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Development of 3D porous structures for buccal drug delivery

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À minha avó São

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Abstract

Advances in polymer science and engineering has led to the development of new polymeric systems for controlled drug delivery in the oral cavity. In this work, pH responsive scaffolds were prepared by two distinct methods: freeze-drying and gelation process followed by scCO2-assisted drying. The objective was to obtain two distinct porous structures, a gum and a candy to treat oral diseases. The scaffolds of chitosan (CHT), xanthan gum (XG) and polymer m (PM) were prepared with and without crosslinkers (N,N'-methylene-bis-acrylamide (MBA), tetramethylenediamine (TEMED) and ammonium persulfate (APS)) in order to find the optimal features for the purpose of this work. Structures with a wide range of pore sizes and different mechanical properties, swelling ability and controlled biodegradability were obtained depending on the scaffold composition.

Microarchitectural analysis by scanning electron microscopy demonstrated that it was possible to obtain a wide range of pores, depending on the fabrication process and the polymers used. The ability of these structures to release a low molecular weight drug (ibuprofen, Ibu) and a large model protein (BSA) was investigated. Additionally, a mathematical model was adjusted to the experimental release profiles in order to describe the drug release mechanisms.

The tunable morphological and mechanical proprieties together with the ability for the controlled release of bioactive molecules make these structures attractive for pH sensitive systems to treat oral illness.

The preliminary studies indicated that the porous scaffolds showing the best performance as pH sensitive buccal drug delivery systems were: xanthan gum blended with polymer m (XGPM) for the candy scaffold, and xanthan gum blended with chitosan crosslinked with MBA, TEMED and APS (TXGCHT) for the gum scaffold. The quantity of drug release was 60 mg/ g of structure and 75 mg/g of structure, for XGPM and TXGCHT respectively.

Keywords: Chitosan, Oral illness, pH sensitive scaffolds, Polymer m, Xanthan gum

Resumo

O avanço da ciência de polímeros e da engenharia levou ao desenvolvimento de novos sistemas poliméricos para a libertação controlada de fármacos na cavidade oral. Neste trabalho, foram desenvolvidos monólitos sensíveis ao pH por dois métodos distintos: por liofilização e através do processo de gelificação seguido de secagem assistida por CO_2 supercrítico. O objetivo era obter duas estruturas porosas distintas, uma na forma de rebuçado e outra na forma de pastilha para o tratamento de doenças orais. Os polímeros usados para a produção dos monólitos foram o quitosano (CHT), a goma de xantana (XG) e o polímero m (PM), estes foram preparados com e sem reticulante (N,N'-metileno-bis-acrilamida (MBA), tetrametilenodiamina (TEMED) e persulfato de amónio (APS)), de modo a avaliar as características ótimas para o propósito deste trabalho. Foram obtidas estruturas com uma vasta gama de tamanho de poro com diferentes propriedades mecânicas, capacidade de inchamento e biodegradabilidade controlada, dependendo da composição do monólito.

A análise microarquitetural por microscopia eletrónica de varrimento demonstrou que era possível obter uma elevada gama de poros, dependendo do processo de fabricação e os polímeros utilizados. A capacidade destas estruturas libertarem princípios activos de forma controlada foi investigada para dois fármacos modelo, um de baixo peso molecular (ibuprofeno, Ibu) e outro de maior peso molecular, uma proteína modelo (BSA). Além disso, foram ajustados modelos matemáticos aos perfis de libertação experimentais, de modo a esclarecer os mecanismos de libertação dos fármacos nas diferentes matrizes poliméricas.

A possibilidade de ajustar as propriedades morfológicas e mecânicas das estruturas, juntamente com a libertação controlada de moléculas bioativas produziram estruturas relevantes para sistemas sensíveis ao pH para o tratamento de doenças orais.

Os estudos preliminares indicaram que as estruturas porosas com melhor desempenho para a aplicação em vista foram: a mistura de goma xantana com o polímero m (XGPM) para a opção de rebuçado e goma xantana com quitosano reticulada com MBA, TEMED e APS (TXGCHT) para a opção de pastilha. A quantidade de libertação de droga foi de 60 mg/g de estrutura e de 75 mg/g de estrutura, para XGPM e TXGCHT respetivamente.

Palavras-Chave: Doenças orais, Goma Xantana, monólitos sensíveis a pH, polímero m, Quitosano.

List of Contents

1. INTRODUCTION 1

1.1. ORAL FLORA AND DISEASE 1

1.2. POLYMERS 7

1.3. POLYMERS PROCESSING 11

1.4. IMPREGNATION ..... **ERRO! MARCADOR NÃO DEFINIDO.**

1.5. THESIS ORGANIZATIONAL OUTLINE 14

2. MATERIALS AND METHODS 17

2.1. MATERIALS 17

2.2. METHODS 17

3. RESULTS AND DISCUSSION 25

3.1. SCAFFOLDS PRODUCTION 25

3.2. SCAFFOLDS CHARACTERIZATION 28

3.3. RELEASE STUDIES 44

4. CONCLUSION 49

5. REFERENCES I

6. APPENDIX VIII

List of Figures

FIGURE 1.1- CONVERSION REACTION OF R-IBUPROFEN INTO S-IBUPROFEN 6
FIGURE 1.2- CHITOSAN MOLECULE 9
FIGURE 1.3- XANTHAN GUM MOLECULE 10
FIGURE 1.4- ACRYLAMIDE MONOMER 11
FIGURE 2.1-CASTING SOLUTION 18
FIGURE 2.2-LYOPHILIZER 18
FIGURE 2.3-FLASKS WITH THE 2 ML SAMPLES 18
FIGURE 2.4-ScCO$_2$ APPARATUS; (1) CO2 BOTTLE, (2) CRYOSTAT, (3) PUMP, (4) BACK PRESSURE VALVE, (5) THERMOSTATIZED BATH 19
FIGURE 2.5--HIGH PRESSURE CELL 19
FIGURE 2.6- FTIR-ATR EQUIPMENT AND COMPUTER 20
FIGURE 2.7- MECHANICAL TESTER AND SCAFFOLD 21
FIGURE 2.8- DEGRADATION AND RELEASE APPARATUS 22
FIGURE 3.1- FREEZE-DRY SCAFFOLDS; A) CHT SCAFFOLDS; B) XG SCAFFOLDS AND C) PM SCAFFOLDS. I) 3% WT/WT POLYMER; II) 2% WT/WT POLYMER AND III) 1% WT/WT POLYMER 26
FIGURE 3.2- SCCO2 ASSISTED SCAFFOLDS: A) SCAFFOLD IN GELATION STEP; B) SCCO2 PM 3% WT/WT SCAFFOLD WITH BSA AND C) FAILED SCCO2 PM SCAFFOLD 28
FIGURE 3.3-SEM IMAGES OF CROSS-SECTION OF THE POLYMERS NATIVE SCAFFOLDS PRODUCED BY FREEZE-DRYING (A) CHT 2% WT/WT, (B) CHT 3% WT/WT ,(C) XG 2% (WT.%),(D) XG 3% WT/WT,(E) PM 2% WT/WT,(F) PM 3% WT/WT 29
FIGURE 3.4- SEM IMAGES OF CROSS-SECTION OF THE CROSSLINKED SCAFFOLDS PRODUCED BY FREEZE-DRYING (A) CHT WITH MBA 2% WT/WT, (B) CHT WITH MBA 3% WT/WT (C) CHT WITH TEMED AND APS 2% WT/WT, (D) CHT WITH TEMED AND APS 3% WT/WT, (E) XG 2% WT/WT WITH MBA, (F) XG 30
FIGURE 3.5- SEM IMAGES OF CROSSECTION OF POLYMERS MIXTURES OBTAIN BY FREEZE-DRYING: (A)CHT+GUM 2% WITHOUT CROSSLINKER; (B) PM+ GUM 2% WITHOUT CROSSLINKER; (C) CHT+GUM 2% WITH MBA; (D) PM+ GUM 2% WITH MBA; (E) CHT+GUM 2% WITH MBA, TEMED AND APS; (F) PM+GUM 2% WITH MBA, TEMED AND APS 31
FIGURE 3.6- SEM IMAGES OF CROSSECTION OF SCCO2 ASSISTED SCAFFOLDS: (A) TCHT 3% WT/WT AND (B) PM 3% WT/WT 32
FIGURE 3.7- FTIR-ATR OF A) CHT SCAFFOLD VS CHT CROSSLINKED WITH MBA VS CHT CROSSLINKED WITH TEMED AND APS; B) NATIVE XG SCAFFOLD VS XG CROSSLINKED WITH MBA VS XG CROSSLINKED WITH TEMED AND APS C) OF NATIVE PM SCAFFOLD VS PM CROSSLINKED WITH MBA VS AND PM CROSSLINKED WITH TEMED AND APS 33
FIGURE 3.8- FTIR-ATR OF A) NATIVE XGPM SCAFFOLD VS XGPM CROSSLINKED WITH MBA VS XGPM CROSSLINKED WITH TEMED AND APS AND B) NATIVE XGCHT SCAFFOLD VS XGCHT CROSSLINKED WITH MBA VS XGCHT CROSSLINKED WITH TEMED AND APS 34
FIGURE 3.9- FTIR-ATR OF TCHT SCAFFOLD VS CHT IMPREGNATED WITH IBU VS CHT IMPREGNATED WITH BSA 35
FIGURE 3.10- SWELLING RATE OF NATIVE SCAFFOLDS PREPARED BY FREEZE-DRYING METHOD: A) CHT PH5.5; B) CHT PH 7.4; C) PM PH 5.5 AND D) PM PH 7.4 36

