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Enzymatic degradation of natural polysaccharide-based layer-by-layer devices

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UMinho | 2015 Matias Joaquin Cardoso **Enzymatic degradation of natural polysaccharide-based layer-by-layer devices**

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Dissertação de Mestrado
Mestrado em Bioquímica aplicada

Trabalho efetuado sob a orientação do
Professor Doutor João Filipe Colardelle da Luz Mano
e do
Professor Doutor João Carlos Marcos

Novembro de 2015

DECLARAÇÃO

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Título da dissertação:

Enzymatic degradation of natural polysaccharide-based layer-by-layer devices

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Ano de conclusão: 2015

Mestrado em Bioquímica aplicada

Área de especialização: Biomedicina

Universidade do Minho, ___/___/_____

Assinatura: _____

ACKNOWLEDGMENTS

The realization of this master thesis has significant support and encouragements without which it would not have become a reality and which I will be grateful.

I would like to express my gratitude in particular to my supervisor Professor Doutor João Filipe Colardelle da Luz Mano, for the excellent guidance, caring, encouragement and the opportunity given to work in his exceptional group. His inspiration, curiosity and the incredible support, belief and enthusiasm through all the investigation, are the main reasons for this achievement.

A special recognition to Rui R. Costa, for all the support, patience and especially for his availability to described during this year the true meaning of being a researcher. Acknowledge all the generous hours committed to this work aiming to present a well-structured and constructive research work, adding an advantage to the scientific field.

A special gratitude to all my professors, especially for the two course directors during this two years, Professor João Carlos Marcos and Professor Pedro Santos for their support and availability to solve some problems when it was needed.

Another special appreciation goes to all researchers and staff of 3B's in particular to Sofia Caridade for the support during the struggle of mechanical properties and to Catarina Custódio and Margarida Costa for their support on the microscopy analyses.

To all my friends, colleagues that supported me and believed in the development of the work. Inestimable is the support from my wonderful family, especially to my father, an everlasting gratitude.

ABSTRACT

In the last two decades, the layer-by-layer (LbL) assembly technique has been used in different areas, such as materials science, and biology. One of the major trending usages is in the area of biomedical sciences, in particular by the design of nanostructured polyelectrolyte multilayer films (PEM) with various morphologies and dimensions that closely resemble a specific assembly substrate, such as thin coatings, membranes and capsules. To this purpose, natural polymeric-based materials have been utilized as biomaterials for specialized LbL devices. These materials present several advantages, such as biocompatibility, bioactivity and in some cases biodegradability, which are hard to find in synthetic materials. Natural polysaccharides are good candidates for the construction of devices with different biomedical applications, such as implantable porous scaffolds, prostheses and other tridimensional structures applicable in tissue engineering and regenerative medicine. Drug delivery vehicles are one of the main utilizations of such materials. In fact, these materials have to present physical, chemical, mechanical and biological properties to promote efficient action. Among these materials, marine origin polysaccharides have important biological properties such as biocompatibility, biodegradability, bioactivity, as well as adhesive and antimicrobial actions. Because of such properties, they are suitable candidates as a biomaterials for different biomedical applications, and can be processed into various drug delivery carriers including particles, capsules, hydrogels or polyelectrolyte films. Among the most common polysaccharides used in the pharmaceutical field are alginate, carrageenan, fucoidan, chitosan and hyaluronic acid, which can be obtained from different marine organisms.

Polysaccharides have been used as building materials for LbL assembly. Chitosan/hyaluronic acid LbL-based PEMs were produced with different dimensional scales to study their enzymatic degradation. Ultrathin films showed a high sensibility to hyaluronidase degradation, which induces topography modifications. Freestanding membranes can also be degraded by hyaluronidase. The films were degraded in the presence of hyaluronidase, leading also to a loss of mechanical properties. LbL microcapsules were prepared in order to study the enzyme triggering release. Results, suggest that these enzyme loaded microcapsules could be suitable as delivery carriers for different applications. Knowing the enzymatic degradation of LbL structures represents a step forward towards the translation of such technology to preclinical assays concerning a new generation of implantable biomedical devices. Furthermore, enzymes were shown to be suitable triggers to promote the release of bioactive agents from LbL-based carriers and could be useful to tune the release rate within desired administration requirements.

RESUMO

Layer-by-Layer (LbL) define-se como técnica que permite a montagem de estruturas por deposição de diferentes camadas pelas interações entre vários materiais, entre elas as interações electroestáticas. A utilização de diferentes materiais permite a construção de estruturas de diferentes tamanhos, morfologia e em alguns casos podem apresentar atividade biológica. Geralmente, essas características é lhe conferida pelos tipos de materiais utilizados. A utilização de materiais inteligentes podem levar à construção de estruturas com a capacidade de apresentarem uma resposta a diferentes estímulos externos, como por exemplo, a degradação enzimática.

Nesse aspeto, os polissacarídeos de origem marinha apresentam inúmeras vantagens, como por exemplo, biocompatibilidade, biodegradabilidade, bioatividade, biodisponibilidade, baixa toxicidade, assim como propriedades anti adesivas e antimicrobianas. Propriedades difíceis de encontrar em materiais de origem sintética. Devido a estas características, estes materiais são utilizados na construção de estruturas com aplicabilidade biomédica, como por exemplo, membranas implantáveis para a regeneração de tecidos ou a construção de capsulas para a libertação controlada de agentes bioativos. Estes materiais apresentam propriedades físico-químicas, mecânicas e biológicas para promover uma ação e libertação eficiente. Estes materiais podem ser utilizados na construção de diferentes dispositivos, como por exemplo, partículas, capsulas ou hidrogéis. Os polissacarídeos de origem marinho mais utilizados são: o alginato, o quitosano, o sulfato de condroitina e o ácido hialurónico.

Estes polissacarídeos têm sido utilizados como principal constituinte de estruturas produzidas por LbL. Diferentes estruturas utilizando o quitosano e o ácido hialurónico foram obtidas para estudar a sua degradação enzimática. Filmes ultrafinos demonstraram uma grande sensibilidade à degradação enzimática, apresentando alterações na sua estrutura após contacto com a enzima mesmo a baixas concentrações. Aumentando o número de camadas foi possível obter membranas manejáveis. A ação enzimática nestas membranas demonstrou a perda de massa causada pela degradação que pode levar ao rompimento da mesma. Forma produzidas por LbL capsulas contendo enzima para demonstrar se esta consegui induzir a libertação de biomoléculas através da degradação interna da cápsula. Resultados sugerem que a degradação da enzima promove uma maior libertação do agente. Fazendo deste sistema, adequável à libertação controlada de agentes bioativos.

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LIST OF ABBREVIATIONS

A

AFM – atomic force microscopy

ANOVA – analysis of variance

B

BSA – bovine serum albumin

C

°C – Celcius degree

Ca^{2+} - calcium ion

$CaCl_2$ – calcium chloride

$CaCO_3$ – calcium carbonate

CHT – chitosan

D

$\delta 3$ – viscous depth penetration

ΔD – dissipation values

Δf – normalized frequency

DDS – drug delivery system

DMA – dynamics mechanical analysis

DNA – deoxyribonucleic acid

E

E' – storage modulus

EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

EDTA – ethylenediaminetetraacetic acid

ELR – elastin-like recombinamers

F

FITC – fluorescein isothiocyanate

FITC-BSA – albumin fluorescein isothiocyanate conjugate

G

α-β-GP – α-β-glycerophosphate

H

h – hours

h_0 – thickness of the quartz crystal

H_2O_2 – hydrogen peroxide

HA – hyaluronic acid

Hase – hyaluronidase

HA-Tyr – Hyaluronic acid-tyramine

HPCH – Hydroxypropyl chitosan

HRP – horseradish peroxidase

HTCC – N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride

Hz – Hertz

K

k – total of thin viscoelastic layers

Kg – kilograms

L

λem – emission wavelengths

λexc – excitation wavelengths

LbL- Layer-by-layer

M

μ – elastic shear modulus of the overlayer

μg – microgram

μL – microliter

μm – micrometer

M – molar concentration

m3 – cubic meter

mf – final mass

MHz – megahertz

mi – initial mass

min – minutes

mL – milliliter

N

η3 – viscosity of the bulk liquid

N – Newton

Na_2CO_3 – Sodium carbonate

NaCl – Sodium chloride

nm – nanometers

NOCC – N,O-carboxymethyl chitosan

O

O – oxygen

P

Pa – Pascal

PBS – phosphate buffered saline

PEI – polyethylenimine

PEG – poly(ethylene glycol)

PEM – polyelectrolyte multilayer

PLL – poly-L-lysine

PNIPAAm – Poly(N-isopropylacrylamide)

Q

QCM – quartz-crystal microbalance

QCM-D – quartz-crystal microbalance with dissipation monitoring

R

$\rho 0$ – density of the quartz crystal

$\rho 3$ – density of liquid

Ra – average roughness

ROS – reactive oxygen species

Rq – root mean squared roughness

S

SD – standard deviation

SEM – scanning electron microscopy

T

tan δ – loss modulus

TEMED – Tetramethylethylenediamine

W

w/v – weight/volume

ω – angular frequency of the oscillation

X

$\chi 2$ – total error

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SECTION I – GENERAL INTRODUCTION

Chapter 1 – Marine origin polysaccharides in drug delivery systems

Chapter 1 – Marine origin polysaccharides in drug delivery systems

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Abstract: Oceans are a vast font of resources. In them, we find various compounds with wide biotechnological and biomedical applicability. The exploitation of the sea as a renewable source of biomaterials can have a positive impact on the development of new systems and devices for biomedical applications. Marine polysaccharides are among the most abundant materials in the seas, thanks to which a decrease of the extraction costs is possible. Polysaccharides such as alginate, carrageenan and fucoidan can be extracted from algae, whereas chitosan and hyaluronic acid can be obtained from animal sources. Most marine polysaccharides have important biological properties such as biocompatibility, biodegradability, and anti-inflammatory activity, as well as adhesive and antimicrobial actions. Moreover, they can be processed into various shapes and sizes and may exhibit response over external stimuli, such as pH and temperature. Due to these properties, these biomaterials have been studied as raw material for the construction of carrier devices for drugs, including particles, capsules and hydrogels. The devices are designed to achieve a controlled release of therapeutic agents in an attempt to fight against serious diseases, and to be used in advanced therapies, such as gene delivery or regenerative medicine.

1. Introduction

Marine origin organisms are a vast source of different compounds with diverse biological properties and bioactivity. Recently a growing interest in many scientific areas that study the diverse applications of marine origin compounds has been found, justified by their large biodiversity and simplicity of the extraction and purification processes (Pomponi, 1999; Silva, T. H., Duarte, Moreira-Silva, Mano, & Reis, 2012). Marine origin biomaterials have wide applicability in biomedicine because of their noncytotoxic characteristics, biodegradability and biocompatibility. These biological properties have allowed the discovery of a broad range of novel bioactive compounds with pharmacological properties and constitute a fundamental cornerstone of the pharmaceutical industry (Molinski, Dalisay, Lievens, & Saludes, 2009; Munro et al., 1999; Silva, Tiago H et al., 2012). Some of these compounds have been studied for cancer treatment due to their antitumoral properties (Jimeno, J. et al., 2004; Newman & Cragg, 2014; Schwartsmann, da Rocha, Berlinck, & Jimeno, 2001), among which are polypeptides extracted from tunicates (Rinehart, 2000) and sponges (Suarez-Jimenez, Burgos-Hernandez, & Ezquerra-Brauer, 2012). Many of these compounds are already used clinical trials, such as Aplidin (Jimeno, J. M., 2002) and Ecteinascidin 743 (Valoti et al., 1998).

Marine polysaccharides are mostly used in food and cosmetic industries, but are also widely present in pharmaceutical sciences, with an increasing interest to integrate them as materials for the incorporation of bioactive agents (Laurienzo, 2010). Marine algae are the main source of marine origin polysaccharides, but they can also be obtained from animal sources, such as the skeletons of crustaceans and cartilaginous fish tissue. There are also some polysaccharides that can be extracted from marine microorganism, like some prokaryotes (Senni et al., 2011). Marine polysaccharides can be described as a large complex group consisting of different macromolecules with different biological properties (Costa, L. S. et al., 2010; Ngo & Kim, 2013). Polysaccharides may exhibit different chemical structures and different biological properties such as biocompatibility, biodegradability, adhesive properties and the ability to form hydrogels. Among marine polysaccharides there is one group that stands out: sulfated polysaccharides (Wijesekara, Pangestuti, & Kim, 2011). In comparison with other marine polysaccharides, they exhibit bioactivities that include antioxidant (Barahona, Chandia, Encinas, Matsuhiro, & Zuniga, 2011), anticoagulant (Ciancia, Quintana, & Cerezo, 2010), anticancer (Sithranga Boopathy & Kathiresan, 2010), antiviral (Bouhlal et al., 2011), anti-allergic (Vo, Ngo, & Kim, 2012), anti-adhesive, anti-angiogenic and anti-inflammatory actions (Cumashi et al., 2007). The systematic study of

some of these materials for drug delivery systems (DDSs) allowed discovering new chemical modification methods aiming to harness such biological activities and change their affinity to specific drugs. Considering the latter, it has been possible to increase the ability to incorporate drugs and increase the efficacy of their release, either by chemical reactions or by interactions with other natural or synthetic polymers (d'Ayala, Malinconico, & Laurienzo, 2008).

The interest in the study of marine polysaccharides for DDSs with therapeutic purposes relies in the possibility of developing novel approaches of less invasive and more personalized treatments. Several experiments have already shown that many of these biomaterials allow loading lower drug dosages, which may lead to a drastic reduction of the side effects caused by the drugs. These materials can be used as a signaling marker that could lead the delivery of a carrier to a specific location and widening the function of DDSs as diagnostic instruments (Allen & Cullis, 2004; Brannon-Peppas & Blanchette, 2012). These systems have a wide applicability also in gene therapy, which is usually limited by the health risk of associated with viral vectors (Thomas, C. E., Ehrhardt, & Kay, 2003). In contrast, biomaterials have been shown to offer numerous advantages for the encapsulation of genetic material and others therapeutic agents, by ensuring stabilization and protection, also increasing its solubility and promoting a sustained release as well their biocompatibility and in some cases biodegradability (Nitta & Numata, 2013; Silva, T. H. et al., 2012). In this review, we focus on the use of marine origin polysaccharides as raw materials for the construction of DDSs (**Figure 1.1**). We identified alginate, chitosan, carrageenan, hyaluronic acid and chondroitin sulfate as the major marine polysaccharides used currently in – or being considered for – the pharmaceutical industry. The various means to modify and adapt these biopolymers to achieve drug protection and delivery, stimuli-responsiveness and targeting capability will be discussed.

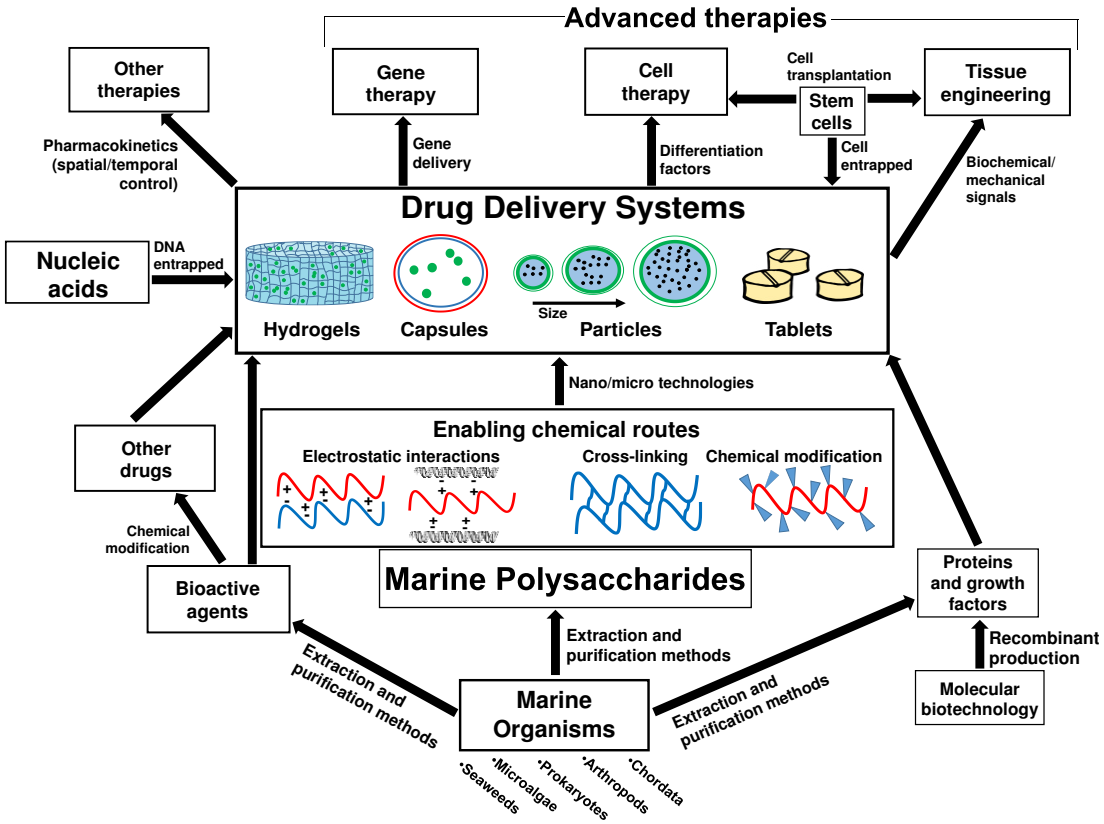

Figure 1.1. Interrelations of marine origin polysaccharides in DDSs applied in advances therapies.

2. Polysaccharides from marine algae

Among the vast marine organisms diversity, algae are the main source of marine origin polysaccharides. There are also some polysaccharides that can be extracted from marine prokaryotes like microalgae. These microorganisms can have a controlled growth in bioreactors and can lead to the extraction of high quantities of extracellular polysaccharides. Red algae are the most used sources of polysaccharides but it is possible obtain polysaccharides from green or brown algae. Seaweeds are a multicellular different type of marine algae and are also a major source of polysaccharides. The latter are also divided in groups: red, green and brown. Nowadays, despite the large quantity of marine algae that reach and deposit in the coast regions, the extraction of polysaccharides remains a relatively unexploited industry.

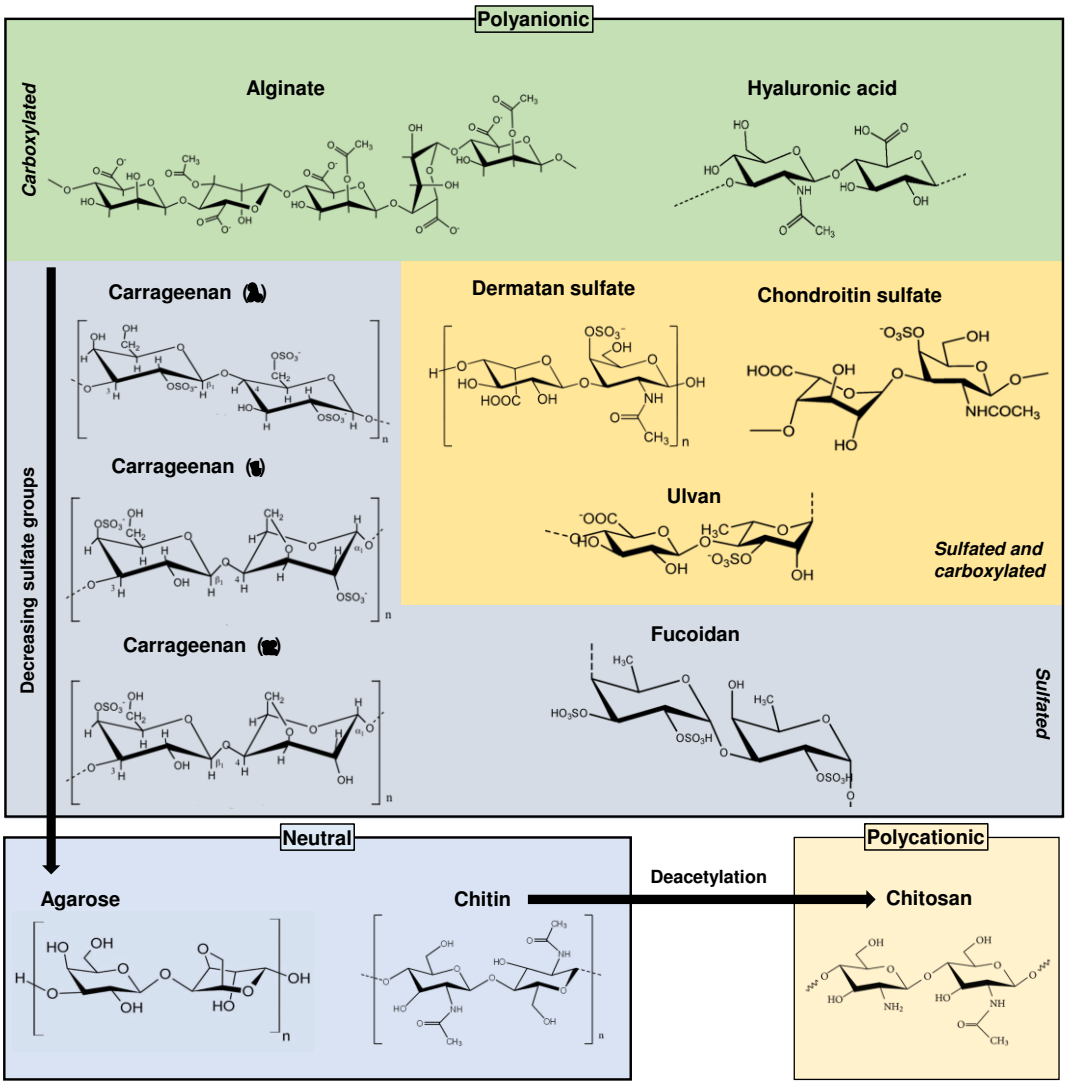

Figure 1.2. Structure of main marine origin polysaccharides, depending on ionic nature and the presence of carboxylate and sulfate groups.

