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**Encapsulation of Single hMSCs  
in Polyelectrolyte Shells  
Preliminary Studies**

Tese de Mestrado em Bioquímica Aplicada

Trabalho efectuado sob a orientação da

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This Master Thesis was the first research work conducted at CQM - Centro de Química da Madeira (Universidade da Madeira) which explored the LbL technique. In this context, it allowed the gaining of experience in the method and, even if many questions remained to be answered (the presented results resume the experimental work that was possible to be done in the available period of 12 months), it will certainly serve as a basis for the launching of other research works in the field at CQM. For the Student, it was an opportunity to learn how to access and analyse research literature, design a research project, interpret experimental data and communicate science. In terms of laboratory skills, the Student learned the basic techniques of animal cell culture, procedures of chemical synthesis used to label polymers with fluorescent dyes, how to use the optical inverted microscope (including the fluorescence microscope) and the microplate reader (fluorescence spectroscopy, UV/vis spectroscopy), as well as several biochemical assays used to analyse cell viability.

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## ABSTRACT

The main objective of this Thesis was to encapsulate single viable cells within polyelectrolyte films using the Layer-by-Layer (LbL) technique. Most of the experiments used human mesenchymal stem cells (MSCs) whose characteristics (capacity of self-renewal and potential to differentiate into several types of cells) make them particularly interesting to be used in biomedical applications. Also, most of the experiments used alginate (ALG) as the anionic polyelectrolyte and chitosan (CHI) or poly(allylamine hydrochloride) (PAH) as the cationic polyelectrolyte. Hyaluronic acid (HA) was also tested as an anionic polyelectrolyte.

At the beginning of the work, the experimental conditions necessary to obtain the encapsulation of individual cells were studied and established. Through fluorescence microscopy visualization by staining the cell nucleus and using polyelectrolytes conjugated to fluorescent dyes, it was possible to prove the obtainment of capsules containing one single cell inside. Capsules aggregation was an observed problem which, despite the efforts to design an experimental process to avoid this situation (namely, by playing with cell concentration and different means of re-suspending and stirring the cells), was not completely overcome.

In a second part of the project, single cells were encapsulated within polyelectrolyte layers made of CHI/ALG, PAH/ALG and PAH/HA and their viability was evaluated through the resazurin reduction assay and the Live/Dead assay. In these experiments, during the LbL process, polyelectrolyte solutions were used at a concentration of 1mg/mL based on literature. In general, the viability of the encapsulated cells was shown to be very low/absent.

Then, as a consequence of the lack of viability of cells encapsulated within polyelectrolyte layers, the LbL technique was applied in cells growing adherent to the surface of cell culture plates. The cells were cultured like in a sandwich, between the surface of the cell culture dish and the polyelectrolyte layers. Also here, the polyelectrolyte solutions were used at a concentration of 1mg/mL during the LbL process. Surprisingly, cell viability was also absent in these systems.

A systematic study (dose-effect study) was performed to evaluate the effect of the concentration of the individual polyelectrolytes (ALG, CHI and PAH were studied) in cell viability. Experiments were performed using cells growing adherent to the surface of cell culture plates. The results pointed out that a very high (cytotoxic) concentration of polyelectrolytes had been in use. Also, in general, PAH was much more cytotoxic than CHI, whereas ALG was the less cytotoxic polyelectrolyte.

Finally, using alginate and chitosan solutions with adequate concentrations (low concentrations: 50ng/mL and 1µg/mL), the encapsulation of single viable cells was again attempted. Once again, the encapsulated cells were not shown to be viable.

In conclusion, the viability of the encapsulated cells is not only dependent on the cytotoxic characteristics (or combined cytotoxic characteristics) of the polyelectrolytes but it seems that, when detached from the culture plates, the cells become too fragile and lose their viability very easily.

**Keywords:** Layer-by-layer technique; single cell encapsulation; chitosan, alginate; poly(allylamine hydrochloride), mesenchymal stem cells.

## RESUMO

O principal objectivo deste projecto foi o encapsulamento individual de células viáveis no interior de camadas formadas por polielectrólitos usando a técnica “Layer-by-Layer” (LbL). As células estaminais mesenquimatosas humanas foram utilizadas na maioria dos procedimentos, uma vez que apresentam características únicas de auto-renovação e diferenciação. A maioria das experiências usaram, sobretudo, o alginato (ALG) como polielectrólito negativo e o quitosano (CHI) e o poli(hidroclorato de alilamina) (PAH) como polielectrólitos positivos. O ácido hialurónico (HA) também foi testado como polielectrólito negativo.

No início do trabalho, foram estudadas as condições experimentais para a formação de cápsulas com uma única célula no seu interior. Usando a Microscopia de Fluorescência, através da marcação com sondas fluorescentes dos polielectrólitos e dos núcleos celulares, foi possível provar que se obtiveram cápsulas com uma única célula no seu interior. Um dos problemas observados durante o processo foi a agregação das cápsulas. Apesar dos esforços desenvolvidos para desenhar um procedimento experimental que evitasse esta situação (por exemplo, variando a concentração celular e os diferentes modos de ressuspender e agitar as células), não foi possível ultrapassar completamente este problema.

Na segunda parte do trabalho, as células encapsuladas foram submetidas a testes de viabilidade, nomeadamente ao teste de redução da resazurina e ao teste “Live/Dead”. Nestas experiências, foram usadas cápsulas constituídas por CHI/ALG, PAH/ALG e PAH/HA, utilizando-se sempre uma concentração de 1mg/mL, com base em valores da literatura. Em geral, a viabilidade das células encapsuladas foi bastante baixa/nula.

Seguidamente, em consequência da baixa viabilidade obtida nos ensaios anteriores, o método foi aplicado em células aderentes a uma placa de cultura. As células foram cultivadas, como numa “sandwich”, entre a superfície da placa de cultura e as camadas de polielectrólitos. Neste procedimento, foi também utilizada uma concentração de 1mg/mL para as soluções poliméricas. Surpreendentemente, também neste caso as células não se mostraram viáveis.

Foi, então, feito um estudo sistemático (estudo “dose-efeito”) para avaliar o efeito da concentração dos polielectrólitos quando presentes individualmente (foram feitos estudos com o ALG, o CHI e o PAH). Estas experiências foram realizadas com as células aderentes à placa de cultura. Os resultados mostraram que estava a ser usada uma concentração muito elevada (tóxica) de polielectrólitos para encapsulamento das células. A partir destes estudos, também foi possível concluir que o PAH é mais tóxico que o CHI e que o ALG é o polielectrólito menos tóxico.

Finalmente, usando soluções de alginato e quitosano com concentrações adequadas (concentrações baixas: 50ng/mL e 1µg/mL), foi novamente efectuado o encapsulamento de células individuais. Verificou-se que, tal como anteriormente, as células encapsuladas não se mantiveram vivas.

Concluindo, a viabilidade das células encapsuladas não depende apenas das características citotóxicas dos polielectrólitos (ou da combinação das suas características citotóxicas), mas parece que, uma vez retiradas da superfície da placa de cultura, as células ficam demasiado frágeis e perdem a sua viabilidade muito facilmente.

**Palavras-chave:** Técnica camada-sobre-camada; encapsulamento de células únicas; quitosano, alginato, poli(hidrocloreto de alilamina), células estaminais mesenquimatosas.

# CONTENTS

<b>ACKNOWLEDGEMENTS</b> .....	<b>i</b>
<b>ABSTRACT</b> .....	<b>iii</b>
<b>RESUMO</b> .....	<b>v</b>
<b>LIST OF ACRONYMS</b> .....	<b>x</b>
<b>LIST OF FIGURES</b> .....	<b>xi</b>
<b>LIST OF TABLES</b> .....	<b>xv</b>
<b>CHAPTER 1 – Introduction</b> .....	<b>1</b>
1.1    The Layer-by-layer technique .....	1
1.2    Cell Encapsulation .....	3
1.3    Encapsulation of single cells via the Layer-by-layer technique .....	5
1.4    Polyelectrolytes used in the LbL technique (special emphasis for those used in the Thesis) .....	7
1.4.1    ALGINATE AND CHITOSAN .....	8
1.4.2    HYALURONIC ACID .....	11
1.4.3    COLLAGEN .....	12
1.4.4    POLY(ALLYLAMINE HYDROCHLORIDE) .....	14
1.5    Mesenchymal Stem Cells (MSCs) .....	14
1.6    Objectives & General Strategy of the Thesis .....	16
<b>CHAPTER 2 – Materials and Methods</b> .....	<b>20</b>
2.1    Cells and cell culture .....	20
2.2    Equipments, materials and reagents .....	20
2.3    Preparation of solutions .....	21
2.4    Alginate and chitosan fluorescent labelling .....	22
2.5    Cell encapsulation .....	23
2.6    Observation of the encapsulated cells in the inverted fluorescence microscope 24	
2.7    Evaluation of the viability of the encapsulated cells .....	26

2.7.1	Live/Dead Assay.....	26
2.7.2	MTT reduction assay.....	28
2.7.3	Resazurin reduction assay.....	28
2.8	Cells cultured over the 2D-surface of the cell culture dish but under polyelectrolyte layers – evaluation of cell viability.....	30
2.9	Evaluation of the polymers cytotoxicity.....	31
<b>CHAPTER 3 – Results and Discussion.....</b>		<b>32</b>
3.1	Establishment of the best experimental conditions to obtain the encapsulation of single cells.....	32
3.2	Evaluation of the viability of cells encapsulated within CHI/ALG bilayers... 36	
3.3	Evaluation of the viability of cells encapsulated within PAH/ALG bilayers . 38	
3.4	Evaluation of the viability of cells encapsulated within PAH/HA bilayers.... 41	
3.5	Evaluation of the viability of cells cultured over the 2D-surface of the cell culture dish but under polyelectrolyte layers.....	43
3.6	Evaluation of polyelectrolyte cytotoxicity .....	49
3.7	Cell encapsulation using polyelectrolyte solutions of lower concentration . 56	
<b>Chapter 4 – General Conclusions .....</b>		<b>58</b>
<b>REFERENCES .....</b>		<b>59</b>
	Credits List.....	70
<b>APPENDICES .....</b>		<b>71</b>
<b>Animal cell culture (technical aspects) .....</b>		<b>71</b>
1.1	Introductory aspects .....	71
1.2	Cell Culture.....	73
1.2.1	Primary Culture and Subculture, Primary Explant.....	77
1.2.2	Cell Lines.....	80
1.2.3	Cell Growth Curve .....	81
1.2.4	Cell Medium.....	82
1.2.5	Cell Counting .....	83
1.3	Aseptic technique .....	84
1.4	Equipments in cell culture.....	85

1.4.1	Autoclave.....	85
1.4.2	Laminar flow hood .....	86
1.4.3	CO <sub>2</sub> Incubator.....	87
1.5	Cryopreservation .....	87
1.6	Inverted Fluorescence Microscope.....	88
<b>References .....</b>		<b>90</b>
	Credits List .....	91

# LIST OF ACRONYMS

**ALG** – Alginate

**ALS** - Amyotrophic Lateral Sclerosis

**CHI** – Chitosan

**DAPI** – 4',6-diamidino-2-phenylindole

**DASPMI** - Dimethylaminostyrylmethylpyridiniumiodine

**DTAF** – Fluorescein dichlorotriazine

**D-pepep** - Dimethylpepep

**ESCs** - Embryonic stem cells

**FDA** – Fluorescein diacetate

**HA** – Hyaluronic acid

**HBSS** - Hank's Balanced Salt Solution

**hMSCs** – human Mesenchymal Stem Cells

**LbL** – Layer-by-layer

**MMW** – Medium molecular weight

**MW** – Molecular weight

**MTT** – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

**PAH** – Poly (allylamine)-hydrochloride

**PBS** – Phosphate Buffered Saline

**PI** – Propidium iodide

**PLL** – Poly-L-Lysine

**PSS** – Poly(sodium 4-styrenesulfonate)

**RITC** – Rhodamine-B-isothiocyanate

## LIST OF FIGURES

Figure 1 - Schematic of polyelectrolyte capsule fabrication by layer-by-layer (LbL) assembly [4].	3
Figure 2 - Schematic representation of conjugating nanoparticles to bone marrow-derived MSCs via high molecular weight polyelectrolyte chains: poly-L-lysine, chitosan and hyaluronan [68].	6
Figure 3 - Schematic illustration of the encapsulation process [13].	7
Figure 4 - Alginate and Chitosan structures [8].	9
Figure 5 - Alginate composition: A - Mannuronic acid residues chain; B - Guluronic acid residues chain; C - both residues (randomly) [80].	10
Figure 6 - Scheme showing the deacetylation of chitin to produce chitosan. The deacetylated units are marked with a purple circle; the acetyl group is marked with a blue circle and the acetylated units with a green one.	11
Figure 7 - Hyaluronic acid structure.	12
Figure 8 - Collagen structure.	13
Figure 9 - Poly(allylamine hydrochloride) structure (Mw~56,000Da).	14
Figure 10 - The origin, isolation, and specialization of stem cells.	15
Figure 11 - Culture-expanded human mesenchymal stem cells exhibit a spindle-shaped fibroblastic morphology following culture expansion <i>ex vivo</i> . Under appropriate inducing conditions, the culture will demonstrate adipogenic differentiation, chondrogenic differentiation or osteogenesis [99].	16
Figure 12 - General strategy followed in the work.	19
Figure 13 - DTAF structure [107].	24
Figure 14 - RITC structure.	25
Figure 15 - Left: DAPI structure; Right: DAPI bounded to DNA.	25
Figure 16 - Fluorescence excitation and emission profiles of DAPI bound to dsDNA [110].	26
Figure 17 - FDA (on the left)) and PI (on the right) structures.	27
Figure 18 - Confocal images of rat lung slice co-stained with the LIVE/DEAD® viability/cytotoxicity assay kit for animal cells [113].	27
Figure 19 - MTT is reduced to formazan (s) by a mitochondrial reductase.	28
Figure 20 - Conversion of resazurin to resorufin by metabolically active cells results in the generation of a fluorescent product [114].	29
Figure 21 - The CellTiter-Blue® Cell Viability Assay protocol [114].	30
Figure 22 - General procedure used in the encapsulation of cells.	33

Figure 23 - Images obtained in the Fluorescence Microscope. Rat MSCs were encapsulated within 1 bilayer of CHI/ALG-DTAF; A: Green fluorescence from ALG-DTAF; B: Blue fluorescence from DAPI; C: merged images of both A, B. The nuclei (in blue) are inside the capsules (green).....34

Figure 24 - Images of NIH 3T3 cells were encapsulated within 1 bilayer of CHI/ALG-DTAF. The images were obtained in the Fluorescence Microscope for an initial number of cells of A:  $1.5 \times 10^5$ , B:  $3.0 \times 10^5$  and C:  $7.5 \times 10^5$ . Green fluorescence from ALG-DTAF is observed. The step of washing between polyelectrolytes adsorption was done with NaCl 0.15M and the time of contact between the cells and the polyelectrolyte solutions was 10 min. ....35

Figure 25 – Evaluation of the metabolic activity of NIH 3T3 cells encapsulated within 1 and 2 bilayers of CHI/ALG using the resazurin reduction assay after 0h (A) and 3 days (B) of cell culture.....37

Figure 26 – Evaluation of the viability of NIH 3T3 cells encapsulated within 1 (A) and 2 bilayers (B) of CHI/ALG using the Live/Dead assay at the beginning of cell culture (0h). Dead cells are red under the fluorescence microscope.....37

Figure 27 - Images obtained in the Fluorescence Microscope. hMSCs were encapsulated within 1 bilayer of PAH/ALG-DTAF without (A) and with an inner collagen layer (B). The step of washing between polyelectrolytes adsorption was done with NaCl 0.15M and the time of contact between the cells and the polyelectrolyte solutions was 10 min. Green fluorescence is due to ALG-DTAF and blue fluorescence to DAPI (the merged images are shown). The nuclei (in blue) are inside the capsules (green). ....39

Figure 28 - Evaluation of the viability of hMSCs cells encapsulated within 1 bilayer of PAH/ALG (A) and 1 bilayer of collagen/PAH/ALG (B) using the Live/Dead assay at several cell culture times (0h, 1 day and 3 days). Dead cells are red and live cells are green under the fluorescence microscope. ....40

Figure 29 - Evaluation of the metabolic activity of hMSCs encapsulated within 1 and 2 bilayers of PAH/HA using the resazurin reduction assay after 0h and 1 day in culture. Experiments were done with a percentage of 20% (A) and 40% (B) of FBS in the cell culture medium. ....42

Figure 30 - Evaluation of the viability of hMSCs encapsulated within 1 (A) and 2 bilayers (B) of PAH/HA using the Live/Dead assay after 0h, 1 day and 3 days in culture. Experiments were done with a percentage of 20% of FBS in the cell culture medium.....43

Figure 31 - Experimental procedure used in the assays (A); Scheme showing cells adherent to the plastic surface of the cell culture dish and having layers of polyelectrolytes over them (B); .....45

Figure 32 - Evaluation of the metabolic activity of hMSCs cultured under 1, 2 and 4 bilayers of PAH/ALG using the resazurin reduction assay after 0h (A) and 1 day (B) in culture. ....46

Figure 33 - Evaluation of the viability of hMSCs cultured under 1 (A) and 2 (B) bilayers of PAH/ALG using the Live/Dead assay after 0h, 1 day, 3 days and 7 days in culture. Dead cells are red and live cells are green under the fluorescence microscope. ....	47
Figure 34 - Evaluation of the viability of hMSCs cultured under 4 (C) bilayers of PAH/ALG using the Live/Dead assay after 0h, 1 day, 3 days and 7 days in culture. Dead cells are red and live cells are green under the fluorescence microscope. ....	48
Figure 35 - Bright field images of cells cultured under 2 (A) and 4 (B) bilayers of PAH/ALG. The skinny arrows exemplify the nuclei of the cells and thick ones the extracellular membrane. ....	48
Figure 36 - Effect of polyelectrolyte concentration on hMSCs viability (“dose-effect” studies) obtained through the resazurin reduction assay. Cell viability was analysed along 7 days. The value of RFU for control is highlighted with a pink circle. The same control was used for the three polymers study, thus there is an overlap of values. ....	52
Figure 37 - Effect of PAH concentration on hMSCs viability obtained through the Live/Dead assay. Cell viability was analysed along 7 days. Red cells are dead; green cells are alive. ....	53
Figure 38 - Effect of ALG concentration on hMSCs viability obtained through the Live/Dead assay. Cell viability was analysed along 7 days. Red cells are dead; green cells are alive. ....	54
Figure 39 - Effect of CHI concentration on hMSCs viability obtained through the Live/Dead assay. Cell viability was analysed along 7 days. Red cells are dead; green cells are alive. ....	55
Figure 40 - Evaluation of the viability of hMSCs encapsulated within 1 bilayer of CHI/ALG using the Live/Dead assay after 0h, 1 day and 3 days in culture. Experiments were done with solutions with concentrations of CHI and ALG of 50ng/mL and 1µg/mL. Red cells are dead. ....	57
Figure 41 - Cell culture. The cells spread over the Petri dish surface. ....	73
Figure 42 - Substrate-cell and cell-cell interactions. ....	74
Figure 43 - Cell Cycle phases. ....	74
Figure 44 - Mitosis phases. ....	75
Figure 45 - Mitosis of a fibroblast (skin cell). ....	75
Figure 46 - Scheme about Culture of animal cells. ....	78
Figure 47 - Schematic procedure of an explants (human bronchial epithelial cells grown from explants). ....	79
Figure 48 - How can a cell line be derived? ....	80
Figure 49 - Growth curve of bacteria (for instance). ....	81
Figure 50 - Alpha-MEM; Serum; Antibiotics: Penicilin and Sptreptomicin, respectively. ....	83

Figure 51 - Neubauer chamber.....	84
Figure 52 - Scheme where it is possible to see the counting area. The blue cells are non-viable (when stained with <i>trypan blue</i> , and visualized in a Microscope), the other ones are viable [6]. .....	84
Figure 53 - Autoclave Equipment. ....	86
Figure 54 - Laminar Flow Chamber. ....	87
Figure 55 - Incubator representation. ....	87
Figure 56 - N <sub>2</sub> (l) bottle. ....	88
Figure 57 - Left: Fluorescence Microscopy; Right: Fluorescence Microscopy mechanism [10].....	89

## LIST OF TABLES

Table 1 - Characteristics of some polyelectrolytes used for cell encapsulation [64].	8
Table 2 - Chain composition and body distribution of collagen types [88].	13
Table 3 - Effect of the type of stirring used in the cell re-suspension steps on cell aggregation (+++, ++ and + represents high, moderate and low levels of cell aggregation, respectively).	36
Table 4 - Cell viability results using the resazurin reduction assay. hMSCs were encapsulated within 1 bilayer of PAH/ALG polyelectrolytes and resazurin was added only at 0h. Resorufin fluorescence was measured after 3h, 6h and 24h of cell culture. Results are expressed as a percentage of the control.	41
Table 5 - Evaluation of the metabolic activity of hMSCs cultured under 1 bilayer of PAH/ALG using the resazurin reduction assay after 0h, 1 day, 3 days and 7 days in culture. PAH concentrations in solution of 0.1mg/mL and 0.5mg/mL were studied.	49
Table 6 - Effect of polyelectrolyte concentration on hMSCs viability (“dose-effect” studies) obtained through the resazurin reduction assay. Results are shown as a percentage of the control values. Cell viability was analysed along 7 days.	51
Table 7 - Cell viability results after 0h, 1 day and 3 days in culture using the resazurin reduction assay. hMSCs were encapsulated within 1 bilayer of CHI/ALG polyelectrolytes. Experiments were done with solutions with concentrations of CHI and ALG of 50ng/mL and 1µg/mL. Results are expressed as a percentage of the control.	56

## CHAPTER 1 – INTRODUCTION

### 1.1 *The Layer-by-layer technique*

The classical layer-by-layer technique (LbL) was proposed by G. Decher in 1997 [1] and is based on the alternating adsorption of oppositely charged species (usually polyelectrolyte species) onto a charged template which can be a surface (2-dimensional template) or a particle (3-dimensional template; usually, a nano or microparticle). The process is illustrated in Figure 1 using a 3-dimensional template. Basically, for a positive template, the process begins with the initial electrostatic adsorption of a negatively charged polymer, followed by the adsorption of a positively charged one [2-31]. By repeating this procedure, a multilayer growth is possible, thus creating a film or a shell over the template with precision of thickness and roughness of less than 1 nm [2, 32].

The driving force of multilayer assembly has been extended to non-electrostatic interactions, including hydrogen bonding, covalent bonding, hydrophobic interactions, between others [3, 33-36]. The creation of multilayered polymer structures is a very versatile process and depends on the ability of the charged species to be adsorbed onto the top of an oppositely charged layer [1, 3-5, 37, 38]. Therefore, the templates and the polyelectrolyte pairs are very important components in the layer-by-layer technique [1, 3-5, 37, 38].

When 3-dimensional templates are used, the removal of the internal template after the LbL deposition can give rise to empty cavities thus creating nanocapsules or macrocapsules [2-31] - this is also represented in Figure 1. These hollow and stable capsules contain an interior cavity and polymer walls which can be loaded and functionalized with a variety of substances such as dyes, drugs, nanoparticles and biomolecules [2, 4, 10, 13, 15, 39, 40]. To produce capsules, many templates can be used: biological cells, polystyrene latex particles, inorganic crystals (e.g.  $\text{MnCO}_3$ ,  $\text{CaCO}_3$ ,  $\text{CdCO}_3$ ), gold particles, colloidal particles, between others [4, 25, 29, 30, 31, 41-46]. The template should be stable under the LbL method and it also should be removable from the inside of the capsule, without providing any effect in terms of morphology, stability of the assembled layers on top of it [4]. The method of dissolving the core template depends on its nature and properties. There are works describing the dissolution of the core using ethylene diamine tetraacetic acid (EDTA) or a salt solution ("controlled precipitation" technique); another technique includes the  $\text{pH} < 3$  and a solvent, usually acetone and

ethanol, which makes the shell permeable to larger molecules (“permeability regulation” technique) [27, 47]. Other reagents can be used for core template dissolution, such as: tetrahydrofuran (THF), hydrochloric acid (HCl) and hydrofluoric acid (HF) [4, 27, 30]. The hollow capsules wall properties, like thickness, permeability, stability and biocompatibility can be designed and adjusted, as it is desired [5, 7, 24, 36, 38, 48].

These hollow capsules have attracted interest to be used as encapsulation systems, for example, as drug carrier systems (for instance dexamethasone [49, 50], insulin, vitamin K<sub>3</sub> [51]), reaction vessels and nanoreactors (to prepare organic/inorganic hollow materials) [3, 15, 26, 27, 30, 38, 40, 43, 45, 48, 52], catalysis [3, 5, 26, 30, 36] and biosensing [5].

Other applications of hollow capsules include tools for implants and diagnostics [5]. Sometimes, a serious problem arises: the cytotoxicity of polyelectrolytes or of their metabolic products, after degradation [53, 54].

According to the literature, the polyions’ functional groups influence the cytotoxicity [54]. However, the literature also refers that there is a strong difference in toxicity if the polymer is inside the cell [54] or is only in contact with the membrane [55]. So, the same polyelectrolyte can induce both cell death or provide the adhesion of the cells to surfaces [55]. Furthermore, the investigations of de Rosa *et al.* [55] gave evidence that the surface charge also plays a crucial role in cell survival and adhesion of cells.

Thanks to versatility of this technique, the two compartments of the capsules (shells and cavity) can be easily manipulated in order to create different types of systems, according to the specific requirements for a certain application [56, 57]. Compared to other traditional strategies, the LbL adsorption technique is an easy and inexpensive process for multilayer formation, and allows the incorporation of different types of materials in the film structures. Thus, this method can be regarded as a versatile bottom-up nanofabrication technique [57].

However, this technique has some drawbacks, such as: (i) high permeability to small molecules; (ii) aggregation, specifically of small capsules (< 1 mm in diameter); (iii) the reproducibility of results is low and (iv) long-term stability unknown [58].

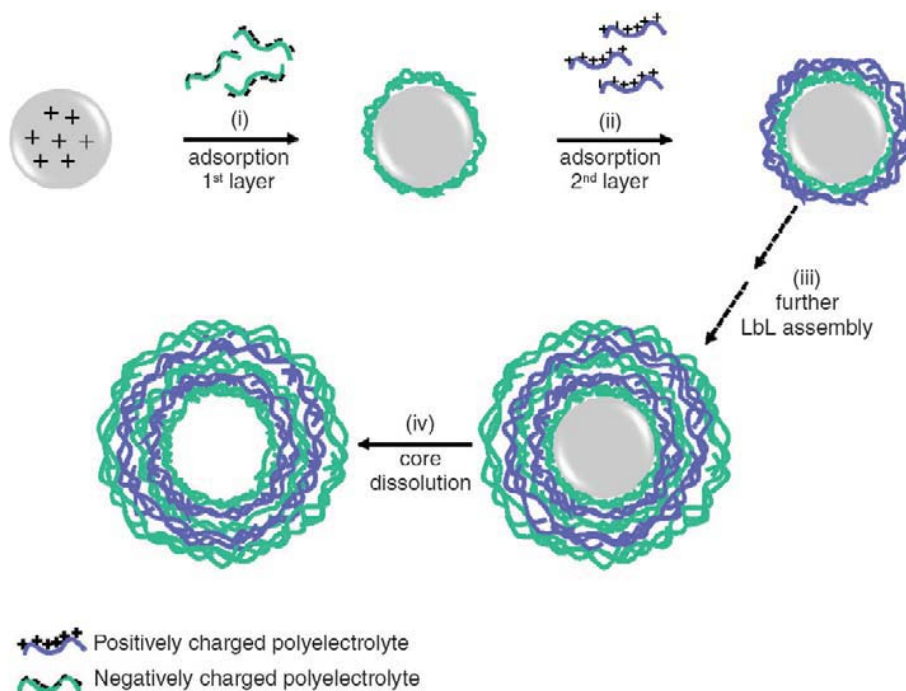


Figure 1 - Schematic of polyelectrolyte capsule fabrication by layer-by-layer (LbL) assembly [4].

## 1.2 Cell Encapsulation

Cell encapsulation has the goal of entrapping viable cells within semi-permeable ‘membranes’, which should be permeable for transport of molecules essential for cell survival [59-61]. This membrane must protect the inner cells from both mechanical stress and host’s immune system, while allowing the diffusion of oxygen, nutrients and waste [59, 62].