FIGURE 3.11-SWELLING RATE OF CROSSLINKED SCAFFOLDS OBTAIN BY FREEZE-DRYING: A) CHT WITH MBA PH5.5; B) CHT WITH MBA PH 7.4; C) PM WITH MBA PH 5.5, D) PM PH 7.4 WITH MBA, E) CHT WITH TEMED AND APS AT PH 5.5 AND F) CHT WITH TEMED AND APS AT PH 7.4.....37
FIGURE 3.12-SWELLING RATE OF POLYMERS MIXTURES PRODUCED BY FREEZE-DRYING: A) XGPM 2% WT/WT AT PH 5.5, B) XGPM 2% WT/WT AT PH 7.4, C) XGCHT 2% WT/WT AT PH 5.5 AND D) XGCHT 2% WT/WT AT PH 7.4.....38
FIGURE 3.13- SWELLING RATE OF $ScCO_2$-ASSISTED SCAFFOLDS WITH BSA OF TCHT 3% WT/WT AND PM: 3% WT/WT A) AT PH 5.5 AND B) AT PH 7.4.....38
FIGURE 3.14-ZETA POTENTIAL ANALYSIS OF THE SELECTED SCAFFOLDS.....41
FIGURE 3.15- DEGRADATION STUDIES A) CANDY OPTION (PM 3% WT/WT AND XGPM2% WT/WT) AND B) GUM OPTION (TCHT 3% (WT.%) AND TXGXHT 2% WT/WT).....42
FIGURE 3.16-BRIGHT-FIELD MICROGRAPHS OF HUMAN DERMAL FIBROBLASTS AFTER 24 HOURS IN CONTACT WITH DIFFERENT CONCENTRATIONS OF PM POLYMER. 43
FIGURE 3.17- CELL VIABILITY AFTER 24 HOURS IN CONTACT WITH PM AT DIFFERENT CONCENTRATIONS. DATA IS PRESENTED AS THE AVERAGE OF THREE REPLICATES WITH STANDARD DEVIATION. ***P<0.01, ONE-WAY ANOVA AND BONFERRONI'S POST-HOC TEST. 43
FIGURE 3.18- BSA RELEASE PROFILE OF FREEZE-DRYING OBTAIN SCAFFOLDS A) PM 3% WT/WT, B) TCHT 3% WT/WT AND MATHEMATICAL MODULATION OF THE BEST METHOD (POWER LAW) C) PM AND D) TCHT 44
FIGURE 3.19-BSA RELEASE PROFILE OF FREEZE-DRYING OBTAIN SCAFFOLDS A) XGPM 2% WT/WT, B) TXGCHT 2% WT/WT AND MATHEMATICAL MODULATION OF THE BEST METHOD C) XGPM AND D) TXGCHT 45
FIGURE 3.20- BSA RELEASING PROFILE OF SCCO2-ASSISTED SCAFFOLDS AT PH 5.5 AND 7.4: A) PM 3% WT/WT AND B) TCHT 3% WT/WT AND MATHEMATICAL MODULATION C) PM WITH POWER LAW AND D) TCHT HIGUSHI MODEL.....46
FIGURE 6.1-FREEZE-DRY SCAFFOLDS CROSSLINKED WITH MBA; A) CHT SCAFFOLDS; B) XG SCAFFOLDS AND C) PM SCAFFOLDS. VIII
FIGURE 6.2- FREEZE-DRY SCAFFOLDS CROSSLINKED WITH TEMED AND APS; A) CHT SCAFFOLDS; B) XG SCAFFOLDS AND C) PM SCAFFOLDS. I) 3% WT/WT POLYMER; II) 2% WT/WT POLYMER AND III) 1% WT/WT POLYMER IX
FIGURE 6.3- FREEZE-DRY SCAFFOLDS OF POLYMERS MIXTURES; A) XGPM 2% WT/WT SCAFFOLDS; B) XGCHT 2% WT/WT SCAFFOLDS I) NATIVE SCAFFOLDS, II) CROSSLIKED WITH MBA AND III) CROSSLINKED WITH TEMED AND APS IX
FIGURE 6.4- FTIR-ATR ANALYSIS OF SCAFFOLDS IMPREGNATED WITH BSA AND IBU A)PM B)XGPM C)TXGCHT.....X
FIGURE 6.5- SWELLING RATE OF SCAFFOLDS OBTAIN BY FREEZE-DRYING METHOD AT PH 9: A) CHT NATIVE SCAFFOLDS; B) PM NATIVE SCAFFOLDS; C) CHT CROSSLINKED WITH MBA D) PM CROSSLINKED WITH MBA AND E) CHT CROSSLINKED WITH TEMED AND APS XI
FIGURE 6.6- IBU RELEASE PROFILE OF FREEZE-DRYING OBTAIN SCAFFOLDS A) PM 3% WT/WT, B) TCHT 3% WT/WT AND MATHEMATICAL MODULATION OF THE BEST METHOD (POWER LAW) C) PM AND D) TCHT XI
FIGURE 6.7- IBU RELEASE PROFILE OF FREEZE-DRYING OBTAIN SCAFFOLDS A) XGPM 2% WT/WT, B) TXGCHT 2% WT/WT AND MATHEMAT-ICAL MODULATION OF THE BEST METHOD (POWER LAW) C) XGPM AND D) TXGCHT.....XII

List of Tables

TABLE 1.1-PH IN VARIOUS TISSUES AND CELLULAR COMPARTMENTS (ADAPT FROM VARIOUS SOURCES) 2
TABLE 1.2-ANTIFUNGAL AGENTS USED IN THE TREATMENT OF ORAL CANDIDIASIS (ADAPT FROM [29]).... 4
TABLE 3.1- COMPRESSIVE MODULUS (KPA) OF DRY SCAFFOLDS 39
TABLE 3.2- MODELATION VALUES FOR HIGUSHI AND THE POWER LAW 47

1. Introduction

Pharmaceutical industry in later years had been searching for innovative methods for treatment of oral diseases. Drug delivery technology had accomplish that with the progress in polymer science. The use of polymers in the pharmaceutical industry started as drugs additives or mechanical supporters. Nowadays they are used to increase the drugs efficiency and more functional.

1.1. Oral flora and disease

The treatment of oral mucosa diseases was almost not existing until the 1940s, since the most common types were caused by fungus. The scientific community disregarded the illness caused by yeasts because before the HIV-era it was believed that fungus infections were too rare to justify a significant research by the pharmaceutical industry. Another factor that delayed the antifungal development was the “apparent” lack of selective fungal target, not present in other cells.[1][2]

The first treatments to be available were polyene antibiotic amphotericin B, some azole derivatives, the allylamines–thiocarbamates and 5-flucytosine. The agents acted by interfering with the structural or functional integrity of the fungus plasma membrane, by physical disruption or by blocking the biosynthesis of the membrane sterols.[3][1] [4]

In recent years, with the expansion of basic and clinical researches new and more specialized options, such as azoles compounds and candis, have been available. Antifungal resistant drugs have increased due to a more exposed population to factors that favour mouth problems. This can be caused by highly processed food, by excess cleaning and by bacteria control of the environment, which makes the fungus drug resistant. Another factor is the excess of medication that makes the natural defence system of the human body deficient. For this reason, the development of new and innovative ways to treat a large number of illnesses is needed. However, the research in this area has focused in finding different active principles instead of making the carriers of the ones that already exist more effective. A good option to make a carrier more effective is the development of sensitive pH carriers. One of formats used is cap-

sules, which have a large number of applications, such as in genic therapy, by electrostatic interactions. One of the most studied polymers for this application is poly(ethylenimine), which has shown to be a viable option for polycation-DNA complex to treat Willebrand's disease. [2], [5], [6] [7] [8] [9]

The herpes simplex virus (HSV) belongs to the *herpesviridae* family. It is a very common human virus, causing a range of diseases, but most known from uncomplicated mucocutaneous infection. Recent advances in technology and knowledge have led to insights into disease pathogenesis and management. Nowadays, from a commercial point of view, there are a few methods of treatment available. The most common cure of herpes is using drugs, which can be topical, oral or intravenous. These treatments usually use acyclovir either in pill or ointment format, which is a synthetic acyclic purine-nucleoside analogue, and have helped to control symptoms.[10][11] [12][13], [14]

Mucous membrane pemphigoid (MMP) describes a heterogeneous group of chronic, inflammatory, mucous membrane-dominated, sub epithelial blistering diseases that manifest in several areas in the human body, namely in the oral region. MMP affects the mucous membranes, with or without skin. Although scarring is a usual symptom, this is not always obvious in the oral mucosa. One of the most common lesions in the general population derived from MMP is aphthous ulcers, with a frequency of 5 to 25% and three-month recurrence rates as high as 50%. Although non-lethal, this type of disease affects patient's quality of life, since it may lead to difficulty in swallowing, eating and/or speaking. While most of these afflictions are small and heal until 10 days, larger ulcers can last for weeks or even months. As a result, therapy for recurring ulcers should not only address the healing process but also its prevention.[15][16] [17]–[21]

Table 1.1-pH in various tissues and cellular compartments (adapt from various sources)

Tissue/cellular compartment	*pH*
Blood	7.35-7.45
Stomach	1.0-3.0
Duodenum	4.8-8.2
Colon	7.0-7.5
Early endosome	6.0-6.5
Late endosome	5.0-6.0
Lysosome	4.5-5.0
Golgi	6.4
Tumour, extracellular	6.5-7.2
Mouth	6.8-7.5

In the case of thrust problems, the major cause of oral and esophageal infection is *Candida albicans*. Its clinical significance, which is not life threatening but causes significant morbidity in patients, has increased with time. The usual treatment of candidiasis is with anti-

fungal, such as azole, but long-term usages causes the appearance of drug-resistant fungus or side effects. The most common drug for this type of illness is the active principle fluconazole, which is an antifungal derivate from triazole. Usually, it comes as pills of 50 mg, 100 mg, 150 mg or 200 mg of active principle. However, some fluconazole-resistant fungus have been found. Another problem is the toxicity of long-term treatment with this type of pharma's, especially in the liver, endocrine system and serum cholesterol.[22]

