern perfusion cultures of CHO cells involving a range of cell retention systems including spin filters, tangential flow filtration, continuous centrifugation and acoustic-filters. Gugerli *et al.* reported a maximum cell density of $2.8 \cdot 10^7$ cells/$mL_{culture}$ for CHO SSF3 cells using an acoustic filter (Gugerli, 2003). Clincke et al, using a tangential flow filtration system obtained a maximum cell density of $2 \cdot 10^8$ cell/ mL with a μ of 0.01167 d^{-1} (Clincke et al., 2013a, 2013b). The cell density obtained in this work ($1.94 \cdot 10^7$ cells/ $mL_{culture}$ with a μ of 0.028 d^{-1}) therefore clearly shows that microcapsules have potential as a new cell retention system, since this corresponds to 69% of the maximum cell density obtained by Gugerli *et al*. and almost 10% of the cell density obtained by Clincke *et al*. (2013a, 2013b), moreover, the growth rate of the cell in encapsulated culture is similar to the growth rate of the cells in suspension, and twice as high as the growth rate published by Clincke et al. (2013a, 2013b). These results were obtained despite the limited mechanical stability, which prevented full capsule colonisation being obtained. Through the development of microcapsules from alternative materials to alginate-PLL with stronger polyelectrolyte interactions which allow the cells to grow freely but are sufficiently stable to resist bioreactor shear stresses and the medium composition, a maximum cell density of $1.8 \cdot 10^8$ cells/$mL_{culture}$ should be obtainable with 25% capsule: media ratio. This value is very close to that reported by Clincke *et al*. (2013a, 2013b). Furthermore, by increasing the microcapsule: medium ratio in the reactor to the maximum of 75% microcapsules volume the maximum cell density that it should be possible to obtain is between 3-4 x 10^8 cells/$mL_{culture}$. It is important to point out that both the cell density per mL of culture and the colonization of the microcapsules depend on the amount of microcapsules used in the reactor. As the media can support the growth of a known amount of cell in batch mode, decreasing the amount of capsules will lead to a lower initial cell density per mL culture at the beginning of the culture, while the initial cell concentration per mL capsule will remain constant, therefore the cells will grow until

they reach the maximum cell density that the medium can support, leading to a higher cell density per mL capsules while the final cell density per mL culture will remain the one that the media can support. The degree of microcapsule colonisation would be expected to be higher with a smaller volume of capsules in the culture than with a high volume of capsules in the culture for a batch culture. As the cells are retained within the microcapsules, they cannot grow further than the volume available within the core of the microcapsules. The microcapsules are therefore limiting the maximum cell density possible in the culture due to the physical entrapment of the cells. Increasing the amount of microcapsules in the culture would therefore result in an increase in the cell density with respect to reactor volume.

3.6. Conclusion

It has been shown that APA microcapsules could be used to culture mammalian cells in bioreactors and that microcapsule stability can be maintained during a batch culture, however the poor stability and dependence on medium composition will become a problem during long duration perfusion cultures where gelling ions and PLL will be replaced by non-gelling ions and washed-out through the feed. In order to reduce capsule breakage and run a perfusion culture, the culture medium composition needs to be adapted to an appropriate gelling/non-gelling ion ratio to maintain capsule stability. The improvement in capsule stability by addition of calcium during the culture is however, not important enough to counteract the replacement of PLL by non-gelling ions. As the addition of PLL during the culture is not feasible, APA microcapsules cannot be stabilized sufficiently to use them in a perfusion cultures. However it could be an option to grow adherent cells in CSTR bioreactor in batch or fed-batch modes, which do not involve medium replacement. Since the medium composition has such a large influence on microcapsule stability, it will nevertheless be difficult to implement and very risky to use APA microcapsules as a microreactor for mammalian cell culture in CSTR bioreactors. Nevertheless microencapsulation as a perfusion tool shows a good potential since the maximal cell density obtained in suspension perfusion cultures with CHO cells was approximately $2 \cdot 10^8$ cells/mL using an alternating tangential flow system. This cell concentration could theoretically be reached with a reactor containing only 50% by volume microcapsules. Other alginate-polyelectrolyte microcapsule combinations should be investigated to overcome the instability issues with perfusion cultures employing high medium feed rates over extended time intervals.

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4. Optimisation and characterisation of Alginate-poly-L-ornithine microcapsules for cell culture purposes

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Abstract

Alginate-poly-L-lysine-alginate microcapsules have been widely used in cell microencapsulation, however the lack of mechanical resistance and stability under culture conditions has stimulated the study of alternative polycations. In this chapter, microcapsules coated with poly-L-ornithine (PLO) were developed, characterized and compared with alginate-PLL-alginate microcapsules in order to understand whether these new microcapsules could be used as a new retention system for perfusion cultures. The microcapsules could be easily retained in a standard stirred tank bioreactor without the need for a cell separation device resulting in high cell densities with a maximum of $1.1 \cdot 10^8$ cell/$mL_{capsules}$, corresponding to a colonisation of 14.6% of the internal capsule volume, representing a two-fold increase over microcapsules produced from PLL. Unlike PLL-containing microcapsules, the PLO-containing ones were shown, through measurements of microcapsule integrity and mechanical resistance, to be stable under perfusion culture conditions. However, despite the continuous change of media apparently having little effect on microcapsule stability, the microcapsules were shown to be unsuitable for perfusion cultures, since the higher level of colonisation of the microcapsules by the cells (14.6%) resulted in microcapsule breakage.

4.1. Introduction

The immobilization of living cells in microcapsules has been widely investigated for the past decades with potential applications ranging from bio-artificial organ to high cell density cultures (Bartkowiak and Hunkeler, 2000). The protocol developed by Lim and Sun (Lim, 1983; Lim and Sun, 1980) has been dominant for cell microencapsulations for over 30 years and consists of the application of a polyamino acid layer on alginate microspheres gelled with a divalent cation such as Ca^{2+} (Bartkowiak and Hunkeler, 2000). Calcium alginate microcapsules coated with a polycation have been widely investigated for applications such as cell transplantation, enzyme immobilisation, drug release systems and high cell density cultures (Gåserød et al., 1999, 1998). Uncoated calcium alginate beads can be destabilised by chelators such as lactate, phosphate and citrate; or by non-gelling cations such as sodium or magnesium ions that will exchange with the calcium bound to the alginate. The polycation membrane is therefore required to bind with the negatively charged alginate through electrostatic interactions to stabilise the microcapsules (Gåserød et al., 1998). When microcapsules are used for high cell density cultures, the microcapsule membranes must be strong enough to resist the osmotic swelling pressure build-up within the microcapsules, bioreactor shear stresses and the effect of the medium composition. Numerous papers describe alginate polycation microcapsules permeability and mechanical resistance, however the capsule stability when the microcapsules are exposed to culture environments is rarely reported (Gåserød et al., 1998). The authors previously showed that alginate-PLL microcapsules could be used to culture mammalian cells in bioreactors and that microcapsule stability could be maintained during a batch culture, however the poor stability and dependence on medium composition became a problem for extended duration perfusion cultures where gelling ions and PLL are continuously replaced by non-gelling ions and washed-out with the medium outlet (Demont et al. 2015) Consequently in order to achieve microencapsulation of high cell density cultures, there is therefore a need to develop microcapsules from alternative materials to alginate-PLL with stronger polyelectrolyte interactions, which allow the cells to grow freely but are sufficiently stable to resist the medium and conditions present in bioreactors (Demont et al. 2015). Since microcapsules are intended to be used in bioreactor cultures, they must be capable of long-term stability in cell culture media or in physiological environments particularly since these media are usually rich in ions,

which can destabilize the integrity of the alginate gels or the polyelectrolyte interactions involved in the microcapsule integrity (Breguet et al., 2007; Gugerli, 2003). Some studies suggested that poly-L-ornithine (PLO) may have some advantages for microencapsulation such as a reduction of swelling and an increase in mechanical strength compared to the traditional use of poly-L-lysine (PLL) for coating alginate microcapsules (Darrabie et al., 2005), although other studies reported that replacing PLL by PLO did not result in a significant improvement (De Castro et al., 2005). Many procedures have been developed to produce microcapsules however; most are descriptions of the production process and lack details of characterization of the microcapsules properties. As a result there is no consensus in the properties of PLO-based microcapsule and many results have proven difficult to reproduce. This shows the importance of quantitative microcapsule characterisation (de Vos et al., 2009; Tam et al., 2011).

The present investigation aims to investigate the possibility to produce microcapsules suitable for perfusion culture using PLO to coat alginate beads and to compare them with alginate PLL microcapsules. The variation of molecular weight cut- off, burst force with time, the microcapsule stability in culture medium and integrity was quantitatively evaluated for encapsulated cells under batch and perfusion culture conditions to avoid lab-to-lab variation and allow reproducibility. The kinetics and stoichiometric parameters of CHO-DP12 cells have been determined for suspension batch cultures, encapsulated batch and perfusion cultures.

4.2. Material and Methods

Material and methods used in this chapter are described in section 2.2. Specific experiments set-up are described below.

4.2.1. Shake flask encapsulated cultures and stability tests

20mL of cell containing microcapsules or empty microcapsules were cultured in shake flask (Corning Inc, Corning, NY, USA), with a working volume of 100mL and a microcapsule volume representing 25% of the medium volume. The culture was then incubated at 37°C, under a humidified atmosphere of air containing 5% CO_2 and an agitation of 100rpm. For perfusion cultures, the microcapsules were allowed to settle

before a known volume of medium was aseptically removed with a sterile pipette and replaced with the same volume of fresh medium. Perfusion was performed once daily. Each culture and stability test carried out in shake flask were carried out only once

4.2.2. Bioreactor encapsulated cultures

Encapsulated cells were grown in a Minifors (Infors, Bottmingen, Switzerland) bioreactor, with a working volume of 1.2L for PLO microencapsulated culture and in a modified Mettler Toledo RC1 (Mettler-Toledo, Columbus, OH, USA) 2L biocalorimeter, with a working volume of 1.4L for PLL microcapsules. The bioreactor was equipped with a pH and temperature control with air sparging. The bioreactor was operated in perfusion mode at a temperature of 37°C, agitation of 100 rpm with a sparged airflow of 0.01 vvm and a headspace airflow of 0.2 vvm and 0.1 vvm CO_2. The pH was maintained at 7.2 by addition of NaOH and CO_2 to the headspace. For perfusion cultures, the volume was maintained constant by the use of a feed system, which added and removed media at the same rate. A 200µm nylon mesh was installed on the medium outlet port to avoid microcapsule removal in the outlet stream. A microcapsule volume of 25% of the medium volume was used for all bioreactor cultures. For encapsulated perfusion cultures with PLL-containing microcapsules in the bioreactor, a feed of $CaCl_2$ was applied in parallel to the NaOH pH control to maintain a 1:50 gelling/non-gelling ions ratio, the initial culture media was also supplemented with 1.31mmol/L $CaCl_2$. Each culture carried out in bioreactor were carried out only once.

4.2.3. Perfusion feeding strategy

Prior to perfusion, sampling of the cultures was performed. Based on the predetermined cellular growth rates, specific metabolite consumption and production rates, the concentration of glucose and glutamine required by the cells to maintain the growth rate was calculated. The adjustments in glucose and glutamine concentration in the fresh medium were made to meet the concentrations that would be required by an anticipated cell number which would be present in the culture on the following day based on the growth rate displayed by the cells. The required amount of anhydrous glucose (Sigma Aldrich, Saint Louis, Missouri, USA) and L-glutamine (Sigma Aldrich, Saint Louis, Missouri, USA) powder were then weighed and added to fresh medium.

The medium was then sterile filtered (0.2µm Steritop filters, Merck Millipore, Ireland) before being added to the culture.

4.2.4. Encapsulation

The encapsulation method used was a modification of the technique originally developed by Lim and Sun (2,3,13) undertaken under completely sterile conditions using a vibrating nozzle encapsulation device (Encapsulator Biotech, EncapsBioSystem Inc, Greifensee, Switzerland or Inotech IE-50R, Inotech, Greifensee, Switzerland) as described elsewhere (14). Calcium alginate beads were first formed by extrusion of 300mL pre-sterilised (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland) sodium alginate solution (1.5% w/v) (Manucol DH, FMC biopolymers, UK; Büchi Alginate, Büchi, Switzerland) through the encapsulator nozzle into an aqueous solution of $CaCl_2$ 110mM (Sigma, Saint Louis, Missouri, USA). After 5 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules incubated in the polyelectrolyte solutions (0.05% w/v poly-L-lysine 30-70kDa or 0.15%w/v poly-L-ornithine (Sigma, Saint Louis, Missouri, USA)) for 30 min at room temperature. The microcapsules were then washed with saline buffer containing 10mM MOPS followed by incubation in 0.03% sodium alginate for 10min. After washing with saline buffer containing 10mM MOPS, the solid alginate core of the microcapsules was liquefied by incubation for 5min in 0.05M sodium citrate (Sigma, Saint Louis, Missouri, USA). After core liquefaction with citrate, the microcapsules were washed with saline buffer followed by a wash with cell culture medium before transfer to cell culture medium to be inoculated. When cells were encapsulated, the cells ($0.7 \cdot 10^6$ $_v$cells/$mL_{alginate}$) were carefully mixed with the alginate solution prior to extrusion and bead formation followed according to the method above.

4.2.5. Microcapsule characterisation and culture analysis

Homogenous culture samples containing microcapsules were regularly removed from the bioreactor to determine intra/extra-capsular cell number, concentration of key culture metabolites, microcapsule size, mechanical resistance, molecular weight cut-off (MWCO) and membrane thickness using methods described in sections 2.2.2 and 2.2.3.

The microcapsules colonisation was estimated by first determining the amount of capsules within 1mL of capsules and then by calculating the amount of cell per capsule. The theoretical maximum cell concentration within the capsule was estimated afterwards by dividing the microcapsule volume by the volume of a cell. The microcapsule colonisation was then estimated by dividing the maximum theorical cell concentration within one capsule by the real cell concentration within one capsule. It was therefore assumed that both the cells and the microcapsules were capable of deformation to fill up the free spaces and were therefore not behaving like solid spheres.

4.3. Results – Part I: determination of the working window – Optimisation of the Alginate-PLO microcapsules

Alginate-PLO microcapsules have been reported to have some advantages for microencapsulation such as a reduction of swelling and an increase in mechanical strength compared to alginate-PLL microcapsules (Darrabie et al., 2005), however other studies also reported that replacing PLL by PLO did not result in a significant improvement (De Castro et al., 2005). As no detailed information is available about how the microcapsules where prepared and the types of alginates that were used, it was decided to investigate and optimize the composition of the microcapsules in detail before characterizing them under culture conditions. Two alginates of different M/G ratio were investigated in order to determine the optimal alginate composition to produce alginate-PLO microcapsules, then the optimal PLO concentration to produce resistant microcapsules was determined. Once the microcapsules have been developed, they were tested under culture condition with respect to their mechanical resistance, size and molecular weight cut off (MWCO) evolution.

4.3.1. Optimisation of the microcapsule parameters for PLO-containing microcapsules

4.3.1.1. Identification of the optimal alginate

PLO is known to have a higher affinity for high M alginates. Two different alginates were therefore tested to determine the optimal alginate to use, an alginate from Büchi and Manucol DH alginate with a M/G ratio of 1.56. The identification of the optimal alginate was determined by determining the kinetics of the adsorption of PLO on alginate beads produced with two alginates, respectively Manucol DH and alginate from Büchi. The PLO was labelled with FITC which allowed the spectrophotometric measurement of the residual concentration of FITC-labelled PLO in the solution as a function of time, the residual concentration being represented as a ratio between the absorbance at time t (A) and the initial absorbance (A_0). The results in Figure 4.1 show that Büchi alginate has a slightly higher affinity for PLO than Manucol DH alginate and will therefore be used to continue the experiments.

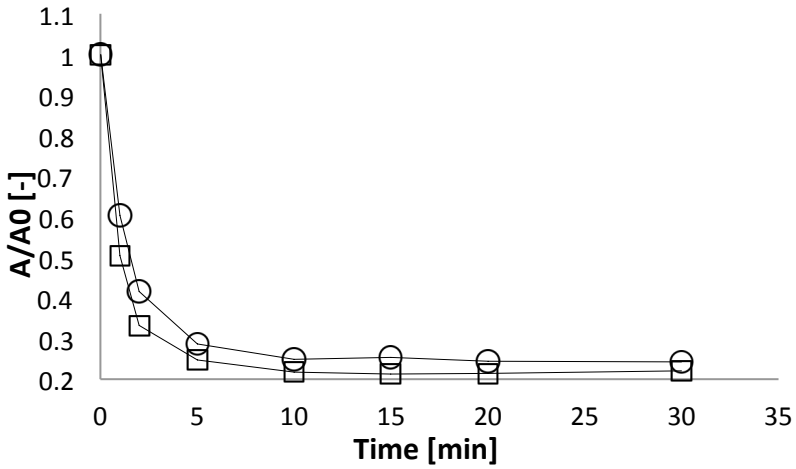

Figure 4.1: Representation of the residual concentration of FITC-labelled PLO in the solution as a function of time. The residual concentration of PLO is represented as a ratio between the absorbance at time t (A) and the initial absorbance (A_0). The absorbance was measured at 498nm for both Buchi (squares) and Manucol (spheres) alginate.

4.3.1.2. Determination of the optimal PLO concentration

In order to determine the optimal concentration of PLO to produce stable alginate-PLO microcapsules, different PLO concentrations were used to produce alginate-PLO microcapsules. The microcapsules were then incubated in saline buffer containing 10mM MOPS under agitation during 18 days in order to measure their stability.

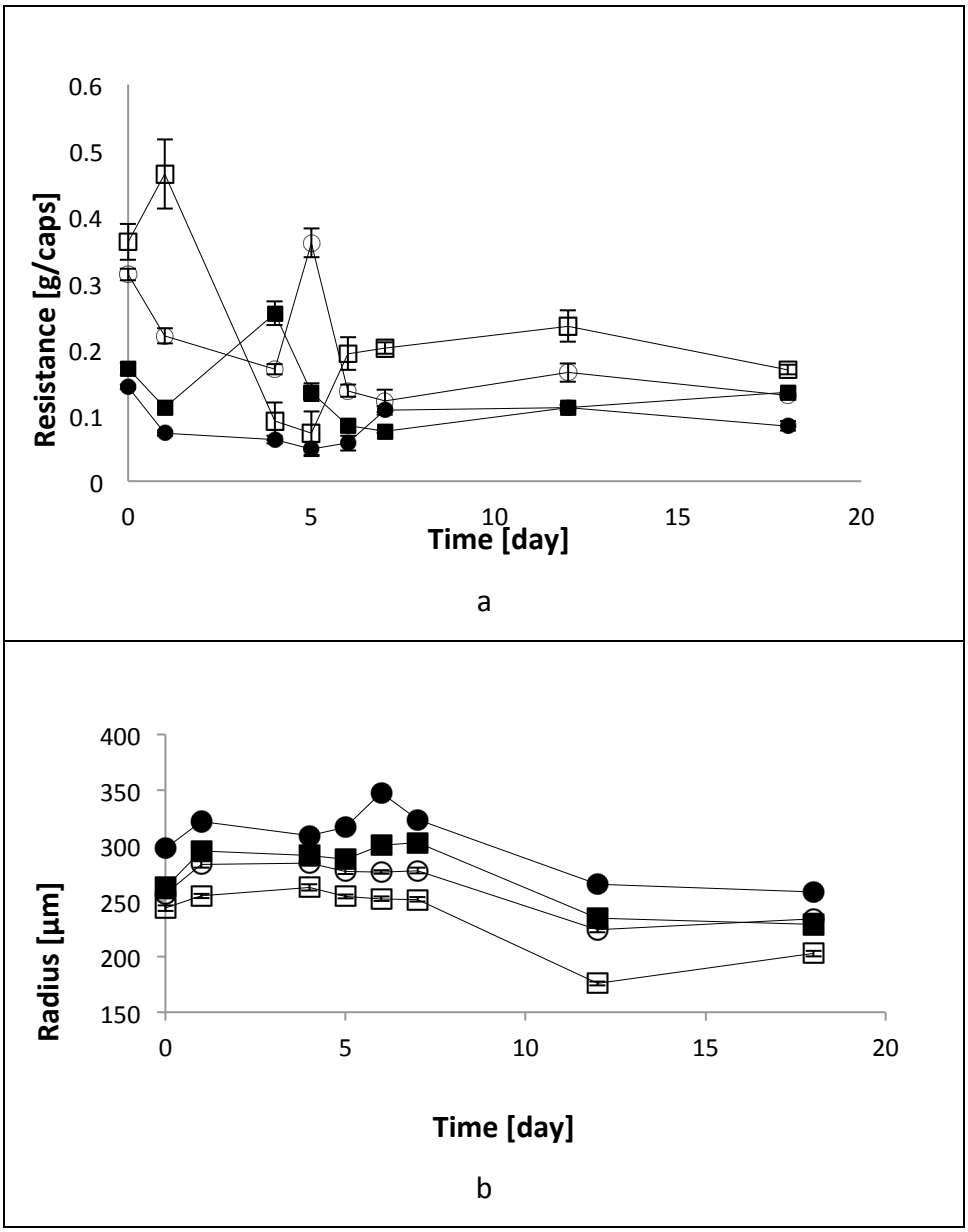

Figure 4.2 : Evolution of the mechanical resistance (a) and size (b) of PLO microcapsules made with a range of concentrations of PLO when incubated in MOPS buffer for 18 days. The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean. Concentrations of 0.15% (w/v) (empty squares), 0.1% (w/v) (empty spheres), 0.075% (w/v) (full squares) and 0.05% (w/v) of PLO (full spheres) were compared.

The size of the microcapsules is shown to be inversely proportional to the PLO concentration, showing that the more PLO is present, the less swelling occurs (Figure 4.2b). The mechanical resistance of the microcapsules was expected to be proportional to the PLO-concentration until a certain point when the capsules would start to become brittle as reported for PLL microcapsules by Breguet et al. (2007). Figure 4.2a shows that microcapsule mechanical resistance is indeed proportional to the PLO concentration, however no loss in strength is observed in the tested concentration range. Despite a decrease in mechanical resistance of respectively 45% and 57% in the first 6 days of the cultures, microcapsules made of 0.15% and 0.1% of PLO remain

stable after day 6 at a mechanical resistance of 0.16 and 0.13 g/capsule. The microcapsules made with 0.075% and 0.05% of PLO also show a decrease in mechanical resistance until day 6 and then remain stable. The mechanical resistance values of the capsules made with 0.05% PLO were below 0.1g/capsule and were therefore considered to be damaged. In order to determine the optimum concentration between 0.15%, 0.1% and 0.075% of PLO, adsorption kinetics of FITC-labelled PLO were performed and results (Figure 4.3) showed that the adsorbed concentration is proportional to the initial PLO concentration, meaning that a higher concentration was absorbed with a PLO concentration of 0.15%. As a result, this concentration was chosen for the following experiments.

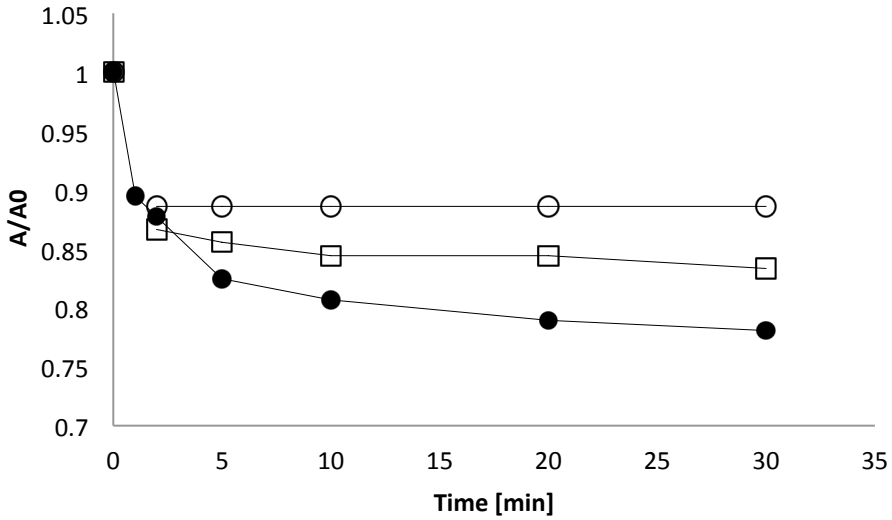

Figure 4.3 : Representation of the residual concentration of FITC-labelled PLO in solution as a function of time. The residual concentration of PLO is represented as a ratio between the absorbance at time t (A) and the initial absorbance (A_0). The absorbance was measured at 498nm for 0.15% (w/v) (full spheres), 0.1% (w/v) (empty squares) and 0.075%(w/v) (empty spheres) of FITC-labelled PLO.