Bioencapsulation is a great method to create the appropriate interaction between cells and the microenvironment, leading to desired assemblies [6, 63]. Depending on the choice of material to create the capsule, and whether membranes are prefabricated or fabricated around viable cells, the cells will be submitted to different entrapment conditions [60]. An optimal equilibrium must be maintained among the several capsule properties to hold cell survival in a certain set of experimental conditions. As different types of cells have different metabolic requirements, the permeability of the capsules can depend on the choice of the cells [60]. In this way, the microenvironment of the all system can mimic the extracellular matrix or the culture conditions, where the cells to be encapsulated normally live [62].

The encapsulation of cells in appropriate matrices has huge clinical applications, playing a very important role in cell and transplantation therapies. This approach is used to create bioartificial organs in order to treat many human disorders, like diabetes [59, 60, 64],

Parkinson's disease [59, 64], kidney failure [60, 64], hepatic failure [59] and amyotrophic lateral sclerosis (ALS) [64]. Other applications are related to cartilage replacement [59, 64], construction of replacement heart [64] and urinary valves [64], and artificial organs [62, 64].

The cell encapsulation systems are also denominated as "immunoprotective devices", which means that these systems can protect the cells from immunodestruction caused by host antibodies and T-cells [60, 62, 64]. Encapsulation is not only a method for supporting/immobilizing and protecting cells, but can be also used as a vehicle for drug delivery. Each of these applications demands materials with specific physical, chemical, biomechanical, biological and degradation properties to provide an efficient therapy [59]. These encapsulation devices can constitute physically environments for study and control of biochemical processes [7, 12, 42, 63, 65].

Several methods for cell encapsulation, entrapment and coating within polymers and hydrogels have been investigated in the past. Beyond the LbL method, there are many others: "Gelation" or solidification, "Chemical Crosslinking", "Ionic Crosslinking", "Formation of an insoluble complex", "One-stage Process", between others [64].

There are two different strategies for cell encapsulation: (i) microencapsulation and (ii) macroencapsulation. The first one is defined as the involucre of individual cells or small cell aggregates in a semipermeable membrane while the second corresponds to the utilization of hollow materials to deliver bigger aggregates of cells or multiple cells. The microencapsulation systems are usually small in its size and they are not taken from the patient, the devices are eliminated by the kidneys or by alternative ways. The macroencapsulation, on the other hand, has a superior mechanical integrity, but the obtained structures are not available for transport. Besides, these systems have a big disadvantage, because they can create problems from host proteins aggregating on their surfaces [60, 64].

Many mammalian cells show an anchorage-dependent behaviour; this happens because in order to expose the best viability and the correct metabolic functionality, the cells must adhere to a surface or material. Therefore, the inner layer of the encapsulation material should provide suitable adhesion signals to the encapsulated cells. Moreover, the interaction of encapsulation matrices with the surrounding tissue *in vivo* is important as well, so that the outer layer of the matrix shouldn't induce a host inflammatory response [64, 66]. Summarizing, it is very important to control and provide cell adhesion, even inside or outside the encapsulation matrix [64].

### 1.3 Encapsulation of single cells via the Layer-by-layer technique

The LbL technique can be very useful and attractive for cell encapsulation applications, because it requires mild conditions and can be combined with shells functionalization [6, 67]. In particular, the encapsulation of living single cells inside polyelectrolyte layers can be very challenging has recognized by the few papers that were published in this subject.

Alberto Diaspro and his colleagues [12] used the LbL method to encapsulate single yeasts (*Saccharomyces cerevisiae*, the common baker's yeast). Their goals were to encapsulate living yeasts inside polymeric shells (made by poly(allylamine hydrochloride), PAH, and poly(sodium styrene sulfonate), PSS), using the LbL method. They used the fluorescent lipophilic cationic dye dimethylaminostyrylmethylpyridiniumiodine (DASPMI) to verify the metabolic activity of the yeasts and the Flow Cytometry technique to check the efficiency of cell encapsulation. They concluded that the polyelectrolyte coating didn't influence cell metabolism and, in addition, that yeasts could even suffer division.

T. Svaldo-Lanero and his co-workers [14] also used yeasts (*Saccharomyces cerevisiae*) and the LbL technique for encapsulation. They studied yeasts viability, mechanical properties and duplication capability, when encapsulated in polymeric shells made of PAH and PSS. To evaluate cell viability, they used dimethylpep (D-pep), a red dye which stains nuclear and mitochondrial DNA in living cells. In their study, they concluded that the internal cell structure was preserved and the duplication capability was verified.

The encapsulation of animal cells using the LbL technique was also attempted. Nalinkanth G. Veerabadran and his co-workers [6] reported the encapsulation of rat Mesenchymal Stem Cells (MSCs) by using the LbL method. The polyelectrolyte shells were made of hyaluronic acid and poly(L-lisine). In this study, they demonstrated the ability to individually encapsulate animal cells within polymeric shells, as well as their capacity to survive until 7 days (by the MTT assay and the two-colour fluorescence Live/Dead Assay, assays that were also used in the present Master thesis). Before encapsulation, the PLL/HA capsules were characterized using Atomic Force Microscopy (AFM),  $\zeta$ -potential measurements, Crystal Microbalance (QCM) monitoring and contact-angle measurements.

Although the authors concluded that the LbL technique was a successful approach for single cell encapsulation, they also experienced some difficulties in evaluating cell viability using the MTT method, for which they used a time period greater than what would conventionally be used.

A very recent publication of Boon C. Heng and co-workers [68] described the partial coating of MSCs with polyelectrolytes (Figure 2). It is known that the binding of nanoparticles directly to the cell membrane can influence the cellular function by obstructing cell surface receptors. So, their goals were to investigate the use of polymer bilayers (hyaluronan (HA), poly-L-lysine(PLL) and chitosan (CHI); several concentrations were tested) to bind nanoparticles to MSCs, thus they developed a technique to obtain only half of the cell surface conjugated with nanoparticles via polyelectrolyte chains. They investigated both PLL/HA and CHI/HA pairs (used to create the polyelectrolyte bilayers) and verified that the best results were obtained by the chitosan/hyaluronan pair.

Furthermore, they investigated the re-attachment and proliferation of the trypsin-dissociated nanoparticle-conjugated MSCs. They concluded that they were able to re-attach and proliferate cells over a period of 7 days after the freeze-thawing process.

However, nanoparticles distribution was not uniform among the daughters.

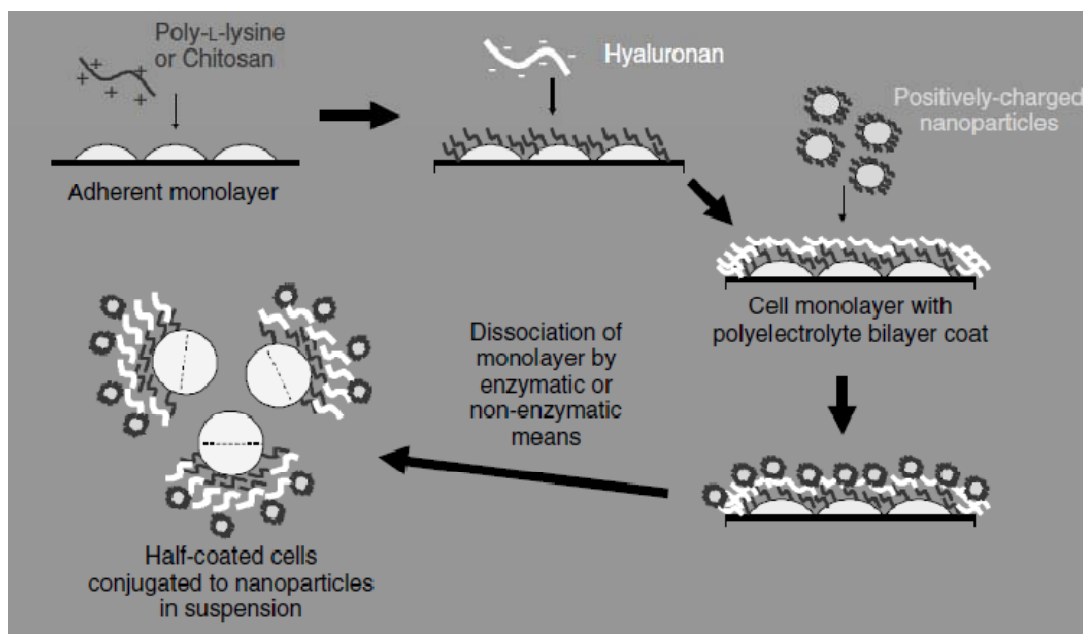


Figure 2 - Schematic representation of conjugating nanoparticles to bone marrow-derived MSCs via high molecular weight polyelectrolyte chains: poly-L-lysine, chitosan and hyaluronan [68].

Another work that deserves to be mentioned is that from Oliver Kreft and his co-workers [13], although their goal was not to encapsulate living cells. In this case, they used erythrocytes as templates for capsules formation. The shells were made of PSS and PAH and, at the end, the human erythrocytes were dissolved, in order to obtain hollow capsules. The aim was to obtain capsules for encapsulation of DNA and HSA ('human serum albumin'; coupled to a fluorescent dye: TRITC) using the LbL method. They reported that

the capsules were impermeable to DNA and HSA at the beginning (a) but, during drying, the permeability was 'switched on' and both compounds accumulated in the capsule interior and precipitated upon total drying (b, c); after re-suspension, the filled capsules were impermeable again (d) (Figure 3).

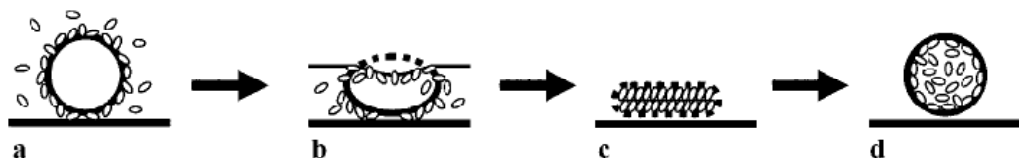


Figure 3 - Schematic illustration of the encapsulation process [13].

#### **1.4 Polyelectrolytes used in the LbL technique (special emphasis for those used in the Thesis)**

A polymer is a large molecule composed by repeating structural units, usually linked by covalent bonds. They can be synthetic for instance, such as polystyrene, PVB, PVC [69], between others, or called biopolymers, which include polysaccharides, polypeptides and polynucleotides [13, 42, 47, 56, 58, 61, 70-72]. Polymers can work as polyelectrolytes - species with charged or chargeable groups, when dissolved in polar solvents [73].

Additionally, these materials can be divided into those where the charge density depends on pH (weak polyelectrolytes) and those for which the charge density is independent of pH (strong polyelectrolytes) (see examples in Table 1) [64].

In the biomedical area, many polymers have been investigated, like PAH, PSS, HA, PLL, CHI, alginate (ALG), between others (see examples in Table 1) [5, 9, 31, 58, 64, 74].

The capsules constituted by PAH and PSS are the best characterized polyelectrolyte multilayered systems in the literature [10]. The poly(sodium styrene sulfonate) (PSS) is a strong polyanion and poly(allylamine hydrochloride) (PAH) is a relatively weak polycation, sensitive to pH changes, a fact that can have an influence in the inter-polyelectrolyte interactions [11-14]. This pair of polyelectrolytes has been extensively used for the fabrication of multilayers on flat and colloidal surfaces [74]. The success of these materials in *in vivo* applications requires the maintenance of not only the cell viability, but also of the desired cellular metabolism [64]. For this purpose, it is possible to employ natural, polyelectrolyte thin-films coatings, which can modulate the cell permeability and influence cellular function, without compromising the cell viability [6].

The capsules can also, beyond polysaccharides, polypeptides and polynucleotides, be made by lipids, dyes and inorganic nanoparticles, proteins [11, 13, 25, 28, 29, 33, 39, 40, 42, 43, 45-48, 58, 74, 75]. The capsule wall composition has a relevant role for the creation of functional capsules, because their permeability depends on the chemical structure and the molecular weight of the polyelectrolyte layers [4].

Table 1 - Characteristics of some polyelectrolytes used for cell encapsulation [64].

System	Notes
Alginate	Strong polyanion
Carrageenan	Strong polyanion
Poly(styrene sulfonate)	Strong polyanion
Carboxymethylcellulose	Polyanion
Cellulose sulphate	Polyanion
Heparin	Polyanion
Poly(methylene-co-guanidine)	Polyanion
Poly(diallyldimethyl ammonium chloride)	Strong polyanion
Chitosan	Weak polycation, pKa of primary amine 6,3 – 6,8
Poly(L-lysine)	Weak polycation, pKa of primary amine ~10,5
Poly(allylamine) Hydrochloride	Weak polycation, pKa of amine group ~8,5
Poly(vinylamine) Hydrochloride	Weak polycation

#### 1.4.1 ALGINATE AND CHITOSAN

Since 1998, polyelectrolyte microcapsules have attracted a great interest, because their properties, including size, composition, shape, thickness, permeability, stability and stiffness can be tailored easily [7, 33, 37, 47, 56]. Polysaccharides are particularly used due to their hydrophilic and protective properties and to their biodegradable and compatible capacities as well [30, 61, 76].

Natural polysaccharides such as alginate and chitosan (Figure 4) have been investigated for applications in several functions: drug delivery, coating membranes and for biomaterials research [8, 30, 41]. The use of alginate in the encapsulation of animal cells has been very successful, due to the absence of toxic components in this polymer [77, 78].

The negatively charged carboxylic acid groups of manuronic and guluronic acid units present in alginate molecule can interact electrostatically with the positively charged amino groups of chitosan, producing a polyelectrolyte complex. Alginate is one of the most studied anionic polyelectrolytes in assemble with chitosan, because the formed complex is still biodegradable and biocompatible [76, 79].

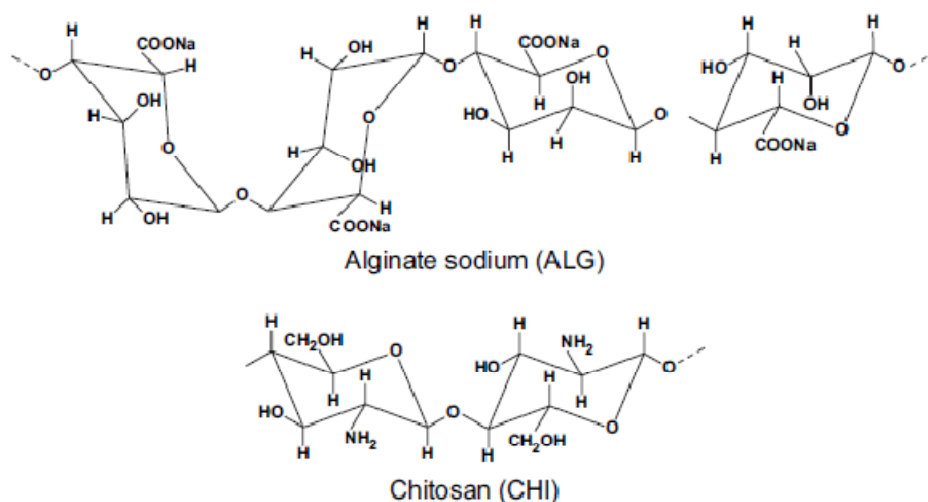


Figure 4 - Alginate and Chitosan structures [8].

Alginate is an anionic polymer obtained from marine brown algae [8, 61, 79]. It is a linear binary copolymer composed by two kinds of residues: (1→4)-linked  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G) (Figure 5) [30, 45, 61, 76, 77, 79].

The distribution of these monomers along the alginate chain is very important, because it will influence its properties [66, 77]. The properties of each monomer of alginates have previously been reported, and the  $pK_a$ -values for mannuronic acid and for guluronic acid are 3.38 and 3.65, respectively [76].

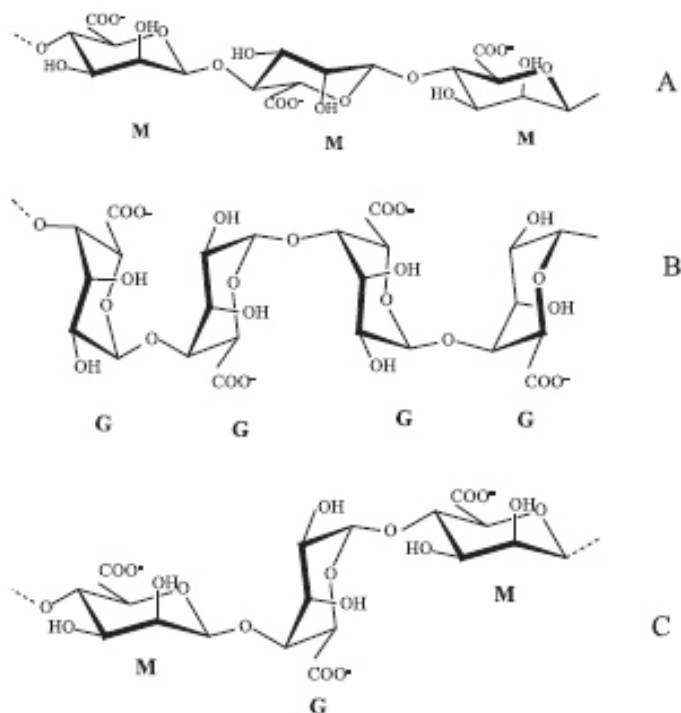


Figure 5 - Alginate composition: A - Mannuronic acid residues chain; B - Guluronic acid residues chain; C - both residues (randomly) [80].

Chitosan (Figure 6) is a natural cationic polymer obtained from the deacetylation of chitin (a copolymer of  $\beta(1\rightarrow4)$  linked N-acetyl-D-glucosamine [81]) [8, 30, 65, 79, 82, 83], which is a product found in crustacean shells [30, 65, 68, 76, 81, 83]. In this reaction (made by chitin-deacetylase enzyme), some units of chitin lost their acetyl group, creating deacetylated units. So, it is a linear binary copolymer composed by  $\beta(1\rightarrow4)$ -D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Both residues are randomly distributed along the chitosan chain [65, 76, 79, 84].

Relatively to its solubility, chitosan is a weak base and is insoluble in water and organic solvents, but it is soluble in dilute aqueous acidic solutions ( $\text{pH} < 6.5$ ), which can make the glucosamine units soluble:  $\text{R-NH}^{3+}$ . Chitosan gets precipitated in alkaline solution or with polyanions and forms a gel at lower pH [83].

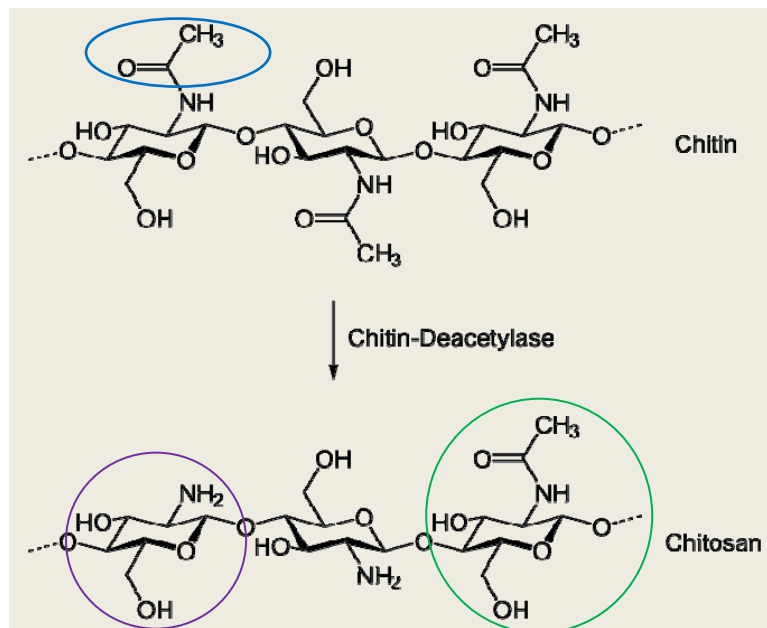


Figure 6 - Scheme showing the deacetylation of chitin to produce chitosan. The deacetylated units are marked with a purple circle; the acetyl group is marked with a blue circle and the acetylated units with a green one<sup>1</sup>.

Chitosan shows great biological properties such as: biocompatibility, biodegradation in the human body, and immunological, antibacterial and wound-healing activity. In recent studies, chitosan has shown capacities to be used as a support material for many applications: gene delivery, cell culture, and tissue engineering. Therefore, chitosan is receiving a huge attention as a new functional material [65, 79, 81, 82, 85, 86].

#### 1.4.2 HYALURONIC ACID

Hyaluronic acid (HA) is also a polysaccharide. It is a glycosaminoglycan (GAG), because one of the sugars used in its structure is modified with an amino group (-NH<sub>2</sub>). It is a polymer of disaccharides, themselves composed by D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating  $\beta$ -(1,4) and  $\beta$ -(1,3) glycosidic bonds (Figure 7) [6, 15, 87]. HA is present in almost all biological tissues and body fluids. HA presents important physiological functions in living organisms, which make it an attractive biomaterial for many medical purposes [87]. It presents a good biocompatibility and biodegradability, therefore, it can be used for several drug delivery applications [15].

<sup>1</sup> The references of all figures are presented in the **Credits List** (at the end of this thesis), except for those which were taken from articles, identified with numbers.

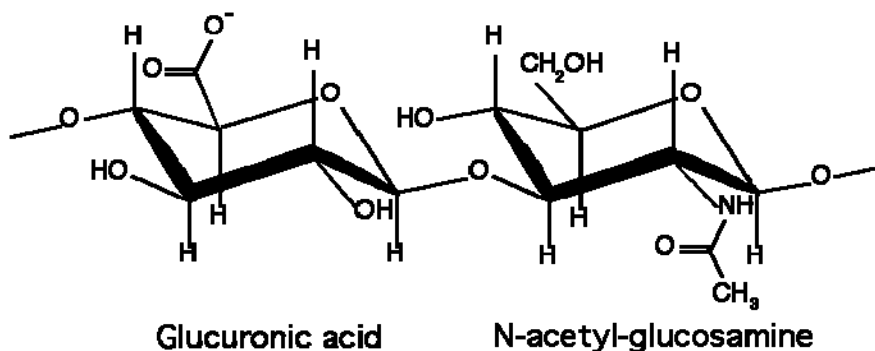


Figure 7 - Hyaluronic acid structure.

In clinical medicine, HA is used as a diagnostic marker for many diseases, such as rheumatoid arthritis, cancer and liver disorders. It is also used in ophthalmological and ontological surgeries and cosmetic regeneration and reconstruction of tissue. This polymer can immobilize water in tissue and change dermal volume and compressibility. It can also influence cell proliferation, differentiation, and tissue repair, between other biological functions [87].

### 1.4.3 COLLAGEN

Collagen represents the most common structural protein in the vertebrate body (approximately 30%). There are at least 13 types of collagen, which have been isolated in respect to the length of the helix and the nature and size of the non-helical areas (Table 2) [88].

Type I collagen is the most found in animals, namely in the skin, tendon, and bone.

Structurally, it is a compound of three chains, two of which are identical:  $\alpha 1(I)$ , and one  $\alpha 2(I)$  chain with different amino acid composition. Basically, the collagen molecule contains three polypeptide  $\alpha$ -chains, each with more than 1000 amino acids (Figure 8) [88].

Collagen can be processed into tubes, sheets, powders, sponges, fleeces, injectable solutions and dispersions, all of which have found use in medical purposes. Moreover, these systems have been used for drug delivery for several applications, including ophthalmology, wound and burn dressing, tissue engineering and tumour treatment [88].

Table 2 - Chain composition and body distribution of collagen types [88].

Collagen Type	Chain composition	Tissue distribution
I	$(\alpha 1(I))_2\alpha 2(I)$ , trimer $(\alpha 1(I))_3$	Skin tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dermis
II	$(\alpha 1(II))_3$	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	$(\alpha 1(III))_3$	Large vessels, uterine wall, dermis, intestine, heart valve, gingival (usually coexists with type I except in bone, tendon, cornea)
IV	$(\alpha 1(IV))_2\alpha 2(IV)$	Basement membranes
V	$\alpha 1(V)\alpha 2(V)(3(V))$ or $(\alpha 1(V))_2\alpha 2(V)$ or $(\alpha 1(V))_3$	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva
VI	$\alpha 1(VI)\alpha 2(VI)\alpha 3(VI)$	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	$(\alpha 1(VII))_3$	Skin, placenta, lung, cartilage, cornea
VIII	$\alpha 1(VIII)\alpha 2(VIII)$ chain organization of helix unknown	Produced by endothelial cells, Descemet's membrane
IX	$\alpha 1(IX)\alpha 2(IX)\alpha 3(IX)$	Cartilage
X	$(\alpha 1(X))_3$	Hypertrophic and mineralizing cartilage
XI	$1\alpha 2\alpha 3\alpha_1$ or $\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$	Cartilage, intervertebral disc, vitreous humour
XII	$(\alpha 1(XII))_3$	Chicken embryo tendon, bovine periodontal ligament
XIII	Unknown	Cetal skin, bone, intestinal mucosa

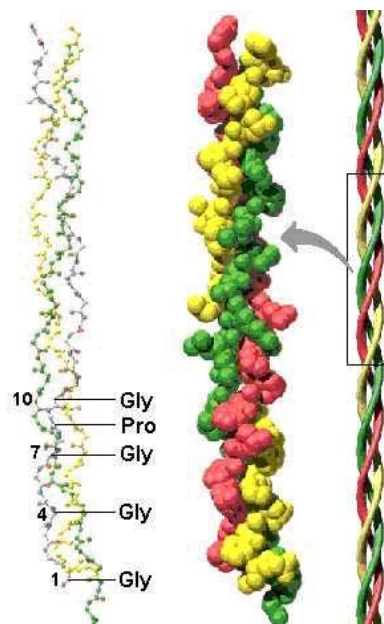


Figure 8 - Collagen structure.

#### 1.4.4 POLY(ALLYLAMINE HYDROCHLORIDE)

The PAH (poly(allylamine hydrochloride)) is another polyelectrolyte which can be used in the encapsulation field [8-10, 58, 64]. The PAH, also denominated as PAA or PAAH is a cationic polyelectrolyte prepared by the polymerization of allylamine (Figure 9) [10].

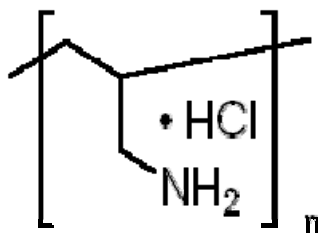


Figure 9 - Poly(allylamine hydrochloride) structure (Mw~56,000Da).

The PAH is one of the most used polymer for the fabrication of capsules [10-14, 70]. In particular, PAH has been used as a model cationic polyelectrolyte for DNA and proteins for preparation of hollow microcapsules using the Layer-by-layer method [89].

It is a weak polycation, with a pKa of amine group ~ 8.5 [64, 90, 91]. Being a weak polycation, the change of pH or ionic strength will alter the PAH conformation [64, 92, 93].

Not only the conformation, but also the thickness (of the layers or capsules made by this polymer) is sensitive to solution pH, due to the fact that the charge density of a weak polyelectrolyte can be varied by the changing of pH. The PAH is positively charged at low and neutral pH, but at high pH, it leads to the dissociation of H<sup>+</sup> ions and forms a structure with uncharged amine groups [94].

### 1.5 Mesenchymal Stem Cells (MSCs)

Nowadays, “Stem cells” is a very important topic in Biology. It is thought that their unique properties can provide a huge number of answers to some questions in biology, as well as possible treatments for several degenerative diseases and applications in tissue engineering [67, 95, 96]. Stem cells are unique, because they have the capacity to self-renewal and to differentiate into a several types of cells (including chondrocytes, osteoblasts, adipocytes, nervous cells, blood cells) (Figure 10) [6, 67, 95-97].

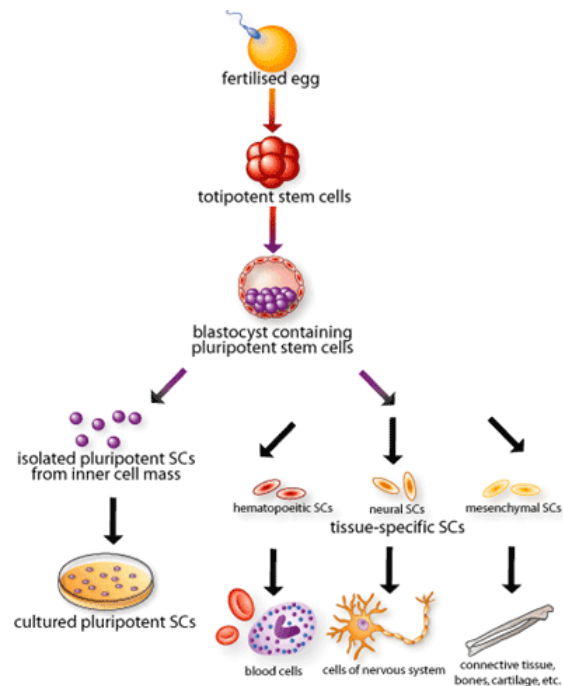


Figure 10 - The origin, isolation, and specialization of stem cells.

