Universidade do Minho
Escola de Engenharia

Ana Cristina Jesus Costa

Study of stability of multilayers films based on natural polymers

outubro de 2015

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Study of stability of multilayers films based on natural polymers

Dissertação de Mestrado
Mestrado em Propriedades e Tecnologias de Polímeros

Trabalho efetuado sob a orientação da
Professora Doutora Natália Alves

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Declaração

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Thanks!

Abstract

The use of natural polymers in areas such as tissue engineering has increased thanks to relevant properties exhibited by these as biocompatibility or biodegradability, leading to the development of new therapies in this area.

Tissue engineering has the main objective to regenerate or replace damaged tissues having as methodology the cell culture on support structures, scaffolds. These should be optimized favoring cell growth and avoiding immune responses when implanted.

In the present work, multilayer films of chitosan and alginate were prepared using the layer-by-layer technique. In order to investigate the stability of these films to the action of enzymes, *in situ* tests were made through the microbalance quartz crystal (QCM-D) technique, at distinct pH. Moreover, the degradation for long times (until 30 days) was analysed for the first time by the QCM-D technique. The morphology of the produced films after degradation was analyzed by SEM. The experiments allowed to conclude that the constructed films $(CHT/ALG)_7$+CHT are stable when they were immersed for 3 days in PBS as well as in enzymatic solutions, at either pH 5.5 or 7.4 and at 25^{o}C. It was also found that mass loss due to degradation is only evident after 7 days in the presence of lysozyme.

Resumo

A utilização de polímeros naturais em áreas como a engenharia de tecidos tem aumentado, graças às propriedades relevantes apresentadas por estes tais como biocompatibilidade ou biodegradabilidade, levando ao desenvolvimento de novas terapêuticas nesta área.

A Engenharia de tecidos tem como principal objetivo regenerar ou substituir tecidos lesados tendo como metodologia o cultivo de células sobre estruturas de suporte, os scaffolds. Estes devem ser otimizados de forma a favorecerem o crescimento celular e evitando respostas imunitárias quando implantados.

Neste trabalho, filmes multicamada de quitosano e alginato, foram preparados utilizando a técnica de layer-by-layer. De forma a investigar a estabilidade destes filmes sob a ação de enzimas, foram realizados testes *in situ* através da técnica de microbalança de cristais de quartzo (QCM-D), variando o pH. Além disso, a degradação para longos períodos (até 30 dias) foram analisados pela primeira vez pela técnica de QCM-D. A morfologia dos filmes produzidos após a degradação foi analisada por SEM. Através das análises efetuadas foi possível concluir que os filmes $(CHT/ALG)_7$ + CHT são estáveis quando estes são imersos durante 3 dias em solução de PBS, assim como em soluções enzimáticas a pH 5.5 ou 7.4, a 25 °C. Verificou-se também que a perda de massa devido à degradação só é evidente após 7 dias na presença de lisozima.

Contents

List of Abbreviations and Acronyms

ALG - Alginate

AFM – Atomic force microscope

CHT – Chitosan

DNA – Deoxyribonucleic acid

LbL – Layer-by-Layer

NaCl – Sodium chloride

PBS – Phosphate buffered saline

PEC – Polyelectrolyte Complex

QCM-D – Quartz crystal microbalance with dissipation monitoring

SEM – Scanning electron microscope

TE – Tissue Engineering

List of Figures

List of tables

Chapter 1

Introduction

Chapter 1 General Introduction

1.1 Motivation

In 1993, Langer and Vacanti presented for the first time the concept of Tissue Engineering as being “an interdisciplinary field that applies the principles of engineering and life science toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ”. Tissue engineering is based in three components: cells, scaffolds and grow factors and the objective is replacing or restoring function in diseased or injured tissues. Tissue engineering emerged as an alternative to tissue and organ reconstruction.

Scaffold is one of the most important components of tissue engineering. It has the function support to cells culture, under certain conditions, until obtaining a functional tissue. In parallel the implant material should degrades and the by-product shall be metabolized out from the body. This is a great challenge involving the design of a scaffold due to the number of variables that need to be considered.

A large variety of polymers, with different structures and properties, has been used for many different applications. Chitosan, hyaluronic acid or sodium alginate are examples of polymers that exhibit relevant properties of biomaterials such as biocompatibility and biodegradability, and where the synergy between the materials is very important to build materials which later can be used in the biomedical area, including tissue engineering.

In this work, the use of natural polymers in the biomedical area will initially be explained in chapter 1, focusing essentially on tissue engineering which is the main propose for the chitosan and alginate film studied in this thesis. It also addresses the importance of degradation in polymers being more detailed with the polymers used.

A description of all the material and techniques used in film stability studied in this work is made in chapter 2. Already in Chapter 3 presents, the description of the work performed, the results obtained and their discussion.

Finally, in chapter 4 are presented all conclusions obtained with this experimental work and some proposals for future works.

1.2 Polysaccharides

Replacement of fossil feedstocks with renewable ones is one of the main challenges of modern plastics industry. Natural polymers represent a specific class of materials among polymers based on natural resources. Typical examples are cellulose, hemicelluloses, lignin, silk and starch. Another class of materials consists of the natural-based or bio-based synthetic polymers, the monomers of which derive from renewable resources. [1,2]

Polysaccharides are macromolecules formed by the union of several monosaccharides. In nature, polysaccharides have various resources: from algal origin (e.g. alginate), plant origin (e.g. pectin, guar gum), microbial origin (e.g. dextran, xanthan gum) or animal origin (chitosan, chondroitin).[3]

Figure 1.1 - Polysaccharides resources.

Polysaccharides have been found to participate in many biological processes, such as cell–cell communication, embryonic development, infection of bacteria and/or virus, and cellular immunity.[4,5] The biological effects that polysaccharides can exert are limited to therapeutic activities for diseases of humans and animals, and toxic activity responsible for causing human and animal disease. Although polysaccharides have been used for decades in various industrial applications, e.g. pharmaceuticals, biomaterials, foodstuff and nutrition, and biofuels, growing understanding and deeper investigations

of the importance of polysaccharides in life science are driving the development of polysaccharides for novel applications.[6]

The use of natural polysaccharides and/or their derivatives are especially attractive because of their availability, renewability and low toxicity. Moreover, the usual biodegradability and biocompatibility of these natural polymers, coupled with their capacity for chemical modification confers them ideal properties for their use in biomedical applications such as tissue engineering scaffolds or drug release systems. [7–10]

1.3 Tissue engineering

Tissue engineering has evolved since the appearance of the idea of combining cells and scaffolds to create artificial tissues with the objective to develop biological substitutes to replace, restore and regenerate defective tissues and organ of the human body. Tissue engineering emerged as a potential alternative therapeutic process to treat severely injured patients with minimally invasive techniques. Commonly, TE strategies utilize combination of cells, biodegradable scaffolds, and bioactive molecules to recapitulate natural processes of tissue regeneration.[11,12] Cooperative interplay of the components is imperative to achieve biologically functional engineered tissue and this method is contingent upon the development and implementation of advanced biomaterials.

In tissue engineering the main objective are the scaffolds because these should present adequate biological, chemical, physical and mechanical properties adapted the physiological conditions *in vivo*. Therefore, in their design must be taken in account several factors such as:

(I) Biocompatibility – the scaffold must be biocompatible; cells must adhere, function normally, and migrate onto the surface and eventually through the scaffold and being proliferative. After implantation, the scaffold must induce a negligible immune reaction in order to prevent it, causing such a severe inflammatory response.

(II) Biodegradability – scaffolds does not work as a permanent implant, so it's important to allow the body's own cells, over time, to eventually replace the implanted scaffold. Therefore, scaffolds must be biodegradable to allow cells to produce their own extracellular matrix. The by-products of this degradation should be non-toxic and able to exit the body without interference with other organs.

(III) Mechanical Properties – scaffolds should have mechanical properties consistent whit the anatomical site where they will be implanted. Producing scaffolds with adequate mechanical properties is one of the greatest challenges. In case of engineered bone and cartilage, the implanted scaffold must have sufficient mechanical integrity to function from the time of implantation to the completion of the remodelling process.

(IV) Scaffold architecture – scaffolds should have an interconnected pore structure and high porosity, to ensure cellular penetration and adequate diffusion of nutrients to the cells within the construct and to extra-cellular matrix formed by these cells. Additionally, a porous interconnected structure is required to allow diffusion of waste products out of scaffolds, and the products of scaffolds degradation should be able to exit the body without interference with other organs and surrounding tissues.

(V) Manufacturing technology – is important that scaffold to become clinically and commercially viable, therefore it should be cost effective and it should be possible to scale-up from making one at time in a research laboratory to small batch production.[13]

In Tissue engineering, many polymers have been investigated for the scaffolds construction, including synthetic polymers such as poly(glycolic acid) (PGA) or poly(lactic acid) (PLA).[14] These turned out to be a good solution but also have some disadvantages such as the risk of rejection when implanted due to reduced biocompatibility. Thus, the natural polymers are the best option to the scaffolds construction. These present the ability to promote the cell adhesion and growth as well as the fact that they are biologically active. Examples of these materials are collagen, alginate or chitosan. However the use of scaffolds fabricated from a single-phase biomaterial caused some problems due to their poor mechanical properties, which can make them difficult to use.