4.3.1.3. Determination of the adsorption kinetics of PLO on alginate beads

In order to measure the amount of PLO adsorbed on the alginate microbeads, the kinetics were determined by measuring the absorbance of FITC-labelled PLO remaining in the solution against time. This absorbance was then converted into a concentration using a standard curve (Appendix 4.1). A mass balance was established using the kinetic results and the membrane thickness measured by confocal microscopy (Figure 4.13).

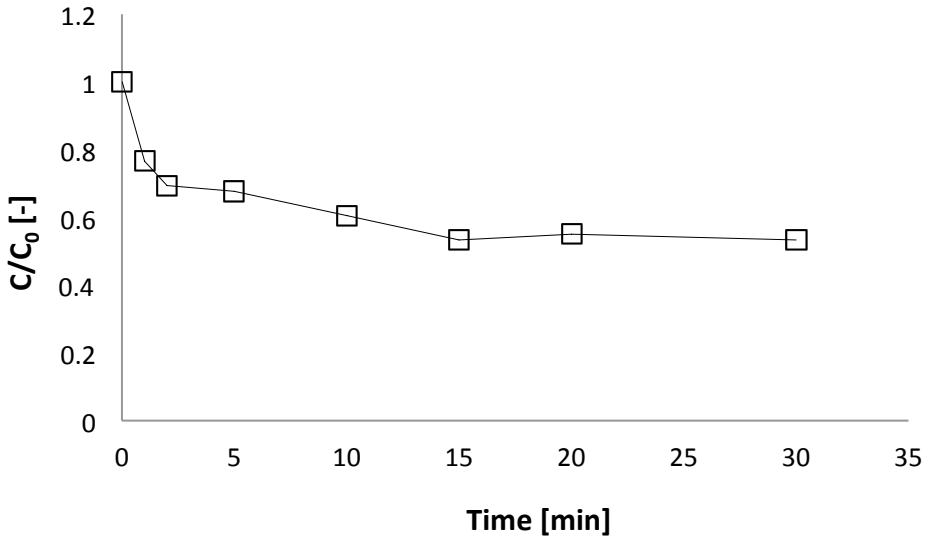

Figure 4.4: Adsorption kinetics for PLO on alginate microbeads. The ratio between the concentration at time t (C) and the initial concentration (C_0) is represented as a function of time

The results of the mass balance calculations (Table 4.1) show that PLO microcapsules have a membrane density of 9.73 10^{-6} mg/µm^3.

Table 4.1 : Mass balance calculation of the PLO adsorbed on alginate microbeads. The mass of the polyelectrolyte adsorbed on the microbeads m^*_{ads}, the initial concentration in the solution $C_{l,0}$ and the residual concentration in the solution at the equilibrium C^*_l were calculated from the volume of the solution (40mL). The density of the membrane ρ was calculating using the volume of the membrane $V_{membrane}$, which can be calculating with the membrane thickness T_{memb} and the microcapsule radius.

	$C_{l,0}$ [g/L]	$Q_{l,0}$ [mg]	C^*_l [g/L]	Q^*_l [mg]	m^*_{ads} [mg]	T_{memb} [µm]	$V_{membrane}$ [µm^3]	ρ [mg/µm^3]
PLO	1.505	60.22	0.803	32.11	28.11	7.140	2.89 10^6	9.73 10^{-6}

4.3.2. Characterisation of PLO-containing microcapsules under culture conditions

In order to assess the microcapsule stability under culture conditions and exclude any effects due to cells, microcapsules that did not contain cells were produced and incubated in cell culture conditions. The mechanical resistance, the size, the membrane thickness and intensity and the molecular weight cut-off of the microcapsules were then analysed as a function of time.

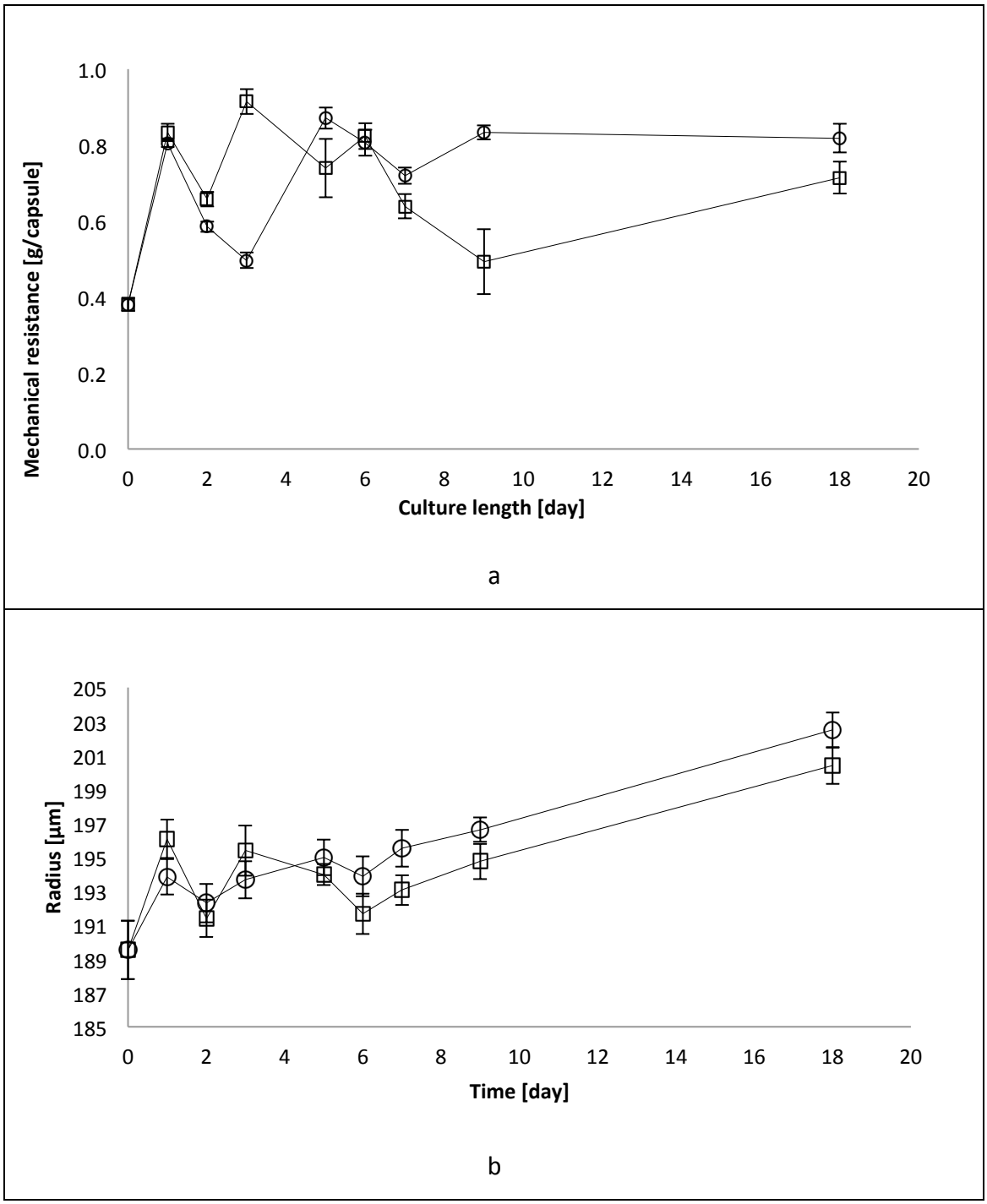

Figure 4.5 : Mechanical resistance (a) and radius (b) evolution during the stability test of microcapsules made of PLO in batch (spheres) and perfusion (squares) cultures conditions in CHO-DHFR medium. The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean. For perfusion cultures conditions, the feed was started at day 5.

Figure 4.5a shows that the mechanical resistance of the microcapsules increases from 0.4 to 0.8 g/capsule between day 0 and day 1 and then remains stable around 0.7g/capsules throughout the batch culture phase. In perfusion culture, 29% loss in microcapsule mechanical resistance can be observed at day 9 with a measured mechanical resistance of 0.5g/capsules. This apparent loss in stability is however most certainly due to imprecisions in the measurement since the standard error of the mean

is relatively high (±0.1g/capsule) and the mechanical resistance at day 18 once again increase to 0.7g/capsules. It can therefore be concluded that PLO-containing microcapsules are stable for 18 days under both batch and perfusion culture conditions when no cells were encapsulated. Figure 4.5b also shows that the mode of culture has no apparent effect on the size of the microcapsules.

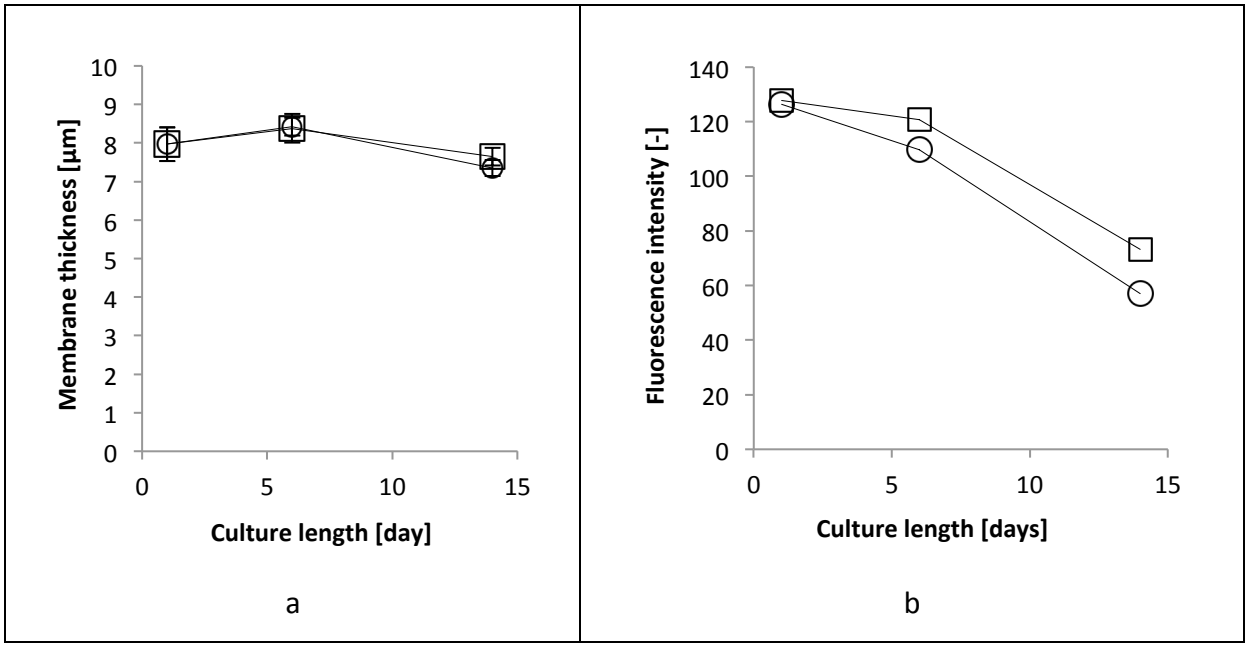

Figure 4.6 : Evolution of the membrane thickness (a) and membrane intensity (b) during the stability test in CHO DHFR$^-$ media in batch (squares) and perfusion (spheres) conditions. The membrane thickness is given in µm (n=10) and the error bars represent the standard error of the mean.

During the stability test under culture conditions, confocal microscopy was used to measure the evolution of the membrane thickness and the evolution of the fluorescence of the FITC-PLO membrane. The measurement of the membrane thickness (Figure 4.6a) shows that the membrane of the PLO microcapsules remained stable around 7.97±0.44 µm throughout the batch and perfusion cultures. Moreover, no effect of the culture mode on the membrane thickness can be observed. A loss in membrane fluorescence intensity of 43% in batch and 55% in perfusion can be observed (Figure 4.6b). The membrane intensity being linked with the amount of PLO in the membrane, this reduction in intensity means that some of the PLO leaked from the microcapsule membrane. This leakage however does not seem to be important enough to influence the microcapsule resistance since no significant loss in mechanical resistance was observed (Figure 4.5a)

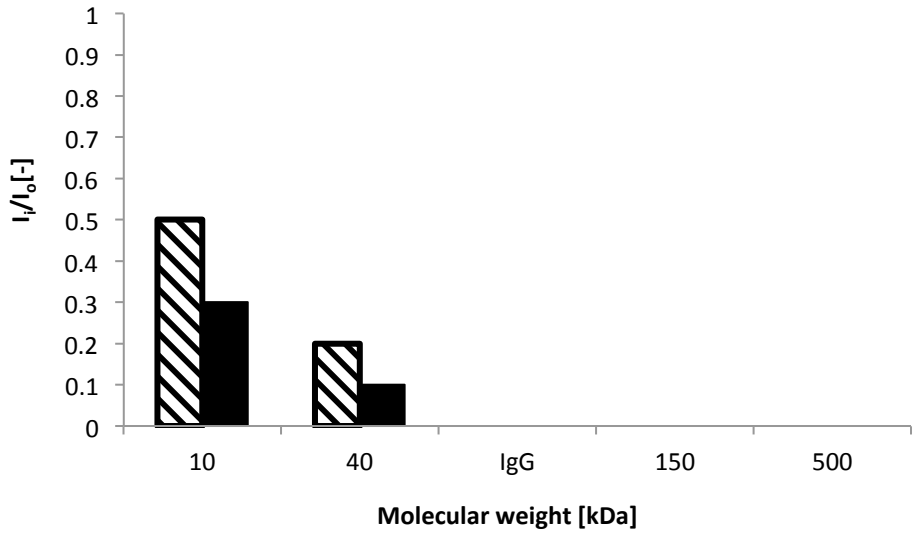

Figure 4.7: Evolution of the molecular weight cut off of the PLO microcapsules under perfusion culture condition at day 0 (dashed-lined) and day 9 (full).

The permeability of the PLO membrane is an important parameter to characterize as it will define whether the target product, in this case an IgG, will exodiffuse though the capsule membrane or accumulate within the core. Microcapsules were incubated in FITC-dextran and FITC-IgG solutions and the difference in fluorescence intensity inside (I_i) and outside (I_o) of the microcapsules was measured. The closer to 1 is the ratio I_i/I_o, the larger are the membrane pores. Figure 4.7 shows that the permeability of the PLO-containing microcapsules decreases between day 0 and day 9, that the largest molecular weight dextran passing through the membrane is of 40kDa and that IgG does not diffuse in. This result is not positive since it would have been preferred that IgG passes freely through the membrane in order that IgG secreted by cells within the core would exodiffuse, thereby faciliting subsequent separation and purification. However, even though IgG is a protein of 150kDa, it is not the molecular weight that is important in diffusion studies but the Stoke's radius or hydrodynamic radius. The Stoke's radius of IgG is of 52.9A, which situates the IgG molecule between the 40 and 70 kDa dextrans on the Stoke's radius scale. Since IgG is a protein (polyelectrolyte) and not an inert molecule such as dextran, diffusion into/out of microcapsules may also be hindered by interactions between the IgG and the polyelectrolyte membrane. Furthermore, the cell influence on microcapsules has not been taken into account in the experiment which may change the permeability of the PLO membrane.

4.3.3. Determination of whether cells can grow in PLO-containing microcapsules

CHO-dp12 cells were encapsulated within microcapsules made with PLO with an initial concentration of 0.7 10^6 cells/$mL_{alginate}$ and cultured in perfusion in CHO-$DHFR^-$ medium in a shake flask.

4.3.3.1. Characterisation of the PLO-containing microcapsules

The mechanical resistance, size and molecular weight cut-off of microcapsules was analysed during the flask perfusion culture and the results reported in Figure 4.8.

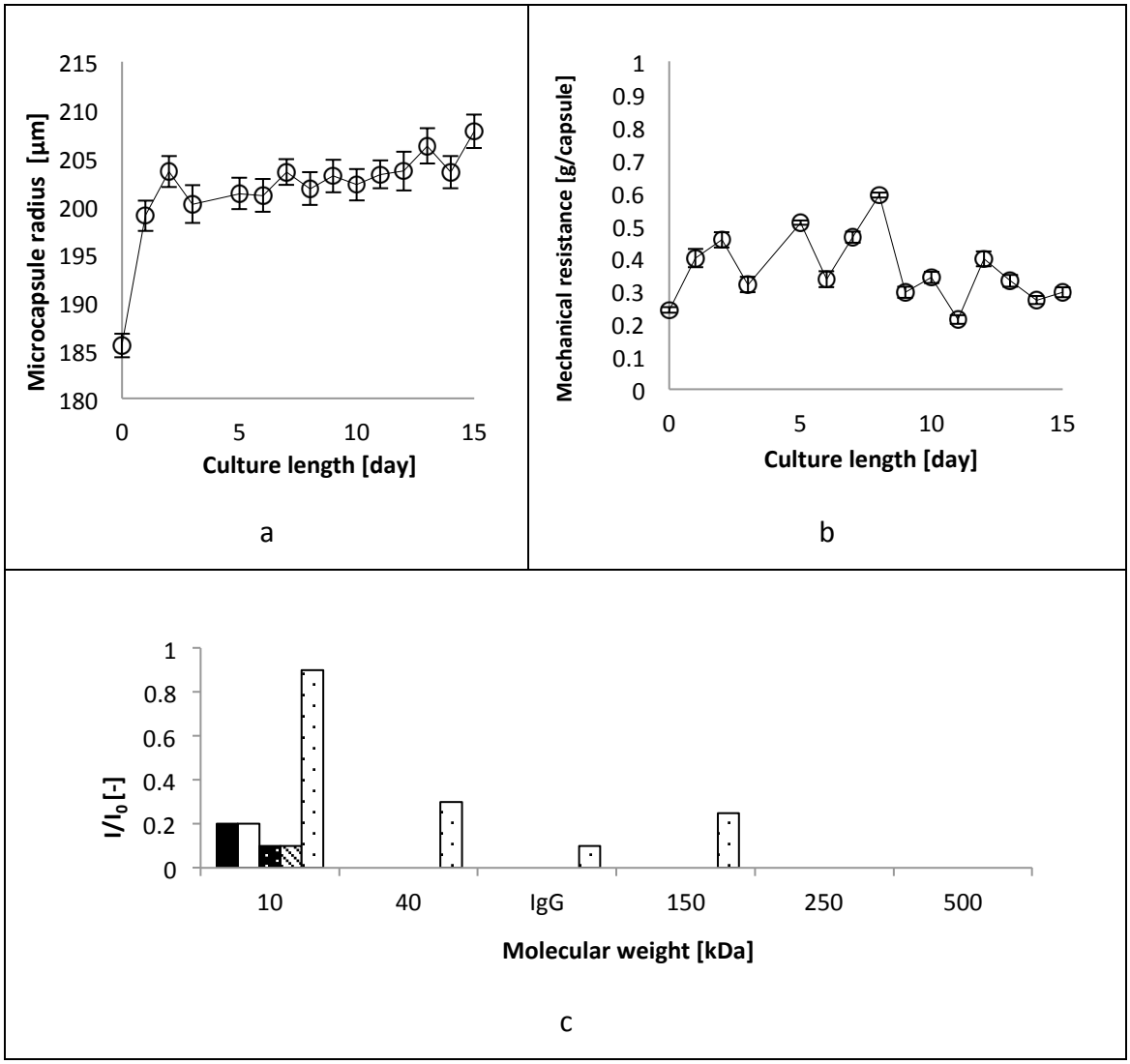

Figure 4.8: Evolution of the size (a), the mechanical resistance (b) and the molecular weight cut off (c) of the microcapsules throughout the perfusion flask culture. The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean. The molecular weight cut off was measured at day 0 (full symbol), day 2 (empty symbol), day 7 (full symbol with white dots), day 9 (dash-lined symbol) and day 15 (empty symbol with black dots).

A swelling of 5% can be observed between inoculation and the first day of the culture, the microcapsules size then remains stable until the end of the culture. (Figure 4.8a). As observed previously with empty microcapsules (microcapsules with no cells) the mechanical resistance of the microcapsules increases between the inoculation and the first day of the culture and then remains stable until the end of the culture. It is also interesting to note that the mechanical resistance of microcapsules containing cells is approximately 50% of the mechanical resistance of those with no cells. The permeability of the microcapsules was analysed throughout the culture and shows that until day 9, the microcapsules are only permeable to a 10kDa dextran, they then become permeable to IgG and dextran up to 150kDa (Figure 4.8c). This change in permeablity is not due to microcapsules breakage as the integrity of the microcapsules is high after 15 days of culture (Figure 4.10)

4.3.3.2. Cell growth in PLO-containing microcapsules

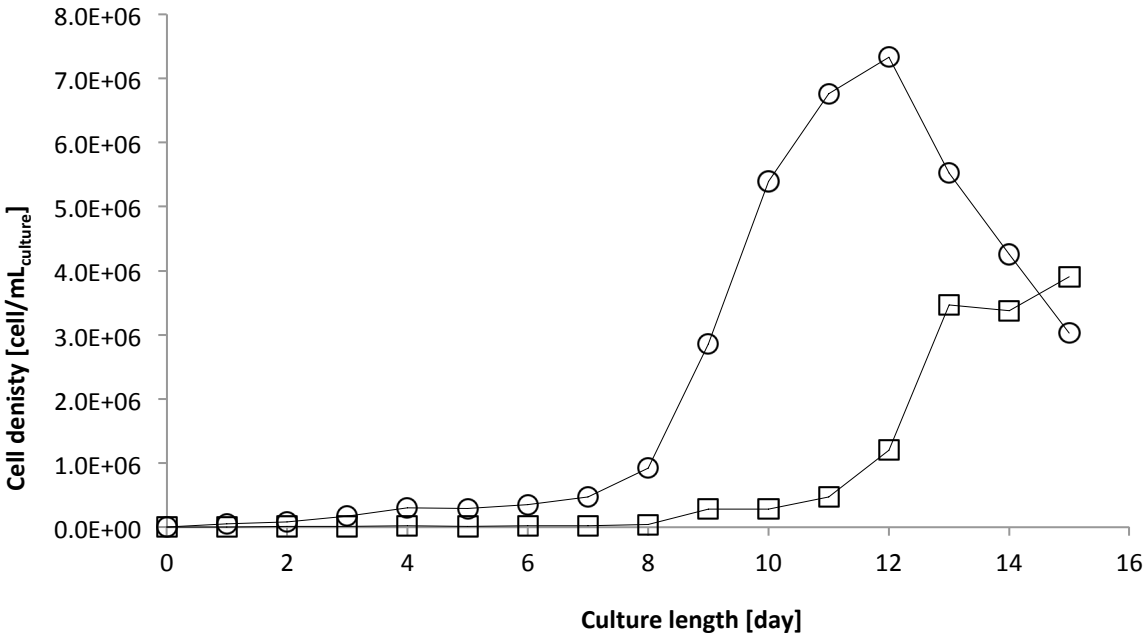

Figure 4.9 : Evolution of the cell density of the microencapsulated flask perfusion culture in PLO microcapsules. The feed of the perfusion culture started on day 5, the viable cells are represented by spheres and the dead cells by squares.