These cells are usually classified in embryonic stem cells (ESCs) and adult stem cells. The first ones are pluripotent due to their capability to origin all kinds of cells. The ESCs can be obtained from the early mammalian embryo at the blastocyst stage and using specific culture conditions, they have the capacity to expand unlimited *in vitro* and differentiate. On the other hand, adult stem cells are multipotent and are derived from many tissues such as bone, brain, adipose tissue, umbilical cord blood, blood vessels, blood, between others [95, 97].

The “mesenchymal stem cells” (a term used by Arnold Caplan for the first time in 1991) often receive other designations such as “marrow stromal cells”, “precursors of non-hematopoietic tissue”, “colony forming unit fibroblasts” or “multipotent adult progenitor cells” [98]. MSCs are multipotent adult stem cells, nonhematopoietic which can be derived from mesoderm and neuroectoderm. This cell type can be found in most postnatal organs and tissues, namely in the bone marrow (BM). They are able to differentiate not only into cells of mesodermal origin such adipocytes, chondrocytes, osteocytes (Figure 11), tenocytes, skeletal and myocytes, but also into representative lineages of the three embryonic layers, such as neurons (from ectoderm) and hepatocytes (from endoderm) [97, 99-101].

The adult mesenchymal stem cells (MSCs), besides being multipotent, are easily isolated and cultured *in vitro* and, a great advantage, is that the use of this kind of cells do not raise ethical issues related with their origin. These cells have been considered an important tool for several clinical applications, due to some of their characteristics: they

present an optimal expansion potential and genetic stability; there are well established protocols for their isolation and new sources keep on showing up, beyond the already existing ones. In addition, MSCs are able to migrate to areas of tissue damage in immune privileged conditions, presenting immunosuppressive properties. All these advantages have provided many successful MSCs transplantations [97, 99, 100]. Other applications are bone, cartilage, tendon and skeletal muscle repair [101].

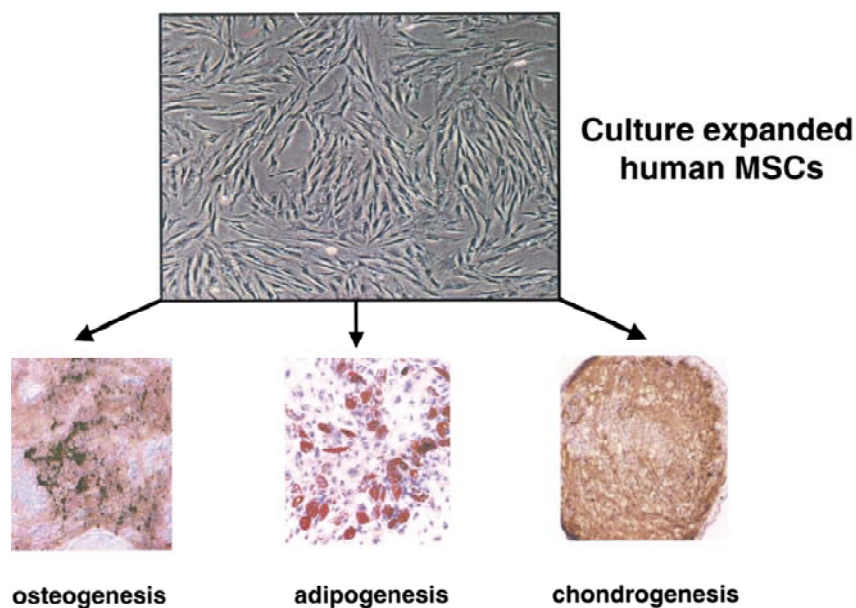


Figure 11 - Culture-expanded human mesenchymal stem cells exhibit a spindle-shaped fibroblastic morphology following culture expansion *ex vivo*. Under appropriate inducing conditions, the culture will demonstrate adipogenic differentiation, chondrogenic differentiation or osteogenesis [99].

## 1.6 Objectives & General Strategy of the Thesis

The principal aim of this Thesis was to encapsulate single viable cells within polyelectrolyte films using the Layer-by-Layer (LbL) technique. Due to their characteristics and potential to be applied in the biomedical field, human mesenchymal stem cells (MSCs) were used in the great majority of the experiments. The first experiments used alginate (ALG) as the anionic polyelectrolyte and chitosan (CHI) as the cationic polyelectrolyte as they are natural polymers known to be biocompatible. As a consequence of the lack of viability of the cells encapsulated within CHI/ALG capsules, other polyelectrolytes were also tested for the same purpose: poly(allylamine hydrochloride) (PAH) and hyaluronic acid (HA).

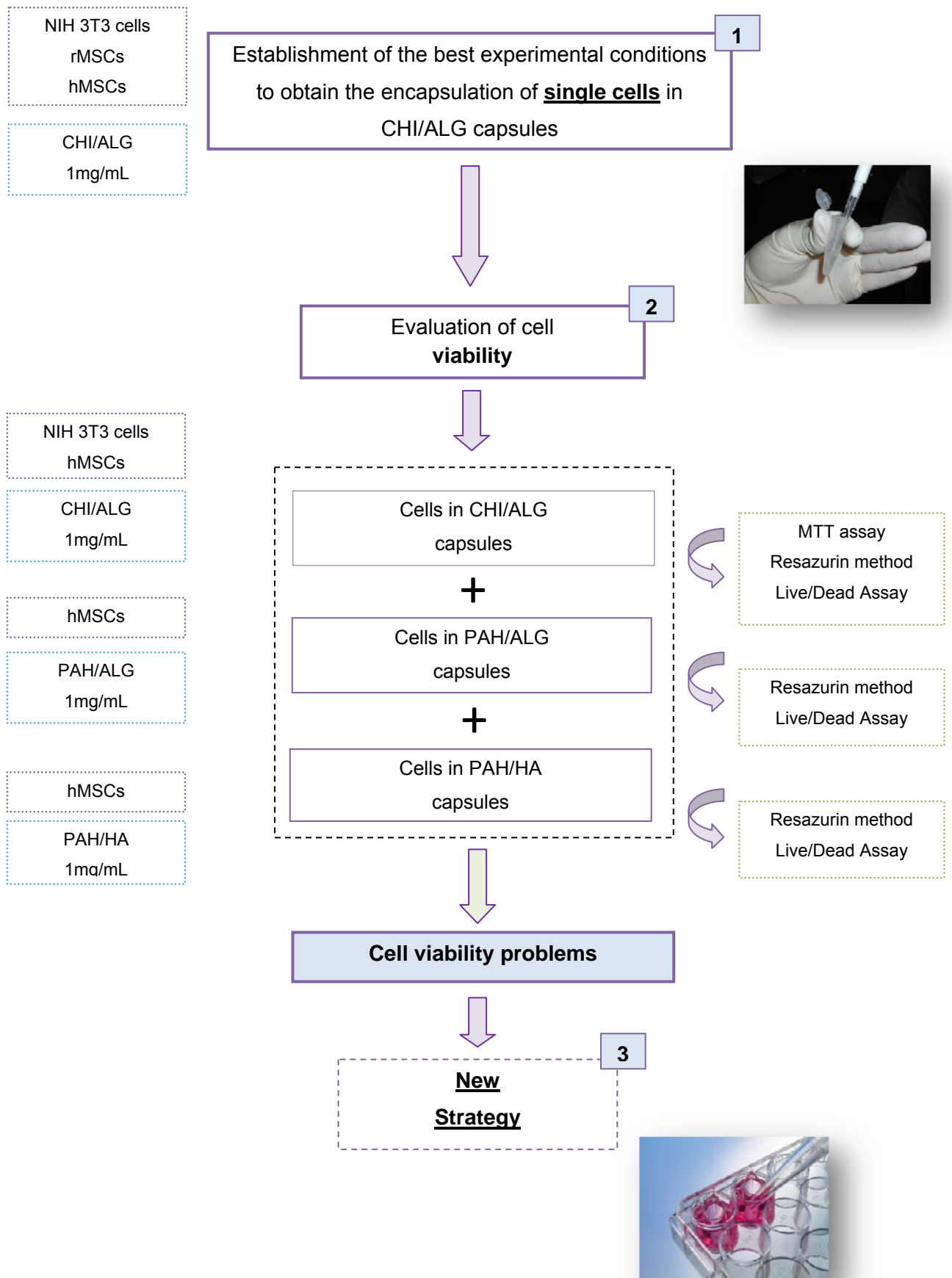
Figure 12 shows the general strategy followed in the present Thesis. First, the experimental conditions necessary to obtain the encapsulation of individual cells were studied and established.

Several type of cells were used in these studies (NIH 3T3, rMSCs and hMSCs) and polyelectrolyte solutions of 1mg/mL (this concentration was chosen based on literature) were used in the encapsulation process. Single cell encapsulation was assessed by fluorescence microscopy.

Second, the viability of cells encapsulated within CHI/ALG layers was evaluated (assessed both by the resazurin reduction assay and the Live/Dead assay) and, as the results were not satisfactory, cells were also encapsulated the pairs of polyelectrolytes PAH/ALG and PAH/HA. NIH 3T3 and hMSCs were used in these experiments, together with polyelectrolyte solutions of 1mg/mL.

Once again, cell viability was very low/absent. The LbL technique was then applied in cells growing adherent to the surface of cell culture plates. The cells (hMSCs) were cultured like in a sandwich, between the surface of the cell culture dish and the polyelectrolyte layers made of PAH/ALG. Also here, the polyelectrolyte solutions were used at a concentration of 1mg/mL during the LbL process. Surprisingly, cell viability was also absent in these systems. This set of results pointed out the need for a systematic study (dose-effect study) concerned with the evaluation of the effect of the concentration of the individual polyelectrolytes (ALG, CHI and PAH were studied) in cell viability. Experiments were performed using cells (hMSCs) growing adherent to the surface of cell culture plates and polyelectrolyte concentrations varying from 0 to 500µg/mL. As a conclusion from these studies, very high (cytotoxic) concentration of polyelectrolytes had been in use. Also, in general, PAH was much more cytotoxic than CHI, whereas ALG was the less cytotoxic polyelectrolyte. So, as a last experiment, alginate and chitosan solutions with adequate concentrations (low concentrations: 50ng/mL and 1µg/mL), were applied in the encapsulation of single hMSCs. Once again, the encapsulated cells were not shown to be viable.

The next chapters describe in more detail the strategy here presented. Although the objective main goal of the Thesis was not fully accomplished, the work developed under its scope allowed interesting observations and to draw some conclusions.



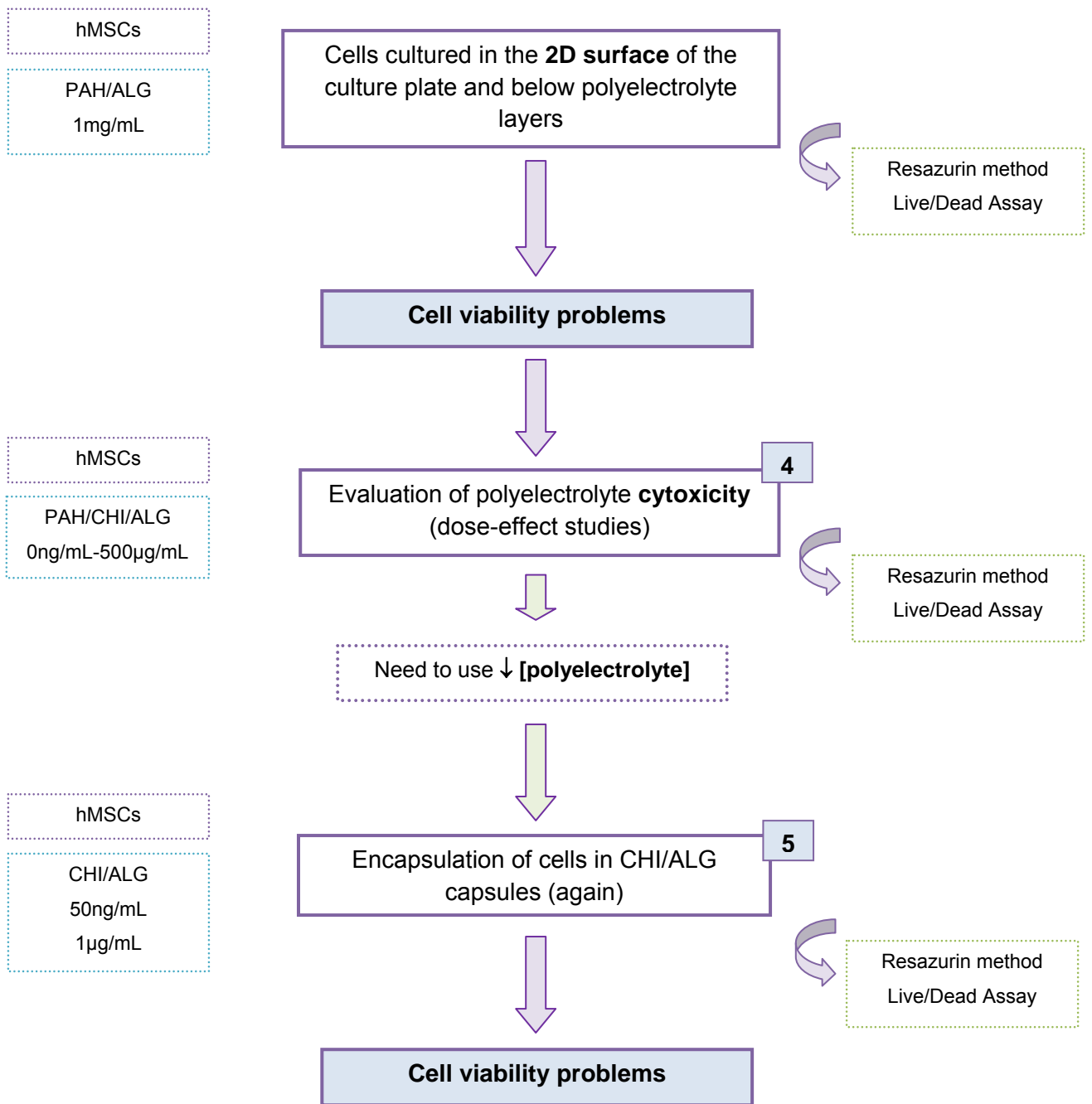


Figure 12 - General strategy followed in the work.

## CHAPTER 2 – MATERIALS AND METHODS

### 2.1 *Cells and cell culture*

Fibroblasts (NIH 3T3) and mesenchymal stem cells (MSCs) derived from the bone marrow of rats and humans were used in the experiments. The NIH 3T3 cell line was gently offered by INEB (University of Porto). Rat MSCs were isolated from the femora of 8-week-old male Wistar rats (Charles River Laboratories, Spain). Following euthanasia by pentobarbital 20% (v/v), the bones were aseptically excised, cleaned of soft tissue, and washed in a saline solution. The bone metaphyseal ends were cut off and the marrow was flushed out from the mid shaft with  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM) using a syringe.

The human cells were isolated from the bone marrow present in the trabecular bone of healthy adults which was obtained during surgery interventions after trauma. Only tissue that would have been discarded was used, with the approval of the Ethical Local Committee. After establishment of a primary culture and expansion of the cells in culture (cell passages were done using trypsin-EDTA from GIBCO<sup>®</sup>), cells were frozen using the standard procedures [66]. After, when needed, cells were thawed and placed in culture also using the normal procedures [66].

All cell cultures were incubated at 37°C, in a humidified atmosphere of 95% air and 5% carbon dioxide. NIH 3T3 cells were cultured in D-MEM (by GIBCO<sup>®</sup>) and the MSCs (both from rat and human origin) were cultured in  $\alpha$ -Minimum Essential Medium Eagle ( $\alpha$ -MEM, GIBCO<sup>®</sup>) with 10% Fetal Bovine Serum (FBS, GIBCO<sup>®</sup>) and 2% of an antibiotic-antimycotic solution (GIBCO<sup>®</sup>, with 10,000 units penicillin/ml, 10mg streptomycin/ml and 25 $\mu$ g amphotericin B/ml). All cell culture dishes were from NUNC.

### 2.2 *Equipments, materials and reagents*

An incubator (NUAIRE, Autoflow IR Direct Heat CO<sub>2</sub> incubator), a laminar flow hood (NUAIRE, Class II A/B3), an inverted optical microscope (OLYMPUS, CK40), an inverted fluorescence microscope (NIKON, TE2000), a microplate reader (PerkinElmer VICTOR<sup>3</sup><sup>™</sup>) an autoclave (ajc<sup>®</sup>) and a rotary evaporator (Buchi, R210) were used in the experimental work when needed.

The dialysis membranes were from Spectrum®labs and the filters used for solution sterilization were from VWR™ with a pore size of 0.22µm.

Five different polymers were used in this work: alginate (MMW (50-120kDa), SIGMA®); chitosan (85% deacetylated, SIGMA®), PAH ( $M_w \sim 56000$ Da, Aldrich®), hyaluronic acid ( $M_w=1.63 \times 10^6$ kDa, BioChemika®,) and collagen (Native Calf Skin Collagen Type I, IBFB, Germany).

PBS (GIBCO®, without calcium or magnesium), calcium chloride (ACROS®), magnesium chloride (MERCK®), magnesium sulphate (MERCK®), potassium chloride (Riedel-de Haën®), potassium phosphate monobasic (MERCK®), sodium bicarbonate (MERCK®), sodium chloride (MERCK®), sodium phosphate dibasic (Riedel-de Haën®), glucose (MERCK®), acetic acid (Riedel-de Haën®), EDTA (MERCK®) were used in the preparation of several solutions during the experimental work.

DAPI (SIGMA®), DTAF (SIGMA-ALDRICH®) and RITC (FLUKA®) were used in the staining procedures.

The methods applied in the evaluation of cell viability used resazurin (ALDRICH®), the MTT reagent (SIGMA®), PI (SIGMA-ALDRICH®) and FDA (SIGMA®).

### **2.3 Preparation of solutions**

The polymeric solutions (of alginate, PAH and HA) were prepared in NaCl 0.15M at 1mg/mL. The pH of each of these solutions was between 7 and 8 (alginate: pH=7.4; PAH: pH=7.2; HA: pH=7.28). The CHI was dissolved (1%) (w/v) in a solution containing acetic acid (0.1M) and NaCl (0.15M). The CHI was allowed to dissolve overnight with a gentle stirring. Then, the pH of the solution was adjusted to 4.1 (without suspended particles formation) and the solution was filtrated under vacuum. All the polymer solutions were sterilized (with a 0.22 µm membrane and a syringe, inside the laminar flow chamber) and kept in the fridge at 4°C. All the glassware and other materials were sterilized in an autoclave, to avoid any kind of contamination.

The collagen was prepared from a mother solution (2mg/mL, prepared in 0.1M acetic acid) using a NaCl solution (0.15M) and sterilized as well, with a final concentration of 100µg/mL.

The α-MEM (stock) was prepared adding 10.08g of the commercial powder and 2.2g of NaHCO<sub>3</sub> to 1L of distilled water. The pH was checked and the solution was sterilized. To prepare supplemented α-MEM (200mL), 2mL of the antibiotic-antimycotic

solution, 20mL of serum and 178mL of  $\alpha$ -MEM (stock). This mixture was maintained in the fridge at 4°C.

For the Phosphate Buffered Saline (PBS) solution preparation, 9.55g of PBS powder was dissolved in 1L of distilled water. The pH was checked and the solution was sterilized.

The Hank's Balanced Salt Solution (HBSS, 1x) was prepared by adding the following reagents (with their final concentrations in solution): calcium chloride (140mg/mL); magnesium chloride (100mg/mL); magnesium sulphate (100mg/mL); potassium chloride (400mg/mL); potassium phosphate monobasic (60mg/mL); sodium bicarbonate (350mg/mL); sodium chloride (8000mg/mL); sodium phosphate dibasic (48mg/mL) and glucose (1000mg/mL) [102]. The pH was established to 6.65 and the solution was sterilized with a 0.22 $\mu$ m membrane.

The resazurin reagent was prepared by mixing 0.02g of resazurin with 200mL of PBS (1x). This solution was sterilized (with a 0.22 $\mu$ m membrane and a syringe, inside de laminar flow hood chamber), distributed in smaller tubes and kept at -20°C.

The MTT reagent was prepared adding 100mg of MTT to 20mL of PBS. This solution was sterilized (with a 0.22 $\mu$ m membrane and a syringe inside de chamber), distributed in smaller tubes and kept at -20°C.

For the Live/Dead assay, two specific dyes were used: the PI and the FDA. The first one was used at 1mg/mL (it was not prepare in the lab) and FDA with a concentration of 5mg/mL in acetone.

## ***2.4 Alginate and chitosan fluorescent labelling***

To prepare the DTAF-labelled ALG solution, 15.0mL of ALG solution (1mg/mL) was mixed with 8mg of DTAF (the pH was adjusted to a value superior than 11 with NaOH) and it was allowed to react overnight, at room temperature, with gentle stirring, covered with foil. The DTAF-labelled ALG was separated from free DTAF by extensive dialysis against water. The solvent of the final solution was evaporated in the rotary evaporator, during 1 hour, approximately. Then, the obtained material was dissolved in 14mL of NaCl (0.15M) and distributed in several 1mL eppendorfs (the final concentration in solution was 1mg/mL). These tubes were kept at -20°C, covered with foil [103].

To prepare the RITC-labeled CHI solution, 20mg of RITC (1mg/mL in methanol) were added to 20mL of CHI (1%)(w/v) and the mixture was allowed to react during three hours, at room temperature, protected from the light and with gentle stirring. After, NaOH

(0.5M) was added to increase the pH to 10 (pH=10.56) in order to obtain a precipitate. Then, the solution was transferred to eppendorfs and centrifuged during 15 minutes at 15000g; the supernatant was removed and the pellet was washed with distilled water, and centrifuged again. This procedure was repeated until obtaining no fluorescence in the supernatant (6-7 centrifugations were done). After the centrifugations, the pellet was dissolved in 20mL of acetic acid (0.1M) and submitted to a dialysis process (against water, during three days, protected from the light; the water was changed daily). The dialysis used a cellulose membrane previously treated according to the manufacturer instructions (a solution of sodium bicarbonate 2% and EDTA 1mM was used; the membrane was put inside a reservoir with a high volume of this solution, during 30 minutes, at 80°C). After the dialysis, the solution was dried in a rotary evaporator and dissolved in 40mL of a solution containing acetic acid (0.1M) and NaCl (0.15M) [104, 105]. The final solution had a concentration of 5mg/mL and it was kept at 4°C, distributed in eppendorfs. To use this solution for cell encapsulation, it was necessary to dilute it to 1mg/mL.

## 2.5 Cell encapsulation

First, cells were trypsinised (during 10 minutes) from the cell culture plates where they were growing and counted in the inverted optical microscope using a hemacytometer (dilution 3:1, when necessary). After counting, depending on the amount of cells needed in the experiment, the suitable volume of cell suspension was transferred to an eppendorf, the suspension was centrifuged, cells were washed with a NaCl solution (0.15M) and centrifuged again (2500rpm, 8 min, 25°C).

Different pairs of polymers were used in the LbL method to encapsulate cells (CHI/ALG, PAH/ALG and PAH/HA). Previous fluorescent labelled alginate and chitosan were also used, depending on the experiments. After centrifugation, the cells were re-suspended with 500µL of the solution containing the cationic polymer and left during a certain period of time (depending on the experiment) at 37°C. After, the cell suspension was centrifuged (5 min, at 2500rpm) and the remaining pellet was washed with a solution of NaCl (0.15M) to remove the excess of un-adsorbed polyelectrolyte. The anionic polymer was adsorbed in the same manner (but the centrifugation speed was increased to 5000rpm (since the alginate solution presented some viscosity). This process was repeated until a layering scheme of cells/(P<sup>+</sup>/P<sup>-</sup>)<sub>n</sub>, was produced, where n represented the number of bilayers and P<sup>+</sup> and P<sup>-</sup> the cationic polymer and anionic polymer, respectively. At the end, the encapsulated cells were re-suspended in 500µL of NaCl 0.15M or 500µL of cell culture medium and observed at the inverted fluorescence microscope or analysed for cell viability,

respectively. Another counting was done using the hemacytometer to confirm the number of cells (encapsulated cells) in solution.

In part of the experiments, collagen was also used as a first conditioning layer before deposition of the first layer of the cationic polyelectrolyte. The idea behind collagen use was that the cell-matrix interactions could be mimetized (supplying survival signals), thus improving cell viability [106].

## 2.6 Observation of the encapsulated cells in the inverted fluorescence microscope

Cells were observed in the inverted fluorescence microscope to verify if single cell encapsulation was achieved. As a tool to observe the contour of the polyelectrolyte layers surrounding the cells, DTAF-labelled alginate was used as the anionic polymer. DTAF (fluorescein dichlorotriazine) is a “green” dye which reacts with amino groups, but also with thiol groups and hydroxyl groups present in polysaccharides (covalent links) or other alcohols in aqueous solution at a pH above 9 (Figure 13). DTAF presents an  $\lambda_{\text{Absorption}} = 492\text{nm}$  and  $\lambda_{\text{Emission}} = 517\text{ nm}$  [107].

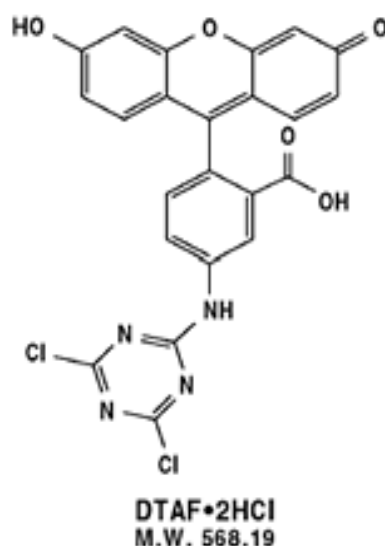


Figure 13 - DTAF structure [107].

As an alternative, RITC-labelled chitosan was used as the cationic polymer. RITC (rhodamine-B-isothiocyanate) is a “red” dye (Figure 14).

The synthesis of RITC-labelled chitosan was based on the reaction between the isothiocyanate group of RITC and the primary amino groups of chitosan. The RITC compound presents an  $\lambda_{\text{excitation}} = 570 \text{ nm}$  and  $\lambda_{\text{Emission}} = 595 \text{ nm}$  [108, 109].

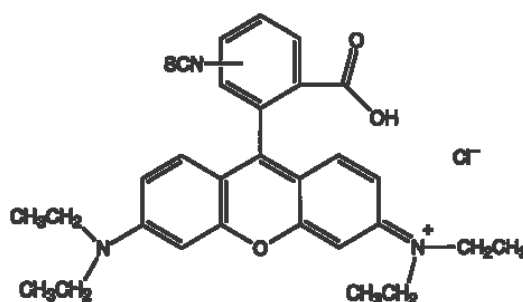


Figure 14 - RITC structure.

On the other hand, the cell nucleus was stained with DAPI. DAPI (4',6-diamidino-2-phenylindole) molecule is a low molecular weight fluorescent dye (Figure 15), which has the capacity to penetrate easily in the polyelectrolyte capsule and in the cell membrane and link to the nuclear and mitochondrial DNA. It is the most famous dye used in experiments involving cells. When it is bounded to a double-stranded DNA, DAPI has its absorption at 358 nm (ultraviolet) and its emission at 461 nm (blue) (Figure 16) [12].

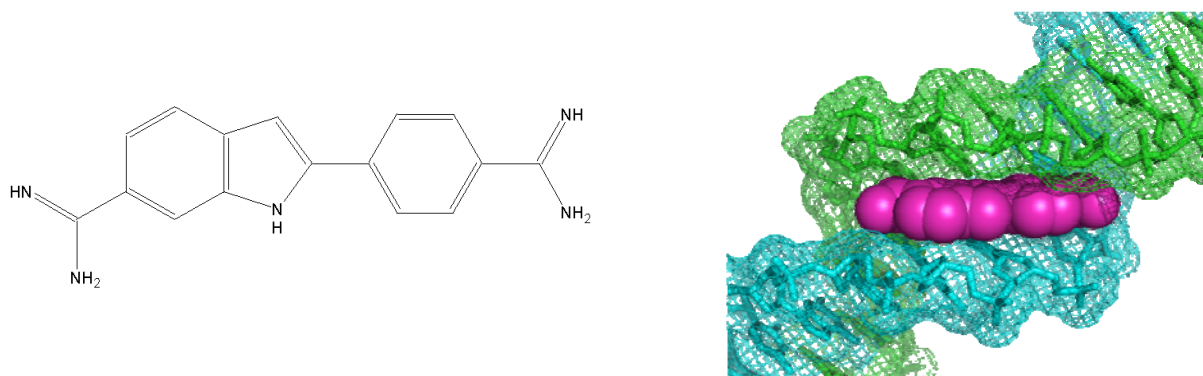


Figure 15 - Left: DAPI structure; Right: DAPI bounded to DNA.