For patients with a complex clinical condition, such as HIV or cancer, a common option is mycostatin. The active principle is nystatin, which has a topical application (oral lozenge, oral tablet, oral suspension, compounding powder, oral capsule). The recommended dosage is between 1 to 6 ml, four times a day.[1][3]

Lactoferrin (LF) is an iron-binding glycoprotein present in exocrine secretions, as milk and saliva, as well in neutrophil granules. This protein has a number of biological functions, including antimicrobial and immunomodulatory effects *in vitro* and *in vivo*. It can inhibit the *in vitro* growth of *C. albicans*, not only in yeast form but also in hyphal form, which is important for pathogenesis of this fungus. It has also been reported that orally administered bovine LF improves survival rate of host or reduces the number of pathogenic viruses in tissues of animals with bacterial infection. There are a number of causes for this type of problems, such as:

- Trauma and stress;
- Systemic diseases and nutritional deficiencies;
- Food allergies;
- Infection;
- Genetic predisposition;
- Immune disorders;
- Drug induction [5], [23]

Trauma and stress are the most common factors in the appearance of ulcers or thrusts. Injury to the oral mucosa can result from self-biting, dental procedures, toothbrush bristles and sharp-edged foods. Emotional and environmental stress may justify 60% of first time aphthous ulcers cases and involve 21% of recurring episodes.[24][25][26], [27]

Systemic diseases involving immune and nutritional deficiencies have been associated with the development of oral problems. Nutritional deficiencies involving iron, folic acid, zinc and vitamins are twice as common in patients with MMP as in healthy persons, occurring in up to 20% of the patients. MMP has been linked with gastrointestinal problems, including Crohn's disease, ulcerative colitis and celiac disease.[24]

Food allergies, like cow's milk and wheat protein (celiac disease) have been present in patients with MMP. Strict elimination diets involving the specific food, to which the person is allergic, have resulted in resolution or improvement of the symptoms.[28]

Regarding genetic predisposition the evidence is not strong, but correlations relating the appearance and genetics or the effects of personality and stressors in the domestic and work environments can be made.[28][24]

MMP is more common in patients with immune disorders, such as cyclic neutropenia, inflammatory bowel disease, Behçet's disease and HIV. People that suffer from this type of illness have evidence of antibody dependent cytotoxicity and elevated serum immunoglobulins.[28]

Drug induction ulcers can be caused by antineoplastic medication, which can accelerate the detachment of oral epithelial cells. Burning and reddening of the oral mucosa appear within

hours of drug administration. Erosions and ulcers can occur in both keratinized and non-keratinized epithelium. Precautionary oral hygiene measures may help avoid superimposed infections. Patients who have “scalded mouth” present angiotensin-converting-enzyme inhibitors. [28]

Table 1.2-Antifungal agents used in the treatment of oral candidiasis (adapt from [29]**)**

Drug	Form	Dosage
Amphotericin B (Fugilin)	Lozenge,10 mg	Dissolved in the mouth 3/4 times a day after meals during 2 weeks.
	Oral suspension	Placed in the infected area after food 4 times a day for 2 weeks.
Nystain (Mycostain, Nystan)	Cream	Apply to the affected area 3/4 a day
	Pastille	Dissolve one pastille slowly after meals 4 times a day, usually for 7 days
	Oral suspension	Apply after meals 4 times a day, for 7 days, and continue use for several days after postclinical healing
Clotrimazole (Mycelex)	Cream	Apply to the affected area 2/3 times daily for 3–4 week
	Solution	5 ml 3/4 times daily for 2 weeks minimum
Miconazole (Daktarin)	Oral gel	Apply to the affected area 3/4 times daily
	Cream	Apply twice per day and continue for 10–14 days after the lesion heals
Ketoconazole (Nizoral)	Tablets	200 mg tablets taken once/twice daily with food for 2 week
Fluconazole (Sporanox)	Capsules	100 mg capsule once daily for 1–2 weeks
Itraconazole (Sporanox)	Capsules	100 mg capsules daily, taken immediately after meals for 2 weeks

The type of drugs available for mouth illnesses can be divided in three groups: pills, ointments and drops. Pills have the disadvantage of not being very specialized thus causing side effects, such as damages to the stomach. Ointments and drops are more specialized but have low yield and are uncomfortable to the patients.[1]–[3][29][30]

Different strategies have been studied for controlled oral delivery, such as mucoadhesives, enzymatic inhibitory and penetration enhancer proprieties and the design of novel formulations, which besides improving patient compliance favour an intimate and prolonged contact of the drug with the absorption mucosa. [31]–[33] [34], [35], [36]–[38]

PH sensitive drug delivery systems have been also applied in triggered drug release in cancer targeting, which in the case of the mouth, can be lip, tongue, cheek or throat. This type of treatment can work in two different ways, one in the extracellular tissue, since cancer cells have a slightly lower than the normal pH of 7.4 (table 1.1). Other method is through the targeting of lysosomes inside the cell. In this area one of the first reported works was performed by Hiroshi Maeda et. al., in which uses styrene and maleic acid conjugated with proteins were used to attack cancer cells as an alternative to chemotherapy. [39]

The most used carriers for endosomolytic delivery are the polyanions and amphoteric polymers. The works of S. C. W. Richardson et. al. have proven that poly(amidoamine)s can be used as endosomolytic vectors, since they have the potential to act as a synthetic alternative for funogenic peptides and thus to promote endosomal escape.

Ibuprofen (Ibu) and bovine albumin serum (BSA) are two models drugs widely used in drug delivery. These two molecules were chosen for impregnation and liberation studies due to their different characteristics, with especial emphasis on their size - while ibuprofen is a small drug (around 50 µm), BSA, being a protein, has a large size (about 150 µm). This will help to define the type of medication applied with each 3D structure.

Ibuprofen (Ibu)

Ibuprofen is one of the most widely used analgesic-anti-pyretic-anti-inflammatory drugs. It is probably one of the least toxic anti-inflammatory in the market, being rarely associated with deaths from accidental or deliberate ingestion or with serious adverse reaction.[40][41]Ibuprofen (R/S) is the most used drug at a commercial and research level. It has a daily dose up to 2,400 mg prescription and 1,200 mg for non-prescription cases. Its composition is half as the S(+) enantiomer, which is pharmacologically active as a prostaglandin (PG) synthesis inhibitor and the other half as R(-) ibuprofen which is less active as a PG synthesis inhibitor but which may have pharmacological properties relevant to the anti-inflammatory actions of ibuprofen. About 40–60% of the R(-) form of ibuprofen is metabolically converted to the S(+) form (Figure 1.1) in the intestinal and liver tract after oral absorption.[41]

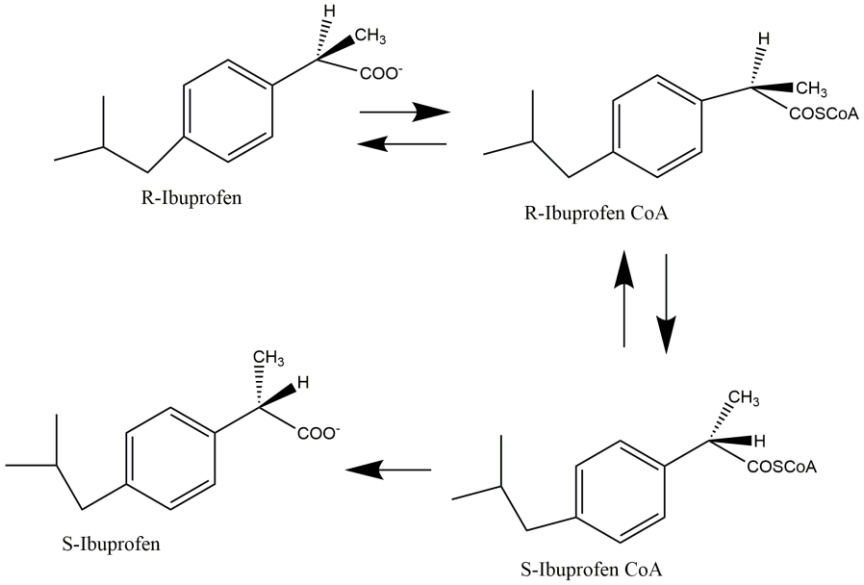

Figure 1.1- Conversion reaction of R-ibuprofen into S-ibuprofen

Bovine Serum Albumin (BSA)

Serum albumin (SA) is the most common plasma protein in mammals. It is synthesized in the liver and it is released in the plasma as a non-glycosylated protein, reaching a concentration of 0,6mM, while contributing to the colloid osmotic pressure. SA has a great capacity for binding ligands; it is a reservoir of the signalling agent nitric oxide and serves as a transporter for a diverse range of metabolites, drugs, nutrients, and other molecules. In mammals, SA, is the major circulatory protein involved in the handling of Ca^{2+} and Mg^{2+} controlling the ionized or "biologically active" levels of these metals in the blood. These properties give SAs a wide range of clinical, pharmaceutical, and biochemical applications.[42][43]

These proteins are relatively large (molecular weight of around 66kDa) and negatively charge proteins. SAs are heart-shaped and comprise three helical domains, each comprising two subdomains.[44]

Bovine serum albumin is a model protein in a number of studies. It is structurally well characterized, freely available in its native conformational state, suitable for immunodiagnostic procedures, cell culture media and clinical chemistry, and of great importance in food containing bovine milk or meat. BSA contain 583 amino acids of which one Cys and seventeen $(Cys)_2$ residues, which are important from a structural point of view.[45]

1.2. Polymers

In a controlled release system, the active agent in the structure is incorporated into a carrier, which is generally a polymeric material. The characteristics of the polymer is one of the main factors determining the release rate of the substance. The other element is the environment, such as body fluids and consequently their stimulus as, in the case of this work, pH.[46][47]–[49]

The significance of this type of systems relies on a uniform and continuous drug release in a fixed, predetermined pattern for a desired period. This should result in a uniform drug concentration over time, requiring smaller dosages, and causing fewer side effects. The application of polymers is not only limited to drug carrier systems for controlled release, but they can also be used as structures for bone screws and tissue engineering.[48], [49]

Conventional drug delivery products provide sharp increases in systemic drug concentrations that can easily reach potentially toxic levels, followed by a relatively short period at the therapeutic level after which, drug concentration drops until new administration occurs. Controlled delivery drugs arise when a system polymer/drug is designed to release the drug in a predetermined manner. One of the purposes of these controlled release structures is to attain delivery profiles that yield the therapeutic systemic concentration of the drug over a longer period, avoiding the large fluctuations in drug concentration and reducing the need for frequent administrations.