One solution to this problem is the combination of materials, where the properties of materials used can complement each other. One example is the combination between chitosan and alginate, which has been extensively studied in biomedical application, mainly in tissue engineering. Iwasaki *et al*. reported the use of alginate based chitosan hybrid polymeric fibers for scaffolds in cartilage tissue engineering.[15] Similar studies present chitosan-alginate scaffolds manufactured for cartilage tissue engineering.[16,17] Alves et. al. reported the self-assembling and crosslinking of polyelectrolyte multilayer films of chitosan and alginate[18] which presents the ability to this two polymers to form a novel biocompatible multilayer film based in alternative depositions of this. Baysal et. al. present chitosan/alginate crosslinked hydrogels: preparation, characterization and application for cells growth purposes.[19]

One of the factors that have enabled the development of tailored biomaterials using these polymers has been their potential to form a polyelectrolyte complex (PEC).[20] Polyelectrolyte complex are the association between oppositely charged particles through electrostatic interactions.[21]

Chitosan and alginate form a polycomplex membrane via ionic cross-linking (Figure 1.2) that exhibited tensile strength and yet lower elongation break, than polymers. This membrane also showed lower methanol permeability as compared to both chitosan and alginate alone.[22]

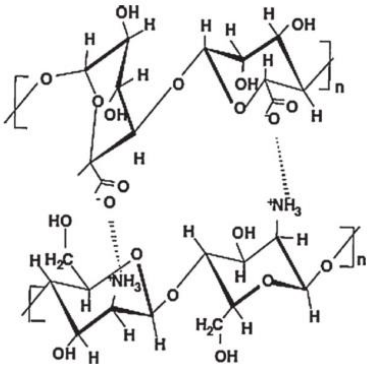

Figure 1.2 -- Polyelectrolyte complex of chitosan and sodium alginate.[22]

The combination of biocompatible, suitable mechanical and degradation properties constitutes one of the main advantages for the use of these two polymers, showing that they have potential to be used as scaffolds in tissue engineering.

1.4 Degradation

Degradation is understood as a chemical reaction, which causes the division of the polymer chains by physical or chemical agents, consequently leading to the modification of properties.

Two types of polymer degradation could occur, depending on the variables involved in the process: (1) in absence of living organisms – abiotic degradation; (2) in presence of microorganisms – biodegradation.

The abiotic degradation depends on abiotic factors such as sunlight, heat or humidity and from there can occurs hydrolysis or oxidation reactions can occurs.

Biodegradation is the degradation that occurs in natural polymers. This happens by the action of enzymes produced by microorganisms such as fungi or bacteria, which are both present in environment or animal organisms, and causes the conversion of organic compounds in more simple organic compounds.[23] So, enzymes will hydrolyze the polymer chains and there are variables that affect the biodegradation process such as temperature, light and pH.[24]

The degradation of polymers in biomedical applications is very important because it is necessary to ensure that no component or by-product of the degradation of polymers used can induce inflammatory or toxic reactions in the body.

1.4.1 Chitosan Degradation

When implanted in the human body, chitosan matrices are degraded due to the presence of lysozyme, in a process that involves two steps. When the matrix is immersed in the lysozyme solution, the water permeated into the matrix will cause swelling, known as hydration process. This is the first stage of degradation process. Due this

phenomenon, bonds cleavage will also occur resulting in the degradation of chitosan. When the swelling is maximum continual degradation leads to weight loss (second stage), the matrices tends to be thinner, and swelling degree decrease.[25] So, degradation and swelling are directly related, as illustrated in Figure 1.3.

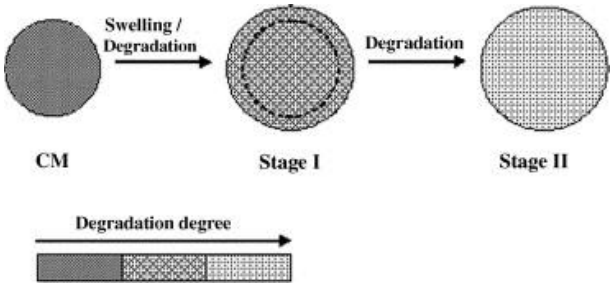

Figure 1.3 - Degradations stages[25]

Several studies have been published about the degradation of chitosan by enzymatic hydrolysis, in particular by the action of lysozyme.[26–30] In fact, it has been demonstrated that chitosan is degraded under the action of lysozyme, which exists in many organisms including the human body.[29] In this case, the normal concentrations of this enzyme, can range from 4 to 13mg/L in serums and 450 to 1230mg/l in case of tears.[25] It has been estimated that at least a normal subject produces 500 mg of lysozyme per day, but the lifetime of the protein in plasma is very short: approximately 75% is eliminated within 1h, mainly through clearance via the kidneys and only traces are detectable in the urine of healthy subjects. [30,31]

The products resulting from the degradation of chitosan are biocompatible chitosan oligosaccharides of variable length, as illustrated in Figure 1.4.[32]

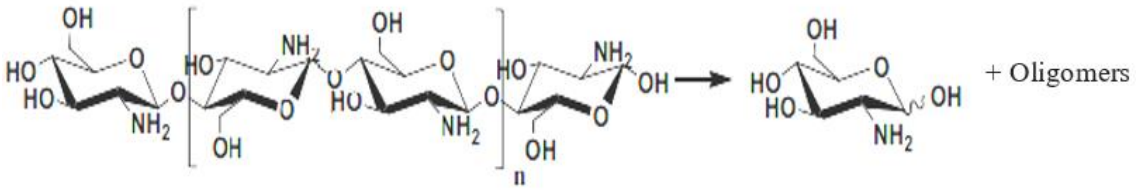

Figure 1.4 - Schematic illustration of chitosan hydrolysis.[33]

The activity of lysozyme increases with decreasing pH or increasing temperature in a certain range, which causes the environmentally dependent degradation behavior of chitosan- based biodegradable materials.[30,34]

1.4.2 Alginate Degradation

Alginate is synthetized abundantly in organisms as cell walls and the intracellular matrix of browns algae or bacteria. One of the most important areas where alginate is used is in biomedical applications in the form of hydrogels[35], microparticles[36], films[37] and fibers[38]. Products originated by the degradation of alginate exhibit biological functions as promotion of root growth in higher plants, enhancing penicillin production in *Penicillium chrysogenum*, promotion of proliferation of cells and bringing down blood pressure in human.[39]

Alginate degradation occurs via β-elimination of glycosidic bonds as illustrate in Figure 1.5. [40–42] A double bond is formed between the C4 and C5 carbons of the six-membered ring from which the 4-O-glycosidic bond is eliminated, depolymerizing alginate and simultaneously yielding a product containing 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid as the non-reducing terminal moiety.[43–45] Glycosidic linkages of alginate are susceptible to a variety of degradation mechanisms, including acid-, alkaline- and enzymatic catalyzed hydrolysis.

For this work, the form of degradation studied will be the enzymatic degradation by alginate lyase. This enzyme are specific for alginate degradation and the mechanism is similar to that of alkaline degradation to glycuronans.[46]

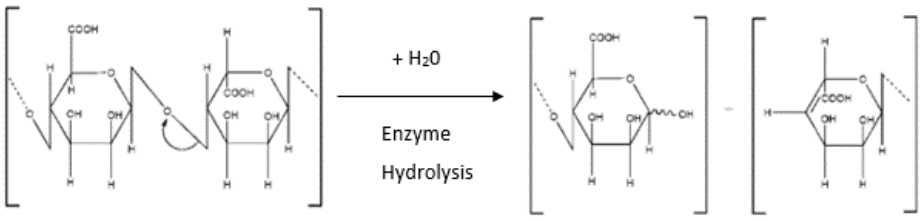

Figure 1.5 - Alginate degradation mechanism by alginate lyase (β-elimination.)[40]

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Chapter 2

Materials and Methods

Chapter 2 - Materials and Methods

2.1 Chitosan (CHT)

Chitosan is a linear, semi-crystalline polysaccharide, composed of (1→4)-2-acetamido-2-deoxy-b-D-glucan (N-acetyl D-glucosamine) and (1→4)-2-amino-2-deoxy- b-D-glucan (D-glucosamine) units, illustrated in Figure 2.1. This polysaccharide is not extensively present in the environment but can be easily obtained by deacetylation of a natural polymer- chitin. [1]

Chitin can be found in the shells of crustaceans, cell walls of green algae and yeasts and is therefore the second most abundant biopolymer in nature. [1,2]

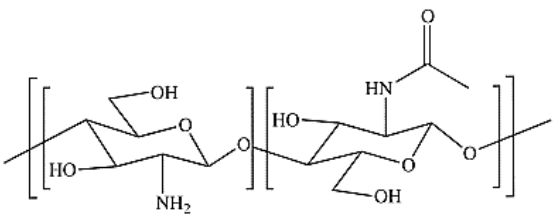

Figure 2.1 - Chemical structure of chitosan.

The presence of amino groups in the chitosan structure differentiates chitosan from chitin, and gives to this polymer many peculiar properties.[1] Chitosan, because of the semi-crystalline structure of chitin with extensive hydrogen bonds, is normally insoluble in aqueous solutions above pH 7. However when in dilute acids (pH<6), the free amino groups are protonated and the molecule becomes soluble (Figure 2.2), which opens prospects to a wide range of applications, particularly in the field of cosmetics. [3,4]

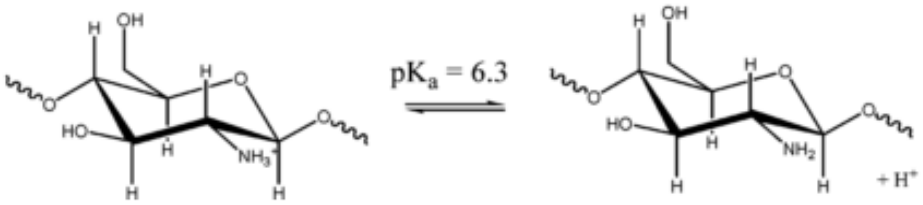

Figure 2.2- Protonation of chitosan.

Chitosan has a wide range of applications, as can be seen in Table 1. [5] This is caused by easy access to this natural resource, low cost and because it has a great advantage of being prepared in different forms such as hydrogels, microspheres and films.[6]

Principal application for chitosan	
Agriculture	Defensive mechanism in plants Stimulation of plant growth Seed coating, Frost protection Time release of fertilizers and nutrients into the soil
Water & waste treatment	Removal of metal ions Ecological Polymer (eliminate synthetic polymers) Reduce odors
Food & beverages	Not digestible human(dietary fibre) Blind lipids (reduce cholesterol) Preservative, fungistatic, antibacterial coating for fruits Protective
Cosmetics	Treat acne Improve suppleness of hair Reduce static electricity in hair Tone skin Oral care (toothpaste, chewing gun)
Biopharmaceutics	Immunological, antitumoral Haemostatic and anticoagulant Healing, bacteriostatic

Table 2.1- Principal applications of chitosan.[2]

Chitosan exhibits good biocompatibility and biodegradability and has been widely explored for applications in controlled drug delivery and tissue engineering.[7] It has been reported that it can stimulate the activity growth factors and play an important role as a structural component of scaffolds for tissue regeneration. [8] The molecular weight and degree of N-deacetylation (DD) are thought to be the two most important determinants of the properties of chitosan.