As shown in Figure 4.9, the encapsulated CHO-dp12 were grown in batch phase for 5 days before initiation of a medium feed. The growth of the encapsulated cells in PLO-containing microcapsules can be divided to 4 distinct phases (Figure 4.9): a lag phase of

6 days followed by an exponential phase of 4 days, a stationary phase of 2 days and a decline phase of 3 days. A loss in cell viability is observed from day 12 despite the good condition of the microcapsules Figure 4.10. A decrease of the culture pH was observed from day 10 to day 15 as the colour of the media containing phenol red turned from red to orange/yellow. This may be the cause of the loss in cell viability and a system with pH control will be needed in order to increase the cell density and the culture length.

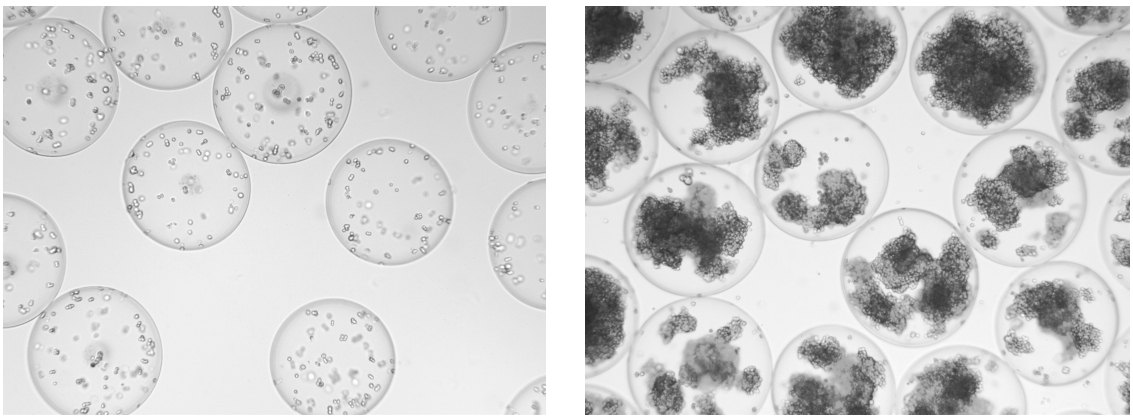

Figure 4.10: evolution of the encapsulated CHO-dp12 in alginate-PLO microcapsules between the beginning (left) and the end (right) of the culture. The microcapsules have been magnified 40x and have a radius of 185.5±1.2 µm at the beginning of the culture and 207.8±1.8 µm at the end of the culture.

PLO microcapsules were shown to be a potential solution to grow cells in perfusion mode as they remain stable in a 15-day perfusion culture in shake flasks. Despite the small molecular weight cut-off of the microcapsules, it appears that IgG diffuses out of the microcapsules after 9 days of culture, which is ideal for DSP purposes. In order to obtain a higher cell density however, the microcapsules will need to be cultured in a bioreactor in order to control the pH of the culture and to improve the aeration of the culture in order to avoid cell death due to a drop in pH. The stability in bioreactor will therefore need to be assessed.

4.4. Results – Part II: comparative study of polyelectrolyte microcapsules produced with PLL and PLO as cell retention systems

4.4.1. Characterisation and comparison of PLO-containing microcapsules with PLL-containing microcapsules

For this work Poly-L-ornithine was studied and alginate-PLO microcapsules were compared to the results obtained with the classical and well-characterised alginate-PLL microcapsules. Alginate-PLO microcapsules were produced using alginate from Büchi since previous experiments had shown that more PLO was adsorbed to this alginate compared to Manucol DH alginate (Figure 4.1), which has routinely been used for PLL microcapsules (Demont et al. 2015). The optimum PLO concentration of 0.15% PLO was determined by performing stability tests on microcapsules produced with different PLO concentrations (from 0.05% to 0.15% w/v) in saline buffer containing 10mM MOPS (Figure 4.2).

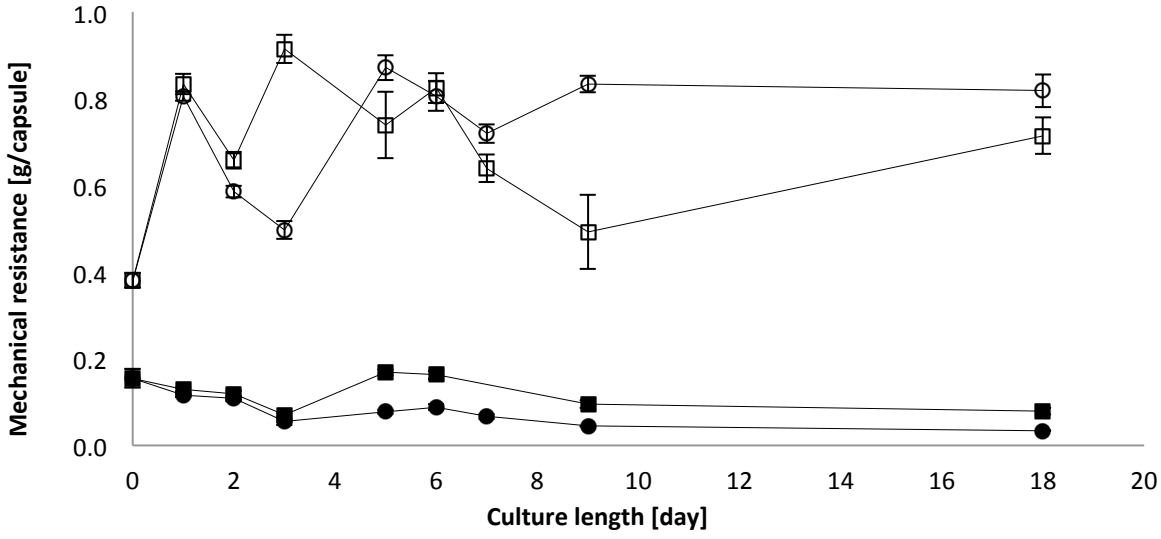

Figure 4.11: Stability test of microcapsules made of PLL (filled symbols) and PLO (empty symbols) in simulated batch (spheres) and perfusion (squares) cultures in CHO-DHFR medium (microcapsules which had not been inoculated with cells yet were operated under perfusion conditions as if they had). The mechanical resistance is given in g/capsule (n=5), the error bars represent the standard error of the mean. For perfusion-simulated cultures, the feed was started at day 5 for both PLL and PLO

Since the aim is to use microcapsules as a cell retention system for bioreactor perfusion cultures, mechanical strength is a major concern so that the microcapsules can withstand the physical stresses occurring during the culture. As reported by De Castro et al (2005), a wide number of parameters are involved in microcapsule strength, including microcapsule size, membrane density and membrane thickness (Gugerli, 2003). Microcapsule strength also depends on the core strength; therefore, the alginate core properties were maintained constant between PLL-containing and PLO-containing microcapsules to compare the stability of microcapsules made with PLL and with the newly developed PLO. Figure 4.11 shows the evolution of the mechanical resistance of microcapsules made of PLO and of PLL when incubated in cell culture medium. The results (Figure 4.11) show that upon incubation of the microcapsules made from PLO in culture medium the mechanical resistance actually increased by over 87% during perfusion operation and 115% in batch operation. Indeed in both cases, once the initial change had occurred, the mechanical stability remained relatively constant over 18 days. By contrast, with PLL-containing microcapsules, upon incubation in culture medium the mechanical resistance actually fell by 49% during perfusion operation and 78% in batch operation over the same time period. However the results also clearly indicate that the PLO-containing microcapsules showed on average 17-fold higher mechanical resistance during batch operation and 14-fold higher resistance during perfusion operation compared with PLL-containing microcapsules. In an earlier work (Demont et al. 2015), the decrease in strength of the PLL-containing microcapsules was explained being due to a competition between non-gelling ions, gelling ions and PLL for the alginate chains, creating a leak of PLL and gelling ions from the microcapsules membrane followed by their wash-out in the medium outlet during perfusion culture. By measuring the membrane intensity throughout the culture using confocal microscopy, the decrease in PLL and PLO concentration could be investigated and showed that a loss in intensity of around 50% can be observed for PLO-containing microcapsules (Figure 4.6), while for PLL-containing microcapsules the decrease was approximately 75% (Appendix 4.2 and 4.3). Since less PLO leaks from microcapsules compared to PLL microcapsules, this suggests that the affinity of PLO for alginate is higher than that of PLL, which would explain the higher measured stability of PLO-containing microcapsules.

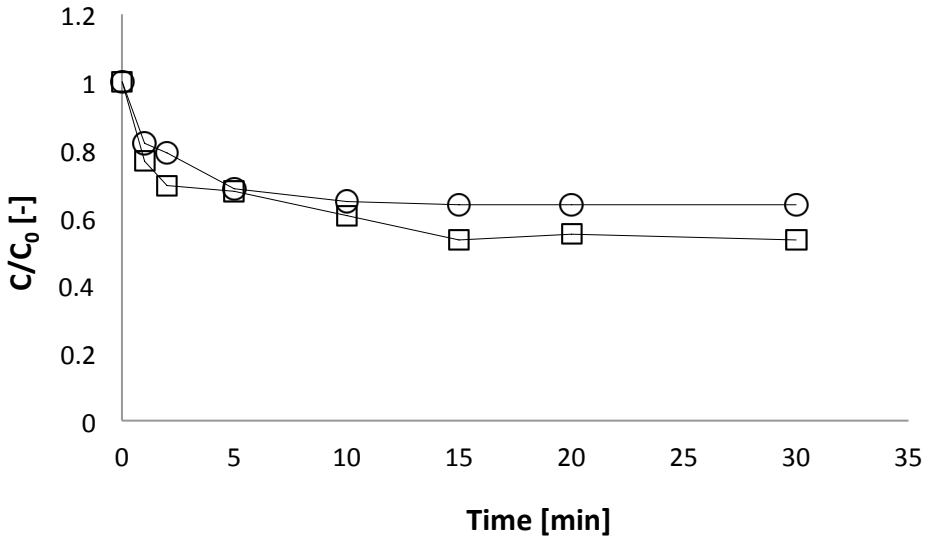

Figure 4.12: Adsorption kinetics for PLL (spheres) and PLO (squares) on alginate microbeads. The ratio between the concentration at time t (C) and the initial concentration (C_0) is represented as a function of time

In order to compare PLO and PLL containing microcapsules, the kinetics of adsorption of the two polycations (Figure 4.12) was determined based on a mass balance calculation, which enabled the membrane density to be established. The membrane thickness of the microcapsules was measured using confocal microscopy as a function of duration of the perfusion culture (Figure 4.13). The results (Figure 4.12) show that a higher percentage of the initial concentration of PLO (46.7%) was adsorbed in the membrane of the microcapsules compared to PLL (36.3%), which would explain the higher stability of PLO microcapsules. Moreover, the mass balance calculations and the confocal imaging also showed that, since the PLO-containing microcapsule membrane was thinner than for PLL-containing microcapsules (Figure 4.13), the density of the membrane is 8.8-fold higher for PLO-containing microcapsules, with a membrane density of 9.74×10^{-3} µg/µm^3 compared with PLL-containing microcapsules (1.09×10^{-3} µg/µm^3). These results further explain the higher stability of microcapsules made with PLO.

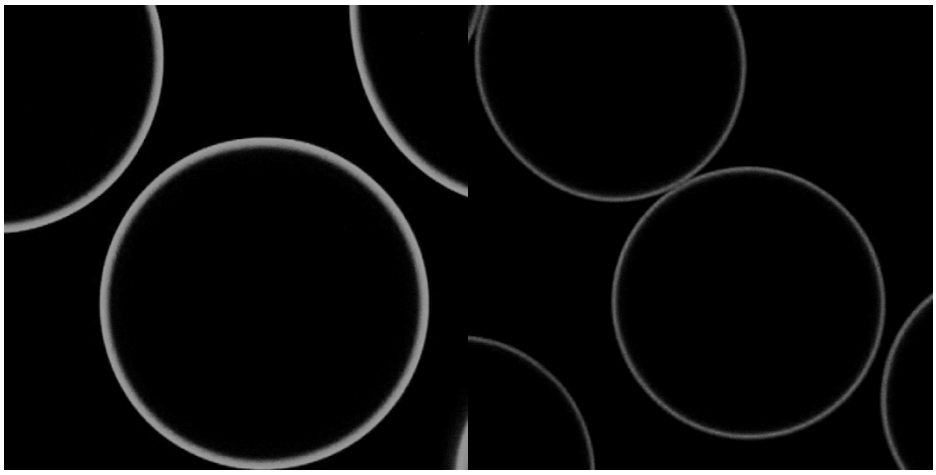

Figure 4.13: Confocal scanning microscopy of microcapsules made with PLL (left) and PLO (right). The PLL microcapsules have an average radius of 270µm and an average membrane thickness of 17.31±0.44µm while PLO microcapsules have an average radius of 150µm. and an average membrane thickness of 7.97±0.44µm.

As shown in a previous work (Demont *et al*., 2015) and confirmed by this work, PLL microcapsules are not suitable for encapsulated cell cultures unless stabilised by the addition of Ca^{2+} to the medium. Such medium supplementation would provide for sufficient stabilisation to allow for batch and fed-batch cell cultures but not for perfusion operation due to the leakage of PLL from the microcapsules and wash out from the bioreactor. However, microcapsules made with PLO were shown to be stable in batch and perfusion simulations for 18 days in the absence of Ca^{2+} supplementation (Figure 4.11). The leakage of PLO to the medium was significantly reduced compared to PLL and the membrane density of PLO microcapsule was higher which explains the improved stability.

4.4.2. Perfusion cultures of CHO-dp12 cells in PLO-containing microcapsules compared with PLL-containing microcapsules

CHO-dp12 cells were encapsulated within microcapsules made with PLL and PLO and cultured in perfusion in CHO-DHFR$^-$ medium in a laboratory bioreactor. Throughout the culture, the microcapsules appeared to be spherical and uniform with a definite membrane. The size of the PLO-containing microcapsules remained stable throughout the culture with a radius of approximately 190µm while PLL-containing microcapsules showed swelling from day 0 to day 4 after which the radius stabilized at approximately 230µm (Figure 4.14b). Both PLL and PLO-containing microcapsules were extruded

using the same nozzle and conditions, however the PLL-containing microcapsules had an initial radius, which was 15.8% larger than the PLO-containing microcapsules, which shows that some swelling occurred immediately after production. The thickness of the membrane for PLL-containing and PLO-containing microcapsules remained unchanged throughout the culture with values of 17µm and 7µm respectively. The molecular weight-cut off of the microcapsules was analysed throughout the culture and showed that IgG would be unable to diffuse through the PLO membrane until day 4 (Table 4.2), while it would diffuse through the PLL membranes freely from day 0 (Figure 3.9).

Table 4.2: Evolution of the membrane permeability during the perfusion culture of CHO DP12 cells in PLO 0.15% microcapsules. The test was done at the beginning (day 0), during (day 5 and 7) and at the end of the culture (day 11). The results represented are the ration between the fluorescence intensity inside and outside the microcapsules (Iin/Iout)

	Day 0	**Day 5**	**Day 7**	**Day 11**
4 kDa	0.4	0.5	0.5	0.5
10 kDa	0.2	0.4	0.4	0.5
40 kDa	0	0	0	0.2
70 kDa	0	0	0	0
150 kDa	0	0	0	0
250 kDa	0	0	0	0
500 kDa	0	0	0	0
IgG	0	0.05	0.1	0.15

The higher density of PLO in the membrane probably resulted in tighter cross-linking with alginate and the formation of smaller pores than for PLL capsules. It therefore appears that there is a correlation between molecular weight cut- off and polymer density in the membrane.

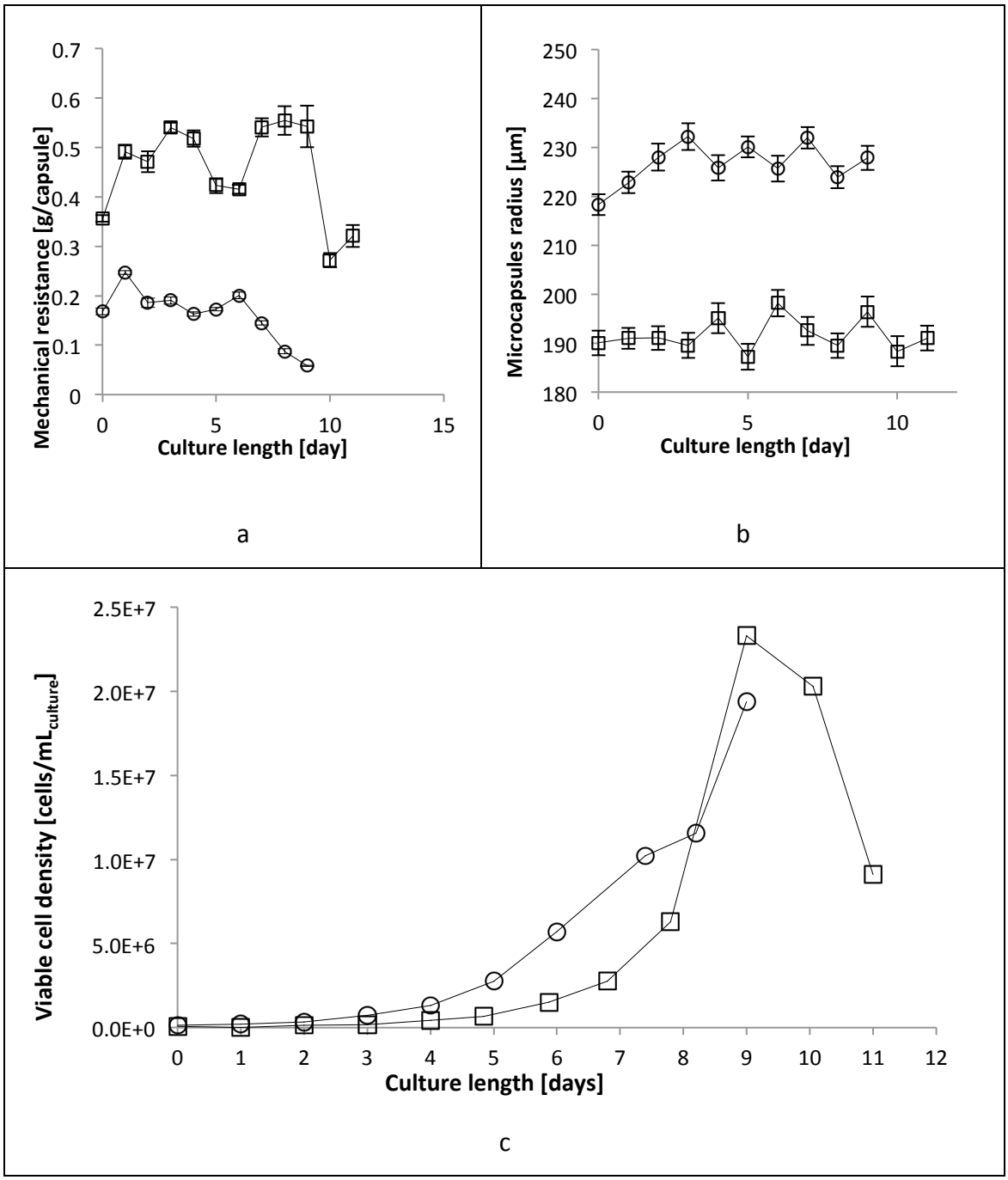

Figure 4.14 : (a) evolution of the mechanical resistance of the PLL (spheres) and PLO (squares) microcapsules under culture conditions. (b) evolution of the size of PLL (spheres) and PLO (squares) microcapsules throughout the perfusion culture. (c) evolution of the viable cell density of the microencapsulated perfusion culture in PLL (spheres) and PLO (squares) microcapsules. The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean. The feed of the perfusion culture started on day 5 for both PLL and PLO perfusion cultures.

The mechanical resistance of the PLO-containing microcapsules in perfusion operation was constant, with a value of 0.5g/capsule until day 10 of the culture followed by an abrupt drop of mechanical resistance (Figure 4.14a). This can be correlated to the sudden capsule breakage at day 10 as shown in Figure 4.15. For PLL-containing microcapsules however, the microcapsules were not stable enough to resist batch

culture operation (Figure 4.14a) in which a continuous drop in mechanical resistance was observed. In order to overcome this loss of mechanical resistance Ca^{2+} were added to the cell culture medium. However PLL-containing microcapsules were still not suitable for perfusion culture as stability decreased once the medium feed began, due to leaching of PLL and its washout in the continuous medium outlet (Demont *et al*., 2015). The growth pattern of the immobilised cells was studied during the cultures. As observed on Figure 4.14c, the encapsulated perfusion culture in PLL-containing microcapsules grew in batch mode for 5 days before initiation of a medium feed of 1.7L d^{-1}, whereas the perfusion culture for PLO-containing microcapsules was grown in batch mode for 5 days before initiation of a medium feed of 1L d^{-1}. The growth of the encapsulated cells in PLO-containing microcapsules can be divided into 3 distinct phases (Figure 4.14c): a lag phase of 3 days followed by an exponential phase of 7 days and a decline phase of 1 day. The decline phase was however not due to a decrease in cell growth and viability but to a washout of cells due to the abrupt breakage of the microcapsules on day 10 (Figure 4.15), since 80% viability was observed at the end of the culture. In the encapsulated perfusion in PLL-containing microcapsules, 2 growth phases can be observed (Figure 4.14c), an exponential phase and a linear growth phase. The linear growth phase is however not due to a decrease in cell growth and viability, but to a loss of cells into the medium outlet throughout the culture due to microcapsule breakage since a viability of more than 90% was found at the end of the culture. Unlike the PLL-containing microcapsules, PLO-containing microcapsules do not show a gradual loss in stability during culture but an abrupt loss in microcapsules strength at day 10. It can therefore be assumed that this loss in stability is not due to a loss in PLO in the medium outlet. The earlier stability tests, performed in cell culture medium, showed that PLO-containing microcapsules were stable for 18 days when no cells were encapsulated (Figure 4.11). It is therefore likely that the destabilisation of the capsules at the end of the perfusion culture is due to the presence of a high concentration of cells, which destabilises the polyelectrolyte membrane due to the surface charges on the cell membranes coupled with an increase in intracapsular pressure.

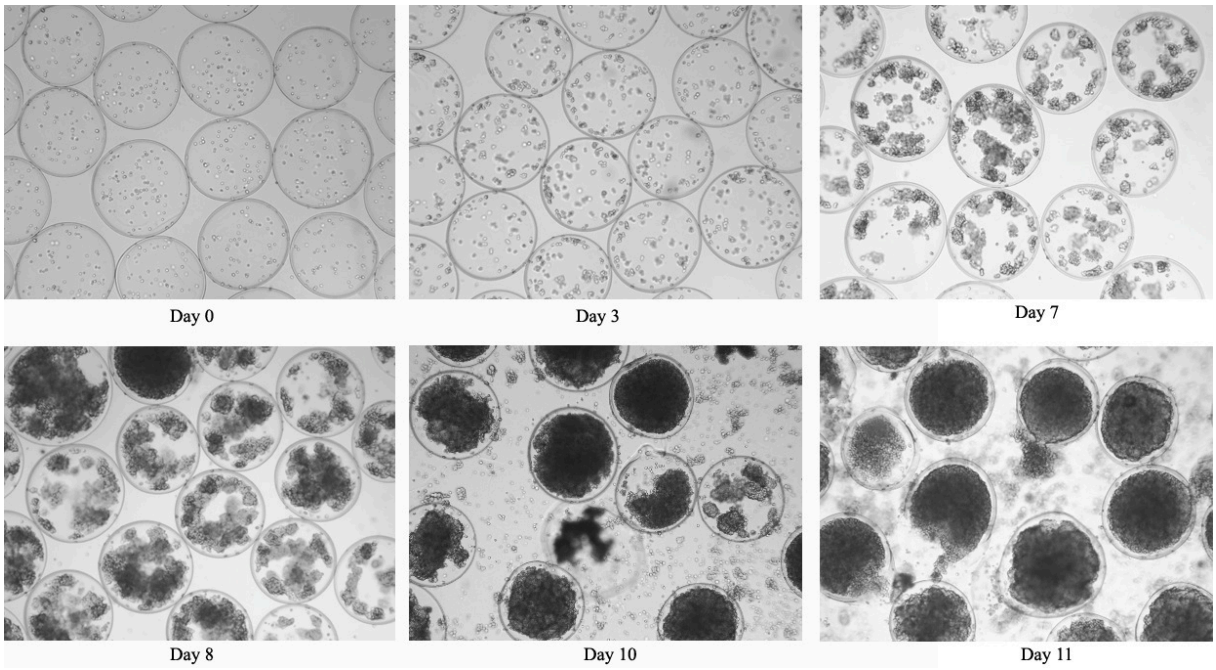

Figure 4.15 : Images of the growth profile of CHO-dp12 encapsulated in alginate-PLO microcapsules at day 0, 1, 7, 8, 10 and 11.

