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Characterization of polysaccharide multilayered capsules for tissue engineering applications

Master thesis

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Characterization of polysaccharide multilayered capsules for tissue engineering applications

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“The path to wisdom is not being afraid to make mistakes.” (Paulo Coelho)

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Abstract

Cell encapsulation has been widely studied as an alternative therapy for almost every human diseases and disorders. This technique enables the inclusion of various types of living cells inside spherical systems which, among other capabilities, mimic the environment provided by the extracellular matrix. This new therapeutic approach has already proved to be successful either in vitro or in vivo studies, thus becoming one of the most promising tools in tissue engineering and regenerative medicine.

The main goal of this thesis was to explore some of the potential of cell encapsulation using simple and versatile techniques that can be performed in physiological and friendly conditions to the cells.

In a first approach, cells were encapsulated in liquid-core capsules using a three step methodology: (i) the precipitation of a polymer solution of alginate into a bath of calcium chloride (ionotropic gelation), (ii) deposition of polyelectrolyte multilayers onto the surface of the beads, in a process called layer-by-layer. (iii) use of EDTA to liquefy the alginate core. Two different natural-based polymers were used, alginate, the most studied copolymer for cell encapsulation and chitosan, a polymer widely explored in a variety of biomedical applications. Both polymers were proved to be biocompatible, biodegradable and can be manipulated under physiological conditions.

All the capsules produced exhibited spherical shape, smooth surface and liquid-core. The results shown that encapsulated cells were viable and

proliferating few days after the alginate-chitosan multilayer buildup, which suggests that the develop capsules posses a semipermeable membrane which allows the correct diffusion of nutrients and metabolites.

A preliminary study was started to test the feasibility of culturing anchorage-dependent cells in PLLA solid microparticles previously treated with human serum fibronectin followed by the encapsulation of the whole set in alginate-chitosan liquid-core capsules. The results are still very incipient but very promising.

Resumo

O encapsulamento de células tem sido amplamente estudado como alternativa terapêutica para quase todas as doenças e distúrbios que afectam a humanidade. Esta técnica permite a inclusão de vários tipos de células vivas dentro de sistemas esféricos que, entre outras capacidades, mimetizam o ambiente criado pela matriz extracelular. Esta nova abordagem terapêutica já deu provas do seu sucesso em estudos quer *in vitro* ou *in vivo*, tornando-se um dos instrumentos mais promissores na engenharia de tecidos e medicina regenerativa.

Esta tese teve como principal objectivo a exploração de alguns dos potenciais de encapsulamento de células recorrendo a técnicas simples e versáteis passíveis de serem realizadas em condições fisiológicas e favoráveis para as células.

Numa primeira abordagem, foram encapsuladas células em cápsulas de núcleo líquido usando uma metodologia trifásica: (i) precipitação de uma solução de polimérica de alginato num banho de cloreto de cálcio (gelificação ionotrópica), (ii) deposição de multicamadas polieletrólitas na superfície das partículas, num processo denominado layer-by-layer (iii) liquefacção do núcleo de alginato com recurso a uma solução de EDTA. Dois polímeros naturais diferentes foram utilizados, o alginato, o copolímero mais estudado para encapsulamento de células e o quitosano, um polímero amplamente explorado nas mais variadas aplicações biomédicas. Ambos os polímeros já mostraram

ser biocompativeis, biodegradaveis podendo ser manipulados em condições fisiológicas.

Todas as cápsulas produzidas exibem uma forma esférica, lisa e núcleo liquefeito. Os resultados mostraram que as células encapsuladas eram viáveis e proliferavam poucos dias após a construção das multicamadas de alginato-quitosano, o que sugere que as cápsulas desenvolvidas possuem uma membrana semipermeável que permite a difusão adequada de nutrientes e metabólitos.

Um estudo preliminar foi iniciado com o intuito de testar a viabilidade do cultivo de células dependentes de ancoragem em micropartículas sólidas de PLLA previamente tratados com fibronectina humana seguido do encapsulamento de todo o conjunto em cápsulas líquidas de alginato-quitosana. Os resultados são ainda muito incipientes, mas bastante promissores.

Abbreviations

A

AC – alginate/chitosan capsules

ADC – anchorage dependent cells

ALG/CHI multilayers – 8 polyelectrolyte alginate/chitosan multilayer

APA – alginate/poly(L-lysine) capsules

B

Ba^{2+} - barium cation

BSA – bovine serum albumin

C

Ca^{2+} - calcium cation

CHO –

CO_2 – carbon dioxide

D

DMEM – Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic acid

E

ECM – extracellular matrix

EDTA - Ethylenediamine tetraacetic acid

F

FBS – fetal bovine serum

H

HPC – high polyelectrolyte concentrated solution

I

IG – ionotropic gelation

L

LbL – layer by layer

LPC – low polyelectrolyte concentrated solution

L929 – mouse fibroblastic lung cell line

M

Mg^{2+} - magnesium cation

Min - minutes

MTS -

MWCO – molecular weight cut off

N

NaCl – sodium chloride

NaOH – sodium hydroxide

O

O.D – optical density

P

PBS – phosphate saline buffer

PEC – polyelectrolyte coating

PLL – poly(L-lysine)

PLLA – poly(L-lactic) acid

Q

QCM – D – quartz crystal microbalance with dissipation

R

RGD – Arginine – Glycine – Aspartic acid

S

SaOs-2 – human sarcoma osteoblastic cell line

SEM – Scanning Electron Microscopy

Symbols

$\Delta f\,(n)$ – normalized frequency

ΔD – dissipation

μ - CT – micro computerized tomography

w/v – weight/volume

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Chapter I: Bioencapsulation in liquid-core capsules- General Introduction

1.1 – Motivation

During the last decades the biomedical field has been experiencing a new approach regarding the development of systems to encapsulate living cells or tissues. Each of these systems through their possible application follows a specific pattern that results from a combination of materials with particular physical, chemical and biological behaviors.

For these systems be considered ideal for biomedical application is necessary to provide properties such as biocompatibility, adequate mass transport properties and mechanical characteristics, and, in some cases, biodegradability. The size, shape and chemical composition of such devices should also be adequate to be possible their implantation within the host organism without triggering any excessive immune response.

Numerous encapsulation techniques have been developed over the years. These techniques are generally classified as microencapsulation (involving small spherical vehicles and conformably coated tissues) and macroencapsulation (involving larger flat-sheet and hollow-fiber membranes)[1] A wide range of polymeric materials, both natural and synthetic, has been proposed for these encapsulation systems, being the alginic acid, PLL and chitosan the most representative polymers used in this approach.

Cell encapsulation makes possible a number of biological advantages formerly impartibly, in particular enables the transplantation of xenogenic animal and human cells, allows the transplantation of genetically modified cells without the commitment of the entire genome of the host and is a process in which factors potentially toxic are reduced once the capsule acts as an insulation system.

Regarding their structural and mechanical properties, encapsulated systems have been first proposed for immunoprotection of grafted cells in living organs.[2] Nowadays, much more applications have been proposed, ranging from therapeutic treatments, cell delivery molecules until cellular biosensors.[3]

This review will focus on different methodologies to obtain liquid-core capsules for cell encapsulation. First we will make a general overview about the bioencapsulation and the properties required to create a suitable cell encapsulation system. Second we will describe the techniques that allow obtaining liquid-core capsules under mild conditions. Finally, we will introduce a new methodology to encapsulate living cells in liquid-core capsules using the LBL technology.

1.2 – Bioencapsulation

Bioencapsulation or encapsulation of living cells and biological active compounds within a semipermeable membrane is a promissing technique which provides added possibilities for complex cell-based therapies, once it allows

allogenic and xenogenic cell transplantations without the further need of immunesuppressive therapy.[4-9] The semipermeable membrane have two main functionalities: on one hand it protects the inner cells from both mechanical stress and the host immune system, by preventing the direct contac with immune cells (immunoglobulins and other immunological constituints)[10-12], on the other hand it allows the free and bidirectional exchange of nutrients, oxygen, bioactive products and cell metabolites into the host physiological environment.[13, 14]

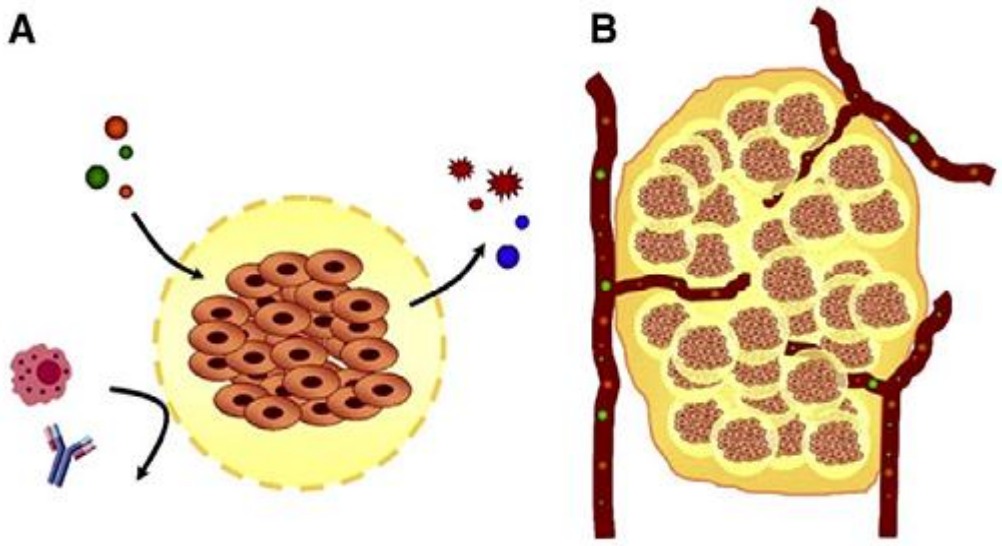

Figure1. 1: schematic representation of a typical bioencapsulation system. (**A)** semipermeable membrane allowing the free bidimensional diffusion of nutrients and metabolites. **(B):** microcapsules used for cell transplantation in a tissue engineering approach. Adapted from [10]

Since it was first introduced in 1964 by Chang[15], encapsulation of living cells in spherical vehicles have become the most investigated technique for producing biological agents such as monoclonal antibodies by immobilized hybridoma cells[16], enzymes and peptides entrapping genetically modified

cells.[8] Researches in pharmacology recently set up these devices as promising systems for continuous cells delivery of therapeutic products in vivo.[6, 13, 17]

Over the past decade, a wide variety of spherical cell encapsulation vehicles have been developed. Considering their structural properties, they can be classified in three different categories. The first one comprises currently designated as “microparticules” - vehicles with a solid-core, where the cells enclosed are surrounded by a solid gel matrix. Usually in solid gel matrix only few and small porous are formed inside the solid network inhibiting the cell proliferation by exerting a microscopic stress around the enclosed cells.[18, 19] However, the mechanical stability is high in solid microparticles which makes them suitable for long term *in vivo* studies or in cases where it is necessary to control the quantity of therapeutic protein provided to the body from the transplanted cells.[17, 20]

The second category includes vehicles with a liquid core matrix called “microcapsules”. Two main types of capsules can be produced from this concept point of view. (1) Hollow-core capsules, where the cells are surrounded by liquid that permeates through the capsule membrane from the surrounding medium or body fluid[17, 21] and (2) liquid-core capsules, where the original solid core is solubilized giving rise to a an aqueous liquid matrix, which surrounds the entrapped cells[22-25]. In both microcapsules cell entrapped are not subjected to above mentioned microscopic stress so, the cells faces a more preferable environment for cellular proliferation and preferred protein secretion.[26, 27]

The third category includes systems, generally designated as "macrocapsules", which are normally much larger than microcapsules and possess a planar or cylindrical geometry. Macrocapsules generally possess diameters >1mm and are very useful as a basic research tool for primary understanding of fundamental immunological an biological behavior of the implanted devices.[1, 28]

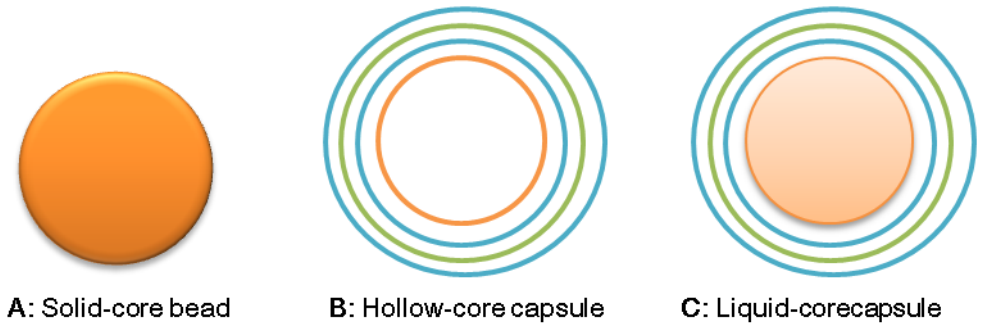

Figure1. 2: schematic representation of the different types of microcapsules

From a biological and structural point of view, microcapsules have (1) higher surface-to-volume ratio providing considerable improve in mass transport and (2) lower volume implant comparatively with macrocapsules. On the other hand macrocapsules have high loading capacity and high rate of recovery.