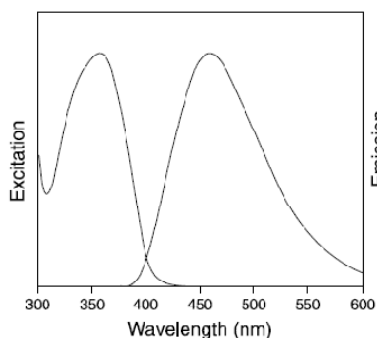


Figure 16 - Fluorescence excitation and emission profiles of DAPI bound to dsDNA [110].

The use of this dye is very advantageous to verify the cell structure preservation, which means that the intact cells will exhibit areas with a well-defined bright fluorescent, while the corrupt ones will show a diffuse blue emission [12].

A stock solution 5mg/mL of DAPI was prepared in distilled water and kept at 4°C, protected from light. 1 $\mu$ L of a 2500x dilution of the stock DAPI solution was added to the 500 $\mu$ L of medium used in the wells of 48 wells culture plates containing the encapsulated cells. After 10 minutes at room temperature, the mixture was centrifuged and 500 $\mu$ L of NaCl solution (0.15M) was added for washing and re-suspension. Finally, the sample was observed in the inverted fluorescence microscope ( $\lambda_{\text{absorption}} = 358 \text{ nm}$ ;  $\lambda_{\text{emission}} = 461 \text{ nm}$  [12].

## 2.7 Evaluation of the viability of the encapsulated cells

The viability of the encapsulated cells was evaluated by qualitative (Live/Dead assay) and quantitative means (the MTT reduction assay and the Resazurin reduction assay). The viability tests done with the encapsulated cells were performed after 0h, 1day and 3 days in culture; in some cases after 7 days.

### 2.7.1 Live/Dead Assay

In this method, two different dyes are used: fluorescein diacetate (FDA) and propidium iodide (PI) (Figure 17).

The first dye identifies the living cells and the other one identifies the dead ones. The FDA (nonpolar ester) passes through the cell membrane and is cleaved by

intracellular esterases. This cleavage results in the fluorescent compound “fluorescein”, which emits in the green range, when the stained cells are metabolically active. Fluorescein (which is a polar compound) stays accumulated inside the cell, exhibiting green fluorescence, when irradiated with blue light in a fluorescence microscope (Figure 18) [111, 112].

The PI passes through damaged cell membranes, having the capacity to intercalate with the DNA and RNA, creating a bright red fluorescent complex, visualized in the nuclei of dead cells, when seen in the fluorescence microscope. If the cells are alive, this dye is excluded by their healthy membrane. The intensity of the red fluorescence observed can depend on the degree of nuclear membrane disruption (Figure 18). The PI and other dyes (like trypan blue), are considered DNA-intercalating dyes, and they estimate cell membrane transport properties [111, 112].

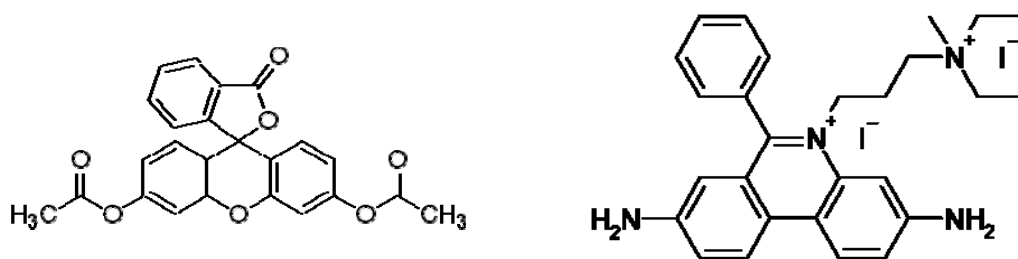


Figure 17 - FDA (on the left) and PI (on the right) structures.

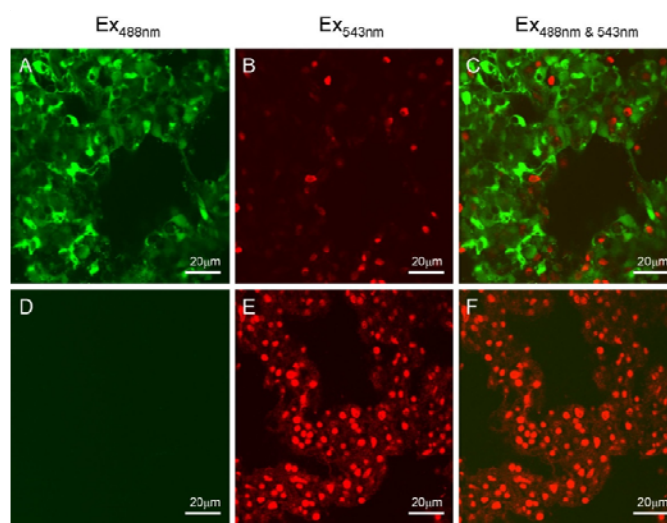


Figure 18 - Confocal images of rat lung slice co-stained with the LIVE/DEAD<sup>®</sup> viability/cytotoxicity assay kit for animal cells [113].

For the Live/Dead assay, 1 $\mu$ L of FDA and 10 $\mu$ L of PI were added to the encapsulated cells present in 500 $\mu$ L of NaCl 0.15M. After 10 minutes, the mixture was centrifuged and the pellet was washed twice with NaCl 0.15M. The final sample was observed in the inverted fluorescence microscope.

### 2.7.2 MTT reduction assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method also informs about the metabolic state of the cells. This dye presents a yellow colour and, if the cells are alive, there will be a reduction reaction by the mitochondrial reductases present inside the cells, resulting in a solid compound with purple colour, called formazan (Figure 19) ( $\lambda = 570\text{nm}$ ) [6]. The obtained compound, being solid, must be dissolved in a suitable solvent, for instance, DMSO (dimethyl sulfoxide). After the dissolution, the amount of the formazan produced can be determined with a spectrophotometer [66].

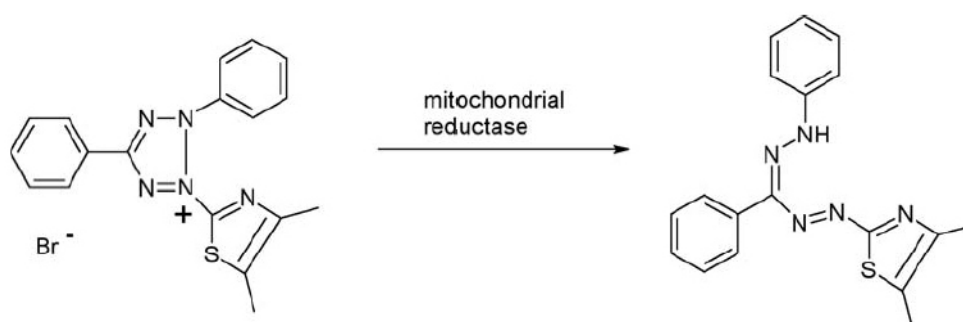


Figure 19 - MTT is reduced to formazan (s) by a mitochondrial reductase.

In the experiments, the MTT reagent was added directly to each well (50 $\mu$ L for 500 $\mu$ L of cell culture medium containing the encapsulated cells). After 3-4 hours, cells were disrupted with DMSO (dimethyl sulfoxide) and absorbance was measured using a microplate reader at 570nm (100 $\mu$ L of each solution was added to a well of a transparent 96 wells culture plate).

### 2.7.3 Resazurin reduction assay

This method is a fluorometric method for estimating the number of viable cells. It uses the indicator dye Resazurin to measure the metabolic capacity of cells. It is an indicator of cell viability [114].

If the cells are viable, they become able to reduce resazurin into resorufin (as a result of the action of several different redox enzymes), which is highly fluorescent (Figure 20). The nonviable cells lose their metabolic capacity, so they do not reduce the indicator dye, and because of that, there is not a fluorescent signal. Resazurin presents a dark blue color and has a little intrinsic fluorescence until it is reduced to resorufin, which is pink and highly fluorescent ( $\lambda_{\text{Excitation}} = 579 \text{ nm}$ ,  $\lambda_{\text{Emission}} = 584 \text{ nm}$ ) [114].

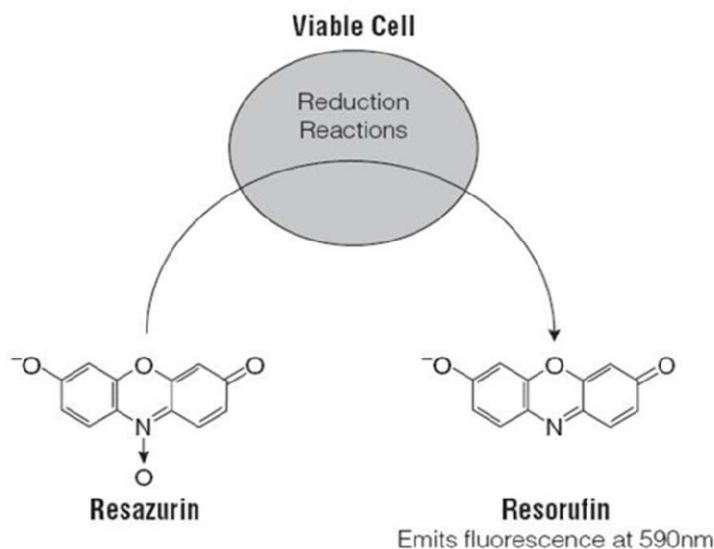


Figure 20 - Conversion of resazurin to resorufin by metabolically active cells results in the generation of a fluorescent product [114].

Under most experimental conditions, the fluorescent signal from resazurin is proportional to the number of viable cells. There is a linear relationship between cell number and fluorescence. The linear range and lower limit of detection are dependent on the cell type and the ability to reduce resazurin. The procedure is shown below (Figure 21). Resazurin is added directly to each well, then the plates are incubated at 37°C to allow cells to convert resazurin to resorufin, and the fluorescent signal is measured in a spectrophotometer [114].

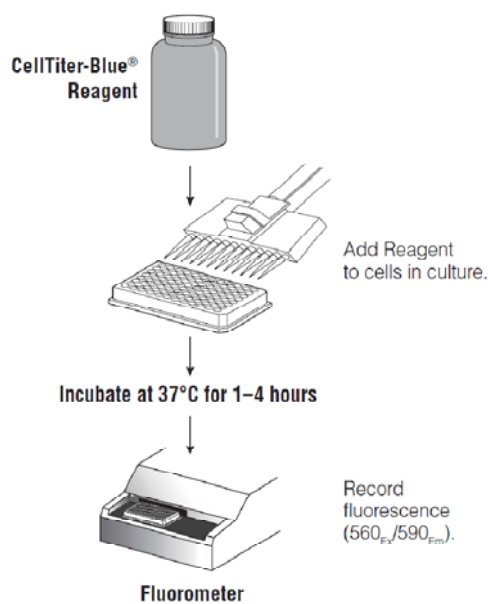


Figure 21 - The CellTiter-Blue® Cell Viability Assay protocol [114].

In the experiments, 10 $\mu$ L of resazurin solution (prepared at a concentration of 0.1% in PBS) was added per 100 $\mu$ L of medium present in the well containing the encapsulated cells. Then, the plates were incubated at 37°C, during 3-4 hours, to allow cells to convert resazurin to resorufin. The fluorescent signal was measured in a microplate reader (100 $\mu$ L of each solution was added to a well of an opaque 96 wells culture plate).

## **2.8 Cells cultured over the 2D-surface of the cell culture dish but under polyelectrolyte layers – evaluation of cell viability**

Only human MSCs were used in these studies, as well as the pair of polyelectrolytes PAH/ALG. Cells were seeded in the surface of cell culture wells at the desired cell density. After 24h in culture, the cell culture medium was removed and a solution containing the cationic polyelectrolyte was putted in contact with cells during 10 minutes, at 37°C. Then, the culture was washed twice with 0.15M NaCl and the process was repeated with the anionic polyelectrolyte. This process was repeated until a layering scheme of cells/(P<sup>+</sup>/P<sup>-</sup>)<sub>n</sub>, was produced, where n represented the number of bilayers and P<sup>+</sup> and P<sup>-</sup> the cationic polymer and anionic polymer, respectively. For the control, 0.15M NaCl without polyelectrolytes was used throughout the process.

Cell viability was determined after 0h, 1 day, 3 days and, for certain cases, 7 days in culture. Both the Live/Dead assay and the Resazurin reduction assay were used, according

to the procedures above described. In the Resazurin test, the solution of resazurin is added at each time point. In some experiments, resazurin was only added at 0h, but resorufin fluorescence was still measured at different time points (as the low/absence of cell viability could be associated with difficulties in the diffusion of resazurin throughout the polyelectrolyte layers, this assay allowed us to put aside this possibility).

## **2.9 Evaluation of the polymers cytotoxicity**

The low/absence of cell viability led us to make a systematic study (dose-effect study) of the effect of polyelectrolyte concentration on cell viability. This study was done with human MSCs and using the Live/Dead assay and the Resazurin reduction assay (along 0h, 1 day, 4 days and 7 days). The concentrations investigated were: 500µg/mL, 100µg/mL, 50µg/mL, 10µg/mL, 1µg/mL, 500ng/mL, 250ng/mL and 50ng/mL (dilution from a 'mother solution' of 1mg/ml). The assays were done for PAH, CHI and ALG.

Cells were seeded at the desired concentration in cell culture plates and let adhere and growth during 24h before contact with the polyelectrolyte solution. For the Resazurin method, 96 wells culture plates (for adherent cells), 20µL of polymer solution/well and  $3.0 \times 10^4$  cells/cm<sup>2</sup> were used; washing with NaCl (0.15M) was done 3 times after a contact time of 10 minutes between the polymer and the cells. After, 10µL of resazurin solution was added per well and, 4h later, resorufin fluorescence was measured. The Live/Dead Assay was performed in 48 wells culture plates (for adherent cells), adding 200µL of polymer solution per well and using a cell density of  $1.8 \times 10^4$  cells/cm<sup>2</sup>. Again, washing with NaCl (0.15M) was done 3 times after a contact time of 10 minutes between the polymer and the cells. In the assay, 1µL of FDA and 10µL of PI solutions were added to 500µL of medium. The final sample was observed in the inverted fluorescence microscope.

## CHAPTER 3 – RESULTS AND DISCUSSION

### ***3.1 Establishment of the best experimental conditions to obtain the encapsulation of single cells***

The first part of the work consisted in the establishment of adequate experimental conditions to achieve the encapsulation of individual cells. The general procedure used for cell encapsulation was based on the Layer-by-Layer technique and is schematised in Figure 22. Mesenchymal stem cells and the fibroblastic line NIH 3T3 were used in these initial experiments. The pair of electrolytes chosen was chitosan and alginate and, based on the work of Xia Tao and co-workers [8], solutions of 1mg/mL were used in the LbL process. To visualize the capsules in the fluorescence microscope, DTAF-labelled alginate (which emits in the green area of the spectrum) was used or, in alternative, RITC-labelled chitosan (which emits in the red area of the spectrum). The nuclei of the cells were visualized in the samples by staining them with the fluorescent dye DAPI (which emits in the blue area of the spectrum). The DAPI, being a low-molecular-weight dye, had the capacity to pass through the capsule easily, and to bind to the nuclear and mitochondrial DNA present in the cell [12]. The objective was to have only one cell inside one capsule.

Experimental variables such as (i) the time of contact between polyelectrolytes and cells, (ii) the way cells were washed between polyelectrolytes adsorption, (iii) cell concentration and (iv) the method of re-suspending and stirring the cells after centrifugation were studied.

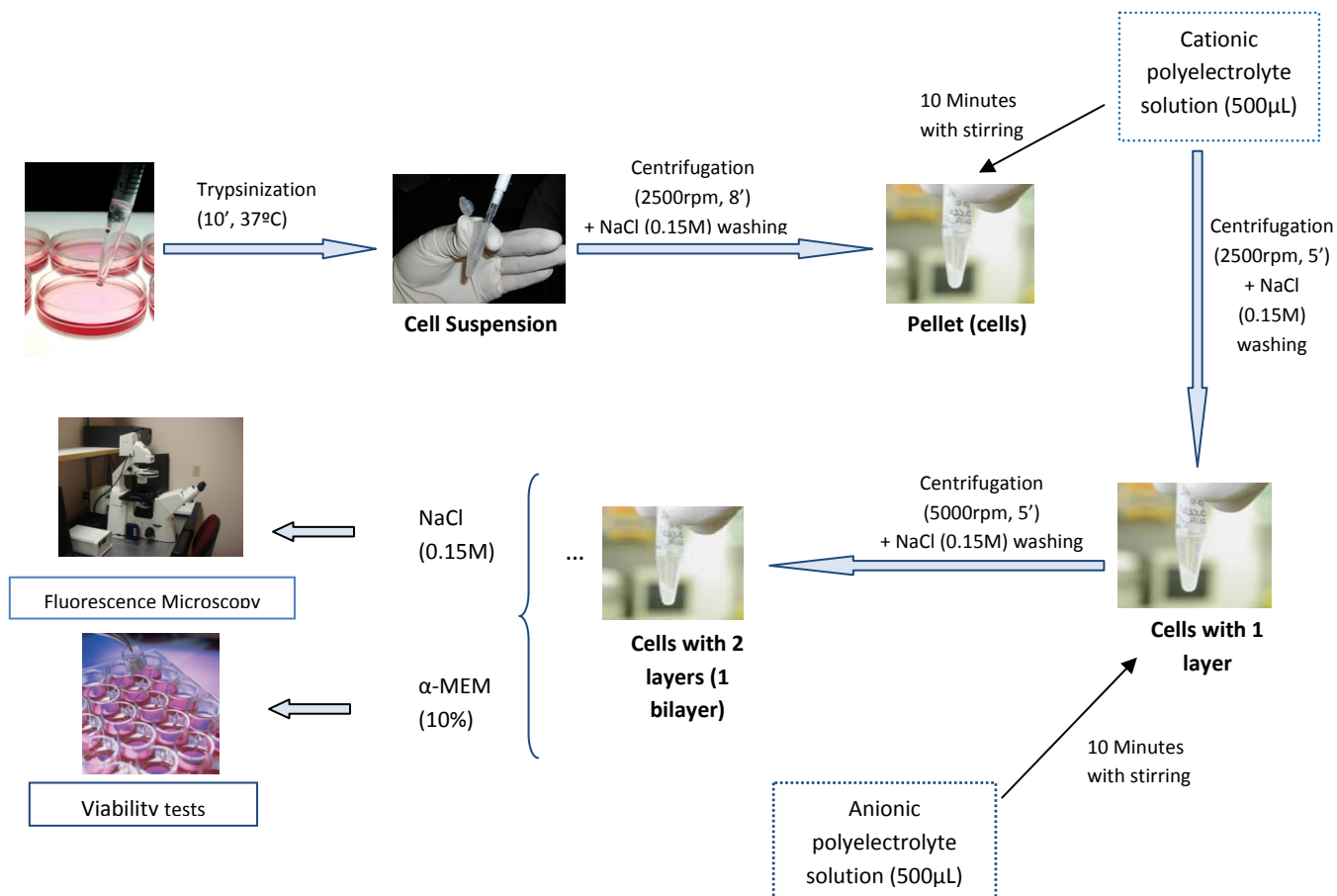


Figure 22 - General procedure used in the encapsulation of cells.

The tested times of contact between cells and polyelectrolytes were 5 minutes and 10 minutes but no influence was noted in the obtainment of encapsulated single cells. Based on these results, a 10 minutes contact time was chosen to be used in all other experiments.

The washing step just after polyelectrolyte adsorption is a normal step in the LbL technique [6, 12, 115]. This washing is used to remove the excess of polyelectrolyte (not absorbed) and will, in principle, improve the observation of the samples in the Fluorescence Microscope by minimizing blur.

In the present work, three procedures were tested: (i) cells were not washed between polyelectrolytes deposition; (ii) washing was done with a solution of NaCl at 0.15M; and (iii) washing was done with Hank's Balanced Salt Solution. NaCl at 0.15M was used because it is a very simple solution having an isotonic concentration [66]. HBSS was used based on the work of Verrabadran and co-workers [6]. The results obtained with these three approaches were similar but, anyway, it was decided to keep the step of washing with NaCl 0.15M between polyelectrolytes deposition.

Figure 23 illustrates the type of images obtained with these systems using fluorescence microscopy. Here, rat MSCs were encapsulated within 1 bilayer of CHI/ALG-DTAF. The step of washing between polyelectrolytes adsorption was done with NaCl 0.15M and the time of contact between the cells and the polyelectrolyte solutions was 10 minutes. The blue fluorescence signals emitted by cell nuclei are clearly seen and their co-localization with the green fluorescence signals arising from the polyelectrolyte capsules is evident.

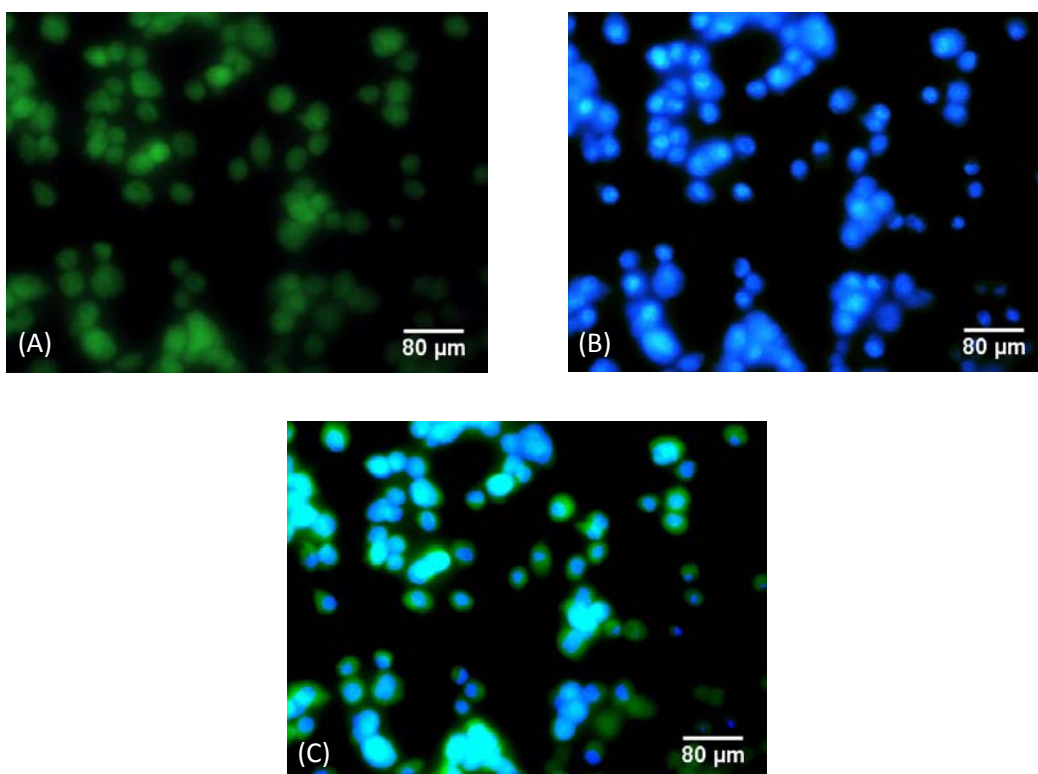


Figure 23 - Images obtained in the Fluorescence Microscope. Rat MSCs were encapsulated within 1 bilayer of CHI/ALG-DTAF; A: Green fluorescence from ALG-DTAF; B: Blue fluorescence from DAPI; C: merged images of both A, B. The nuclei (in blue) are inside the capsules (green).

These first set of experiments were done without measuring the number of cells in solution. As many aggregates were noticed in the observed samples, we hypothesized that maybe this problem could be related with a high cell concentration. An experiment was then done using different amounts of cells in the encapsulation process (Figure 24). The initial quantity of cells placed in the eppendorfs was  $1.5 \times 10^5$ ,  $3.0 \times 10^5$  and  $7.5 \times 10^5$  cells. At the end of the encapsulation process, as the capsules containing the cells were re-suspended in  $500 \mu\text{L}$  of NaCl 0.15M, the encapsulated cell concentrations were  $3.0 \times 10^5 \text{ cells/mL}$ ,  $6.0 \times 10^5 \text{ cells/mL}$  and  $15.0 \times 10^5 \text{ cells/mL}$ , respectively. Results showed that capsules aggregation increased with the number of initial cells used.

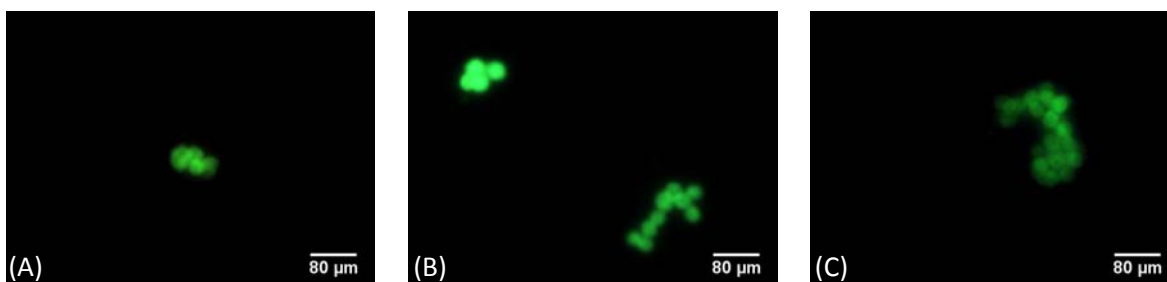


Figure 24 - Images of NIH 3T3 cells were encapsulated within 1 bilayer of CHI/ALG-DTAF. The images were obtained in the Fluorescence Microscope for an initial number of cells of A:  $1.5 \times 10^5$ , B:  $3.0 \times 10^5$  and C:  $7.5 \times 10^5$ . Green fluorescence from ALG-DTAF is observed. The step of washing between polyelectrolytes adsorption was done with NaCl 0.15M and the time of contact between the cells and the polyelectrolyte solutions was 10 min.

However, the mentioned aggregation problem was not completely solved by decreasing the cell number. In fact, Veerabadran and co-workers [6] used a much higher starting quantity of cells ( $5 \times 10^6$  cells in 1mL) in their encapsulation studies and obtained the capsules more or less isolated. It must be said, however, that the experimental procedure used by Veerabadran and co-workers is not presented in detail in their publication, not allowing a full comparison of the results. For instance, they do not refer how much volume of polyelectrolyte solution was added to the cell suspension.

Therefore, believing that the cell concentration was not the unique factor affecting the aggregation of the encapsulated cells, several experiments were done to evaluate the effect of the type of stirring used during the cell re-suspension steps inherent to the process. Table 3 resumes the results obtained in experiments done with initial cell numbers of  $1.5 \times 10^5$  and  $5.0 \times 10^5$  and by encapsulating NIH 3T3 cells within 1 and 2 bilayers of polyelectrolytes. Stirring was performed by simply passing the cell suspension through the small tip of a micropipette several times, or by using a vortex mixer, an orbital mixer or a spinning magnet inside the tube containing the cell suspension. Moderate levels of cell aggregation were obtained with the use of a micropipette or the vortex mixer. In all other situations, the level of cell aggregation was high. Although the use of a vortex mixer seemed to be one of the best strategies to obtain separated encapsulated cells, due to concerns related with cell viability (cells could not resist to the strong forces generated during vortexing), the micropipette was used for stirring in all the following experiments.

Table 3 - Effect of the type of stirring used in the cell re-suspension steps on cell aggregation (+++, ++ and + represents high, moderate and low levels of cell aggregation, respectively).