2.2 Alginate (ALG)

Alginate is a natural polysaccharide, consisting of linear chains of α-L-glucuronic acid (G) and β-D-mannuronic acid (M) and is found in marine brown algae.[9] It is the water solubility and present negative charge attribute for the carboxylic acid groups presents on its units – Figure 2.3.

Alginate is a biomaterial that has found numerous applications in biomedical science and engineering due to its favorable properties, including biocompatibility, biodegradability and non-toxicity. The fact that it is very abundant and provide a low cost are also very important factors.[10,11]

Alginate can be easily modified in any form such as hydrogels, microspheres, microcapsules, sponges, films and fibers. This property can increase the applications of alginate in various fields such as tissue engineering and drug delivery.

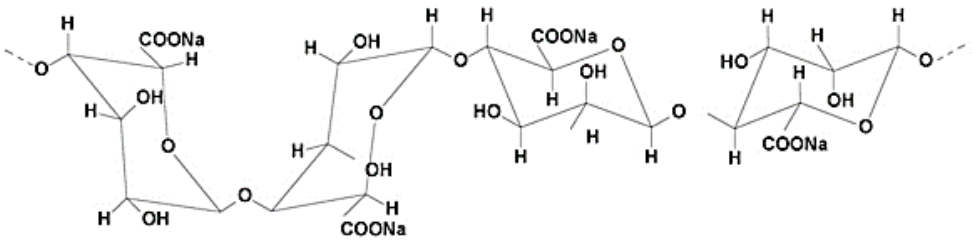

Figure 2.3 - Alginate Sodium (ALG). [12]

Alginate is also a known compound for its properties in the formation of scaffolds, and is therefore a very important compound in the treatment of organ failures or losses.[13]

The combination of chitosan and alginate is also widely known as alginate exhibits polyanionic behavior and when combined with a polymer who exhibits polication behavior can give highly stable films.[14]

2.3 Lysozyme

Lysozyme was discovered in 1992 by Alexander Fleming. This protein (Figure 2.4) can be found in many organisms like virus, insects, amphibious, reptiles, birds and mammals which are producer in many tissues and fluids such as breast milk, saliva, tears and it's productions is greater in polymorphonuclear leukocytes in humans, where lysozyme is very important in the defense against infections.

It has been reported that its anti-viral activity against the virus VIH type 1(test *in vitro*) may be associated with a positive charge and the interactions established on the surface of the virus when negatively charged.[15]

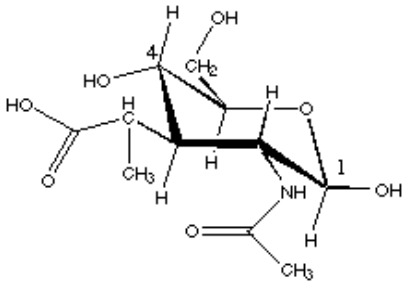

Figure 2.4 - Lysozyme structure.

2.4 Alginate lyase

Alginate lyases, also known as alginases or alginate depolymerases, catalyze the degradation of alginate by a β-elimination mechanism. Alginate-degrading enzymes with various substrate specificities have been isolated from many sources, including marine algae, marine mollusks, and a wide range of microorganisms. Alginate lyase activity has been reported in extracts obtained from several species of brown algae. These enzymes are promising for removing and modifying alginate.[16]

2.5 Techniques

2.5.1 Layer-by-Layer

Layer-by-layer (LBL) assembly technology, which can conduct the formation of thin films on distinct substrates by adsorbing oppositely charged polyelectrolytes or other species, has been widely used in the membrane fabrications and surface modifications.

Hammond 2012, reported the review about building biomedical materials layer-by-layer, presenting specific advantages of the use of Layer-by-Layer assembling versus traditional polimeric blends encapsulation. Also presented the impact and potencial of this technique in biomedical areas such as drug delivery or tissue engineering.

This technique is presented as an easy, facile, robust, reproducible, flexible, and efficient way of modifying membrane surfaces and fabricating highly ordered nanostructured thin films and nanocomposites with regular thickness, compositions, structures and properties.

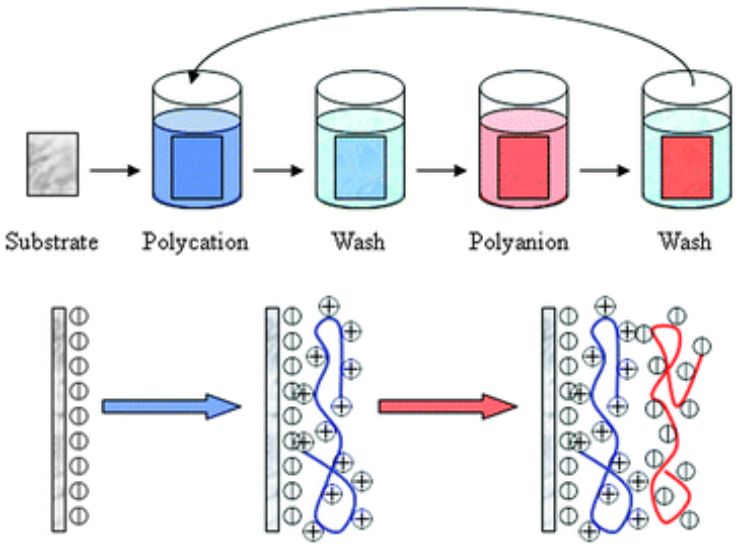

Figure 2.5- Layer-by-Layer film deposition.[17]

The LBL assembly process is comparatively simpler and environmental friendly as it shown in figure 2.5. The surface (glass, metal…) exhibits negative charge when in solution because surface oxidation and hydrolysis. When immersed in a solution of positive charged polyelectrolyte, electrostatic interaction reactions will occur which

cause the change of liquid surface charge becomes positive (polycation). The next step is to wash the surface for removing the polyelectrolyte that is not adsorbed to ensure the efficiency of the assembly process. Then the substrate is again immersed in a polyelectrolyte solution but now with negative charge and once again, it is washed.

Although the LBL assembly process requires many steps, these steps are always repeated and simple, including alternate immersion and washing procedures without chemical reactions. Therefore, this method allows control the number of layers required.

This technique was used in experimental work in two different situation. In first case, the depositions of seven bilayers more one last of chitosan, was realized in a glass support, to then be placed in contact with the enzyme solution in order to verify the mass lost. Weighing of the support was performed in three different times: before deposition, after deposition and finally after the defined degradation time.

The same thing as done but using as support the QCM crystals in order to use the quartz crystal microbalance offline, i. e., using the equipment only for measuring the frequency and dissipation of the crystals in a specific period of time (before deposition, after deposition and after degradation time). This is a innovate form to use QCM technique to study the degradation of very thin films.

2.5.2 Quartz Cristal Microbalance with dissipation monitoring (QCM-D)

The quartz crystal microbalance with dissipation monitoring (QCM-D) measure mass variations in the nanometric scale, thus allowing study the adsorption variations in layers of ultra-thin films or molecules. It is a very important when it's necessary to confirm the construction of films. This technique has attracted attention from researchers because it allows *in-situ* study of obtained results in real time, which is a huge advantage for film construction processes.[18]

This technique is based on the piezoelectric effect of quartz crystals. This effect is caused by the intrinsic properties of anisotropic and minerals such as quartz, which reacts to mechanical disturbances whit the creation of an electric field. Thus, when the crystal

oscillator is pressed on one of its faces, will cause a deformation of the crystal lattice and will cause the rearrangement. This will induce opposite charges on their faces, so generating an electric field within the crystal.[19]

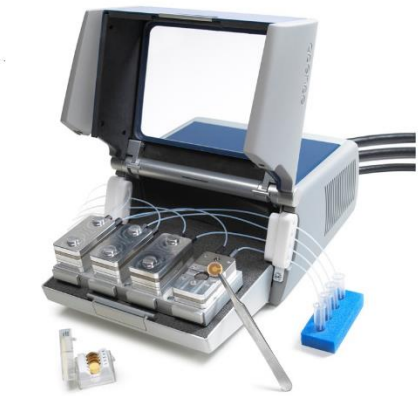

Figure 2.6 – Quartz Cristal equipment. [20]

When the crystal is coupled to the oscillator circuit and is applied an electrical current that has appropriate frequency characteristics and magnitude to the geometry of the crystal, it will oscillate in a manner called shear (wave propagation is perpendicular to the electric field). The range of frequencies that can be used in the crystals can be very high, which ensures high precision in the determination of the variations - Figure 2.6.

In 1959, G. Sauerbrey presented a mathematical relationship where a ratio between the mass change rigidly coupled to the crystal and the change in resonance frequency of a quartz crystal when subjected to oscillations by applying alternating current signals is established. According Sauerbrey relation, adsorption of mass on a thin quartz sensor surface, induces a decrease in which for a rigid substance translates into the increase in mass.[21]

$$\Delta m = \frac{-C.\Delta f}{n}$$

Where, C is the mass sensitivity constant (17.7 $ng.cm^{-2}.Hz^{-1}$ at 5 MHz) and n is the overtone number. The quartz crystal microbalance with dissipation monitoring allows to simultaneously measuring the changes in the resonant frequency and in the viscoelastic properties (dissipation) when a film is adsorbed at the crystal surface. The measurements can be conducted at the fundamental frequency and at several overtones. When exist any viscoelastic behaviour in adsorbed layer, the Saurbrey model

can't be applied and the QCM-D response of a viscoelastic film has been modelled using a Voigt based model. In this case the changes in the resonant frequency (Δf) and in the dissipation factor (ΔD) according to Voinova and co-workers[22] are:

$$\Delta f \approx -\frac{1}{2\pi\rho_0 h_0}\left\{\frac{\mathfrak{n}_B}{\delta_B} + h_L\rho_L\omega - 2h_L\left(\frac{\mathfrak{n}_B}{\delta_B}\right)^2 \frac{\mathfrak{n}_L\omega_L}{\mu_L^2 + \omega^2 n_L^2}\right\}$$

$$\Delta D \approx \frac{1}{\pi f\rho_0 h_0}\left\{\frac{\mathfrak{n}_B}{\delta_B} + 2h_L\left(\frac{\mathfrak{n}_B}{\delta_B}\right)^2 \frac{\mu_L\omega_L}{\mu_L^2 + \omega^2 n_L^2}\right\}$$

where ω is the angular frequency of the oscillation and $\rho_0$and $h_0$are the density and thickness of the crystal, respectively.