Along with the type of vehicle selected for cell encapsulation many other parameters has to be considered. The immunological isolation conditions as well as the materials used to produce the semipermebale membrane are closely related with the further application of the system and will be crucial for its success after implantation. Over the last 20 years several natural and synthetic

polymers have been studied as potential devices for bioencapsulation[29-33] being the microcapsules for pancreatic islets encapsulation the most researched device applied.[34-36] Nowadays a new range of implantable systems based on biencapsulation has been applied in a wide range of therapies approaches such as hypoplastic anemia[37], immunoisolation in cancer therapies[38-40], treatment of hyperparathyroidism[41] and in central nervous system disorders.[42] The great majority of these therapies involve the production and release of *de novo* proteins like growth factors [21], DNA [43, 44], enzymes[45, 46], and antibodies.[16]

Molecular weight cut of (MWCO), i.e., the upper limit of capsule permeability, is another important property with regard to bioencapsulation since it specifies the inflow and outflow of the products across the membrane.[1, 20] The encapsulation fabrication process, which will be discussed later in this review, is another factor of great importance in bioencapsulation.

1.3. – Liquid-core capsules for cell encapsulation

The encapsulation of living and functional cells or bioactive compounds usually requires a matrix capable of providing a water-rich environment. Most commonly, this matrix is a hydrogel frequently produced in a spherical geometry.[29]

Hydrogels are networks of water-soluble polymers that can be produced by different crosslinking methods compatible with cell viability which usually

involves non-covalent crosslinking.[47, 48] Generally hydrogels present reversible structures which can be disrupted in response to changes in temperature, ion concentration and pH.[49] These polymers provide some advantages properties for living cells encapsulation such as (1) softness and malleable environment that reduce the friction between surfaces of the capsules and the surroundings (2) hydrophilic behavior, which theoretically reduces the surface tension between the capsule and the surrounding liquid medium, thus reducing the affinity of proteins and host cell adhesion on the surface of the material and (3) enables a selective permeability to the extent that it only allows the diffusion of low molecular weight substances at the expense of others high molecular weight.[1, 48, 50, 51] Moreover, due to the ease with which they can be manipulated hydrogels usually are tailored to be biocompatible and biodegradable for biological applications.[52]

Microparticles with solid-core were the first and most common way of producing bioencapsulation systems based on hydrogels. There are a plenty of studies where solid-core microparticles were used to encapsulate bone marrow stem cells[53], cartilage precursors[54], hepatocytes[55], myoblasts[8] among others. Due to their small implant site which requires nothing more than a small and non-invasive surgical procedure and their feasibility to conveniently adjust to the implantation site microcapsules became a very attractive alternative as cell carriers for tissue engineering. However, in a solid spherical structure, the

cells in the inner zone are subjected to a lower intake of oxygen and nutrients thus becoming necrotic.[12, 18]

In recent years liquid-core capsules has been receiving great attention as an alternative for cell encapsulation instead of solid-core microcapsules. The, higher diffusivities of oxygen, nutrients, and metabolites in a liquid than in a gel seem to be a strong reason for the higher cellular growth and survival in liquid-core microcapsules.[24, 49] Sun et al. have demonstrate that cell enclosed in liquid-core capsules are more resistant to hyperosmotic stress, oxidative stress and heat shock stress than cells encapsulated in solid-core capsules.[24] In a different study, Breguet and its coworkers demonstrate that CHO cells growth more when encapsulated in a batch of liquid-core capsules.[27]

Up to now there are very few techniques that allow the encapsulation of living cell in liquefied capsules. Interfacial phase polymerization was the first technique proposed to encapsulate cells in liquid-core. In this approach liquid-core capsule are formed using monomers dissolved in the respective immiscible phases. Briefly, a polymeric aqueous solution containing cells can be dispersed into an oil phase by stirring. The capsule is immediately formed by addition of another organic solvent soluble into the continuous organic phase (oil).[46] However, this technique requires always the use of organic solvents that can compromise cell viability and it is only used for encapsulation of microbial cells or biocatalysts.[22] To overcome these adversities, new alternatives for cell encapsulation have been proposed. The next topic will be presented two

techniques that currently allow encapsulating living cells in liquid-core capsules under physiological conditions without the further use of harsh treatments.

1.3.1. Techniques for cell encapsulation under mild conditions

In the design of novel capsules the perfect adaptation of the construct device to the cell properties are the basis for cell survival and encapsulation success. Methodologies that can ensure a microenvironment that could mimic the extracellular matrix or the culture conditions, in which the cells are used to live, must be preferred. Ionotropic gellation[56] and capsule wall formation by polyelectrolyte[49] deposition are two simple techniques widely used in cell encapsulation studies in liquid-core capsules.

Ionotropic gelation (IG) is a phenomenon that occurs when a polyelectrolyte solution (anionic or cationic) is brought into contact with an multivalent counterion in a receiving bath[57] - see Fig.2A. The gelation is instantaneous and the resulting structure depends on both molecular and physico-chemical properties of polyelectrolyte and also on the type of counterion used in the process.[58] Usually the gelation takes place at the interface between the both solutions therefore theoretically the capsule interior remains liquid or semi-liquid[49]. However, the most studied polymer for cell encapsulation is the alginate which in a presence of a divalent counterion bath such as calcium or barium forms rigid beads.[59-61] In order to obtain liquid-core

gels from these solid beads a treatment with divalent chelator like EDTA or sodium citrate is always required - see Fig.2C.

Ionotropic gelation is a gentle gelation and cell compatible process which can be performed under room temperature at mild conditions so the living cells can be suspended in the polymer solution even before the encapsulation procedure takes place.

Ionotropic gels generally suffer degradation by slow exchange of divalent and monovalent cations, leading to the dissociations of ionic bridges[59].so, most of the time, these gelified structure must be stabilized using adjuvant polymers, such as poly(L-lysine)[43, 62], poly(ethyleneglycol)[11]our chitosan[6, 63] among others. Orive et al have produced alginate and poly(methylene-co-guanidine) (PMCG) microcapsules for cell encapsulation. According to their study, PMCG is a polycationic methylol amid which improves the uniformity of size beads, wall thickness and mechanical stability of the cell encapsulation devices.[20]

Capsule wall formation by PE deposition or PE coatings (PEC) is a simple coating methodology based on the alternatively dipping of oppositely charged polymers (polycationic or polianionic) in order to form a polyelectrolyte film (see Fig.2B).[64] The success for such technique relies on the choice of the oppositely charged polyelectrolytes and in the deposition conditions[65]. Several polyelectrolytes can be used to form layered structures onto the surface of solid or liquid materials by means of simple electrostatic adsorption. Thickness of this

PECs can range from few nanometers up to several micrometers depending on the number of deposited layers.[44]

PEC is the major contributor for the success of bioencapsulation. These coatings can be performed around encapsulated cells for liquid-core capsules fabrication[16, 19, 20] or directly around the cells[5, 66-68] for "single" cell coating. The formation of a polyelectrolyte membrane will provide and additional mechanical stabilization and reduces nonspecific hosts response, thus enhancing the biocompatibility.[57, 65]

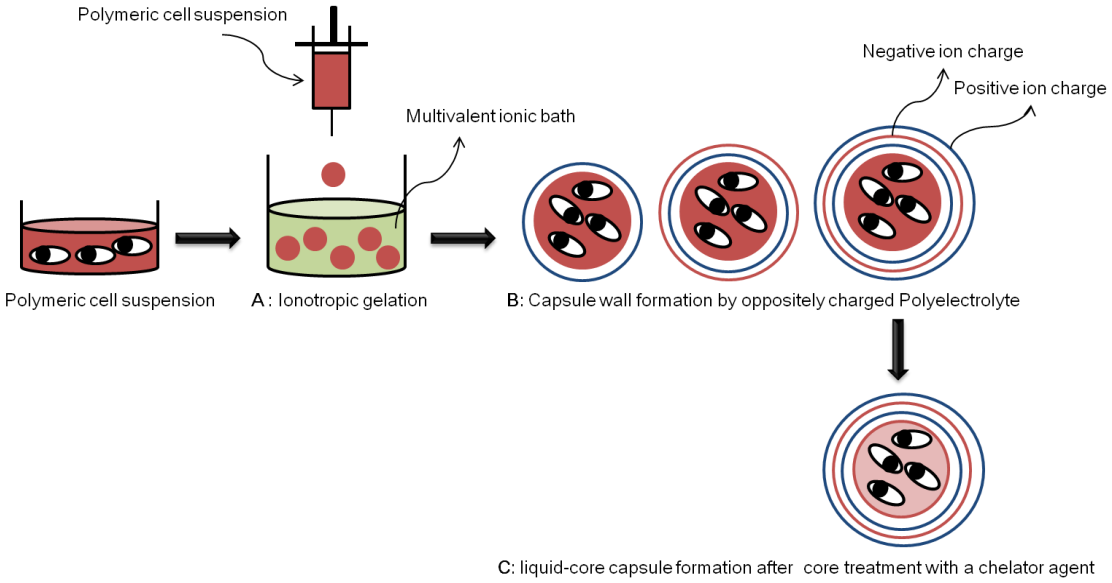

Figure1.3: under mild conditions. A: ionotropic gelation of a polymeric cell suspension in a multivalent ionic bath B: Polyelectrolyte coating for capsule wall formation; C: liquid-core capsule obtained after core treatment with a chelator agent.

The versatility and universal character of PEC does not impose any limitation on the type of polyelectrolyte applied. Until now, several charged macromolecules such as synthetic and natural polymers, proteins[7],

immunoglobulins[69] and nucleic acids[43] have been used as films building blocks. Diaspro et al have encapsulated single living yeast cell by alternate adsorption of labeled polyelectrolytes.[70] Germain et al coated MELN cells using oppositely charged polyelectrolytes, proving that it is possible to directly coat living mammalian cells with more than 5 bilayers in a cell friendly environment.[3]

Table 1 summarizes a few studies where IG and PEC were combined to produce liquid-core capsules under mild conditions.

Technique	Core Polymer	Core liquefaction method	Cell encapsulated	References
Ionotropic gelation	Alginate/Ca^{2+}	Citrate	CHO C2C12 myoblast	[20, 27, 41, 71]
Ionotropic and thermal gelation	Alginate – Agarose/Ca^{2+}	Citrate	C2C12 myoblast	[16, 19]
Ionotropic geraltio	Alginate/Ca^{2+}	Thermal liquefaction	CRFK cells	[72]

Table1.1: Cell encapsulation approaches using ionotropic gelation and polyelectrolyte coating.

1.4. – Layer-by-layer electrostactic assembly as a vehicle to obtain liquid core capsules

As mentioned above, the coating of microparticles is a powerful tool in obtaining encapsulated systems with better physical and chemical properties. The deposition of polyelectrolyte multilayer films [64] represents an alternative solution for biomaterial coating.[73] Matching cells to multilayers assemblys is a relatively recent activity that has revealed some of the most promising properties of multilayered membranes, where hydrophobicity[74, 75]

composition[76, 77], and stiffness[78] may be tuned by the components, conditions, and sequence of layering. The adhesion, proliferation and differentiation of cells are directed in a large extent by the multilayer constructs.