2.1 Alginate

Alginate is a polysaccharide extracted from the brown seaweed, composed by a sequence of two (1→4)-linked α-L-guluronate and β-D-mannuronate monomers (**Figure 1.2**). Alginate is biocompatible, has low toxicity and high bioavailability as well. These are the main advantages that make alginate one of the biopolymers with the widest biomedical applicability. One of the most common applications of alginate is their use as an excipient in DDSs, namely acting as a stabilizer agent in pharmaceutical formulations (Tonnesen & Karlsen, 2002). Alginate has carboxyl groups which are charged at pH values higher than 3-4, making alginate soluble at neutral and alkaline conditions. Such pH sensitivity promotes greater protection for drugs with preferential absorption in the intestinal tract: the more acidic environment of the

stomach does not disturb the stability of the alginate carrier, whereas in the intestine (where the pH is alkaline) the solubility of this biopolymer – as well as the drug release – is promoted (George & Abraham, 2006). Thus, solubility and pH sensitivity make alginate a good biomaterial for the construction of DDSs (Chen, S. C. et al., 2004).

Alginate is widely used for its biocompatibility, low toxicity, high bioavailability and lower extraction and purification costs as compared with other biopolymers, and for the capability to be processed in the form of hydrogel matrices, beads and particles (Beneke, Viljoen, & Hamman, 2009; Gombotz & Wee, 2012; Laurienzo, 2010; Sudhakar, Kuotsu, & Bandyopadhyay, 2006). Alginate is also used as an excipient in pharmaceutic tablets to promote greater protection and stabilization of the drug. Sodium alginate is the type of alginate mainly used in the pharmaceutical industry in the manufacture of tablets, especially when the drug is not soluble in water. Sodium alginate may be used for the purpose of extending the drug release (Tonnesen & Karlsen, 2002). Studies using tablets containing ibuprofen demonstrated that it is possible to control the absorption ratio of the tablets. By using sodium alginate with different chemical structure and degree of viscosity, Sirkia *et al*. obtained carriers that triggered either an immediate ibuprofen release or prolonged it, proving that the chemical structure of alginate may influence the release rate of the bioactive agent (Sirkia, Salonen, Veski, Jurjenson, & Marvola, 1994).

Alginate has been used in the construction of microcapsules for controlled drug release following different techniques, including emulsion (Lima, Sher, & Mano, 2012; Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001; Zhang, Y., Wei, Lv, Wang, & Ma, 2011), multiple-phase emulsion (Ribeiro, Neufeld, Arnaud, & Chaumeil, 1999; Tonnesen & Karlsen, 2002) and calcium cross-linked encapsulation (Lin, Liang, Chung, Chen, & Sung, 2005). Using superhydrophobic surfaces it is possible to produce polymer particles suitable as DDSs. This method allows loading of drugs into spherical structures with an encapsulation efficiency close to 100% (Costa, A. M., Alatorre-Meda, Alvarez-Lorenzo, & Mano, 2015; Costa, A. M., Alatorre-Meda, Oliveira, & Mano, 2014; Song, Lima, & Mano, 2010). Taking its anionic nature, alginate can be assembled with polycations into different structures using layer-by-layer (LbL) (Becker, Johnston, & Caruso, 2010; Costa, N. L., Sher, & Mano, 2011; Zhao, Q. et al., 2007). This technique may be useful as a biomimetic approach applied in deconstructing and reconstructing the physiological conditions found in native biological environments, such as the human body (Mano, 2015). LbL has also been explored in several biomedical applications, such as biomimetic coatings, cell encapsulation and porous structures for cell seeding (Costa, R. R. & Mano, 2014). Over the years

chemical modification methods have been employed to promote a better loading efficacy of bioactive substances. The incorporation of proteins could usually be performed in alginate hydrogels complexed with other kinds of naturally occurring polymers such as the polycation chitosan (Augst, Kong, & Mooney, 2006; d'Ayala et al., 2008; George & Abraham, 2006; Tonnesen & Karlsen, 2002). There are other types of structures that can be obtained through LbL besides spherical and capsules: polyelectrolyte freestanding films have been shown to be suitable drug reservoirs of biomolecules, such as growth factors and antibiotics (Chen, D. et al., 2014). This type of films exhibit a good cell adhesion, possibility of cargo entrapment and fast release by variations of electrostatic interactions strength, and also promote a sustained release due to the slow film degradation (Caridade et al., 2013; Fujie et al., 2009; Fujie, Okamura, & Takeoka, 2007; Jiang, C. & Tsukruk, 2006; Okamura, Kabata, Kinoshita, Saitoh, & Takeoka, 2009). Such multilayer systems can be also used as barriers with controlled mass transporter properties (Silva, J. M. et al., 2014).

Alginate has the ability of cross-linking with Ca^{2+} ions through an ionotropic gelation process, usually above pH 6 (Mandal, Basu, & Sa, 2010). Ca^{2+} is not the only ion capable of promoting ionotropic gelation of alginate: Ba^{2+} or Zn^{2+} ions may also be used for that propose (Russo, Malinconico, & Santagata, 2007). Gelling of sodium alginate promoted by Ca^{2+} ions can solve pH dependent limitations related to the hydration, dilation and erosion of the carrier. Virtually any drug may be entrapped during such mild cross-linking process, and its subsequent release may be dependent on several factors, such as cross-linking extension (Oliveira, M. B. & Mano, 2013). In acidic environments, an alginate gel has a higher degree of viscosity, which may interfere with the elution of the bioactive agent from the device, thereby limiting drug release when the pH is low (Pillay & Fassihi, 1999a, 1999b; Xu, Y., Zhan, Fan, Wang, & Zheng, 2007). Giunchedi *et al.* reported that using sodium alginate, hydroxypropyl methylcellulose (HPMC), calcium gluconate, and ketoprofen as a model drug in the preparation of tablets by direct compression in different combination and ratios may prolong drug release, in particular in tablets with 20% of HPMC (Giunchedi, Gavini, Moretti, & Pirisino, 2000). Alginate hydrogels also have applications in wound healing treatments through the construction of structures used for wound dressings. Several studies show that the bioavailability of drugs encapsulated in alginate hydrogels is greater than if free drug was applied directly at the lesion site, thus increasing the efficacy of healing (Hamidi, Azadi, & Rafiei, 2008). Due to their gelling capability, alginate is also used in the construction of hydrogels that are widely used in tissue regeneration treatments and cell encapsulation (Augst et al., 2006; Lee, K. Y. & Mooney, 2001).

Hydrogels obtained from alginate, in particular, present some similar features of the extracellular matrix, thus being appropriate materials to be used in tissue engineering and regenerative medicine applications (Hamidi et al., 2008).

Alginate is also used in the construction of microparticles with the ability to incorporate different bioactive agents, particularly proteins. Alginate microparticles have the capability of retaining large amounts of drug and also promoting protection of the cargo from any proteolytic attack. There are different mechanisms of release of a bioactive agent from the delivery device, such as through variations of temperature and pH values, and the use of biodegradable materials or enzymatic degradation, among other chemical and physical stimuli-responsive methods (George & Abraham, 2006; Gombotz & Wee, 2012; Mano, 2008; Mura, Nicolas, & Couvreur, 2013). These parameters are difficult to control and program, since they can vary significantly. However, new release mechanisms from microparticles have been developed, that depended on the effect of fully controlled external stimuli, such as ultrasound-triggering. Duarte *et al.* developed a type of alginate microparticles which were shown to have perfluorocarbon breakthrough capacity when subjected to vibration by ultrasound waves (Duarte, Unal, Mano, Reis, & Jensen, 2014). Results showed a disruption of these microparticles after 15 minutes of exposure, suggesting that such structures are promising DDSs controlled externally by acoustic stimuli (**Figure 1.3**).

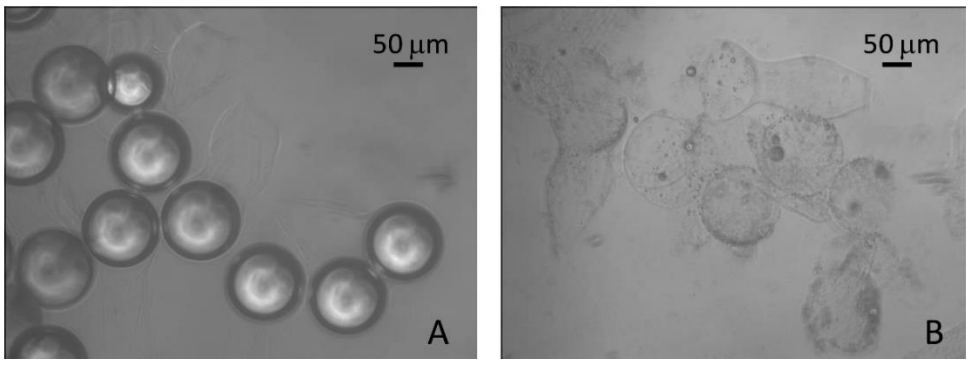

Figure 1.3. Optical microscope images of alginate microspheres before (A) and after (B) ultrasound exposure. Reprinted with permission from (Duarte et al., 2014), Copyright © 2014 American Chemical Society.

Alginate has the ability to create complexes with other biomaterials by electrostatic interactions, chemical modification or cross-linking. This capability can be exploited for building hybrid but more versatile DDSs. Capsules constructed from chitosan/alginate-PEG complexes are reliable models for encapsulating proteins, such as albumin, one of the most common model proteins used in controlled

release studies (Chandy, Mooradian, & Rao, 1998). The construction of alginate spherical structures with other types of synthetic materials can be a good strategy to extend the versatility of these systems. Using poly(*N*-isopropylacrylamide) (PNIPAAm) to take advantage of its thermosensitive properties (Schmaljohann, 2006) in combination with alginate can lead to devices capable of delivering biomolecules with a dual stimuli-responsive dependence (both pH and temperature) (Prabaharan & Mano, 2006). Studies using indomethacin as a model drug reported that chitosan-alginate-PNIPAAm beads showed lower release rates with decreasing temperatures (Shi, Alves, & Mano, 2008). The same occurs when there is a decrease in pH, indicating that it is possible to control the permeability of the particles by controlling both pH and temperature. This approach can lead to the development of DDSs capable of promoting higher control over the release of drugs, proteins and others biomolecules with pharmaceutical interest. Following a similar concept of polymer conjugation, alginate can also undergo complexation with natural polymers, like chitosan, to enhance the absorption and cargo protection in oral delivery, for example, for the administration of insulin (Sarmento et al., 2007; Zhang, Y. et al., 2011).

Alginate may be used in the construction of capsules for cell encapsulation often associated with cytotherapy treatments or simply the creation of cellular microcultures in more complex systems where the use of a conventional bioreactor is not possible. In this context, a new approach to the construction of alginate-based capsules for the incorporation of different types of cells has been presented (Correia, Reis, & Mano, 2013; Correia, Sher, Reis, & Mano, 2013). Cells were encapsulated in alginate liquefied particles, coated with multilayer of alternating chitosan and alginate. Along with the cells, poly(lactic acid) microparticles were co-encapsulated to provide anchorage points so that cell survival is promoted. Results demonstrated a high viability of the encapsulated cells and usefulness of these capsules as culture systems. This type of system has wide applicability not only for the cell culture but also in other biomedical applications, since it will allow the encapsulation of different types of cells in combination with other biomolecules such as, for example, growth factors and other cytokines.

Alginate has been used to construct DDSs (especially nanoparticles) for gene therapy treatments. The very first systems for the gene delivery were based on genetic material encapsulated within viral vectors. These have several limitations such as the possibility to trigger an immune and inflammatory reactions, infections and mutations. These systems also have high costs of production due to complexity in the processing of viral vectors (Thomas, C. E. et al., 2003). Taking advantage of the capability of natural polymers to form complexes with DNA, safer DDSs could be synthesized to deliver genetic material. The

most commonly used polymers in the construction of DNA load vehicles are usually of synthetic origin, for example polyethylenimine (PEI), poly-L-lysine (PLL), poly(L-ornithine) and poly(4-hydroxy-L-proline ester) (Thomas, M. & Klibanov, 2003). The use of these synthetic materials has allowed the synthesis of complexes *via* electrostatic interactions between the polymer and the DNA, allowing the creation of a stable complex and the possibility of size adjustment. One of the major limitations of using synthetic materials is their often adverse biological effect in the body. PEI, for example, exhibits elevated levels of cytotoxicity (Pack, Hoffman, Pun, & Stayton, 2005). In contrast, most natural materials are biocompatibile, biodegradable (in some cases) and show similar capacity to form ionic bonds, therefore providing ensuring good protection for genetic material (Liu, Jiao, Wang, Zhou, & Zhang, 2008; Panyam & Labhasetwar, 2003; Quick, Macdonald, & Anseth, 2004). Krebs *et al.* developed a calcium phosphate-DNA nanoparticle system incorporated in alginate-based hydrogel for gene delivery to promote bone formation. Results showed a DNA sustained release from the alginate hydrogel around 45% of DNA released after approximately 75 days. *In vivo* studies, through the injection of alginate hydrogels containing calcium phosphate nanoparticles and osteoblast-like cells in mice, showed evidence of bone formation (Krebs, Salter, Chen, Sutter, & Alsberg, 2010).

2.2. Carrageenan

Carrageenan is a sulfated polysaccharide present in red algae, which structure consists in a linear sequence of alternate residues forming $(AB)_n$ sequence, where A and B are units of galactose residues. These residues may or may not be sulfated. They are linked by alternating α-(1→3) (unit A) and β-(1→4) (unit B) glycosidic bonds (**Figure 1.2**). Unit A is always in D-conformation, while unit B can be found either in D- or L- configuration. The sulfated groups give it a negative charge, which categorizes carrageenans as polyanions (Rinaudo, 2008). Carrageenans are classified according to their degree of sulfation: they can be kappa (κ), iota (ι), and lambda (λ), if they have one, two or three sulfate groups respectively. The extraction process is straightforward, consisting in the immersion of the raw material in alkaline solution so that a gel forms. Then follows an extraction step, where the gel is immersed in water heated at 74 °C. Depending on the type of carrageenan and desired degree of purification, it is possible to execute additional purification steps, such as dialysis. The process finishes with filtration, precipitation and drying (Rinaudo, 2008). All carrageenans are water soluble, and the number of sulfated groups influences the gelation capacity. Carrageenans κ and ι can form gels in the presence of cations more readily than carrageenan λ because of the low number of sulfated groups. This characteristic has been

used in many areas, such as food industries (using carrageenan as an emulsifier and stabilizer), as well as in the cosmetic and pharmaceutical industries (Li, L., Ni, Shao, & Mao, 2014). Contrary to what happens with other biomaterials of marine origin, the use of carrageenan as an excipient in the pharmaceutical industry is not common, so there are not many studies on their applications, characteristics and functions as such. As an example, a study was conducted where two types of carrageenan (κ and ι) were analyzed in terms of compression behavior and their capability of tablet formation (Picker, 1999). Results showed that both carrageenans are suitable excipient for controlled release. Carrageenans are also present in various biomedical applications due to their anticoagulant properties (Silva, F. R. F. et al., 2010), antitumor, immunomodulatory (Zhou et al., 2004), anti-hyperlipidemic (PanlasiguiPhD, Baello, DimatangalBSc, & DumelodMSc, 2003) and antioxidant activities (de Souza et al., 2007). They also have a protective activity against bacteria, fungi and some viruses (Campo, Kawano, da Silva, & Carvalho, 2009; Carlucci, Ciancia, Matulewicz, Cerezo, & Damonte, 1999). Due to the latter, carrageenans have been suggested for possible treatments of respiratory diseases, such as the famous bird flu, and is also being tested for killing other viruses, such as the dengue fever, hepatitis A, HIV (Schaeffer & Krylov, 2000) and herpes viruses (Carlucci, Scolaro, Noseda, Cerezo, & Damonte, 2004). Studies showed that carrageenan, and derivatives of degradation have different levels of toxicity, but do not endanger the health of the patients (Campo et al., 2009; Prajapati, Maheriya, Jani, & Solanki, 2014). These properties make carrageenan a promising biomaterial for biomedical applications.

The use of carrageenan as an excipient in the manufacture of devices for oral delivery depends mostly on their physicochemical properties, such as water solubility and jellification capability. Carrageenan load capacity depends largely on the sulfation extent, which affects its mechanical properties and its dissolution rate. These factors may affect the release of the cargo, prolonging or accelerating its release (Bornhoft, Thommes, & Kleinebudde, 2005). To obtain a greater control of the drug release profile – regardless of other conditions, such as carrageenan type and pH – it is necessary to associate or conjugate other polymers. The addition of polymers such as hydroxypropyl methylcellulose (HPMC), a temperature sensitive semi-synthetic polymer, can solve problems related to pH erosion and provide higher protection to the drug, thus promoting a sustained release that does not depend on pH (Bonferoni et al., 1998). However, the opposite response may be desired (i.e. pH-triggered degradation) and, for that, pH responsive polymers may be conjugated. By varying the pH, it is possible to control not only the loading but also the release mechanisms of carrageenan/alginate interpenetrated networks (Mohamadnia,

Zohuriaan-Mehr, Kabiri, Jamshidi, & Mobedi, 2007). The use of stimuli-responsive materials offers another perspective for drug and gene delivery where the carrier may be an active trigger and function as a therapy optimizer. Using temperature-sensitive materials for nanocarriers construction can promote a controlled release at temperatures above 37 °C. Such a system could be helpful in situations as common as a fever. However, it is possible to use other nanocarriers in situations of hyperthermia, where the drug would be available in a localized region (Ganta, Devalapally, Shahiwala, & Amiji, 2008; Meyer, Shin, Kong, Dewhirst, & Chilkoti, 2001).

Carrageenan in the pharmaceutical industry is basically used as a raw material for the construction of DDSs, cell capsules for cell therapies and cartilage regeneration applications (Popa et al., 2013; Silva, T. H. et al., 2012). The use of carrageenan-based hydrogels as a vehicle for the controlled delivery of biomolecules can be a good strategy especially for cargo stabilization Popa *et al.* showed that κ-carrageenan hydrogels are adequate environments to encapsulate different types of human cells achieving chondrogenic differentiation (Popa, Caridade, Mano, Reis, & Gomes, 2015). This system proved to have potential for cartilage regeneration strategies, not only due to the referred differentiation but also because these hydrogels can be easily injectable *in situ* and may be used as reservoirs for growth factors (Rocha, Santo, Gomes, Reis, & Mano, 2011). Carrageenan-based hydrogels, along with other materials of marine origin, have also proved to be suitable good devices for cell encapsulation (Gasperini, Mano, & Reis, 2014; Luna, Gomes, Mano, & Reis, 2010). New methods on the production of spherical beads and fibrillar carrageenan/alginate based hydrogel have been developed. Fibrillar hydrogels obtained by wet spinning showed great potential for applications as a cell carrier for cell delivery systems (Popa, Gomes, & Reis, 2011). Knowing the biological properties of carrageenan, it is hypothesized that carrageenan-based devices are suitable DDSs for the delivery of not only bioactive agents but also of cells for cytotherapies.

Carrageenan is also used in the construction of multilayer structures (Oliveira, S. M., Silva, Reis, & Mano, 2013), microcapsules (Yeo, Baek, & Park, 2001) and micro/nanoparticles (Rodrigues, da Costa, & Grenha, 2012). These structures are mainly built *via* electrostatic interactions between carrageenan and a polycation. For example, Grenha *et al.* developed carrageenan/chitosan nanoparticles through a simple construction method by ionic interactions between polycationic groups of chitosan and polyanionic ones of carrageenan (**Figure 1.4A**) (Grenha et al., 2010). This method has the advantage of avoiding the use of organic solvents and harmful cross-linkers. These nanoparticles had a diameter size between

350-650 nm. Using albumin as a model protein, *in vitro* release tests demonstrated a prolonged release over time, with a 100% of albumin release after three weeks (**Figure 1.4B**). Having a slow release rate is important since it enables the reduction of the encapsulated dose and also provides continuous long-term release without the need for repeated administrations. Cytotoxicity tests demonstrated that these devices present low toxicity. These results are a good indicator that these structures may be feasible for the encapsulation of agents with therapeutic purposes.

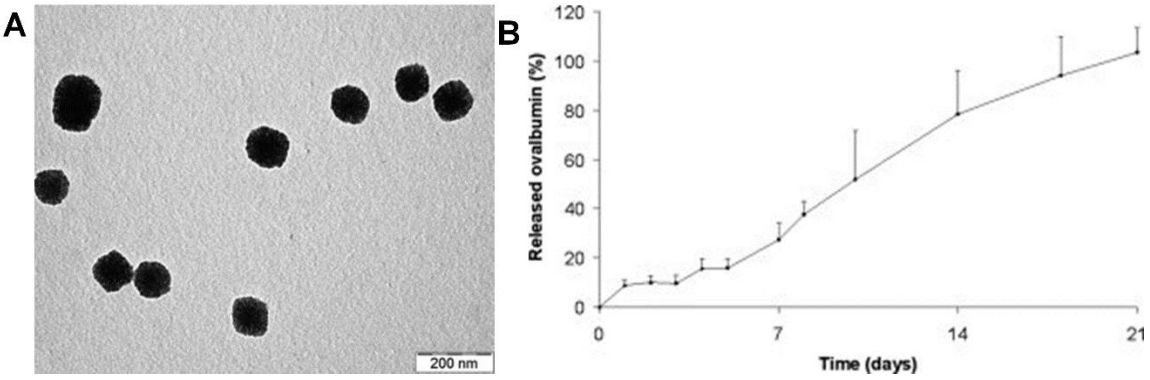

Figure 1.4. TEM micrograph of chitosan/carrageenan nanoparticles (A). Ovalbumin release profile from chitosan-carrageenan nanoparticles (B). Adapted with permission from (Grenha et al., 2010), Copyright © 2009 Wiley Periodicals, Inc.

Agarose is another marine biomaterial with a structure similar to carrageenan, present in the cell wall of red algae. Its structure comprises monosaccharide residues connected alternately in the conformation $(AB)_n$. The units consist of galactose residues linked by α-(1→3) (unit A) and β-(1→4) (unit B) linkages (**Figure 1.2**). The main difference between carrageenan and agar is that the carrageenan unit A is always in the D- conformation, while in the agar unit A can only be in the L- conformation (Usov, 1998). Unlike carrageenan, agarose is not classified according to the sulfation degree, since the best known type of agarose is a neutral type without any sulfated group. Agarose is widely used in food industry and also in microbiology in the form of gel to be used as culture medium in the form of agar. Agarose is associated with several biomedical applications especially as hydrogels for the release of bioactive agents, taking advantage of its ability to jellify, biocompatibility and native biodegradability (Hoare & Kohane, 2008; Rossi et al., 2011).