The viscosity of the bulk liquid is $\mathfrak{n}_B$, $\delta_B = \left[\left(\frac{2\mathfrak{n}_B}{\rho_B\omega}\right)^2\right]^{1/2}$ is the viscous penetration depth of the shear wave in the bulk liquid and ρ_B is the liquid's density. The thickness, density, viscosity and elastic shear modulus of the adsorbed layer are represented byh_L, ρ_L , $\mathfrak{n}_L$ and μ_L, respectively.

2.5.3 Scanning Electron Microscope (SEM)

The scanning electron microscope (SEM) is an instrument frequently used in scientific research because it allows producing high-resolution images of a sample surface and when applied in thin films allows to determinate the thickness, identify impurities and defects, or study the adhesion, corrosion and fracture in them.

The images obtained have a very high range of magnification in a nanometric (nm) and micrometer (µm) range.

The equipment consists on an optoelectronic column (electron cannon and reducing electron beam system), a scanning unit, a vacuum chamber (where sample is placed) and a system image viewing as in Figure 2.7.

The operating principle of this technique is based on the impact of an electron beam on a surface and the collection of electronic signals emitted by the target material.

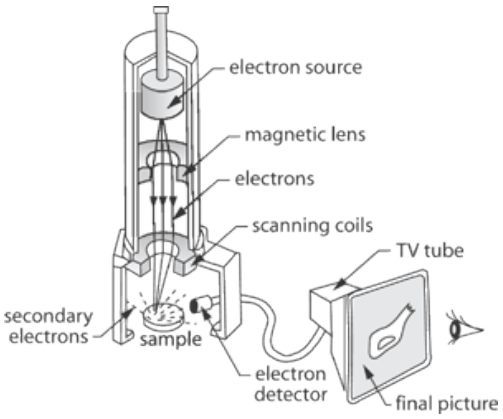

Figure 2.7 - Scanning electronic microscope equipment.

The samples will be sequentially subjected to an accelerated electron beam, focused by an electromagnetic lens system so that the beam is focused on a small diameter in a small area of the sample. The interaction of the electron beam with the sample, results in the issuance of several types of radiation such as secondary electrons, backscattered electrons, characteristic X-rays, etc. – Figure 2.8. When be captured, these particles give information about the characteristics of the samples, such as composition, crystal structure or the topography of the sample.

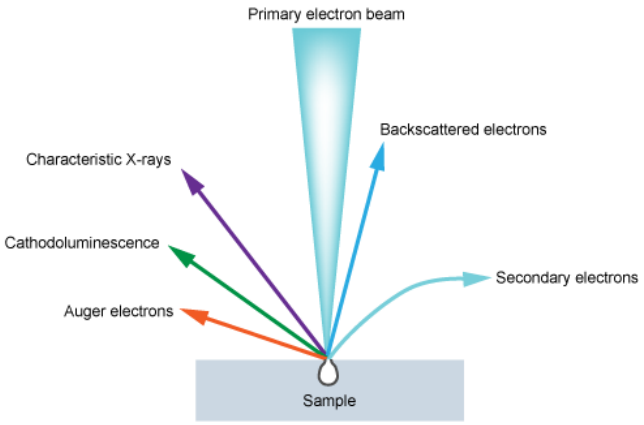

Figure 2.8 - Interaction volume in SEM.

For the formation of the final SEM image, some signals, are important such as secondary electrons and backscattered electrons. The first are a result of energy transfer between electrons emitted and the bombarded electrons on the sample surface and allows the detection of the sample surface topography.

Backscattered electrons are originated from elastic interactions, they are a result of the angular deviation of the incident electrons caused by the sample surface. These electrons give information about the sample surface roughness.

To be subject to SEM, samples must meet the following conditions: (1) provide good surface conductivity, if any conductivity is verified shall be applied an ultra-thin coating that can be Au or C; (2) supporting vacuum since this technique utilizes electron beam; (3) Stable physical and chemical interaction with the electromagnetic beam.

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Chapter 3

Study of stability of multilayer films based on natural polymers

Chapter 3 - Study of stability of multilayer films based on natural polymers

The stability study of films containing biodegradable polymers is essential to optimize their properties envisaging biomedical applications. The present work explores the stability of polysaccharide multilayer films under the action of two different enzymes, lysozyme and alginate lyase. First, multilayer films of chitosan and alginate were prepared using the layer-by-layer technique. In order to investigate the stability of these films to the action of enzymes, *in situ* tests were made through the microbalance quartz crystal (QCM-D) technique, at distinct pH. Moreover, the degradation for long times (until 30 days) was analyzed for the first time by the QCM-D technique. The morphology of the produced films after degradation was analyzed by SEM. The QCM-D experiments allowed to conclude that the constructed films $(CHT/ALG)_7$+CHT are stable when they were in contact for 3 days in PBS as well as in enzymatic solutions, at either pH 5.5 or 7.4 and at 25°C. The produced films for LbL, when immersed for 3 days in PBS or lysozyme (0.013 $mg.mL^{-1}$ and $1mg.mL^{-1}$) at either pH 5.5 and 7.4 at 25°C analyzed by SEM, present more porosity at higher concentration.

Keywords: chitosan, alginate, multilayer films, stability, enzymatic degradation.

3.1 Introduction

The use of natural polymers in substitution of the synthetic ones has been considered very important, since they are based on renewable resources, which are available in large quantities in the environment, and their commercial value is much lower when compared to the synthetic polymers. In particularly, for biomedical applications, natural biodegradable polymers such as gelatin, collagen, chitosan and alginate have been extensively studied for biomedical applications.[1]

One of the most studied polymers for biomedical purposes is chitosan (CHI), which is a N-deacetylated chitin, poly(D-glucosamine), and presents interesting biological properties such as biocompatibility, biodegradation, antibacterial and wound-healing activity.[1] In similarity with CHI, biomedical studies on alginate (ALG) have also intensified

due to its non-toxicity, biocompatibility and non-immunogenicity, which are very important for their tissue regeneration applications.[2] ALG is comprised of (1→4)-linked β-D-mannuronate (M) and α-L-guluronate (G), and these uronic acids are arranged in block structures, which may be homopolymeric (poly-M block and poly-G block) or heteropolymeric (random sequence with MG random block).[3]

In the present work films containing CHI and ALG were produced through the layer-by-layer technique (LbL)). Such method has been widely used for the preparation of different multilayer nanostructured systems.[4] The basic principle of this technique relies on the fabrication of nanostructured multilayered films by assembling a stratified organization of polyanions, polycations or bipolar amphiphilic on the material surfaces.[5] In the LbL assembly, a thin film is grown up from a substrate by its alternating exposure to aqueous solutions containing species with opposite multivalent attractive affinities. Upon each exposure, one component deposits to overcompensate the surface affinity, causing reversal of that affinity and allowing the deposition of a layer of opposite affinity.[6] Films created by LbL assembly are inherently two-component composites and it can be designed to provide practically any type of functional performance. [7] Combinations of CHI and ALG have been widely used as drug delivery systems, where ALG beads are coated with CHI.[8,9] LbL films containing both polysaccharides could find application in the coating of substrates for different biomedical applications, including in tissue engineering and in the delivery of bioactive agents constructed with each chitosan/alginate multilayer films[10] could also offer unique properties for the immobilization of antibodies with retaining its binding activity. [11]

The stability study of films containing biodegradable polymers is essential to optimize their properties envisaging biomedical applications. In particular, for chitosan/alginate films, it is important to understand the degradation mechanism of each polymer individually. In case of polysaccharides, the degradation occurs by enzymatic hydrolysis[12]. The principal agent is lysozyme, that exists in many organisms including in the human body with concentrations from 4 to 13mg.L^{-1}.[12–16] This enzyme acts by hydrolysis in β 1-4 glycosidic linkages between N-acetylglucosamine monomers.[16,17] In the case of alginate, normally the degradation occurs by action of alginate lyase through β-elimination of glycosidic bonds[18]. Various alginate lyases have been found and isolated

from algae, marine microorganisms and marine invertebrates.[19] However, the enzymatic activity is affected by several factors such as pH, temperature and ionic strength. Attending to these effects, in the present work it was performed a stability study of the multilayer films containing chitosan and alginate, based in enzymatic reactions strictly controlled in terms of pH and temperature. As far as we know, this is the first time that such a study is conducted for CHI/ALG multilayer films. A detailed study of the degradation behavior under distinct conditions was conducted by quartz crystal microbalance with dissipation monitoring (QCM-D). The changes in the film morphology due to distinct degradation conditions were analyzed by scanning electron microscopy (SEM).

3.2 Materials and methods

3.2.1 Materials

Medium molecular weight chitosan (CHT), sodium alginate (ALG), lysozyme from chicken egg white (~100000 units.mg^{-1}), alginate lyase (~10000 units.g^{-1}), sodium chloride, acetic acid and phosphate buffered saline tablets were purchased from Sigma-Aldrich. All reagents were used without further treatment, unless the chitosan which was previously purified by recrystallization following the procedure described in detail by Couto and co-workers[20].

3.2.2 Methods

3.2.2.1 Quartz Crystal Microbalance with dissipation monitoring (QCM-D)

The formation of multilayer CHT/ALG was realized in situ by QCM-D (Q-Sense E4 system), for monitoring the alternately adsorption of chitosan and alginate onto the gold-coated crystals.

LbL films were produced using fresh solutions prepared in 0.15M of sodium chloride: 0.5mg.mL^{-1} CHT solution with 1% (v/v) of acetic acid and 0.5 mg.mL^{-1} ALG solution. The multilayered systems were assembled at pH of 5.5 adjusted with NaOH and HCl solutions. CHT was used as polycation and ALG was the polyanion.