	Micropipette (during 'min, until 10')	Vortex (10s)	Orbital mixer (37°C, 300rpm)	Spinning magnet (10', 750rpm)
<b>1.5x10<sup>5</sup> cells</b> (3x10 <sup>5</sup> cells/mL) 1 bilayer	++	++	*	*
<b>5.0x10<sup>5</sup> cells</b> (1x10 <sup>6</sup> cells/mL) 2 bilayers	++	*	+++	+++

\* Not studied

### 3.2 Evaluation of the viability of cells encapsulated within CHI/ALG bilayers

After knowing that it was possible to obtain single encapsulated cells through the LbL technique, the next step was to verify if cells remained viable inside the capsules.

The first set of experiments used NIH 3T3 cells encapsulated within 1 and 2 bilayers consisting of CHI and ALG. A low cell concentration was used to minimize aggregation ( $4 \times 10^4$  cells in each well of a 48-well culture dish) and cell viability was studied using the MTT test. Cell viability just after the cell encapsulation process (at 0h of cell culture) was 0% in relation to the values obtained with non-encapsulated cells. The treatment applied to non-encapsulated cells was equal to the one applied to the encapsulated cells, except that no polymers were present in the solutions. Thinking that, for some reason, the MTT test was not suitable to evaluate the metabolic activity of the encapsulated cells, two other cell viability tests were used: the resazurin reduction assay and the Live/Dead assay. Figure 25 shows the results obtained with the first method. Cell viability was extremely low at the beginning of cell culture (0h) and, after 3 days, decreased to 0%.

The Live/Dead assay results were in accordance with these findings. As can be seen in Figure 26, NIH 3T3 cells appear red under the fluorescence microscope due to propidium iodide incorporation, revealing that cells were damaged (not viable). Results are shown for cell viability analysis at 0h of cell culture but identical images could be seen after 1 day in culture (data not shown). These experiments were also performed with hMSCs. Results have also shown the lack of cell viability since the beginning of the cell culture.

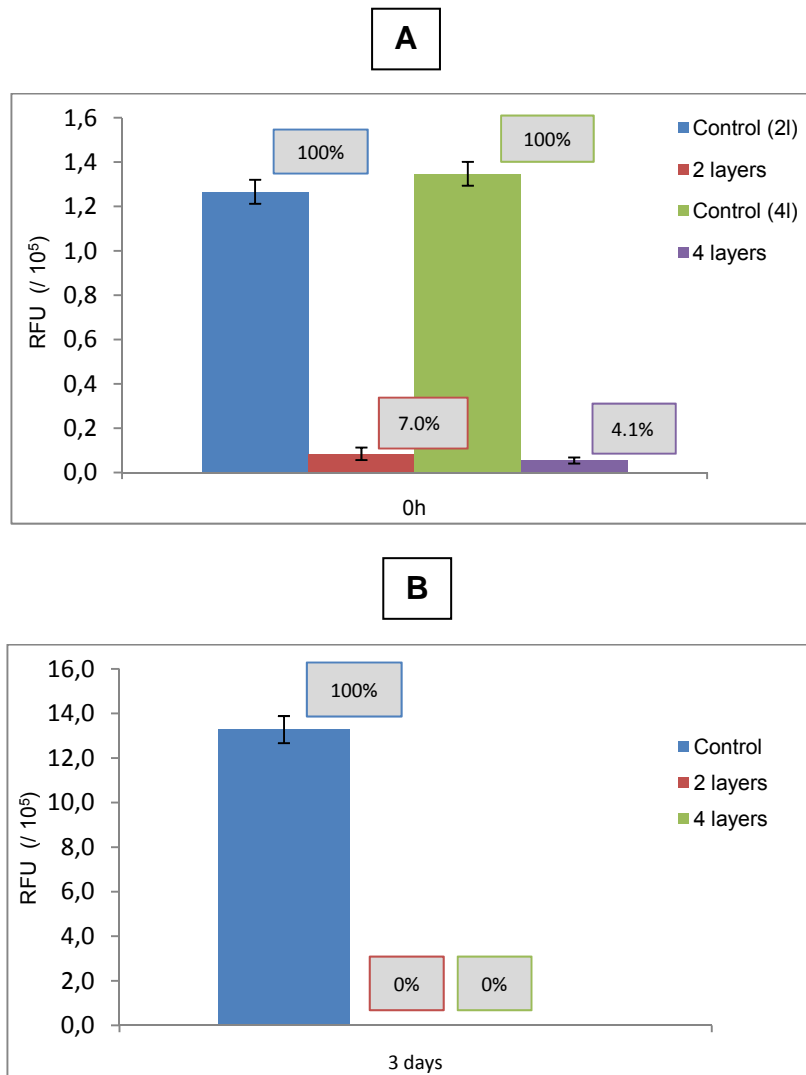


Figure 25 – Evaluation of the metabolic activity of NIH 3T3 cells encapsulated within 1 and 2 bilayers of CHI/ALG using the resazurin reduction assay after 0h (A) and 3 days (B) of cell culture.

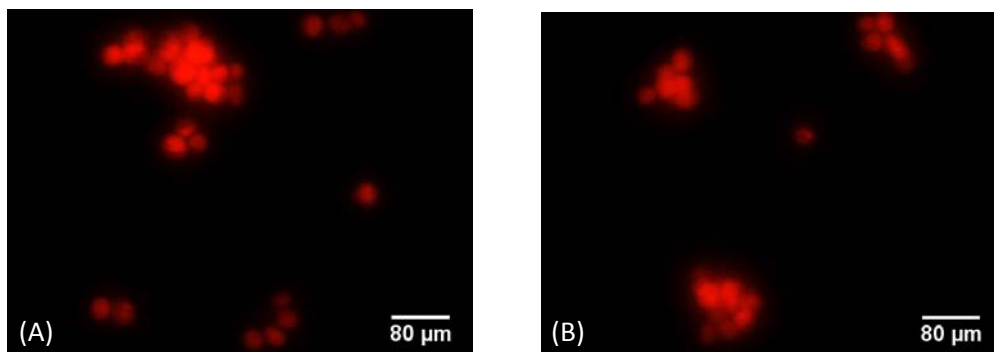


Figure 26 – Evaluation of the viability of NIH 3T3 cells encapsulated within 1 (A) and 2 bilayers (B) of CHI/ALG using the Live/Dead assay at the beginning of cell culture (0h). Dead cells are red under the fluorescence microscope.

### **3.3 Evaluation of the viability of cells encapsulated within PAH/ALG bilayers**

As the obtained results were not satisfactory for the pair of electrolytes CHI/ALG (cell viability was null), it was decided to change CHI by another cationic polyelectrolyte. In fact, chitosan was not soluble at the physiological pH and had to be dissolved in a solution containing acetic acid (pH=4.1).

So, maybe the cells were not able to support the low pH of the chitosan solution and died.

Poly(allylamine hydrochloride) was then chosen as the new cationic polyelectrolyte for cell encapsulation through the LbL technique. For starting, the obtainment of single encapsulated cells with the pair of electrolytes PAH/ALG was checked using hMSCs. Figure 27 (A) clearly shows that the blue fluorescence signals emitted by cell nuclei are co-localized with the green fluorescence signals arising from the polyelectrolyte capsules. So, also in this situation, single cell encapsulation was obtained.

In order to improve cell viability inside the capsules made of PAH/ALG, another strategy was studied. Before the formation of the PAH/ALG layer, cells were putted in contact with a solution containing collagen type I. Collagen type I is a negative polymer but can establish linkages with the cell surface through specific receptors (such as the integrins which exist at the MSCs surface) [116]. The objective was to allow cells to closely interact with a matrix protein, providing them the necessary cell survival signals which could help maintain their viability. Indeed, the animal cells used in the present work are adherent cells (anchorage dependent cells) and the lost of cell-matrix interactions inherent to the procedure applied in the LbL technique is an important issue which cannot be forgotten. By loosing cell-matrix interactions, cells can enter apoptosis and lose their viability.

Although not concerned with single cell encapsulation, an interesting work of Golnaz Karoubi and co-workers [115] has shown that capsules supplemented with matrix proteins (fibronectin and fibrinogen) re-introduced the cell-matrix interactions and, in consequence, provided the necessary signals to rescue the cells to suffer apoptosis, increasing therefore their viability. Collagen has very important roles in human body, being the major constituents of several types of specialized extracellular matrices. Studies of collagen-binding integrins in several *in vitro* assays show that they participate in cell migration, cell adhesion, control of collagen synthesis, matrix metalloproteinase (MMP) synthesis, and influence some processes such as cell differentiation, cell proliferation, angiogenesis, between others [115, 116].

Collagen is known to be a promising material, thus, it has been applied in many applications in the tissue engineering area, due to its excellent biocompatibility and biodegradability [117, 118].

The use of an inner collagen layer, in close contact with the MSC surface, did not had influence on the obtainment of single encapsulated cells, as can be seen in Figure 27 (B).

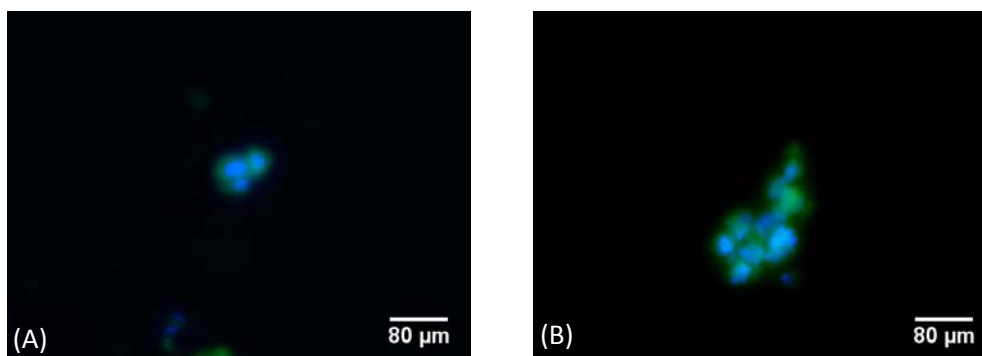


Figure 27 - Images obtained in the Fluorescence Microscope. hMSCs were encapsulated within 1 bilayer of PAH/ALG-DTAF without (A) and with an inner collagen layer (B). The step of washing between polyelectrolytes adsorption was done with NaCl 0.15M and the time of contact between the cells and the polyelectrolyte solutions was 10 min. Green fluorescence is due to ALG-DTAF and blue fluorescence to DAPI (the merged images are shown). The nuclei (in blue) are inside the capsules (green).

The viability studies using these new encapsulated cells (PAH/ALG and Collagen/PAH/ALG) and the Live/Dead assay were more promising. As can be observed in Figure 28, the hMSCs appeared red under the fluorescence microscope due to propidium iodide incorporation only at the beginning of cell culture (0h); after that (1 day and 3 days in culture), the cells seemed to recuperate and appeared green due to fluorescein diacetate intake, revealing that cells were metabolic active (viable). The presence of collagen did not have an influence on these results. However, it was noticed that the presence of collagen in the capsules turned the manipulation of the pellets easier during the LbL experimental process.

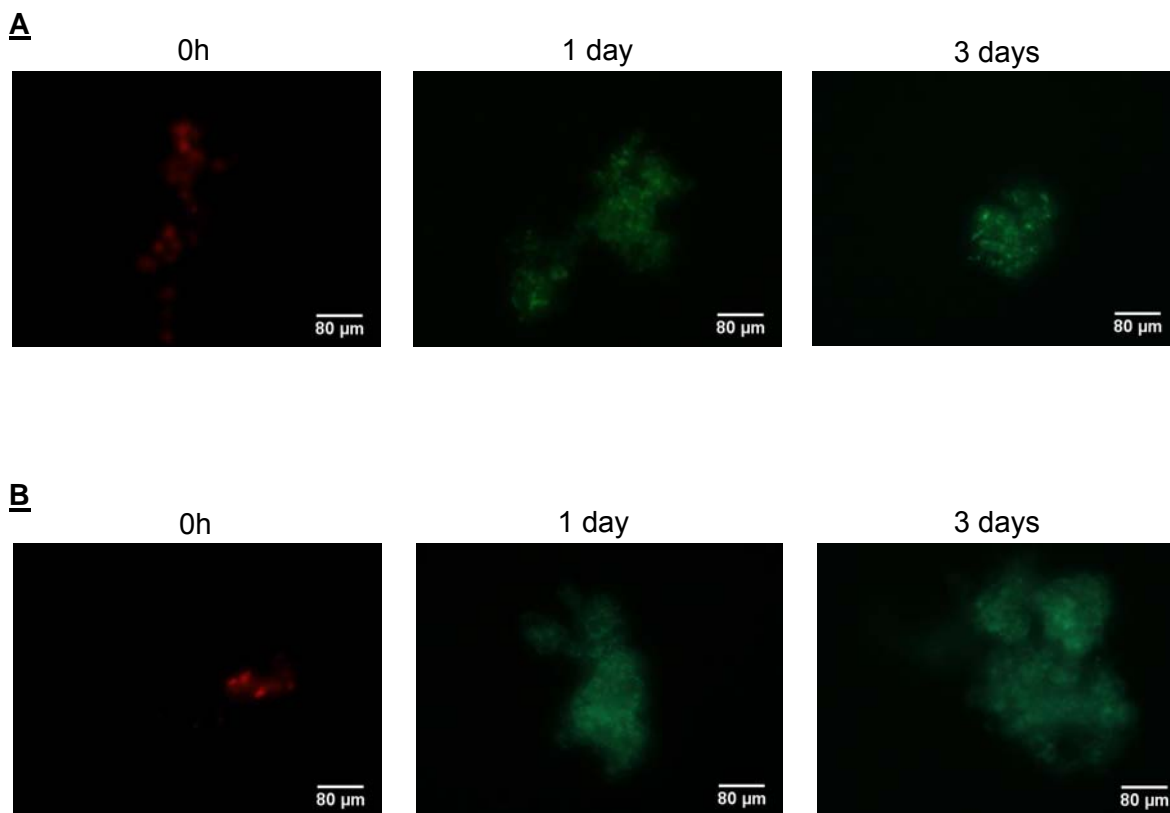


Figure 28 - Evaluation of the viability of hMSCs cells encapsulated within 1 bilayer of PAH/ALG (A) and 1 bilayer of collagen/PAH/ALG (B) using the Live/Dead assay at several cell culture times (0h, 1 day and 3 days). Dead cells are red and live cells are green under the fluorescence microscope.

Cell viability was also evaluated through the resazurin reduction assay for cells encapsulated within PAH/ALG. The viability of the encapsulated cells at the beginning of the cell culture (0h) was approximately zero. To put aside the possibility of existing diffusion problems preventing resazurin to reach the cells, in a first experiment, it was decided to measure resorufin fluorescence 3h (the normal period, as described in the “Materials and Methods” chapter), 6h and 24h after resazurin addition to the cell culture medium. Thus, resazurin was only added once in the cell culture medium and enough time was given for the molecules to reach the interior of the cells. Results are summarized in Table 4 and show that resazurin diffusion through the polyelectrolyte layers was not a problem. Instead, it must be the encapsulation process that affects the cell metabolic activity. In opposition to what was expected, the results of the resazurin reduction assay were not in agreement with those obtained through the Live/Dead assay for which cells looked viable after 1 day and 3 days in culture.

To enhance the probability of cell survival, it was also decided to perform an experiment with increasing concentrations of fetal bovine serum (FBS) in the cell culture medium. The FBS contains several growth factors and survival signals which could improve cell survival [66].

However, experiments done with 20% and 40% of FBS in cell culture medium haven't conducted to different results in terms of cell viability.

Table 4 - Cell viability results using the resazurin reduction assay. hMSCs were encapsulated within 1 bilayer of PAH/ALG polyelectrolytes and resazurin was added only at 0h. Resorufin fluorescence was measured after 3h, 6h and 24h of cell culture. Results are expressed as a percentage of the control.

	Control	Encapsulated cells (1 bilayer)
After 3h	100.0±0	1.0±1.9
After 6h	100.0±0	2.0±1.6
After 24h	100.0±0	0±1.1

### **3.4 Evaluation of the viability of cells encapsulated within PAH/HA bilayers**

Another attempt was made for obtaining single encapsulated viable cells, this time changing alginate by hyaluronic acid. hMSCs were, then, encapsulated in the interior of 1 or 2 bilayers consisting of PAH/HA. Cell viability studies were done using a percentage of serum in the cell culture medium of 20% (v/v) or 40% (v/v).

Based on the resazurin reduction assay, at 0h, cell viability was about 22% and 29% of that present in the control for cells encapsulated within 1 and 2 bilayers of PAH/HA, respectively. After 1 day, there were no signals of cell metabolic activity. When the percentage of serum was raised to 40%, at day 0, those values increased to 32% and 44%, respectively, but also decreased to zero after 1 day. These results are exposed in Figure 29.

Surprisingly, the results obtained with the Live/Dead assay showed dead cells at 0h and live cells for later times (Figure 30). Again, this two cell viability evaluation methods seem to not correlate with each other.

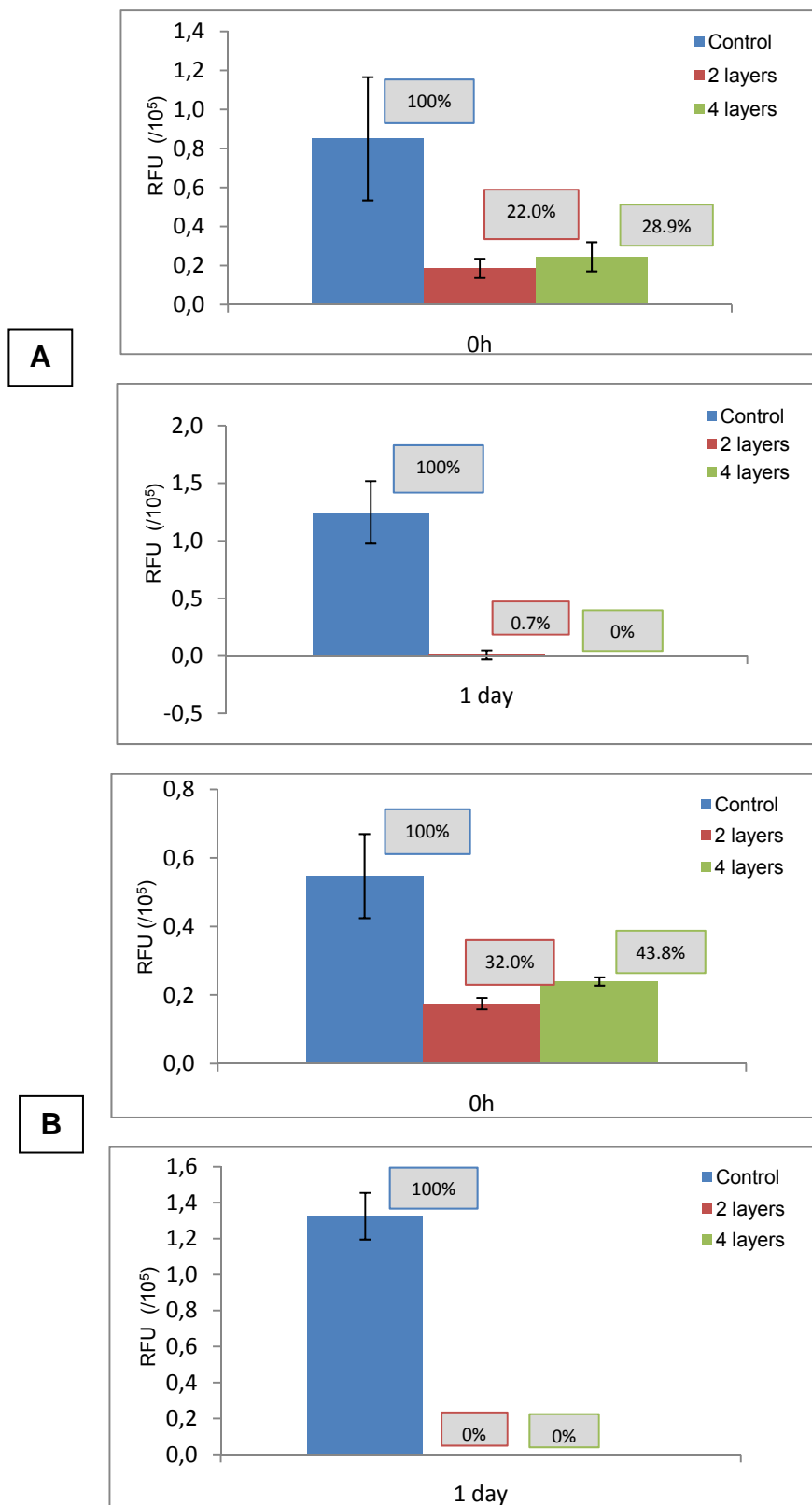


Figure 29 - Evaluation of the metabolic activity of hMSCs encapsulated within 1 and 2 bilayers of PAH/HA using the resazurin reduction assay after 0h and 1 day in culture. Experiments were done with a percentage of 20% (A) and 40% (B) of FBS in the cell culture medium.

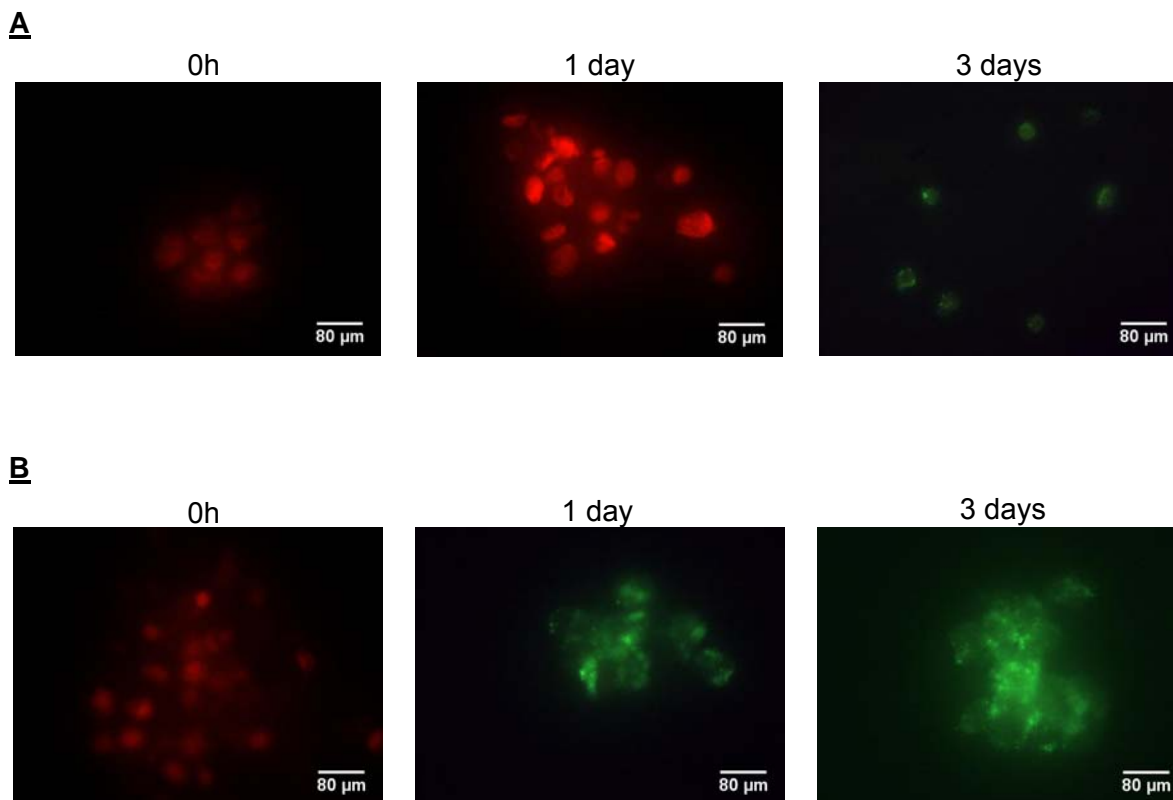


Figure 30 - Evaluation of the viability of hMSCs encapsulated within 1 (A) and 2 bilayers (B) of PAH/HA using the Live/Dead assay after 0h, 1 day and 3 days in culture. Experiments were done with a percentage of 20% of FBS in the cell culture medium.

### ***3.5 Evaluation of the viability of cells cultured over the 2D-surface of the cell culture dish but under polyelectrolyte layers***

This last experiment (using PAH/HA systems) showed that it was possible to increase the viability of cells encapsulated inside polyelectrolyte layers by playing with the type of polyelectrolytes used in the LbL procedure and, also, by providing cells with survival factors such as those present in serum. However, the improvement verified with the pair of polyelectrolytes PAH/HA and using 40% of FBS in the cell culture medium was not brilliant.

So, instead of further exploring the PAH/HA systems, it was decided to try to understand why it was so difficult to maintain cell viability inside the polyelectrolyte capsules. So, it was hypothesized that as NIH 3T3 cells and MSCs are adherent cells, they would always need a substrate to attach in order to survive and that the lack of viability was mainly related with the fact that cells were cultured in suspension. According to this idea, cells cultured attached to the surface of a cell culture dish and coated in their top with layers of polyelectrolytes (also using the LbL method) would be able to survive.

The next step of this Master thesis was, then, to culture hMSCs attached to the surface of cell culture plates (a monolayer of cells) but having layers of the polyelectrolyte pair PAH/ALG deposited over them (see Figure 31 (A, B)). Cells were seeded at a concentration of  $2.6 \times 10^4 \text{ cell/cm}^2$  and cultured during 7 days. Experiments were done by depositing 1, 2 and 4 bilayers of polyelectrolytes over the cells. The experimental conditions used for encapsulation of single cells were maintained, namely, the time of contact between cells and the polyelectrolyte solutions was 10 minutes and the concentration of the polyelectrolyte solutions was 1mg/mL). Cell viability was assessed using the resazurin reduction assay and the Live/Dead assay.

Surprisingly, cell viability was also absent in these “two-dimensional” experiments where hMSCS were cultured like in a sandwich, between the surface of the cell culture dish and the polyelectrolyte layers. Results can be seen in Figure 32, Figure 33 and Figure 34.

The data obtained from the resazurin reduction assay is shown only for 0h and 1 day of cell culture (for the other time points the results were identical). An important observation is that, by looking to the decreasing values of the metabolic activity associated with the different control experiments when the number of bilayers is increased, one can see that cell viability was strongly affected by the LbL process itself (maybe by the time that cells spent out the incubator). It must be reminded that the experiments done with different layers of polyelectrolytes had different controls (each control passed through all the procedures suffered by the sample except that no polymers were added to the solutions).

Figure 35 shows the morphology of hMSCs (bright field images) cultured with 2 and 4 bilayers of polyelectrolytes over them and after 7 days in culture. Cells presented an irregular morphology, characteristic of damaged cells.