As shown in Figure 4.15 and Figure 4.16, a gap of around 37µm appears between the microcapsule core, containing cells, and the inner border of the capsule membrane. Confocal microscopy analysis revealed that the gap is not part of the PLO membrane, since the PLO membrane measured only measure 7µm (Figure 4.16). Since no noticeable change in microcapsule size was observed this suggest that the internal core, containing the cells, must have contracted. It would therefore follow that the alginate concentration in the core is higher than in the gap and that the polyelectrolyte interactions within the core increased. Since there was no change in calcium chloride concentration during the culture, the Ca^{2+} concentration remained stable therefore this suggests that it must be the cells themselves interacting with the alginate, causing the alginate core to contract. This is confirmed by confocal microscopy, which shows that no cells are observed in the gap (Figure 4.16), and clearly indicates that the microcapsule core contracted due to cell interactions with the alginate at high cell density.

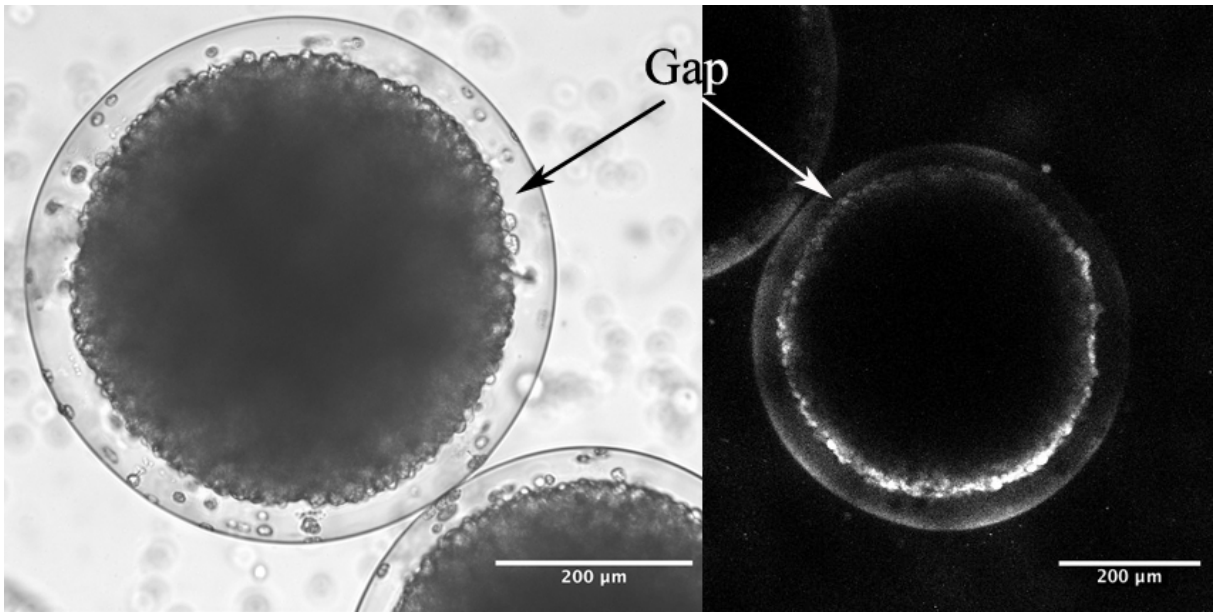

Figure 4.16: light microscope (left) and confocal microscope (right) images of the microcapsules just before they break. The cells were dyed with calcein and propidium iodide for confocal microscopy and the PLO membrane was labelled with FITC. A dark space of 37µm width between the PLO membrane and the cells can be observed on confocal microscopy picture.

Further confirmation was obtained by the incubation of the high cell density containing- microcapsules in calcium chloride. Under these conditions, the microcapsule diameter remained unchanged, whereas the alginate relaxed and the core expanded to refill the microcapsule volume, the gap disappearing (Figure 4.17). This shows that Ca^{2+} ions compete with the cells for alginate at high cell density.

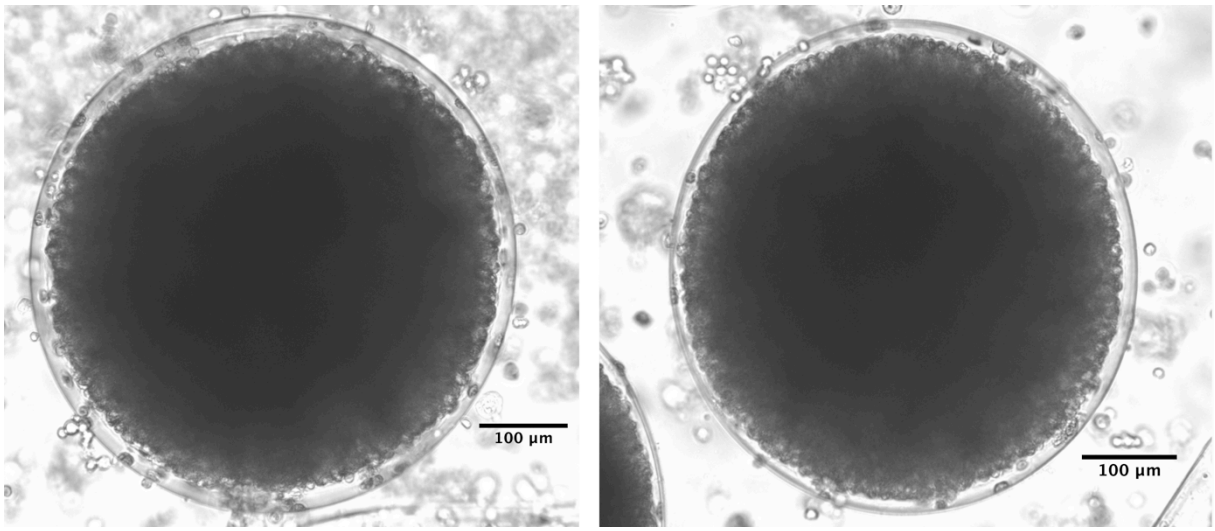

Figure 4.17 : Microcapsules after addition of $CaCl_2$ 100mM for 15 (left) and 30 minutes. No noticeable change in microcapsule diameter is seen, however a reduction in the size of the gap is observed, due to a relaxation of the core containing the cells.

4.4.3. Cell growth in alginate-PLO microcapsules in perfusion culture

Since the cells interact with the alginate, they form a very dense mass in the core of the microcapsules. The maximum cell density that can be contained in one capsule is calculated to be 8.69 x 10^{-4} cells/µm^3, whereas in the perfusion cultures a cell density of only 1.27 x 10^{-4} cells/µm^3 was obtained if the total capsule volume is considered (Table 4.1). If only the volume of the core containing the cells is taking into account, a cell density of 2.57 cells/µm^3 is obtained (Table 4.1), which corresponds to 29% of the maximum theoretical cell density. Such a cell density has not been obtained previously in microcapsule cultures, it is therefore important to understand if this has an effect on the cell metabolic activity.

Table 4.3: Kinetic and stoichiometric parameters of encapsulated CHO cells perfusion culture in PLL and PLO microcapsules

Value	Units	Encapsulated-PLL perfusion	Encapsulated-PLO perfusion
Microcapsule radius	µm	227	150
µ	d^{-1}	0.67	0.73
Culture duration	h	216	264
Final viability	%	91.7	80
$X_{culture,\ max}$	cell/$mL_{culture}$	1.94 x 10^7	2.33 x 10^7
$X_{capsule,\ max}$	cell/$mL_{capsule}$	3.67 x 10^7	1.1 x 10^8
Max cells	cell/capsule	1.8 x 10^3	1.56 x 10^3
Colonisation	%	5.33	14.6
$q_{lactate}$	mole $cell^{-1}$ day^{-1}	2.34 x 10^{-9}	6.27 x 10^{-10}
q_{NH3}	mole $cell^{-1}$ day^{-1}	3.0 x 10^{-7}	6.1 x 10^{-11}
q_{IgG}	pg $cell^{-1}$ day^{-1}	5.59	2.28
C_{IgG}	$mg_{IgG}/mL_{culture}$	0.11	0.0013
Max density of cells	cells/µm^3	4.65 x 10^{-5}	1.27 x 10^{-4} / 2.57 x 10^{-4}

The determination of kinetic values is complicated due to microcapsule breakage, as a result the total cell number has been underestimated due to cell leakage, the kinetic values are therefore only estimations. C_{IgG}, is the concentration of IgG in the bioreactor at the end of the culture.

The encapsulated cell perfusion culture with PLO-containing microcapsules grew to a maximum cell density of 2.33 x 10^7 cells/$mL_{culture}$ with a growth rate of 0.73 d^{-1}, while the encapsulated perfusion culture in PLL microcapsules grew to a maximum cell density of 1.94 x 10^7 cells/$mL_{culture}$ with a growth rate of 0.67 d^{-1}. These results therefore show that there was no negative effect of the encapsulation of cells in PLO-

containing microcapsules compared with PLL microcapsules. In perfusion culture, the intact PLO-containing microcapsules were totally retained within the bioreactor and became colonized with cells to a maximum concentration of 2.33 x 10^7 cells/$mL_{culture}$ (1.1 x 10^8 cells/$mL_{capsule}$) (Table 4.1). These values are significantly higher than those reported by (Breguet et al., 2007). The lactate ($q_{lactate}$) and NH_3 (q_{NH3}) productivity is lower for PLO microencapsulated perfusion cultures than for PLL microencapsulated culture (Table 4.1) despite the higher density of cells in the capsule core in PLO-containing microcapsules. This would suggest a higher utilisation of glutamine and glucose via respiration in PLO microcapsules and that the density of cells has no influence on cell metabolism. However, the IgG productivity (q_{IgG}) was smaller for PLO microencapsulated cultures compared with PLL microencapsulated cultures (Table 4.1) suggesting that a higher density of cells might inhibit IgG production. However, the values are estimations only since the total cell number has been underestimated due to cell leakage and microcapsule breakage.

4.5. Discussion

In the present study, the possibility of using alginate-PLO-Ca^{2+} system to encapsulate cells in order to grow them in CSTR bioreactors has been evaluated and compared with the well-characterised alginate-PLL-Ca^{2+} system. It has been shown previously (Breguet et al., 2007) that due to the mild encapsulation process, the alginate-PLL-Ca^{2+} system was found to possess the required properties to allow cell encapsulation without damaging the cells. Such microcapsules however, do not possess the necessary stability to protect against the conditions prevailing in the bioreactor due to the dependence of stability on the medium ionic composition, bead size and type of alginate (Demont et al. 2015) As a strategy to improve microcapsule stability, PLL was replaced by PLO which has a similar structure to PLL and has been reported to produce more biocompatible and resistant microcapsules than PLL (Darrabie et al., 2005; Tam et al., 2011). The results show that the PLO has a slightly higher affinity for Büchi alginate than for Manucol DH with a 1.56 M/G ratio. This result confirms that PLO has a higher affinity for high M alginate (Santos et al., 2010). As expected, the mechanical resistance of the microcapsules made with PLO was proportional to the PLO concentration, however unlike PLL microcapsules that become brittle above a

concentration of 0.05% PLL (Breguet V., 2007), no loss in resistance was observed in the tested range of concentrations for PLO microcapsules. The PLO coating improved microcapsule stability under culture conditions, nearly eliminated bead swelling after citrate chelation of the microcapsule core, significantly increased the mechanical strength of the microcapsules and reduced the pore size of the microcapsules due to a higher membrane density. These findings are in agreement with published data (Darrabie et al., 2005 ; Rosiński et al., 2005). The PLO monomer is one methyl group shorter than the PLL monomer. This difference probably results in a higher affinity of PLO for the alginate membrane, which would result in an increase in membrane strength and density. The decrease in pore size compared to PLL-containing microcapsules did not influence the cell metabolic activity, since nutrients such as glucose and glutamine were shown to diffuse freely into the microcapsules from the beginning of the culture (Rosiński et al., 2005), while IgG diffused out of the microcapsules from day 4.

In bioreactor culture, the PLO-alginate microcapsules were found to be stable under perfusion operation despite the continuous change of medium, unlike the PLL-alginate microcapsules. The problem with perfusion culture is however not completely resolved since the culture remained time-limited because of microcapsule breakage. Since microcapsules were shown to be stable in perfusion- simulated culture conditions (microcapsules which had not been inoculated with cells yet were operated under perfusion conditions as if they had) for 18 days, it is safe to assume that only the presence of high cell density has changed and that this must be the factor destabilising the microcapsules. Assumptions can be made as to whether this is due to a destabilisation of the polyelectrolyte membrane due to the presence of cells or to the pressure applied on the membrane by the cells when they are growing, however it can be conclude that PLO-containing microcapsules cannot be used in perfusion culture or for cultures with cell densities higher than 1.56×10^3 cells/capsule, which correspond to a colonisation of 14.6%.

The maximum cell density attained was $2.33 \cdot 10^7$ cells/$mL_{culture}$, whereas the maximum theoretical cell density that could be attained, assuming complete capsule colonisation and stability was estimated to be $1.6 \cdot 10^8$ cells/$mL_{culture}$ for a culture in which the

microcapsules represent only 25% of the total culture volume. Accordingly, the cell density attained represents only 14.6% capsule colonisation. Abundant literature reports have been found regarding perfusion cultures of CHO cells involving a range of cell retention systems including spin filters, tangential flow filtration, continuous centrifugation and acoustic-filters. A maximum cell density of $2.8 \cdot 10^7$ cells/$mL_{culture}$ for CHO SSF3 cells using an acoustic filter was reported (Gugerli, 2003). Clincke *et al*., using a tangential flow filtration system achieved a maximum cell density of $2 \cdot 10^8$ cell/ mL (Clincke et al., 2013a, 2013b). The cell density obtained in this work ($2.33 \cdot 10^7$ cells/ $mL_{cultures}$ therefore clearly shows the potential of microcapsules as a new cell retention system, since this corresponds to 82% of the maximum cell density obtained by Gugerli *et al*. and 11.5% of the cell density obtained by Clincke *et al*. (2013a, 2013b). These results were obtained despite the limited mechanical stability preventing full microcapsule colonisation to be obtained. Through the development of microcapsules from alternative materials with stronger polyelectrolyte interactions or with covalent interactions which are stable enough to resist bioreactor shear stresses and the medium composition but would still allow the cells to grow freely, a maximum cell density of $1.6 \cdot 10^8$ cells/$mL_{culture}$ should be obtainable with 25% capsule: media ratio. This value is very close to that reported by Clincke *et al*. (2013a, 2013b) Furthermore, as previously mentioned by Demont et al. 2015, the cells are entrapped in the microcapsules and can thus not grow further than the volume available within the core of the microcapsules. The microcapsules are therefore limiting the maximum cell density possible in the culture due to the physical entrapment of the cells. Increasing the amount of microcapsules in the culture would therefore result in an increase in the cell density with respect to reactor volume (Demont et al. 2015). An increase of the microcapsule: medium ratio in the reactor to the maximum of 75% microcapsules volume should therefore increase the maximum cell density to a value between 3-4 x 10^8 cells/$mL_{culture}$.

4.6. Conclusion

The present work reports on the optimisation and characterisation of polyelectrolyte microcapsules to assess if the use of polymers such as PLO in the microcapsule membrane formation has a positive effect on the microcapsule resistance in perfusion

culture compared to microcapsules based on PLL membranes. Several experiments have been performed to assess the stability properties of the microcapsules in perfusion culture conditions, resulting in the conclusion that polyelectrolyte interactions are not strong enough, under the tested conditions, to produce microcapsules that can be used for perfusion culture in bioreactors. There is therefore a need to develop more stable microcapsules and to investigate different sources of polymer with stronger polyelectrolyte interactions or with covalent interactions, which allows the cells to grow freely but are sufficiently stable to resist bioreactor shear stresses and the medium composition. Chitosan and cellulose sulphate have been reported to produce strong and stable microcapsules (Bartkowiak and Hunkeler, 2000; Gåserød et al., 1999, 1998; Gugerli, 2003; Kaiser et al., 2014; Werner et al., 2013) however, nobody has shown the long- term effects of medium changes on microcapsules made of these polymers. There is therefore no evidence that such polymers could be used to produce microcapsules that can be use in perfusion cultures. Another solution would be to investigate microcapsules made with covalent membranes such as PGA (Breguet et al., 2005; Hurteaux et al., 2005; Levy and Edwards-Levy, 1996; Rachik et al., 2006) or genipin (Chen et al., 2009, 2006; Hillberg et al., 2013).

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5. Stabilization of polyelectrolyte microcapsules using different cross-linking methods

Abstract

The present work reports on the possibility to produce microcapsules with improved stability compared to the widely characterized alginate-PLL microcapsules, using microcapsules with covalent membranes created by (1) the cross-linking of alginate-PLL microcapsules with genipin or (2) the transacylation of alginate-PLL microcapsules containing PGA. Results showed that PGA-containing microcapsules with a transacylation time of 10 seconds had an initial mechanical resistance twice as high as for alginate-PLL microcapsules, however under culture conditions, the mechanical resistance decreased continuously throughout the culture due to the sensitivity of PGA to the solution pH. The scale-up of microcapsule production also appears to be difficult since the initial mechanical resistance of the PGA-containing microcapsules tends to decrease dramatically as a function of the volume of the capsules to be produced. Genipin microcapsules produced with a cross-linking reaction time of 6 hours were shown to be stable during 15 days with only 30% loss in mechanical resistance, against 87% loss of mechanical resistance for alginate-PLL microcapsules over the same period of time. Genipin- containing microcapsules were however, shown to be unsuitable for perfusion culture, as it was revealed that under the conditions tested , genipin induced cell death.

5.1. Introduction

The immobilization of living cells in microcapsules has been widely investigated for the past decades with potential applications ranging from bio artificial organ to high cell density cultures (Bartkowiak and Hunkeler, 2000). Microcapsules are frequently produced following the protocol established by Lim and Sun (Lim, 1983; Lim and Sun, 1980) over 30 years ago, which consists of the application of a polyamino acid layer (polycation) on alginate (polyanion) microspheres gelled with a divalent cationic solution such as $CaCl_2$ (Bartkowiak and Hunkeler, 2000). The reaction between the polyanion and the polycation depends on their charge density which is controlled by the chemical structure and pI of the polyelectrolytes and by the pH of the solution ((Breguet et al., 2005)). At neutral pH, alginate is negatively charged, while polycations such as poly-L-lysine are positively charged. Several polyion systems such as alginate-poly-L-lysine (Breguet et al., 2007; de Groot et al., 2003; Gugerli et al., 2002; Ma et al., 2012), alginate-poly-L-ornithine (Darrabie et al., 2005; Thanos et al., 2007), alginate-chitosan (Bartkowiak and Hunkeler, 2000, 1999; Gåserød et al., 1999, 1998) or cellulose sulfate-PDADMAC (Bohlmann et al., 2002; Bučko et al., 2005; Weber et al., 2004) have been reported to produce polyelectrolytes membranes. Demont et al. (2015a, 2015b) previously showed that polyelectrolyte interactions between alginate-PLL and alginate-PLO are not strong enough, under the tested conditions, to produce microcapsules that can be used for perfusion culture in bioreactors. Chitosan and cellulose sulphate have been reported to produce strong and stable microcapsules (Bartkowiak and Hunkeler, 2000; Gåserød et al., 1999, 1998; Gugerli, 2003; Kaiser et al., 2014; Werner et al., 2013) however, very few reports on the long- term effects of medium changes on microcapsules made of these polymers can be found. There is therefore no evidence that such polymers could be used to produce microcapsules that can be used in perfusion cultures.

In order to increase the mechanical resistance and culture stability of microcapsules, several reactions have been reported to covalently link the membrane polymer (Breguet et al., 2005; Chen et al., 2009, 2006, 2005; Dusseault et al., 2005; Gattás-Asfura et al., 2011; Gugerli, 2003; Hillberg et al., 2013). Amongst those methods, crosslinking the PLL-membrane with genipin or transacylating alginate beads were

chosen due to polymer availability and mechanism simplicity. Genipin has been reported to have the capacity to cross-link molecules containing residues with primary amine groups and has been used to replace chemical crosslinkers to covalently link biomaterials (Butler et al., 2003; Chen et al., 2009, 2006, 2005; Hillberg et al., 2013; Muzzarelli, 2009). The alginate transacylation reaction is based on the method developed by Levy and Edwards-Levy (1996) which reports membrane formation between propylene-glycol-alginate (PGA) and natural protein. The reaction takes place under alkaline conditions, when the amino groups become uncharged ($-NH_2$).

The present investigation aims to (1) characterise and compare genipin-containing and PGA-containing microcapsules in culture conditions, (2) to investigate the possibility to stabilise alginate-PLL microcapsules using either the crosslinking reaction with genipin or the transacylation reaction with the PGA-containing system (3) to investigate whether the produced microcapsules are suitable for perfusion cultures in bioreactor from a stability and a cell perspective. The variation of molecular weight cut- off, burst force with time, the microcapsule stability in culture medium and integrity was quantitatively evaluated under perfusion culture conditions to avoid lab-to-lab variation and allow reproducibility. The kinetics parameters of CHO-DP12 cells have been determined for suspension batch cultures and encapsulated batch cultures.

5.2. Material and methods

Material and methods used in this chapter are described in section 2.2. Specific experiments set-up are described below.

5.2.1. Shake flask encapsulated cultures and stability tests

20mL of cell containing microcapsules or empty microcapsules were cultured in shake flask (Corning Inc, Corning, NY, USA), with a working volume of 100mL and a microcapsule volume representing 25% of the medium volume. The culture was then incubated at 37°C, under a humidified atmosphere of air containing 5% CO_2 and an agitation of 100rpm. Each culture and stability test carried out in shake flask were carried out only once

5.2.2. Genipin microcapsules formation

Alginate (Manucol DH, FMC biopolymers, UK) beads were first formed by extrusion of 100mL pre-sterilised (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland) sodium alginate solution (1.5% w/v) through the encapsulator nozzle into an aqueous solution of $CaCl_2$ 110mM (Sigma, St-Louis, USA). After 5 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules incubated in the polyelectrolyte solution (0.05% w/v poly-L-lysine 30-70kDa (Sigma, Saint Louis, Missouri, USA)) for 30 min at room temperature under agitation. The microcapsules were then washed with saline buffer followed by incubation in 3L 0.03% sodium alginate for 10min under gentle agitation. After washing with MOPS buffer solution (8.5g/L NaCl, 2.09g/L MOPS, pH7), the microcapsules were incubated in 300mL genipin solution (1mg/mL genipin in MOPS buffer solution) for 0 to 6 hours at 37°C under agitation. The solid alginate core of the microcapsules was then liquefied by incubation for 5min in 0.05M sodium citrate (Sigma, Saint Louis, Missouri, USA). After core liquefaction with citrate, the microcapsules were washed with MOPS buffer twice followed by a wash with cell culture medium before being transferred to cell culture medium to be inoculated. When cells were encapsulated, the cells ($0.7 \cdot 10^6$ $_v$cells/$mL_{alginate}$) were homogenised in the alginate solution and the bead formation was made according to the method above.