The type of the polymeric chain can define the kind of function the polymer will have. It may act as a bioactive (a polymeric drug) or as an inert structural component of a conjugate (a polymeric micelle or a non-viral vector). The categories of polymeric structures used in pharmacy are:

a) Polymeric drug (or sequestrant);
b) Polymer-protein conjugates (in which polymer-DNA-complex is included);
c) Polymer-drug conjugate;
d) Polymeric micelle.

The first two categories have been clinically tested, typically have a tripartite structure; and the polymer is a linker and is bioactive. However, the more elaborate multicomponent systems have additional features for cell-specific targeting, to regulate intracellular trafficking and nuclear localization, and to allow the incorporation of drug combinations. An example of a polymeric drug is poly(allylamine), which is a known polymeric sequestrant for oral administration. It is designed to bind phosphate, lowering the serum phosphorus and parathyroid hormone to treat end-stage renal failure patients, and it works as control in cholesterol absorption by binding complex bile acids. [50][51]

In contrast, polymeric micelles bind to the drug non-covalently. They have been studied as a pluronic block copolymer and incorporates doxorubicin, such as the polymer-drug-conjugate. They are also able to circumvent p-glycoprotein-mediated resistance as it was shown in the works of Valery Alakhov and her team.[50], [52]

Modern chemistry has been producing more complex polymer structures, as multivalent polymers, branched polymers, graft polymers, dendronized polymers, block copolymers, stars

and hybrid glycol- and peptide derivatives. Their potential advantages will lead to the development of polymers therapeutics in the future. Within these advantages there are:

1. More defined chemical composition;
2. Tailored surface multivalency;
3. Creation of defined three-dimensional architecture within either a synthetic water-soluble macromolecule or by the creation of a new supramolecular system.[50]

The norm in pharmaceutical compounds is to manufacture homogeneous drug substances, which are composed of a single defined species. By contrast, polymers are heterogeneous and can present special challenges for characterization.

New synthetic methods and dendrimer chemistry are moving towards the production of synthetic macromolecules that, as proteins, are monodisperse. However, depending on the mechanism of polymerization, some synthetic polymers have advantage of very narrow polydispersity.

Synthetic polymers have not been the only ones used in the pharmaceutical field. Controlled drug delivery products, using natural, biocompatible or biodegradable polymers, have got more attention in the last years. The use of these compounds as drug delivery systems have the advantage of possible control of the drug release rate and consequently a more controlled therapeutic action. Other reason for the application of natural polymers is commercialize plant based ones in pharmaceutical, due to a wide range of material, properties and molecular weight. Because of this reasons, natural polymers have also been used particularly in drug delivery systems.[53], [54]

One of the natural polymers used to that end are natural gums. They can be used as they are, or be modified to meet the necessary requirements for drug delivery. They have been applied in sustained release tablets and as drug retarder. In this work, three different polymers were studied and investigated: chitosan, xanthan gum and polymer m. [54][55][53]

Chitosan (CHT)

Chitosan is a linear polysaccharide composed of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamide (acetylated unit). It is made by deacetylation of chitin, which is an element in the exoskeleton of crustaceans. Because of its unique chemical and biologic properties, CHT is used in biomedical and pharmaceutical applications. It is a cationic polysaccharide ($pKa \approx 6.3$) soluble at acidic pH, and bioadhesive, which increases retention at the site of application and readily binds to negatively charged surfaces. Biodegradability and biocompatibility of CHT has been exploited for use in controlled drug delivery systems.[56]

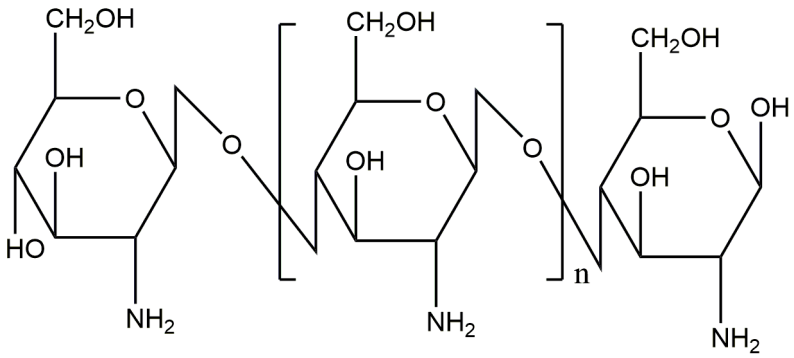

Figure 1.2- Chitosan molecule

CHT has been studied for a large number of biomedical and pharmaceutical applications, such has tissues engineering, bone graft and for the conjugation with DNA and other types of molecules. It is also an alternative for drug delivery system such as the hybrid CHT collagen scaffolds sensible to pH and temperature variations.[57][58]

Chitosan has several applications as an oral matrix system, for immobilization and encapsulation of drugs, since it is a mucoadhesive material and can be used as an adsorption promoter. Furthermore, CHT interacts with mucus and mucosal surfaces, because it interacts with the charged sugar groups, and therefore can be used as a bioadhesive material. This feature has been used to full extent in the development of gastro retentive bioadhesive microsphere formulations as well as an agent in nasal delivery and other mucosal routes.[59][60][61][33][62] [63] [64] [59] [63]

CHT chains length can be controlled by acid hydrolysis or the enzymatic degradation. The N-acetylD-glucosamine and D-glucosamine groups are randomly or block distributed throughout the polymeric chain. The OH and NH_2 groups can be used in the bonding process, to drugs or other polymers. Moreover, it provides a strong electrostatic interaction with sialic acid groups of mucus, due to the cationic polyelectrolyte nature of chitosan, or with the negatively charged epithelial surfaces. The properties which are considered essential for mucoadhesion are the CHT chain flexibility, due to the linearity of the molecule, and also the fact that its conformation being highly dependent on ionic solubilisation vehicle. The mucoadhesive properties as well as the importance of the adsorption enhancing ability of chitosan have been previously demonstrated. Furthermore, some human enzymes, such as lysozyme, metabolize it, being considered as a biodegradable polymer. Chitosan-based drug delivery systems have been widely investigated for pharmaceutical applications because it is a natural polysaccharide, it is water soluble when is positively charged, and may be conjugated with negatively charged polymers, macromolecules and polyanions in an aqueous environment. [65]–[68] [63][69]–[71] [60]

Xanthan Gum (XG)

Another extensively investigated polymer is xanthan gum, this natural compound is a water soluble heteropolysaccharide. It has a high molecular weight and is an anionic polymer, it is produced by fermentation of a pure culture bacterium *Xanthomonas compestris* and its bigger use is in the food industry as a viscosity controller due to its high viscosity in aqueous media. XG has received attention in the medical field as it has been found to retard drug release. In addition, natural gums are being used in pharmaceutical applications as diluents, binders, disin-

tegrates in tables (which is a feature useful in the case of this thesis), thickeners in oral liquids, protective colloids in suspensions, gelling agent in gels and in suppository. Other widely found applications of natural gums are in cosmetics, textiles, paints and papermaking.[72]–[74]

The most novel concept application of XG is as a base for pharmaceutical gel. It can be used either alone or in combinations with other gums. Gelling is a result of inter and intra molecular links within polymer chains to manufacture three- dimensional structures, like in scaffolds and membranes, within which the water molecules are entrapped. The gelation mechanism of XG is different, since the macromolecular chains are hydrogen bonded and, consequently, junction zones are formed between hydrogen bonded segments of chains. The same principle can be apply to scaffolds, making XG and its mixtures with other polymers elastic.[55][75], [76]

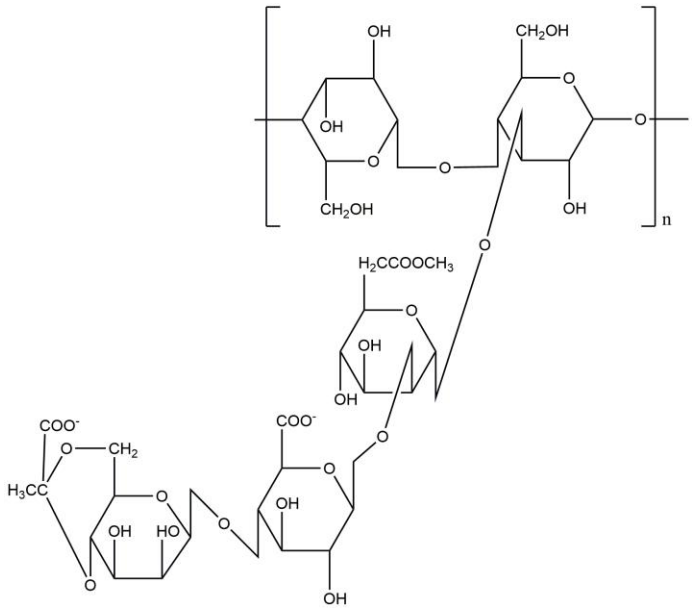

Figure 1.3- Xanthan gum molecule

Besides having a large range of applications in the food industry, it has been studied as drug delivery system, especially as a hydrogel. It is also one of the main components of the TIMERx^{TM}, which is a system for oral drug delivery. It is able to encapsulate soluble and insoluble drugs, and it is flexible for a variety of controlled release profiles depending on the quantity of XG and the other main component of the formulation. [77][78], [79]