2.3 Fucoidan

Fucoidan is a sulfated polysaccharide found in many species of brown algae. It is a polymer chain of (1→3)-linked α-l-fucopyranosyl residues (**Figure 1.2**), although it is possible to find alternating (1→3) and (1→4)-linked α-l-fucopyranosyl residues. The structure of fucoidan and its composition depend largely on the extraction source, especially the type of algae. The extraction can be processed by precipitation using salts or organic solvents, followed by a purification step by chromatography (Li, B., Lu, Wei, & Zhao, 2008). Recently it was reported that fucoidan has antitumor activity dependent on the degree of sulfation and can inhibit tumor cell proliferation and growth (Anastyuk et al., 2012; Ermakova et al., 2011). However, it was also reported that fucoidan is toxic not only to tumor cells but also to normal healthy cells, thus hindering its use in anticancer therapies. Fucoidan has also shown anticoagulant and anti-inflammatory properties (Cumashi et al., 2007), as well as anti-adhesive and antiviral properties (Kim, S. K., Ravichandran, Khan, & Kim, 2008; Sezer, A. & Cevher, 2011).

Like other marine-origin polysaccharides, fucoidan can also be used as a raw material for the construction of DDSs. A typical way of processing fucoidan DDSs is by electrostatic interactions with chitosan, to make microspheres, so-called fucospheres (Sezer, A. D. & Akbuga, 2006), which have been suggested for burn treatments. Particles with sizes ranging between 367 and 1017 nm were shown to trigger both *in vitro* and *in vivo* a decrease of the normal burn treatment time due to the increase of regeneration and healing of epithelial tissue (Sezer, A. D. et al., 2008). Taking advantage of the great bioactivity of fucoidan, and the ability to complex with other materials like chitosan, other approaches can be pursued. Nakamura *et al.* developed a chitosan/fucoidan microcomplex hydrogel for the delivery of heparin binding growth factors (Nakamura et al., 2008). This hydrogels showed high affinity with growth factors and were able to promote growth factor activity and also a controlled release. *In vivo* studies showed a neovascularization promoted by the growth factors released from the chitosan/fucoidan hydrogel. Another approach to take advantage of chitosan/fucoidan interactions as DDSs is described by Huang and Li, who developed a novel chitosan/fucoidan nanoparticles with antioxidant properties for antibiotics delivery (**Figure 1.5A**) (Huang & Li, 2014). These nanoparticles presented a spherical morphology and diameter of 200-250 nm. Results showed a highly anti-oxidant effect by reducing concentration of reactive oxygen spices (ROS), using gentamicin as a model drug, release studies showed a controlled release around 99% of gentamicin in 72 h (**Figure 1.5B**). The antioxidant chitosan/fucoidan

nanoparticles could thus be effective in delivering antibiotics to airway inflammatory diseases, where the amount of ROS it significantly high.

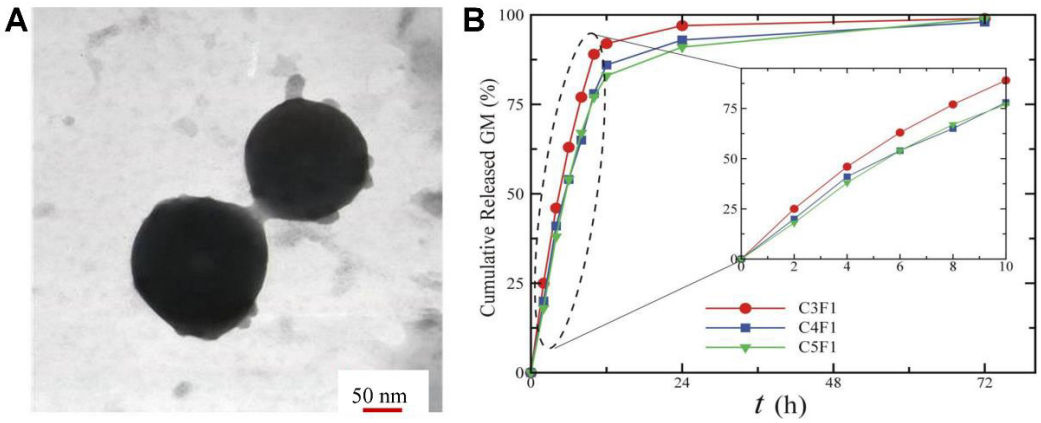

Figure 1.5. TEM image of chitosan/fucoidan nanoparticles (A). Gentamicin release kinetics from chitosan/fucoidan particles (B). Adapted with permission from (Huang & Li, 2014), Copyright © 2014 distributed under a Creative Commons Attribution License.

There are several other ways of processing fucoidan-based DDSs. Some of them are built using LbL, particularly for fucoidan-chitosan pH sensitive capsules for insulin controlled release (Sato, K., Takahashi, & Anzai, 2012). Pinheiro *et al.* used polystyrene nanoparticles with a diameter approximately 100 nm as a template for the deposition of a fucoidan-chitosan multilayered coating (Pinheiro et al., 2015). After construction of the coating, the polystyrene core was removed, being thus possible to incorporate into the capsule numerous bioactive agents. Using PLL as a model molecule, results showed that the release profile was pH dependent and also that the release occurred by diffusion. These results indicate the sensitivity of these particles to pH variations found along the gastro-intestinal tract and the possibility of using these particles as DDSs for oral administration.

2.4. Ulvan

Ulvan is a sulfated polysaccharide extracted from the green algae *Ulva lactuca*. Ulvan consists in a polymer chain of different sugar residues like glucose, rhamnose, xylose, glucuronic and iduronic acid with α- and β-(1→4) linkages (**Figure 1.2**). Because of the large number of sugars in its composition, ulvan may exhibit variations in the electronic density and charge distribution, as well as variations of molecular weight. Since it contains rare sugars, ulvan is a natural source for obtaining them upon depolymerization, instead of resorting to chemical synthesis. The extraction process is simple, consisting

in adding organic solvent over the feedstock followed by successive washing steps with hot water, filtration and centrifugation (Lahaye & Robic, 2007). Ulvan has several properties of biological interest, such as exhibiting antiviral, antioxidant, antitumor, anticoagulant, anti-hyperlipidemic and immune system enhancing activities. Ulvan also presents low cytotoxicity levels in a wide range of concentrations (Alves, A., Sousa, & Reis, 2013a). Ulvan is typically used in the food and cosmetic industries, but because of their biological properties, it has a great potential for the development of new DDSs, such as being used as an active principle in pharmacological formulations (Ahmed & Ahmed, 2014). Because of their ability for complexing with metal ions, ulvan can also be used as a chelating agent in the treatment against heavy metal poisoning (Alves, A., Sousa, & Reis, 2013b). Furthermore, the capacity to process ulvan as nanofibers and membranes has been useful for tissue engineering and regenerative medicine, for example in wound healing treatments (Dash et al., 2014).

Like other marine origin polysaccharides, ulvan may undergo chemical modifications to synthesize thermostable hydrogels. The addition of other functional groups is also possible so that temperature and light responsive hydrogels are conceived. In this case, ulvan was modified with methacrylate groups to allow jellification by photopolymerization through the irradiation with ultraviolet light (Morelli & Chiellini, 2010). This is a useful approach to develop cell encapsulation strategies for cytotherapy applications. Ulvan is also used in the construction of membranes, due to electrostatic interactions with other cationic polymers (Toskas et al., 2012). Through chemical modification, ulvan and chitosan can also be used as a polymeric component of bone cement, especially due to their mechanical properties (Barros et al., 2013).

Ulvan has been used in construction of nanocarriers for biomolecules. Alves *et al*. constructed a two-dimensional ulvan-based structure for drug delivery by chemical cross-linking for wound healing (Alves, A., Pinho, Neves, Sousa, & Reis, 2012). Using dexamethasone as a model drug, there was a rapid release in the first hour (around 49%), followed by a slower and sustained release, around 75% up to 14 days. Additionally, it is also possible to obtain three-dimensional ulvan-based structures. In this context, ulvan/chitosan particles were produced for the encapsulation and release of dexamethasone (Alves, A., Duarte, Mano, Sousa, & Reis, 2012). These particles were incorporated in three-dimensional poly(D,L-lactic acid) porous scaffolds for bone tissue regeneration. *In vitro* release assays demonstrated a steady release in the first 3 hours (around 52%), followed by a sustained cumulative release up to 0% in the next 21 days.

3. Polysaccharides from marine animals

There are other marine sources beside algae and microorganisms: marine animals are also an excellent font for polysaccharides. In this section, the most important animal origin polymers used in DDSs will be presented. There are two main categories of polymers: chitin-derived polymers and glycosaminoglycans (GAGs), polymers that consist of a disaccharide that possesses an amino sugar.

3.1 Chitosan

Chitosan is a linear polysaccharide derived from chitin, one of the most abundant natural polymers of our ecosystem (Bansal, Sharma, Sharma, Pal, & Malviya, 2011). Chitosan is obtained by the deacetylation of chitin, resulting in a compound with randomly distributed D-glucosamine residues (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (**Figure 1.2**) (Alves, N. M. & Mano, 2008; Rinaudo, 2006). Chitosan, as well as chitin, can be degraded by enzymes such as chitinase and lysozyme (Varum, Myhr, Hjerde, & Smidsrod, 1997). Chitin is the main component of the exoskeleton of arthropods and crustaceans such as crabs, shrimps and lobsters, and can also be extracted from some fungi and nematodes. Chitin is not water soluble, and thus it is usually converted into soluble derivatives including chitosan (soluble in acidic conditions) and carboxymethyl chitosan (soluble in a wide range of acidic and alkaline solutions). Chitosan has amine groups sensitive to pH variations, being positively charged in acidic environments and neutral in alkaline pH values (pK_a close to 6) (Mano, 2008). Chitosan is one of the marine origin polysaccharide most widely used and studied for biomedical applications, in particular in the construction of nanoparticles, beads and capsules for controlled drug delivery systems, and also membranes, films and scaffolds for tissue engineering and regenerative medicine (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Couto, Hong, & Mano, 2009; Prabaharan & Mano, 2005a; Sinha et al., 2004).

Chitosan has antimicrobial activity, a useful property to build films that prevent wound infection (Kim, I. Y. et al., 2008; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). It also shows antitumor and anti-inflammatory activity (Chung, Park, & Park, 2012; Qin, Du, Xiao, Li, & Gao, 2002). All of these biological properties make chitosan an excellent candidate for constructing devices that require the contact with biological environments, and as excipients for DDSs (Ilium, 1998; Singla & Chawla, 2001). For chitosan-based DDSs, electrostatic interactions between the polysaccharide and a bioactive agent are a key to drug stabilization, protection and acceleration (or deceleration) of its release. This means that, if

a drug is anionic, positively-charged polymers (like chitosan) are used as excipient, and vice-versa. The release profile and rate of biomolecules from within chitosan-based carriers may depend on the morphology, size, density, cross-linking degree, as well as the deacetylation degree of chitosan and physicochemical properties of the bioactive agent. The release will also be affected by the pH and by the presence or absence of enzymes. The release may occur in different ways: (i) release from the surface of DDSs, (ii) passive diffusion, and (iii) erosion of the DDS. Deacetylation degree of chitosan can be also used as a degradation control parameter (Agnihotri et al., 2004; Bhise, Dhumal, Paradkar, & Kadam, 2008). Another mechanism of release exploited for chitosan-based carriers is triggered by enzymatic degradation (Felt, Buri, & Gurny, 1998). It is also possible to increase the binding capacity of poorly water-soluble drugs by introducing different chemical modifications onto chitosan. Hydroxypropyl chitosan (HPCH), obtained from the reaction between chitosan and propylene epoxide under alkali condition, can be grafted with carboxymethyl β-cyclodextrin mediated via a water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Prabaharan & Mano, 2006). Hydrophobic drugs can be encapsulated due the presence of hydrophobic groups present in HPCH. Also, due to the free amine groups that can be protonated at lower pH values such DDSs can be pH-responsive. Using ketoprofen as a hydrophobic drug model, *in vitro* release results showed that this chitosan derivate structure has a great potential as a biodegradable delivery system for hydrophobic drugs in a pH-sensitive controlled release (Prabaharan & Mano, 2005b; Prabaharan, Reis, & Mano, 2007).

Chitosan chemical modification can be a good strategy to increase the effectiveness of release and attribute other properties such as drug protection and stabilization (Alves, N. M. & Mano, 2008). For example, the introduction of thiols groups increases the solubility of chitosan in water, maintaining the pH dependence of chitosan particles (Jayakumar, Reis, & Mano, 2007). *N,O*-carboxymethyl chitosan (NOCC, also known as carboxymethylated chitosan) is a water soluble derivative that retains a fraction of the amine residues and its polycationic properties under acidic conditions (Chen, S. C. et al., 2004). Ketoprofen-loaded beads of NOCC and a PNIPAAm with a telechelic amine group (PNIPAAm-NH_2) were developed for the study of controlled release system. Release studies taking in acidic and physiological conditions at 21 and 37 °C, showed that these particular beads are sensitive to temperature and pH variations (Prabaharan & Mano, 2007). Acetylated chitosan grafted with fatty acid like palmitoyl is another strategy to develop chitosan-based excipients to entrap and release hydrophobic drugs (Jiang, G. B., Quan, Liao, & Wang, 2006; Le Tien, Lacroix, Ispas-Szabo, & Mateescu, 2003; Martin, Wilson, Koosha, &

Uchegbu, 2003). Methacrylamide chitosan, a water-soluble modified chitosan, also suitable for photo-cross-linking, has been used for the construction of delivery carriers. Wijekoon *et al.* developed a fluorinated methacrylamide chitosan hydrogel for oxygen delivery in wound healing (Wijekoon, Fountas-Davis, & Leipzig, 2013). During the methacrylation process, different fluorinated ligands were added to chitosan to obtain different fluorinated methacrylamide chitosans. Hydrogels were constructed by photo-cross-linking. This new biocompatible, injectable moldable photo-cross-linked chitosan-based hydrogel allowed to control both the capacity and rate of oxygen delivery, maintaining beneficial oxygen level up to five days.

The reactivity of chitosan with other materials may also promote sustained release and cargo stabilization and protection. This can be achieved using different methods, such as graft copolymerization with synthetic polymers like poly(ethylene glycol) (PEG) and PEI (Felt et al., 1998; Prashanth & Tharanathan, 2007). Several studies showed the ability of chitosan to enhance and prolong the absorption of hydrophilic drugs taken by oral (Thanou, Verhoef, & Junginger, 2001) and pulmonary (Andrade et al., 2011) administration routes. Chemical modification of chitosan with PEG is a way of improving the biocompatibility of chitosan, especially to reduce chitosan toxicity, as well as to enhance protein adsorption, cell adhesion, growth and proliferation (Casettari et al., 2012; Zhang, M., Li, Gong, Zhao, & Zhang, 2002). Prego and coworkers showed that chitosan-PEG nanocapsules for oral delivery of peptides exhibited low cytotoxicity and enhanced intestinal absorption capability (Prego, Fabre, Torres, & Alonso, 2006). Other studies showed that this approach can also be applied to deliver other drugs such as insulin (Jintapattanakit et al., 2007; Mao, Germershaus, et al., 2005; Mao, Shuai, et al., 2005; Zhang, X. et al., 2008). Taking advantage of the jellification capability of some copolymers containing chitosan, Bhattarai and coworkers presented an injectable PEG-grafted chitosan hydrogel for controlled release (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005). These hydrogels were liquid at room temperature and a gel at physiological temperature. Using albumin as a protein model, i*n vitro* release studies at 37 °C showed a high release in the first 5 hours, up to 50-60% followed by a sustained release for the next days with a cumulative release up to 80%.

Hydrogels based on cross-linked chitosan may have the ability to promote a sustained release upon nasal administration. Hydrogels were constructed by joining *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) and PEG with the addition of a small quantity of α-β-glycerophosphate (α-β-GP) as a gelling agent (Wu, Su, & Ma, 2006). These hydrogels are pH sensitive and have the particularity of

being liquid at room temperature and exhibit higher rigidity at 37 °C. Wu and coworkers developed these hydrogels as smart devices for the controlled release of biomolecules through nasal administration as drops or spray. Once applied, the solution is exposed to physiological temperature, becoming a viscous hydrogel which can be absorbed by mucosa. Because of their ease of production and administration, this new formulation was tested as a loading device for the controlled release of insulin. Assays in rats showed an increased absorption in the nasal cavities and a decrease in blood glucose, without any evidence of cytotoxicity. These results demonstrated the great potential of these hydrogels as carriers for the controlled release of bioactive agents, especially hydrophilic biomolecules (Wu, Wei, Wang, Su, & Ma, 2007). Nasal administration is less compliant for the patient, causing no discomfort and pain, leading to a reliable management and patient satisfaction (Nazar et al., 2011). Furthermore, the fact that this type of hydrogels are liquid at room temperature also enhances their ease of application as a DDS for parenteral administration (Tahrir, Ganji, & Ahooyi, 2015).

As with other marine polysaccharides, chitosan can form stable and highly dense complexes capable of providing stability and protection to drugs, and in some cases are able to respond to other types of external stimuli. One such example is the conception of LbL microcapsules made by complexation of chitosan with negatively charged elastin-like recombinamers (ELRs), a recombinant polypeptide with intrinsic response towards temperature (Costa, Rui R., Martín, Mano, & Rodríguez-Cabello, 2012). Novel thermoresponsive ELR/chitosan microcapsules were developed for the delivery of active molecules (Costa, R. R., Custodio, Arias, Rodriguez-Cabello, & Mano, 2013). Using bovine serum albumin (BSA) as a model molecule, the results showed a greater BSA retention at physiological temperature (37 °C), when compared to room temperature (25 °C). Studies with cells also showed a low cytotoxicity for such structures. The pH response of these microcapsules was not studied, but the results are a good indicator that chitosan can bond with other sources of stimuli-responsive biomaterials, including unconventional ones such as genetically engineered polypeptides. While thermal responses are perhaps the most exploited mechanism integrated in smart DDSs, it is debatable whether their sensitivity would be enough to treat, for example, a common fever, where the body temperature varies just 1-2 °C. Besides, not all people have exactly the same body temperature. Therefore, conjugating two or more physiological parameters could be a solution for diseases that require administration based on triggers operating within tight ranges.

Being a polycationic polysaccharide, chitosan can form complexes with nucleic acids for gene therapy. There are different studies that use chitosan for gene transfection (Borchard, 2001; Leong et al., 1998; Roy, Mao, Huang, & Leong, 1999; Sato, T., Ishii, & Okahata, 2001). The formation of complexes between the polymer and the nucleic acids depends on many intrinsic factors, such as the deacetylation degree, the molecular weight, as well external factors like temperature and pH, and represent crucial factors on the efficiency of transfection (Saranya, Moorthi, Saravanan, Devi, & Selvamurugan, 2011). The positive charge of chitosan allows interacting with the negatively charged peptidoglycans present in the cell membrane, facilitating the entry of a chitosan/DNA complex into the cell by pre-established endocytic pathways (Cho, Kim, & Park, 2003; De Smedt, Demeester, & Hennink, 2000). The amount of genetic material available to react with chitosan is also very important: an improper ratio can lead to the dissociation of the complex or to a lack of synthesized complexed particles, resulting in low transfection rate (Saranya et al., 2011). It has been reported that using chitosan overcomes some of these limitations: chemically modifying chitosan can increase the affinity with the DNA to yield a more stable complex, which can lead to an increase in the transfection efficacy (Kean, Roth, & Thanou, 2005). The modifications can also increase chitosan solubility and thus offer greater protection to the cargo from the degradative action of DNases on DNA (Saranya et al., 2011). Chemical derivatives opened a new range of possibilities to construct DDSs for the intracellular release of the genetic material, with wide applicability in the treatment of various genetic diseases. As previously mentioned, copolymerization of chitosan with other polymers can lead to a better control over the release of biomolecules. Following this line, Forrest and coworkers presented a PEI-PEG-chitosan-copolymer for gene delivery with good loading capacity and high transfection efficacy, as well as low toxicity that makes these particles good candidates for *in vivo* gene delivery (Kievit et al., 2009).

3.2. Hyaluronic Acid

Hyaluronic acid is a linear polysaccharide consisting of an alternating chain disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic linked by β-(1→3) and β-(1→4) glycosidic bonds (**Figure 1.2**) (Burdick & Prestwich, 2011). Hyaluronic acid is a major component of extracellular matrix and is present in the synovial fluid, vitreous humor and cartilage tissue. Due to its high viscoelasticity, hyaluronic acid has an important role in several biological functions and also as an excellent material for different biomedical applications. Namely, it is involved in tissue regeneration, cell proliferation, differentiation and migration (Kogan, Soltes, Stern, & Gemeiner, 2007). Because of its presence in the synovial fluid in joints,

hyaluronic acid can be used as a biological marker to diagnose diseases associated with rheumatoid arthritis (Lee, H., Lee, Kim, & Park, 2008). Due to its biocompatibility and biodegradability, hyaluronic acid has also been proposed for tissue engineering applications for manufacturing wound healing structures (Chen, W. Y. & Abatangelo, 1999) and as a supplement for patients with arthritis (Kogan et al., 2007). Nowadays, hyaluronic acid production is done on a large scale using different methods and sources, such as bacterial fermentation (Kim, S. J., Park, & Kim, 2006; Rangaswamy & Jain, 2008; Vazquez, Montemayor, Fraguas, & Murado, 2009). Hyaluronic acid may also be extracted directly from marine animal sources, such as cartilage and also from the vitreous humor of several fish species (Murado, Montemayor, Cabo, Vazquez, & Gonzalez, 2012). His biodegradability is mediated by the action of hydrolases, such as hyaluronidase, which breaks the glycosidic bond between two residues (Zhong et al., 1994). In the human body, hyaluronic acid is present in various biological fluids, allowing its use as a biomarker to monitoring its movement in biological fluids (Lokeshwar et al., 2001; Rousseau & Delmas, 2007).