5.2.3. PGA microcapsules formation

Propylene-glycol alginate (PGA) (Kelcoloid S, FMC biopolymers, UK) was dissolved in 20mM MOPS buffer (4.18g/L MOPS, 8.5g/L NaCl, pH7) to make a 5% stock solution. The pH of the solution was constantly adjusted to pH7 as it tends to drop below 4 during PGA dissolution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland). This solution must be prepared fresh, as PGA is not stable in aqueous solution above pH 4.

Sodium alginate (Manucol DH, FMC biopolymers, UK) was dissolved in 10mM MOPS buffer (2.09g/L MOPS, 8.5g/L NaCl, pH7) to make a 2% stock solution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland).

BSA (Sigma, Saint Louis, Missouri, USA) was dissolved in 10mM MOPS buffer (2.09g/L MOPS, 8.5g/L NaCl, pH7) to make a 40% stock solution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland).

PEG 6000 (Sigma, Saint Louis, Missouri, USA) was dissolved in 10mM MOPS buffer (2.09g/L MOPS, 8.5g/L NaCl, pH7) to make a 50% stock solution. The solution was then sterilised using 3 filtration steps (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland).

Beads were first formed by extrusion of a solution composed of 1% alginate, 1,8% PGA, 4% BSA, 1% PEG through the encapsulator nozzle (300µm) into a 4% BSA (Sigma, Saint Louis, Missouri, USA), 5% $CaCl_2$ dihydrate (Sigma, Saint Louis, Missouri, USA), 0.2% Tween 20 (Sigma, Saint Louis, Missouri, USA) aqueous solution. After 10 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules incubated in the polyelectrolyte solution (0.05% w/v poly-L-lysine 30-70kDa (Sigma, Saint Louis, Missouri, USA) 4% BSA (Sigma, Saint Louis, Missouri, USA), 5% $CaCl_2$ dihydrate (Sigma, Saint Louis, Missouri, USA)) for 30 min at room temperature under agitation. The microcapsules were then transacylated by raising the pH to 11 by addition of a predetermined volume of 2M NaOH with a syringe. The reaction was stopped after 10 seconds by neutralization with a predetermined volume

of 2M MOPS, the beads were then agitated for 10min before being washed with 10mM MOPS buffer. The solid core of the microcapsules was then liquefied by incubation for 5min in 0.05M sodium citrate (Sigma, Saint Louis, Missouri, USA). After core liquefaction with citrate, the microcapsules were washed with MOPS buffer twice followed by a wash with cell culture medium before being transferred to cell culture medium.

5.2.4. Microcapsule characterisation and culture analysis

Homogenous culture samples containing microcapsules were regularly removed from the bioreactor to determine intra/extra-capsular cell number, concentration of key culture metabolites, microcapsule size, mechanical resistance, molecular weight cut-off (MWCO) and membrane thickness using methods described in sections 2.2.2 and 2.2.3.

5.3. Results – Part I: Determination of the working window

Alginate polyelectrolytes microcapsules have been reported to be unsuitable for perfusion culture as they were not strong enough under the conditions tested by Demont et al. (2015). There is therefore a need to develop more stable microcapsules by investigating new polymers with stronger interactions. As both genipin and PGA enable the production of microcapsules with a covalently linked membrane, they were chosen to stabilise PLL microcapsules. In the first part of this chapter, the parameters to produce the microcapsules were investigated and optimised in order to improve microcapsule stability and strength.

5.3.1. Optimisation of PLL-genipin microcapsules

Alginate-poly-L-lysine microcapsules were reported to be unsuitable for perfusion cultures since they are very sensitive to medium composition, mainly the presence of non-gelling ions that have a higher affinity for alginate than poly-L-lysine and Ca^{2+}, leading to the leakage of poly-L-lysine and Ca^{2+}, and to capsule rupture under perfusion conditions. As genipin is known to cross-link primary amines, such as those present in the poly-L-lysine, it was decided to investigate whether the poly-L-lysine membrane could be stabilised through genipin cross-linking.

It was previously reported by Chen et al. (2006) and Hillberg et al. (2013) that there was little impact of genipin concentration on the cross-linking of chitosan or PLO microcapsules. They also reported that the cross-linking was dependent on time and temperature, a higher temperature improving the cross-linking reaction (Chen et al., 2006; Hillberg et al., 2013). Taking these findings into account, it was therefore decided to use a genipin concentration of 1mg/mL as Hillberg et al. (2013) reported it as optimal, while the cross-linking temperature was set at 37°C due to the presence of the cells.

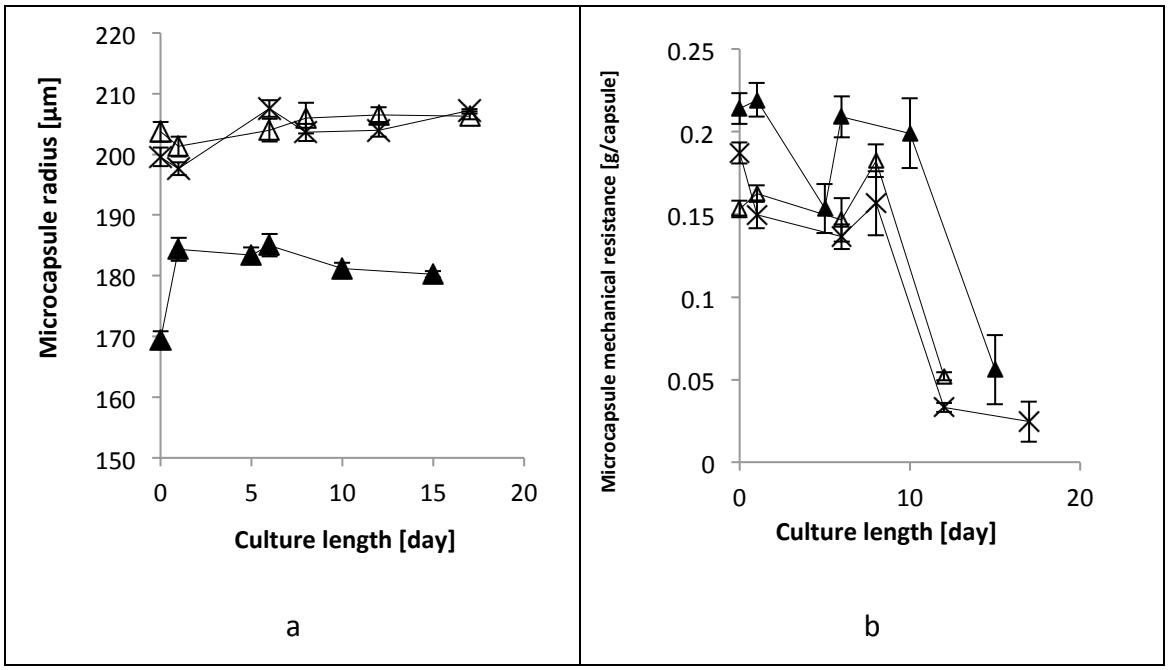

Figure 5.1 : a) Evolution of the microcapsule size under perfusion culture conditions when the core liquefaction with citrate happens before (empty triangles) and after (full triangles) incubation in genipin for 2 hours, and comparison with alginate-PLL microcapsules (crosses). b) Evolution of the microcapsule mechanical resistance in perfusion culture conditions when the core liquefaction with citrate happens before (empty triangles) and after (full triangles) incubation in genipin for 2 hours, and comparison with alginate-PLL microcapsules (crosses). The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean. The feed started at day 5 when the citrate was applied after the genipin cross-linking and at day 6 when the citrate was applied before the genipin cross-linking.

In order to optimize microcapsule strength, the first parameter to be investigated is whether to perform the core liquefaction with citrate before or after the genipin cross-linking. The results show (Figure 5.1) that when the core liquefaction with citrate is performed after genipin cross-linking there is a reduction of the microcapsule swelling and an increase in microcapsule initial mechanical resistance. Moreover, the microcapsules remain stable longer in perfusion than PLL microcapsules or microcapsules that were treated with citrate before the cross-linking. It was therefore decided to perform the core liquefaction after genipin cross-linking.

The second parameter that needed to be investigated is the cross-linking time. Microcapsules with a range of cross-linking times (2hours, 4 hours and 6 hours) were therefore prepared, incubated in cell culture medium and treated as if a perfusion culture was performed. The microcapsule size and mechanical resistance was analysed throughout the culture.

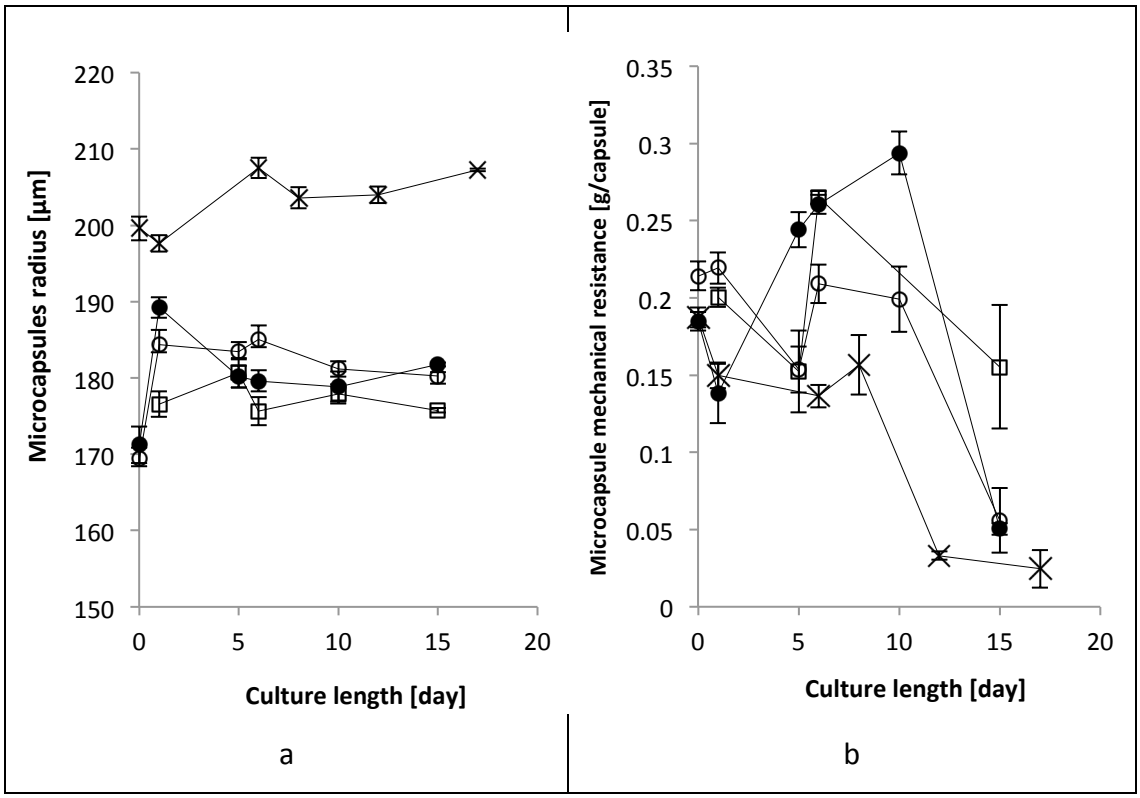

Figure 5.2 : a) Evolution of the microcapsule size in perfusion culture conditions when the core liquefaction with citrate happenned after incubation in genipin for 2 (empty spheres), 4 (full spheres) and 6 (empty squares) hours and comparison with alginate-PLL microcapsules (crosses). b) Evolution of the microcapsule mechanical resistance under perfusion culture conditions when the core liquefaction with citrate was carried out after incubation in genipin for 2 (empty spheres), 4 (full spheres) and 6 (empty squares) hours, and comparison with alginate-PLL microcapsules (crosses). The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean. The medium feed started at day 5.

The results in Figure 5.2 show that the longer the cross-linking time, the less swelling occurred. Moreover, microcapsules produced with genipin have an initial radius, which is smaller than that of alginate-PLL microcapsules due to a reduction in swelling. The mechanical resistance for 2 hours of cross-linking is similar to that of alginate-PLL microcapsules while that for 4 and 6 hours cross-linking is higher than for alginate-PLL microcapsules. The microcapsules are however, more stable with genipin cross-linking as alginate-PLL microcapsule strength begins to decrease at day 6 while for genipin-containing microcapsules the microcapsule resistance remains stable until day 10. The most stable microcapsules appear to be the microcapsules cross-linked for 6 hours since they only a show a loss of resistance of 30% within 15 days while alginate-PLL microcapsules have a loss of resistance of 87% during the same period of time.

5.3.2. Optimisation of Alg-BSA-PGA-PLL microcapsules

5.3.2.1. Determinaton of the optimal composition and production conditions for microbeads.

The optimal polymer composition reported by Levy et al (1996, 1999) and Breguet et al. (2005) to produce large microbeads through a syringe pump extrusion system was: 2% PGA, 1.0-1.5% alginate and 4% BSA. However, this polymer solution cannot be applied to the vibrating nozzle extrusion method, as it is too viscous to be extruded. Breguet et al. (2005) therefore adapted the polymer composition in order to be able to extrude it through a 400µm nozzle, thus producing smaller microbeads. The optimal concentration determined by Breguet et al. (2005) was of 1.2% alginate, 1.8% PGA, 4% BSA and 1% PEG. In order to extrude this polymer solution through a 300µm nozzle, the polymer composition had to be slightly adapted and the optimal determined composition to be extruded through a 300µm nozzle was of 1% alginate, 1.8% PGA, 4% BSA and 1% PEG. The microcapsule shape was also improved by extruding the polymer mixture in a 5% $CaCl_2$ dihydrate bath (340mM) instead of the usual 100mM $CaCl_2$ bath. It was moreover found that 0.2% Tween 20 had to be added to the calcium chloride bath to reduce the surface tension, avoiding therefore the production of tailed microbeads (Figure 5.3).

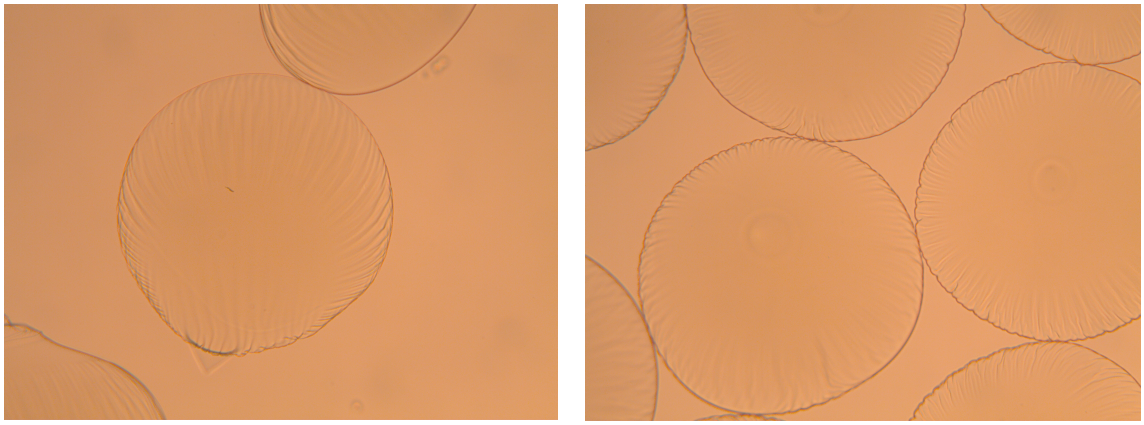

Figure 5.3 : Microbeads produced with 1% alginate, 1.8% PGA, 4% BSA and 1% PEG in 5% $CaCl_2$ (left) and in 5% $CaCl_2$ + 0.2% Tween 20 (right)

In order to reduce the BSA concentration gradient between the beads and the solution and therefore to avoid the leaching in BSA previously reported by Breguet et al (2005) and Levy et al. (1996, 1999), BSA (4% w/v) was added to the solution of the gelling step

and of the PLL coating step. The transacylation was then performed in the PLL coating solution.

5.3.2.2. Determination of the optimal transacetylation time

It was previously reported by Breguet et al. (2005) and Marison et al. (2004) that the transacetylation reaction takes place at pH values between 9 and 10, however, fixing the pH to 11 would ensure the deprotonation of most of the amino groups of PLL (Breguet et al., 2005; Marison et al., 2004). The transacylation reaction pH was therefore set at 11 while the transacylation time will have to be determined.

The effect of transacylation time on the capsule mechanical resistance is shown in Figure 5.4. It can be observed that microcapsule mechanical resistance increases with transacylation time up to 20 seconds before decreasing again. Microcapsules produced with a transacylation time of 10 and 20 seconds have a mechanical resistance, which is twice the mechanical resistance of the PLL microcapsules (i.e. microcapsules with no transacylation).

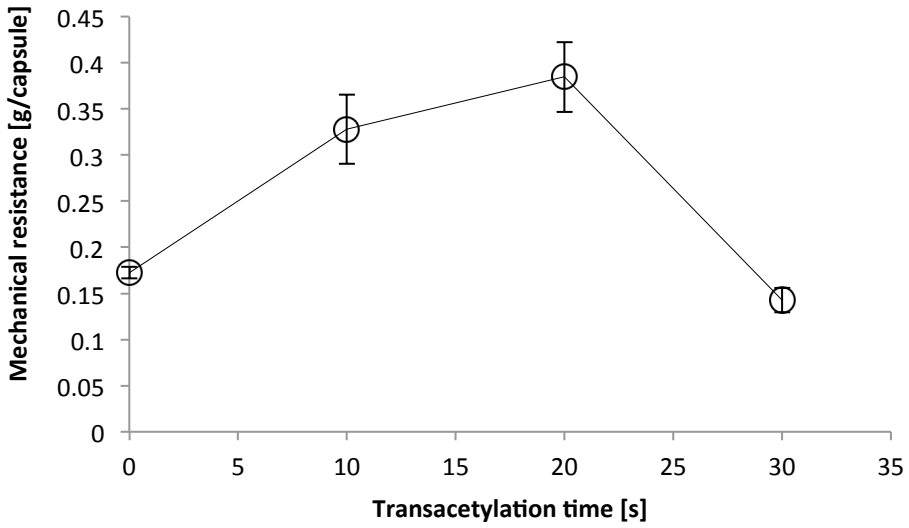

Figure 5.4 : Mechanical resistance of capsules made from 1.2% alginate, 1.8% PGA, 4% BSA, 1% PEG as a function of transacylation time for a constant incubation time in PLL of 30min. The mechanical resistance is given in g/capsule (n=5), the error bars represent the standard error of the mean.Stability test in culture conditions

In order to optimise microcapsule resistance and to limit the potential deleterious effects of NaOH on the encapsulated cells, it was decided to set the transacylation time to 10 seconds.

5.3.2.3. Stability test in culture conditions

In order to assess the microcapsule stability under culture conditions, 30mL of microcapsules that do not contain cells were produced and incubated under cell culture conditions. The mechanical resistance and the size of the microcapsules were then analysed as a function of time.

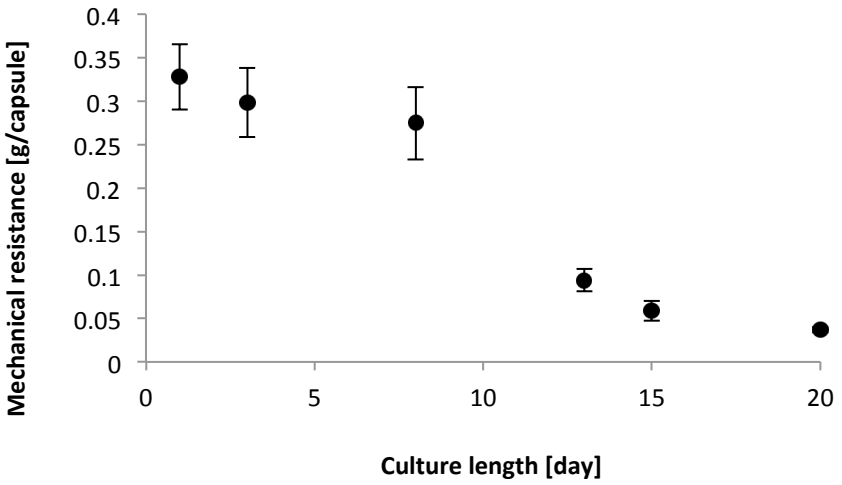

Figure 5.5 : Mechanical resistance of microcapsules as a function of time (full sphere) under perfusion culture conditions. The mechanical resistance is given in g/capsule (n=5), the error bars represent the standard error of the mean. The feed was started at day 7.

The evolution of the mechanical resistance of the PGA microcapsules produced with 10 seconds transacylation shows a decrease in stability during the first 15 days of the culture, before stabilising around 0.037g/capsule (Figure 5.5). The microcapsule radii seem to remain stable up to day 7 when the microcapsules start to swell (Figure 5.6). Both the swelling of the microcapsules and the loss in mechanical resistance are different with PGA-containing microcapsules than that observed with PLL microcapsules where the capsules have a tendency to swell until day 1 before the radius remains stable and to lose mechanical resistance abruptly once the feed is started (Figure 5.2).

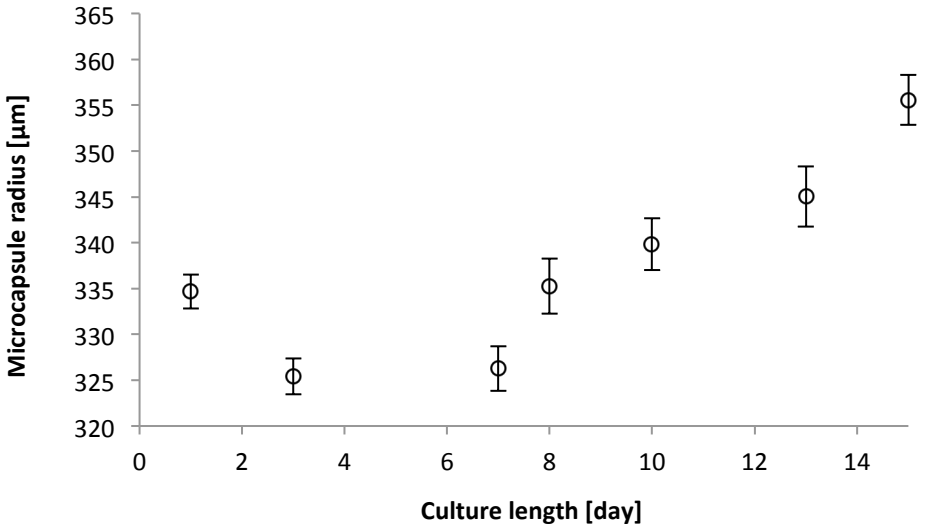

Figure 5.6 : Radius of microcapsules as a function of culture time under perfusion culture conditions. The microbead radius is given in µm (n=30), the error bars represent the standard error of the mean. The feed was started at day 7 .

When a larger volume of microcapsules was produced (100mL), it was observed that microcapsule initial mechanical resistance was significantly reduced (0.056 ± 0.006 g/capsule) compared with the stability test results (0.328 ± 0.038 g/capsule). A similar observation can be made with the initial microcapsule radius which is bigger when a larger volume of microcapsules was produced (564.1 ± 7.7 µm) than for the 30mL of capsules produced in the stability test (334.7 ± 1.9µm).

5.3.3. Part I Results summary

In this section, microcapsules made with genipin or PGA were optimised in order to see whether they could strengthen and stabilise alginate-PLL microcapsules by creating covalently linked membranes.

Genipin was shown to reduce microcapsule swelling and to improve the mechanical resistance of the microcapsules which appears to be influenced by the cross-linking time. Microcapsules cross-linked for 6 hours were shown to remain stable for 15 days with only 25% loss in mechanical resistance.

PGA-containing microcapsules with a transacylation time of 10 seconds were shown to have an initial mechanical resistance which is double that of alginate-PLL microcapsules, however, unlike alginate-PLL microcapsules, the mechanical resistance does not decrease abruptly with the start of the medium feed, but decreases all along the culture. The scale-up of microcapsule production also appears to be problematic as the initial mechanical resistance of the PGA-containing microcapsules tends to decrease dramatically with an increase of the volume of the capsules to be produced. Under the tested conditions, PGA-containing microcapsules are therefore not suitable to stabilise alginate-PLL microcapsules and cannot be used to grow cells under bioreactor cultures.