Polymer M (PM)

Polymer M is a water-soluble polymer based on a hydrolysed acrylamide developed by GEO. In general, polyacrylamides are formed by cross-linked acrylamide monomers. Drug-loaded polyacrylamides have been used in *in vitro* and *in vivo* studies with the objective of producing super porous hydrogel by using acrylamide based matrices [80], [81], despite its toxicity to humans. Polyacrylamides have been applied as a molecular lysozyme imprinting system, and as a hydrogel scaffold for cartilage in tissue engineering.[82], [83]

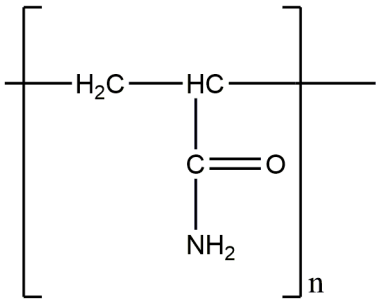

Figure 1.4- Acrylamide monomer

In this thesis, polyacrylamide-based polymers were developed and used to prepare scaffolds. These three-dimensional structures are hydrophilic crosslinked polymeric networks and are capable of retaining water within their matrices and swell without dissolving.

The formation of scaffolds depend on the polymer crosslinking degree, which can be done via the establishment of covalent, ionic, or hydrogen bonds. Lately, a lot of work has been done about synthesis and characterization of pH- and temperature-sensitive structures by co-polymerization and crosslinking. Interpenetrating polymer networks (IPNs) have been gaining a lot of focus lately, since they have a wide range of application such as implants, dialysis membranes and wound dressings. This suggests an enormous potential as drug delivery systems [84][56]. One of the options considered was the use of poly(acrylic acid-co-acrylamide)/O-carboxymethyl chitosan as a hydrogel for effective peroral delivery of peptide and protein drugs. In addition, it can be used as wound dressing, using an antibacterial drug or epidermal growth factor. Other main application of IPNs is tissue engineering.[85][86]

The definition of IPNs is essentially the combination of two or more polymers, each in a network form, at least one of which is synthesized and/or crosslinked in the immediate presence of the other.

There have been an interest in developing controlled drug delivery systems using matrix of natural and synthetic polymers due to the combination of the characteristics of the two types of polymers, because while the natural polymers are non-toxic, biodegradable and biocompatible, the synthetic polymers have the best mechanical proprieties.[56]

1.3. Polymers Processing

In this thesis, two different processing techniques to produce polymeric porous scaffolds were investigated: (i) freeze drying method and (ii) phase inversion followed by supercritical CO_2 drying method.

1.3.1. Freeze Drying Method

This method has been widely used in the preparation of porous materials for biological applications, tissue engineering and pharmaceutical applications with the aim of improving

drugs stability [87]. Different types of porous materials have been successfully prepared by freeze-drying. At a scientific research level, one of the most common structures obtained by lyophilisation are drug delivery systems, such as poly(N-isopropylacrylamide) and N-succinyl chitosan grafted polyacrylamide hydrogel for oral insulin delivery. This technique is a dehydration process, which enables liquid compounds, which have previously been frozen, to be dried under vacuum. [88], [89]

The freeze-drying method consists of three stages:

1. Freezing;
2. Primary drying; and
3. Secondary drying.

The freezing step is realized by contacting a liquid sample with or placing it in a cold bath. The frozen sample is then placed in a freeze dryer to remove the frozen solvent by sublimation. During the freeze-drying process, the polymer has to be kept below its glass transition temperature, so the solvent can be removed under vacuum. Porous scaffolds are shaped by the removal of the solvent. Thus, the frozen solvent acts as porogen to produce porous materials[90].

The freeze-drying process can provide certain advantages, since other methods require large amounts of organic solvents and lengthy washing or etching procedure. The use of this technique can be applied in aqueous solutions, which are used to prepare porous materials. Water is an environment-friendly solvent and the use of ice crystals as porogens is green and sustainable. This is particularly beneficial for biological applications. Another advantage of this process is the ability to provide free-impurity samples, with no solvent residues, which make the purification process not necessary. It is also possible to produce materials with a variety of pore morphologies and nanostructures by freeze-drying only by changing process conditions. The pore sizes can be influenced by freezing temperature, pressure and the composition of the casting solution. Decreasing the temperature, smaller pores are obtained; the usual temperature scale used in this process varies between -70^{o}C and -10^{o}C, which may lead to pore sizes of 0.6 µm and 350 µm, respectively. These materials can be used in a wide range of applications such as bioengineering, drug delivery, catalyst support and separation. These sponge type hydrogels also have been used as wound dressings being widely implemented in pharmaceutical and food industries [87][55], [91] [92] [55], [93].

Ones of the most common scaffolds obtained by freeze-drying are chitosan-based. It has been showed that chitosan network properties are dependent of the drying process. This method also present some disadvantages, especially when working with proteins. The process generates a number of freezing and drying stresses, such as, solute concentration, formation of ice crystals, pH changes, etc. All of these stresses can denaturate the proteins, which may be prevented using specific stabilizers to protect the protein during the freeze drying process.[94][95], [96] .[91][97] Also, even if the lyophilisation process is successful, the long-term storage stability of the proteins may be limited especially at high storage temperatures. In a number of cases, protein stability in solid state has been worse than in the liquid state, depending on the storage conditions and the composition of the formulation.[90][98], [99]

1.3.2. Supercritical CO_2

Other approaches to process polymers is supercritical CO_2 ($scCO_2$) (Tc=31ºC, Pc=73.8 bar) assisted methods, which have been the focus of bioengineering research especially for the production of porous scaffolds with controlled pore structure. CO_2 can be easily removed without leaving any residues. $ScCO_2$ presents liquid-like densities and gas-like viscosities and diffusitives, which are idyllic for penetrating porous structures, becoming this method very attractive for biomedical purposes. This procedure may be optimized by adjusting and controlling a number of parameters (such as pressure, temperature, flow and depressurization rate) to provide a wide range of morphological characteristics. [100]

CO_2 is a great option in the pharmaceutical field since it is inexpensive, non-toxic, non-flammable and is available in high purity from different sources [101]. $ScCO_2$ has been used as a solvent, anti-solvent and plasticizer in synthesis, modification or purification of polymers and in processing, respectively. The solubility of the polymers in $scCO_2$ and vice-versa are fundamental to obtain the desired structures, which have been advantageous in pharmaceutical area since the drugs and the used polymers present low solubility in $scCO_2$. However, when considering the CO_2 solubility in the polymers the reverse happens, CO_2 is very often soluble in the polymer matrix which leads to the decrease of glass transition (T_g) or melting temperatures (T_m) of the polymers used, thus reducing the polymers melt viscosity[31], [32], [34], [37], [102]–[106].

Although CO_2 is a moderate solvent for most non-polar molecules and some polar, it is also a good solvent for low molecular weight molecules. One of low molecular weight chemicals is Ibu, which is soluble at $scCO_2$. The polarity can be a factor for the limitation of solubility in CO_2, since it has a quadrupole moment and no permanent dipole. Furthermore, CO_2 can act as both a Lewis acid or basis.[107]–[109]

CO_2 presents high solubility in many polymers. This often limited the drug used in the impregnation process. In addition, it is limited by the mutual mass transport of SF into solvent and the transport of the solvent into the SF. [110]

Due to that versatility of $scCO_2$ technology, polymers can be foamed as a plain material, in the form of blends and as composites with the addition of inorganic particles. The addition of compressed CO_2 to the polymeric casting solution can have changes in its properties, including viscosity, permeability, interfacial tension and glass transition temperature.[100], [111]

When the process ends, the temperature is constant, the pressure is reduced, the CO_2 phase changes from supercritical to gas and nucleation and growth of gas bubbles occurs in the rubbery polymer generating pores. At the same time, the polymer T_g increases as a result of plasticizer concentration reduction and the porous structure is fixed in the glassy state. During the last stage it is needed a precise control of depressurization in order to control foam three dimensional structures.[111][110]

1.4. Impregnation

The use of $scCO_2$ can also be extended to impregnation of active molecules into porous structures. Under supercritical fluid conditions, the impregnation procedure allows more rapid and homogeneous structures than in normal liquid media. Its advantages for the development of drug impregnated polymeric materials for many biomedical applications have been reported. In

the preparation of controlled-release, this method can also act as a selling and plasticizer agent for polymers, dilating the matrices and helping drug diffusion into them. Furthermore, drug loading and depth penetration can be easily controlled and drugs will be homogeneously dispersed, in short treatment times and leaving no harmful solvent residues.[103][106], [110]

It has been used as poly(methacrylate-co-methacrylic acid) membranes for protein permeation, and poly(N-isopropylacrylamide) coated chitosan scaffolds for a controlled drug delivery system.[112][113]

There are some techniques of drug impregnation; this study will be focused on bulk loading and in supercritical CO_2 impregnation. The bulk impregnation occurs when the polymeric structure is formed trapping the drug inside the system, usually can be through mixing the drug into the polymer mixture, which can contain monomers, co-monomers, initiators, etc.[114][56] Also, the drugs can be either soluble or insoluble. The other option using this method, and the one used in this case, the drug is added to an already synthesized polymer or polymer mixture. It is more common in organic solutions or dispersions, it mixed depends on the polymer and drug solubility.[115] It can have same disadvantages, the drug can be heterogeneously, and it may react as a crosslinker with the polymeric structure. Other disadvantage of this method is the lack of control of the drug integrity during the process.[56]

Alternatively, it is possible to apply $scCO_2$, which can be swell the polymer matrix and serve as a carrier for the drug component. However, it has been shown that the supercritical fluid impregnation results in ibuprofen being molecularly dispersed in a polymer matrix where all drug molecules are H-bonded to the polymer and without the presence of ibuprofen crystals.[109]

In addition, the sustained release in which the drug is release over a period of time in a controlled fashion has also been revealed as a milestone of this type of technology. The polymers above were chosen not only because they are pH sensitive but also because 3D porous structure can be produced and ideally encapsulate a number of drugs. Their porosity and textural properties have fuelled their use as drug delivery systems and their chemical composition; make them good candidates to a candy or gum delivery system. This material can be employed to build scaffolds for controlled drug delivery systems for the treatment for a various number of diseases, but this thesis will concentrate on mouth infections.[57], [64], [116][55][117]

1.5. Thesis organizational outline

The objective of this work comprises the development of two distinguished porous structures (gum and candy formats) combining natural and synthetic polymers, in order to be evaluated as possible oral medical devices to treat muco-oral diseases.