The layer-by-layer (LbL) assembly was first introduced in early 1990's by Decker and co-workers[77, 79] for the preparation of nanoscaled controlled films for biomedical applications.[68, 80] The LbL methodology may be employed in the production of capsules through the sequential deposition of polymers assembled by complementary interactions (e.g., electrostatic, hydrogen bonding, and covalent linkages) on template particles, followed by removal of the templates. This facile yet versatile technique can incorporate a large variety of polymers.[79], templates[69] and also allows the buildup of multilayers from nutrients, ligands, and genetic material.[81]

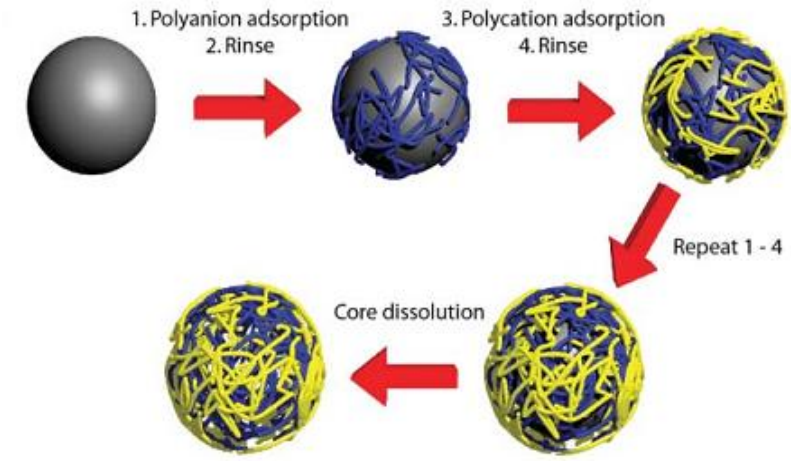

Figure1.4: layer-by-layer under spherical templates. Adapted from [79]

Mechanical stable capsules can be obtained by LbL assembly.[45, 80, 82] The multilayers produced will act as a shielding structure with the ability to protect bioactive molecules in a define volume while creates a special defined microenvironment for the loadings.[83] In the case of liquid-core capsules LbL allows obtaining structures with improved mechanical strength whose toughness of the membranes will be greater the higher the number of multilayers deposited. To date only few studies about the encapsulation of cells in liquid-core capsules constructed using the LBL technique has been reported (see table 1). So far, those capsules were made with no more than 4 layers.[27]

In principle such methodology could be use to produce capsules containing cells coated with nanostructured multilayers proving that the cells can be incorporated inside the initial template particles and that they can be eliminated under mild conditions.[67,68]. This possibility could allow to produce coatings with tunable thickness (and thus controllable permeability) and surface characteristics that could be very attractive for cell isolation and transplantation.[16,69]

Our group recently investigated the combination of two different techniques to achieve a suitable cell encapsulation system for further use in tissue engineering and regenerative medicine. Merging encapsulation techniques with LbL self assembly, liquid core capsules whose walls are composed by nanostructured multilayers were successfully produced. Cells were entrapped inside the conceived capsules and cultured for a certain period

of time to evaluate its biological ability. With this formulation it is expected to obtain a permeable and tight mechanical structured system which could be able to ensure the cell viability and provide effective cell immunoisolation. Moreover, it is also expect that with this versatile technique some membrane features like mechanical properties and biological behavior could be tuned by varying the composition and number of layers, or by introduction of bioactive molecules.

1.5. Conclusion

Biencapsulation in liquid-core capsules is gaining enormous interest in the field of tissue engineering and regenerative medicine. The combination of basic encapsulation methodologies like ionotropic gelation with layer-by-layer technique may lead to obtain capsules with versatile and tunable mechanical and biological properties. Although to ensure the cell viability inside these devices, material properties must be controlled to facilitate the desired metabolic functionality. Many efforts have been made along the past few years to develop tunable materials that will impart the correct biological and mechanical signals to the cells. Up to now, liquid-core capsules prove to be a suitable system in which the cells can grow and proliferate correctly. However a lot of work remains to be done in this field, which makes it an attractive research area with increasing interest for the future.

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Chapter II – Materials and Methods

Polysaccharides are a very important group in the field of water soluble polymers where they play a major role as "*thickening, gelling and emulsifying hydrating and suspending"* materials. Most of these polymers give rise to physical gels at mild conditions under specifically thermodynamic circumstances.[1] Natural-based polysaccharides have been widely recognized for biomedical applications due to their similarity to biological macromolecules namely the extracellular matrix (ECM) ones. This property may represent an immunological advantage for these polymers because when implanted in human host, they may avoid the stimulation of immune cells and consequent citotoxic reaction. Moreover, they can be frequently degraded *in vivo*.[2, 3]

In this chapter, the key features of the materials used in the development of this work as well as all the procedure for their manipulation and characterization will be described in detail.

2.1. Polymers used

2.1.1. Alginate

Alginate has been widely employed as polymeric matrix in cell immobilization procedures.[4] It is considered a suitable polymer for microcapsules fabrication since it is not harmful for host tissue and because of its high ability to form rigid gels under mild and physiological conditions in the presence of few amounts of divalent cations.[5-7] In tissue engineering

applications alginate has shown to provide mechanical integrity while simultaneously transmits initial mechanical signals to cells and surrounding tissues.[8]

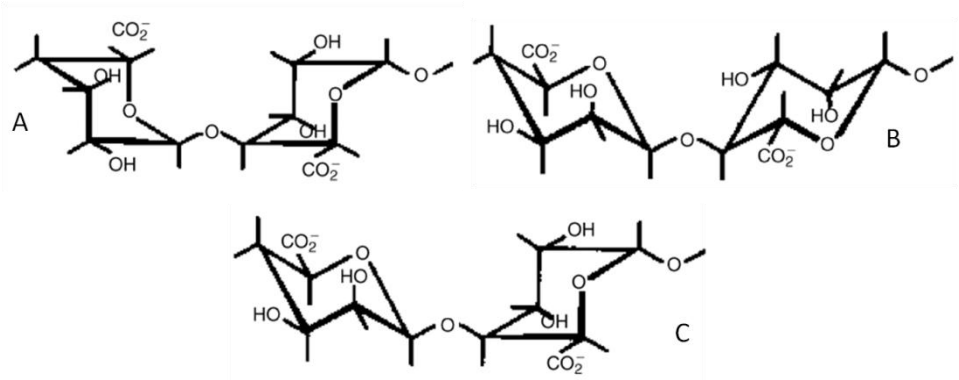

Figure2.1: Chemical structure of alginate. (A) α-L-guluronic acid (G-blocks) (B) β-D-mannuronic acid monomers (M-blocks) (C) interspaced M and G blocks. Adapted from [1]

Alginate is a naturally derived polysaccharide very abundant in structural components of marine brown algae and capsular components of soil bacteria.[3] The term alginate is referred to a range of polysaccharide block copolymers composed of both (1) sequential regions of β-D-mannuronic acid monomers (M-blocks) and α-L-guluronic acid (G-blocks) (2) interspaced M and G blocks – see Fig.2.1.[1, 9, 10] This polymer can be reversible gelled in aqueous solutions when mixed with divalent cations (Ca^{2+}, Mg^{2+}, Ba^{2+}) [11, 12]. At mild conditions, divalent cations interacts with the carboxyl groups located on the polymer backbone, namely G-blocks, creating a three dimensional network by ionic inter-chain bridges. The ratio of M/G contents as well as the individual distribution of both M and G units along the chain will establish the physical properties of alginate in

aqueous solutions.[1] Also the stiffness of the alginate chains and further calcium complex formation depends on the mannuronic and guluronic ratio and distribution along the polymer chain.[13]

Alginate gel matrix surface can be modified by income of macromolecules which are capable to establish ionic interactions with its carboxilate ions.[10] This surface modification may provide additional mechanical integrity to the matrix.[14] Alginate is been largely used as a matrix to prepare microcapsules to be used as cell immobilization matrix[15-18], involving different cell sources such as stem cells[19-22], pancreatic islets[23-25] and a wide range of human and microbial cells.[26-31] Moreover alginate microcapsules have been used in means of cell delivery vehicles[32-35], cells transplantation context[36-40] and even in food and flavors encapsulation.[41]

Alginate in its purest form could be an extremely biocompatible barrier.[42] The pure form of this polymer has shown to have a high content of impurities and mitogenic fractions, which may lead to overgrowth fibrotic tissue when implanted in animal models.[12] Purification substantially reduces the host response to the material. On the other hand, an additional coating with polycationic in the outer shell of alginate microcapsules could lead to a better biocompatibility of the implanted material.[14] Besides this drawback, alginate is one of the most studied and applied polymer in the field bioencapsulation, due to its promising properties as a natural-based polymer.[43]

For this work, low viscosity sodium alginate seaweed from brown algae was purchased from Sigma-Aldrich, Portugal. Alginate solutions prepared with different concentrations. A 1% (w/v) alginate solution was obtained by polymer dissolution in 0.15M sodium chloride (NaCl Sigma-Aldrich, Portugal) aqueous solution. The alginate solutions used for the layer-by-layer polyelectrolyte assembly were prepared with both 0.05% (w/v) and 0.1% (w/v) in 0,15M NaCl aqueous solutions. The pH was adjusted up to 7 with 1M of sodium hydroxide (NaOH) or 1M citric acid ($C_6H_8O_7$). All the polymer solutions were prepared in distilled water, autoclaved (121ºC, 30min) and vacuum-filtered in a 22µm pore-size filter (Schleicher & Schuell Microscience, Germany) to sterilize. The solutions were then stored at 4ºC for further use.

2.1.2. Chitosan

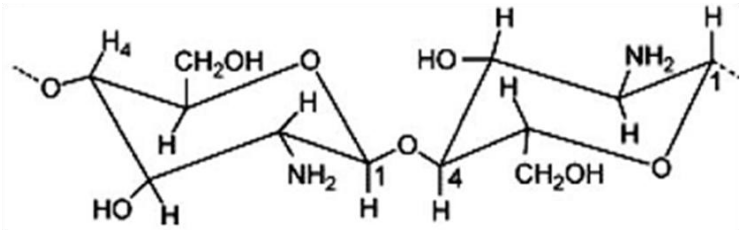

Figure2. 2: chemical structure of chitosan. Adapted from [44]

Chitosan is a natural and linear copolymer of D-glucosamine and N-acetyl-D-glucosamine linked in β (1-4) (Fig.2.2). Usually it derives from chitin, the main organic polymer found in crustaceous shells, cuticles of insects and cell walls of some fungi.[1, 45, 46] Considering its wide and variable application,

chitosan has been formulated as powders, gels, films and spherical micro and nanoparticules, among others.[46] Nowadays chitosan have been investigated as surface modifier for a wide range of biomedical applications.[47] Song et al used this polymer to enhance the biocompatibility of self-assembled membranes[48], Antipov and co-workers used chitosan as core-shell structures[33, 49] and many other groups has been exploring the chitosan as a local drug delivery[50, 51] and bioactive nanocoatings for endovascular devices.[52]

Solubility is a crucial characteristic for chitosan. As a naturally positively charged polymer with a high charge density in acidic medium chitosan usually forms insoluble complexes with water-soluble polyanionic species at neutral conditions.[53] Qin et al (2006) reported that at physiological pH water-insoluble chitosan molecules can precipitate and stack on the microbial cell surface, thereby forming an impermeable layer around the cell which can be fatal for living cells. The formation of such layer can block the transport of essential moieties and may also destabilize the cell wall causing its severe leakage leading ultimately to cell death.[45] So, improving the chitosan solubility will largely facilitate its application in medicine and food industry.[54] Moreover, Chung et al (2003) confirmed that chitosan possess a broad spectrum of antimicrobial activities but, regarding its low solubility at neutral pH, its application can be limited.[55] On the other hand, soluble chitosan showed higher antimicrobial activity against *E. coli, B.subtilis and S. aureus* than crude chitosan[56] have found out that water soluble chitosan promote growth of

C.albicans at physiological pH, even though the optimal ph for *C.albicans* growth is 5.5, thus proving the low cell – toxicity. Moreover, it has been reported in several studies that water soluble chitosan had better physiological performance in antitumor activity and immune-enhancing in vivo effects. [57, 58] Considering aforementioned, water soluble chitosan seems to be a suitable formulation to be applied in systems for cells.

Currently, many companies are now suggesting different procedures to enhance the chitosan solubility[1], among them pure chitosan for medical application are the most interesting ones, since they are able to be prepared under physiological pH at room temperature. Protosan UP Chitosan from NovaMatrix (FMC Norway) is an example of a water soluble chitosan which can be manipulated under mild conditions.[59]

For this work, Protosan Ultrapure Chitosan (Novamatrix, FMC Norway) was used according to previous results obtained by Martins et al (2009).

Ultrapure chitosan PROTOSAN UP CL213, viscosity 107mPa.s, molecular weight M_w=2,7x10^5g/mol and degree of deacetylation DDA=83% was used to prepare the polyanionic LbL polyelectrolyte solutions. Briefly, both 0.05% (w/v) and 0.1% (w/v) chitosan solutions were obtained in a 0,15M NaCl aqueous solution. After complete dissolution, the solutions were vacuum-filtered with 0.45μm) pore size filter (Schleicher & Schuell Microscience, Germany. The pH was adjusted up to 7 using 1M NaOH or 1M citric acid and the solutions were stored at 4ºC before microcapsule production.

2.2. Methods

2.2.1. - Building and characterization of alginate-chitosan multilayers by Quartz-Crystal Microbalance with Dissipation Monitoring (QCM - D)

In order to evaluate the LbL assembly of alginate and chitosan polyelectrolytes, a Q-Sense E4 system (Q-Sense AB, Sweden) was used. This system enables the monitoring of small masses deposition on top of its gold-coated crystals. The crystals are previously cleaned in an ultrasound bath at 30ºC, successively immersed in acetone, ethanol and isopropanol to ensure that all the impurities are eliminated. Then the crystals are rinsed with distilled water and dried with nitrogen gas. Before starting the deposition processes, an equilibration step with the rinsed solution is recommended, for approximately 60 min, to establish the frequency and dissipation baselines. The polyelectrolyte solutions were prepared at 0.05% (w/v) and 0.1% (w/v) in 0,15M NaCl. Deposition occurred at room temperature (approximately 25ºC) at pH 7.0 in a constant flow rate of 100µL/min. Both chitosan and alginate solutions were pumped alternatively for 10min each, followed by a 5min wash step with rinsed solution of NaCl. The resonant frequency ($\Delta f/n$) and energy dissipation (ΔD) was monitored in real time.