5.4. Results – Part II: CHO-dp12 cultures in covalent membrane microcapsules

As microcapsules are intended to be used to culture cells in perfusion culture, it was important to investigate whether they can handle cell culture conditions with no negative effect on cell viability and on microcapsule stability. The possibility to grow cells in genipin- and PGA- containing microcapsules in flask cultures will therefore be investigated in this section.

5.4.1. Flasks perfusion culture with genipin microcapsules

It was shown in section 5.2.1 that cross-linking alginate-PLL microcapsules improved microcapsule stability and reduced microcapsule swelling. It is however important to investigate the effect of the microcapsules on cell viability and the effect of cell growth on microcapsule stability.

5.4.1.1. Cell growth in genipin-containing microcapsules

In order to understand the effect of genipin on cell growth, CHO-dp12 cells were encapsulated in genipin-containing microcapsules with different cross-linking times. Figure 5.7 shows that CHO-dp12 cells do not survive in genipin microcapsules and that cell viability decreases quickly. The cross-linking duration appears to have an influence on cell viability as the cells encapsulated in microcapsules cross-linked for 2 hours grow up to day 2 before losing viability, while cells encapsulated in microcapsules cross-linked for 4 and 6 hours appear to have a similar growth profile and survive until day 1 before losing viability.

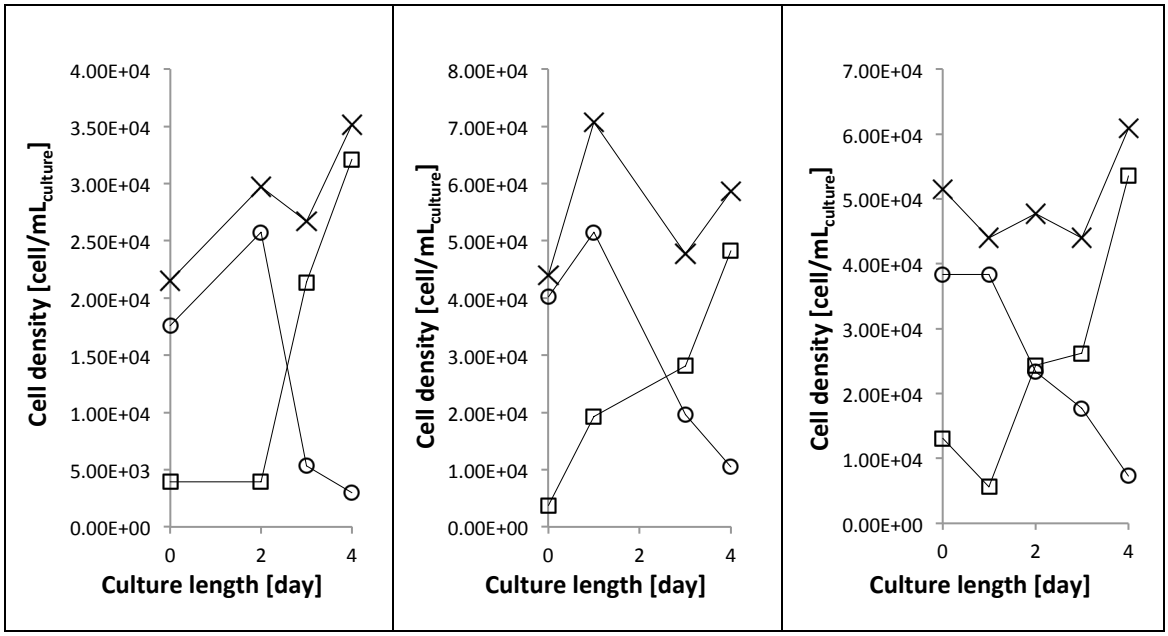

Figure 5.7 : Evolution of the total cell density (crosses), viable cell density (spheres) and dead cell density (squares) in encapsulated flask cultures with genipin- containing microcapsules cross-linked for 2 (left), 4 (middle) and 6 hours (right)

Genipin microcapsules are therefore not suitable to grow CHO-dp12 cells as they appear to have a negative influence on cell growth. In order to confirm the genipin

toxicity, CHO-dp12 cells were seeded at an initial cell density of 0.3 10^6cells/$mL_{culture}$ in a shake flask containing medium supplemented with 1mg/mL genipin. The results of this control showed that the cells did not survive in the flask containing genipin.

5.4.1.2. Characterisation of the genipin-containing microcapsules

The microcapsule mechanical resistance, size, membrane thickness and molecular weight cut-off was analysed during the flask perfusion culture.

Confocal analysis showed that neither the microcapsule membrane intensity nor the membrane thickness increased with the cross-linking time and that the membrane intensity and thickness remained stable during the 4 days of the culture (Figure 5.8), suggesting that genipin prevents the leakage of PLL from the microcapsule membrane. Figure 5.7 also shows that the cells are fluorescent and therefore, have been cross-linked by the genipin.

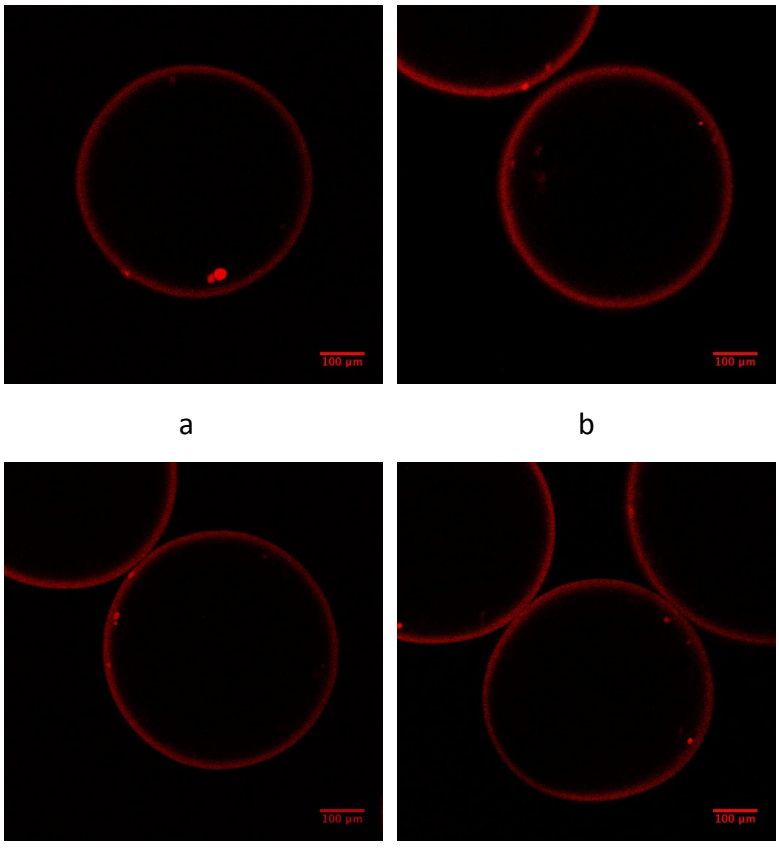

Figure 5.8 : Evolution of genipin microcapsules incubated in genipin for 2 hours (a and b) and 6 hours (c and d) between day 1 (a and c) and day 4 (b and d) of the perfusion culture.

The molecular weight cut-off analysis shows that cross-linking the PLL-membrane with

genipin does not influence microcapsule permeability. Microcapsule mechanical resistance and size evolution until day 2 are similar than those observed during the stability test.

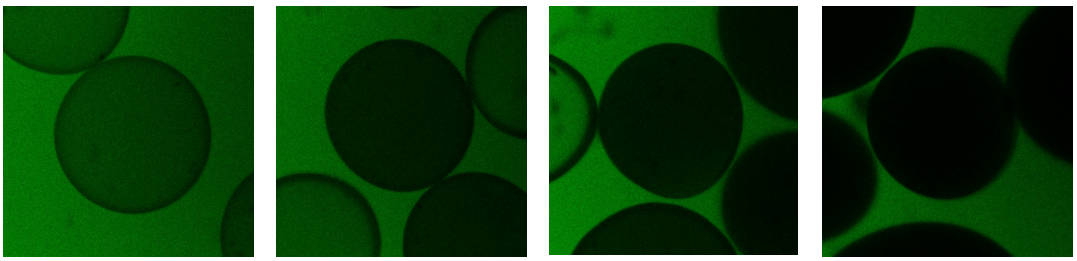

Figure 5.9 : Permeability of genipin-containing microcapsules at day 1 of the culture for 10, 40, 70 and 500kDa FITC-labelled dextran (left to right)

5.4.1.3. Genipin-containing microcapsules - Results summary

In this chapter, it has been shown that by cross-linking PLL with genipin, the leakage of PLL can be prevented as the intensity of the PLL-genipin membrane remained stable during the 4 days of culture while it was reported that PLL microcapsules showed a loss in membrane intensity of 75% without cross-linking. Genipin was shown to reduce microcapsule swelling and the mechanical resistance of the microcapsules was influenced by the cross-linking time. Microcapsules cross-linked for 6 hours were shown to remain stable for 15 days with only 25% of mechanical resistance loss. In the tested condition, genipin was however not suitable to grow CHO-dp12 as it was shown that the cells did not survive longer than 4 days, probably due to the fact that they have been cross-linked.

5.5. Discussion

The success of live cell encapsulation is highly dependent on the ability of microcapsules to protect the cells and to withstand culture conditions. It has been shown previously (Breguet et al., 2007) that due to the mild encapsulation process, the alginate-PLL-Ca^{2+} system possessed the required properties to allow cell encapsulation without damaging the cells. Such microcapsules however, do not possess the necessary stability to protect against the conditions prevailing in the bioreactor due to the dependence of stability on the medium ionic composition, bead size and type of alginate (Demont et al. 2015). Aiming to produce microcapsules with improved stability, microcapsules with covalent membranes produced by: (1) the cross-linking of

alginate-PLL microcapsules with genipin; or (2) the transacylation of alginate-PLL microcapsules containing PGA were investigated.

PGA-containing microcapsules showed promising results for cell encapsulation in Breguet et al. (2005), Marison et al. (2004) and Munin et al. (2006) reports, however very little literature concerning this type of microcapsule has been reported since. Results showed that PGA-containing microcapsules with a transacylation time of 10 seconds had an initial mechanical resistance which is twice as high as that for alginate-PLL microcapsules however, with a transacylation time higher than 20 seconds the mechanical resistance rapidly decreased to a value below that of alginate-PLL microcapsules. It was also shown that unlike alginate-PLL microcapsules, the mechanical resistance of PGA-containing microcapsules transacylated for 10 seconds did not decrease abruptly with the start of the feed, but continuously decreased throughout the culture. This result is in contrast to the results obtained by Breguet et al. (2005), which showed that the stability of PGA-containing microcapsules decreased by only 10% within a month of initiation of a culture. McNeely and Pettitt (1973) however reported that PGA solutions were stable at room temperature at pH 3-4 and can be used up to pH 6. In more alkaline media however, alginate esters are depolymerized by a pH- dependent elimination reaction (McNeely and Pettitt, 1973). Because the calcium ions are removed with citrate from the microcapsules, the PGA and the alginate in the core of the capsules can be considered to be in solution. Since microcapsules are cultured at pH 7 in cell culture media, a depolymerisation due to pH is almost certainly occurring within the microcapsules, thereby liberating the propylene glycol cross-linked to PLL from the alginate chains of the PGA. A similar reaction is probably occurring when the transacylation time is higher than 20 seconds, where most of the propylene-glycol sites have been cross-linked with PLL, with the result that the remaining OH^- ions depolymerize the PGA, thereby reducing microcapsule stability. Due to the nature of the polymer, reported to be unstable at pH higher than 6, it can safely be concluded that PGA microcapsules cannot are unsuitable to culture mammalian cells that need to be cultured at pH 7-7.2.

Results also showed that the scale-up of PGA-containing microcapsule production is difficult since the initial mechanical resistance of the PGA-containing microcapsules

tended to decrease dramatically with an increase of the volume of the capsules to be produced. For such a short reaction time of 10 seconds transacylation, it is important that the mixing time of the NaOH within the solution remains constant with scale-up as an increase in mixing time would result in a reduction of transacylation time. Unfortunately, as shown by the results and explained by Doran (2013), the relationship between mixing time and power consumption makes it rarely possible in practice, since the power required to achieve equal mixing in a larger volume does not increase linearly (Doran, 2013). In order to produce a larger amount of PGA-containing microcapsules, the microcapsule transacylation time would therefore have to be adapted to the mixing time.

Genipin-containing microcapsules were reported to improve microcapsule stability and biocompatibility when genipin was use to cross-link microspheres made of chitosan, gelatin or poly-L-ornithine (Chen et al., 2009, 2006, 2005; Hillberg et al., 2013; Liang et al., 2003). No studies however have been found concerning the stabilisation of alginate-PLL microcapsules using genipin, despite the fact that alginate-PLL microcapsules are the most widely characterised microcapsules for both cell implants and recombinant protein production. It was previously reported by Chen et al. (2006) and Hillberg et al. (2013) that there was little impact of genipin concentration for the cross-linking of chitosan or PLO microcapsules. They also reported that the cross-linking was dependent on the time and on the temperature of the reaction, a higher temperature improving the cross-linking reaction (Chen et al., 2006; Hillberg et al., 2013). Taking these findings into account, it was therefore decided to use a genipin concentration of 1mg/mL, since Hillberg et al. (2013) reported this to be the optimal concentration to cross-link PLO, which has a similar structure than PLL. The cross-linking temperature was set at 37°C due to the presence of cells. Results showed that, as expected, the cross-linking reaction generated a fluorescent PLL-genipin conjugate that can be visualized by confocal laser scanning microscopy. This result is similar to those observed by Chen et al. (2005, 2006, 2009) with chitosan microcapsules cross-linked with genipin. Commercially available alginates have been shown to be highly sensitive to the presence of monovalent ions, such as Na^+ or K^+, during the gelling process using Ca^{2+} (Draget et al., 1998; Martinsen et al., 1989; Beate Thu et al., 1996; B. Thu et al., 1996). Draget *et al*. (1998) showed that the addition of KCl and NaCl in the

buffer solution results in decreased alginate gel strength, with the effect more pronounced for NaCl than for KCl (Draget et al., 1998). As a result, alginate-containing microcapsules have a tendency to swell and to be destabilised in the presence of these ions. Chen et al. (2009) suggested that the formation of a strong membrane around the alginate core might minimize swelling and stabilise the alginate-containing microcapsules. In this study, the morphological changes and the stability of microcapsules exposed to cell culture media have been investigated. Results show that as expected, and improvement of mechanical resistance and a reduction of microcapsule swelling can be observed when alginate-PLL microcapsules were cross-linked with genipin. These results are in agreement with those observed by Chen et al. (2009) with alginate-chitosan microcapsules cross-linked with genipin.

Under the conditions studied however, genipin was shown to be not suitable for the growth of CHO-dp12 since the cells did not survive more than 4 days. This result is in contrast with results reported by Paul, (2010) and Hillberg (2013) who both found an optimal cell viability within microcapsules cross-linked with genipin. Numerous papers however report the toxicity of genipin on mammalian cells such as HepG2 cells, hepatoma cells, leukemia cells, HeLa cells or lung cancer cells (Cao et al., 2010; Feng et al., 2011; Khanal et al., 2012; Kim et al., 2005; Mailloux et al., 2010; Yang et al., 2013). These publications also report that genipin affected cells in a dose- dependent manner, inducing apoptosis and cell cycle arrest in G1 (Cao et al., 2010) or G2/M phases (Feng et al., 2011). According to these studies, it also seems that JNK pathway is playing an important role in apoptosis induced by genipin and that JNK may contribute to the increased amount of p53, which is known to induce cell apoptosis (Cao et al., 2010; Feng et al., 2011; Khanal et al., 2012; Kim et al., 2005; Mailloux et al., 2010; Yang et al., 2013). The highest genipin concentration tested in these studies is 10 times lower than the genipin concentration used to cross-link the alginate-PLL microcapsules, the genipin concentration present in the media can therefore be interpreted as toxic for the cells.

5.6. Conclusion

The present work reports on the possibility to produce microcapsules with improved stability compared to the widely characterized alginate-PLL microcapsules, using microcapsules with covalent membranes created by: (1) the cross-linking of alginate-PLL microcapsules with genipin; or (2) the transacylation of alginate-PLL microcapsules containing PGA. Experiments have been performed to assess the stability properties of the microcapsules under perfusion culture conditions.

The stability of PGA-containing microcapsules was shown to be dependent on the media pH, PGA being unstable at pH higher than 6. It can therefore be safely concluded that PGA microcapsules cannot be used to culture mammalian cells, as they need to be cultured at pH 7-7.2. They could however offer a stable solution to perform encapsulated cultures of cells that are usually grown at lower pH such as yeast, bacteria or microalgae.

Genipin microcapsules were shown to prevent the leakage of PLL, to reduce microcapsule swelling. The microcapsule mechanical resistance was shown to be influenced by the cross-linking time, the optimal conditions being obtained with microcapsules cross-linked for 6 hours. Under perfusion culture conditions, these microcapsules showed a loss of resistance of 30% within 15 days while alginate-PLL microcapsules had a loss of resistance of 87% during the same period of time. It was however revealed that genipin is not suitable to grow CHO-dp12 as these cells did not survive longer than 4 days within the microcapsules. It may be possible to use genipin microcapsules to grow other types of cells such as HeLa cells, HEK-293 or BHK however, CHO cells have been reported to be used to produce 70% of the recombinant proteins in 2014, and are therefore the most important animal cell calls to encapsulate (Butler and Spearman, 2014). Moreover, several reports about cell apoptosis induced by genipin could be found in the literature. It can therefore safely be concluded that despite the good stability properties, genipin cannot be generally used to cross-link microcapsules intended to grow mammalian cells. Genipin-containing microcapsules could however, be considered in the field of drug delivery.

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6. An understanding of potentials and limitations of alginate- based microcapsules for the cultivation of anchorage- dependent cells

Abstract

Many valuable recombinant human proteins are obtained from the cultivation of anchorage dependent cells and therefore require large-scale process to obtain the required amount of product. Since anchorage-dependent cells need surfaces for attachment, they are commonly grown on microcarriers in suspension cultures. In this chapter, the possibility to use alginate-based microcapsules as a support to culture CHO-K1, an adherent cell line, was investigated by encapsulating the cells in alginate beads, alginate-poly-L-lysine beads and alginate-poly-L-lysine microcapsules. Results show that CHO-K1 were unable to grow in alginate beads however, in the presence of poly-L-lysine (PLL), the cells grew to a density of $8x10^5$ cells/$mL_{culture}$ in 7 days in alginate-PLL microcapsules with a growth rate of $0.016h^{-1}$. This cell density is similar to that obtained for non-encapsulated cell cultures however, the specific growth rate, μ, is 50% lower than for non-encapsulated cell cultures.

6.1. Introduction

Large-scale culture of anchorage- dependent cells is important in the biopharmaceutical industry since many recombinant human proteins, such as erythropoietin, tissue plasminogen activator or viral vectors used for gene therapy are currently produced using anchorage-dependent cell lines (Kwon and Peng, 2002). In order to increase product concentrations, high cell density culture of anchorage dependent cell lines is required. This can be done by implementing anchorage dependent cultures into a suspension (fluidised-bed) culture system, which has a high production efficiency per reactor volume and is easy to characterize and to control (Kwon and Peng, 2002). This implementation can be achieved by culturing anchorage-dependent cells on microcarriers made of various materials including dextran, polystyrene, polyacrylamide, cellulose, gelatin, collagen or chitosan (Gröhn et al., 1997; Kwon and Peng, 2002). It has been reported that most commercially available microcarriers were designed to meet requirements for large-scale industrial production of biological products however, some disadvantages, such as the need for enzymatic digestion for cell harvesting, the limitation in size and geometry and the insufficient biocompatibility, were also described (Gröhn et al., 1997). The requirement for surface structures allowing cell attachment and spreading is also well documented together with the role of collagen or other connective tissue proteins in cell attachment and growth. This explains therefore why collagen-based microcarriers are widely used in biotechnology (Gröhn et al., 1997). Collagen-based microcarriers are usually prepared from a suspension of crude collagen extract, spherical beads being formed by discharging a suspension of collagen fibres into liquid nitrogen, followed by dehydration and cross-linking reactions of the gel beads with formaldehyde or glutaraldehyde vapours (Tsai et al., 1998). These procedures employ harsh conditions and are therefore not appropriate for *in-situ* cell entrapment for subsequent cell culture (Tsai et al., 1998). Alginate on the other hand is biocompatible and beads can be formed under mild conditions by simply adding alginate droplets to solutions containing divalent cations. Moreover, beads or pore sizes can be adjusted by varying the alginate concentration, composition or molecular weight, or by coating alginate beads with polyelectrolytes (Andersen et al., 2015).

In the previous chapters, alginate-polyelectrolyte microcapsules were shown to be unsuitable for perfusion culture of CHO-dp12 due to their extreme sensitivity to medium composition. However, they have been shown to be stable in batch and fed-batch cultures when the colonization remained less than 14% of the microcapsule volume. Since CHO-dp12 are suspension-adapted cells, there is no advantage in encapsulating them to cultivate them in batch or fed-batch modes since no medium is removed and no cell retention system is consequently needed. Therefore, here we investigated the possibility of using alginate-based microcapsules as a type of microcarrier to culture CHO-K1, an adherent cell line which does require a surface on which to grow in all culture modes.

6.1. Material and Methods

Material and methods used in this chapter are described in section 2.2. Specific experiments set-up are described below.

6.1.1. Shake flask encapsulated cultures and stability tests

20mL of cell containing microcapsules or empty microcapsules were cultured in shake flask (Corning Inc, Corning, NY, USA), with a working volume of 100mL and a microcapsule volume representing 25% of the medium volume. The culture was then incubated at 37°C, under a humidified atmosphere of air containing 5% CO_2 and an agitation of 100rpm. Each culture and stability test carried out in shake flask were carried out only once

6.1.2. Encapsulation

The encapsulation method used was a modification of the technique originally developed by Lim and Sun (Lim and Sun, 1980; Lim, 1983, 1982;) undertaken under completely sterile conditions using a vibrating nozzle encapsulation device (Encapsulator Biotech, EncapsBio, Switzerland or Inotech IE-50R, Inotech, Switzerland) as described elswhere (Serp et al., 2000). Alginate (Manucol DH, FMC biopolymers, UK) beads were first formed by extrusion of 300mL pre-sterilised (AP20, AP15, 0.2µm Steritop filters, Merck Millipore, Ireland) sodium alginate solution (1.5% w/v) containing cells ($0.7 \cdot 10^6$ $_v$cells/$mL_{alginate)}$ through the encapsulator nozzle into an

aqueous solution of $CaCl_2$ 110mM (Sigma, St-Louis, USA). After 5 min incubation at room temperature the $CaCl_2$ solution was removed and the microcapsules were washed with saline buffer (0.85% NaCl in 10mM MOPS, Sigma, St-Louis, USA) followed by incubation in 1.2L 0.05% (w/v) poly-L-lysine 30-70kDa (Sigma, St-Louis, USA) for 30 min at room temperature under agitation. The microcapsules were then re-washed with saline buffer followed by incubation in 1L 0.03% sodium alginate for 10min under agitation. After washing with saline buffer, the solid alginate core of the microcapsules was liquefied by incubation for 15min in 50mM sodium citrate (Sigma, St-Louis, USA). After core liquefaction with citrate, the microcapsules were washed with saline buffer followed by a wash with cell culture media before being transfer to cell culture media to be inoculated.

6.1.3. Microcapsule characterisation and culture analysis

Homogenous culture samples containing microcapsules were regularly removed from the bioreactor to determine intra/extra-capsular cell number, concentration of key culture metabolites, microcapsule size, mechanical resistance, molecular weight cut-off (MWCO) and membrane thickness using methods described in sections 2.2.2 and 2.2.3.