The construction consisted in sequential adsorptions onto the QCM crystals, which were excited at 15, 25, 35, 45 and 55 MHz. The system started with 0.15M NaCl solution, then, the polyelectrolyte solutions were injected into the cell during 10 min using a peristaltic pump, beginning with CHT. A rinsing step of 5 min with a 0.15 M NaCl solution was included between the adsorptions of each polyelectrolyte, since it is essential for a stable construction of multilayer films. These experiments were performed at room temperature (25ºC) and, for LbL construction, a flow rate of 50 $\mu L.min^{-1}$ was used. Then, the injection of enzymatic solutions was performed using a lower flow rate.

After the LbL construction, the enzymatic solutions were injected using a lower flow rate, 6.71 $\mu L.min^{-1}$. Lysozyme solutions with two distinct concentrations ($1mg.mL^{-1}$ and $0.013mg\ mL^{-1}$) were prepared in 0.1M PBS solution for pH values of 5.5 and 7.4. The alginate lyase solution with concentration 0.1 $mg.mL^{-1}$ was also prepared in 0.1M PBS solution for pH value of 5.5. As a control condition, a 0.1M PBS solution was also injected for pH values of 5.5 and 7.4.

The final graphics obtained were obtained through the Voigt model in the Q-Tools software from Q-Sense.

3.2.2.2 Construction of multilayers films

The construction of $[CHT/ALG]_7$+CHT films onto glass slides was performed using manual dip coating technique. The substrates were dipped alternately in the polyelectrolyte solutions in order to obtain films using exactly the same conditions described for the QCM-D experiments.

Similarly with the QCM-D experiments, after the LbL construction, the multilayer films were immersed in the enzymatic solutions and in the control solution (0.1 M PBS solution), during a certain period of time (up to 30 days). At the end of each period of time, the samples were removed from those solutions and washed with distilled water. Finally, they were dried at room temperature for further characterization.

3.2.2.3 Scanning electronic microscopy

The analysis of the surface morphology of the constructed films on glass substrates, before and after the injection of enzymatic solutions, was performed using scanning electron microscopy, SEM (model JSM-6010LV,JEOL, Japan) with the secondary electron image mode and an accelerating voltage of 10 kV on previously gold coated samples.

3.3 Results and Discussion

3.3.1 QCM-D experiments

The construction of $(CHT/ALG)_7$+CHT films was monitored *in situ* with QCM-D. In Figure 3.1, it was represented the variation of the normalized frequency $\Delta f_5/5$ and dissipation, ΔD_5, which confirmed a successful build-up of these multilayer films. As expected, the frequency decreases after each injection of polyelectrolyte solution, as a consequence of the increase of the mass deposited onto the surface of the gold-coated quartz crystals. On the other hand, ΔD_5 increased due to the non-rigid adsorbed layer structure of the film that is forming onto the crystal surface. In addition, there is a small change of both $\Delta f_5/5$ and ΔD_5 during the washing step, showing that the adsorbed layers are relatively stable.

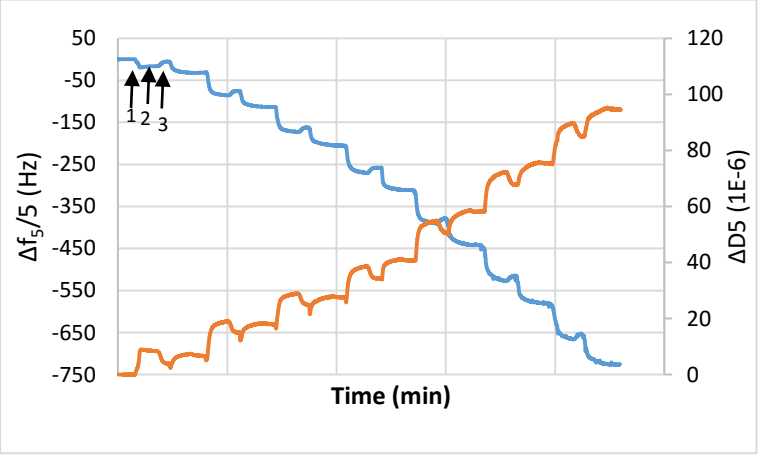

Figure 3.1 - Normalized frequency ($\Delta f_5/5$) and dissipation changes (ΔD_5). 1) Chitosan injection 2) washing step (NaCl) 3) Alginate injection.

Based on these QCM-D results, the cumulative thickness of these films was estimated using a Voigt viscoelastic model instead of the Sauerbrey model, also used in literature to model QDM-D data.

Figure 3.2 shows that the thickness of CHT/ALG multilayers increases as the number of layers increases, presenting a linear growth, as previously reported.[10] The linear growth observed indicates that each CHI layer is effectively adsorbing on the top of the previous ALG layer.

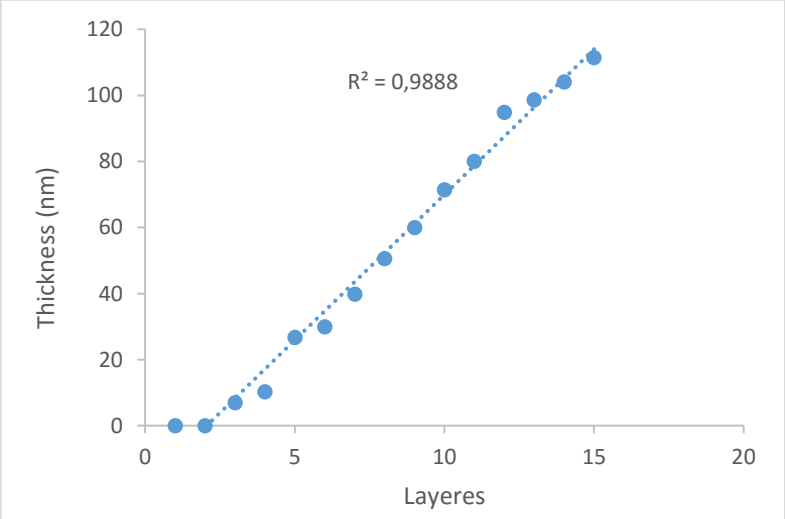

Figure 3.2 - Cumulative thickness evolution of polymeric film as a function of the number of deposited layers. The line represents a linear regression with R^2=0.9888.

With the QCM technique, it was also possible to evaluate *in situ* the degradation of CHI/ALG films by lysozyme. Thus, the enzymatic solution was injected for 72 hours and the degradation of the film was evaluated taking into account the evolution of the normalized frequency and dissipation, and especially, based on the estimated cumulative thickness after the injection of enzymatic solution. All studies were performed at 25 ^{o}C and the effect of pH on enzymatic degradation was also evaluated for two distinct values, 5.5 and 7.4. In order to complement the analysis of the stability of the films, the graphics of thickness for each of the cases are presented. These graphics were obtained through the Voigt model in the Q-Tools software from Q-Sense.[22]

Initially it was conducted a test with the concentration of 1mg/mL de lysozyme without PBS with pH=5.5 and the result is presented in Figure 3.3. The result didn't any alteration in films stability to the action of the lysozyme prepared in these conditions.

Then and follow the literature, a lysozyme solution with the same concentration was prepared with PBS in order to roughly simulate the hydration conditions in the human body, when the materials are implanted.[1,23]

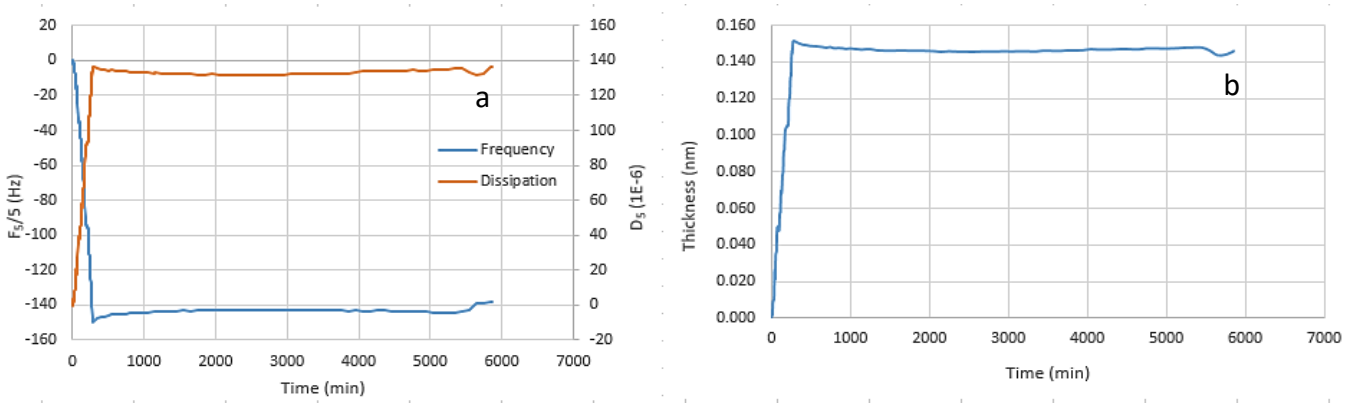

Figure 3.3 - a) QCM monitoring with normalized frequency ($\Delta f_5/5$) and dissipation change (ΔD_5) during $(CHT/ALG)_7$+CHT build up and degradation with 1mg/mL of lysozyme solution at pH=5.5, and respective B) cumulative thickness evolution estimated using Voigt viscoelastic model.

Figure 3.4 – 3.5 present the stability of the build-up of $(CHT/ALG)_7$+CHT films to the action of PBS at different pH (5.5 and 7.4) as control. Regarding these results, it can be seen the $\Delta f_n/n$ variation is equal for both conditions. By comparing these results it is possible to say that PBS does not cause any change in the film, i.e., the thickness remains unchanged throughout the injection of PBS.

Figures 3.6 and 3.7 compare the stability of the build-up of $(CHT/ALG)_7$+CHT films to the action of lysozyme with a concentration of 1mg/mL prepared in PBS at a different pH (5.5 and 7.4). The results show similar $\Delta f_n/n$ variation similar in both graphics and the action of this enzyme is not relevant in the thickness of the film.

So, it can be concluded that, for the time period of 72 h, the produced CHI/ALG films are quite stable in the presence of lysozyme. Lin at al. reported that chitosan with a 62% deacetylation degree, when in contact with lysozyme, presented degradation right from the first day and after 28days there was only 75% of the total mass.[24] In fact, the combination of ALG with CHI should be responsible for the system stability when compared with chitosan alone.