2.2.2. Microcapsules production and characterization

There are two main methods to produce alginate/chitosan capsules in mild conditions. The first one is the one-step procedure where the capsules is produce by simply letting the alginate droplets fall into a aqueous solution of calcium chloride with chitosan. The other is the two-step procedure where the calcium-alginate bead is produced by dropping an alginate solution into a gelling solution of calcium chloride. The beads were then coated with chitosan.[14, 60, 61]

For this work, it will be used only the two stage procedure, once it has been shown that it may result in the binding of 100 times more chitosan than the one stag procedure.[60]

2.2.2.1. Production of calcium-alginate microparticules

The Calcium-alginate microparticules were produced by ionotropic gelation method as described in literature [61, 62]. Briefly, 1% (w/v) alginate solution was prepared in 0.15M NaCl. By adding nongelling sodium ions to the gelling solution, beads with more homogeneous surface will be produced.[63] Then, 1ml of the polymer solution was extrude through a 27G needle (B Braun, AG- Germany), using a syringe pump (B Braun, AG – Germany), into 30 ml of two calcium chloride ($CaCl_2$ Sigma-Aldrich, Portugal) solution containing 0.5% (w/v) or 0.75% (w/v) and 0.15M NaCl. The distance between the needle tip and the gelling solution was 10mm. The resulting microparticules were allowed to

gel for 20min under stirring. Afterwards the microcapsules were rinsed in 0.15M NaCl solution and thoroughly dried using a gaze. All the solutions were prepared using distilled water.

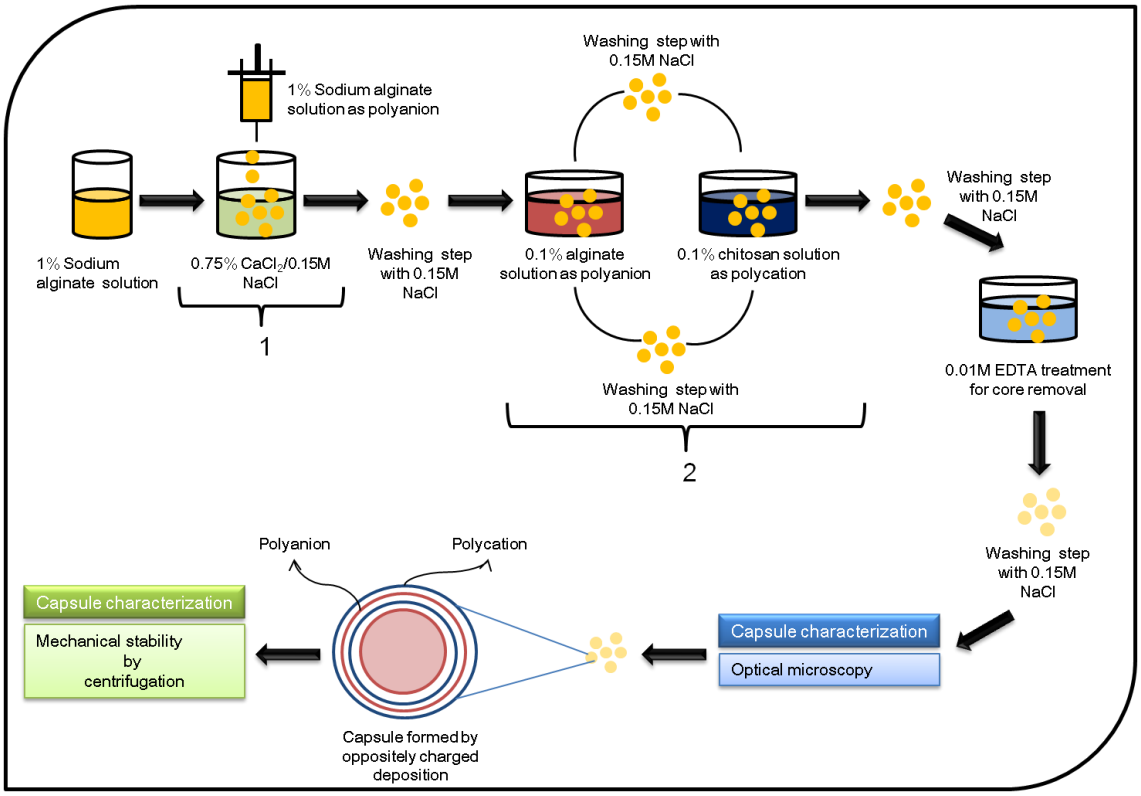

Figure2.3: schematic representation of the procedure used for capsule production using ionotropic gelation (1) and layer-by-layer polyelectrolyte assembly (2).

2.2.2.2. LbL self assembly deposition

The calcium-alginate beads were immersed in 20ml of both chitosan CL213 0.05% (w/v) and 0.1% (w/v) for 10min. After rinsed in NaCl solution and dried, the microcapsules were immersed in 20ml of both alginate solution with 0.05% (w/v) and 0.1% (w/v) for another 10min. This process was repeated until reaching 8 polyelectrolyte multilayers.

The liquid core alginate-chitosan capsules were obtained after 5min treatment with 0,05M ethylenediaminetetraacetic acid (EDTA, Sigma -Aldrich, Portugal). EDTA is an acid that acts as a metal ions sequestering agent through complexation. Due to its hexadentate chemical structure, EDTA binds preferentially to divalent cations, such as calcium. Under these conditions, EDTA in aqueous solution will chelate calcium cations by removing them from the alginate beds, thus leading to a liquefied core. Finally the resulting alginate-chitosan microcapsules were rinsed in NaCl and stored at 4ºC in the same buffer solution for further physical characterization. A few microcapsules were also immersed in DMEM in order to access the mechanical stability and the permeability to the culturing medium at 37ºC. All this process was performed under mild condition.

2.2.2.3. Morphological characterization

Morphological characterization is very important to assess the structural features of a given system. Aspects like size, shape and general topological characteristics are quite simple to obtain using only specific microscopy techniques. Stereolight microscope (Zeiss-Stemi 2000-C KL 1500 LCD, 459315) was used to evaluate the size and topological aspect of the produced microcapsules. Scanning Electron Microscopy (Nova Nano SEM 200 - FEI Company, US), SEM, analysis enables to investigate the presence of the

LbL coating and the organization of the polyelectrolyte films deposited around the alginate-chitosan microcapsules.

2.2.2.4. Assessment of alginate-chitosan microcapsules mechanical stability by centrifugation

A short term stability assay was performed to investigate the mechanical behavior of alginate-chitosan microcapsules when submitted to hash mechanical conditions. The procedure was based on Coradin et al[64] and Haque et al[65] with slightly modifications. Briefly, alginate-chitosan microcapsules were placed in different centrifuge tubes filled with 0.01M EDTA and distilled water. Calcium alginate (Ca-alginate) cores could be decomposed by EDTA treatment within the polyelectrolyte multilayer microcapsules[66, 67], which therefore allows partial release of alginate molecules from the alginate-chitosan capsules. Hence, by adding a few amount of EDTA in a solution with the liquid core alginate-chitosan capsules, more Ca^{2+} ions will be chelated therefore more alginate molecules will be removed from the capsules. Two rotational stress tests were performed on the alginate-chitosan capsules with EDTA. The first one was carried out for 60min at 200rpm in a centrifuge at 25ºC. After this rotating period, the same amount of capsules was centrifuged for additional 15min at 4500rpm at the same temperature. The number of intact capsules was counted every 15min, during all the rotational stress test. The triplicates for each tested condition were made. By conjugating chemical stress,

caused by an excess of EDTA, with mechanical rotational stress higher destabilization of the multilayered membrane will be achieved which may increase the rate of membrane disintegration.

2.2.3. *In vitro* studies

For in vitro studies a human osteoblasts-like cells (SaOs-2 cell line, European Collection of Cell Cultures, UK) was used. SaOs-2 like-cells was grown as monolayer cultured in 75cm^3 culture flasks using Dulbecco's Modified Eagle Medium low glucose (DMEM. Sigma-Aldrich, Portugal) and sodium bicarbonate (Sigma-Aldrich, Portugal) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Germany), 1% antibiotic/antimycotic (Invitrogen, Portugal) at 37ºC in a 5%CO_2 incubator. The culture medium was changed every three days and cells were left to grown until confluence. As a cell line, SaOs-2 cells were prepared to be immortal and able to maintain their phenotypic characteristics for long periods in culture and so can be used in a wide range of in vitro tests, namely primarily test with materials.

2.2.3.1. Cell encapsulation

Immobilization of cells was performed using SaOs-2 cell line in both alginate-chitosan (solid and liquefied) capsules. Capsules and cell encapsulation was carried out under mild and sterile conditions in a flow chamber. All the solutions were prepared and manipulated as described above.

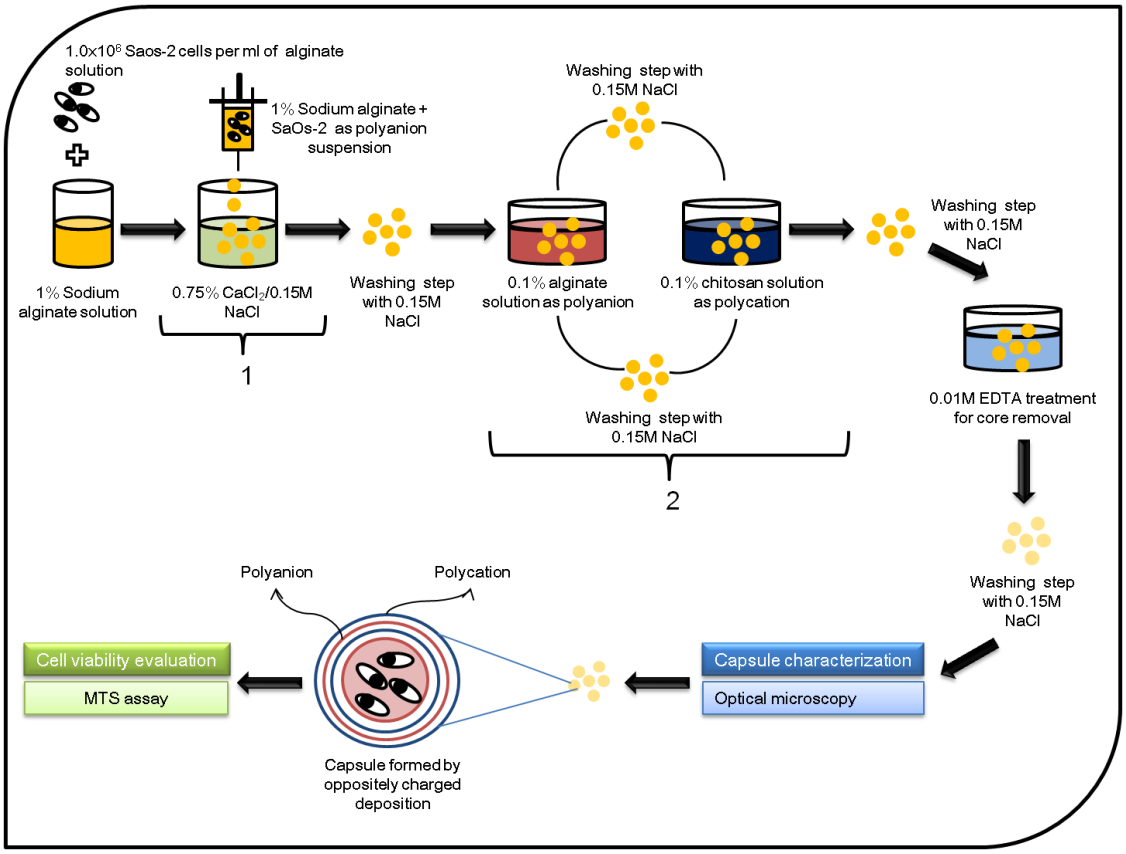

Figure2.4: schematic representation of cell encapsulation procedure in liquid-core capsules.

SaOs-2 was culture in culture 75cm^3 flasks for few days until reaching confluence. SaOs-2 monolayer was initially harvested using trypsin-EDTA (Invitrogen, Portugal). Cells were counted using a Neubauer Chamber after previous staining with Trypan Blue (vital staining). Approximately 1x10^6 cells/ml were suspended in 1% (w/v) alginate solution. The cell suspension was extruded through a 27G needle using a syringe pump into a calcium chloride solution. The resulting capsules with cells within were left in $CaCl_2$ solution for 20 min under stirring at room temperature. A batch of alginate microcapsules with cells were placed in 24 well plates with DMEM and incubated in a 5% CO_2

incubator at 37ºC. The following LbL self assembly procedure was performed has described above (section 2.2.2.2). The encapsulated cell was incubated in a 5% CO_2 incubator at 37ºC and culture for 1, 3 and 7 days. The culture medium was changed every 3 days.