Hyaluronic acid has the ability to interact with several proteins. This can be used as a diagnostic tool, in particular due to existence of membrane receptors specific for hyaluronic acid. It is the case of CD44, a receptor that is highly expressed when there is an increase in cell proliferation. Determining an increased expression level of CD44 by means of hyaluronic acid devices can be an excellent marker for the early diagnosis of cancer (Kramer et al., 2011). Hyaluronic acid hydrogels can be used as reservoirs of bioactive agents obtained via various methods of constructions (Luo, Kirker, & Prestwich, 2000). Nanoparticles based on the interaction of hyaluronic acid with metals, such as gold, have been widely used as markers for diagnosing diseases such as rheumatoid arthritis and cancer due to the ability of some of these devices to emit fluorescence (Leroy et al., 2004; Lokeshwar et al., 2000; Mohammad & Xuefei, 2011; Rousseau & Delmas, 2007).

Like other polyanions, hyaluronic acid can be complexed with polycations such as chitosan to form nanoparticles (Oyarzun-Ampuero, Brea, Loza, Torres, & Alonso, 2009) and microspheres (Lim, Martin, Berry, & Brown, 2000). Recent studies presented a new approach for the treatment of ocular disorders. Hyaluronic acid/chitosan nanoparticles have been synthesized by means of electrostatic interactions to develop nanoparticles for the delivery of genes to the cornea and conjunctiva (Contreras-Ruiz et al., 2011; de la Fuente, Seijo, & Alonso, 2008). Results indicated an appropriate size distribution (100-230 nm) and internalization of these particles by endocytic processes mediated by membrane receptors. This result

reveals the great biomedical applications potential of these nanoparticles as gene delivery device for treating diseases at the level of the human conjunctiva and other ocular diseases.

Cross-linked chitosan spheres can serve as templates for the alternating adsorption of hyaluronic acid and chitosan multilayers (Grech, Mano, & Reis, 2008). *In vitro* release using gentamycin sulfate as a model drug indicated a sustained release from the microspheres, compared to the release from uncoated cores. These results show that a LbL coating can promote stabilization to the cargo and for that reason allows an enhanced sustained release.

Hyaluronic acid may be used to coat liposomes. Liposomes are pH sensitive lipid-based structures, and have been used as carriers for the controlled release of bioactive agents for cancer treatments (Simoes, Moreira, Fonseca, Duzgunes, & de Lima, 2004). One useful application of such pH sensitiveness is for the intracellular delivery of peptides. Jiang *et al.* presented a new liposome coated with hyaluronic acid-based carrier for anticancer drug delivery (Jiang, T. et al., 2012). In this case, the coating protected the liposome and the cargo against attacks by proteins present in the bloodstream. Entering the tumor extracellular matrix, where the hyaluronidase degrades the outer layer of hyaluronic acid, exposes the liposome to pH changes existing in the cytoplasm, enabling the intracellular drug release. A high antitumor activity was also detected during *in vivo* tests.

Hyaluronic acid hydrogels with dual stimuli-responsiveness can be made, namely towards pH and temperature variations. Hydrogels were obtained from hyaluronic acid and PNIPAAm with TEMED as a cross-linker (Santos, Alves, & Mano, 2010). Using gentamicin as a model drug, *in vitro* release assays at 37 °C and pH 7.4 showed an initial release of around 25% in the first 60 min, followed by a sustainable release up to 30% over the following 20 hours. These results also showed that the release rate increases with increasing hyaluronic acid ratio in the hydrogel composition. These structures showed sensitivity to variations in temperature, showing potential as a device for biomolecules loading with smart controlled release system.

There are other interesting types of hyaluronic acid conjugate-based hydrogels. Hyaluronic acid-tyramine (HA-Tyr) conjugates can be obtained by the enzymatic oxidative reaction of tyramine moieties using H_2O_2 and horseradish peroxidase (HRP). These hydrogels are highly biodegradable, which can be controlled by the cross-linking degree (Kurisawa, Chung, Yang, Gao, & Uyama, 2005), and can encapsulate drugs. It was reported that the concentration of H_2O_2 has an influence in the mechanical

strength of the hydrogel and on the release rate of drugs (Lee, F., Chung, & Kurisawa, 2009). It was also reported that, in contact with hyaluronidase, the entrapped protein can be released continuously and completely from a hydrogel due to the polymer network degradation. On the same line of work, a new hyaluronidase incorporated-hyaluronic acid–tyramine hydrogel was developed for the delivery of trastuzumab, an antibody drug against breast cancer. *In vitro* release studies showed an antibody tunable release accompanied by the hydrogel degradation controlled by the concentration of hyaluronidase, as well as trastuzumab-dependent inhibition on the proliferation on cells (Xu, K., Lee, Gao, Tan, & Kurisawa, 2015).

3.3. Chondroitin Sulfate

Chondroitin sulfate is a sulfated glycosaminoglycan composed of a single chain of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine linked by β-(1→3) and can be sulfated in different carbon positions (**Figure 1.2**). It is usually extracted from the cartilage of bovine and porcine cattle but can also be extracted from some marine animals, like the whale and shark. However, due to ecological reasons, the extraction of protected species is currently quite limited. There are nonetheless other non-mammalian marine animal sources, such as the ray, the salmon fish, the sea cucumber, some cnidarians and mollusks (Silva, T. H. et al., 2012). Chondroitin sulfate has anticoagulant properties and has been suggested as a natural substitute for heparin, one of the most widely used anticoagulants (Lindahl, Lidholt, Spillmann, & Kjellen, 1994; Teien, Abildgaard, & Hook, 1976). In the pharmaceutical industry, this polysaccharide has been used as an active principle in drugs with anticoagulant properties, as a supplement to prevent arthritis (Clegg et al., 2006), and as hydrogels for cartilage tissue regeneration (Wang et al., 2007). Therefore, chondroitin sulfate is a suitable material to build DDSs. Studies with chondroitin sulfate/chitosan nanoparticles have indicated a large retention capacity of proteins and polypeptides, like growth factors (Santo, Gomes, Mano, & Reis, 2012). Release assays showed a sustained release of the cargo in the order of 65% in the first 30 days. Studies *in vitro* performed on human adipose derived stem cells stem showed the ability of these nanoparticles to enter the cells promoting osteogenic differentiation. Cell internalization proved to be dependent on the particles concentration in the culture media, as well as on the incubation time.

While 3D hydrogels and spherical objects are common designs for DDSs, a recent study showed that porous tubular structures can be constructed from hydroxyapatite and chondroitin sulfate for the delivery

of chemotherapeutics (Guo et al., 2014). Results for doxorubicin hydrochloride release showed a high encapsulation capacity around of 91% of efficacy due to the tubes geometry and porosity. *In vitro* release assays at different pH values (5, 6, and 7.4) revealed a pH dependent controlled release. These results revealed the potential use of these structures as controlled drug delivery devices for chemotherapy treatments, not only because of their pH dependent release, but also due to the long-term sustained release that eliminates the need for regular administration.

Electrostatic interactions between different materials can be used for the construction of DDSs with the ability to incorporate different bioactive agents, also to enhance the cargo loading and to promote a sustained controlled release (Zhao, L., Liu, Wang, & Zhai, 2015). Despite the numerous advantages of using natural materials, synthetic polymers are still commonly used in the pharmaceutical industry, though they can be conjugated with natural ones. For example, chondroitin sulfate/PEG hydrogels was developed and proposed for a variety of biomedical applications, such as in wound healing and regenerative medicine (Strehin, Nahas, Arora, Nguyen, & Elisseeff, 2010). This type of hydrogels proved to be biocompatible, since no inflammatory response when implanted has been observed, and is also biodegradable by enzymatic activity.

4. Other marine-origin sulfated polysaccharides

There are several types of glycosaminoglycans with different biological properties but, due to their low bioavailability, difficult extraction and production, they are not widely used in pharmaceutical sciences. However, due to their biological properties, sulfated glycosaminoglycans, including dermatan sulfate, heparan sulfate and keratan sulfate, can be used as active agents in supplements.

4.1 Dermatan sulfate

Dermatan sulfate is a glycosaminoglycan with a linear disaccharide chain containing units of hexosamine, N-acetyl-galactosamine or glucuronic acid linked by β-(1→4) or (1→3) (**Figure 1.2**). In some cases, this compound may present residues of L-iduronic acid, being the main structural difference between dermatan sulfate and chondroitin sulfate. Dermatan sulfate is extracted mainly from ray skin and can be used as a stabilizer for growth factors and cytokines. Recent studies have shown anticoagulant activity for dermatan sulfate without causing the possible complications present in the treatments made with heparin (Davenport, 2012; Mourao & Pereira, 1999; Vitale et al., 2013). Dermatan sulfate

anticoagulant character inhibits thrombin, showing no effect on factor X of the clotting cascade. It also has no interaction in platelet function. Thus, dermatan sulfate is a good alternative for heparin (Trowbridge & Gallo, 2002). Thanks to its anticoagulant and antithrombotic activities, dermatan sulfate is seen as a potential substitute for heparin (Teien et al., 1976).

4.2 Heparan sulfate

Heparan sulfate is another glycosaminoglycan which structure is very similar to heparin. It consists in a linear chain of alternating D-glucuronic acid or iduronic acid and D-glucosamine residues, which can be sulfated or acetylated. The distribution of sulfated residues can set some of the biological properties of heparan sulfate. The number of sulfated groups can influence the affinity with other proteins and so may influence their biological properties (Kreuger, Spillmann, Li, & Lindahl, 2006). For example, heparan sulfate can block DNA topoisomerase activity in cell nucleus (Kovalszky et al., 1998), and also has a role in the control of cell cycle and proliferation. Regarding the latter, heparin sulfate/cell complexes are often associated with increased cell proliferation which can lead to processes of oncogenesis. Thus, heparan sulfate has a significant role in the development of cancer, which is being associated with the increase of cell proliferation, angiogenesis in tumors, cancer cells differentiation and metastasis formation (Sasisekharan, Shriver, Venkataraman, & Narayanasami, 2002). However, the effect of heparan sulfate on tumor cells may depend on the glycosaminoglycan structure, the type of tumor cell and/or the tumor microenvironment (Stewart & Sanderson, 2014).

Independently of its role in cancer, this sulfated polysaccharide is also biodegradable, particularly by enzymatic action of heparanase (Vlodavsky, Ilan, Naggi, & Casu, 2007). Due to the presence of sulfated groups, it may bind to a number of different proteins and regulate biological processes such as coagulation and regulation. Heparan sulfate has the ability to bind to various polypeptides, such as the complex formed by the cellular receptor and growth factors (Lindahl & Kjellen, 2013). Chemical modification of heparan sulfate can interfere with its anticoagulant activity and can have therapeutic effects in tumors. Regardless of the heavy involvement of heparan sulfate in different stages of tumor formation, it is possible that this polymer could be helpful as a new diagnostic method in the discovery and in the development of new drugs for cancer treatments, as well as in the development of DDSs with sensing capability. (Knelson, Nee, & Blobe, 2014). Due to the biological properties of heparan sulfate, it

is not unreasonable to state that the production of heparan sulfate-based DDSs based could be a strategic approach to develop new chemotherapeutic strategies.

4.3 Keratan sulfate

Keratan sulfate is a glycosaminoglycan composed of a base unit of a disaccharide. This unit normally consists in galactose residues and *N*-acetylglucosamine bonded by β-(1→4) linkages. The extremities of keratan sulfate have a protein binding region at the extremities. There are three different classes of keratan sulfate which differ in the nature of the protein binding region. Class I is known for its presence in the cornea and in small cartilage. The protein binding occurs between the *N*- of a *N*-acetylglucosamine and an asparagine. In Class II, also present in small cartilage, the protein binding is made between the *O*- of *N*-acetylglucosamine with either a serine or a threonine. Finally, in Class III (first isolated from nervous tissue), the protein binding occurs in the *O*- of the mannose residue to a serine or threonine (Uchimura, 2015). The presence of keratan sulfate in corneal tissue is related to the maintenance of the moisture level of the corneal tissue, which may influence its levels of transparency. Studies at the cellular level have shown that keratan sulfate has anti-adhesive properties. In nervous tissues, keratan sulfate can prevent the growth of axons, and in cartilage it may decrease the immune response in diseases such as osteoarthritis (Funderburgh, 2002). However, keratan sulfate presents an inhibitory action in nerve regeneration after nerve injury (Geisert, Bidanset, Del Mar, & Robson, 1996; Ueno et al., 2015).

5. Conclusions

Marine origin polysaccharides have been widely used to synthesize DDSs. The fact that they are biocompatible, nontoxic and often biodegradable and stimuli-responsive makes these polymers suitable raw materials for the construction of increasingly complex loading devices with a release that can be potentially controlled. We showed that such devices can be constructed using different methods and can be synthesized in various shapes, such as membranes, particles, capsules and hydrogels, capable of protecting different bioactive agents like proteins and nuclei acids. Each and every polymer exhibits several chemical and biological properties, making marine origin biomaterials and their derivatives excellent materials not only for the construction of load devices but also for other pharmaceutical formulations as excipients or even active compounds in some food supplements. Natural-origin biomaterials allow incorporating a wide variety of proteins, drugs and nucleic acids, which for many new

drugs would not be possible with many synthetic materials, which may be even toxic for the body. The release of bioactive agents may occur through various mechanisms, which may be controlled by using stimuli-responsive polymers to promote a fast or a sustained release. Because these materials are often biocompatible and biodegradable, their use may augment the efficiency of encapsulation and promote the protection of a bioactive agent.

Nowadays, it is already possible to find systems able to control the release of therapeutic molecules for the treatment of genetic diseases. Despite the great knowledge and wide use of marine polysaccharides in the pharmaceutical industry, some challenges remain unsolved, such as the efficient targeted delivery, the perfect control over the release rate to fit within a therapeutic window, and the adaptability to administration routes that are more patient compliant (e.g. oral instead of intravenous). Therefore, further investigation will be required to improve the isolation and purification of marine origin biopolymers, as well as the synthesis of their chemical modification and processing into the various possible matrices shapes. It is expected that in the short-term such control will lead to more efficient loading, higher degrees of control over the release and improved DDS designs, that could be used in advanced therapies. This could be possible by looking into the interactions between polymer, drugs and native biological tissues, as well the intelligent response of the polysaccharides and targeting capability. Future strategies should also combine the possibility of controlled release from this type of devices with diagnostic capability (theranostics approaches) where platforms involving nanotechnologies and image should be taken into consideration.

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SECTION II – EXPERIMENTAL SECTION

Chapter 2 – Materials and methods

Chapter 2 – Materials and methods

1. Aims and prime scope of the work

This work aims to understand the enzymatic degradation of different LbL-based structures designed mostly for biomedical applications. The interest in the study and development of new devices for biomedical applications has increased in the last years. Hence, were developed different techniques related to micro/nanotechnology. These techniques and methods have been used due to its wide applicability in biomedicine, low cost and simple production methods. Among these various techniques, LbL is one of the most explored and studied. This technique is based on modification of the surface by alternately absorption of at least two different materials deposited onto a substrate. LbL allows the construction of different structures. Two-dimensional structures, as thin films and freestanding detachable films, these structures are often designed for regenerative medicine and tissue engineering applications. Three-dimensional structures, such as microcapsules, with main interest in the construction of multifunctional capsules for different applications, for example, in drug delivery systems, biosensors and the constructions of three-dimensional structures for cell growth. One of the prime drawback of this techniques is the choice of suitable materials, for the last years it has been reported that the use of biomaterials could help to overcome some biocompatibility limitations.

Marine origin biomaterials like chitosan and hyaluronic acid proved to be an excellent materials due to their biocompatibility and also their biodegradability. The degradation of these polysaccharide-based structures can be mediated by different pathways. One of the most common process is the degradation by specific enzymes. In the present work, we studied the degradability properties of different chitosan/hyaluronic acid based LbL polyelectrolytes multilayer assemblies by the action of hyaluronidase. Using different characterization techniques, such as quartz-crystal microbalance, atomic force microscopy, dynamics mechanical analysis, we study the influence of hyaluronidase concentration in chitosan/hyaluronic acid based PEM thin films and freestanding membranes. At last, we introduce a hypothesis for an enzyme-responsive LbL PEM polysaccharide-based DDSs device. This system is mainly based on the degradation from the inside of enzyme-drug-loaded capsules for controlled release systems.

2. Materials

Chitosan

Chitosan is a linear polysaccharide derived from chitin, one of the most abundant natural polymers of our ecosystem (Bansal, Sharma, Sharma, Pal, & Malviya, 2011). Chitosan is obtained by deacetylation of chitin, resulting in a compound with randomly distributed D-glucosamine residues (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Alves & Mano, 2008; Rinaudo, 2006). Chitosan, as well as chitin, can be degraded by enzymes such as chitinase and lysozyme (Varum, Myhr, Hjerde, & Smidsrod, 1997). Chitosan is one of the marine origin polysaccharide most widely used and studied for biomedical applications, in particular in the construction of nanoparticles, beads and capsules for controlled drug delivery systems, and also membranes, films and scaffolds for tissue engineering and regenerative medicine (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Couto, Hong, & Mano, 2009; Prabaharan & Mano, 2005; Sinha et al., 2004).

Hyaluronic Acid

Hyaluronic acid is a linear polysaccharide consisting of an alternating chain disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic linked by β-(1→3) and β-(1→4) glycosidic bonds (Burdick & Prestwich, 2011). Hyaluronic acid is a major component of extracellular matrix and is present in the synovial fluid and cartilage tissue. Due to its high viscoelasticity, hyaluronic acid has an important role in several biological functions and also as an excellent material for different biomedical applications. Due to its biocompatibility and biodegradability, hyaluronic acid has also been proposed for biomedical and tissue engineering applications for manufacturing wound healing structures (Chen & Abatangelo, 1999), his biodegradability is mediated by the action of hydrolases, such as hyaluronidase.

Like other polyanions, hyaluronic acid can be complexed with polycations such as chitosan to form nanoparticles (Oyarzun-Ampuero, Brea, Loza, Torres, & Alonso, 2009) and microspheres (Lim, Martin, Berry, & Brown, 2000).

Hyaluronidase

Hyaluronidase is an enzyme that degrades hyaluronic acid. In mammalians, hyaluronidases are classified in different groups according to biochemical analysis (Meyer, 1947). In the human body,

hyaluronidase is found in different organs like, skin, eye, liver, kidney, uterus and placenta and also in fluids like blood or sperm (Meyer & Rapport, 1952). The ability of hyaluronidase to promote the depolymerisation of hyaluronic acid, makes this enzyme crucial in several biological process in which this polymer is involved. Hyaluronidases are also responsible to control hyaluronic acid metabolism and homeostasis (Menzel & Farr, 1998). In particular, the effect on the control of elasticity and viscoelastic properties of the extracellular matrix (Girish, K. & Kemparaju, 2007). Due to his biological properties, hyaluronidase is also used as a therapeutic agent (Girish, K. S., Kemparaju, Nagaraju, & Vishwanath, 2009). Hyaluronidase has the ability to interact with other glycosaminoglycans, studies show that hyaluronidase present/display enzymatic activity in other glycosaminoglycans like chondroitin sulfate and chitosan (Girish, K. & Kemparaju, 2005; Saitoh et al., 1995).

3. Methods

I- Study of enzymatic degradation of chitosan/hyaluronic acid multilayer thin films

Build-up and enzymatic degradation analysis of thin films by quartz-crystal microbalance

The build-up and enzymatic degradation of chitosan/hyaluronic acid multilayer thin films was followed *in situ* by quartz crystal microbalance with dissipation monitoring system. Trough technique, it is possible to observe the build-up of the films in real time. This technique it is based on applying an alternating electric field across the quartz crystal through the gold metal electrodes covering the quartz surface. Based on the piezoelectric effect, an alteration of mass at the surface is converted to oscillation of frequency values (Δf). The dissipation monitoring also allows to measure the damping properties of the film (i.e. viscoelasticity) of the top layer (ΔD) (Dixon, 2008; Martins, Merino, Mano, & Alves, 2010). (**Figure 2.1**). A major advantage of this technique is its capability to be operated under conditions that simulate conditions of biological environments, such as the presence of enzymes. Quartz Crystal Microbalance (QCM) technique is suitable to obtain, for example, stable and catalytically active protein layers for developing successful biosensing devices (Casero, Vazquez, Parra-Alfambra, & Lorenzo, 2010). A major advantage of these technique is its ability to provide important information due to its ability to perform measurements under physiological conditions close to the native environment of the specimens.

Nowadays, the application of these technique relies especially into the characterization of the immobilization of enzymes on different modified and unmodified surfaces as well as on the study of protein interactions.

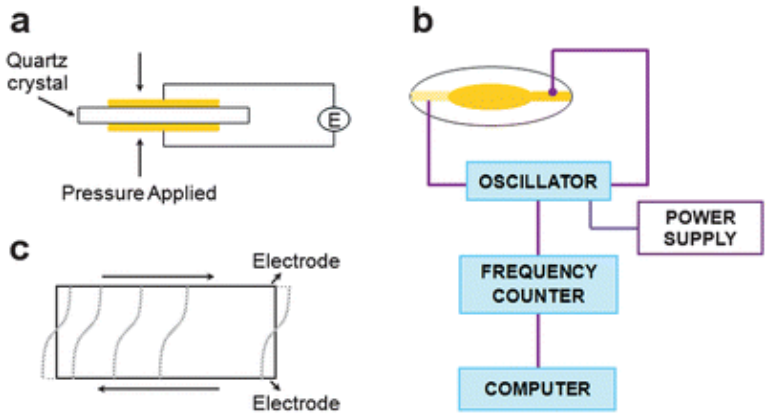

Figure 2.1. (a) Piezoelectric effect: a pressure exerted on a quartz crystal results in an electric field (E) between deformed surfaces. (b) Experimental setup of the quartz crystal microbalance. (c) Schematic representation of a quartz crystal oscillating in the fundamental thickness shear mode. Reprinted with permission from (Casero et al., 2010). Copyright © 2010 Royal Society of Chemistry.