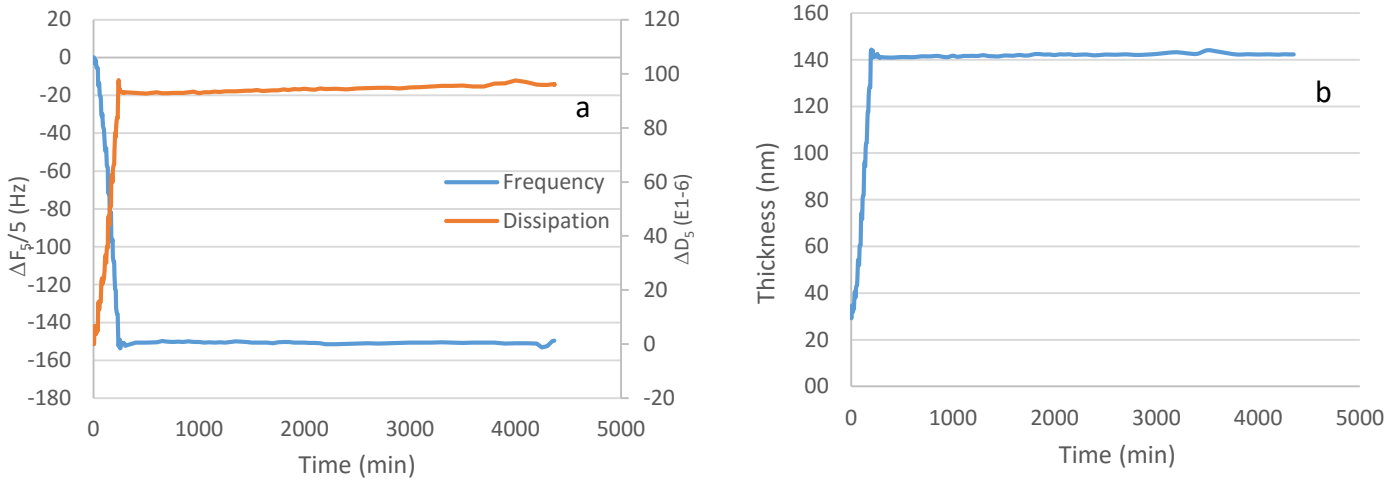

Figure 3.4 - a) QCM monitoring with normalized frequency (Δf5/5) and dissipation (ΔD5) change during (CHT/ALG)7+CHT build up and degradation of PBS at pH=5.5, and respective b) cumulative thickness evolution estimated using a Voigt viscoelastic model.

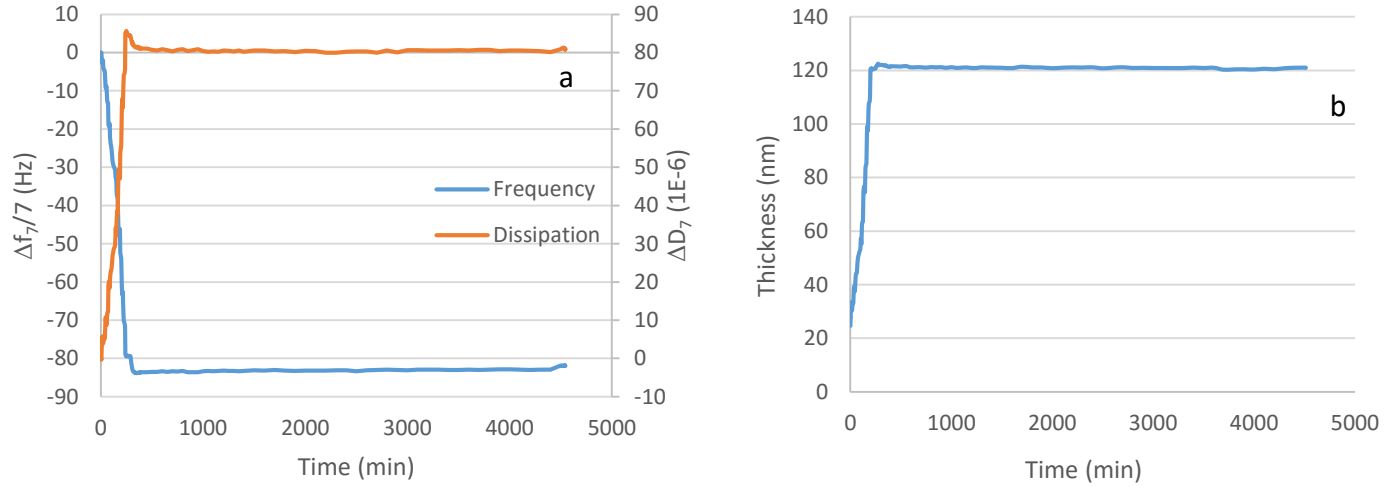

Figure 3.5 - a) QCM monitoring with normalized frequency (Δf5/5) and dissipation (ΔD5) change during (CHT/ALG)7+CHT build up and degradation of PBS at pH=7.4, and respective b) cumulative thickness evolution estimated using a Voigt viscoelastic model.

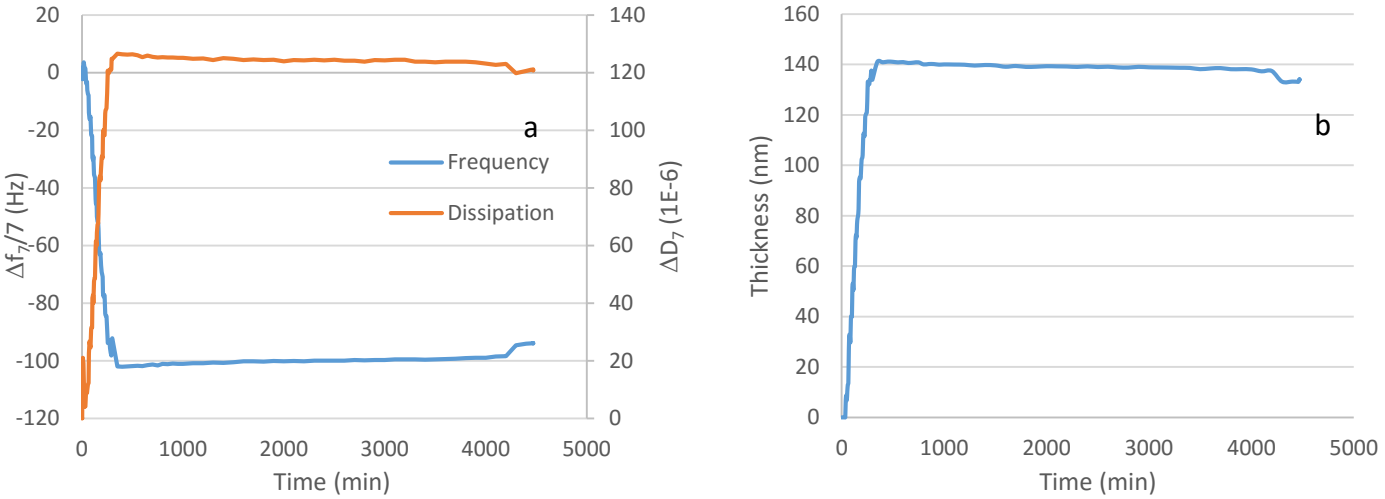

Figure 3.7 - a) QCM monitoring with normalized frequency ($\Delta f_7/7$) and dissipation (ΔD_7) change during $(CHT/ALG)_7$+CHT build up and degradation of 1mg/mL lysozyme solution in PBS at pH=5.5, and respective b) cumulative thickness evolution estimated using a Voigt viscoelastic model.

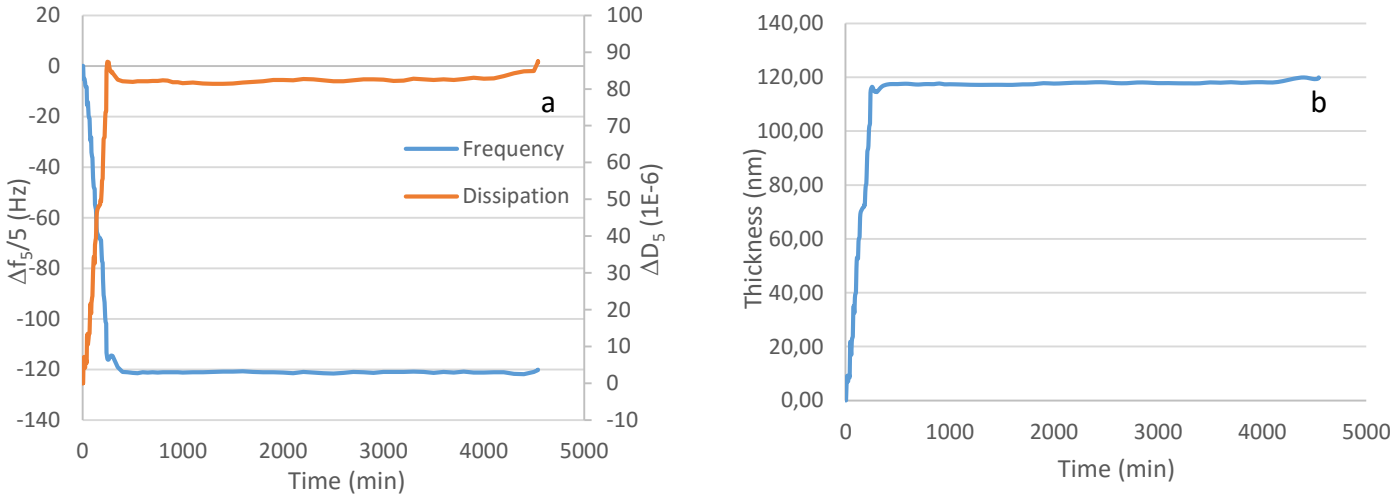

Figure 3.6 - a) QCM monitoring with normalized frequency (Δf5/5) and dissipation (ΔD5) change during (CHT/ALG)7+CHT build up and degradation of 1mg/mL lysozyme solution in PBS at pH=7.4, and respective b) cumulative thickness evolution estimated using a Voigt viscoelastic model.

The study of stability of the build-up $(CHT/ALG)_7$+CHT *in situ* was also carried out with the alginate lyase enzyme. This was performed under the same condition used with lysozyme. These results are presented in Figure 3.8.