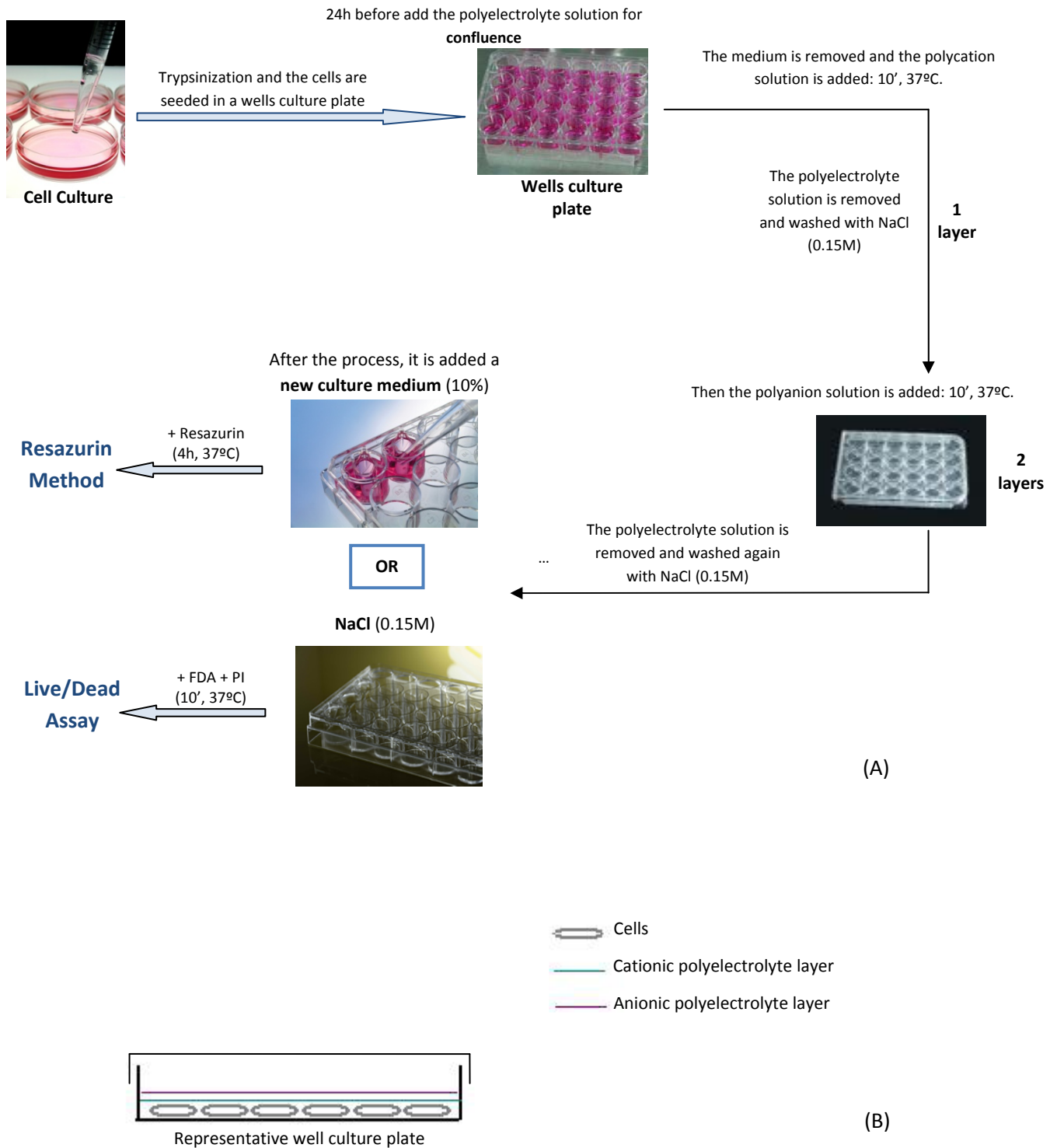


Figure 31 - Experimental procedure used in the assays (A); Scheme showing cells adherent to the plastic surface of the cell culture dish and having layers of polyelectrolytes over them (B);

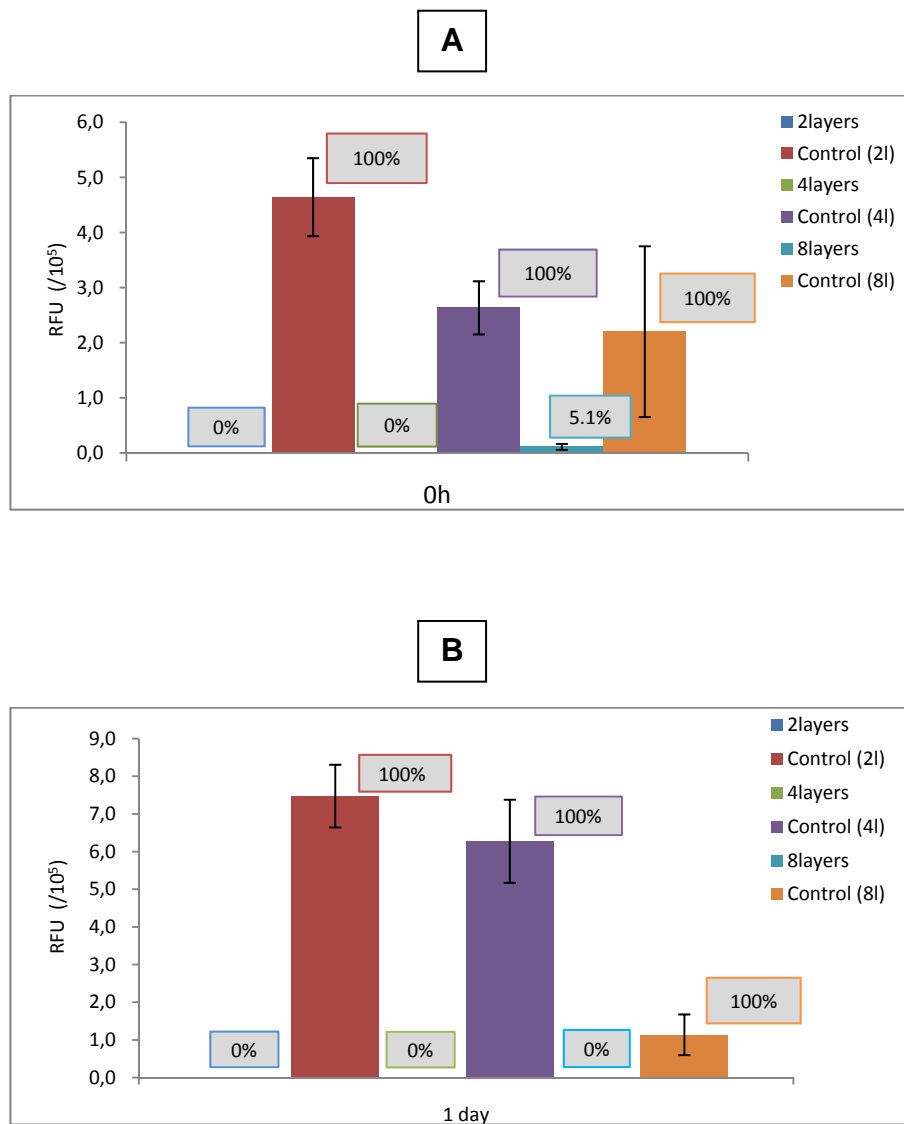


Figure 32 - Evaluation of the metabolic activity of hMSCs cultured under 1, 2 and 4 bilayers of PAH/ALG using the resazurin reduction assay after 0h (A) and 1 day (B) in culture.

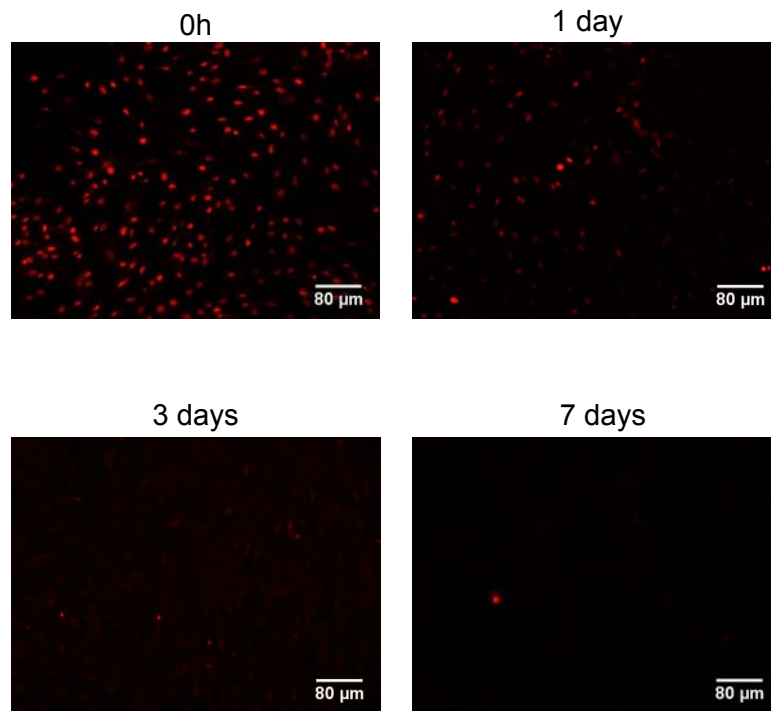
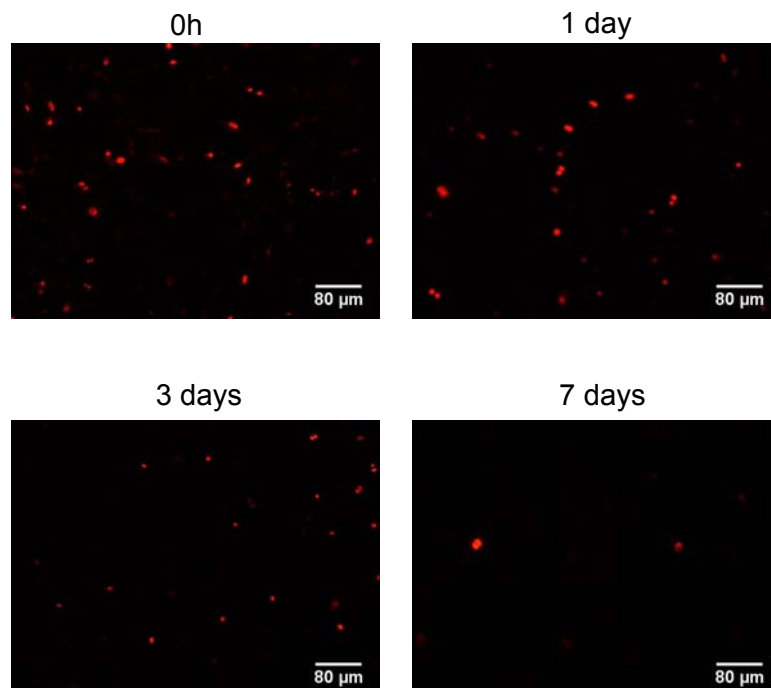
**A** For 2 layers:**B** For 4 layers:

Figure 33 - Evaluation of the viability of hMSCs cultured under 1 (A) and 2 (B) bilayers of PAH/ALG using the Live/Dead assay after 0h, 1 day, 3 days and 7 days in culture. Dead cells are red and live cells are green under the fluorescence microscope.

**C** For 8 layers:

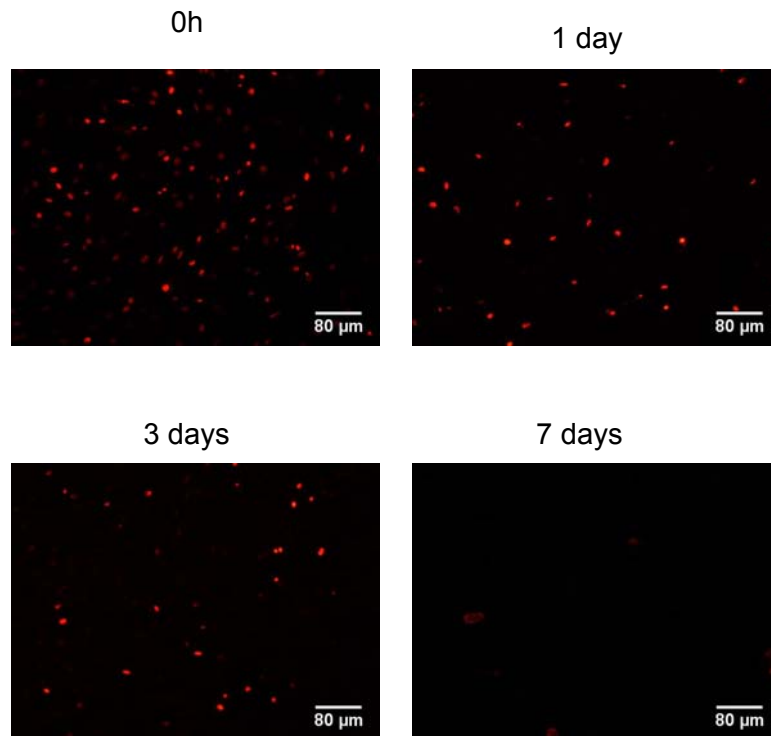


Figure 34 - Evaluation of the viability of hMSCs cultured under 4 (C) bilayers of PAH/ALG using the Live/Dead assay after 0h, 1 day, 3 days and 7 days in culture. Dead cells are red and live cells are green under the fluorescence microscope.

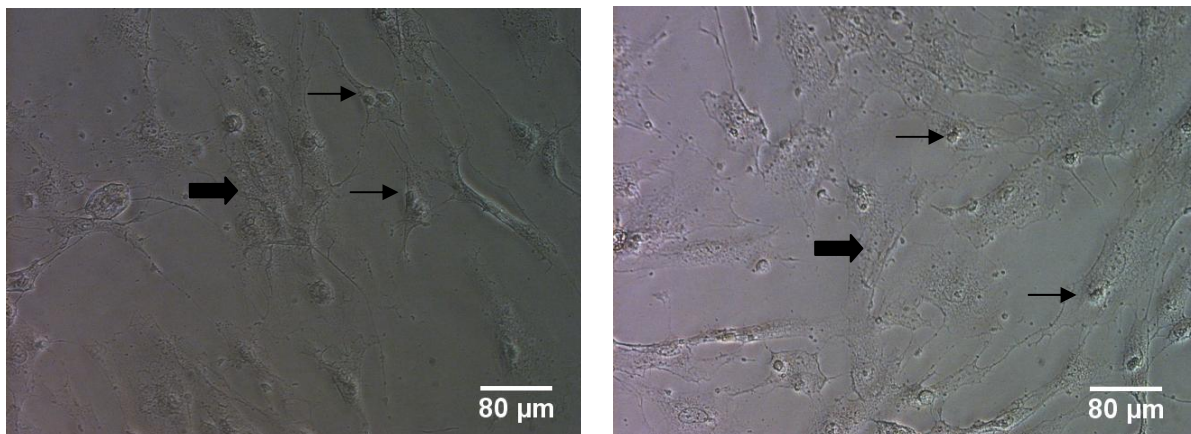


Figure 35 - Bright field images of cells cultured under 2 (A) and 4 (B) bilayers of PAH/ALG. The skinny arrows exemplify the nuclei of the cells and thick ones the extracellular membrane.

After this experiment which was also unsuccessful in terms of cell viability, the possibility that high (cytotoxic) concentrations of polymers were being used was raised. In fact, as already referred, 1mg/mL polyelectrolyte solutions were being applied based on the concentrations used by other authors also working with MSCs [6].

As cationic polymers are usually much more cytotoxic than anionic ones [86, 119], another experiment was performed with only 1 bilayer of polyelectrolytes over the cells and using lower concentrations of PAH in solution (0.1mg/mL and 0.5mg/mL); on the other hand, the amount of alginate that will adsorb at the cell surface will be dependent on the quantity of cationic polymer initially adsorbed. Therefore, it was reported that the 'density' of the polyelectrolyte bilayer adsorbed to the cells can be controlled by varying just the concentration of PAH itself [68]. The concentration of alginate was maintained. The results are shown in Table 5. Also in this case, cell viability was severely affected.

Table 5 - Evaluation of the metabolic activity of hMSCs cultured under 1 bilayer of PAH/ALG using the resazurin reduction assay after 0h, 1 day, 3 days and 7 days in culture. PAH concentrations in solution of 0.1mg/mL and 0.5mg/mL were studied.

	Control	100µg/mL	500µg/mL
0h	100.0±0	0±3.6	0±2.5
1 day	100.0±0	6.0±2	5.0±2
3 days	100.0±0	3.0±0.3	2.6±0.4
7 days	100.0±0	4.0±0	4.5±0.6

### 3.6 Evaluation of polyelectrolyte cytotoxicity

After all the experiments done before, it was evident that a systematic study would have to be performed to evaluate the effect of polyelectrolyte concentration on cytotoxicity.

A set of experiments was then programmed for individually testing the cytotoxicity of CHI, PAH and ALG using hMSCs in culture. As explained in more detailed in the Materials and Methods chapter, cells were seeded in cell culture plates and, 24h later, they were covered with solutions containing the polyelectrolyte under study at the desired concentration during 10 minutes. After, the cultures were washed with a saline solution and left to growth until 7 days. The dose-response effects were quantitatively studied using the resazurin reduction assay and are presented in Table 6 and Figure 36 in respect to control values. The Live/Dead assay was used as a qualitative mean to evaluate the effect of the polyelectrolyte concentration on cell viability. These results are shown in Figure 37 to Figure 39.

The cell metabolic activity results depend on the period of time that cells remain in culture. Just after the end of polyelectrolyte exposure (0h), the cell metabolic activity presents the lowest values in respect to the control values. After culturing cells for some time, cells seem to adapt to the new situation and recover from the suffered injury, proliferating in culture and raising their metabolic activity.

In general, the results from the resazurin reduction assay show that PAH is much more cytotoxic than CHI and ALG. As expected, ALG is the less cytotoxic polyelectrolyte.

As example, for a dose of 500 $\mu$ g/mL of polyelectrolyte, at 0h, cell viability was 0%, 53% and 20% of the control values for, respectively, PAH, ALG and CHI. This information is very important to understand the lack of viability verified for the encapsulated cells. In fact, the LbL method applied to encapsulate the cells used polyelectrolyte concentrations of 1mg/mL, a value which corresponds to severe cytotoxic effects. Furthermore, the encapsulated cells are simultaneously putted in contact with 2 kinds of polyelectrolytes and, thus, the cytotoxic effects can be even more potentiated. So, future work in these systems should have this in consideration and much lower polyelectrolyte concentrations must be used.

The results from the Live/Dead assay correlate well with the results obtained through the resazurin reduction assay. However, at 0h, for the PAH at the highest concentrations, cells should look red (since the percentage of metabolic activity obtained in the resazurin reduction assay was near zero) but appear green under the fluorescence microscope. For the CHI, the contrary happens: cells should look green (since low but positive values were obtained for the percentage of metabolic activity using the resazurin reduction assay) but appear red under the fluorescence microscope. This is in line with some of the results obtained with the encapsulated cells for which the cell viability results assessed by the two methods were frequently in disagreement.

Indeed, the two methods are often used to evaluate cell viability but they are based in different principles: the resazurin method is dependent on the activity of intracellular enzymes (metabolic activity) and the Live/Dead assay is dependent on the extension of cell membrane damage (PI, being hydrophilic, is excluded from the membrane of viable cells, whereas FDA, being non-polar, can pass through them). A possible explanation for the disagreement between these two methods (which mainly occurs at the beginning of cell culture, just after the end of cell/polyelectrolyte contact) is that cell membrane deterioration does not occur exactly at the same time as enzymes lose their activity. So, the resazurin reduction assay correlates well with the Live/Dead assay only for later times after polyelectrolyte exposure.

Table 6 - Effect of polyelectrolyte concentration on hMSCs viability ("dose-effect" studies) obtained through the resazurin reduction assay. Results are shown as a percentage of the control values. Cell viability was analysed along 7 days.

Polymer	PAH				ALG				CHI			
	0h	1 day	4 days	7 days	0h	1 day	4 days	7 days	0h	1 day	4 days	7 days
500µg/mL	0.3±0	1.9±1	0±3	0±2	53.3±8	59.8±7	105.5±13	89.9±4	20.4±2	19.2±7	24.9±28	52.5±20
100µg/mL	0.4±1	2.2±1	0±1	1.1±2	47.0±3	55.7±4	95.9±7	84.6±3	53.0±3	38.8±10	15.3±18	40.8±24
50µg/mL	2.5±1	2.8±1	6.8±6	37.3±19	52.0±10	60.1±6	91.3±7	92.7±7	52.4±9	44.0±9	23.1±9	55.5±12
10µg/mL	33.7±9	37.3±5	84.1±9	72.5±14	53.9±6	61.9±3	99.9±6	84.4±9	71.2±15	83.0±10	104.2±3	94.5±6
1µg/mL	52.8±12	63.0±6	96.3±5	94.8±4	60.4±8	75.6±11	90.8±10	81.9±5	63.0±7	84.2±12	101.3±2	101.8±2
500ng/mL	61.3±1	73.8±4	95.0±11	87.0±8	59.1±6	67.8±3	93.9±9	85.6±7	63.8±6	81.3±8	98.6±7	98.0±5
250ng/mL	64.9±4	73.9±3	93.0±6	92.0±5	72.4±17	72.4±4	97.6±7	84.7±2	78.5±7	87.1±7	100.4±5	103.0±9
50ng/mL	58.0±7	76.3±5	101.0±6	90.0±6	85.2±18	96.0±18	103.1±4	100.4±2	72.1±9	89.6±7	102.9±18	99.8±7
0ng/mL	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0

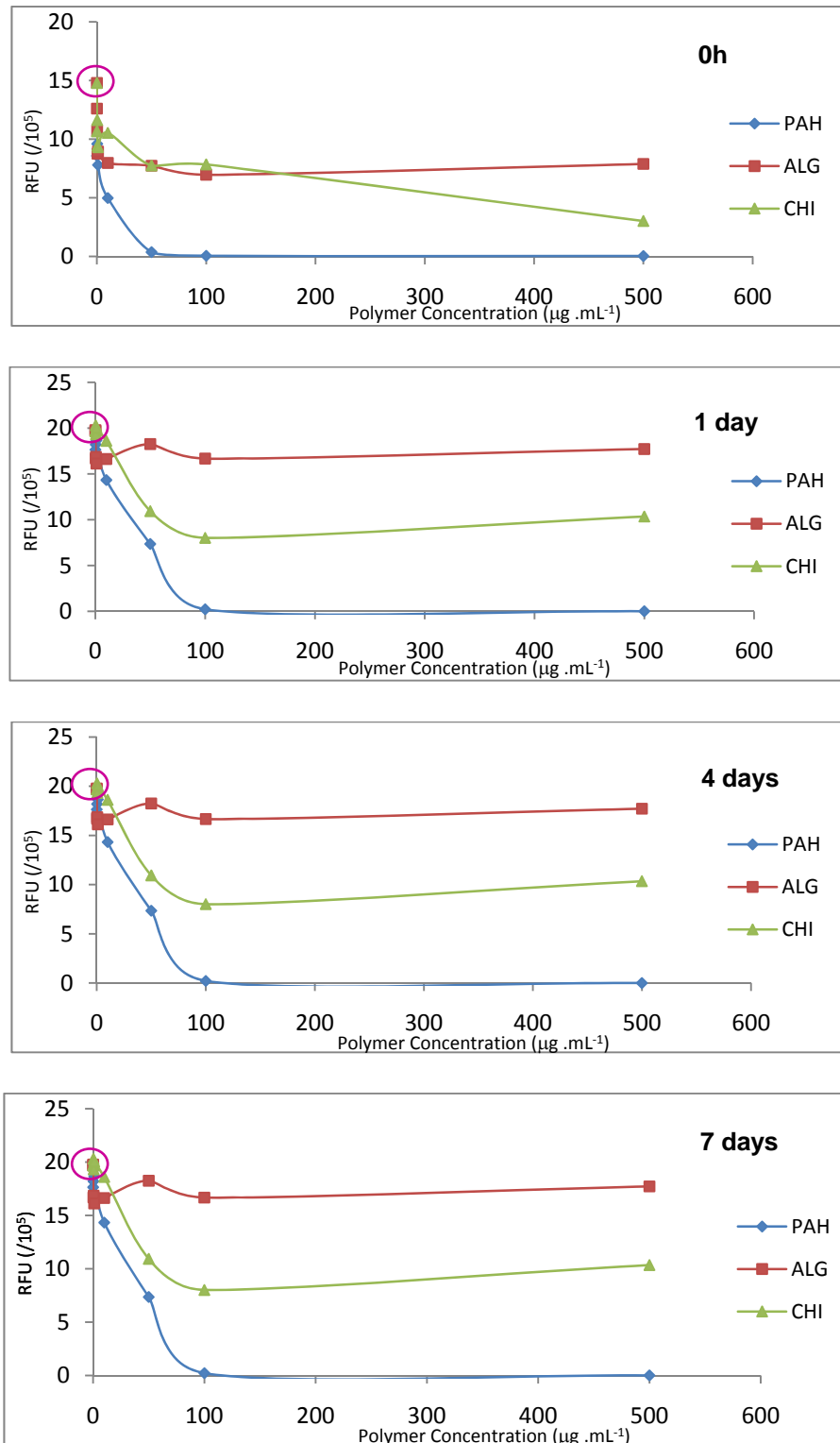


Figure 36 - Effect of polyelectrolyte concentration on hMSCs viability (“dose-effect” studies) obtained through the resazurin reduction assay. Cell viability was analysed along 7 days. The value of RFU for control is highlighted with a pink circle. The same control was used for the three polymers study, thus there is an overlap of values.

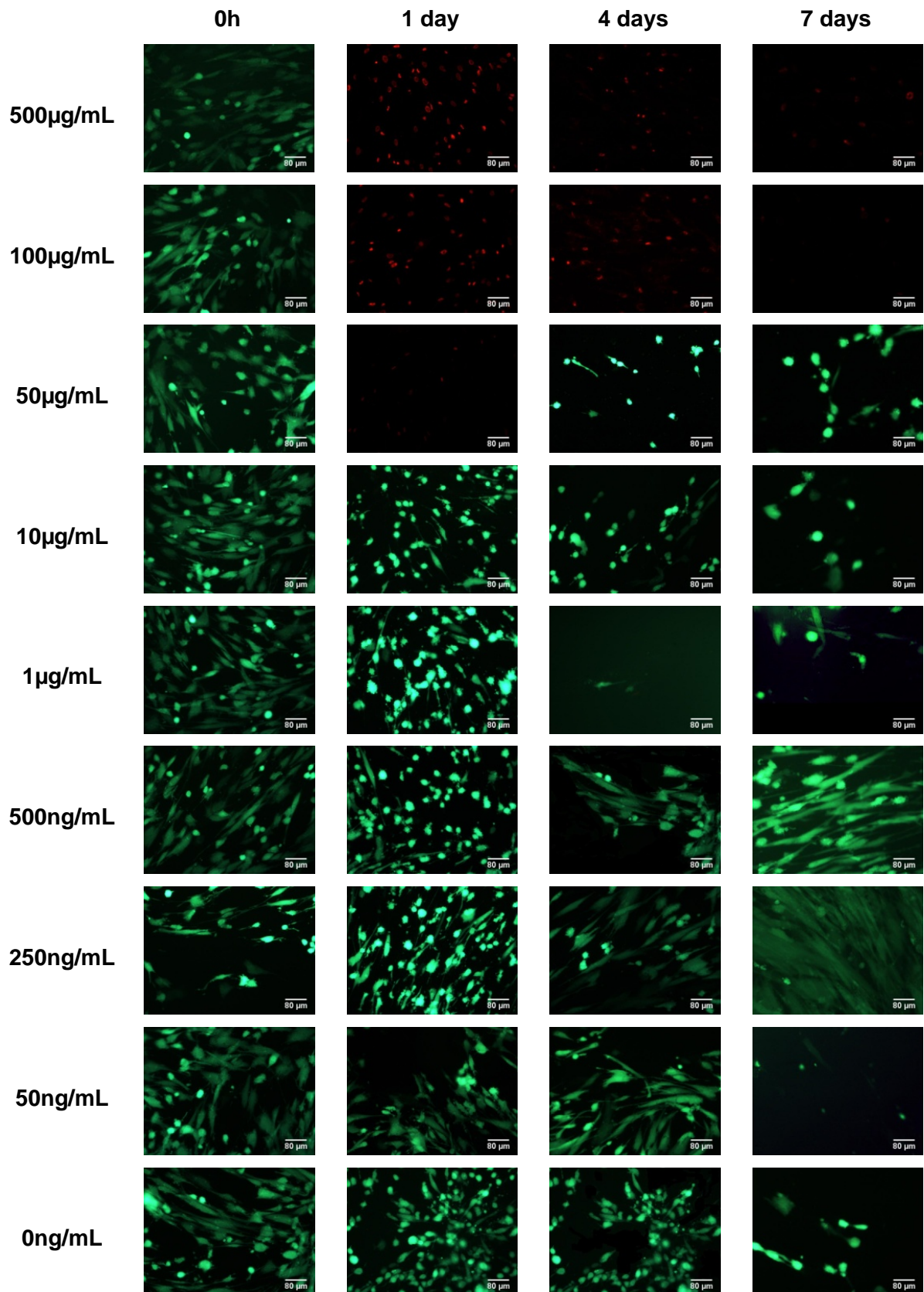


Figure 37 - Effect of PAH concentration on hMSCs viability obtained through the Live/Dead assay. Cell viability was analysed along 7 days. Red cells are dead; green cells are alive.