A quartz-crystal microbalance with dissipation monitoring (QCM-D) (Q-Sense, E4 model, Sweden) and gold-coated AT-cut quartz sensors (Q-sense, ref. QSX 301) was used to follow the adsorption of CHT and HA, with simultaneous excitation of multiple overtones: 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th}, and 13^{th}, corresponding to: 5, 15, 25, 35, 45, 55, and 65 MHz, respectively. Adsorption took place at 25 °C using polyelectrolyte solutions prepared at 0.5 $mg.mL^{-1}$, NaCl 0.15 M, pH=5.5, intercalated with a rinsing step with NaCl 0.15 M **(Figure 2.2**). All deposition and rinsing solutions were flushed for 10 min at a constant flow rate of 50 $\mu L.min^{-1}$. Monitoring of the LbL assembly proceeded until three bilayers of CHI/ALG – $(CHT/HA)_3$ – or three bilayers with an extra layer of CHT – $(CHI/HA)_3$-CHI – were assembled. After reaching the required number of layers, the temperature was raised to 37 °C (heating slope: 1 °C per minute). Then, the Hase solution at different concentrations (1, 10, 50, 100, 1000 and 2000 $\mu g.mL^{-1}$) was flushed for 12 min at a constant flow rate of 25 $\mu L.min^{-1}$, after which the flow was stopped and Hase was left to incubate for 24h. After 24h followed a rinsing step time of 20 min to remove remains of Hase and film debris. The registered frequencies for each overtone (Δf) are already normalized to the fundamental resonant frequency of the quartz crystal.

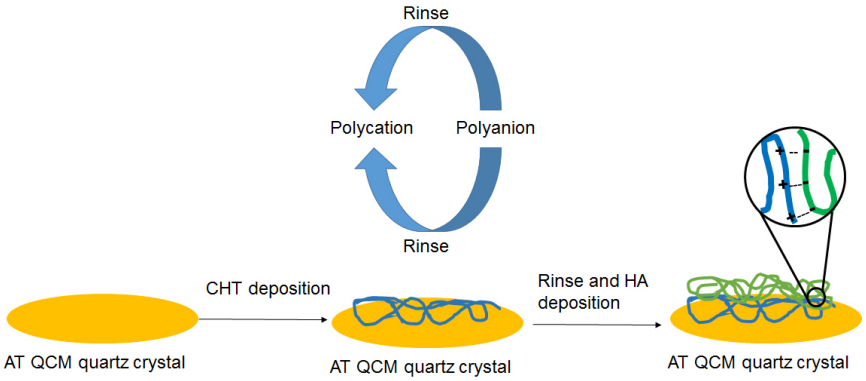

Figure 2.2. Schematics of layer-by-layer adsorption of chitosan (CHT) and hyaluronic acid (HA) based on an electrostatic mechanism deposition. The procedure is based on the contact of the substrate with a solution with an oppositely charged polymer. In this example, an aqueous solution of CHT is deposited onto a substrate (AT QCM quartz crystal), followed by a solution containing HA. Each adsorption stage is separated by a rinsing step to remove loosely adsorbed molecules.

Estimation of thin films thickness

To estimate the film thickness, the Voigt-based model was used (Voinova, Rodahl, Jonson, & Kasemo, 1999), contained in the software QTools (version: 3.1.25.604) provided by Q-Sense, based on equations (1) and (2),

$$\Delta F \approx -\frac{1}{2\pi\rho_0 h_0}\left\{\frac{\eta_3}{\delta_3}+\sum_{j=k}\left[h_j\rho_j\omega-2h_j\left(\frac{\eta_3}{\delta_3}\right)^2\frac{\eta_j\omega^2}{\mu_j^{\,2}+\omega^2\eta_j^{\,2}}\right]\right\} \quad (1)$$

$$\Delta D \approx \frac{1}{2\pi f\rho_0 h_0}\left\{\frac{\eta_3}{\delta_3}+\sum_{j=k}2h_j\left(\frac{\eta_3}{\delta_3}\right)^2\frac{\mu_j\omega}{\mu_j^{\,2}+\omega^2\eta_j^{\,2}}\right\} \quad (2)$$

where, considering a total of k thin viscoelastic layers, ρ_0 and h_0 are the density and thickness of the quartz crystal, η_3 is the viscosity of the bulk liquid, δ_3 is the viscous penetration depth of the shear wave in the bulk liquid, ρ_3 is the density of liquid, μ is the elastic shear modulus of an overlayer, and ω is the angular frequency of the oscillation. The model requires three parameters, namely solvent density, solvent viscosity and film density, to be fixed. The solvent viscosity was therefore fixed at 0.001 Pa (the same as for water) and film density at 1200 kg.m^{-3}. The solvent density was varied by trial and error between 1000 and 1015 kg.m^{-3} until the total error, χ^2, was minimized until the total error, χ^2, was minimized. Calculation were made using at least three overtones (5th, 7th, 9th).

Construction and enzymatic degradation analysis of thin films by atomic force microscopy (AFM)

In order to analyze the effect of enzymatic degradation upon thin films surface, was used a high definition microscopy technique, the atomic force microscopy (AFM). This technique allows to determinate a roughness of a sample surface and also to obtain a topography characterization of a sample surface in a nanometer scale. Among diverse features, which may be attached to type of interaction of the tip with the surface, which may be in contact or intermitted contact (tapping). This technique also provide other important data that a simple image, such as parameters that described the surface topography. These parameters are, the average roughness (Ra), the arithmetic average of the absolute values of the roughness profile and the root mean square of average roughness (Rq), that is used to study temporal surface modification as well as spatial differences (Raposo, Ferreira, & Ribeiro, 2007). Atomic force microscopy (AFM, Dimension Icon, Brunker, France) was used to analyze the effect of Hase on the $(CHI/HA)_3$ ultrathin films surfaces. The analyzed films were built by dipping microscopy glass slides (1x1 cm^2) alternately in the polyelectrolyte solutions. The glass slides had been previously cleaned with acetone, ethanol and 2-propanol, then were rinsed with water and dried with nitrogen gas. Adsorption took place at room temperature for 10 min using polyelectrolyte solutions prepared at 0.5 $mg.mL^{-1}$, NaCl 0.15 M, pH=5.5, rinsing step with NaCl 0.15 M (**Figure 2.3**). Then, the Hase solution at 50 $\mu g.mL^{-1}$ prepared in NaCl 0.15 M, pH=5.5 was added (3 mL) and the samples were incubated at 37 °C for different pre-established time points (3, 6, 9 and 24h). Thereafter, the samples were rinsed with ultrapure water to remove enzyme and salt remains and let drying at room temperature. Thin films topographies were acquired with a 512x512 pixel resolution, analyzing areas of 5x5 µm to calculate the average and the root mean squared roughness (Rq). At least three measurements were performed on different specimens (n=3).

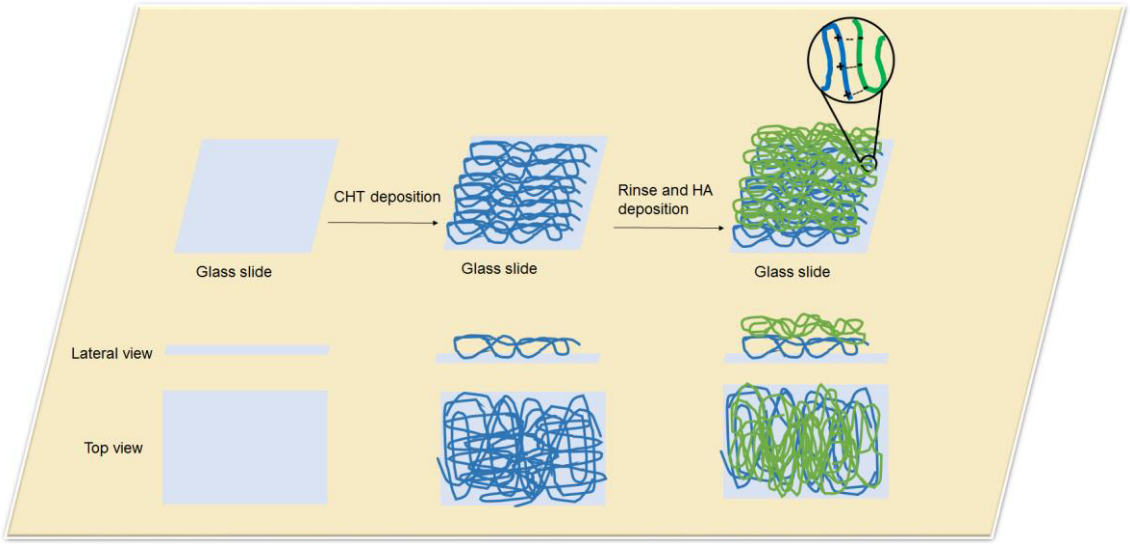

Figure 2.3. Schematics of layer-by-layer adsorption of chitosan (CHT) and hyaluronic acid (HA) based on an electrostatic mechanism deposition. In this example, an aqueous solution of CHT is deposited onto a glass substrate, followed by a solution containing HA. Each adsorption stage is separated by a rinsing step to remove loosely adsorbed molecules.

II- Study of enzymatic degradation of chitosan/hyaluronic acid freestanding films

Production of chitosan-hyaluronic acid freestanding films by dipping robot

By increasing the number of layers it is possible to obtain membranes a few micrometers thick. This new structure allows to access to relevant macroscopy information, such as weight loss or mechanical properties. For the production of robust films, are require automatized mechanisms that allows to obtain PEM with a high numbers of layer. This process can be easily automatized using a robot, that control the deposition time as well the number of cycles that determined thhe number of layer. Through this process CHI/HA PEM freestanding membranes were produced using an in-house automatized robot specially designed for fabrication of multilayers membranes. CHI and HA at 2 $mg.mL^{-1}$, NaCl 0.15 M, pH=5.5 were used to obtain robust and detachable films. The multilayer films were fabricated on a polypropylene substrate that promotes an easily detachable membrane (Caridade et al., 2013; Silva et al., 2014). Polypropylene substrates were immersed alternatively in CHI and HA solutions for 6 min with an intermediate rising step in NaCl 0.15 M, pH=5.5 for 4 min. This cycle was repeated until 100 bilayers were absorbed, after which the membranes were dried at room temperature and stored until use (**Figure 2.4**).

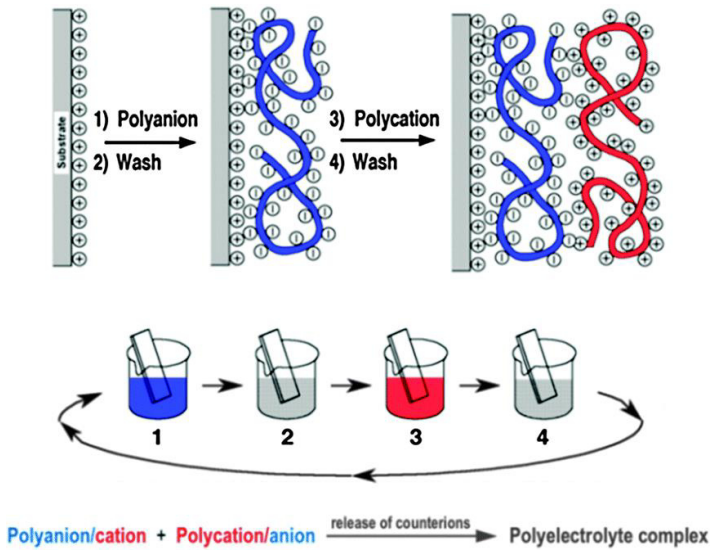

Figure 2.4. Schematics of layer-by-layer vertical adsorption based on an electrostatic mechanism deposition. In this example, an aqueous solution of a polyanion is deposited onto a glass substrate, followed by a solution containing polycation. Each adsorption stage is separated by a rinsing step to remove loosely adsorbed molecules. Reprinted with permission from (Rongé et al., 2014). Copyright © 2014 Royal Society of Chemistry.

Enzymatic degradation tests

In order to investigate the enzyme degradation of CHI/HA based membranes were designed polypropylene holder (80x30 mm^2) has a frame in the middle (13x13 mm^2) to promote contact between the membrane and the enzymatic solution, and also to prevent self-folding and stickiness against the container of the enzyme solution specially for the experiment. $(CHI/HA)_{100}$ membranes (20x20 mm^2) previously weighted (initial mass, m_i), were mounted into the holders **(Figure 2.5)**. The holder was placed in a cylindrical tube containing 40 mL of Hase at 50 $\mu g.mL^{-1}$ prepared in NaCl 0.15M pH=5.5. Then, the holders were placed at 37 °C and retrieved after predetermined time-points: 3, 6, 9 and 24h. Control samples were placed in tubes containing only NaCl 0.15M, pH=5.5. Following the retrieval, the holders were meticulously rinsed with ultrapure water, followed by drying at room temperature. Dry membranes were detached from the holder and weighted (final mass, m_f), and used to calculate the weight loss using equation 3.

$$\text{Weight loss}(\%) = \frac{m_i - m_f}{m_i} \times 100 \quad (3)$$

For the study of long-term degradation, membranes with the same dimensions were mounted in the holders and immersed in 40 mL of Hase at 50 µg.mL^{-1} prepared in PBS (pH=7.4). Then, the holders with the samples were placed at 37 °C and retrieved after predetermined time-points: 6, 9, 18, 24, 48, 72 and 120h **(Figure 2.5).** Follow the retrieval, the holder were meticulously rinsed with ultrapure water, followed by drying at room temperature. Dry membranes were detached from the holder and weighted (final mass, m_f) and used to calculate the weight loss using the same equation (3). All these procedures were made in triplicates samples (n=3).

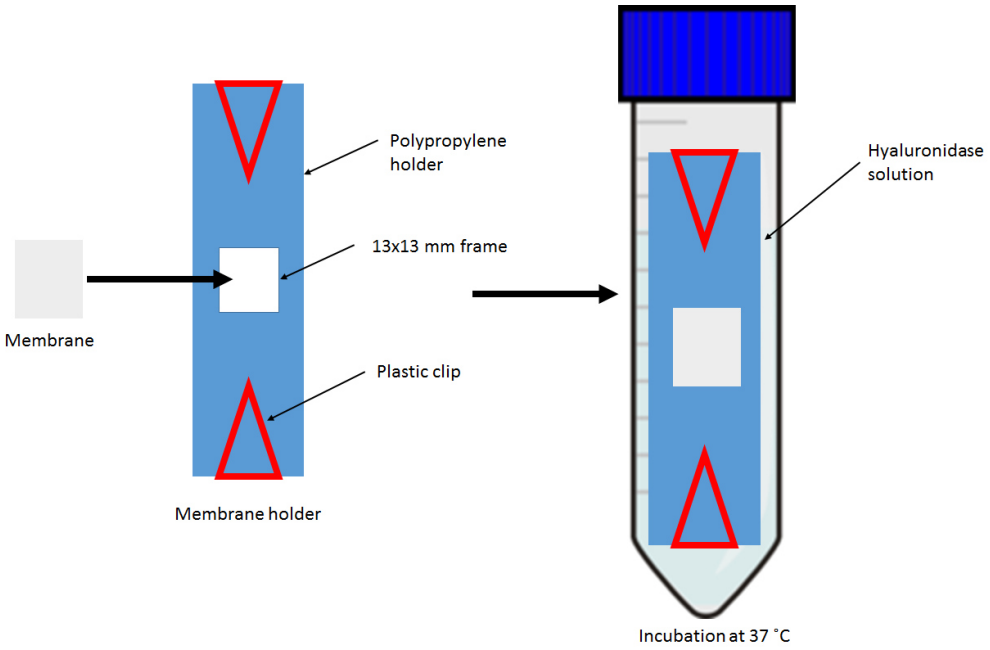

Figure 2.5. Schematic of PEM membranes setup into propylene holder for enzymatic degradation assays.

Dynamic Mechanical Analysis (DMA): real-time online degradation monitoring

In order to study the influence of the enzymatic degradation on the mechanical properties of the CHT/HA membranes, DMA analysis were performed. This technique is widely used to characterize materials properties like measurements of elastic and damping, associated to the excitation and relaxation as a function of temperature, time, frequency, stress or a combination of these parameters. This technique is based on the application of a repetitively sinusoidal deformation to a sample with known geometry, the sample generates a response that it is measure by DMA apparatus. The main parameter are reported as a storage modulus (E'). The storage modulus is the measure of the sample's elastic behavior. Another parameter it is the ratio of the loss modulus, which is a measure of how well a material can get rid of energy and is reported as the tangent of the phase angle (tan δ). This technique allows to

be operated under conditions that simulate biological environments, such as the presence of enzymes and the possibility to work at 37 °C (Menard, 2008). Real-time online degradation assays permit *in situ* monitoring the influence of the enzymatic degradation in the mechanical properties of CHT/HA membranes.

To promote the stability of the freesandings and allow proper handling, the freestandings were lightly cross-linked using 1% w/v glutaraldehyde for 1 min (Jameela & Jayakrishnan, 1995; Larkin, Davis, & Rajagopalan, 2010). The freestandings were subsequently rinsed with ultrapure water to remove glutaraldehyde remains, then air-dried at room temperature until tested. All of the viscoelastic measurements were performed using a TRITEC2000B DMA from Triton Technology (United Kingdom), equipped with the tensile mode. The measurements were carried out at 37 °C. The distance between the clamps was 5 mm and the membrane samples were cut with about 10 mm width. Samples were always analyzed immersed in a liquid bath placed in a Teflon reservoir. Membranes were previously immersed in NaCl 0.15M, pH=5.5 solution and the geometry of the samples was then measured. Samples were clamped in the DMA apparatus and immersed in the liquid bath (**Figure 2.7)**. Different bath compositions were used: (i) Hase 50 $\mu g.mL^{-1}$ prepared in NaCl 0.15M, pH=5.5 and (ii) Hase-free NaCl 0.15M, pH=5.5, used as control. After equilibrium at 37 °C, the mechanical/viscoelastic properties of the samples were recorded at 1 Hz during 24h. The experiments were performed under constant strain amplitude (50 µm). A static preload of 1 N was applied during the tests to keep the sample tight.

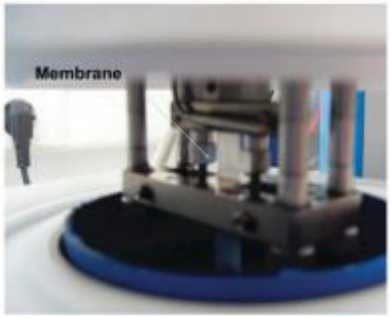

Figure 2.6. Picture showing a membrane in the DMA equipment. Adapted with permision from (Silva, Caridade, Oliveira, Reis, & Mano, 2015). Copyright © 2015 Royal Society of Chemistry.

III- Study of enzymatic degradation of chitosan/hyaluronic acid LbL capsules

Construction of LbL capsules

In order to explore the hypothesis of using an enzyme to control the release of active agents loaded within polysaccharide-based LbL microcapsules. Multilayer micro-capsules for drug delivery applications were produced by assembling coatings onto colloidal sacrificial templates (Caruso, Caruso, & Möhwald, 1998; Costa, Custodio, Arias, Rodriguez-Cabello, & Mano, 2013; Johnston, Cortez, Angelatos, & Caruso, 2006). The degradative effect of enzymes could be exploited to facilitate the release of bioactive agents in drug delivery systems (Borodina et al., 2007; Marchenko et al., 2012; Xu, Lee, Gao, Tan, & Kurisawa, 2015). In this case, Hase function as a trigger to the release of bioactive agent, the enzyme released from the structure is rapidly eliminated from plasma, causing no adverse effects to the body. Following this reasoning, Hase was incorporated within CHT/HA microcapsules alongside FITC-BSA, as a model protein for the controlled release studies. $CaCO_3$ microparticles were often used as sacrificial templates (which can be chelated by EDTA), to assemble CHT/HA-coated capsules (Costa et al., 2013; She, Antipina, Li, & Sukhorukov, 2010; Volodkin, 2014). This method it is well used, for his simplicity and efficiency. To encapsulate 8 mg of FITC-BSA, were prepared a aqueous solutions of sodium carbonate (Na_2CO_3) and calcium chloride ($CaCl_2$) at 1 M. The $CaCO_3$ microparticles synthesis (co-precipitation) was performed under vigorous stirring (≈1000 rpm) and consist by adding 1 mL of Na_2CO_3 into a mixture solution of 1 mL of $CaCl_2$ and 4 mL containing FITC-BSA at 2 $mg.mL^{-1}$ and Hase at 10 $\mu g.mL^{-1}$. After 30 s, stirring was stopped and the suspension of newly synthesized calcium carbonate ($CaCO_3$) microparticles was left to react and precipitate for 15 min. The supernatant was removed and the particles washed twice with ultrapure water to remove residual salts and non-entrapped FITC-BSA and Hase. Each supernatant was retained for determination of entrapment losses. The same method was followed for $CaCO_3$ microparticles entrapping solely FITC-BSA as a control. The $CaCO_3$ sacrificial templates entrapping FITC-BSA and Hase were immersed alternately in CHT and HA solutions at 0.5 $mg.mL^{-1}$, NaCl 0.15M, pH=5.5, for 10 min each under mild agitation, intercalated with NaCl 0.15M, pH=5.5, between polymer immersion steps. To exchange solutions, the agitations was stopped and the particles left to precipitate, after which the aqueous medium was retrieved and replaced by the next one. Centrifugation was avoided to avoid damage and aggregation of the particles each time the samples were to be retrieved from solution. This process was repeated to increase the number of bilayers until three CHT/HA bilayers were assembled. After construction, the $CaCO_3$ core was removed by immersing the coated particles in EDTA 0.2 M pH=7.4 for 30 min, a chelating agent of Ca^{2+} ions **(Figure 2.7)**. All supernatant and polyelectrolyte solutions were

retained for fluorescence measurements and determination of losses during the construction and chelation stages.

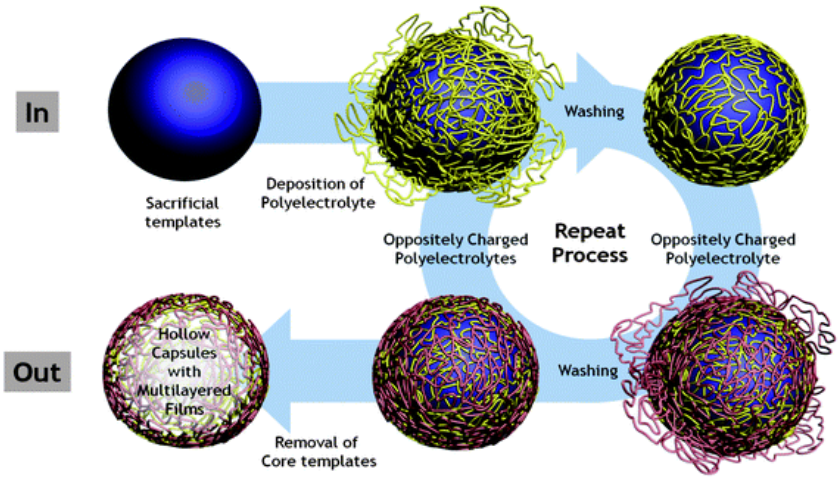

Figure 2.7. A schematic illustration of preparing hollow capsules containing polyelectrolyte multilayer films by the layer-by-layer assembly on sacrificial colloidal templates. Reprinted with permission from (Hong et al., 2011). Copyright © 2011 Royal Society of Chemistry.