2.2.3.2. Microscopy observations

Alginate-chitosan encapsulated cells were observed under light microscopy (Stemi 1000 PG-HITEC Zeiss) using the cell filter. For this analysis any previous treatment was done. All the capsules (solid and hollow) with cells within were permeable to DMEM highlighting the cells inside. With the filter applied, the cells appear as small white dots and bright dispersed throughout the dish.

2.2.3.3. Evaluation of Alginate-chitosan encapsulated SaOs-2 like-cells viability by MTS assay

Cellular viability and proliferation in alginate-chitosan capsules were assessed by MTS using CellTitre 96® (Promega, Madison, USA) a quantitative assay. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium) test has been describe in the literature.[68] Briefly, the metabolically active enzymes in living cells produce dehydrogenases which in contact with substrate-MTS convert yellow tetrazolium compound into a water solubility brown formazan product. The quantity of brown product is directly

proportional to the amount of viable cells. In this study, the MTS solution was prepared using DMEM without phenol red and FBS. The MTS CellTitre 96® reagent was mixed with the medium in a 5:1 ratio. Approximately 300µl was added to alginate-chitosan encapsulated cells and incubated for 3 hours at 37ºC in a 5% CO_2 incubator, protected from the light, in 48 well plates (once the MTS reagent reacts in the presence of an extra light source). After incubation period, 100µl of each 48 well reaction solution were transferred to 96 well plates, in triplicate. The absorbance was read in a microplate reader (BIO-TEK – Synergy HT) at 490nm. The results were express in absorbance obtained for the samples in each culture period.

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Chapter III: Liquefied capsules for cell immobilization coated with multilayered polyelectrolyte films

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Abstract

A cell encapsulation approach is presented based on the development of a nanometeric multilayer coating of polyelectrolytes by the layer-by-layer method on alginate spheres. The alginate core material was liquefied using calcium chelators in order to produce chitosan-alginate multilayered liquefied capsules. All processes were conducted at physiological pH. Human osteoblasts-like cells were immobilized into the liquid core capsules for the proof-of-concept where their viability was found to increase from 1 to 3 days of culture. Such technology has the potential to be used in the biotechnology field where the capsules could act as mini-reactors for cells or other microorganisms, or in a variety of biomedical applications including cell therapy or tissue engineering, in which the multilayered coating can have an immunological protection.

Key words: cell encapsulation, liquid core matrix, alginate, chitosan, immunoisolation, self-assemble, QCM

3.1. Introduction

Bioencapsulation involves the envelopment of tissues or biological active compounds in a semipermeable membrane which protects the enclosed biological structures from potential hazardous processes in the host physiological environment.[1] Various bioencapsulation methods include polyelectrolyte complex coacervation, ionotropic gelation and also polyelectrolyte coating.[2, 3]

Since Chang propose the bioencapsulation as an alternative to create artificial tissues and organs in 1960's [4] a wide range of studies in the field of cells and bioactive compounds immobilization as been carried out. Many semipermeable membranes as well as techniques to improve the capsules properties has been presented as alternative methodologies to encapsulate the most varied types of cells.[5, 6] Such kind of works have demonstrated that bioencapsulation constitute a promising strategy for the treatment of various health disorders, ranging from endocrine or central nervous diseases to cancer.[7] Actually, the most well succeed developed and clinical implemented bioencapsulation devices are those applied in endocrine disorders treatments, namely the alginate/poly(L-lysine) microcapsules for pancreatic islet immunoisolation.[8-10]

The majority of encapsulated encapsulated systems are made by solid matrix/core hydrogels, which were reported to cause some biological limitations which include long term stability [11], reduced cell growth and protein production by exerting stress [7, 12, 13] and low gas an nutrients efficiency capacity.[2]

Liquid/core matrix has been widely investigated as an alternative to solid matrix/core hydrogels, once they appear to allow better cell growth and protein production providing higher diffusion of gases and nutrients along the liquid matrix.[2, 12, 14, 15] Sun et al showed that cells cultured in liquid core microcapsules showed an increase in both intracellular glycerol content and stress tolerance substances, while solid core/matrix did not cause any significant physiological variation. Moreover, cells released from liquid core/matrix were more resistant to hyperosmotic stress, oxidative stress, and heat shock stress than cells liberated from solid core/matrix.[16]

The most common method to obtain capsules with liquid core/matrix is through the deposition of one or two layers of oppositely-charge polymers around the solid core/matrix, followed by the core liquefaction.[17] The obtained membranes could readily be tailored by choosing different combinations of polymers for the sequential deposition [18, 19] thus enabling improvement of capsule properties such as permeability and stability, preventing the content release from the template matrix and ensuring the biocompatibility.[11] Until the present date, the vast majority of the studies using liquid core capsules follow the model proposed by Lim and Sun in 1980's [20] in which the semipermeable

membrane were obtained by deposition of only two oppositely-charged polyelectrolytes, namely alginate and poly(L-lysine) (APA).[5, 21]

Just a few studies on liquid core capsules have reported the use of more than two polyelectrolyte layers. Breguet and co-workers studied the influence of 4 layered membranes in liquid core APA microcapsule on the long term stability and in the proliferation of encapsulated CHO cells. They have concluded that such barrier led to an acceleration of CHO cells metabolic activity but did not improve the colonization potential of the microcapsules.[12] In a previous study, the same group have encapsulated CHO cells in a liquid core capsule which was coated using three kinds of interactions generated in the same membrane: (1) electrostatic bonds between alginate and poly(L-lysine) (PLL), (2) covalent amides bonds between propylene-glycol-alginate (PGA) and PLL, and (3) covalent bonds between bovine serum albumin (BSA) and polyglycolic acid (PGA). They observed that the mechanical resistance of the capsule only decrease by 10% during 1 month in batch mode culture. Cells were encapsulated under harsh conditions but their viability was not affected.[22]

In this work we intent to generalize the concept of immobilizing cells into liquefied capsules coated with polyelectrolyte self-assembled multilayers using mild conditions in all steps. For the proof-of-concept liquid alginate core capsules coated with 8 polyelectrolyte layers of alginate/chitosan were processed in cell friendly environment (Scheme 1). The motivation is also based on the assumption that natural derived macromolecules exhibit several

advantages when used in biomedical applications, including tissue engineering.[23] Two methodologies were used for capsule production, namely ionotropic gelation and polyelectrolyte layer-by-layer deposition. Alginate beads were obtained by ionic crosslink with calcium chloride. A water soluble chitosan with high molecular weight was used as the polycationic solution for the multilayer coating construction with alginate polyanionic solution. Different formulations were tried to obtain liquefied capsules with the best mechanical stability. SaOs-2 osteoblast-like cells were used as a model cell source for the encapsulations studies performed in liquid alginate/chitosan capsules.

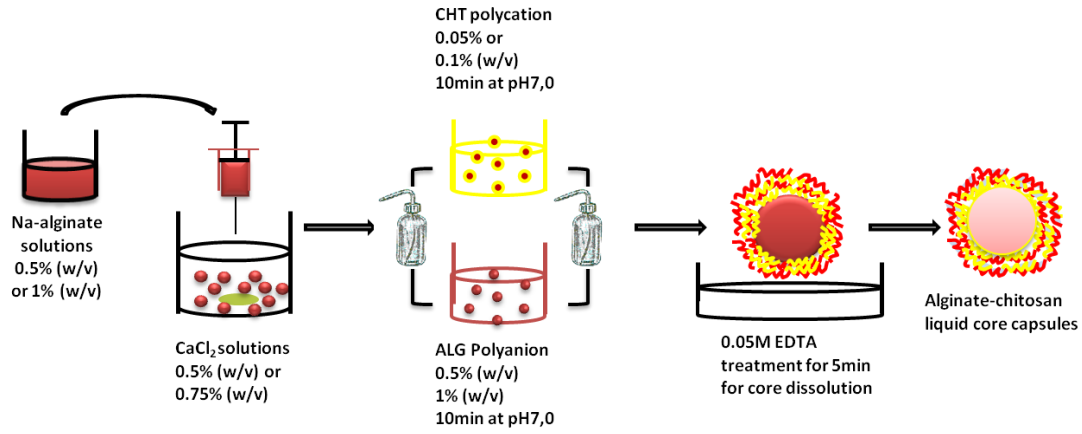

Figure3.1: schematic representation of the three-step alginate-chitosan microcapsules production.

3.2. Materials and Methods

3.2.1. Materials

Water soluble ultrapure chitosan (CHI) salt (PROTOSAN UP CL213, viscosity 107mPa.s, molecular weight M_w=2,7x10^5g/mol, degree of

deacetylation DDA=83%) was purchase from Novamatrix (FMc, Norway). Low viscosity sodium alginate (ALG) was purchase from Sigma-Aldrich (Portugal).

3.2.2. Quartz crystal microbalance with dissipation monitoring (QCM-D)

LbL assembly of alginate-chitosan multilayers was measured by Quartz Cristal Microbalance with dissipation monitoring (Q-Sense E4 system – Q-Sense AB, Sweden). The sequential deposition was carried out using two different polyelectrolyte concentrations: low polyelectrolyte concentration, 0.05% w/v (LPC), and high polyelectrolyte concentration, 0.1% w/v (HPC), alginate and chitosan solutions. Briefly, a baseline was constructed using a 0.15M NaCl solution. Then the bidimensional multilayer films were building by alternating chitosan and alginate onto the gold crystals. The first coating was chitosan (polycation). The polyelectrolyte solutions were injected into the measurement chamber for 10min at a flow rate of 100µL/min and a washing step of 5min with 0.15M NaCl solution was carried out after each polymer adsorption. For all measurements the temperature was set up at 25ºC and the pH of all solutions was adjusted to 7. The experience was performed in triplicates and the results were manipulated using the QTools software (version 3.06.213).

3.2.3. Preparation of Alginate-Chitosan capsules

The alginate-chitosan capsules (AC capsules) were produced using a three step methodology (Scheme 1). Firstly, alginate beads were produced by ionotropic gelation taking place when droplets of 0.5% w/v (formulations A and C) or 1% w/v (formulations B and D) sodium alginate solutions were extruded through a 27G needle into 0.5% w/v (formulations A and C) or 0.75% w/v (formulations B and D) calcium chloride solution and left stirring for 20 min at room temperature. The calcium-alginate beads were recovered by filtration in a 0.22μm filter paper, and rinsed three times in 0.15M NaCl. Secondly, the layer-by-layer assembly was performed over the surface of alginate beads. The sequential deposition was carried out using LPC and HPC alginate and chitosan solutions. Ca-alginate beads were incubated for 10min in CHI solutions, rinsed two times in NaCl and incubated for another 10min in ALG solutions. The procedure was repeated until de 8 alginate/chitosan polyelectrolyte layers (ALG/CHI multilayers) were achieved. Finally, the coated Ca-alginate beads were treated with 0.05M Etilenediaminetetraacetic acid (EDTA cell culture tested – Sigma-Aldrich, Portugal) for 5min to liquefy the AlG core. All the solutions used in this procedure, were prepared in 0.15M aqueous solution NaCl (Sigma-Aldrich, Portugal) and their pH were adjust to 7. Washing steps took place after each polyelectrolyte deposition using 0.15M NaCl as the rinsing solution.

3.2.4. Capsule characterization

3.2.4.1. Micro-computerized tomography (μCT)

The inner structure of the Ca-ALG beads were evaluated by micro-computerized tomography, μ-CT, using a micro-CT scanner (Skyscan 1702, Belgium) with penetrative X-rays of 40 KeV. The X-rays scans were acquired in the high-resolution mode. The CT Analyser® software was used to visualize and to reconstructs the 2D X-ray images of the capsules.

3.2.4.2. Scanning electron microscopy (SEM)

AC capsules were deposited onto carbon tape, placed under cupper stubs and allowed to air dry. The samples were sputtered with gold and observed under Nova Nano SEM 200 (FEI Company, US).

3.2.4.3. Alginate-Chitosan capsules stability test

The ability of AC capsules to sustain the mechanical stress of rotation was determined using a centrifuge.[21, 24] Approximately 50±10 AC capsules were placed in centrifuge tubes containing 0.01M EDTA (in 0.15M NaCl solution). The tubes were rotated for 60min at a speed of 200rpm at 25^{o}C and followed by additional 15min of rotation at a speed of 4500rpm at the same temperature. The number of damaged capsules were observed and counted under a light microscope every 15min of centrifugation.

3.2.5. Cell culture and encapsulation

A human osteoblast-like cell line (SaOs-2 cell line, European Collection of Cell Cultures, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Portugal) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Germany) and 1% antibiotic/antimycotic solution (Gibco, UK). Cells were incubated at 37ºC in a 5% CO_2 atmosphere incubator.

Confluent cells were harvested and suspended in 1% w/v alginate solution, to a final ratio of $1.0x10^6$ cells per ml of alginate. The encapsulation procedure as well as the LbL polyelectrolyte assembly was performed as previously describe for the AC capsules preparation using HPC polyelectrolyte solutions to construct the ALG/CHI multilayer membranes. All the process was carried out in a sterile flow chamber at room temperature under mild conditions.