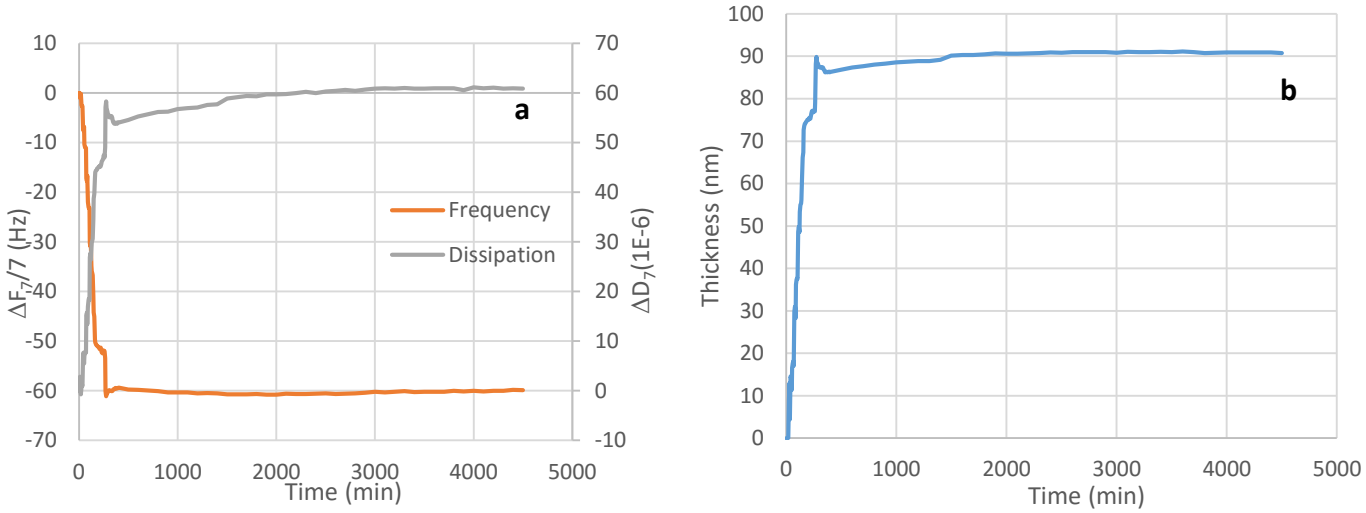

Figure 3.8 - a) QCM monitoring with normalized frequency (Δf7/7) and dissipation (ΔD7) change during (CHT/ALG)7+1 build up and degradation of 0,1mg/mL alginate lyase in PBS, at pH=5.5 b) Thickness change during (CHT/ALG)7+1 build up and degradation of 0,1mg/mL alginate lyase in PBS, at pH=5.5

By looking at Figure 3.8, it can be seen that either $\Delta f_n/n$ or the thickness values didn't change significantly in the presence of this enzyme. So, CHI/ALG films are also stable in the presence of alginate lyase for the time period of 72 h.

Inoue *et al*. 2014 reported the effiency of enzymatic degradation of the alginate by action of alginate lyase.[25] In case of film $(CHT/ALG)_7$+CHT it doesn't happen, being possible to say that the combination of alginate with chitosan improves stability to enzymatic action of this specific enzyme.

Quartz Crystal Microbalance was also used as an innovative technique to measure the frequency and dissipation offline for multilayer films prepared by layer-by-layer on the QCM crystals. The degradation was made with the immersion of these crystals in the enzyme solution during 7, 14 and 30 days and different conditions. The comparison was made in relation to the change in frequency and dissipation of the crystals, before and after the deposition, made by layer-by-layer. The result present refers to 0.013mg.mL^{-1} lysozyme concentration – Figure 3.9.

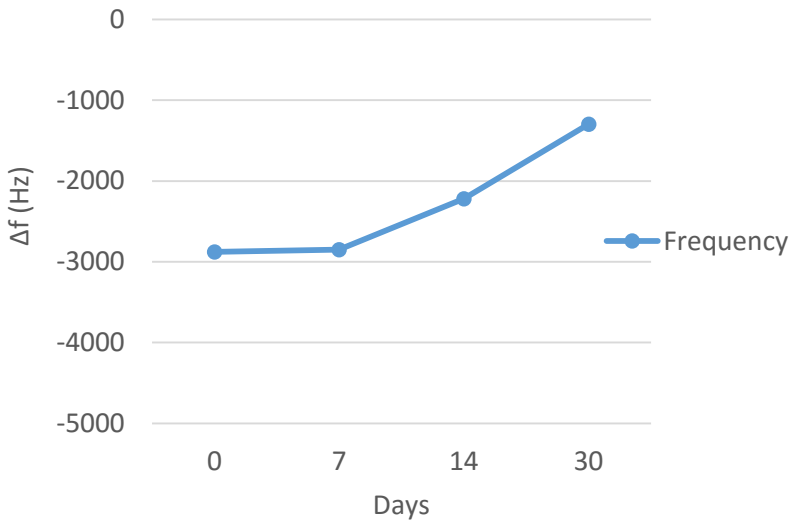

Figure 3.9 - QCM offline monitoring during (CHT/ALG)7+1 degradation of 0.013mg/mL lysozyme solution in PBS at pH=5.5.

The result present the increase of Δf and decrease of ΔD, showing that the enzymatic degradation of the multilayer film in the presence of lysozyme is only visible after 7 days, growing during all the 30 days wherein the films were in contact with the enzyme solution.

3.3.2 Scanning electronic microscopy

The characterization of the surface of the films built through the LBL technique and degraded under different conditions was analysed by SEM. $(CHT/ALG)_7$+CHT were built using as support glass slides. The obtained films were placed in lysozyme for 3 days with different concentration (0.013 $mg.mL^{-1}$ and 1 $mg.mL^{-1}$) and posteriorly, they were washed and dried at room temperature for further SEM analysis. The SEM results obtained for the distinct degradation conditions were illustrated in Figure 3.10

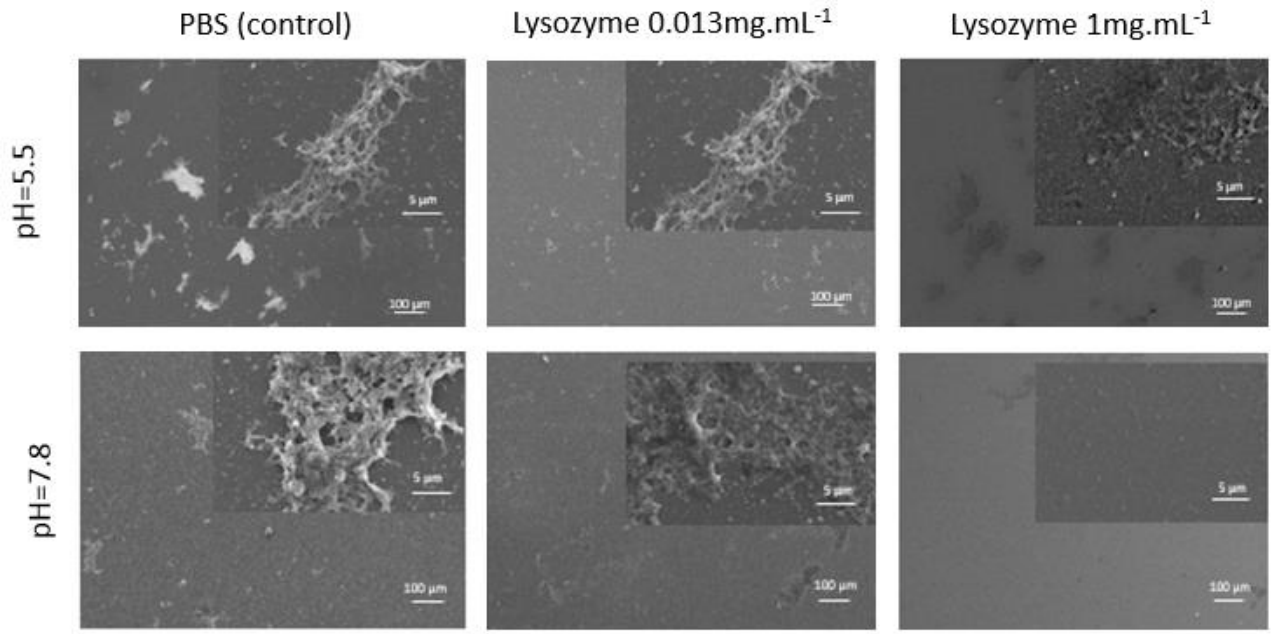

Figure 3.10 - Comparative SEM images for 3 days degradation with lysozyme with different concentrations (0.013mg.mL-1, 1mg.mL-1) at pH=5.5 and pH=7.4.

Attending the surface morphology represented in these SEM results, it is evidenced that multilayer films immersed in the enzymatic solution with higher concentration presented higher porosity at pH=5.5, presented higher degradation when compared with those immersed in PBS solution. Depan *et al*. 2013 report studies of degradation of three-dimensional hybrid porous scaffolds of chitosan comprised of nanostructured carbon (graphene oxide and single-walled carbon nanohorns). The SEM results presented the existence of pores in the micrographs of the analyzed samples resulting from the PBS degradation. [26] Is possible conclude that enzymatic degradation increase the porosity of multilayer films more than the PBS degradation.

3.4 Conclusions

The specific enzymatic hydrolysis with lysozyme and alginate lyase of the Layer-by-Layer assembly prepared from chitosan and alginate was analyzed by QCM-D and SEM.

The QCM-D experiments allowed to conclude that the constructed films $(CHT/ALG)_7$+CHT are stable when they were immersed for 3 days in PBS as well as in enzymatic solutions, at either pH 5.5 or 7.4 and at 25ºC. Moreover, the unconventional use of QCM-D allowed to conclude that the loss of mass due to lysozyme degradation is only visible after 7 days. From the surface morphology of the films evaluated by SEM analysis, it was evidenced that these LbL films immersed during 3 days in 1mg.mL^{-1} lysozyme solution, presented higher porosity than those immersed in 0.013 mg.mL^{-1} lysozyme solution. This behavior occurred for both pH values. The SEM results suggested a higher enzymatic hydrolysis of chitosan (the end layer of the constructed films) with the lysozyme solution with higher concentration. Nevertheless, the degradation should not very significant as mass changes were not detectable by QCM-D and only some changes in the morphology of the films were visible by SEM.