To determinate the protein encapsulation efficiency, was taking in account the ability of FITC emit fluorescence, measurements were performed to establish a calibration curve and calculate FITC-BSA mass losses using microplate reader (BioTek, USA). 150 µL of sample was pipetted into a white 96-well plate in triplicate. PBS was used as blank. The excitation (λ_{exc}) and emission (λ_{em}) wavelengths used were 485 and 528 nm, respectively. The concentration range for the calibration curve was 2 to 40 $\mu g.mL^{-1}$. All supernatants collected during entrapment, construction and chelation were measured and quantified. The encapsulation efficiency was calculated by subtracting the cumulative losses from the initial FITC-BSA mass. Follow the same procedure, fluorescence measurements were used also to determinate the mass of FITC-BSA released from the capsules. Microcapsules consisting of 3 CHT/HA bilayers containing FITC-BSA plus Hase, or solely FITC-BSA, were resuspended in 5 mL of PBS at 37 °C for 14 days. At each predetermined time-point (1, 2, 3, 5, 7, 9, 12 and 14 days) 450 µL was retrieved and refreshed with new PBS. The fluorescence of the retrieved samples was measured following the same quantification procedure described for the determination of protein encapsulation efficacy. All experiments were performed in triplicate (n=3).

In order to obtain microparticles/capsules morphological information, a scanning electron microscopy (SEM, JEOL model JSM-6010LV, Japan), was used to evaluate the morphology of the protein-loaded

$CaCO_3$ particles. Dry particles were pre-coated with a conductive layer of sputtered gold and then observed. For the characterization of CHT/HA multilayer microcapsules at the beginning of the release assay hydrated microcapsules loaded with FITC-BSA and Hase were observed with a TCSP8 confocal laser scanning microscope (Leica, Germany) using samples capsules suspended in a droplet of PBS.

Statistical analysis

Values reported are means±1 standard deviation (SD) of at least three independent experiments. All values were analyzed using ANOVA statistical analysis using Microsoft Excel (version: 15.0.4771.1000) (Microsoft, USA). All results were considered to be statistically significant at p-value less than 0.05.

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SECTION III- EXPERIMENTAL RESULTS

Chapter 3 – Enzymatic degradation of polysaccharide-based layer-by-layer devices

Chapter 3 – Enzymatic degradation of polysaccharide-based layer-by-layer devices

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Abstract: The layer-by-layer (LbL) assembly technique has been used for the design of polyelectrolyte multilayer films displaying increasingly complex shapes and functionalities. The translation of LbL devices to effective clinical assays requires that degradation studies are performed, but such studies have been seldom conducted. Chitosan/hyaluronic acid films in the form of ultrathin films, freestanding membranes, and microcapsules were produced and studied for their enzymatic degradation with hyaluronidase. Ultrathin films were concentration dependent sensitive to hyaluronidase degradation, leading to significant thickness and topography variations. Freestanding membranes exhibited accelerated weight loss up to 120 hours with the presence of the enzyme, being completely degraded after this time-frame. Non-conventional dynamic mechanical analysis performed in real time also demonstrated the gradual loss of mechanical properties in the first moments of hyaluronidase contact. Microcapsules coated with the same multilayer system with around 5 micrometers in diameter loaded with both FITC-BSA as a model protein and hyaluronidase showed that the co-encapsulation of such enzyme with the model protein led to a release of four times more protein than in the absence of hyaluronidase. The results suggest that the degradation of LbL devices may be tuned via embedded enzymes, namely in the control of active agents release for the pharmaceutical industry and in other biomedical applications.

1. Introduction

There have been considerable developments on biomedical polymer- and composite-based structures for various applications, such as carriers in drug delivery systems (Gref et al., 1994; Schmaljohann, 2006), implantable devices (Ramakrishna, Mayer, Wintermantel, & Leong, 2001) and mucoadhesive films (Morales & McConville, 2011). Regardless of their origin and purpose, their performance is often dependent on their stability and integrity when used in biological environments (Langer & Tirrell, 2004; Middleton & Tipton, 2000; Mohanty, Misra, & Hinrichsen, 2000; Piskin, 1995). Polymeric structures can be susceptible to various types of degradation, which include thermal, photo, mechanical and chemical degradation (Göpferich, 1996). Some polymer-based devices may benefit from biodegradability. One such example is a tissue engineering and regenerative medicine scaffold, which aims to be implantable and degrade at the rhythm of the target tissue regeneration, promoting the cell growth and the delivery of growth factors (Hutmacher, 2000; Mano et al., 2007; Rezwan, Chen, Blaker, & Boccaccini, 2006). In other cases, such as large implantable permanent prosthesis, biodegradability is not desired, since such devices work as replacements of native biological structures and should hold for several years (Burg, Porter, & Kellam, 2000). Other examples may include wound dressings, which should protect the wound during the treatment time, and drug carriers for targeted therapies, which ideally retain a drug while in circulation in the bloodstream until cellular uptake and intracellular degradation (Qiu & Park, 2012; Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001; Uhrich, Cannizzaro, Langer, & Shakesheff, 1999).

It is clear that evaluating the biodegradability of a biomedical device is crucial, not only for current medical technologies but also for new cutting-edge tissue engineering ones. It is the case of layer-by-layer (LbL) devices, which are obtained by a simple process of adsorption of two or more biomaterials to a substrate in a sequential fashion (Decher, 1997). The sequential adsorption of biomaterials to a substrate requires the use of materials that are capable of interacting via complemental interactions, such as electrostatic interactions (e.g. oppositely charged polyelectrolytes), to generate nanostructured and multilayered structures exhibiting thickness ranging from several nanometers to a few micrometers (Borges & Mano, 2014; Boudou, Crouzier, Ren, Blin, & Picart, 2010; Hammond, 2012; Tang, Wang, Podsiadlo, & Kotov, 2006). Because it discards the need to use organic and harmful solvents, it is an attractive technique for biomedical applications including biosensors, drug delivery, coating of biomaterials, and tissue engineering. LbL can be used to produce robust coatings even in substrates with complex geometries, such as low energy surfaces, colloidal templates and particle aggregates, making it

possible to synthesize bioactive coatings, membranes, tubular structures, capsules and porous scaffolds (Costa, R. R. & Mano, 2014). However, the biodegradability of LbL devices has been seldom explored (Becker, Zelikin, Johnston, & Caruso, 2009; Borodina et al., 2007; Lee, Jeong, & Park, 2007; Marchenko et al., 2012; Picart et al., 2005; Vázquez, Dewitt, Hammond, & Lynn, 2002), which hinders its transition to translational and preclinical studies (Costa, R. R., Alatorre-Meda, & Mano, 2015; Hammond, 2012). For example, Etienne *et al.* have studied *in vitro* and *in vivo* the enzymatic degradation of multilayer films in the oral environment (Etienne et al., 2005). The degradation of 3D capsules has also been studied by Szarpak *et al.* analyzed the morphology, permeability properties and the enzymatic degradation of microcapsules (Szarpak et al., 2010).

In this work, we will explore the susceptibility to enzymatic degradation of nano- and micro-sized LbL structures based on natural polysaccharides. Natural materials have raised much interest as LbL ingredients. Unlike synthetic materials, natural materials are often biocompatibility, noncytotoxic, biodegradable and exhibit native bioactivity (Dang & Leong, 2006; Hoffman, 2012; Mano et al., 2007). Among them, polysaccharides are especially interesting, as they can be highly hydrated, biocompatible, biodegradable specifically by enzymatic action, and can be chemically modified and cross-linked to fit the degradation rate required by a specific application (Prestwich & Atzet, 2013). Furthermore, as many polysaccharides exhibit charged groups, they can be easily processed into polyelectrolyte multilayer films driven by electrostatic interactions. For example, chitosan (CHT) and hyaluronic acid (HA) represent two abundant natural polysaccharides exhibiting positive and negative charge, respectively (Chua, Neoh, Kang, & Wang, 2008; Croll, O'Connor, Stevens, & Cooper-White, 2006; Manna, Bharani, & Patil, 2009; Zhang et al., 2005). Besides their bioavailability and electrostatic complementarity, CHT and HA are common polyelectrolyte building blocks used to synthesize multilayered structures, thus proving to be good candidates to integrate future LbL-based devices (Neto et al., 2014; Picart et al., 2005; Schneider, Richert, Francius, Voegel, & Picart, 2007). The biodegradability of such devices should take into consideration the existence of enzymes in the human body. For example, hyaluronidase (Hase) is an enzyme that degrades HA. In mammalians, Hases are classified in different groups depending on the cleavage site on the polymer chain, which can be random or specific (i. g. endo-hexosaminidases)(Menzel & Farr, 1998; Meyer, 1947). In the human body, Hase is found in different organs, like in skin, eye, liver, kidney, uterus and placenta, and also in fluids (Meyer & Rapport, 1952).

In the present work, the enzymatic degradability of polyelectrolyte multilayer-based CHT/HA ultrathin films, freestanding membranes (also known as freestandings) and microcapsules will be addressed using Hase. The influence of Hase on the stability of such structures will be compared taking into account the thickness and topography variations in ultrathin films. The results will be extrapolated to freestandings in terms of weight loss and variations of mechanical properties. We also explores the susceptibility of the enzymatic degradation of CHT/HA multilayers to force and control the release of active agents from compartmentalized drug release systems. The use of Hase as a factor to control the release profile of drugs from LbL microcarriers will be introduced based on the quantification of FITC-BSA release.

2. Experimental

2.1. Materials

Medium molecular weight chitosan with 75-85 % degree of deacetylation (DD) (ref. 448877), hyaluronic acid sodium salt from *Streptococcus equi* (ref. 53747), hyaluronidase from bovine testes, Type I-S, lyophilized powder, 400-1000 units/mg solid (ref. H3506), albumin fluorescein isothiocyanate conjugate (ref. A9771), phosphate buffered saline tablets (ref. P4417), sodium carbonate ACS reagent anhydrous (ref. 222321), and ethylenediaminetetraacetic acid (EDTA, ref. E9884) were purchased from Sigma Aldrich. Sodium chloride (ref. 131659), were purchased from Laborspirit (Portugal). Calcium chloride (ref 1.02378); glutaraldehyde 25% (ref 1.04239.0250) were purchased from VWR international (Portugal).

2.2. Methods

I- Study of enzymatic degradation of chitosan/hyaluronic acid multilayer thin films

Build-up and enzymatic degradation analysis of thin films by quartz-crystal microbalance

A quartz-crystal microbalance with dissipation monitoring (QCM-D) (Q-Sense, E4 model, Sweden) and gold-coated AT-cut quartz sensors (Q-sense, ref. QSX 301) was used to follow the adsorption of CHT and HA, with simultaneous excitation of multiple overtones: 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th}, and 13^{th}, corresponding

to: 5, 15, 25, 35, 45, 55, and 65 MHz, respectively. Adsorption took place at 25 °C using polyelectrolyte solutions prepared at 0.5 mg.mL^{-1}, NaCl 0.15 M, pH=5.5, intercalated with a rinsing step with NaCl 0.15 M. All deposition and rinsing solutions were flushed for 10 min at a constant flow rate of 50 µL.min^{-1}. Monitoring of the LbL assembly proceeded until three bilayers of CHI/ALG – $(CHT/HA)_3$ – or three bilayers with an extra layer of CHT – $(CHI/HA)_3$-CHI – were assembled. After reaching the required number of layers, the temperature was raised to 37 °C (heating slope: 1 °C per minute). Then, the Hase solution at different concentrations (1, 10, 50, 100, 1000 and 2000 µg.mL^{-1}) was flushed for 12 min at a constant flow rate of 25 µL.min^{-1}, after which the flow was stopped and Hase was left to incubate for 24h. After 24h followed a rinsing step time of 20 min to remove remains of Hase and film debris. The registered frequencies for each overtone (Δf) are already normalized to the fundamental resonant frequency of the quartz crystal.

To estimate the film thickness, the Voigt-based model was used (Voinova, Rodahl, Jonson, & Kasemo, 1999), contained in the software QTools (version: 3.1.25.604) provided by Q-Sense, based on equations (1) and (2),

$$\Delta F \approx -\frac{1}{2\pi\rho_0 h_0}\left\{\frac{\eta_3}{\delta_3}+\sum_{j=k}\left[h_j\rho_j\omega-2h_j\left(\frac{\eta_3}{\delta_3}\right)^2\frac{\eta_j\omega^2}{\mu_j{}^2+\omega^2\eta_j{}^2}\right]\right\} \quad (1)$$

$$\Delta D \approx \frac{1}{2\pi f\rho_0 h_0}\left\{\frac{\eta_3}{\delta_3}+\sum_{j=k}2h_j\left(\frac{\eta_3}{\delta_3}\right)^2\frac{\mu_j\omega}{\mu_j{}^2+\omega^2\eta_j{}^2}\right\} \quad (2)$$

where, considering a total of k thin viscoelastic layers, ρ_0 and h_0 are the density and thickness of the quartz crystal, η_3 is the viscosity of the bulk liquid, δ_3 is the viscous penetration depth of the shear wave in the bulk liquid, ρ_3 is the density of liquid, μ is the elastic shear modulus of an overlayer, and ω is the angular frequency of the oscillation. The model requires three parameters, namely solvent density, solvent viscosity and film density, to be fixed. The solvent viscosity was therefore fixed at 0.001 Pa (the same as for water) and film density at 1200 kg.m^{-3}. The solvent density was varied by trial and error between 1000 and 1015 kg.m^{-3} until the total error, χ^2, was minimized until the total error, χ^2, was minimized. Calculation were made using at least three overtones (5th, 7th, 9th).

Construction and enzymatic degradation analysis of thin films by atomic force microscopy (AFM)

Atomic force microscopy (AFM, Dimension Icon, Brunker, France) was used to analyze the effect of Hase on the $(CHI/HA)_3$ ultrathin films surfaces. The analyzed films were built by dipping microscopy glass slides (1x1 cm^2) alternately in the polyelectrolyte solutions. The glass slides had been previously cleaned with acetone, ethanol and 2-propanol, then were rinsed with water and dried with nitrogen gas. Adsorption took place at room temperature, using the same conditions of QCM studies. Then, the Hase solution at 50 $\mu g.mL^{-1}$ was added (3 mL) and the samples were incubated at 37 °C for different pre-established time points (3, 6, 9 and 24h). Thereafter, the samples were rinsed with ultrapure water to remove enzyme and salt remains and let drying at room temperature. Thin films topographies were acquired with a 512x512 pixel resolution, analyzing areas of 5x5 µm to calculate the root mean squared roughness (Rq). At least three measurements were performed on different specimens (n=3).

II- Study of enzymatic degradation of chitosan/hyaluronic acid freestanding films

Production of chitosan-hyaluronic acid freestanding films by dipping robot

CHI/HA freestanding membranes were produced using an in-house automatized robot specially designed for fabrication of multilayers membranes. CHI and HA at 2 $mg.mL^{-1}$, NaCl 0.15 M, pH=5.5 were used to obtain robust and detachable films. The multilayer films were fabricated on a polypropylene substrate that promotes an easily detachable membrane (Caridade et al., 2013). Polypropylene substrates were immersed alternatively in CHI and HA solutions for 6 min with an intermediate rising step in NaCl 0.15 M, pH=5.5 for 4 min. This cycle was repeated until 100 bilayers were absorbed, after which the membranes were dried at room temperature and stored until use.

Enzymatic degradation tests

$(CHI/HA)_3$ membranes (20x20 mm^2) were mounted in a polypropylene holder designed especially for the experiment. The polypropylene holder (80x30 mm^2) has a frame in the middle (13x13 mm^2) to promote contact between the membrane and the enzymatic solution, and also to prevent self-folding and stickiness against the container of the enzyme solution. All membranes were first weighted (initial mass,

m_i) and then mounted. The membrane holder was placed in a cylindrical tube containing 40 mL of Hase at 50 μg.mL^{-1} prepared in NaCl 0.15M pH=5.5. Then, the holders were placed at 37 °C and retrieved after predetermined time-points: 3, 6, 9 and 24h. Control samples were placed in tubes containing only NaCl 0.15M, pH=5.5. Following the retrieval, the holders were meticulously rinsed with ultrapure water, followed by drying at room temperature. Dry membranes were detached from the holder and weighted (final mass, m_f), and used to calculate the weight loss using equation 3.

$$\text{Weight loss}(\%) = \frac{m_i - m_f}{m_i} \times 100 \quad (3)$$

For the study of long-term degradation, membranes with the same dimensions were mounted in the holders and immersed in 40 mL of Hase at 50 μg.mL^{-1} prepared in PBS (pH=7.4). Then, the holders with the samples were placed at 37 °C and retrieved after predetermined time-points: 6, 9, 18, 24, 48, 72 and 120h. Follow the retrieval, the holder were meticulously rinsed with ultrapure water, followed by drying at room temperature. Dry membranes were detached from the holder and weighted (final mass, m_f) and used to calculate the weight loss using the same equation (3). All these procedures were made in triplicates samples (n=3).

Dynamic Mechanical Analysis (DMA): real-time degradation monitoring

To promote the stability of the freesandings and allow proper handling, the freestandings were lightly cross-linked using 1% w/v glutaraldehyde for 1 min (Jameela & Jayakrishnan, 1995; Larkin, Davis, & Rajagopalan, 2010). The freestandings were subsequently rinsed with ultrapure water to remove glutaraldehyde remains, then air-dried at room temperature until tested. All of the viscoelastic measurements were performed using a TRITEC2000B DMA from Triton Technology (United Kingdom), equipped with the tensile mode. The measurements were carried out at 37 °C. The distance between the clamps was 5 mm and the membrane samples were cut with about 10 mm width. Samples were always analyzed immersed in a liquid bath placed in a Teflon reservoir. Membranes were previously immersed in NaCl 0.15M, pH=5.5 solution and the geometry of the samples was then measured. Samples were clamped in the DMA apparatus and immersed in the liquid bath. Different bath compositions were used: (i) Hase 50 μg.mL^{-1} prepared in NaCl 0.15M, pH=5.5 and (ii) Hase-free NaCl 0.15M, pH=5.5, used as control. After equilibrium at 37 °C, the mechanical/viscoelastic properties of the samples were recorded

at 1 Hz during 24h. The experiments were performed under constant strain amplitude (50 µm). A static preload of 1 N was applied during the tests to keep the sample tight.

III- Study of enzymatic degradation of chitosan/hyaluronic acid LbL capsules

Entrapment of protein in calcium carbonate particles

FITC-BSA and Hase were co-encapsulated in $CaCO_3$ microparticles following a well-known method (Costa, R. R., Custodio, Arias, Rodriguez-Cabello, & Mano, 2013; She, Antipina, Li, & Sukhorukov, 2010; Volodkin, D., 2014). Shortly, to encapsulate 8 mg of FITC-BSA, aqueous solutions of sodium carbonate (Na_2CO_3) and calcium chloride ($CaCl_2$) were prepared at 1 M. Co-precipitation of both solutions was performed under vigorous stirring (≈1000 rpm) by adding 1 mL of Na_2CO_3 into a mixture solution of 1 mL of $CaCl_2$ and 4 mL containing FITC-BSA at 2 $mg.mL^{-1}$ and Hase at 10 $\mu g.mL^{-1}$. After 30 s, stirring was stopped and the suspension of newly synthesized calcium carbonate ($CaCO_3$) microparticles was left to react and precipitate for 15 min. The supernatant was removed and the particles washed twice with ultrapure water to remove residual salts and non-entrapped FITC-BSA and Hase. Each supernatant was retained for determination of entrapment losses. The same method was followed for $CaCO_3$ microparticles entrapping solely FITC-BSA as a control.

Construction of the multilayer microcapsules

The $CaCO_3$ sacrificial templates entrapping FITC-BSA and Hase were immersed alternately in CHT and HA solutions at 0.5 $mg.mL^{-1}$, NaCl 0.15M, pH=5.5, for 10 min each under mild agitation, intercalated with NaCl 0.15M, pH=5.5, between polymer immersion steps. To exchange solutions, the agitations was stopped and the particles left to precipitate, after which the aqueous medium was retrieved and replaced by the next one. Centrifugation was avoided to avoid damage and aggregation of the particles each time the samples were to be retrieved from solution. This process was repeated to increase the number of bilayers until three CHT/HA bilayers were assembled. After construction, the $CaCO_3$ core was removed by immersing the coated particles in EDTA 0.2 M pH=7.4 for 30 min, a chelating agent of Ca^{2+} ions. All supernatant and polyelectrolyte solutions were retained for fluorescence measurements and determination of losses during the construction and chelation stages.

Determination of protein encapsulation efficiency

Fluorescence measurements were performed to establish a calibration curve and calculate FITC-BSA mass losses using microplate reader (BioTek, USA). 150 μL of sample was pipetted into a white 96-well plate in triplicate. PBS was used as blank. The excitation (λ_{exc}) and emission (λ_{em}) wavelengths used were 485 and 528 nm, respectively. The concentration range for the calibration curve was 2 to 40 $\mu g.mL^{-1}$. All supernatants collected during entrapment, construction and chelation were measured and quantified. The encapsulation efficiency was calculated by subtracting the cumulative losses from the initial FITC-BSA mass.

Quantification of FITC-BSA release

Microcapsules consisting of 3 CHT/HA bilayers containing FITC-BSA plus Hase, or solely FITC-BSA, were resuspended in 5 mL of PBS at 37 °C for 14 days. At each predetermined time-point (1, 2, 3, 5, 7, 9, 12 and 14 days) 450 μL was retrieved and refreshed with new PBS. The fluorescence of the retrieved samples was measured following the same quantification procedure described for the determination of protein encapsulation efficacy. All experiments were performed in triplicate (n=3).

Microscopy characterization of CaCO3 microparticles and multilayer microcapsules

A scanning electron microscopy (SEM, JEOL model JSM-6010LV, Japan), was used to evaluate the morphology of the protein-loaded $CaCO_3$ particles. Dry particles were pre-coated with a conductive layer of sputtered gold and then observed. Hydrated CHT/HA multilayer microcapsules loaded with FITC-BSA and Hase were observed with a TCSP8 confocal laser scanning microscope (Leica, Germany) using samples capsules suspended in a droplet of PBS.