For gum devices the requirements are: pore size diameter of 150 μm, a compression modulus lower than 5kPa, pH sensitive and must release 50 mg drug per gram of support in 5h time.

For candy devices the requirements are: pore size diameter of 50 μm, a compression modulus higher than 5 kPa, pH sensitive and must release 40 mg drug per gram of support in 2.5 h time. These set-points were defined according to drug delivery literature.[118] The following chapters will divide this thesis:

1. Introduction

This chapter was divided in four main subchapters. First, it is discussed the pharmaceutical field and the state of the art concerning the development of drug delivery devices for treatment of muco-oral diseases, both in a commercial and scientific light. The second subchapter was focused in the usage of polymers in drug delivery systems and the ones used in this work. The third discussed the two methods used in polymers processing. Finally, the last subchapter spoke about drug impregnation.

2. Materials and Methods

The materials and methods focus in the material used in the laboratory work, as well as the protocols applied in the polymers processing, characterization and drug delivery.

3. Results and Discussion

This chapter was divided in three main sections. The first discuss the casting solutions produced as well as the methods utilized in the scaffolds production. The second talks about the scaffolds characterization, which discuss the results obtained by SEM, FTIR-ATR, swelling, mechanical analysis, structures degradation, zeta potential and PM biocompatibility. Finally, the last section focus in drug release behavior and its mathematical modulation.

4. Conclusion

In the conclusion a critical evaluation of the work is included, highlighting the structures that are better suited for oral drug delivery application, as well as the envisaged future work.

5. Appendix

The appendix has all the additional information of this study that was not included in the main text. It was divided in scaffolds production, FTIR-ATR, swelling analysis, and drug delivery and modulation.

2. Materials and Methods

2.1. Materials

For the casting solutions, acetic acid (AA, purity ≥ 99%, from ROTH); ammonium persulfate (APS, purity ≥ 98%, Xilong Chemical Co., Ltd.), medium molecular weight CHT (from Sigma Aldrich), N, N'-methylene-bis-acrylamide (MBA, purity ≥ 98%, from Xilong Chemical Co.), PM (30% hydrate polyacrylamide, purity ≥ 99%, from GEOSoil), tetramethylenediamine (TEMED, purity ≥ 99%, from Merk) and XG (purity ≥ 98%, from Sobeltec) were used. The active principles used in the solutions were Ibuprofen (IBU, purity ≥ 99%,from Alfa Aesar) and BSA (purity ≥98%, from Sigma Aldrich). For the swelling, degradation and release studies, citric acid (CA, purity ≥ 99%, from Merk), glycine (Gly, purity, 99%, from Xilong Chemical, Co., Ltd.), trisodium citrate-dihydrate (SC, purity ≥ 99.9%, from VWR Chemicals), sodium hydroxide (SH, purity ≥ 98%, from Xilong Chemical, Co., Ltd.), sodium phosphate monobasic (SPM, purity ≥ 99%), sodium phosphate dibasic (SPD, purity ≥ 99.9%) both from VWR Chemicals and chicken egg white lysozyme (Lys, purity ≥ 90%, from Alfa Aesar) were used.

2.2. Methods

2.2.1. Casting solutions preparation

All polymeric casting solutions were prepared using different polymers: chitosan, xanthan gum and polymer M.

2.2.1.1 Without Crosslinker

Different solutions of chitosan, xanthan gum and polymer M were prepared with concentrations of 1, 2 and 3% (w/v). Xanthan gum and polymer M were dissolved in distilled water at room temperature while chitosan was dissolved in acidic water (1% v/v of acetic acid) at 80 ºC. After polymers solubilisation, samples containing 2 mL of each solution were placed in tubes (with 1.3 cm diameter and 4 cm height) for further freezing and lyophilisation.

2.2.1.2. With crosslinker

N,N'-methylenebis(acrylamide) (MBA) was used as crosslinker. The polymeric casting solutions were prepared as previously mentioned, however with an additional step. After polymers solubilisation, 2% wt/wt of MBA was added. After MBA solubilisation

(around 3h), the solution was immediately placed in tubes of 2 ml (with 1.3 cm diameter and 4 cm height) for further freezing and lyophilisation. Half of the prepared tubes were heated until 50 ºC under stirring during 24 h previously to the freezing procedure.

2.2.1.3. With initiator and catalyst

Casting solutions containing initiator and catalyst were also prepared following the same method described in section 3.2.1.2. but with an additional step: after placing the solution in tubes of 2 mL (1.3 cm diameter and 4 cm height) solutions of TEMED (23 µl) and APS (40 µl), initiator and catalyst, respectively, were added in order to promote the crosslinking process that occurred at 0ºC during 30 min under stirring in a shaker at 150 rpm. Then, the samples were freezed and lyophilized.

2.2.1.4 Preparation of polymer blends

Aqueous solutions containing polymers with a composition of 50:50 wt/wt in a total volume of 100 mL (casting solutions) were prepared. The mixtures were: (i) polymer M and xanthan gum 2 %wt/wt, (ii)chitosan and xanthan gum 2%wt/wt and, (iii) chitosan and polymer M 2% wt/wt.

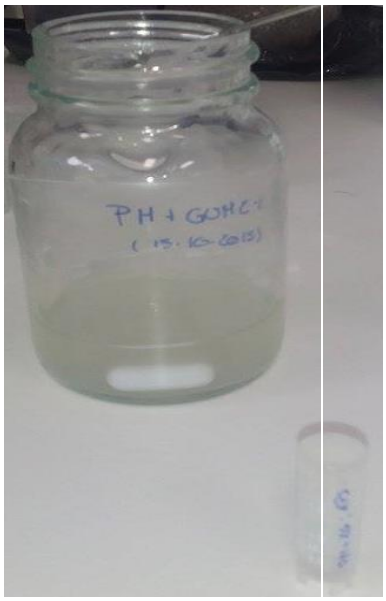

Figure 2.1-Casting solution

Figure 2.2-Lyophilizer

Figure 2.3-Flasks with the 2 ml samples

2.2.2.Scaffolds prepared by freeze drying

The casting solutions were frozen at -20 ºC during 12 h in a freezer. After 12 h, the frozen casting solutions were freeze-drying during 48 h at -50 ºC and 0.70 mPa in a Telstar cryodos-50.

2.2.3. Scaffolds by $scCO_2$-assisted cryogelation

The preparation of the casting solutions was similar to the lyophilisation method. Once prepared, the casting solutions were placed into steel containers having 1.6 cm of internal diameter and 1.3 cm of height, and then frozen during 24 h at -20 ºC to obtain the hydrogel through the gelation

process. Then, the hydrogels were removed from the vessels and immersed in acetone at -20 ºC during 48 h for water-acetone replacement, and finally dried using $scCO_2$.

Firstly, steel containers were loaded with the hydrogels, then they were introduced in the high pressure cell, which was closed and introduced in a thermostatized bath. After, the high pressure cell was filled with CO_2 until the desire pressure was achieved at fixed flow rate. Finally, after reaching the operational pressure, the supercritical stream passes through a back pressure regulator which separates the CO_2 from the acetone. These experiments were performed at 150 bar with a CO_2 flow rate of 10 g/min during 4 h. Finally, the system was depressurized during 10 min and dried porous scaffolds were obtained.

Figure 2.4-$ScCO_2$ apparatus; (1) CO2 bottle, (2) cryostat, (3) pump, (4) back pressure valve, (5) thermostatized bath

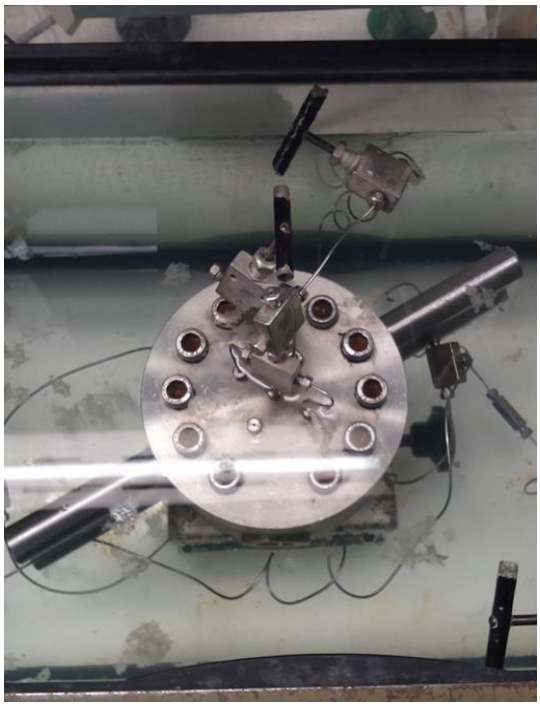

Figure 2.5--High pressure cell

$ScCO_2$ is a continuous process. It starts (Figure 2.4) when the CO_2 leaves the bottle (1) and goes through the line into the cryostat (2). Inside the cryostat the CO_2 temperature drops from the ambient temperature to -15 ºC, becoming liquid. Then leaves the cryostat to the line and enters the pump (3). The pump sets up the operation CO_2 rate. The liquid CO_2 goes from the pump to the pressure cell (5). The pressure cell is maintained above the CO_2 critical conditions with a constant bath temperature. The pressure cell is filled with CO_2, starting the process. Finally, the CO_2 that exits the pressure cell is controlled by the backpressure (4).