3.2.5.1. Cell viability assay

The cell viability was determined using the MTS assay. This assay is based on the bioreduction of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphofenyl)-2H-tetrazolium (MTS – CellTitre 96®, Promega, Madison, USA) into a water-soluble brown formazan product. The optical density (OD) was quantified by UV-spectroscopy, reading the formazan absorbance at 490nm in a microplate reader (Bio-Tek, Synergie HT, USA). Results were compared to the cell viability on the culture plate, as assay

control, and to the AC capsules without cells, stated as sample control. The samples were analyzed in triplicate per each time point.

3.3. Results and discussion

3.3.1. Quartz crystal microbalance with dissipation monitoring (QCM-D)

QCM-D experiments were performed initially to monitor the formation of ALG/CHI multilayers on planar surfaces using LPC and HPC polyelectrolyte solutions. Figure 1 shows the build-up of 8 polyelectrolyte layers using LPC and HPC solutions at pH 7. The decrease of film $\Delta f/n$ – see Fig. 1A - after each polyelectrolyte adsorption step suggests that mass is being deposited at the crystal surface in both studied conditions. Moreover the sequential deposition occurs as a stable process giving rise to film with a continuous increase in the thickness and with a systematic increase of the viscoelastic behavior due to the steady increasing in ΔD – see Fig. 1B. The obtained results were very similar to those achieved in a previous study, where the same chitosan, but with a low molecular weight, was used to construct multilayers with low viscosity alginate at pH 7 and at room temperature.[25] Assuming a simple viscoelastic model and some characteristic values of densities and viscosity of the liquid medium we estimate that in both HPC and LPC multilayered membranes the thickness was around 100nm after the deposition of 8 the layers.

The QCM-D experiments demonstrate that stable ALG/CHI multilayers can be produced by layer-by-layer using high molecular weight chitosan (CL213) and alginate under physiological simulated conditions. Such system was used to coat Ca-alginate beads.

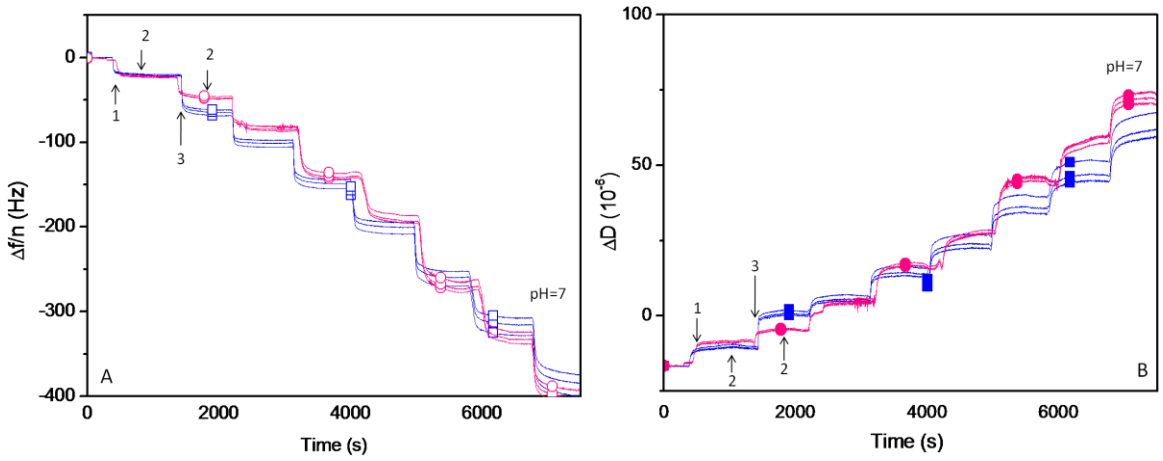

Figure3.2: QCM-D results showing the deposition of 8 ALG/CHI layers films at pH 7 with low polyelectrolyte concentration (LPC –circles) and high polyelectrolyte concentration (HPC – squares). (A) Normalized frequency ($\Delta f/n$) and (B) dissipation (ΔD) variations are recorded as a function of time. Steps 1 and 3 represent chitosan and alginate deposition, respectively, and step 2 is related to rinsing with saline buffer NaCl. Plots represent the 7th, 9th, and 11th harmonics for HPC frequency (solid squares) and dissipation (open squares) and the 7th, 9th and 11th harmonics for LPC frequency (solid circles) and dissipation (open circles) at pH 7.0.

3.3.2. Alginate-Chitosan capsules morphologic characterization

Stereoligth microscopy observations revealed that the Ca-alginate beads exhibit a spherical-like shape with a diameter in the range 2.0-2.5 mm – see Fig.2A.

μ-CT analyze was performed on air dried Ca-alginate beads and revealed a lamellar-like organization of the material – see Fig.2B. The structure

can possibly be a result of the water leaching during the air drying which leaves the corresponding fingerprint in the solid hydrogel. Such kind of lamellar morphology was also observed in the interior of the Ca-alginate dried beads by SEM – see Fig.2C, being consistent with the μ-CT results.

After the multilayer coating and the liquefaction process the AC capsules were obtained. The pH of the polyelectrolyte solutions used in this study was 7. Although chitosan is known to be soluble in low pH conditions, it was shown before that it is possible to increase the pH of these types of soluble chitosan until 7 without polymer precipitation, and that such solutions can be used together with the alginate solution in the build-up of ALG/CHI multilayers.[25] Figure 2D shows a representative liquid core AC capsule exhibiting a well preserved and homogenous multilayered membrane build at mild conditions and pH 7. The dimension of the AC capsules is similar to the Ca-alginate beads.

Polyelectrolyte deposition over the Ca-alginate bead was investigated by means of SEM analysis. Figure 2E shows the organization of the polymeric membranes evolving the liquid core of AC capsules. It is clear the layered-like organization of the membrane that also displays a quite smooth surface. The membrane thickness of polyelectrolyte multilayers build on the Ca-alginate beads with HPC is of the order of the micron-scale which contrasts with the much thinner thickness observed in the ALG/CHI multilayres developed in planar gold substrates as monitored by QCM-D. This could be explained by the

partial diffusion of chitosan within the Ca-alginate beads producing a dense layer stabilized by complexation.

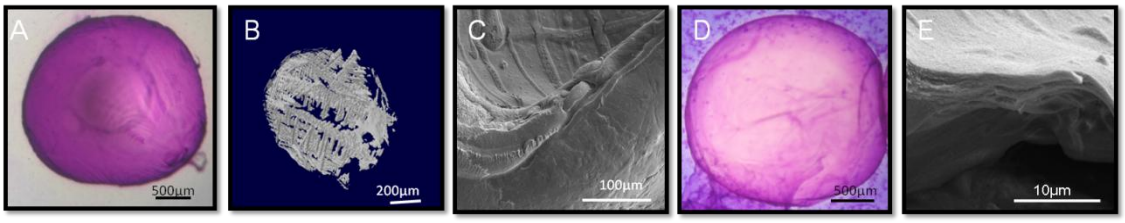

Figure3.3: (A) – Calcium-alginate bead observed by optical microscopy; (B) – alginate organization observed by µ-CT analysis; (C) – SEM of the interior of Ca-ALG beads; (D) – alginate-chitosan liquid core capsule observed by optical microscopy; (E) – SEM of the alginate-chitosan coating organization of the AC capsules;

3.3.3. Alginate-Chitosan capsules stability test

Table 1 shows the four different Ca-alginate beads formulations used as templates for LBL assembly. LPC and HPC solutions were used to coat the surface of Ca-alginate beads previously prepared by precipitation of 0.5% w/v or 1% w/v alginate solution in 0.5% w/v and 0.75% w/v $CaCl_2$ solutions.

Samples	Na-alginate (g/100ml)	$CaCl_2$ (g/100ml)
A	0.5	0.5
B	1	0.5
C	0.5	0.75
D	1	0.75

Table3.1: Concentration of the sodium alginate and calcium chloride solutions used to prepare the different liquid core capsules.

The mechanical stability of the AC capsules subjected to EDTA treatment is resumed in figure 3. All capsules were coated with 8 layers using LPC (solid

symbols) or HPC solutions (open symbols). It is noticeable that the capsules prepared using HPC solutions presented a much better stability with respect to the LPC solutions. This could be due to a more extent of complexation of the first chitosan multilayer with the Ca-alginate bead that can form a more robust layer - see Fig. 2E. Moreover the capsule prepared with higher concentrations of alginate (B and D) are more stable but this effect is mainly seen when the capsules are coated with LPC solutions.

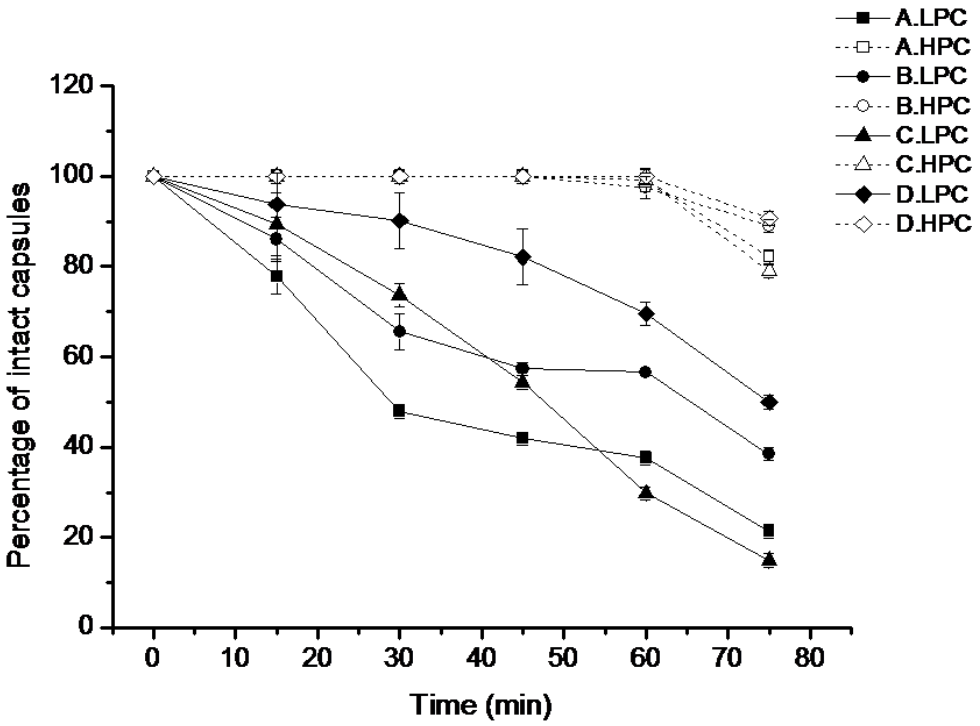

Figure3.4: The effect of the mechanical impact of rotation at 200rpm and 4500rpm on the integrity of the four types of liquid core capsules as function of time.

3.3.4. Cell viability assay

Taking into account the results obtained from the mechanical stability tests, cell encapsulation was only performed in AC capsules whose membranes were build using HPC solutions.

SaOs-2 cells were encapsulated in 8 layers AC capsules to evaluate the *in vitro* biocompatibility of the developed system. Optical microscopy images after 1 day of culture shows that cells were entrapped and uniformly distributed inside the AC capsule – see Fig. 4A1. After 3 days of culture the cells tend to attached to the inner side of the multilayred membrane that is consistent with the adherent nature of the cells – see Fig. 4A2. Previous studies also concluded that cells encapsulated in large liquid microcapsules (>1mm) generally tent to move through the capsules periphery were the gas and nutrient diffusions are more efficient.[15, 26]

The viability of the encapsulated SaOs-2 cells was assessed using MTS assay - see Fig.4B. The results suggested that the cell viability increases 1 day to 3 days of culture. This indicates that cell viability is not affected by the multilayered build up process, even though the time required for their production has been approximately 3h. This can be explained by the fact that mild conditions has been ensured since the beginning with the selection of water soluble and biocompatible natural-based polymers which were manipulated at physiological pH and at room temperature. Moreover, the salt and polyelectrolyte concentrations were appropriate to maintain the capsules and

the encapsulated cells in a adequate ionic strength and osmotic pressure environment. At the 0.05 level, the two population variance is significantly different.