Statistical analysis

Values reported are means±1 standard deviation (SD) of at least three independent experiments. All values were analyzed using ANOVA statistical analysis using Microsoft Excel (version: 15.0.4771.1000) (Microsoft, USA). All results were considered to be statistically significant at p-value less than 0.05.

3. Results and discussion

I- Study of enzymatic degradation of chitosan/hyaluronic acid multilayer thin films

In order to study the enzymatic degradation of CHT/HA polyelectrolyte multilayer structure thin films, the construction of the film, as well as the effect pf Hase, was followed *in situ* using QCM-D. In this technique, it is possible to observe the build-up of the films in real time by applying an alternating electric field across the quartz crystal through the gold metal electrodes covering the quartz surface. Based on the piezoelectric effect, an alteration of mass at the surface is converted to oscillation of frequency values (Δf). The dissipation monitoring also allows to measure the damping properties of the film (i.e. viscoelasticity) of the top layer (ΔD). A major advantage of this technique is its capability to be operated under conditions that simulate conditions of biological environments, such as the presence of enzymes. **Figure 3.1** shows the variations of dissipation (ΔD_5) and frequency ($\Delta f_5/5$) during the construction of CHT/HA films with 3 bilayers and one last layer of CHT $(CHT/HA)_3$-CHT (**Figure 3.1A**). As time progressed, the sequential decrease of $\Delta f_5/5$ values shows a successful film construction, evidencing the deposition of polymer molecules onto the surface, to approximate frequency variations of -375 Hz. An increase of dissipation values was also registered, revealing that the films were not rigid. Furthermore, the variation represents a shift towards a film with a higher viscous component and great ability to absorb water, properties that typical of soft polymeric structures.

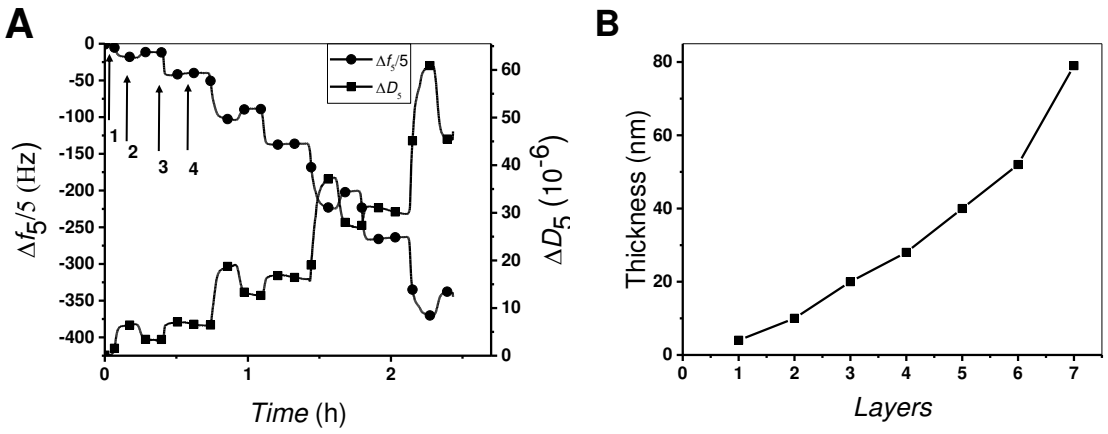

Figure 3.1. (A) QCM results of *in situ* build-up of $(CHT/HA)_3$-CHT thin films with normalized frequency (●) $\Delta f_5/5$ (Hz) and dissipation (■) ΔD_5 (10^{-6}). (1) Addition of CHT; (2) and (4) cleaning steps with NaCl 0.15M; (3) addition of HA. (B) Cumulative thickness variations of $(CHT/HA)_3$-CHT films.

The QCM-D was used to determinate film thickness in every adsorption step by the Voigt-based viscoelastic model. **Figure 3.1B** shows the cumulative thickness evolution of $(CHT/HA)_3$-CHT films, which reached a value of 79 nm, for $(CHT/HA)_3$ films the cumulative thickness reached a value of 75 nm. The QCM-D experiments confirm that CHT can be used with HA to obtain an ultrathin film exhibiting viscoelastic properties. It was reported an exponential growth of PEM CHT/HA films (Etienne et al., 2005; Neto et al., 2014; Richert et al., 2004). The non-linear evolution of the thickness in Fig. 1B seems to be consistent with such hypothesis.

After the films construction, Δf_n were recorded for further 24h upon flushing them with Hase solutions prepared at various concentrations, also adjusted to pH=5.5. This pH value was selected not only to ensure optimal enzyme activity (range between 4.5-6.0) (De Salegui, Plonska, & Pigman, 1967) but also to avoid inducing pH-dependent variations on the film, which would result in the disruption on the Δf and ΔD values. After 24h, the enzyme solution was removed alongside with film remains by flushing with the rinsing solution (NaCl 0.15 M) for 30 min. The variations of $(-\Delta f_5/5)$ for 24h after Hase injection show the various degradations profiles induced by varying Hase concentrations (**Figure 3.2**). A decrease of frequency in absolute value indicates removal of film mass, thus being an indicator of degradation. For $(CHT/HA)_3$ films (**Figure 3.2A**), $(-\Delta f_5/5)$ decreased faster when higher enzyme concentrations were used. In particular, it was observed that most of the film was degraded in the first two hours. For example, $(-\Delta f_5/5)$ decreased about 70% when enzyme concentration was 2000 $\mu g.mL^{-1}$. Afterwards, $(-\Delta f_5/5)$ decrease rate was progressively slower during the remaining hours of the experiment. At the lowest concentrations, the variations were not as faster as in the higher concentrations but is also progressive during the monitored 24 hours. At minimum studied enzyme concentration (1 $\mu g.mL^{-1}$), though a pronounced $-\Delta f_5/5$ decrease was observed at the beginning, the variation was only residual the following hours (about 20% of $(-\Delta f_5/5)$ decrease). Using a Hase solution with a concentration of 50 $\mu g.mL^{-1}$ provided an intermediate $(-\Delta f_5/5)$ decrease among all the tested concentrations. For $(CHT/HA)_3$-CHT films, similar $(-\Delta f_5/5)$ variations were observed (**Figure 3.2B**), although not as pronounced for the two lowest Hase concentrations. This delay could be caused by the last protective film layer, since CHT it is not a specific substrate of Hase (Girish, Kemparaju, Nagaraju, & Vishwanath, 2009).

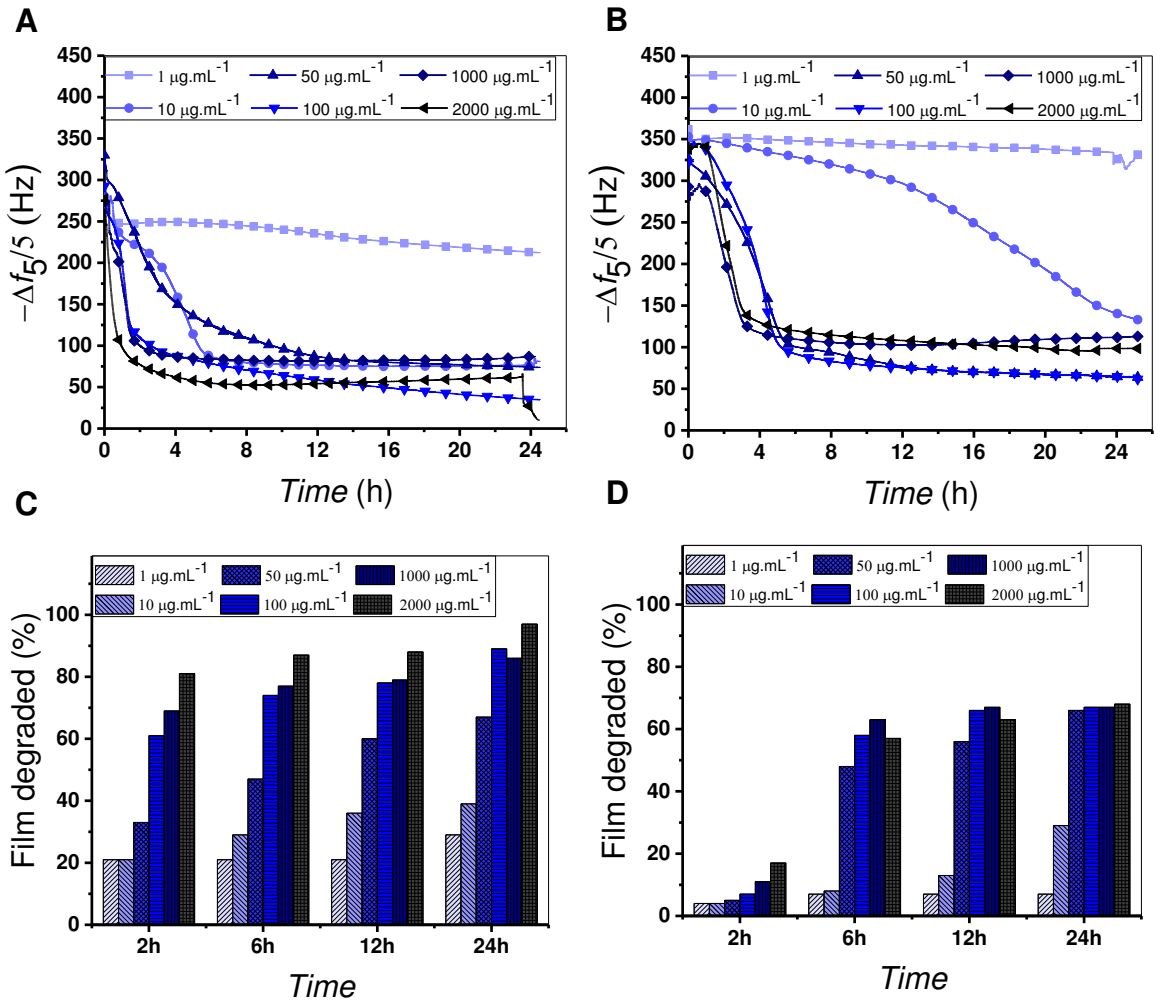

Figure 3.2. Normalized frequency variations for the 5th overtone (-$\Delta f_5/5$) during 24h for (A) $(CHI\text{-}HA)_3$ thin films and (B) $(CHI\text{-}HA)_3$-CHT, after addition of Hase solution at different concentrations (■) 1 μg.mL^{-1}; (●) 10 μg.mL^{-1}; (▲) 50 μg.mL^{-1}; (▼) 100 μg.mL^{-1}; (♦) 1000 μg.mL^{-1}; (◀) 2000 μg.mL^{-1}. Thickness percentage of films degraded after enzyme degradation during time as quantified from the QCM-D data using the Voigt-model for $(CHT/HA)_3$ (C) and $(CHT/HA)_3$-CHT films (D).

In order to quantify more accurately the degradation profile between both films architectures and the various enzyme concentrations used, the Voigt-based viscoelastic model was used (see Equations 1 and 2). The model takes into account the dissipation variations and thus the variations of coupled water mass during the degradation. As observed in **Figure 3.2C-D**, the degradation of $(CHT/HA)_3$ films is more pronounced than $(CHT/HA)_3$-CHT ones. In the case of $(CHT/HA)_3$ films, there was a fast degradation in the first 2h for the three highest concentrations, for which film degradation was higher than 60%. At the same time-point, film degradation was only 20-30% at lower concentrations, less than half of the higher concentrations. After 12h in contact with Hase at higher concentration, the thickness decrease was not as pronounced as in the first 2h. However, for the highest studied concentration, the observed thickness

reduction was 97%, corresponding to a near-full degradation of the film. A more specific analysis of Hase concentration of 50 μg.mL^{-1} indicates a more progressive thickness reduction through time (in accordance to the tendency observed in the QCM-D graphics in **Figure 3.1**): after 24h, the thickness decreased by 67% at this Hase concentration. For $(CHT/HA)_3$-CHT films, the thickness variations follow the same trend as the former architecture. However, the variations reveal that the degradation is not as pronounced as with HA-ending films. For example, for 2000 μg.mL^{-1} Hase the degradation only reached 68%, much lower than the 97% observed for the $(CHT/HA)_3$ films, thus showing that the degradation of LbL films can be controlled not only by its composition but also by the type of polyelectrolyte adsorbed in the last layer. The quantification of the thickness reduction confirms again that the last, CHT layer offers a shielding effect against the enzymatic degradation of the film.

The QCM-D results shows that it is possible obtain a strong knowledge of the enzymatic degradation of polyelectrolyte thin multilayer films depending on the type of the last layer and with the affinity between enzyme and polymer. In order to have a better understanding of films degradation profile, homogeneity and how Hase affects film structure, AFM was used to analyze their topography and roughness. Hase at 50 μg.mL^{-1} was selected for the following experiments since it exhibited a more gradual degradation during 24h, while achieving about two thirds of total thickness loss. In this case only the $(CHT/HA)_3$ film lacking the ending layer of CHT were studied. The roughness parameters values (R_a and R_q) can indicate surface alterations that may indicate film degradation. AFM was performed in $(CHT/HA)_3$ films after predetermined time-points of Hase incubation: 0h (used as a control), 3, 6, 9 and 24h in contact with Hase. The characterization of CHT and HA ultrathin films by AFM was previously described as having an appearance of polymer aggregates called “islets”, which surface topography profile could be homogenized with the assembly of more layers (Richert et al., 2004). **Figure 3.3** shows the surface modification and roughness values variations in $(CHT/HA)_3$ films during the enzymatic degradation.

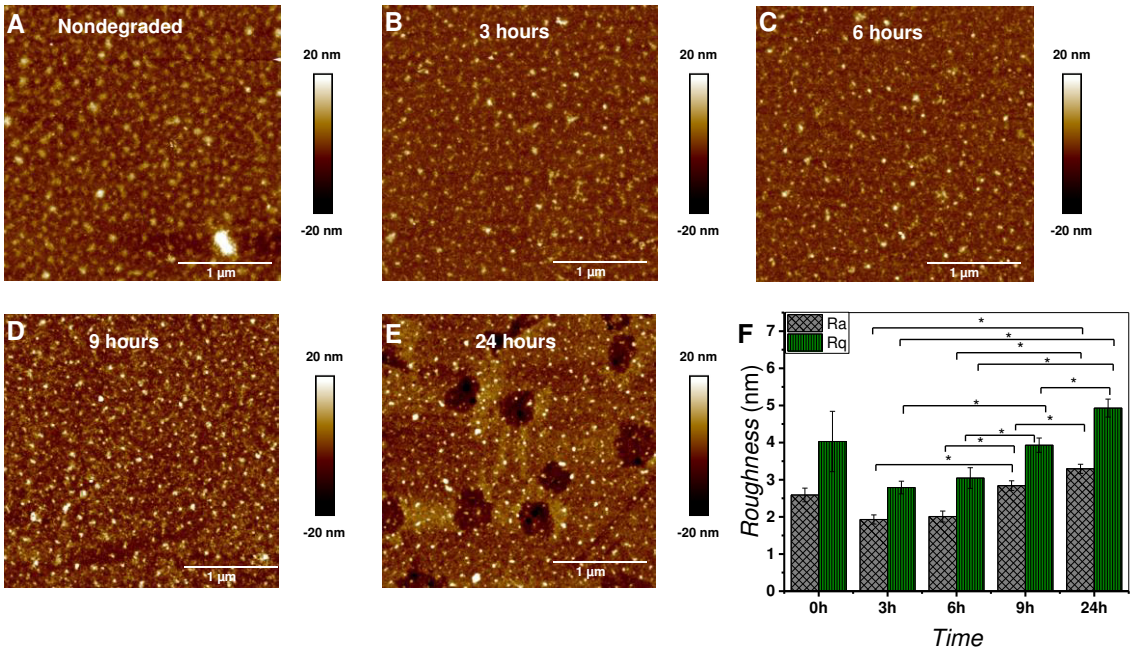

Figure 3.3. AFM images (5x5 µm) of $(CHT/HA)_3$ films (A) prior to degradation, and in contact with Hase solutions at 50 $\mu g.mL^{-1}$ after (B) 3, (C) 6, (D) 9, and (E) 24h. (F) Roughness analysis from root mean squared roughness (Rq) and average roughness (Ra) determined from three independent samples. Data are means±SD. Significant differences were found for *$p<0.05$ ($n=3$).

It was possible to observe a transition from a seemingly homogenous film exhibiting islets (**Figure 3.3A**) into a highly irregular film after 24h in contact with Hase (**Figure 3.3E**). In the first 6h (i.e. time-points 0h, 3h, and 6h) the enzyme action did not translate into a significant difference in surface topography. However, R_q and R_a decreases abruptly in the first 3h, and increases only slightly after 6h (**Figure 3.3F**). This observation suggests that in the first instant that the enzyme comes into contact with the surface the islets are first disturbed by Hase, thus flattening the surface. During the next hours it is possible to observe an increase of roughness values, as well as topography changes. The roughness increase suggests a less homogeneous degradation as the enzyme penetrates through the film leading to a loss of film integrity. In **Figure 3.3E** it is also perceivable that the film exhibited more significant defects, as suggested by the appearance of darker areas, which result in the significant increase of roughness values values. The variation of both roughness parameters follow the same tendency. The dark patches also suggest that Hase enables three-dimensional regulation of the surface structure of multilayer films, rather than solely at the interface of the film with the aqueous surroundings. Given the ability of using enzymes to introduce modifications of the surface structure, it is possible to envisage the production

of multilayer films treated with enzymes as a means to tune mass transport properties and permeability (Silva et al., 2014).

II- Study of enzymatic degradation of chitosan/hyaluronic acid freestanding films

The study of the biodegradability profile of polyelectrolyte coatings at the 2D level lets foresee how more complex LbL devices will behave under similar degradative environments and how their biodegradability is affected by the dimensional scalability of LbL assembly. In terms of dimensional scalability, freestanding membranes are the next level of LbL complexity after ultrathin films, differing only in the augmented film dimensions in the vertical axis that leads to the formation of membranes a few micrometers thick. This geometry in interesting as it permits to access to relevant macroscopy information, such as weight loss or mechanical properties. The study of enzymatic degradation of freestandings has not been much explored, though such structures have been characterized for their physical, chemical and biological properties (Caridade, Monge, Mano, & Picart, 2012; Larkin et al., 2010; Lu & Hu, 2006). Nonetheless, previous studies alert to the native degradation of CHT and HA films in saline solutions (Larkin et al., 2010). Herein, the degradation of freestanding $(CHT/\ HA)_{100}$ films by Hase was studied using films obtained by an automatized dipping method onto polypropylene substrates. We showed that this method of adsorption is reliable to produce robust and detachable membranes (Caridade et al., 2013; Silva et al., 2014)(**Figure 3.4A**). The freestandings were mounted in holders that ensured the enzyme/membrane contact in an area of 13x13 mm^2 and immersed up to 24h in such enzymatic solution at 37 °C (**Figure 3.4B**). Equation 3 was used to calculate weight loss, which is shown in **Figure 3.4C**. The Hase solution was prepared in NaCl 0.15 M, pH 5.5 at 50 $\mu g.mL^{-1}$, as prepared for the AFM assays, so that a comparison could be established between the ultrathin films and these denser membranes.

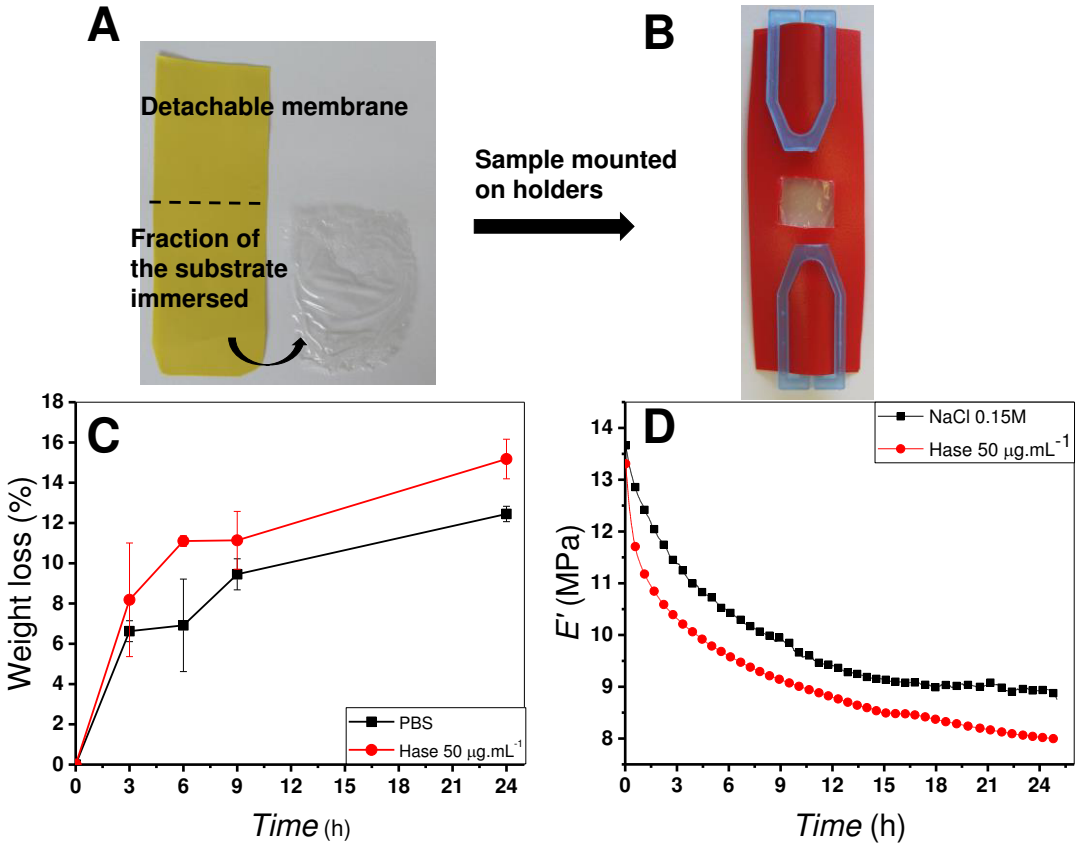

Figure 3.4. (A) Detachable $(CHT/HA)_{100}$ freestanding membranes from polypropylene substrate. (B) Polypropylene membrane holder with the frame that ensured the enzyme/membrane contact. (C) Weight loss of $(CHT/HA)_{100}$ freestanding membranes in Hase at 50 µg.mL^{-1} (●) and in NaCl 0.15M (■) used as a control. Data are means±SD (D) Real-time online DMA storage modulus (*E'*) measurements of 1% (w/v) glutaraldehyde cross-linked membranes immersed in NaCl 0.15M (■) and in Hase at 50 µg.mL^{-1} (●) during 24h obtained under a frequency of 1 Hz.