3.5 References

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Chapter 4 - Conclusion Remarks

The main objective of this thesis was to study the stability of films constructed by chitosan and alginate. To this end, it was subjected to immersion at different times and conditions in solutions with enzymes that are present in human body and which degrade the polymers in question. In this way, it was possible to simulate deployment condition these same materials.

To study the variation of mass of the film in function of time, were used at microbalance quartz crystal, thus allow evaluate the degradation of the film *in situ*. Another type of test was realized by SEM for analysis the morphology of the film after degradation by contact with enzymes.

It was than concluded that the films produced present stability upon contact with lysozyme for 72 hours. When in contact with alginate lyase the same not happened. it was possible register some changes in mass of the film.

As future works, stability of the film can be studies with different concentration of both enzymes, as well as longer degradation times.

Attachments

The results show the mass loss of multilayer films for different periods of time and two different temperatures (25ºC and 37ºC). Would however need repeat the experience in order to consolidate the results as well the realization for more long times as 60 days.

- 25ºC

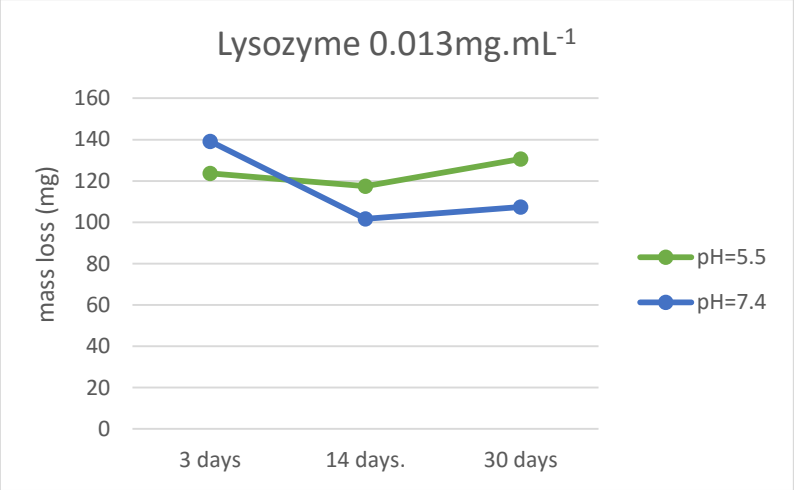

Figure 5.1 - Mass loss of $(CHT/ALG)_7$+CHT film in lysozyme 0.013mg/mL during 3 days, 14 days and 30 days at pH=5.5 and pH=7.4 at 25ºC.

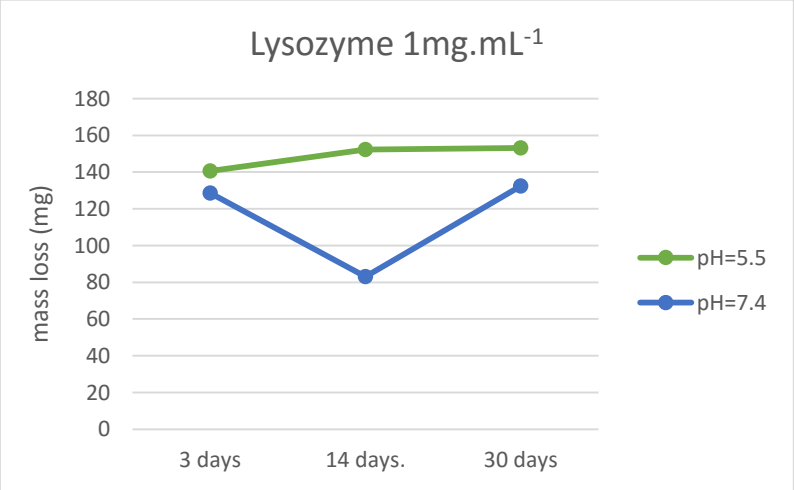

Figure 5.2 - Mass loss of $(CHT/ALG)_7$+CHT film in lysozyme 1mg/mL during 3 days, 14 days and 30 days at pH=5.5 and pH=7.4 at 25ºC.

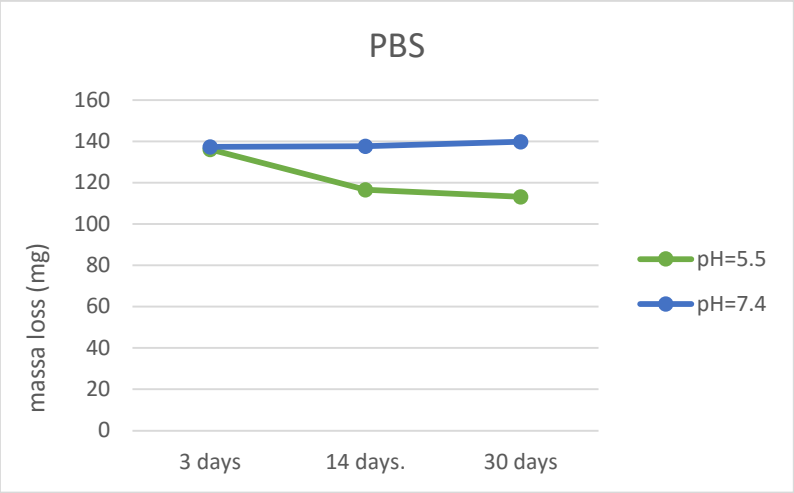

Figure 5.3 - Mass loss of $(CHT/ALG)_7$+CHT film in PBS during 3 days, 14 days and 30 days at pH=5.5 and pH=7.4 at 25°C.

- 37°C

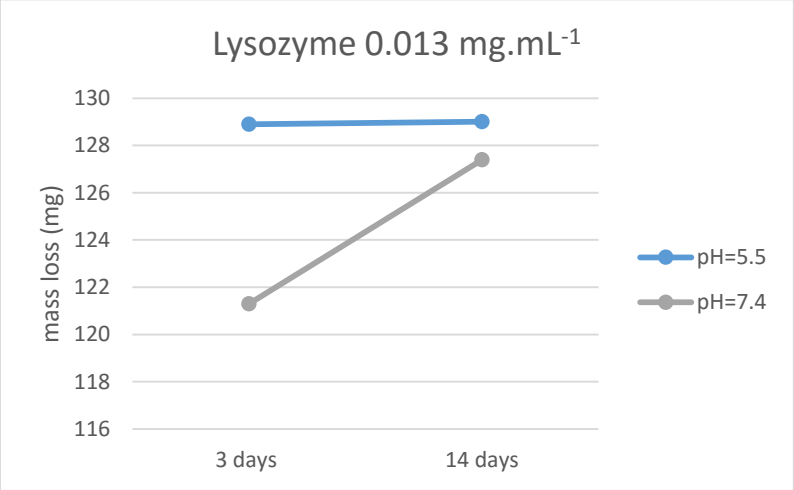

Figure 5.4 - Mass loss of $(CHT/ALG)_7$+CHT film in lysozyme 0.013mg/mL during 3 days and 14 days and 30 days at pH=5.5 and pH=7.4 at 37°C.

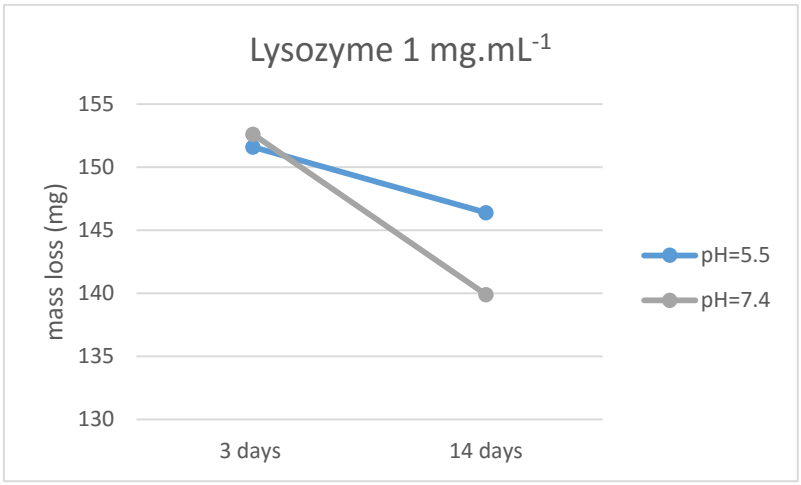

Figure 5.5 - Mass loss of (CHT/ALG)7+CHT film in lysozyme 1mg/mL during 3 days and 14 days and 30 days at pH=5.5 and pH=7.4 at 37ºC.

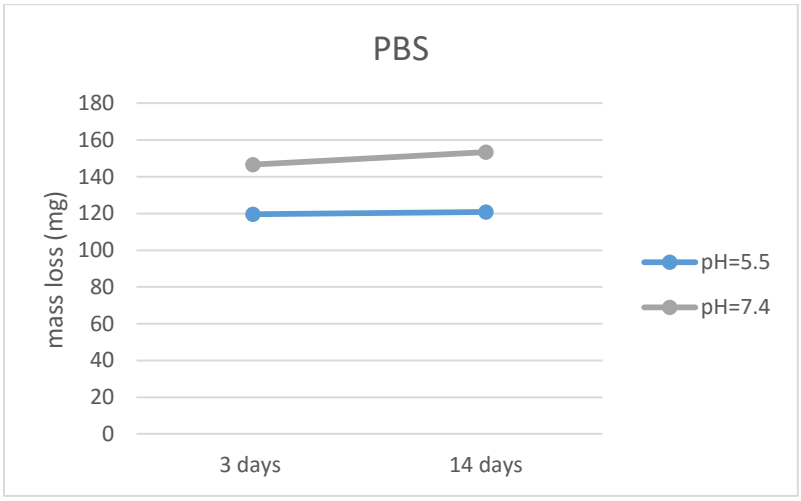

Figure 5.6 - Mass loss of (CHT/ALG)7+CHT film in PBS during 3 days and 14 days and 30 days at pH=5.5 and pH=7.4 at 37ºC.