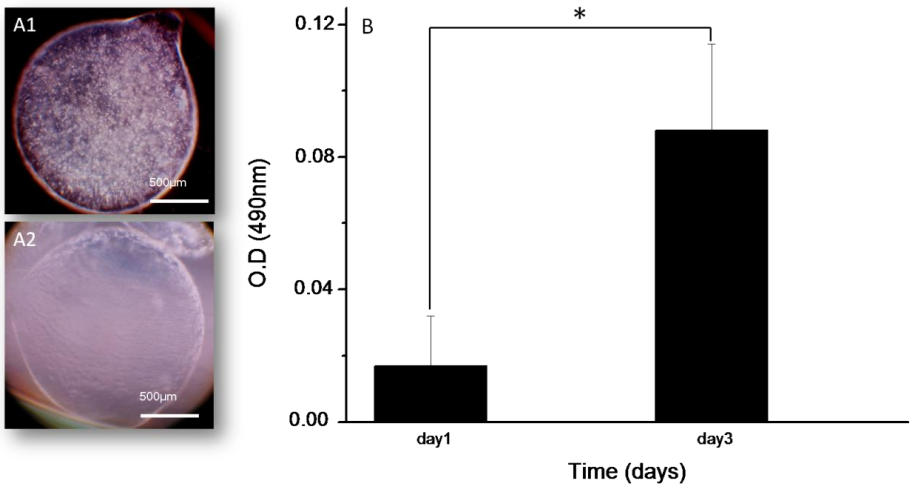

Figure3.5: (A) –Cells encapsulated in 8LbL AC capsules after 1day (A1) and 3 days culture seen by optical microscopy; (B) – MTS viability assay of encapsulated cells aft and 3 days of culture. (*) – Significance level of 95% ($p < 0.05$).

3.4. Conclusions

In this work the possibility of encapsulating cells within a multilayered liquid core capsule without compromising the cell viability was demonstrated. A water soluble chitosan with high molecular weight was used as a polycation to produce multilayered membranes by alternate deposition with oppositely-charged alginate. The polyelectrolyte LbL was performed onto ionotropic gelled Ca-alginate capsules with previously entrapped cells. The main goal of this study was to encapsulate cells in liquid core capsules at the mildest condition

as possible, so, neutral pH was employed for all the polymers which were prepared in physiological saline buffer under room temperature and in sterile conditions. Low and high concentrations of polyelectrolyte solutions were used to prepare capsules multilayered membranes and their mechanical stability was assessed. Results revealed that multilayered membranes prepared with high polyelectrolyte concentration were mechanically more stable and not so dependent from the Ca-alginate initial concentrations. SaOs-2 cells were encapsulated in the capsules with 8 layers and that their viability was not compromised.

3.5. References

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Chapter IV: Preliminary study on the encapsulation of PLLA microparticles seeded with cells onto liquefied capsules

4.1. Introduction

Cell encapsulation in liquid-core matrix with a spherical shape has been a widely applied methodology to entrap living cells and bioactive compounds. Among the numerous biomaterials candidates, hydrogels have shown great promise due to their unique advantages.[1-3] Generally, hydrogels provide highly hydrophilic and bio-inert microenvironment in which the suspended cells are constrained to a round shape environment.[4, 5] This feature may be a drawback since the majority of encapsulated living cells are anchorage-dependent cells (ADCs), which require a surface to attach and spread in order to maintain their natural phenotype.[6, 7]

Non-collagen and un-modified hydrogels are known to be lack of cellular integrin receptors.[8, 9] In normal condition this receptors binds to specific ligands triggering intracellular signaling cascades which prevents cell apoptosis and activates cytoskeletal reorganization.[6, 10] In the absence of these receptors the entrapped ADCs are unable to naturally anchor to polymer matrix.[11] Moreover, physical enclosing by the round-shape liquid-core matrix may submit the ADCs to an undesirable environment which may be ineffectual for the cells spreading in the hydrogels.[12]

In order to improve the cell adhesion and proliferation in hydrogel matrix, inert polymeric chains can be tailored with select biological moieties to yield bioactive materials. The most common procedure to attain this level of bioactivity is the inclusion of Arginine–Glycine–Aspartic acid (RGD) tri-peptide.[2, 13, 14] This specific amino acid sequence is a prototypical oligopeptide ligand which can be found in the adhesive proteins such as fibronectin and vitronectin. Usually this ligand binds to α4β1 integrin receptors expressed on the cell surface enhancing cell adhesion, spreading, proliferation and phenotype expression in *in vitro* cultures.[15-18] Recently, fibronectin or RGD itself has been used to modify polyelectrolyte film surfaces for cell adhesion[19], in cell sheets constructions[20-22], or in the production of polymeric matrix microcariers for ADCs delivery.[23-25]

In this study cells were immobilized in spherical annular Ca-alginate gels that provide and aqueous and three-dimensional environment for the cells. To provide immune isolation and mechanical stability Ca-alginate core was coated with alginate-chitosan multilayers using an identical procedure to what we have previously related – see Chapter 3. In the earlier encapsulation anchorage-dependent cells were entrapped inside liquid-core capsules and coated with alginate-chitosan polyelectrolytes using LbL methodology. Incubation with EDTA leads to the diffusion out of high amount of calcium ions bonded to the polyguluronate sequences. This event is responsible for the loose of the “egg box” structure (solid core) and core liquefaction.[26, 27] In the presence of liquid-

core matrix and regarding the large diameter of the capsules (>1mm) ADCs usually tend to form cell clusters within the core matrix and become deprived of oxygen and nutrients.[28] On the other hand, a few amounts of cells seem to migrate to capsule membrane, and attached on it, since it is the only solid structure available to attach on. As a result, cells are not dispersed throughout the core and became necrotic.

Here, an alternative method for encapsulating living anchorage-dependent cells in liquid-core capsules containing solid particles within is presented. The possibilities to have additional solid surface where the cells can attach and proliferate represent a recent concept for increasing the loading capacity of aqueous capsules.

4.2. Materials and Methods

4.2.1. PLLA particles processing

For this work polylactic acid (PLLA – M_n=69000, from Cargill Dow, USA) microparticles were obtained by milling (Ultra Centrifugal ZM 200, Retsch - Germany. Briefly, some PLLA beads, used as received, were milled four times and their range was estimated using a particle weight separator (Sieve Shaker As 200, Retsch - Germany). The processed microparticles had rough surface morphology and ranged between 125µm - 250µm. For further use in *in vitro* studies, the PLLA microparticles were sterilized in Pronefro (Portugal). The morphology of the PLLA microparticules was confirmed by means of Stereoligth Microscopy and Scanning Electron Microscopy (SEM).

In our group PLLA has been used in various formulations for studies of cell adhesion and proliferation, often showing good results. Material properties or the scaffolding that it will integrate can be controlled through the surface modification[29] or previous treatments applied to the material.[30-32]

4.2.2. Cell culture studies

Both cell line of osteoblastic like cells (SaOs-2) and fibroblastic like cells (L929) were selected for the in vitro studies once they are the most applied cells in preliminary studies with biomaterals. The cell lines were obtained from European Collection of Cell Cultures (ECC, UK) and were cultured in 75 cm^3 culture flasks using Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Portugal) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Germany), 1% antibiotic/antimycotic (Invitrogen, Portugal) and sodium bicarbonate (Sigma-Aldrich, Portugal) at 37ºC in a 5% CO_2 incubator. The culture medium was changed every two days and cells were growth until confluence, before any *in vitro* assay. A fraction of the PLLA microparticles was treated with human fibronectin (Biopur, Swiss) and both treated and untreated PLLA microparticles were seeded with cells and cultured for different periods of time. The cells adhesion and proliferation onto PLLA microparticles were assessed by microscopy and MTS assays. PLLA microparticles with cells seeded onto were encapsulated in alginate-chitosan liquid-core capsules and culture for different periods of time.

4.2.3. Seeding and culturing osteoblast like-cells and fibrobroblast like-cells into PLLA microparticules with and without fibronectin treatment

The aim of this study was to investigate differences in cells adhesion and proliferation onto both PLLA microcaparticles treated with human serum fibronectin and untreated.

The procedure was made according to Custósio et al[33] with slightly modifications. Briefly, PLLA microparticles were placed in 24 well plates in order to have 1mg of sample per each well. 12 wells containing the PLLA microparticles were treated with 1ml of a solution containing 0,15M NaCl and 100µl of human serum fibronectin. The other 12 wells were maintained with 0,15M NaCl. The 24 culture well plate with PLLA samples was incubated for 24h at 37ºC in a 5% CO_2 incubator. After the incubation time, the solutions were removed, and 3,0x10^5 SaOs-2 and L929 cells were seeded – see Figure 4.1. The plate was incubated for 2h at 37ºC in a 5% CO_2. At the end of the 2h for cell and material contact, 1ml of culture medium DMEM were added to each well and the samples were incubated in the same conditions previously described for different periods of time. Microscopy and MTS assay were performed in order to evaluate the cells adhesion, proliferation and viability along 3 days.

4.2.4. Staining procedure for microscopy analysis

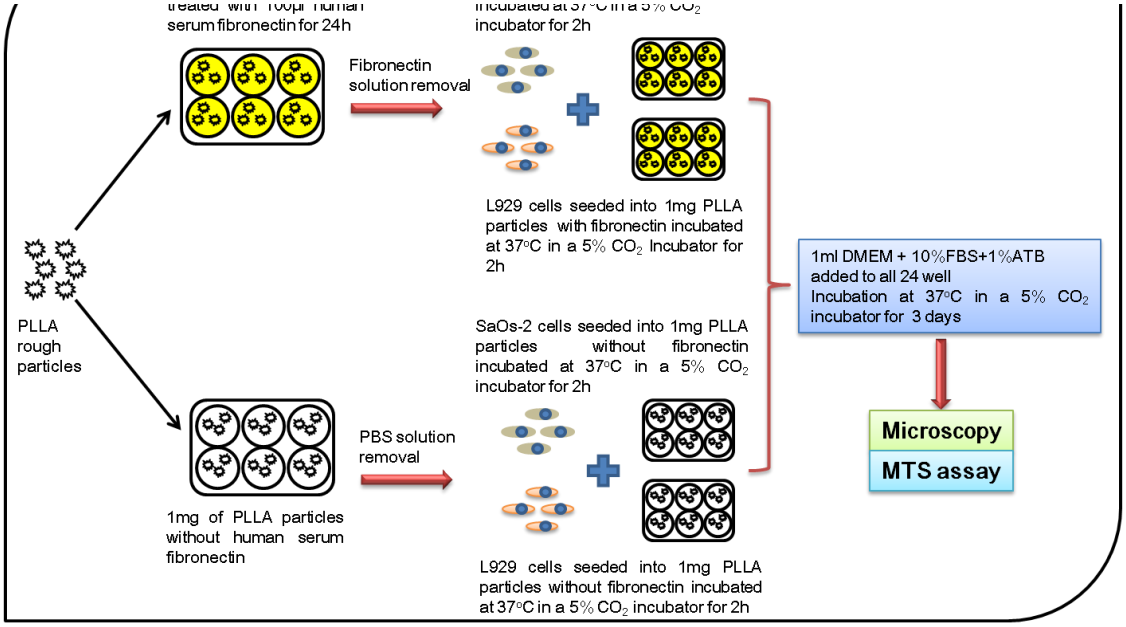
From the 24 well sample culture plaque, one well of each studied condition were used for microscopy analysis. Briefly, each of the four wells were washed two times in PBS and incubated with formalin for 30 min at 37°C in a 5% CO_2 incubator. Formalin was used to fix cells and maintain the culture safe from microbial. After the fixation time, the formalin was removed and the wells were again washed once with PBS. A solution containing 1ml of PBS and 10µl of methylen blue was added to each well and the plaque was incubated for 10 min at the same conditions. Methylen blue was used as a cell dye. The stained samples were analyzed under the conventional microscope.

4.2.5. MTS quantification of viable cells

The cell viability and proliferation under the PLLA microparticules were assessed using the CellTitre 96® MTS reagent (Promega, Madison, USA). For the viability quantification, cell culture medium DMEM was prepared without phenol red and FBS and was mixed with MTS reagent in a (5:1) ratio. Afterwards, 300µl of MTS solution was added to each sample culture well and incubated in the dark for 3h at 37°C in a 5% CO_2 incubator. After the incubation time, 100µl was transferred from each culture well to a 96 well plaque (with the respectively triplicates) and the absorbance was read in the microplate reader (BIO-TEK-Synergy HT) at 490 nm. A blank solution, with MTS solution and samples without cells, was made as assay control. The MTS graph was constructed with the corrected values, i.e., all the values read for samples with

cells menus the assay control. The time points selected for this study were 24h, 48h and 72h.

Figure4.1: schematic representation of the procedure used for seeding and culturing SaOs-2 and L929 cell lines onto PLLA microparticles treated and untretaed with human fibronectin.



4.2.6. Encapsulation of PLLA microparticules with cells in alginate-chitosan capsules

Cell seeding onto PLLA microparticles was carried out as describe earlier in 2.2.1 in both 1mg and 0.05mg of sterilized PLLA microparticles. A low viscosity sodium alginate (Sigma-Aldrich, Portugal) was used to prepare 1.5% alginate solution in 0.15M NaCl. The polymeric solution was vacuum-filtered in a 22µm pore-size filter (Schleicher & Schuell Microscience, Germany) to sterilize. 10ml of the sodium alginate solution were added to PLLA microparticules with cells in order to obtain two different cell/microparticles suspensions with a final ratio of 0.05mg/10ml and 1mg/10ml. Capsules were

produced using a Pasteur pipette instead of a needle, once its diameter allows the flux of both suspensions containing PLLA microparticles. The suspensions were extruded separately to a 1% calcium chloride (Sigma-Aldrich – Portugal) solution prepared in 0.15M NaCl, and left gelling for 20min under stirring at room temperature. The following encapsulation procedure such as the layer-by-layer multilayer construction and the liquid-core capsule production was performed according to what was already described in Chapter 3. The encapsulated cell/microparticles were incubated in a 5% CO_2 incubator at 37ºC and culture for 7days in culture plaques. The culture medium was changed every 2days.