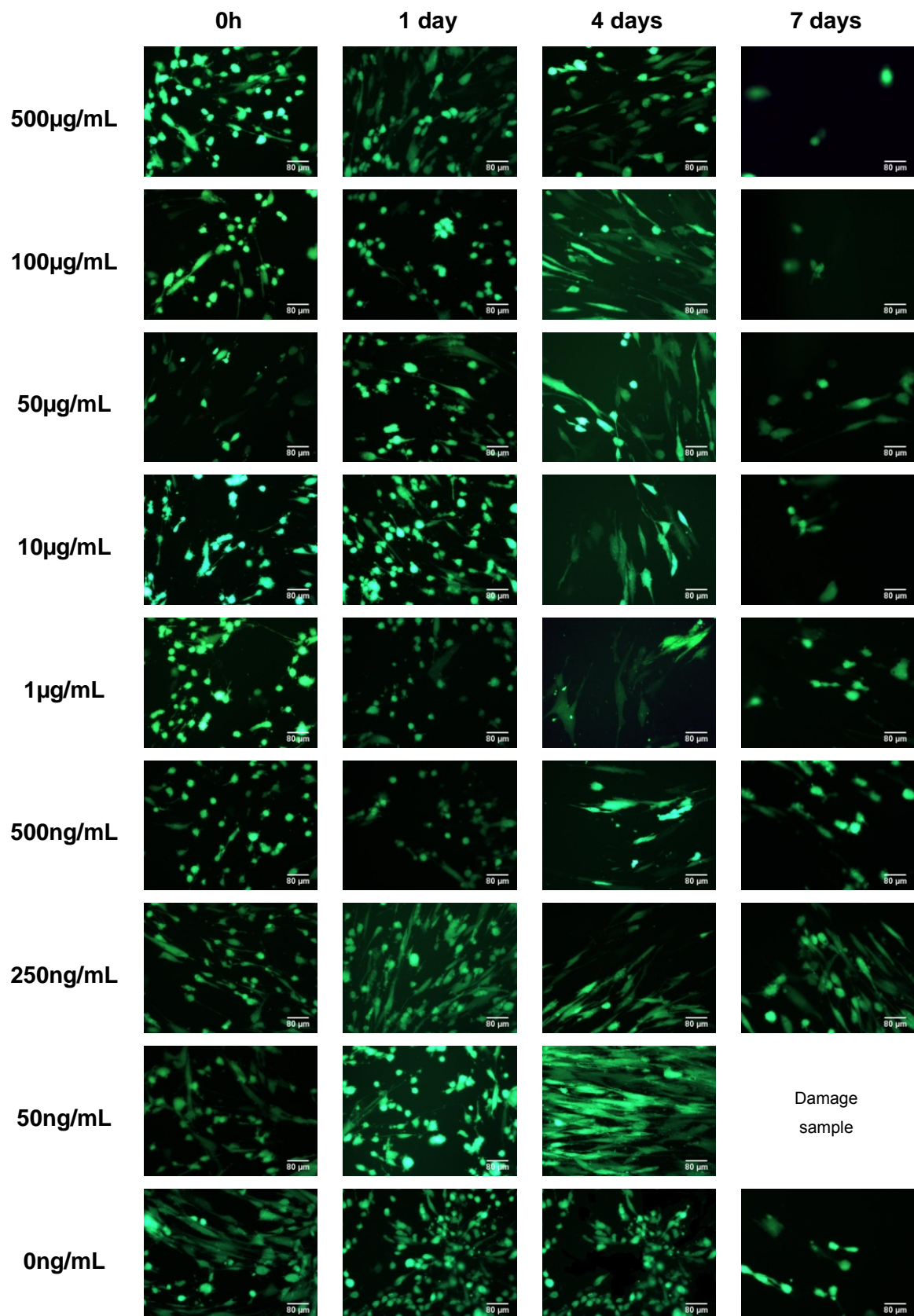


Figure 38 - Effect of ALG concentration on hMSCs viability obtained through the Live/Dead assay. Cell viability was analysed along 7 days. Red cells are dead; green cells are alive.

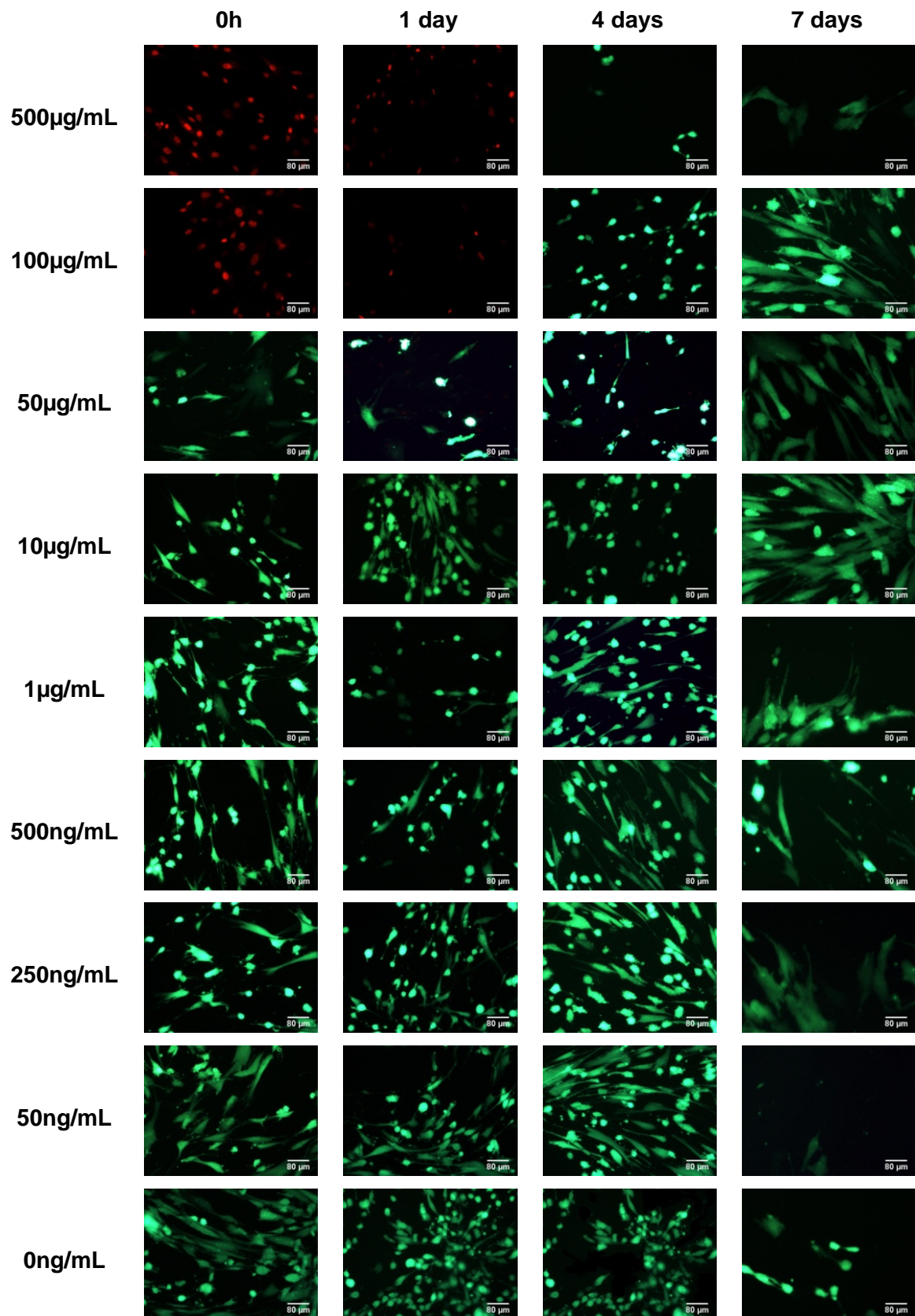


Figure 39 - Effect of CHI concentration on hMSCs viability obtained through the Live/Dead assay. Cell viability was analysed along 7 days. Red cells are dead; green cells are alive.

### 3.7 Cell encapsulation using polyelectrolyte solutions of lower concentration

A last experiment was done in the scope of the Master work which consisted in the encapsulation of single hMSCs within 1 bilayer of CHI/ALG (since these polyelectrolytes presented lower cytotoxicity than PAH) but using, in the LbL process, solutions with low concentrations of polyelectrolytes. Experiments were done with solutions with concentrations of CHI and ALG of 50ng/mL and 1 $\mu$ g/mL (much lower concentrations than the one used in the initial encapsulation experiments). As usual, the viability of the encapsulated cells was studied using the resazurin reduction assay and the Live/Dead assay. Results are shown in Table 7 and Figure 40. Once again, the cells were not able to retain their viability once encapsulated within the polyelectrolyte layers.

Overall, taking in consideration the previous and these last results, one must conclude that the viability of the encapsulated cells is not only dependent on the cytotoxic characteristics (or combined cytotoxic characteristics) of the polyelectrolytes but it seems that, when detached from the culture plates, the cells become too fragile and lose their viability very easily. Even when adherent to the cell culture plates, the simple process of forming multiple polyelectrolyte layers over them, gives rise to a significant lost of metabolic activity (Figure 32). In future experiments, special care should be taken with the manipulation of cells outside the incubator, for instance, maybe it is desirable to reduce the time of contact between the cells and the polyelectrolyte solutions and, globally, to shorten all the process.

Table 7 - Cell viability results after 0h, 1 day and 3 days in culture using the resazurin reduction assay. hMSCs were encapsulated within 1 bilayer of CHI/ALG polyelectrolytes. Experiments were done with solutions with concentrations of CHI and ALG of 50ng/mL and 1 $\mu$ g/mL. Results are expressed as a percentage of the control.

	Control	50ng/mL	1 $\mu$ g/mL
0h	100 $\pm$ 0	2.0 $\pm$ 0	2.1 $\pm$ 1.9
1 day	100 $\pm$ 0	0 $\pm$ 3.4	0 $\pm$ 3.6
3 days	100 $\pm$ 0	0 $\pm$ 0.5	0 $\pm$ 1.1

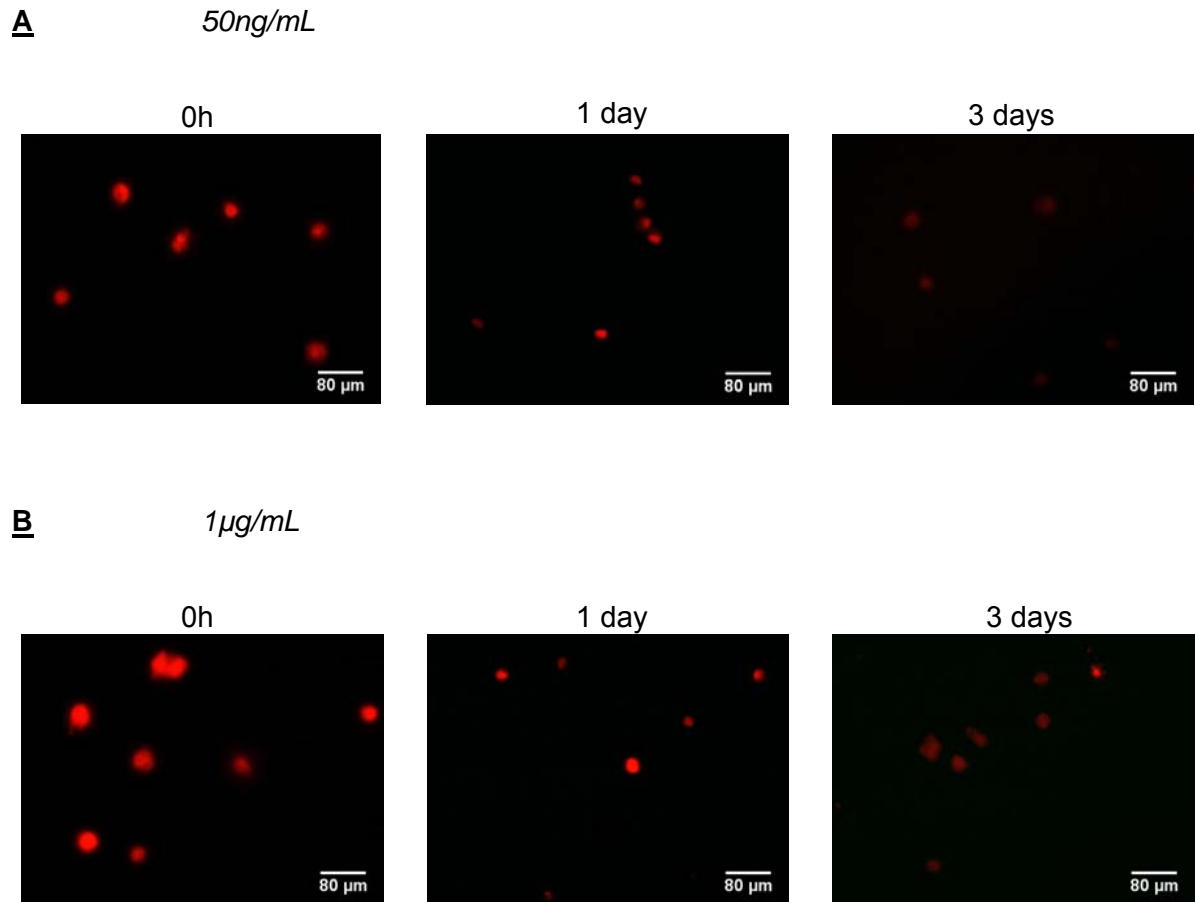


Figure 40 - Evaluation of the viability of hMSCs encapsulated within 1 bilayer of CHI/ALG using the Live/Dead assay after 0h, 1 day and 3 days in culture. Experiments were done with solutions with concentrations of CHI and ALG of 50ng/mL and 1µg/mL. Red cells are dead.

## CHAPTER 4 – GENERAL CONCLUSIONS

The main objective of this project was to encapsulate single viable cells within polyelectrolyte films using the Layer-by-Layer (LbL) technique. Despite the various attempts made to achieve this goal, cells lose their viability once encapsulated, independently of the pair of polyelectrolytes used and of the concentration of the polyelectrolyte solution applied in the LbL process.

However, even if the objective was not fully accomplished, the work developed in the scope of the present Master thesis allowed interesting observations and to draw some conclusions, namely:

- a) It was possible to obtain single encapsulated cells via the LbL technique as proven by fluorescence microscopy, using fluorescent labelled polyelectrolytes and staining the cell nuclei with the fluorescent dye DAPI; this possibility was tested with success for different pairs of polyelectrolytes (CHI/ALG, PAH/ALG and PAH/HA) and for different cell types (NIH 3T3 cells, rat MSCs and human MSCs); capsules aggregation was an observed problem which, despite the efforts to design an experimental procedure to avoid this situation (namely, by playing with cell concentration and different means of re-suspending and stirring the cells), was not completely overcome;
- b) Low concentration polyelectrolyte solutions should be used in future experiments concerned with animal cell encapsulation through the LbL technique (or whenever there is an interaction between the animal cells and the polyelectrolytes); one can conclude this from the “dose-cytotoxic effect” profiles obtained for CHI, ALG and PAH; this results further revealed that the PAH was the more cytotoxic polyelectrolyte, followed by CHI and, then, by ALG when hMSCS were used;
- c) Along the project, cell viability was assessed using the resazurin reduction assay (based on the evaluation of the metabolic activity of the cells) and the Live/Dead assay (based on the extension of cell membrane damage); due to their different nature, the cell viability information obtained by these methods was only correlated for later times after polyelectrolyte exposure;
- d) Overall, results also point out that when detached from the culture plates, the cells become too fragile and lose their viability very easily; in the future, shorter methods (less time outside the incubator, less time in contact with the polyelectrolyte solutions, etc.) must be devised if the objective is to attain single viable cells encapsulated using the LbL technique.

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**Figure 6** - [http://upload.wikimedia.org/wikipedia/commons/thumb/b/bb/Chitosan\\_Synthese.svg/712px-Chitosan\\_Synthese.svg](http://upload.wikimedia.org/wikipedia/commons/thumb/b/bb/Chitosan_Synthese.svg/712px-Chitosan_Synthese.svg). Access on: 15<sup>th</sup> June 2010.

**Figure 7** - <http://www.madsci.org/posts/archives/2001-04/986571103.Bc.r.html>. Access on: 15<sup>th</sup> June 2010.

**Figure 8** - <https://chempolymerproject.wikispaces.com/Collagen-D-apml>. Access on: 15<sup>th</sup> June 2010.

**Figure 9** - [www.sigmaaldrich.com](http://www.sigmaaldrich.com). Access on: 20<sup>th</sup> June 2010.

**Figure 10** - [www.scq.ubc.ca/stem-cell-bioengineering/](http://www.scq.ubc.ca/stem-cell-bioengineering/). Access on: 17<sup>th</sup> April 2010.

**Figure 14** - <http://cool.conservation-us.org/coolaic/sg/bpg/annual/v14/bp14-04.html>. Access on: 1<sup>st</sup> August 2010.

**Figure 15** - <http://commons.wikimedia.org/wiki/File:DAPI.gif> ; [http://en.wikipedia.org/wiki/File:1D30\\_DNA\\_DAPI.png](http://en.wikipedia.org/wiki/File:1D30_DNA_DAPI.png). Access on: 1<sup>st</sup> August 2010.

**Figure 17** - <http://onlinelibrary.wiley.com/doi/10.1002/chem.v9:23/issuetoc>; [http://en.wikipedia.org/wiki/Propidium\\_iodide](http://en.wikipedia.org/wiki/Propidium_iodide). Access on: 16<sup>th</sup> May 2010.

**Figure 19** - [www.biocompare.com/images/bc/006/ArticleImages/71785\\_Ap\\_No\\_Cell\\_3.jpg](http://www.biocompare.com/images/bc/006/ArticleImages/71785_Ap_No_Cell_3.jpg). Access on: 14<sup>th</sup> May 2010.

## APPENDICES

### ANIMAL CELL CULTURE (TECHNICAL ASPECTS)

#### 1.1 *Introductory aspects*

The culture of tissues was made for the first time, at the beginning of this century as a tool to study the animal cell behavior, without the influence of systematic variations. Usually, the term tissue culture includes cell culture of dispersed cells taken from the initial tissue and this process can be made by using mechanical, enzymatic or chemical methods [1].

In 1907, the frog was chosen by Harrison as his first source of tissue, because this animal is a cold-blooded animal, hence the incubation was not necessary. Moreover, he thought that the tissue of this kind of animal, would grow more easily, comparing with mammalian animals, since tissue regeneration was more usual in lower vertebrates. His idea provided a new interest in the cultivation of tissues *in vitro*. However, the warm-blooded animals were also a field of interest, which normal and pathological development were closer to what happens in humans. Among different kinds of tissues, many of them growing well in culture, the embryonated hen's egg was a favourite choice. However, with the experimental development of the animal husbandry, especially rodents, the mammalian come to the forefront as a better material. The rodent tissues have the advantage of producing continuous cell lines and a considerable set of transplantable tumours. The increment of transgenic mouse technology with the well-established genetic studies of the mouse, gave a new impulse to the selection of this animal as a favourite species. The human tumours can also give rise to continuous cell lines like the rodent tissues, which provided a new interest in human tissue, aided later by the classical studies of Leonard Hayflick, molecular geneticists and virologists [1].

The development of the cell culture occurred due to the needs of two major types of medical research: the production of antiviral vaccines and the understanding of neoplasia. The cell culture has also applications in cancer research, in the study of cell interactions and intracellular control mechanisms in cell differentiation and development, neurological and chromosomal analysis, among others [1].

Nowadays, there is a strong interest in growing stem cells *in vitro*. The stem cells have two important characteristics that distinguish them from other types of cells: first, they are unspecialized with the capacity to renew themselves for long periods of time through cell division to at least one daughter cell; secondly, submitted to certain physiological or experimental conditions, they can be induced to differentiate (these signals include chemicals secreted by other cells, physical contact with neighbouring cells, and contact with molecules in the environment), which means that they can divide themselves into cells with special functions. Most specialized cells present in our body (such as muscle cells, blood cells, or nerve cells) do not replicate themselves. The supply of these cells is maintained by stem cells, which replicate very often and then differentiate into specialized cells that are necessary. So, the cells are continuously replenished, when others die. This is called homeostasis [2].

The use of stem cells is very common in bioengineering, and the major goal is to understand and possibly control the differentiation of these cells. If this can be reached, a huge amount of therapeutic applications can be considered. One potential application is the generation of different types of neurons for the treatment of Alzheimer's disease, spinal cord injuries, or Parkinson's disease. The production of heart muscle cells for heart attack survivors could also be possible. Stem cells could also be useful for a number of tissue engineering applications such as production of complete organs including livers, kidneys, eyes, hearts, or even parts of the brain. Other areas that would benefit from a better understanding and control of stem cell proliferation *in vitro* are: drug testing, cancer research, and fundamental research on embryonic development [3].

## 1.2 Cell Culture

The process of growing cells in the laboratory is known as cell culture. The cells divide and spread over the surface of the Petri dish (Figure 41) [1]. But this aspect will be referred and explained more ahead in this chapter.



Figure 41 - Cell culture. The cells spread over the Petri dish surface.

Before starting a cell culture, there are many aspects that should be considered: the culture environment, cell adhesion, proliferation (cell cycle), differentiation and energy metabolism [1].

The environment on the culture influences it in three ways: (i) the nature of the substrate or phase on or in which the cell grow, which could be solid, (as in a monolayer growth on plastic), semisolid, (as in a gel such as collagen or agar), or liquid, (as in a suspension culture); (ii) the physic–chemical and physiological nature of the medium and (iii) the incubation temperature [1].

Concerning cell adhesion, most of the cells from solid tissues grow as adherent monolayers, otherwise they are transformed or cancer cells. It was found that the cells have more probability to attach to or spread on glass that had a slight net negative charge. In addition, it was also found that the cells attach to some plastics as well, such as polystyrene, if the plastics were treated with an electric ion discharge or high energy radiation. Nowadays, it is known that the adhesion of the cells is mediated by specific cell surface receptors, for molecules presented in the extracellular matrix. The receptors involved in cell–cell adhesion are CAMs ( $\text{Ca}^{2+}$  independent) and cadherins ( $\text{Ca}^{2+}$  dependent). The cell substrate interactions are mediated primarily by integrins (e.g

fibronectin, entactin, collagen, laminin) and proteoglycans. The integrins are receptors with signal domain, while the proteoglycans don't (Figure 42) [1].

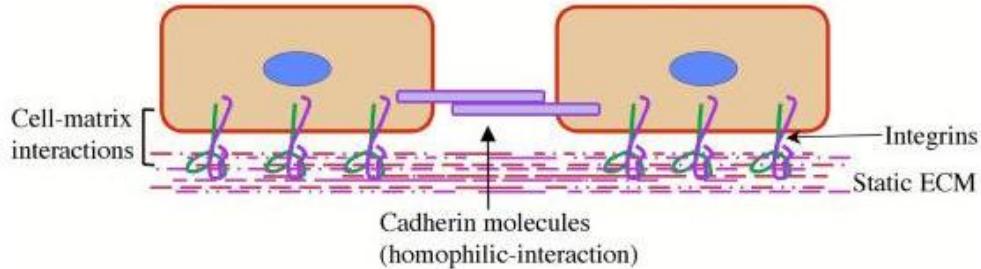


Figure 42 - Substrate-cell and cell-cell interactions.

The cell proliferation is related to the cell cycle. It is composed by four phases: M phase (M=Mitosis), G1 phase (G=Gap), S phase (S=Synthesis) and G2 phase (Figure 43) [1].

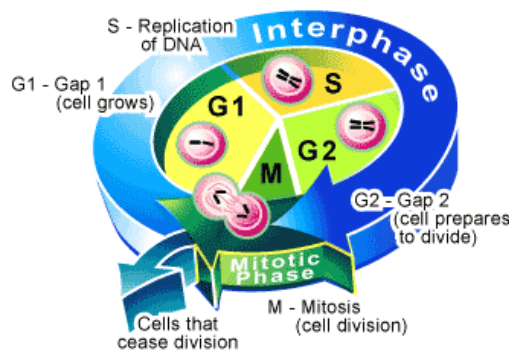


Figure 43 - Cell Cycle phases.

Very briefly, in the M phase, the chromatin concentrates into chromosomes, and the two single chromatids (which constitute the chromosome) separate in each daughter cell [1]. The Mitosis process is a phenomenon in which one cell gives rise to “daughter” cells, and it is constituted mainly by four stages: (a) prophase; (b) metaphase; (c) anaphase and (d) telophase (Figure 44) [4].

In the Prophase, the chromosomes are irregularly distributed in the nucleoplasm. These chromosomes have two molecules of DNA strictly equal. During this phase, the chromosomes are shortened, becoming less thick and compact, and consequently, more visible. In the Metaphase, the chromosomes are located in the equatorial plan, through its centromeres. The Anaphase is characterized by polar rise of each “daughter” chromosomes (each chromatid moves to opposite poles of the cell), resulting in the

separation of the metaphase chromosomes. The Telophase is characterized by the reorganization of the nuclear membrane of the “daughter” cells, which surrounds the chromosomes. This phase is completed by the individualization of the “daughter” cells (Figure 44, Figure 45) [4].

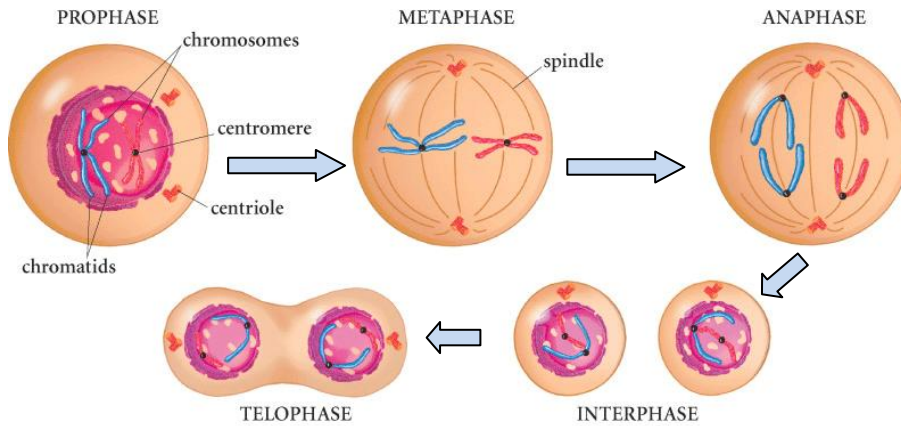


Figure 44 - Mitosis phases.

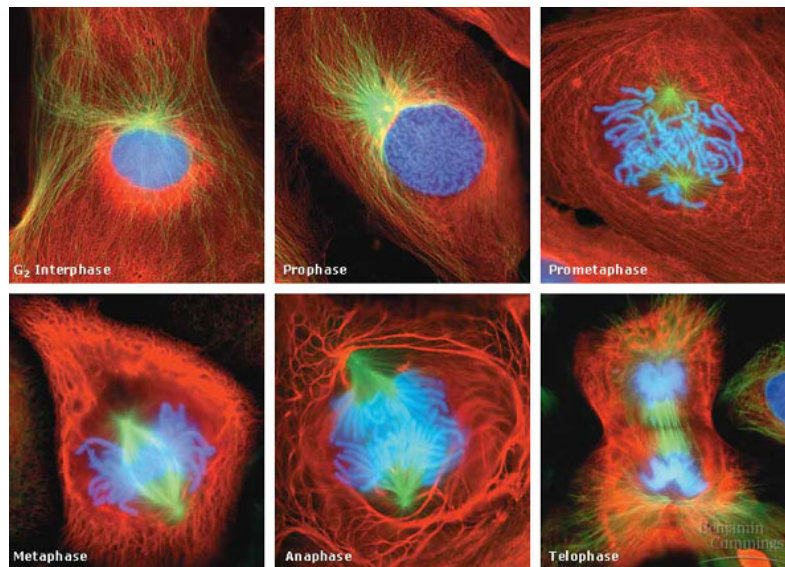


Figure 45 - Mitosis of a fibroblast (skin cell).

In the G<sub>1</sub> phase, there are the synthesis of several molecules (such as enzymes, proteins, RNA) and the formation of many cellular organelles. In the S phase, occurs the DNA synthesis and replication. It is in the G<sub>2</sub> phase, that the cell get ready for re-entry into mitosis [1].

It is possible to control the cell proliferation. If we maintain a low cell density, cells are capable to spread on the plate, which means that they will enter into their cell cycle. On the other hand, if a high cell density remains, the proliferation is inhibited, they start to grow over each other and the result is the change of their shape and the reduction of spreading [1].

For differentiation happen, some conditions are required: a high cell density, an enhanced cell–cell and cell–matrix interactions and the presence of several differentiation factors [1].

The energy metabolism is also important, because the cells need energy sources to live. Most culture media is composed by 4 to 20mM of glucose, which is used by cells as a carbon source for glycolysis, resulting in lactic acid as an end product. Some amino acids can also be used as an energy source, namely the glutamine, by the oxidation to glutamate by glutaminase enzyme, which allows entering to the citric acid cycle by transamination to 2–oxoglutarate [1].

The main advantages of tissue culture are the control of the physiological environment (pH, temperature, osmotic pressure, O<sub>2</sub> and CO<sub>2</sub> tension) and physiological conditions which can be kept relatively constant, but cannot always be defined. Other advantages are the regulation of the matrix, cell–cell interaction and gases diffusion; the availability of selective media, between others. On the other hand, there are limitations, such as the chemical and microbial contamination; the containment and disposal of biohazards; the dedifferentiation and selection; the instability, between others [1].