As observed, CHT/HA freestanding films were degraded mildly in the presence of Hase, showing weight losses of 15% after 24h. For freestandings incubated in Hase-free solutions, the weight loss was significantly lower (12%). A more careful analysis to the weight loss variations of samples immersed in Hase show a large increase especially in the first 6 hours. Between 6h to 9h, there is a stabilization in the degradation profile. This result reflects the same behavior previously observed in AFM for thin films between 3h and 6h, though in that case the lower thickness of the film can justify why such stabilization occurred earlier than in freestandings. After 24h, the weight loss reached 15%. The similarity of values between both studied situations may be justified by phenomena present in both studied conditions: (i) disruption of the HA nanolayers due to the great ability of these films to absorb large amounts of water (ii) hydrolytic degradation of HA (Tokita & Okamoto, 1995), and (iii) interference of the salt counterions in the electrostatic cross-links of the multilayered membranes (Costa, Rui R, Costa, Caridade, & Mano,

2015). These phenomena may explain why the degradation values are so similar, regardless of the presence – or absence – of Hase, and show that the thicker freestandings are less susceptible to degradation than their ultrathin counterparts in the same time-frame.

In order to obtain more information on the stability of these membranes, DMA assays were performed under the same conditions as in the weight loss experiments. To promote the stability of the PEMs and handleability, the films were lightly cross-linked by exposure to a solution containing 1% (w/v) glutaraldehyde for 1 min, a strategy also adopted by Larkin and coworkers (Larkin et al., 2010). DMA results in **Figure 3.4B** show a progressive decrease of the storage modulus of the freestandings in both studied conditions, indicating a loss of mechanical integrity as the assay progressed. As expected, such decrease was more pronounced for samples incubated in Hase, confirming the degradative effect that such enzyme has on such structures. These results suggest that enzyme degradation contributes not only to the weight loss of freestanding multilayer membranes but also to a loss of their mechanical integrity, which is useful to estimate the short-term stability of LbL structures in enzyme-containing aqueous environments.

The use of freestanding membranes in biomedical applications requires longer degradation studies in order to simulate the evolution of the stability and integrity in the long-term, and thus suitability for specific applications. To achieve this goal, freestanding membranes were immersed in Hase at the same concentration and temperature used in the previous weight loss and DMA assays, but with the difference that the Hase and enzyme-free solutions were prepared in PBS (an isotonic solution that simulates the saline environment of the human body). The weight loss for 120h (i.e. 5 days) was calculated using Equation 3 and is shown in **Figure 3.5E**.

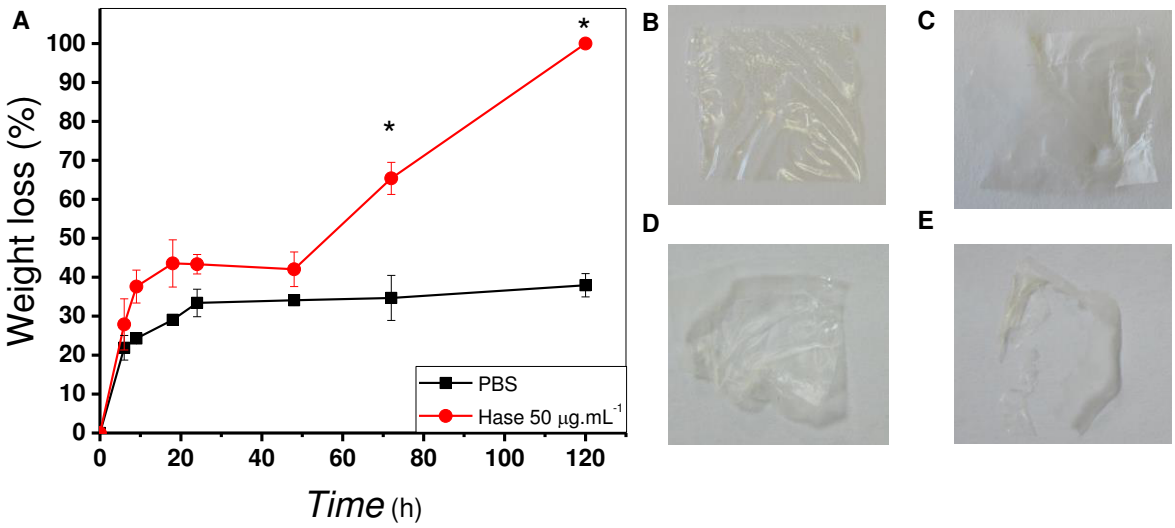

Figure 3.5. (A) Weight loss in Hase at 50 µg.mL^{-1} (●) and in PBS saline buffer (■) used as a control. Data are means±SD. Photographs of CHT/HA freestanding membrane before (B) and after 24 (C); 48 (D) and 72h (E) of Hase incubation. Significant differences were found for *p<0.05.

Long-term degradation results showed that the (CHT/ HA)$_{100}$ freestanding films are fully degraded in the presence of Hase in 120h. The weight loss of films in the presence of the enzymatic solution was significantly higher (100%) when compared with the control (around 40%), evidencing a large contribution of Hase activity on the multilayer structure for times longer than 24h (as was shown previously in **Figure 3.4C**). Meanwhile, it was also possible to observe degradation in the control samples (i.e. immersed in enzyme-free PBS), but weight loss stabilized around 35-40% after the first 24 hours. A closer look at the weight loss evolution in Hase reveals a high degradation rate in the first 18h. Afterwards, there was a stabilization in the degradation around 40% until 48h have passed. After 48 h, the film degradation increased significantly, which was not observed for the control. It is possible that the degradation of the membrane in contact with Hase first leads to a molecular rearrangement phenomenon that prevails more pronouncedly over the stripping and removal of molecules from the structure. As time progresses, the integrity of the structure is compromised enough to lead to the complete disassembly of the membrane and its breakdown (**Figure 3.5B-E**).

These results reveal how enzymes can degrade not only ultrathin films but also PEM freestanding membranes, which is an important factor to consider for biomedical applications. In only 72h, it was possible to destroy a membrane in contact with a Hase solution of 50 µg.mL^{-1}. This concentration is indeed much higher than the Hase basal levels in the human body: in biological environments they are

present at lower concentrations, for example, at 60 $ng.mL^{-1}$ in human serum (Frost, Csóka, Wong, & Stern, 1997), thus the structural modifications induced on LbL structures by Hase in the human body would most likely be less pronounced and take longer.

III- Study of enzymatic degradation of chitosan/hyaluronic acid LbL capsules

There are systems where the degradative effect of enzymes is exploited to facilitate the release of bioactive agents from drug delivery systems (Xu, Lee, Gao, Tan, & Kurisawa, 2015). In this case, the Hase released from the structure is rapidly eliminated from plasma, causing no adverse effects to the body (Menzel & Farr, 1998). The enzymatic degradation of ultrathin films and freestandings has been so far demonstrated herein. Knowing how enzymes can significantly affect LbL-based structures, one could envisage the use of enzymes to develop mechanisms of spatio-temporal control inherent to LbL devices, such as films with tunable mass transport properties and multilayer carriers for the controlled release of bioactive agents. Following this reasoning, Hase was incorporated within CHT/HA microcapsules alongside FITC-BSA, the latter used as a model protein for the proof-of-concept, in order to explore the hypothesis of using an enzyme to control the release of active agents loaded within LbL structures.

Capsules represent the next dimensional level of scalability that LbL coatings have been translated to. Multilayer nano- and micro-capsules for drug delivery applications have been often produced by assembling coatings onto colloidal sacrificial templates (Borodina et al., 2007; Caruso, Caruso, & Möhwald, 1998; Costa, R. R. et al., 2013; De Geest et al., 2009; Johnston, Cortez, Angelatos, & Caruso, 2006; Marchenko et al., 2012). Herein, $CaCO_3$ microparticles were used as sacrificial templates (which can be chelated by EDTA), to assemble CHT/HA-coated capsules. The capsules used herein consist in three CHT/HA bilayers loaded with FITC-BSA and Hase – $[(CHT/HA)_3$-(FITC-BSA/Hase)] – and capsules with no enzyme incorporated used as a control – $[(CHT/HA)_3$-(FITC-BSA)]. The encapsulation of the agents is made during the co-precipitation of sodium carbonate and calcium chloride under vigorous stirring, a method that are very effective for loading proteins (Balabushevich, de Guerenu, Feoktistova, & Volodkin, 2015; Volodkin, D., 2014; Volodkin, D. V., Larionova, & Sukhorukov, 2004). We theorized that encapsulating Hase could increase the release rate of FITC-BSA (**Figure 3.6A**). SEM observations of uncoated $CaCO_3$ particles loaded with FITC-BSA and FITC-BSA/Hase (**Figure 3.6B**) showed diameters around 1-3 μm and smooth surfaces. The similarity between the surface of the two types of particles $CaCO_3$/FITC-BSA and $CaCO_3$/FITC-BSA/Hase let foresee that the incorporation of Hase does not lead to

any morphological change that could influence the LbL assembly. After construction, the core was chelated with EDTA to form hollow microcapsules. The estimated encapsulation efficiency of FITC-BSA encapsulated for $(CHT/HA)_3$-(FITC-BSA/Hase) and $(CHT/HA)_3$-(FITC-BSA) capsules was 78 % and 73 %, respectively.

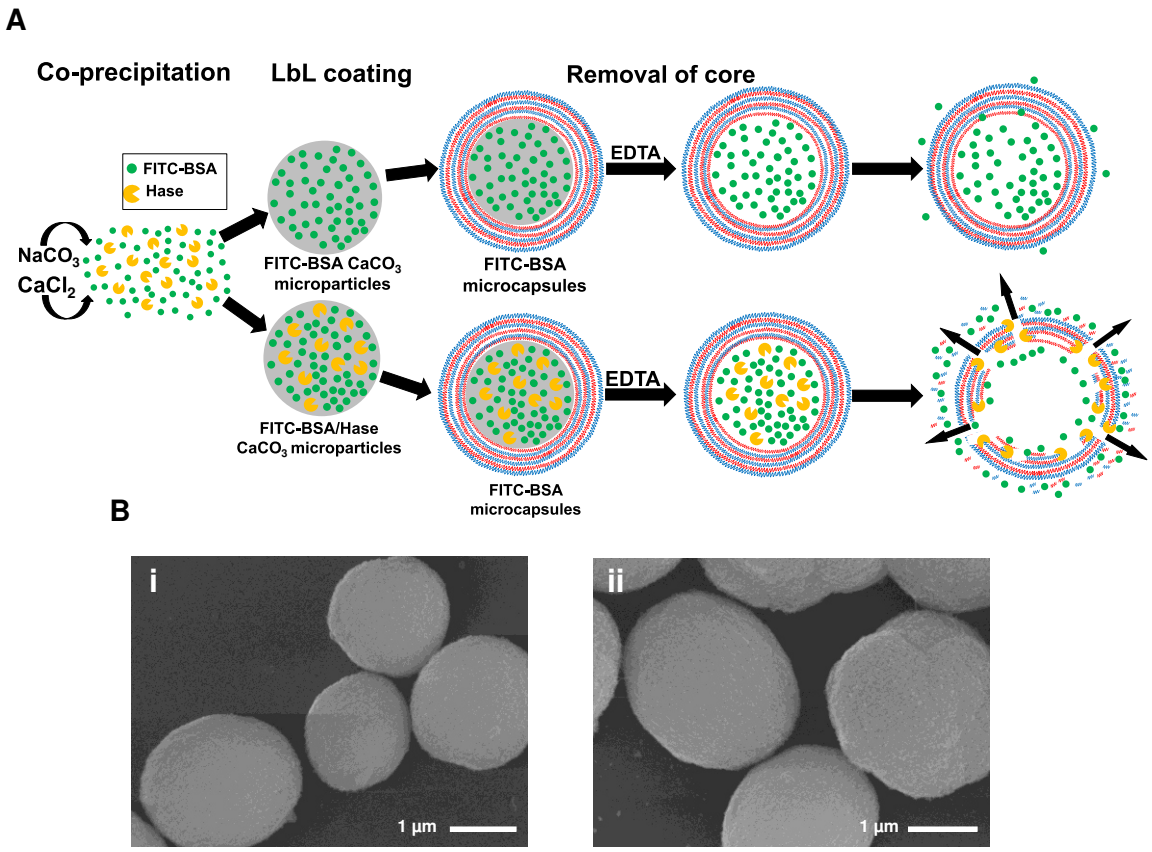

Figure 3.6. (A) Schematic representation of the PEM polysaccharides-based microcapsules. From the co-precipitation to protein/enzyme entrapment to the enzyme-triggered faster release. (B) Micrograph of calcium carbonate microparticles loaded with (i) FITC-BSA and (ii) FITC-BSA/Hase.

The release of FITC-BSA release was followed up to 14 days (**Figure 3.7A**). As expected, there was a higher quantity of FITC-BSA released from Hase-loaded capsules (about 100 µg) than from Hase-free capsules (about 25 µg). The results are complemented by visual assessment using confocal microscopy, showing microcapsules with around 5 µm and a decrease in fluorescence intensity after 14 days (**Figures 3.7B and 3.7C**).

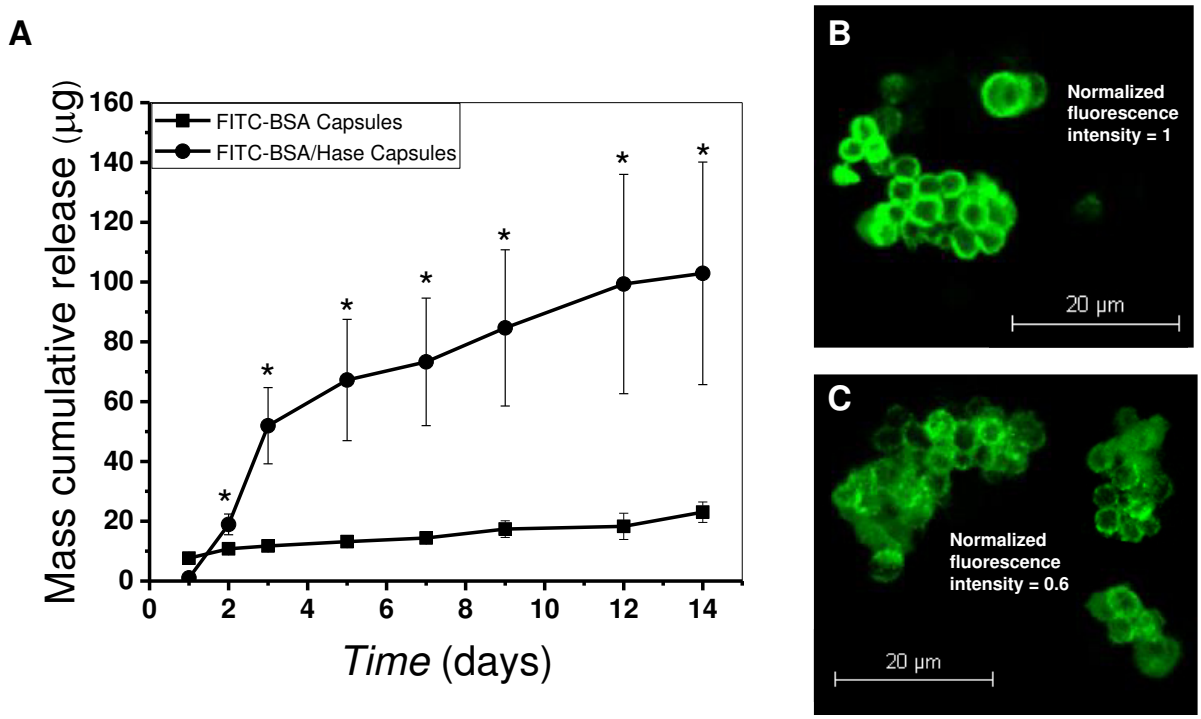

Figure 3.7. FITC-BSA release profile from CHT/HA microcapsules (A). Images of CHT/HA multilayer microcapsules loaded with FITC-BSA and hyaluronidase before (B) and after 14 days (C). Data are means±SD. Significant differences were found for *p<0.05.

The release of FITC-BSA from the capsules was 4 times higher when co-encapsulated with Hase, showing that the enzyme promotes an enhanced release of loaded agents. Furthermore, it is noteworthy that the capsules maintain their spherical shape after 14 days, showing that the amount of Hase encapsulated is high enough to increase the permeability of the multilayer shell without compromising their integrity. The preferential localization of fluorescence near the shell also suggest a high affinity between the BSA and the inner CHT layer, which has been demonstrated elsewhere (Martins, Merino, Mano, & Alves, 2010). In the case of Hase-free capsules, the amount of FITC-BSA released was lower than in enzyme-loaded capsules, as expected. The amount released is therefore solely due to the native HA hydrolytic degradation and counterions interference, as mentioned before, as well as molecule diffusion through the multilayer shell.

This result also suggests that the release profile of multilayer drug carriers can be tunable by the action of enzymes loaded in the encapsulation process, proving that enzymatic degradation can function as a triggering release of biomolecules. This control over the release profile is in accordance to our hypothesis to develop polyelectrolyte capsules coated using LbL assembly and loaded enzymatic triggers. One can

envisage the incorporation of specific enzymes along with desired bioactive agents, which will be released for a prolonged period of time without the need of other triggered release factors, such as temperature or pH variations. In the context of controlled drug delivery technologies, it may be possible to eliminate the need of repeated administrations, since the release rate could be tuned to fit within a proper therapeutic window, or a sustained release of growth factors could be achieved for tissue engineering and regenerative medicine strategies.

4. Conclusion

Different multilayered CHT/HA structures were produced *via* electrostatic interactions to study their biodegradability properties by specific enzymatic action. The study of the degradation profile and how this degradation occurs is important to future applications of different LbL-based structure obtained by using different templates. Ultrathin film degradation was followed using quartz crystal microbalance and observed by AFM. CHT/HA ultrathin films showed a high sensitivity to Hase, inducing significant topography films variations. It was also verified that this sensitivity changes if the last layer ingredient is not a specific substrate of Hase, showing that the degradation of LbL films can be controlled by the nature of the polyelectrolytes used and the films architecture.

By increasing the scale complexity of the films, it was shown that freestandinds retain the enzymatic susceptibility to Hase, but it was also demonstrated that even in its absence, degradation still occurs due to HA native highly hydration ability and counterion interference in the multilayer structure. Taking advantage of Hase as a trigger to control the release of bioactive agents, polyelectrolyte microcapsules were successfully prepared loaded with Hase and a model protein. The Hase-mediated enhanced release proved that this concept could be useful to conceive drug carriers which permeability is controlled *via* the incorporation of specific enzymatic triggers with potential applications in tissue engineering, regenerative medicine and mass transport devices including drug delivery systems.

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SECTION 4 – CONCLUDING REMARKS

Chapter 4 – Conclusions and future outcomes

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Natural based polysaccharides have been widely used to produce devices with biomedical applications. The fact that they are biocompatible, nontoxic and often biodegradable and stimuli-responsive makes these polymers suitable raw materials for the construction of complex loading devices with application in controlled release, for example. These devices can be constructed using different methods and can be synthesized in various shapes, such as membranes, particles, capsules and hydrogels, capable to entrapped different bioactive agents. Natural polysaccharide are preferred than synthetic materials, since their exhibit several chemical and biological properties. Among this biomaterials, marine origin polysaccharides and their derivatives can been seeing as excellent materials not only for the construction of load devices but also for other structures with specific applications. Because these materials are biocompatible and biodegradable, their use may be considered to increase the efficiency of these devices for biomedical applications. Therefore, further investigation will be required to improve the isolation and purification of marine origin biopolymers, as well as the methods of their chemical modification and processing into the various possible structures. This could be possible by looking into the interactions between polymer, drugs and native biological tissues, as well the intelligent response of the polysaccharides and targeting capability. Biodegradability, it is one of the main properties that makes these materials suitable for injectable devices or carriers for drug delivery systems. Future strategies involving nanotechnologies should also combine the possibility of controlled release systems from this type of natural polysaccharides-based devices responsive to an external or internal stimuli.

Polyelectrolyte multilayer structures were successfully produced using biodegradable polysaccharides by LbL deposition technique. These structures with different shape, size and thickness revealed to be susceptible to enzyme degradation. Through different characterization techniques was possible obtain valid information of the degradation profile. Studying the degradation profile and how enzymatic degradation affects the structure of the different devices may lead to greater knowledge of their behavior in the presence of different stimulus and moieties. This feature it is important, since permit make progress to understand in which manner this structures may be applied on different fields.

Chitosan/hyaluronic acid thin films showed a high sensibility to hyaluronidase, that even a lower concentration showed high levels of degradation and surface modifications. This type of nanocoatings could be applied in different areas, like in the construction of biosensors, since this type of materials can

easily absorb proteins and DNA. By increasing the complexity of the structure, was possible obtain robust detachable membranes. These freestanding films showed degradation in the presence of hyaluronidase. These results suggest that biocompatible freestanding films are of high potential interest for several applications, inclusive as drug release since it is possible, through LbL incorporate particles, biomolecules and by crosslinking process it is also possible to incorporate enzymes that could promote the degradation of the film that lead to the controlled release of bioactive agents.

Microcapsules were successfully prepared by LbL with hyaluronidase encapsulated within the microcapsules. The degradation of the capsules catalyzed by the enzyme promote the release of encapsulated protein. The proof of this concept suggest that specific enzymes can be loaded inside microcapsules and so develop responsive-carriers for different applications, such as in DDDs or in biotechnology.

The study of the biodegradability of LbL structures in different moieties and conditions can promote a better understanding on how this structures behave on those different environments and wither these structures are able to maintain his properties. This approach can lead to the development, improvement and characterization of the innumerous structures that can be produced by LbL. The degradation profile of these structures could help to determinate if there are suitable or not for biomedical applications.