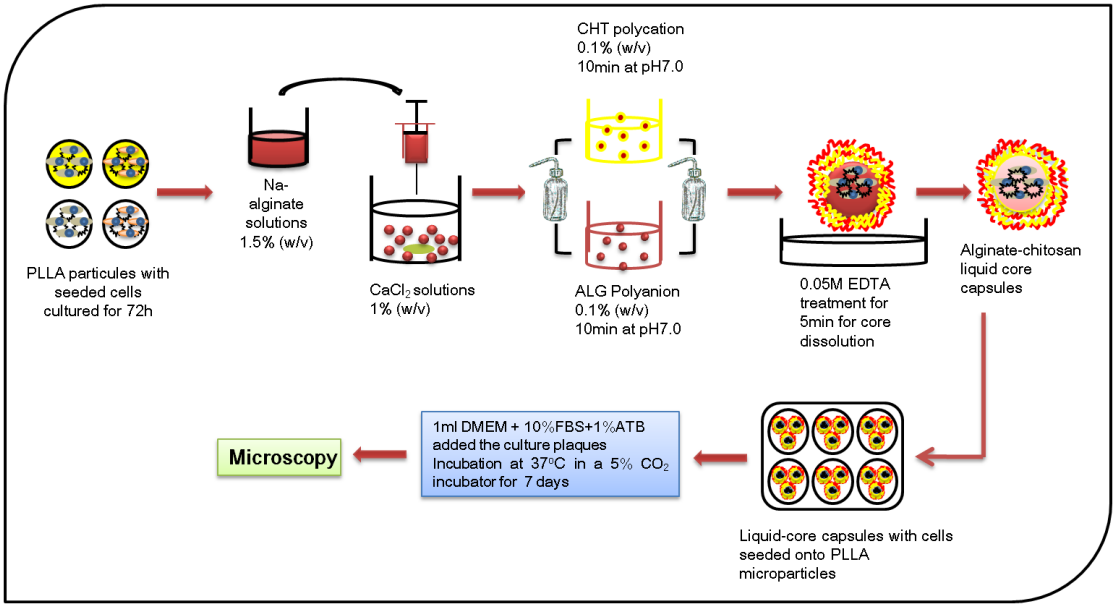


Figure4.2: Schematic representation of the procedure used to prepare liquid-core alginate/chitosan capsules containing cells seeded onto PLLA microparticles.

4.3. Results and Discussion

4.3.1. Cellular adhesion and proliferation

PLLA microparticles were seeded with SaOs-2 and L929 cell lines in order to evaluate the cell/material interaction with respect to cell adhesion and proliferation. Optical microscopy revealed that both cell lines have attached to the surface of both fibronectin treated and untreated PLLA microparticles after the 72h of culture. However, fibroblastic cell line, L929, shows to adhere better than the osteoblastic cell line, SaOs -2 in both tested conditions. Moreover, it seems that PLLA microparticles surface previously treated with human fibronectin have better affinity to the living cells – see Figure 4.3 E.

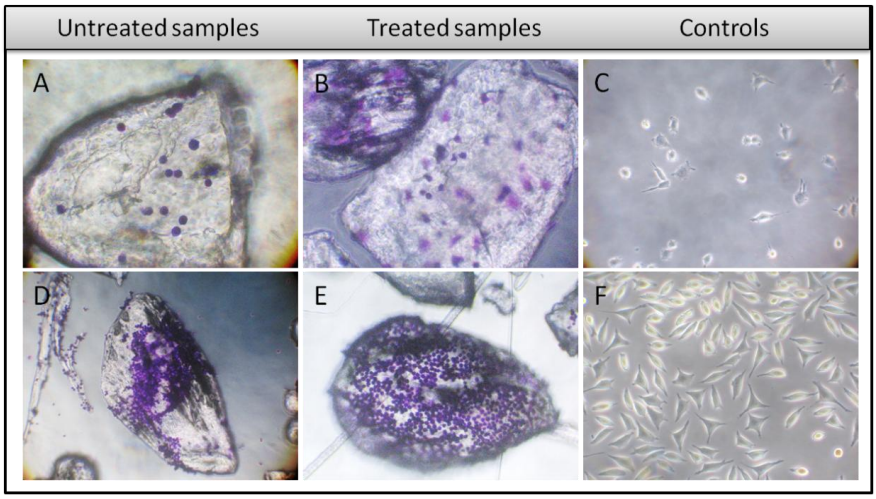


Figure4.3: Optical microscopy cells cultured onto untreated PLLA microparticules (A – SaOs-2 and D – L929) and fibronectin treated PLLA microparticles (B – SaOs-2 and E – L929) after 72h. C and F represents, respectively, SaOs-2 cells and L929 cells cultured for 72h at 37ºC (positive control). A, B, C and F were obtained using a magnification of 40X. D and E were obtained using a magnification of 20X.

Regarding to the cell morphology, it was possible to observe that unlike the controls (cells in culture) the cells seeded onto both PLLA formulations were round and not express pseudopodias. This cell behavior on the PLLA microparticules can be explained by the solid particle formulation which is the result of the processing method applied. Actually, several studies have confirmed that cells adhesion and proliferation strongly depends on the material surface characteristics.[34-38] Other studies have shown that the thickness and rigidity of a substrate can affect cell attachment and alter cell shape.[39-43] Moreover, Salloum et al demonstrate that cell morphology and motility depends more on the hydrophobicity and charge on top of the material than on the thickness of the material.[44] So, to achieve a suitable solid surface which elicits cell adhesion and proliferation, further improvements in the solid particles processing must be taken into account.

4.3.2. MTS quantification of viable cells

Figure 4.4 shows the results obtained by the MTS assay after 72h of culturing cells onto PLLA microparticles treated and untreated with human fibronectin serum. The results show an increasing cell density for both cell lines during the culturing time. However, it is clear the high adhesion and proliferation capacities of the fibroblastic like-cell when compared with osteoblastic like-cells.

Fibroblast adhesion and proliferation seems to happen faster and easily during the first 48h when the PLLA microparticles are treated with human fibronectin serum. On the other hand, after 72h of culture there were no

significant differences between the optical densities (O.D) of cells cultured onto treated and untreated PLLA microparticules. This situation may be explained by (1) the high ability to induce cellular adhesion express by the extracellular matrix proteins, such as fibronectin, which may have a critical role during the first 48h of culture and (2) the anchorage-dependent behavior of the living cells which naturally impels them to attached on a solid surface, which may justified the increasing of adherent and proliferating cells on untreated PLLA microparticles after 72h. These results are in agreement with the optical microscopy observations.

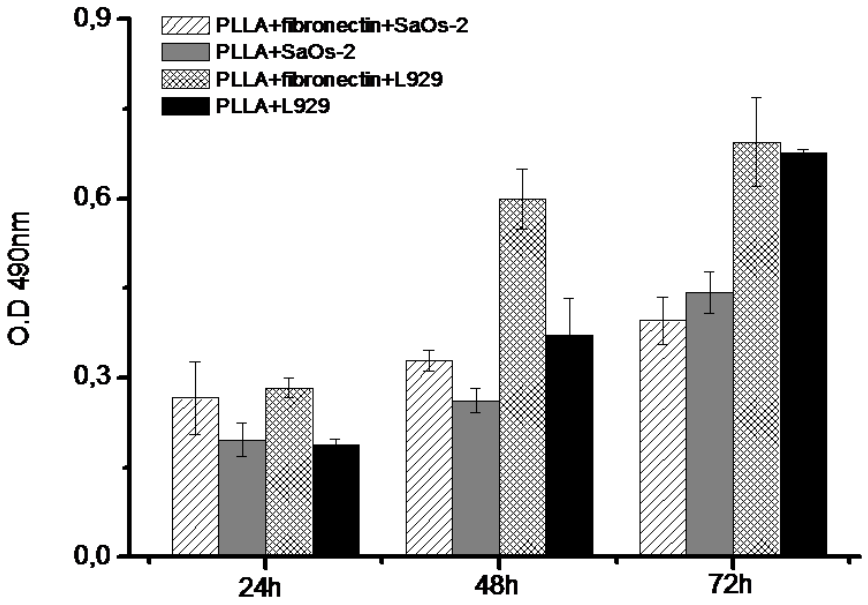


Figure4.4: Cell viability and proliferation of SaOs-2 cells and L929 cells cultured onto untreated and treated PLLA microparticles. The results were obtained using MTS assay after 24h, 48h and 72h.

Nevertheless, the O.D of cells seeded in the PLLA microparticles after the 72h are slightly low in comparison with the amount of cells in culture (positive

control) for the same period of culturing, which may be explained by the loose of PLLA micropartcles during the procedure to preparing samples for MTS assay.

4.3.3. Encapsulation of PLLA microparticules with cells in alginate-chitosan capsules

Figure 4.5 shows the encapsulated PLLA microparticles with cells seeded onto. From the pictures it is only possible to observe the PLLA microparticles within the liquid-core capsules. Due to the considerable diameter of the alginate/chitosan capsules (>3mm) none of the attempted microscopy techniques allowed us to visualize the cells seeded onto the solid PLLA microparticles. Thus, even though the cells are actually present in the encapsulated material, the images obtained cannot confirm it.

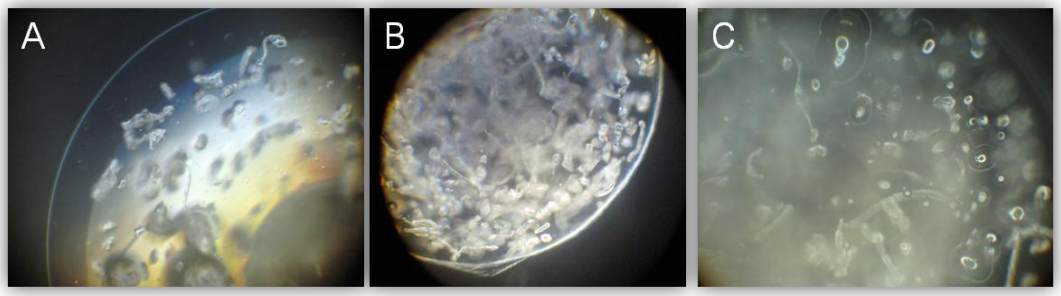


Figure4.5: Alginate-chitosan liquid core capsules containing PLLA microparticules with cells seeded onto. A – Capsule with 0.05mg of PLLA microparticles, magnification of 40X; B – capsule with 1mg of PLLA microparticles, magnification of 20x; C – PLLA microparticles dispersed within liquid-core matrix, magnification of 40x.

4.4. Conclusions

In the field of biomaterials, controlling the surface mechanical properties may be a means of influencing cell behavior including re-colonization, adhesion, and migration.[45] Proteins play an important role in the adhesion, spreading, and growth of cells.[44] For its part, the cell physiology itself (morphology and functionality) affects the mechanism of the adhesion (specific and unspecific)[46] and consequently its proliferation. In this preliminary study we observe that incorporation of solid particles in liquid-core capsules could be a good alternative to provide a solid surface for adherent cells dispersed in a liquefied/viscous environment. Here we suggest PLLA microparticles as the solid surface for cell adhesion, but, in principle, a wide range of bioactive or even inorganic materials can be used as cell support. Once the techniques used for the production of these systems are quite facile and versatile, all the components presented here can be manipulated differently depending on its further application. Therefore, the capabilities of this system are very promising once it allows the applications of a wide range of natural or synthetic biomaterials not only in the buildup of the liquid-core matrix and polyelectrolyte multilayers but also in the development of the solid inclusion particles. Still, the processing techniques of materials may also be the most varied bearing in mind the dimensions of the capsule, the cell type and its application.

4.5. References

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Future remarks

In the field of bioencapsulation a continuous effort has been done in order to prepare liquid-core capsules based on different formulations. However up to now, a lot of works still has to be done in order to establish these liquefied capsules as a potential and viable alternative for cell encapsulation. Some Improvements can be carried out in order to obtain clinically successful devices. They may be for example, (1) stabilization of the chemical and physical boundings between the materials used, whether to form the core or to build the capsule wall, in order to improve the mechanical properties of the capsules; (2) the development of brand new combinations of natural and synthetic polymers with higher toughness and malleability; (3) the inclusion of peptides or bioactive compounds to enhance the biocompatibility; (4) surface modifications of the capsule walls by the construction of different numbers of layers with the most varied types of macromolecules; (5) choice of new materials that can be used as liquid-core templates in cell friendly procedures.

With the continuous advances in genetics, biotechnology, chemical and biological sciences the improvements will lead to progressions in tissue engendering and regenerative medicine. Moreover, bioencapsulation may become one day closer to a realistic proposal to clinical application.