6.1.3.1. Cell apoptosis determination

In order to be able to determine whether the cells within the microcapsules were going into apoptosis, cells were dyed with FITC-labelled annexin V (apoptotic cells) and propidium iodide (dead cells) and incubated at 37°C for two hours followed by confocal imaging. The microcapsules were analysed with a multiphoton confocal microscope (Zeiss LSM 710 and ConfoColor 3). The excitation wavelength was set at 488 nm, since this wavelength can excite both dyes, and the emission between 500 and 600 nm for the FITC-labelled Annexin V and between 600 and 700nm for propidium iodide. The gain was adapted from 500 to 1000 for each emission wavelength; the pinhole was set at 33.5 and the averaging was set at 4. Moreover, in order to perform a z-stack imaging to see the cell distribution within the microcapsules, the top and bottom position of the microcapsules was defined, such

that the width of each slice of image taken was known.

6.2. Results

6.2.1. Reference culture - Determination of the CHO-K1 growth kinetics and stoechiometric parameters in suspension culture

In order to understand whether adherent cells can be cultured within microcapsules, it is first important to perform a reference non- encapsulated culture in flasks to determine the cell growth kinetics and stoichiometric parameters. CHO-K1 were therefore cultured for 8 days under batch conditions in T25 flasks (sufficient to enable removal of 1 flask per day for cell numeration) with an initial cell density of 1 x 10^4 cell/mL, in 5mL DMEM-F12 medium.

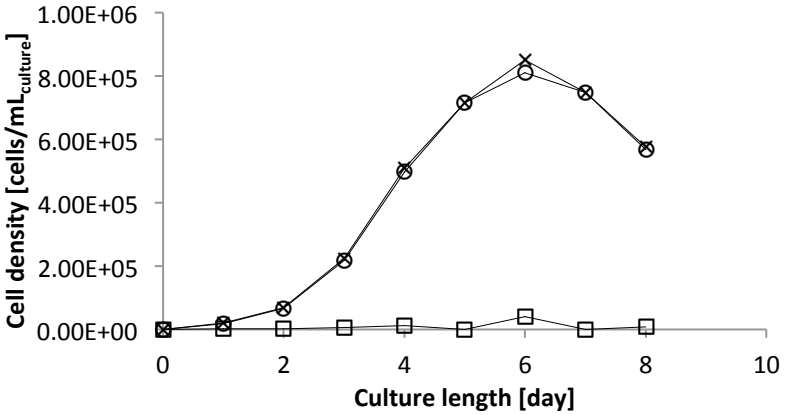

Figure 6.1 : Growth rate kinetics of CHO-K1 in T25 flasks. The total cell density is represented by crosses, the viable cell density by spheres and the dead cells by squares. Cells show a maximum specific growth rate μ of 0.0465 h^{-1}

The cell growth kinetics in Figure 1.1 show a growth rate of 0.0465 h^{-1} and a maximum cell density of 8.5 x 10^5 cells/mL after 6 days of culture and a viability of 95%.

6.2.2. Determination of the possibility to grow adherent cells in alginate-PLL microcapsules

To determine if anchorage-dependent cells could adhere on Ca-alginate gels, CHO-K1 batch cultures were performed in flasks which had been coated with Ca-alginate gel, in Ca-alginate microbeads, in Ca-alginate-PLL microbeads and in Ca-alginate-PLL microcapsules.

6.2.2.1. Culture of adherent cells in flasks coated with alginate

CHO-K1 were cultured under batch conditions for 7 days in cell culture dishes coated with 3mL Ca-alginate gel (sufficient to enable removal of 1 flask per day for cell numeration) with an initial cell density of 5 x 10^4 cell/$mL_{culture}$, in 5mL DMEM-F12 medium in order to determine whether alginate, or leachates from the alginate, inhibited cell growth and whether the cells could adhere and grow on gelled alginate.

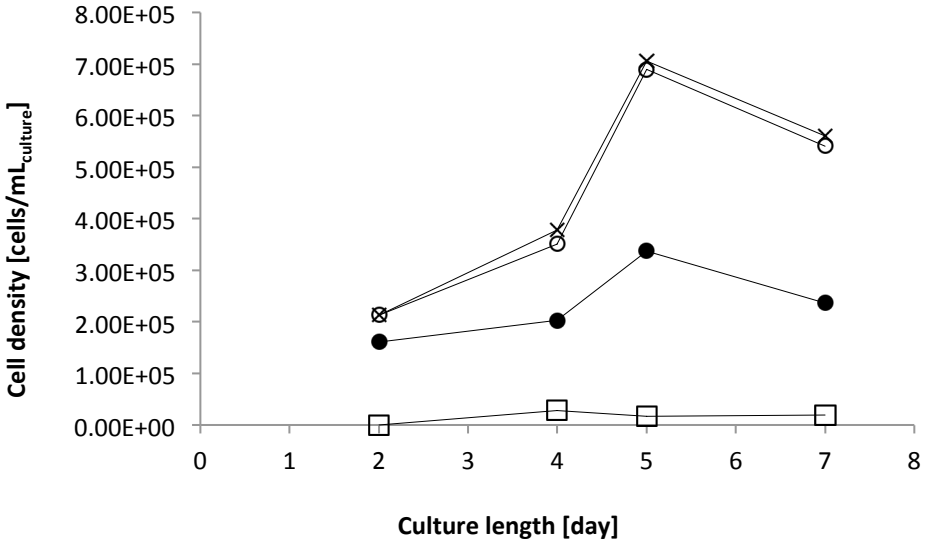

Figure 6.2 : Growth kinetics of CHO-K1 in flask coated with Ca-Alginate gel. Viable cells show a maximum specific growth rate μ of 0.0155h^{-1}. The total cells are represented by a crosses, the total viable cells by empty spheres, the total adherent cells by full spheres and the dead cells by empty squares. The cell counts started at day 2 as the initial cell density was too low to be counted with the trypan blue method and a Neubauer haemocytometer.

In the presence of the alginate gels (Figure 6.2), 50% of cells adhered onto the gel or onto the bottom of the plate, showing a growth rate of 0.0095h^{-1} and attained a maximum cell density of 3.375 x 10^5 cells/mL after 5 days of culture. The remaining 50% of cells adhered together, forming clusters, which grew in suspension, with a growth rate of 0.026h^{-1} reaching a maximum cell density of 3.52 x 10^5 cells/mL after 5 days of culture. It was however shown by seeding a 25mL shake flask with 0.3 x 10^6 cells/mL in DMEM-F12 medium that CHO-K1 were not able to grow in suspension since no cells survived after 24 hours of culture.

The observed growth rates in the presence of alginate are significantly lower than those observed in the reference suspension culture, showing that alginate had an inhibiting effect on the growth of adherent cells. It is however, likely that alginate is

not toxic as the viability remained above 97%.

6.2.2.2. Encapsulated culture of CHO-K1

Adherent cells were encapsulated and grown in batch mode in alginate beads, alginate-PLL beads and alginate-PLL microcapsules in order to investigate the possibility to use microcapsules as a novel microcarrier. It was shown previously that in the presence of alginate, adherent cells have a tendency to adhere together forming clusters and that the growth rate was reduced due to the presence of alginate. It was therefore expected that in alginate-PLL microcapsules, the cells would migrate to adhere together and form clusters since the alginate core had been liquefied. As a result the growth rate would be expected to be lower than that of the reference cultures. However it is still unsure how the cells would behave when entrapped in the Ca-alginate gel of the alginate beads and alginate-PLL microcapsules.

As shown previously in Chapter 3, alginate-PLL microcapsules are stable in batch and fed-batch culture conditions, it is therefore expected that the mechanical resistance of microbeads and the microcapsules would remain stable during this batch culture containing CHO-K1.

6.2.2.2.1. Microcapsule characterisation

In chapter 3, it was shown that PLL-alginate microcapsules were not suitable for perfusion culture due to the extreme sensitivity to medium composition. However, they have been shown to be stable in batch culture and fed-batch culture. It was therefore decided to use them to grow adherent cells in batch or fed- batch as a new type of microcarrier. In order to confirm the stability of alginate-based microcapsules under batch conditions the evolution of the mechanical resistance and size was analysed throughout the batch culture for the alginate microbeads, alginate-PLL microbeads and alginate-PLL microcapsules.

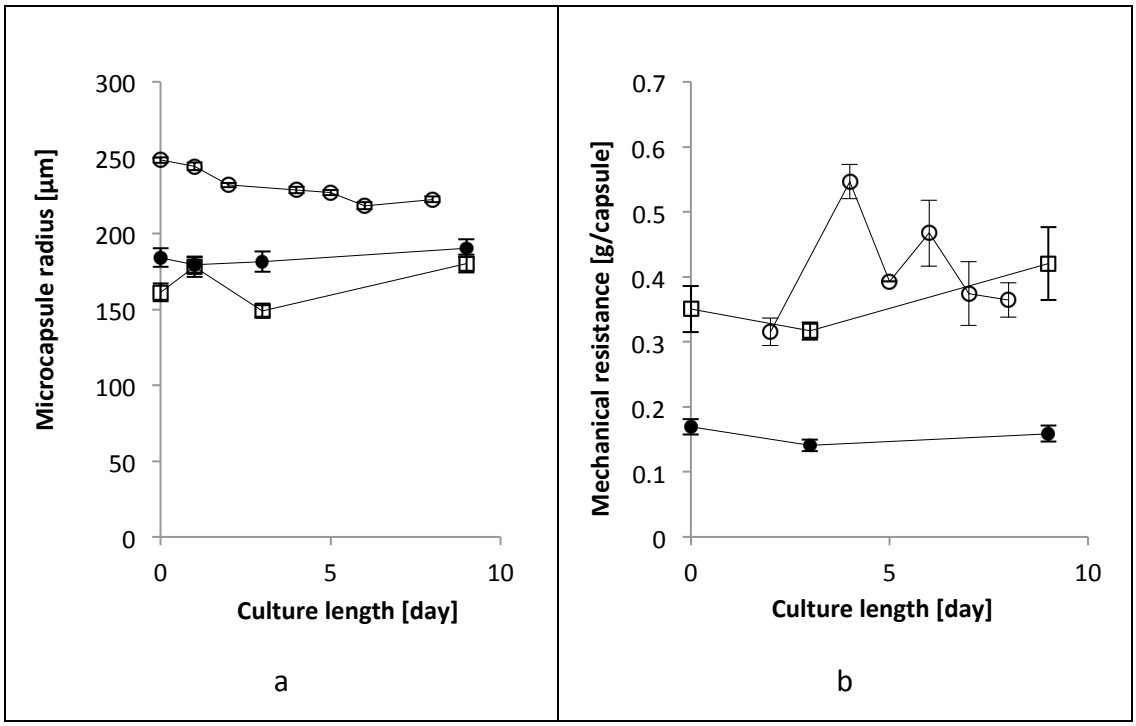

Figure 6.3 : Evolution of the size (a), of the mechanical resistance (b) of alginate microbeads (empty spheres), alginate-PLL beads (empty squares) and alginate PLL microcapsules (full spheres) during the encapsulated batch culture of CHO-K1. The microbead radius is given in µm (n=30) and the mechanical resistance in g/capsule (n=5), the error bars represent the standard error of the mean.

Figure 6.3a shows that the size of the microcapsules remained stable during the 9 days of the culture. The alginate microbeads have a slightly larger size than the alginate-PLL microbeads and microcapsules due to the fact that they were extruded separately. Alginate-PLL microcapsules are slightly larger than alginate-PLL microbeads due to the citrate core liquefaction step. Figure 6.3b shows that the mechanical resistance remains stable for alginate beads, alginate-PLL beads and alginate-PLL capsules throughout the 9 days of the culture, thereby confirming the results obtained in chapter 3 that alginate-PLL microcapsules remain stable under batch and fed-batch culture conditions.

6.2.2.2.2. Cell growth in microencapsulated batch culture

The shake flasks containing DME-F12 medium were inoculated with 25% (v/v) of microcapsules containing CHO-K1 at an initial cell concentration of 2x 10^5 cell/$mL_{Alginate}$. The 3 encapsulated cultures were performed under the same culture conditions.

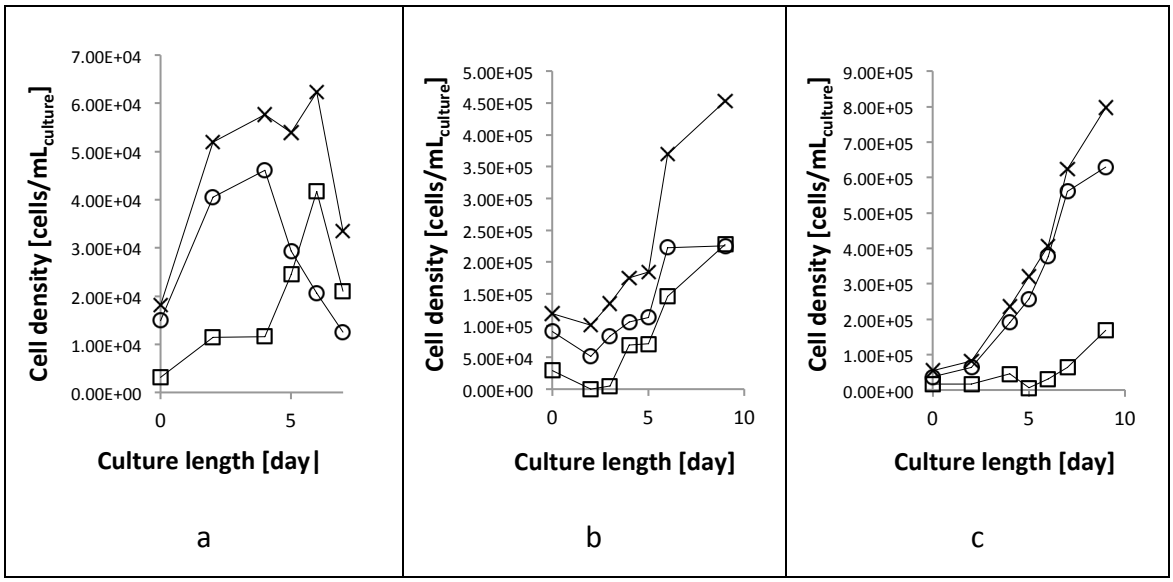

Figure 6.4 : Growth of CHO-K1 cells in encapsulated batch in alginate microbeads (a), alginate-PLL microbeads (b) and alginate-PLL microcapsules (c). The total cell density is represented by crosses, the viable cell density by spheres and the dead cells by squares.

The culture performed in alginate beads shows an exponential phase of one day, followed by a stationary phase of two days and a decline phase. The cells encapsulated in alginate-PLL beads and microcapsules both show an exponential phase of 5 days followed by a stationary phase. The viability at the end of the exponential phase is however lower for the cells in alginate-PLL beads than for the cells in alginate-PLL microcapsules, with a viability of 59% for the beads and 90% for the microcapsules (Figure 6.4).

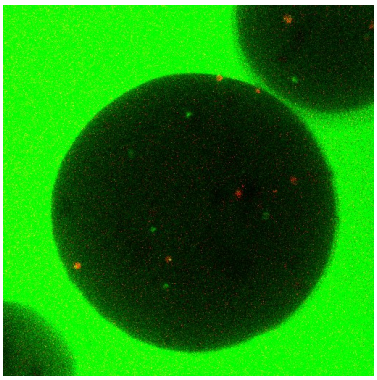

Figure 6.5 : Confocal microscopy analysis of the cell viability and apoptosis in alginate microbeads using propidium iodide and annexin V. The dead cells are shown in red while the living cells entering into apoptosis are shown in green. 500kDa FITC-dextran was used to delimit the microcapsules from the medium.

Confocal microscopy imaging shows that even though some of the cells are still alive in the alginate microbeads until day 3, the cells were entering into apoptosis since annexin V can react with the cells in the microcapsules (Figure 6.5). This phenomenon is not observed in alginate-PLL microbeads and microcapsules.

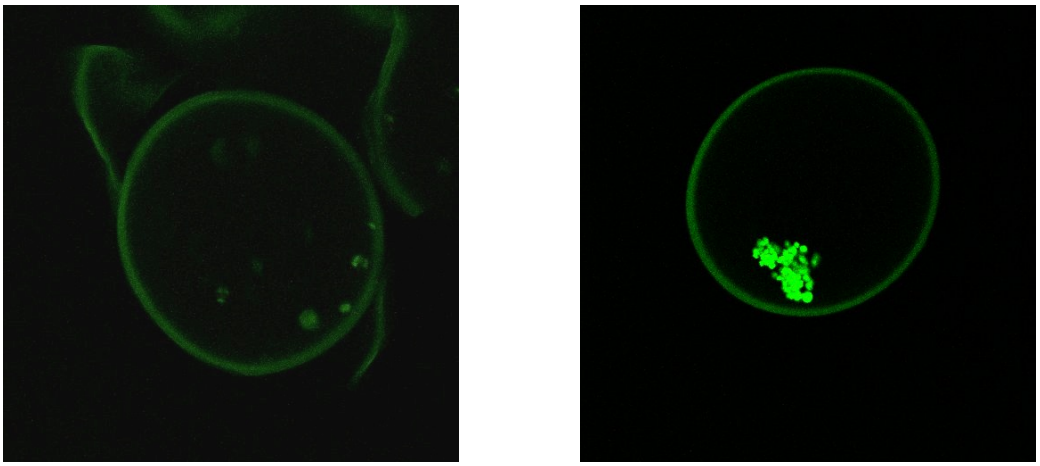

Figure 6.6 : Confocal microscopy image of alginate-PLL beads (left) and microcapsules (right). The cells are dyed with calcein (living cells, green) and propidium iodide (dead cells, red) to investigate dispersion in the capsule and viability. The PLL membrane is labelled with FITC.

When dyed with calcein and propidium iodide (Figure 6.6), the CHO-K1 showed a high viability in both alginate-PLL beads and microcapsules. It can also be observed that in both types of carrier, the cells grow on the edge of the core, close to the PLL membrane.

Table 6.1 : Kinetic and stoichiometric parameters of suspension and encapsulated CHO-K1 cultures

Value	Units	Suspension reference culture	Suspension batch in alginate coated flasks	Encapsulated batch in alginate microbeads	Encapsulated batch in alginate-PLL microbeads	Encapsulated batch in alginate-PLL microcapsules
μ	(h^{-1})	$4.65\ 10^{-2}$	$2.95\ 10^{-2}$	$2.5\ 10^{-3}$	$1.32\ 10^{-2}$	$1.625\ 10^{-2}$
Culture duration	(h)	192	168	192	216	216
$X_{culture,\ max}$	(cell $mL^{-1}_{culture}$)	$8.5\ 10^{5}$	$7.06\ 10^{5}$	$5.76\ 10^{4}$	$4.5\ 10^{5}$	$7.98\ 10^{5}$
q_s	(mmol $cell^{-1}\ h^{-1}$)	$5.11\ 10^{-7}$	$6.92\ 10^{-6}$	$1.13\ 10^{-7}$	$9.89\ 10^{-8}$	$1.77\ 10^{-7}$
$q_{lactate}$	(mmol $cell^{-1}\ h^{-1}$)	$8.41\ 10^{-7}$	$2.62\ 10^{-7}$	$5.94\ 10^{-7}$	$4.3\ 10^{-8}$	$1.2\ 10^{-7}$
$q_{glutamine}$	(mmol $cell^{-1}h^{-1}$)	$1.86\ 10^{-8}$	$1.67\ 10^{-8}$	$3.3\ 10^{-8}$	$2.67\ 10^{-7}$	$2.28\ 10^{-8}$
q_{NH3}	(mmol $cell^{-1}\ h^{-1}$)	$1.48\ 10^{-8}$	$1.21\ 10^{-8}$	$4.2\ 10^{-9}$	$2.09\ 10^{-7}$	$2.24\ 10^{-8}$
$Y_{X/S}$	(cell $mmol^{-1}$)	$9.09\ 10^{4}$	$1.02\ 10^{5}$	$2.2\ 10^{4}$	$1.34\ 10^{5}$	$7.35\ 10^{4}$
$Y_{lactate/S}$	(mmol $mmol^{-1}$)	1.5	1.01	3.39	0.611	0.776

Symbols: $X_{culture,\ max}$ the maximum total cell density obtained per $mL_{culture}$; μ the specific growth rate; q_s the specific consumption of glucose; $q_{lactate}$ the specific productivity of lactate; q_{NH3} the specific productivity of NH_3; $q_{glutamine}$ the specific consumption of glutamine; $Y_{X/S}$ the yield of biomass with respect to glucose; and $Y_{lacatate/S}$ the yield of lactate with respect to glucose.

The cells in the reference suspension culture grew to a maximum cell density ($X_{culture,max}$) of 8.5x 10^5 cells/$mL_{culture}$ with a growth rate μ of 0.0465 h^{-1} while the suspension cells in the flasks coated with alginate grew to a maximum cell density ($X_{culture,max}$) of 7.06x 10^5 cells/$mL_{culture}$ with a growth rate μ of 0.0295 h^{-1}. This result shows that the presence of alginate in the flask appears to have a negative effect by hindering the cell growth. In encapsulated cultures, the cells reached a maximum cell density of 5.76x 10^4 cells/$mL_{culture}$ when entrapped in alginate beads, 4.5x 10^5 cell/$mL_{culture}$ when entrapped in alginate-PLL beads and 7.98x 10^5 cell/$mL_{culture}$ when encapsulated in alginate-PLL microcapsules with growth rates μ of 0.0025 h^{-1}, 0.0132 h^{-1} and 0.01625 h^{-1} respectively (Table 6.1). These results show that adherent cells cannot grow in alginate beads. However, the presence of a PLL layer around the alginate beads allowed cell growth up to a cell density of 4.5x 10^5 cell/$mL_{culture}$ with a growth rate of 0.0132 h^{-1}. Liquefying the alginate core of the microcapsules allowed the cells to migrate and form clusters, favouring growth and achieving a maximum cell density of 7.98x 10^5 cell/$mL_{culture}$ which is similar to the maximum cell density obtained in suspension cultures. The growth rate of the cells encapsulated in alginate-PLL microbeads and microcapsules is 50% lower than the growth rate of the suspension cells grown in the presence of alginate. This reinforces the hypothesis concerning the negative effect of alginate on cell growth.

The yield of lactate to glucose $Y_{lact/S}$ for the culture encapsulated in alginate-PLL beads (0.611 mmol $mmol^{-1}$) and alginate-PLL microcapsules (0.776 mmol $mmol^{-1}$) were 60% and 50% lower respectively than for the reference culture (1.5 mmol $mmol^{-1}$). This result indicates a more efficient utilization of glucose via respiration in the cultures two encapsulated cultures compared to the reference culture.

The slow growth rate of the encapsulated cells and the low colonisation observed may be due to the presence of alginate, however it may also be an indication that the cells were not able to grow freely within the capsules due to diffusional limitations of the cell nutrients. However,the similar values for the specific consumption rates of glucose q_s and the specific productivity of lactate $q_{lactate}$ observed between the different encapsulated and non-encapsulated cultures suggests that there was no significant diffusional limitation and that the low level of colonisation was mainly due to the

presence of alginate (Table 6.1).

6.3. Discussion

In the present study, the possibility of using alginate-based microcapsules to encapsulate adherent cells in order to grow them in CSTR bioreactors has been evaluated. It has been shown previously (Breguet et al., 2007) that due to the mild encapsulation process, the alginate-PLL-Ca^{2+} system possessed the required properties to allow cell encapsulation without damaging the cells. Such microcapsules however, did not possess the necessary stability to protect against the conditions prevailing in the bioreactor under perfusion culture operation due to the dependence of stability on the medium ionic composition, bead size and type of alginate (Demont et al. 2015). Nevertheless it was shown by Demont et al. (2015) that alginate-PLL-Ca^{2+} microcapsules were stable in batch and fed-batch modes, which do not involve medium replacement and could therefore be an option to grow adherent cells in CSTR bioreactor.