For cell culture, there are many culture vessels. The substrate materials include glass and disposable plastic. The glass is the original substrate, due to its optical properties and surface charge, but it has been replaced by synthetic plastic (e.g. polystyrene) in most laboratories. The polystyrene presents a greater consistency and superior optical properties than glass, that's why glass is rarely used, nowadays. However, glass still has some advantages like the price; it can be washed without losing its growth–supporting properties and can be sterilized easily by dry or moist heat. Concerning the disposable plastic (e.g. polystyrene), the use of this kind of material provides a simple and a reproducible substrate for cell culture. These materials have a good optical quality and present a flat growth surface, providing a reproducible and uniform culture. This substrate material doesn't have a charge on its surface which provides a cell growth (hydrophobic), so it is treated by  $\gamma$ -irradiation, chemically or with an electric ion discharge to produce a charged surface. Other examples of plastic substrate materials are polyvinylchloride (PVC), polytetrafluorethylene (PTFE), thermanox (TPX), between others [1].

There many culture vessels such as: multiwell plates (6, 12, 24, and 96 wells), petri dishes, flasks and stirrer bottles with different sizes and capacities [1].

To obtain a cell growth in suspension, it can be used any kind of plate, flask or Petri dishes without treatment for cell attachment, but sterilized [1].

### 1.2.1 Primary Culture and Subculture, Primary Explant

The term “primary culture” is relative to the stage of the culture after the isolation of the cells, but before the first subculture. There are three stages to refer: (i) isolation of the tissue; (ii) dissection and/or disaggregation, and (iii) culture after seeding into the culture vessel (Figure 46). After the isolation of the tissue, the primary cell culture may be formed by two different ways: (1) the cells can migrate from the tissue fragments, adhering to the plate surface and (2) the tissue can be disaggregated mechanically or enzymatically (e.g. trypsin, collagenase, DNase, dispase), in order to obtain a cell suspension, where some of them will attach to the surface plate. Most types of cells grow attached to the plate surface, otherwise, they are transformed cells (implies a spontaneous or induced permanent phenotype change), which survive and proliferate in suspension. The attachment of cells to each other and to the surface plate is caused by the presence of glycoproteins and  $Ca^{2+}$ , in their surface. The presence of adhesion and antitrypsin factors in the serum also contributes to cell attachment [1, 5].

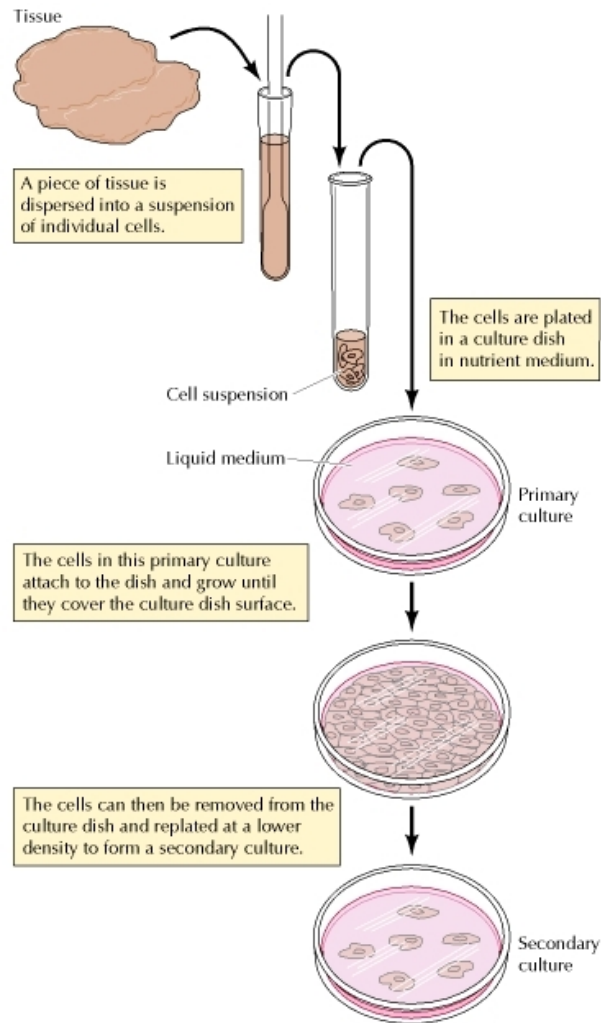


Figure 46 - Scheme about Culture of animal cells.

The first “subculture” (or passage, or transfer) is very important to the cell culture. To proceed with the subculture, the surface plate of the primary culture must be all occupied with cells (confluence). There are other reasons to proceed with a subculture, such as: the exhaustion of culture medium; the time since the last subculture and when the cells are needed for other experimental procedures. The passage number represents the number of times that the culture was subcultured. The color of the medium can indicate if the cells need to be subcultured or not (when the color medium becomes less reddish) [1, 5].

Relatively to the “primary explants”, this technique is especially useful for small amount of tissue, where it is risky to lose most of the cells in the enzymatic or mechanical procedures. It can be any part of the tissue from an animal. Briefly, the tissue is handled in a sterile ambient (Figure 47); usually the tissue should be minced (1), and the pieces are

put in a cell culture dish, containing culture media (2). Over time, the progenitor cells migrate out of the tissue onto the surface of the dish (3). These primary cells can then be further expanded and transferred into new Petri dishes [1, 5].

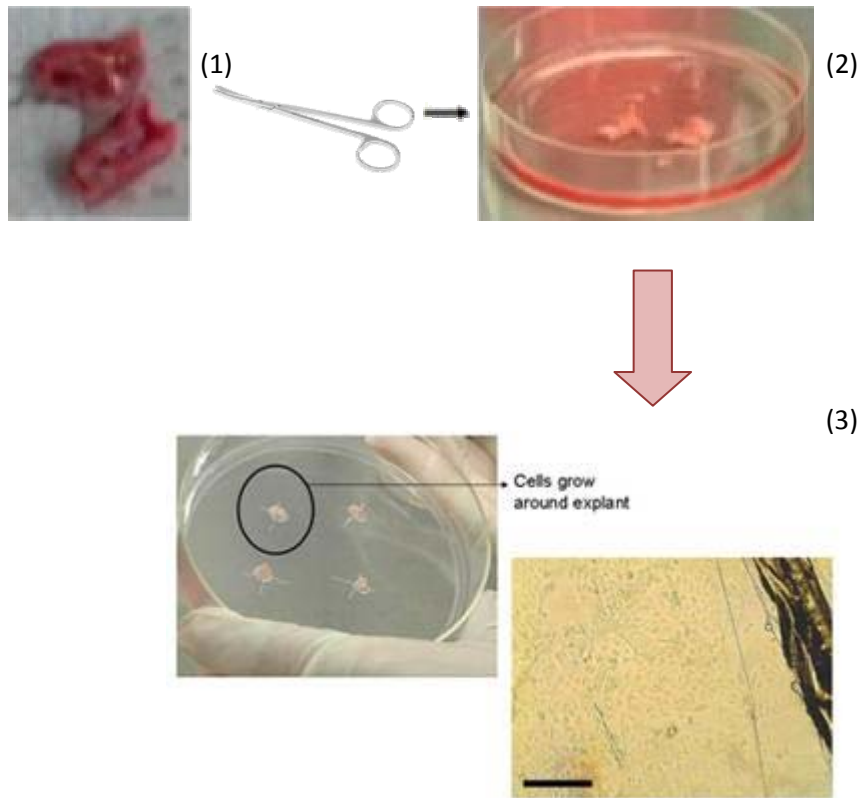


Figure 47 - Schematic procedure of an explants (human bronchial epithelial cells grown from explants).

## 1.2.2 Cell Lines

A cell line represents a primary culture after the subculture. The attainment of a cell line from a primary culture requires: (a) an increase in the total number of cells during several generations and (b) the predominance of cells or cell lineages with the capacity for high growth, resulting in (c) a degree of uniformity in the cell population [1].

The next scheme show the ways how can we create a cell line (Figure 48).

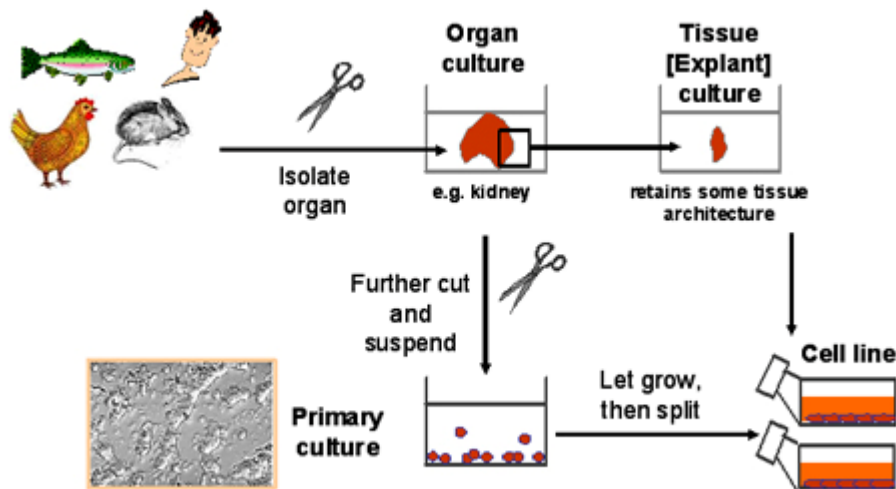


Figure 48 - How can a cell line be derived?

The cell lines can be propagated as an adherent monolayer or in suspension. The monolayer culture means that the cells will grow attached to a substrate (they are anchorage dependent, which means that it is necessary a substrate for cells proliferation). This kind of culture is the most common one, except for hematopoietic cells. On the other hand, the suspension culture doesn't require a substrate for cells growth, they have the ability to survive and proliferate without attachment (anchorage-independent). This type of culture is only applied for certain kinds of cells, such as hematopoietic cells (like was referred before), transformed cells lines and for malignant tumour cells [1].

### 1.2.3 Cell Growth Curve

After the subculture, the cells obey to a pattern growth, which can be represented in a graph (Figure 49), constituted by three stages: (i) lag phase; (ii) exponential or log phase and (iii) stationary or plateau phase [1, 5].

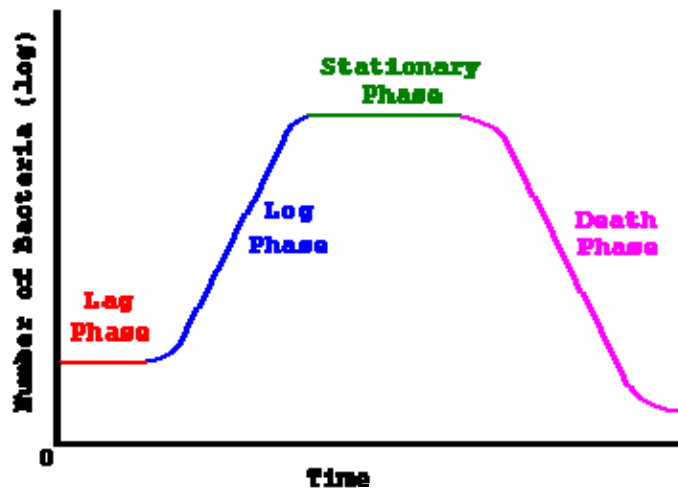


Figure 49 - Growth curve of bacteria (for instance).

Briefly, the lag phase corresponds to the adaptation of the cells, where they replace the adhesion factors lost by the trypsinization process, attach again to the surface plate and spread out.

During spreading, the cells also synthesize again new DNA and structural proteins. The exponential or log phase represents the period of an exponential growth, increasing a lot the number of cells. This growth will depend on the seeding density and the growth rate of the cells, mainly. Relatively to the last phase, the growth rate is reduced, which means that the surface plate is confluent (when all the surface plate is occupied by cells again). In this case, the cells must be passed to another plate, if it doesn't happen the cells will die ("Death Phase") [1, 5].

### 1.2.4 Cell Medium

The cell medium depends of the type of the cells. Eagle's Minimal Essential Medium [Eagle, 1959] is the mostly used, variously supplemented with human, horse or calf serum, protein hydrolysates, and embryo extract [1].

In the development of a media, there are many physicochemical properties to be considered: pH, CO<sub>2</sub> and bicarbonate, buffering, oxygen, osmolality, temperature, viscosity, surface tension and foaming. Giving emphasis to the pH, most of cell lines grow well at 7.4. However, for some types of cells, the pH changes a little bit. For example, for normal fibroblast lines, the optimum pH is between 7.4-7.7, while for the transformed ones, the better pH is between 7.0-7.4. The CO<sub>2</sub> % is also important, because as a buffer, it tends to maintain a relatively constant pH and it can counteract any force that would change it. In this system, the carbon dioxide (CO<sub>2</sub>) combines with water to produce carbonic acid (H<sub>2</sub>CO<sub>3</sub>), which in turn, rapidly dissociates to originate hydrogen ion and bicarbonate (HCO<sub>3</sub><sup>-</sup>), according to the reaction below [1].



The media is constituted by many components: aminoacids (e.g. L-alanine, L-histidine, L-proline; between others), vitamins (e.g. folic acid, biotin, menadione, riboflavin, between others), salts (e.g. CaCl<sub>2</sub>, KCl, MgSO<sub>4</sub>, between others), antioxidants (glutathione), bases and nucleosides (e.g. adenosine, ATP, thymine, cytidine, between others), lipids (e.g. cholesterol, lipoic acid, linoleic acid, between others), glucose, between others components [1].

The amounts of each component will depend of the type of the medium and to which kind of cell is destined. The concentration of the amino acids normally limits the maximum cell concentration that it is possible to be attained, and the balance could influence the cell survival and the growth rate. The glucose is present in most culture medium, as a source of energy. This sugar is metabolized mainly though glycolysis to produce pyruvate, which could be converted to lactate or acetoacetate and can be used in the citric acid cycle to form CO<sub>2</sub> [1, 4].

The final medium also contains serum (10%, usually) which contributes with growth factors for cell proliferation and with adhesion factors, which promote cell attachment. The most used types of serum are from calf, fetal bovine, horse or human. Serum is also a source of minerals (e.g. calcium, potassium), lipids (e.g. fatty acids, phospholipids) and hormones (e.g. insulin, hydrocortisone). The preparation of the culture medium also

includes beyond serum, the *antibiotics* (1%, normally) (usually penicillin and streptomycin), to avoid contamination sources (Figure 50) [1].

Although, if it is used properly equipments like the laminar-flow chamber, jointly with an aseptic technique, the addition of antibiotics in the medium is unnecessary. Even because, the antibiotics have some disadvantages, like: (a) they allow the appearance of antibiotic-resistant organisms; (b) they posses antimetabolic effects that could provide crossreactivity in mammalian cells; (c) they encourage for not utilize an aseptic technique, accordingly [1].



Figure 50 - Alpha-MEM; Serum; Antibiotics: Penicilin and Sptreptomycin, respectively.

### 1.2.5 Cell Counting

A hemacytometer is a graduated counting chamber that can be visualized under a microscope, to determine the concentration of cells in suspension. It is used usually for counting blood cells, but these chambers are widely used also in cell culture to determine the concentration of cells in a suspension. This equipment has some style variations. The most common one is called “Neubauer” type chamber. The instrument is made of ground glass with a central area that is defined by a set of grooves that form an ‘H’ shape. Two counting areas with ruled grids are separated by the horizontal groove of the H. The glass coverslip is held at 0.1 mm above the surface of the counting areas by ground glass ridges on either side of the vertical grooves of the H shape (Figure 51, Figure 52) [6].

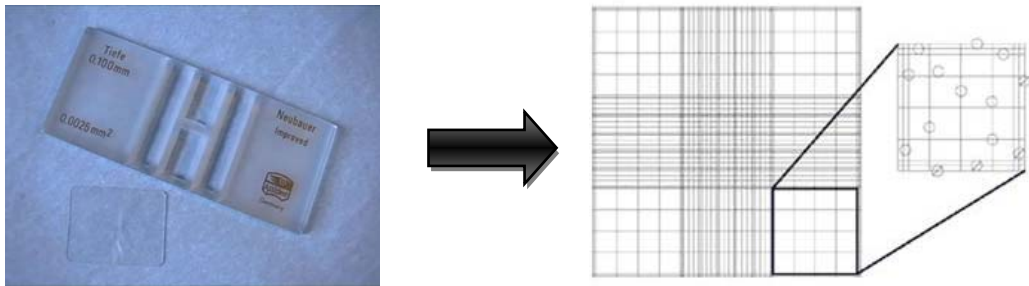
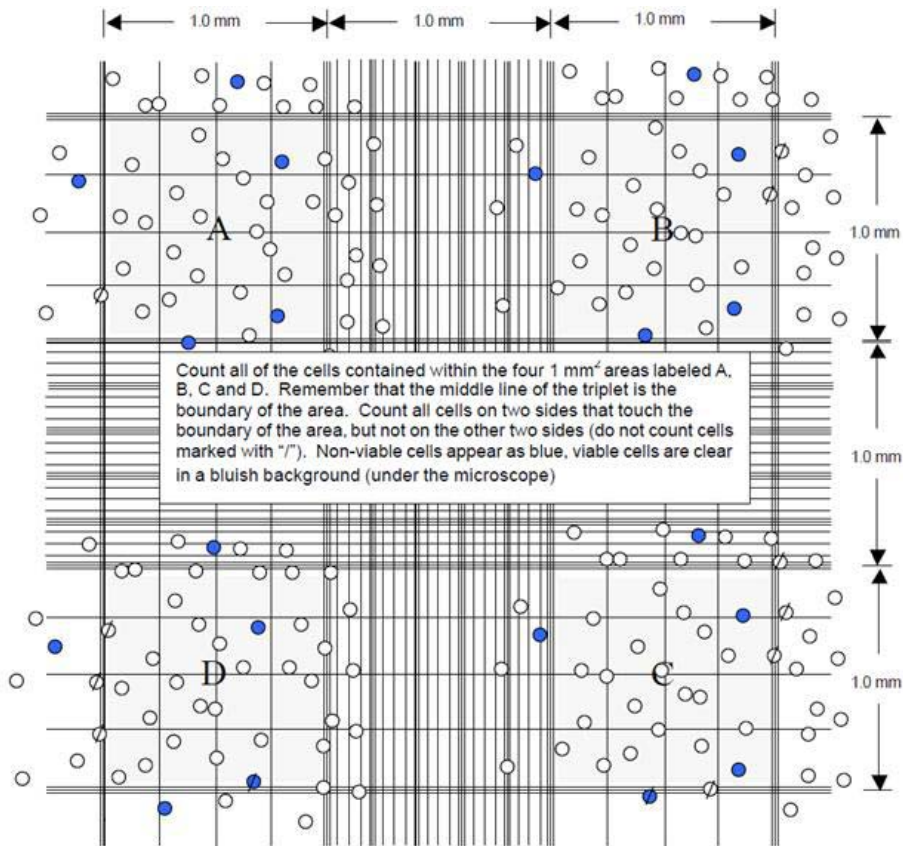


Figure 51 - Neubauer chamber.

Figure 52 - Scheme where it is possible to see the counting area. The blue cells are non-viable (when stained with *trypan blue*, and visualized in a Microscope), the other ones are viable [6].

### 1.3 Aseptic technique

The contamination by microorganisms such as bacteria, mycoplasma, yeast and fungal spores still is the major problem in tissue culture. These microorganisms may be introduced through the operator, the work surfaces, the solutions, the atmosphere, among other sources. A correct aseptic technique should provide a barrier between microorganisms in the environment outside the culture and the “pure” (uncontaminated

culture within its flask or dish). Hence, all the materials that will interact directly with the cells must be sterilized. So, aseptic technique is a combination of procedures in order to reduce the probability of infection [1].

The elements of an aseptic environment include quiet area, work surface, personal hygiene, reagents, media and cultures [1].

It is advisable to choose a quiet area with little or no traffic and no other activity, in absence of laminar flow hood. With this equipment, the picked area should be restricted to tissue culture, free of dust, clean and should not contain other equipments not related to tissue culture [1].

About the work surface, it must be clean every time, it should be swabbed with 70% alcohol. Other rules should be considered such as, mop up any spillage immediately and swab the area with 70% alcohol and only bring the material needed to a particular procedure. After finishing the work, everything should be removed and the work surface should be swabbed down again [1].

Washing hands will reduce adherent microorganisms, which are the most risk for cells cultures. Gloves can be used and swabbed frequently with ethanol. The hair should be tied back, if it is long and the operator shouldn't talk if he's working in an open bench [1].

Reagents and media obtained commercially already have undergone to a strict quality control to ensure that they are sterilized. For the prepared solutions, they should be sterilized in the lab (autoclave). All the reagents bottles should be swabbed with alcohol before using inside the laminar flow hood [1].

About the cultures, they should be handled properly, which means that they must remain the less time possible outside the incubator, because when they are inside the laminar flow chamber, the % of oxygen and temperature decrease and these factors can contribute to the cell death. Other important detail is that the dish or cell plates shouldn't be completely closed, to allow the gas exchange between the cells and the environment [1].

## **1.4 Equipments in cell culture**

### **1.4.1 Autoclave**

The most used equipment to sterilize glassware and other materials is the autoclave (Figure 53). An autoclave is an airtight, steel pressure vessel used to heat substances under high pressure in chemical and industrial processes [7]. This equipment gives an automatic, programming and safety locking, which provides more flexibility [1].



Figure 53 - Autoclave Equipment.

Each autoclave installation is composed of the autoclave itself, along with its auxiliary equipment and systems, including: a vacuum system, a thermocouple system, a heating system, a pressure system, a control panel, a computer or PLC (programmable logic controller) system, an instrumentation system, a datalogging system and safety systems. Of course that there are many models of autoclaves, some of them don't have all the components referred before [7].

Before sterilization, the glass material should be capped with foil, as well as some plastic materials. The most plastic materials are isolated inside plastic little bags, closed hot. All this procedure should be done, using gloves [1].

#### 1.4.2 Laminar flow hood

A laminar flow hood is an equipment where we can work without contamination. The environment inside the chamber is protected because it has constant stable flow of filtered air (HEPA) passing over the work surface (Figure 54) [1].

There are two main kinds of flow: horizontal and vertical. In the first one, the airflow blows from the side facing the operator, parallel to the work surface while whereas in the vertical, the airflow blows down from the top of the chamber onto the work surface and is drawn through the work surface. The horizontal flow hood provides the best sterile conditions for the cell culture and reagents. Besides, it is cheaper than the vertical one. On the other hand, the vertical chamber gives more protection to the operator. The equipment surfaces must be clean regularly, with 70% alcohol, mainly after being used [1].

This equipment also requires a UV light, which should be turned on during 20 minutes before using it and turned off before starting to use the laminar flow hood. This UV light will exterminate the organisms which can be a source for contamination [1].



Figure 54 - Laminar Flow Chamber.

### 1.4.3 CO<sub>2</sub> Incubator

The incubator is the equipment where we can keep the cell culture vessels, in optimum conditions for their maintenance (Figure 55). These conditions are related mainly, to the CO<sub>2</sub> % and the temperature. Usually, the temperature is about 37°C (physiological temperature), and 5% of CO<sub>2</sub> is maintained in the air environment. These equipments should be cleaned weekly or monthly, by removing all the contents (including the trays and the shelves), with usually 70% ethanol [1].



Figure 55 - Incubator representation.

## 1.5 Cryopreservation

Cryopreservation is a process where cells or whole tissues are preserved, by cooling them in low sub-zero temperatures, such as (usually) 77K or -196 °C (the boiling point of liquid nitrogen) (using special dewars) (Figure 56). At these low temperatures, everything that would lead to cell death is stopped (biological activities, including the biochemical reactions). This phenomenon can cause damage to cells, mainly during the freezing stage, problems like: intracellular and extracellular ice formation and dehydration. Many of these effects can be reduced by using cryoprotectors agents [5].

There are many factors which advance a good cell survival, after freezing and defrosting, some of them are: (i) the cell density at freezing should be between  $1 \times 10^6$  and  $1 \times 10^7$  cells/mL; (ii) the presence of DMSO or glycerol (5-10%) to protection; (iii) the slow cooling:  $1^\circ\text{C}/\text{min}$ , down to  $-70^\circ\text{C}$  and then a rapid transfer to liquid nitrogen freezer; (iv) the rapid defrosting, among others [5].



Figure 56 -  $\text{N}_2$  (l) bottle.

## 1.6 Inverted Fluorescence Microscope

An inverted fluorescence microscope is a useful tool to visualize and study cells. Fluorescence techniques are suitable for probing living cells, due to their sensitivity and specificity. An image and a photometric signal can be obtained, from a single cell, so this kind of microscopy has a great potential for qualitative and quantitative analysis related to the structure and functions of the cells. The most important advances which contribute to the use of this technique for single living cells are: (i) probes for specific structures or environmental parameters; (ii) methods for delivering fluorescent probes into living cells and (iii) methods for detecting weak fluorescence signals for living cells [8, 9].

Nowadays, fluorescence microscopy is one of the most important tools for the examination of the cells and cellular constituents. Fluorescent probes and fluorescently marked immunological probes give us the ability to visualize and quantify basic structures within the cells [8, 9]. Concerning its operation, in most cases the sample of interest is labeled with a fluorescent substance known as a fluorophore and then illuminated through the lens with the higher energy source. The illumination light is absorbed by the fluorophores (attached to the sample) and causes them to emit a longer lower energy wavelength light (Figure 57) [8, 10].



Figure 57 - Left: Fluorescence Microscopy; Right: Fluorescence Microscopy mechanism [10].

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## ***Credits List***

**Figure 41** - [http://molprobe.com/images/sub\\_images/](http://molprobe.com/images/sub_images/). Access on: 21<sup>th</sup> April 2010.

**Figure 42** - [www.biosignaling.com/content/figures/1478-811X-3-7-2.jpg](http://www.biosignaling.com/content/figures/1478-811X-3-7-2.jpg). Access on: 25<sup>th</sup> April 2010.

**Figure 43** - [www.scq.ubc.ca/wp-content/cellcycle.gif](http://www.scq.ubc.ca/wp-content/cellcycle.gif). Access on: 25<sup>th</sup> April 2010.

**Figure 44** - <http://rushartsbiology.wikispaces.com/Visuals+-+Unit+5> Access on: 25<sup>th</sup> April 2010.

**Figure 45** - [www.doddhollow.com/biology/lab8.php](http://www.doddhollow.com/biology/lab8.php). Access on: 25<sup>th</sup> April 2010.

**Figure 46** - [www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=cooper&part=A128](http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=cooper&part=A128). Access on: 10<sup>th</sup> May 2010.

**Figure 48** - [www.eawag.ch/organisation/abteilungen/utox/schwerpunkte/projektuebersicht/projekt1](http://www.eawag.ch/organisation/abteilungen/utox/schwerpunkte/projektuebersicht/projekt1) Access on: 10<sup>th</sup> May 2010.

**Figure 49** - <http://web.deu.edu.tr/atiksu/toprak/curve.gif>. Access on: 10<sup>th</sup> May 2010.

**Figure 51** - <http://dk.cryosinternational.com/media/4267/neubauer.jpg>. Access on: 10<sup>th</sup> May 2010.

**Figure 53** - [http://upload.wikimedia.org/wikipedia/commons/c/cc/Autoclave\(1\).JPG](http://upload.wikimedia.org/wikipedia/commons/c/cc/Autoclave(1).JPG). Access on: 10<sup>th</sup> May 2010.

**Figure 54** - [www.aircleansystems.com/images/product/process/laminar-flow-bench-1.jpg](http://www.aircleansystems.com/images/product/process/laminar-flow-bench-1.jpg). Access on: 25<sup>th</sup> April 2010.

**Figure 55** - [www.sanyo-biomedical.co.uk/NewsCO2FDA.htm](http://www.sanyo-biomedical.co.uk/NewsCO2FDA.htm). Access on: 30<sup>th</sup> July 2010.

**Figure 56** - [www.biw.kuleuven.be/dtp/tro/cost871/home.htm](http://www.biw.kuleuven.be/dtp/tro/cost871/home.htm). Access on: 30<sup>th</sup> July 2010.

The work was written using 'Arial' letter type, with size 11, and a spacing of 1.5 between lines. The pages margins are: left: 3cm; right: 2.3cm.

Two different softwares were used: "NIS-Elements AR" (Fluorescence Microscopy) and "Wallac 1420 Workstation" (Microplate Reader). For the treatment of some images obtained from the Fluorescence Microscope, the ImageJ Program was used.