2.2.4. Scanning electron microscopy (SEM)

The morphology of scaffolds was investigated using SEM in Hitachi S-2400 equipment, with an accelerating voltage set to 15 kV. Scaffold samples were frozen and fractured in liquid nitrogen for cross-section analysis. All samples were gold coated before analysis.

2.2.5. FTIR-ATR analysis

The ATR accessory (from Bruker) contained a platinum crystal at a nominal incident angle of 45º, yielding about 12 internal reflections at the sample surface. All spectra (100 scans at 4.0 cm^{-1} resolution and rationed to the appropriate background spectra) were recorded at approximately 25 ºC. The samples weight was about 0.02 g.

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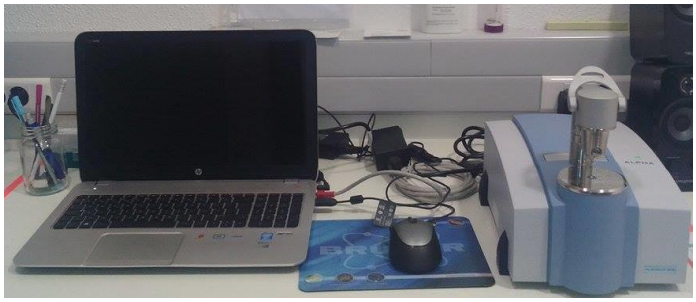

Figure 2.6- FTIR-ATR equipment and computer

2.2.6. Swelling tests

In order to study the swelling behaviour, the scaffold samples (approximately 0.06 g) were immersed in three different swelling solutions: citrate buffer solution (0.1 M) at pH 5.0, phosphate buffer solution (0.1M) at pH 7.4 and glycine-sodium hydroxide buffer solution (0.1M) at pH 9.0. The samples were placed in the swelling solution (about 100 mL) and the weight of the swollen samples was measured against time. Periodically, the samples were removed from the swelling medium, whipped to remove excessive water of the surface and weighted. This procedure was repeated during 24 h. The tests were performed on a shaker (from Biobase) with a rotation of 150 rpm. Equilibrium hydration or swelling degree (W (%)) of the samples was determined as defined by Eq 1:

$$W(\%) = \left(\frac{W_w - W_d}{W_d}\right) \times 100$$

Equation 1- Swelling degree

Where W_w is the weight of sample with water and W_d is the weight of dry sample.

2.2.7. Mechanical analysis

Uniaxial compression was used to determine the mechanical proprieties of the scaffolds using a tensile equipment (MINIMAT firmware v3.1) at room temperature. The compression strength was measured using a mechanical tester at a crosshead speed of 1 mm/min, a full scale load of 20 N and a maximum extension of 90 mm. The samples were cylindrical in shape, with dimensions about: 15 mm in height and 5 mm in diameter. During compression test, the compression modulus was calculated from the slope of the linear portion of the stress-strain curve.

Equation 2

$$Stress = \sigma = \frac{F}{A}$$

Equation 3

$$Strain = \varepsilon = \frac{\Delta L}{L}$$

Where F is the applied force, A the cross-sectional area, ΔL is the change in length and L is the length between clamps.

Figure 2.7- Mechanical tester and scaffold

2.2.8. Zeta potential tests

The electrical charge measurements of particles were performed using a JS94H Microelectrophores apparatus and JS94H-en program at room temperature, with reproducibility being checked by performing three repeated measurements. The polymer solutions were diluted at pH 5.5 and 7.4. The polymer solutions tested were: (i) PM, (ii) CHT (both of 0.5 % (wt.)), (iii) a mixture of PM and XG and (iv) a mixture of CHT and XG (both of 2% (wt.)). To perform the measurements, approximately 700 μL of each solution was loaded into the measurement cell.

2.2.9. Degradation studies

The degradation kinetics of scaffolds (around 20 mg) was followed in a 100 mL phosphate-buffer solution (0.1, PBS, pH 7.4) at 37ºC. The concentration of lysozyme must be selected considering the concentration of human serum (2 μg/ml).

The initial weight of each sample was determined (Wi). After specific periods of time of 72 h, samples were collected from the medium, washed with distilled water, freeze-dried and

weighted. The lysozyme solution was refreshed weakly to ensure continuous enzyme activity. The degradation study lasted for 120 days. The remaining weight allowed the calculation of weight percentage of degradation using the following equation:

$$W(\%) = 100 - \left(\frac{W_i - W_f}{W_i} \times 100\right)$$

Equation 4- Remaining weight

Where W_f is is the dry weight of sample after degradation and W_i is the initial dry weight of sample.

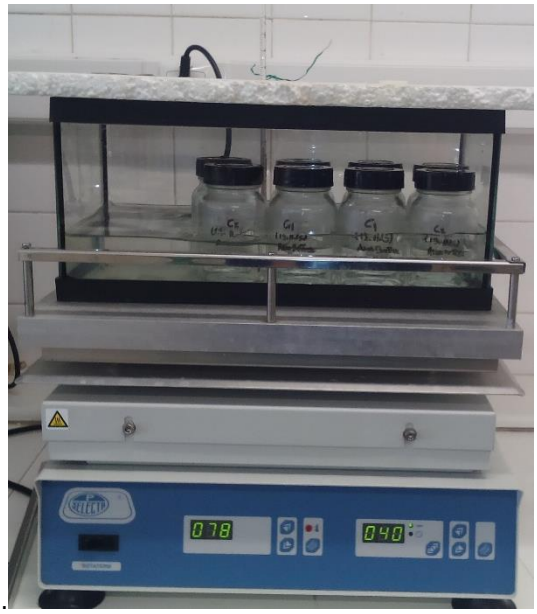

Figure 2.8- Degradation and Release apparatus

2.2.10. Biocompatibility

Human dermal fibroblasts were seeded in 96 well-plates at a density of 5,000 cells per well. The medium used was Dulbecco's modified media (DMEM) supplemented with 10 % wt/wt FBS and 0.5 % wt/wt antibiotics. The cells were incubated overnight in a 5 % wt/wt CO_2 humidified incubator at 37 °C. On the next day, the medium was changed to fresh medium containing different masses of the scaffolds: 5 %wt/wt, 9 %wt/wt, 20 %wt/wt, 33 %wt/wt, 50 %wt/wt, 70 %wt/wt and 80 %wt/wt. The cells were incubated for 24 hours. Cells incubated with medium without polymer were used as controls. Triplicates were performed for each condition. After 24 hours, the viability of the cells was measured by an ATP assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega). This assay quantifies metabolically active cells. The data was normalized by the control. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Bonferroni post-tests using GraphPad Prism 5.00 software.

2.2.11. Release studies

Small portions of the selected scaffolds (around 20 mg) were introduced inside a 100 mL of buffer solutions (0.1M) at different pH (5.5 or 7.4) and at 37 ºC. 1mL aliquots were withdraw periodically from the solutions and collected in Eppendorf's.

The release medium was refreshed with buffer solution (1 mL) after sampling.

In order to determine the ibuprofen (Ibu) and BSA released, the samples were analysed in a Helius Alpha Double-Beam UV/VIS spectrophotometer at 265 and 280 nm[119], respectively. It was performed the calibration curves of Ibu and BSA to the different buffer solutions. For Ibu at pH 5.5 the quantities used were from 9% (wt.) to 1.5% (wt.), due to its low solubility at acidic pH. At pH 7.4, the Ibu masses used were from 28 %(wt.) to 5 %(wt.). To the BSA solutions, the quantities used were from 50%(wt.) to 35%(wt.).

There were also applied two mathematical modulation method: i) Higushi and ii) Power law, [120], [121]which the equations are respectively:

Equation 5- Higushi model

$$\frac{M_t}{M_\infty} = K\sqrt{t}$$

Equation 6- Power law

$$\frac{M_t}{M_\infty} = kt^n$$

Where, M_t and M_∞ are the cumulative absolute amounts of drug released at time t and at infinite time, respectively; k is a constant incorporating characteristics of the models, and n is the release exponent, which might be indicative of the mechanism of drug release.

These two models were chosen in order to understand the releases mechanisms. The Higushi model evaluate the diffusive release mechanism, while the power law relates the drug transport with the swelling and drug diffusion.

3. Results and Discussion

The viable options to alternative drug release systems in the buccal area have been extensively studied. The focus of this work consisted in the development of two types of structures (gum and candy) for oral controlled drug delivery. [21], [23]–[25]

The gum and candy structures were designed for different applications and thus specific morphological and physicochemical properties are envisaged. For instances, both constructs ideally should be pH dependent since a diseased mouth exhibits acidic pH and an healthy one has a neutral pH. In particular, faster drug release should occur at the acidic pH. In terms of degradability, morphology and mechanical properties, the gum scaffold should have porous size around 100-150 μm, with low compression modulus (< 5 kPa) and slow degradation, while, the candy option should have a rigid and degradable structure. Therefore, the set-points defined for the structures under development were: a pore diameter around 50-150 μm, a compression modulus bellow 5 kPa for the gum and abouve to the candy, and swelling rate till 30% for the gum and higer than 30% for the candy. The scaffold should release 50mg of drug per gram of structure in 5h. These set-points were defined according to drug delivery literature[118].