The results of the present study show that when CHO-K1 cells were encapsulated in alginate beads, they grew from about $2x10^4$cells/$mL_{culture}$ to about $6x10^4$ cells/$mL_{culture}$ in 4 days before entering into apoptosis. When grown in alginate-PLL beads, CHO-K1 grew from $1x10^5$ cells/$mL_{culture}$ to about $4.5x10^5$cells/$mL_{culture}$ within 6 days before entering stationary phase and when the alginate core was liquefied to form alginate-PLL microcapsules, CHO-K1 grew from $5x10^4$cells/$mL_{culture}$ to $7.98x10^5$cells/$mL_{culture}$ in 7 days of culture before entering stationary phase. The results therefore show that the presence of PLL provided a more suitable environment for the growth of CHO-K1 compared to alginate.

The results also seem to show that only when in contact with the positively charged PLL membrane, CHO-K1 were capable of proliferation. This result is similar to those obtained by Tsai et al., (2006) in alginate beads containing collagen where they showed that fibroblasts were only capable of proliferation when in contact with the collagen fibres present in the alginate beads. Liquefying the alginate entrapping the cells appeared to improve cell proliferation, allowing cells to migrate closer to the PLL membrane and to form clusters. This confirms the statement of Rowley et al. (1999)

suggesting that as cell adhesion is a prerequisite for cell survival, cells will have to be cultured as multicellular aggregates in alginate hydrogels.

The low viability of CHO-K1 in alginate beads is in agreement with different studies showing that cells such as GH3 rat pituitary tumour cells, 3T3 fibroblasts or osteoblast cells were unable to grow in alginate beads (Hsu et al., 2000; Tsai et al., 2006, 1998; Wu et al., 2007).

This inability of the cells to grow on alginate beads was reported to be due to the cell incapacity of the cells to interact with alginate polysaccharides due to the low protein adsorption of the hydrophilic alginic acid which promotes less cell attachment and spreading (Kwon and Peng, 2002). Cell adhesion is a strict requirement for the survival and the growth of anchorage dependent cells, moreover, many other cellular functions such as migration, proliferation, differentiation and apoptosis are coordinated by cell attachment (Price, 1997; Rowley et al., 1999). Rowley et al. (1999) explained that the interaction between the cells and the biomaterials is mediated by receptors in the membrane, which can recognize the molecules needed for the adhesion at the surface of the material. Many polymers, according to Rowley et al. (1999) thermodynamically promote the adsorption of the molecules required for cell adhesion, leading to an extensive deposit of the protein from the surrounding medium onto the hydrophobic surfaces. Some hydrogel such as alginate, on the other hand, have a hydrophilic nature which discourage protein adsorption (Rowley et al., 1999).

Machida-Sano et al., (2014) investigated the protein absorption capacity and the surface features (wettability, charge, roughness) associated with the protein adsorption abilities of alginate films. They observed that the surface properties of alginate films varied with the type of ions used to cross-link the alginate and that as a consequence, the cell affinity of alginate also varies. According to their research, the ions used to cross-link the alginate have no influence on the film charges, which is negative, however, they have an influence on the morphology and the wettability of the film. It was shown that ferric, aluminium and barium ions produce alginate films supporting a better cell growth and adsorbing a higher amount of protein than other types of ions, calcium ions giving the lowest results as Ca-alginate gels are highly hydrophilic (Machida-Sano et al., 2014). This study therefore confirms that Ca-alginate

gels are not suitable for the growth of adherent cells.

As adherent cells do not possess the receptor required to adhere on alginate gel, adhesion, spreading and therefore proliferation of adherent cells on an alginate hydrogel is not possible (Andersen et al., 2015; Price, 1997). Different solutions have been used to overcome this problem such as preparing microcapsules containing a collagen fibrous network within the alginate core (Hsu et al., 2000; Tsai et al., 2006, 1998; Wu et al., 2007) or using ferric or barium ions to cross-link the alginate gel (Gröhn et al., 1997). Chemically modifying the alginate molecule in order to covalently link the hydrogel with cell adhesion ligands such as RGD-containing peptides (Rowley et al., 1999) was reported to improve cell adhesion. RGD-alginates are commercially available and C2C12 myoblast and MDCK cells were shown to display a rapid proliferation within this type of alginate (Andersen et al., 2015; Rowley et al., 1999). Kwon and Peng (2002) used the property of propylene glycol alginate (PGA) to form an amide linkage with the amino group of proteins in order to produce microcarriers with a gelatin matrix for cell adhesion. Adherent cells such as CHO-K1 and PA317 cells were shown by Kwon and Peng (2002) to exhibit similar growth kinetics on PGA-gelatin microcarriers than when grown on commercial polystyrene microcarriers. The growth rate of CHO-K1 reported by Kwon et al. (2002) was 0.039 h^{-1} which is double the growth rate obtained in alginate-PLL microcapsules (0.01625 h^{-1}, Table 1.1).

6.4. Conclusion

The present work reports on the investigation of the possibility to use alginate-based microcapsules as a type of support to culture an adherent cell line. Several experiments have been performed to compare the growth kinetics and the viability of CHO-K1 in several encapsulated and non-encapsulated culture conditions, resulting in the conclusion that anchorage- dependent cells cannot grow in alginate microbeads under the tested conditions. The presence of PLL however was shown to improve cell growth, the cells reaching a similar cell density in alginate-PLL microcapsules than in non-encapsulated culture, the growth rate being yet 50% lower in encapsulated culture than in the non-encapsulated one. As anchorage dependent cells cannot adhere to the alginate matrix, there is a need to improve the microcapsule composition to promote cell adhesion and therefore improve cell proliferation. Several

solutions have been documented in the literature such as using ferric or barium ions to cross-link the alginate gel (Gröhn et al., 1997; Machida-Sano et al., 2014), chemically modifying the alginate molecule in order to covalently link the hydrogel with cell adhesion ligands such as RGD-containing peptides (Rowley et al., 1999) or producing microcarriers by cross-linking gelatin with propylene-glycol alginate (Kwon and Peng, 2002). Another solution would be to investigate the possibility to grow adherent cells on a BSA matrix within microcapsules made with alginate, propylene-glycol alginate, BSA and PLL such as those reported by (Marison et al., 2004).

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7. Conclusion

Nowadays, the majority of the recombinant proteins are produced using mammalian cells. Due to mammalian cell sensitivity to shear stress and their low-productivity, there is a need for high cell density systems, which allow the production of high concentrations of recombinant proteins. Entrapping the cells within semi-permeable membrane through encapsulation was therefore investigated as it would enable cell growth in a protected environment, reducing the shear stresses encountered in the bioreactor and allowed simple perfusion cultures to be performed since a simple filter mesh is enough to contain the microcapsules within the bioreactor. Microencapsulation of mammalian cells has been used for years and well documented in the cell transplantation field, however a lack of information can be observed concerning microencapsulation for high cell density culture for large-scale production of cell-derived molecules in biotechnology industry. Many procedures have been developed to produce microcapsules however; most are descriptions of the production process and lack details of characterization of the microcapsules properties. As a result there is no consensus in the properties the different microcapsules should have and many results have proven difficult to reproduce and compare. This work therefore aimed (1) to address the need to standardize the characterisation of microcapsules, in order to reduce potential lab-to-lab variation before (2) investigating the possibility to use currently available polymers to develop and characterize a new retention system based on microencapsulation to enable the implementation of high cell density perfusion culture of mammalian cells in STR bioreactor.

The project set about to firstly identify microcapsules key parameter and to gather, optimize and develop suitable methods to standardize microcapsules characterisation in order to allow results being compared with reduced lab-to-lab variation (chapter 2). Particular attention was addressed to the MWCO and the mechanical resistance of the microcapsules as they are key parameters for successful application of the microcapsules, moreover, one method amongst the several existing methods had to

be chosen for both MWCO and mechanical resistance so that comparison between results can be made.

Having completed this initial study, it was necessary to choose a reference for encapsulated cell culture and to characterise this type of microcapsules with the previously defined methods (chapter 3). The widely used and characterised alginate-PLL microcapsules were therefore investigated in detail in order to form a basis for comparison of capsules made from different polymers. Since the microcapsules can be easily retained in the bioreactor without the need for a cell separation device, high cell densities were achieved with a maximum of $4 \cdot 10^7$ cell/$mL_{capsules}$, corresponding to a colonisation of 5% of the internal capsule volume. Microencapsulation as a perfusion tool therefore showed a good potential since the maximal cell density obtained in suspension perfusion cultures with CHO cells was approximately $2 \cdot 10^8$ cells/mL using an alternating tangential flow system (Clincke et al., 2013a, 2013b). The result also indicated a more efficient utilization of glucose via respiration in encapsulated cultures compared with suspension cultures and a stimulating effect of microcapsules on IgG productivity. Measurement of microcapsule integrity and mechanical resistance however showed that alginate-poly-L-lysine microcapsules were not suitable for perfusion cultures since they are very sensitive to media composition, mainly the presence of non-gelling ions that have a higher affinity for alginate than poly-L-lysine and Ca^{2+}, leading to the leakage of poly-L-lysine and Ca^{2+}, and to capsule rupture under perfusion conditions. Alginate-PLL microcapsules stability could however be maintained during batch and fed-batch culture since an improvement in capsule stability by addition of calcium during the culture was observed when no medium replacement was involved.

It may be argued that this work is not novel due to the vast amount of publications already acknowledged focusing on alginate-PLL microcapsules (Lim and Sun, (1980) was cited more than 2100 times), however in starting a project which aims on showing the potential of microencapsulation as a retention system for perfusion culture, it was first necessary to define a reference encapsulation system.

In order to overcome the alginate-PLL microcapsules limitations and to obtain higher cell density culture by performing perfusion encapsulated cultures, alginate

microcapsules coated with poly-L-ornithine (PLO) were developed, characterized and compared with alginate-PLL microcapsules in order to understand whether these new microcapsules could be use as a new retention system for perfusion cultures (chapter 4). The microcapsules were shown to be easily retained in a standard stirred tank bioreactor without the need for a cell separation device resulting in high cell densities with a maximum of $1.1 \cdot 10^8$ cell/$mL_{capsules}$, corresponding to a colonisation of 14.6% of the internal capsule volume, representing a two-fold increase over microcapsules produced from PLL. Unlike PLL-containing microcapsules, the PLO-containing ones were shown, through measurements of microcapsule integrity and mechanical resistance, to be stable under perfusion culture conditions. However, despite the continuous change of media apparently having little effect on microcapsule stability, the microcapsules were shown to be unsuitable for perfusion cultures, since the higher level of colonisation of the microcapsules by the cells (14.6%) resulted in microcapsule breakage.

In moving onward to investigate microcapsule potential for cell culture, there were 3 options from this study: (1) Investigate different sources of polyelectrolytes with reported stronger polyelectrolyte interactions such as chitosan or cellulose sulphate in order to perform a perfusion culture with CHO-dp12 cells. Nobody has however shown the long- term effects of medium changes on microcapsules made of these polymers; there is therefore no evidence that such polymers could be used to produce microcapsules that can be use in perfusion cultures. Moreover, such polymers are quite difficult to find with the required parameters and are therefore quite expensive. (2) Use polyelectrolytes microcapsules made with PLL or PLO to grow adherent cells (CHO-K1) in batch or fed-batch culture where no media change is involved and the cell colonisation will not be higher than 14.6% of the microcapsules. (3) Investigate different source of polymers in order to produce stronger microcapsules with covalent membranes to perform a perfusion culture with CHO-dp12 cells. Since it was already shown that both PLL and PLO were not suitable for perfusion culture of CHO-dp12 and since both chitosan and cellulose sulphate are very difficult to obtain at reasonable conditions, it was chosen to test option 2 and 3 in the next chapters.

The possibility to produce microcapsules with improved stability compared to the

widely characterized alginate-PLL microcapsules, using microcapsules with covalent membranes created by (1) the cross-linking of alginate-PLL microcapsules with genipin or (2) the transacylation of alginate-PLL microcapsules containing PGA was investigated in chapter 5. PGA-containing microcapsules with a transacylation time of 10 seconds were shown to have an optimal initial mechanical resistance, which was twice as high than for alginate-PLL microcapsules. In culture conditions however their mechanical resistance decreased linearly along the culture due to PGA sensitivity to the pH of the cell culture media. The scale-up of the microcapsule production also was problematic as the initial mechanical resistance of the PGA-containing microcapsules tended to decrease dramatically with an increase of the volume of the capsules to be produced. Genipin microcapsules produced with a cross-linking reaction time of 6 hours were shown to be stable during 15 days with only 30% loss in mechanical resistance, against 87% loss of mechanical resistance for alginate-PLL microcapsules in the same period of time. Genipin containing microcapsules were however shown to be unsuitable for perfusion cultures as it was revealed that in the tested conditions, genipin was inducing cell death after 4 days of culture. None of genipin-containing microcapsules or PGA-containing microcapsules are therefore suitable for mammalian cell encapsulation, however due to their mechanical resistance improvement, they might have applications in different field such as microbial encapsulation for the PGA-containing microcapsules or drug delivery for the genipin-containing microcapsules.

The possibility to use alginate-based microcapsules as a support to culture CHO-K1, an adherent cell line, was investigated by encapsulating the cells in alginate beads, alginate-poly-L-lysine beads and alginate-poly-L-lysine microcapsules (chapter 6). The results showed that as suggested in chapter 3, alginate-PLL microcapsules remained stable in batch culture, when no media replacement was performed. CHO-K1 were unable to grow in alginate beads however, in the presence of poly-L-lysine (PLL), the cells grew to a density of 8x 10^5 cells/$mL_{culture}$ in 7 days in alginate-PLL microcapsules with a growth rate of 0.016h^{-1}. This cell density was similar to that obtained for non-encapsulated cell cultures however, the specific growth rate, μ, was 50% lower than for non-encapsulated cell cultures. Moreover, CHO-K1 had a more efficient utilization of glucose via respiration in encapsulated cultures compared with suspension cultures, which is a similar result than those obtained for CHO-dp12 in alginate-PLL and alginate-

PLO microcapsules.

The Alginate-Polyelectrolyte system was chosen due to the mild condition of the process and to the extrusion system. High concentration of alginate can indeed be extruded through the vibrating nozzle system, besides, alginate has the property to form a gel in a rate that is fast enough to keep the spherical shape of the bead and slow enough to allow the bead formation before gelation avoiding thereby the formation of unshaped mass of alginate. Na-Alginate hydrogel are however relatively weak and the forces involved in the formation of the physical hydrogel networks can be disrupted by changing the physical conditions such as the pH, the temperature, the mechanical stress or the ionic forces. This weakness of the alginate gel was mainly shown for the alginate-PLL system and the PGA-containing microcapsules where environmental elements are provoking the microcapsules instability. Despite the progress in better understanding the interactions between the hydrogels material and the cells, material with no negative effects on cells need to be discovered and developed in order to overcome problems such as those shown between adherent cells and Ca-Alginate gel or CHO-dp12 and genipin.

The importance in standardizing the characterisation methods was confirmed since the toolbox created in chapter 2 enabled the comparison of microcapsules produced by several polymers and cultured in different conditions and allowed a better understanding of the main parameters of cell microencapsulation. Moreover each batch of capsules was characterized with the same defined methods, which allowed a direct comparison between different studies.

The potential of microcapsules to run high cell density perfusion cultures was demonstrated in this work since cell densities of $1.94 \cdot 10^7$ cells/ $mL_{culture}$ were obtained with unstable PLL-containing microcapsules and $2.33 \cdot 10^7$ cells/$mL_{culture}$ with unstable PLO-containing microcapsules. Those cell densities are higher than those reported to be obtained in fed-batch culture by Lim et al. (2006) and similar to those reported to be obtained in perfusion culture by Lim et al. (2006) and Gugerli et al. (2003). Encapsulation also was beneficial for the cells since both CHO-K1 and CHO-dp12 showed a more efficient utilization of glucose via respiration in encapsulated cultures compared with suspension cultures and a stimulating effect of microcapsules on IgG

productivity for the CHO-dp12.

There is however still a need to develop more stable microcapsules that can withstand the forces involved in culture conditions. The new polymer system will need to produce microcapsules that are stable enough to last a perfusion culture. A good compromise between strength and elasticity of the membrane will need to be found since strong and fragile microcapsules similar to glass bulbs are not desired, however elastic and solid microcapsules similar to tennis balls are desired. Microcapsules with a membrane sufficiently elastic in order to overcome the pressure produced by the cells when growing, but strong enough to withstand the constraints of the reactor will therefore need to be developed. The microcapsule membrane will also have to be able to allow the supply of essential nutrients and the removal of toxic metabolites together with the free diffusion of the recombinant protein outside of the capsules without interacting with the protein. The polymer will need to allow both suspension cells and anchorage dependant cells to grow freely within the capsule. Several studies can be found comparing the cost of good ($/g) of recombinant proteins produced with several different methods. Lim et al. (2006) showed that in fed batch mode, the cost of good was of 530$/g of produced protein, while in perfusion culture, the cost of good was reduced by 3% (Lim et al., 2006). This study being 9 years old, more recent cost were researched and Pollock et al. (2013) showed that ATF perfusion system has the potential to reduce the cost of good by 20% when compared to fed-batch manufacturing processes (Pollock et al., 2013). It already was shown that microencapsulation could align with the cell densities obtained with ATF perfusion system (2x 10^8 cell/$mL_{culture}$) reported by Clincke et al., (2013a, 2013b) with a reactor containing only 50% by volume microcapsules. The newly developed microcapsules systems will however need to be able to be aligned to the cost reported above in order to be industrially viable. At this moment, research in the field of cell microencapsulation for recombinant protein is quite expensive since 1g of PLL or PLO cost more than 1000$. Other polymers were reported to have stronger polyelectrolytes interactions such as cellulose sulphate or chitosan, they however are also very expensive and only few companies can supply optimal grades products. There is therefore a need for academics, research, industries and polymer suppliers to

collaborate in order to allow this field to develop and to embrace its potential.

Because the cells are encapsulated, they are unavailable for evaluating the cell growth and health of the culture unless a sample of microcapsules is removed which will ultimately affect the outcome of the maximum cell density. There will therefore be a need to find online monitoring tools that can be applied on microencapsulated cultures. Such research was already performed by Cole et al., (2015) and the potential of dielectric spectroscopy and biocalorimetry for online monitoring of encapsulated mammalian cell culture was demonstrated. It would however be interesting to develop the research when stable microcapsules will allow high cell densities cultures.

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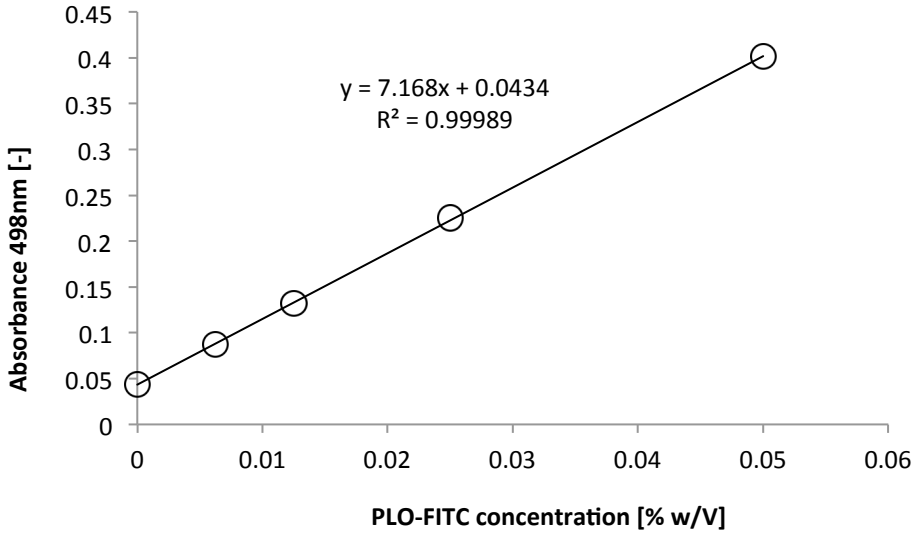
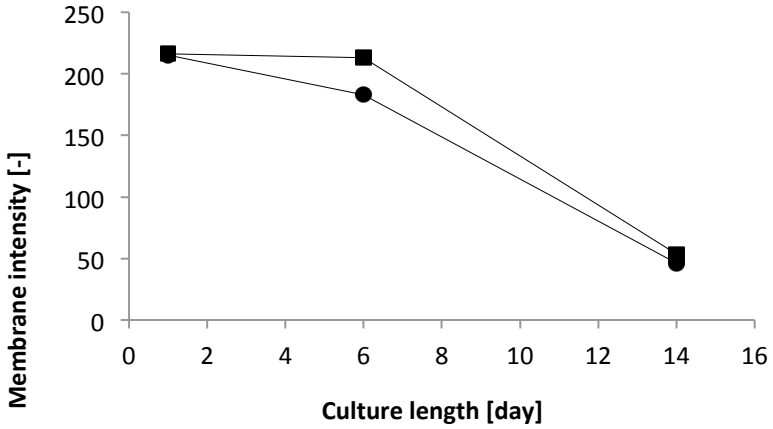
8. Appendices

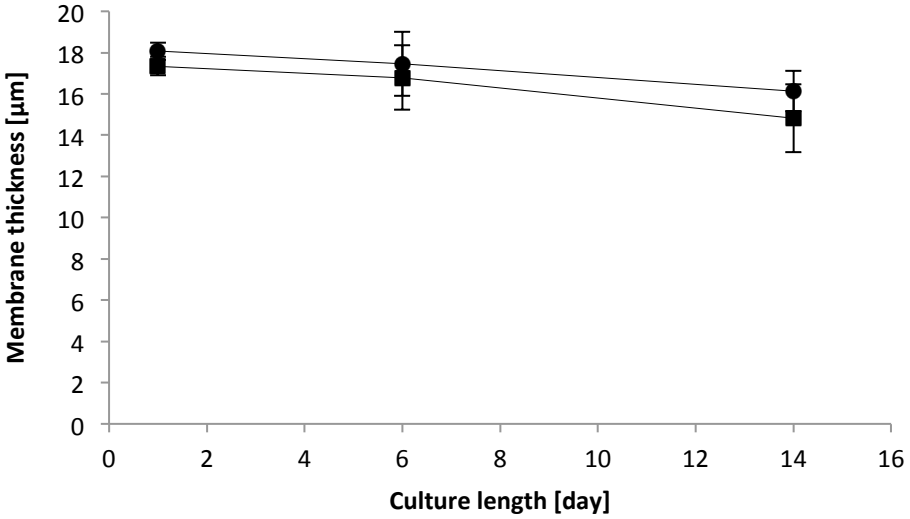
Appendix 4.1 : Alginate determination calibration curve : the absorbance at 498nm of FITC labelled PLO is represented as a fonction of the concentration of the FITC-labelled PLO.



Appendix 4.2 : Evolution of the PLL-membrane intensity during the stability test in CHO DHFR$^-$ media in batch (square symbol) and perfusion (spherical symbol) conditions



Appendix 4.3 : Evolution of the PLL-membrane thickness during the stability test in CHO DHFR$^-$ media in batch (squares) and perfusion (spheres) conditions



Appendix 4.4 : Mass balance calculation of the PLO and PLL adsorbed on alginate microbeads. The mass of the polyelectrolyte adsorbed on the microbeads m^*_{ads}, the initial concentration in the solution $C_{l,0}$ and the residual concentration in the solution at the equilibrium C^*_l were calculated from the volume of the solution (40mL). The density of the membrane ρ was calculating using the volume of the membrane $V_{membrane}$, which can be calculating with the membrane thickness T_{memb} and the microcapsule radius.

	$C_{l,0}$ [g/L]	$Q_{l,0}$ [mg]	C^*_l [g/L]	Q^*_l [mg]	m^*_{ads} [mg]	T_{memb} [µm]	$V_{membrane}$ [µm^3]	ρ [mg/µm^3]
PLL	0.5102	20.41	0.325	12.99	7.42	9.594	$6.81\ 10^6$	$1.09\ 10^{-6}$
PLO	1.505	60.22	0.803	32.11	28.11	7.140	$2.89\ 10^6$	$9.73\ 10^{-6}$