3.1. Scaffolds production

The scaffolds for drug delivery applications were produced using two distinct methods: freeze-drying and $scCO_2$-assisted criogelation. This was performed in an attempt to obtain larger pores, since the aim was to obtain two vastly different porous range according to the shape of the system under study. In detail, for the gum should be around 100-150 μm, and for the candy should be 50-75 μm. The porous structures obtained by both methods were influenced by the conditions employed during each process. This will make it possible to know which process is allow to create a porous structure which fill the requirements for the objective of this thesis.[118]

Different casting solutions were prepared with the selected polymers i) chitosan, ii) xanthan gum and iii) polymer m. The first approach consisted in the preparation of native casting solutions: i) chitosan (CHT), ii) xanthan gum (XG) and iii) polymer m (PM) with different concentrations a) 1% wt/wt, b) 2% wt/wt and 3% wt/wt. CHT was chosen since it has been widely studied as a pH sensitive drug delivery system, namely for oral delivery, such as

mucoadhesives.[59] XG has also been studied considering biomedical applications and as a food addictive. PM was chosen because GEO company pretended to investigate its application in the biomedical area. All the polymers are pH sensitive, biodegradable and biocompatible. [31], [33], [53], [54], [75], [78]

The first method applied to generate porous scaffolds was freeze-drying method. Freeze-drying structures are influenced by a number of factors. They can be influenced by i) the polymer and the solvent concentrations, ii) the polymer nature, iii) the freezing temperature of the scaffold and iv) freeze direction. This method generates open pores microstructures with a high degree of interconnecting pores in the final structure. Herein the conditions used were: freeze at -20^{o}C, during 12h and then the structures were freeze-dried during 48h.

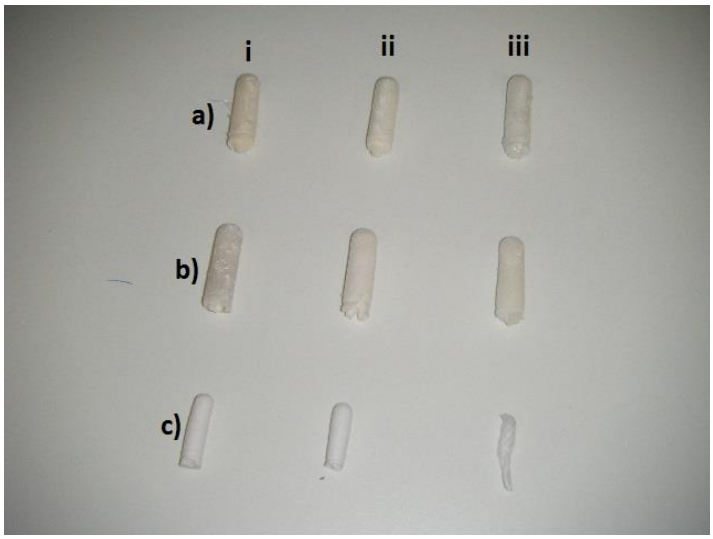

Figure 3.1- Freeze-dry scaffolds; a) CHT scaffolds; b) XG scaffolds and c) PM scaffolds. i) 3% wt/wt polymer; ii) 2% wt/wt polymer and iii) 1% wt/wt polymer

The native scaffolds (Figure 3.1) of CHT (a)) and XG (b)) did not present visually macrostructures difference. Nevertheless, CHT is more regular and homogeneous, while XG was soft. However, the macrostructure of PM 1% wt/wt (c); iii)) presented a very soft and fragile structure.

The next step was the addition of a crosslinker (MBA). This step aimed to improve the structures stability, since native polymer scaffolds tend to be unstable. It is especially notorious for XG scaffolds, which revealed a high tendency for disintegration in water without crosslinker, as well as weak mechanical properties. It was also added TEMED and APS as initiator and catalyst, with the aim of obtain structures with better physical and chemical condition to drug delivery.

All structures prepared with MBA are presented in Figure 6.1, appendix. CHT (a)) kept its macrostructure, while XG (b)) became fragile. PM (c)) scaffolds presented a collapse of the porous network. Regarding the structures prepared with TEMED and APS (Figure 6.2, appendix) the changes were more notorious, CHT (a)) presented a visible loss of formation, due to the effect of plasticizer of the initiator. XG (b)) revealed a huge improvement in its macrostructures, since it showed a ductile and stable behaviour. PM (c)) scaffolds became more stable since they did not collapse; however, the structural integrity became compromised when compared with the native ones. In order to keep improving the chemical and physical stability of porous

structures, the next step was the preparation of casting solutions mixing polymers. The concentration used in the polymers mixtures was 2% wt/wt since it was the concentration that for native scaffolds revealed better scaffolds stability. The casting solutions were prepared mixing xanthan gum and polymer m (XGPM) and, xanthan gum and chitosan (XGCHT). The addition of XG to the PM casting solution improved the integrity of the resulted structure (Figure 6.3 a), appendix), while the addition of XG into CHT casting solution allowed to develop a more ductile structure. Thus, the results suggest that, the addition of MBA allowed to create dense structures, while the addition of TEMED and APS showed the opposite.

This process was used to produce most of the scaffolds, while the $scCO_2$ assisted method and the drug impregnation was used only for the ones that exhibited the best characteristics by freeze drying method.

Summing up, freeze drying method, independently of casting solution composition led to structures with large porous (till 100 μm) heterogeneously distributed [87], [92], [122].

The structures chosen to be processed by $scCO_2$-assisted method were PM 3% wt/wt, XGPM 2% wt/wt (for the candy option), CHT with TEMED and APS 3% wt/wt (TCHT) and XGCHT with TEMED and APS 2% wt/wt (TXGCHT). However, according to parameters used for this method (parameters discussed below), the scaffolds were not possible to be performed. The scaffolds obtain by $scCO_2$ were PM and TCHT impregnated with BSA (80mg)

The structures with well-defined porous network were prepared following an integrated strategy involving: a) gelation process, b) water-acetone substitution and c) $scCO_2$ phase inversion/drying.

The selected scaffolds were reproduced by $scCO_2$-assisted method. They were impregnated with BSA. The freeze time was 24h while the gelation in acetone occurred during 48h at -20ºC. The TCHT 3% wt/wt and PM 3% wt/wt structures were successfully obtained. while, XG based scaffolds presented unstable physical conditions and the characterization was not possible to be performed. To optimize this structure further work should be developed. It has also be prepared solution without protein; however, PM native scaffolds in these conditions do not have physical consistency. Thus, the only characterized scaffolds prepared by gelation processed followed by $scCO_2$ were PM TCHT 3% wt/wt with BSA.

When compared with freeze-drying structures, scaffolds prepared by $scCO_2$ presented complete different macrostructures. In detail, TCHT and PM scaffolds were more rigid, which suggest smaller pores, when compared with it freeze-drying counterparts. These observations suggest that these two methods, freeze-drying method $scCO_2$ combined with gelation step, allowed to prepare porous structures with a wide range of pore size 50 to 160 μm.

These two techniques allowed to obtain a wide range of pores. The freeze-drying obtain structures had a range of pores size 50 to 100 μm. This was due to freeze temperature, polymer concentration. While $scCO_2$-assisted scaffolds had a pore size of 160 μm. The step that is determinant in pores size in this method is the depressurize of high-pressure cell.

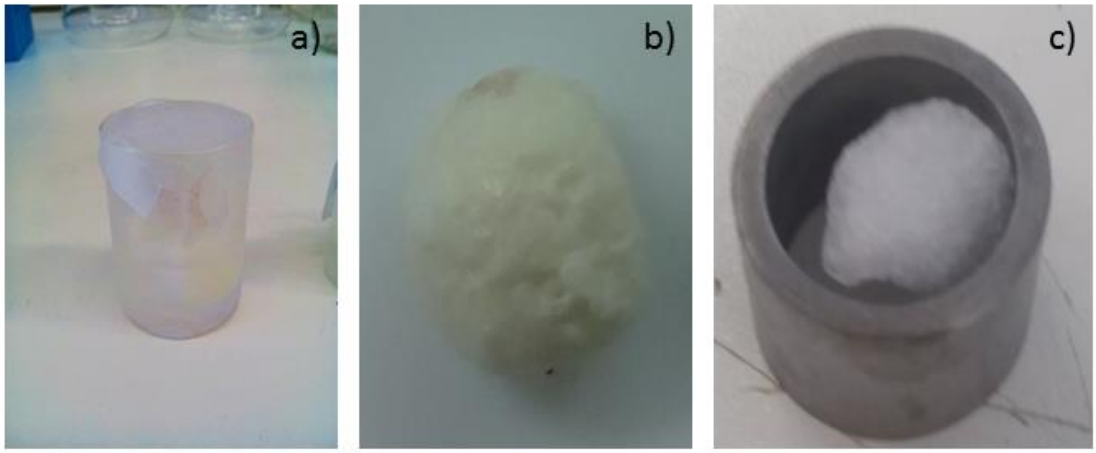

Figure 3.2- ScCO2 assisted scaffolds: a) Scaffold in gelation step; b) ScCO2 PM 3% wt/wt scaffold with BSA and c) Failed ScCO2 PM Scaffold

3.2. Scaffolds characterization

In order to evaluate the characteristics of all structures produced in this work, different techniques were used to evaluate the morphological, mechanical, physicochemical and biological features of the scaffolds.

3.2.1 Scanning electron microscopy (SEM)

Native scaffolds prepared by freeze-drying only with chitosan and polymer m present regular and spherical pores, while polymer m exhibited smaller pores. All native structures were also highly dependable of the polymer concentration, so the Figure 3.3, (E) structure it was more homogenous than Figure 3.3, (F). In contrast, scaffolds made of xanthan gum presented themselves as unaligned and heterogeneous porous structures.

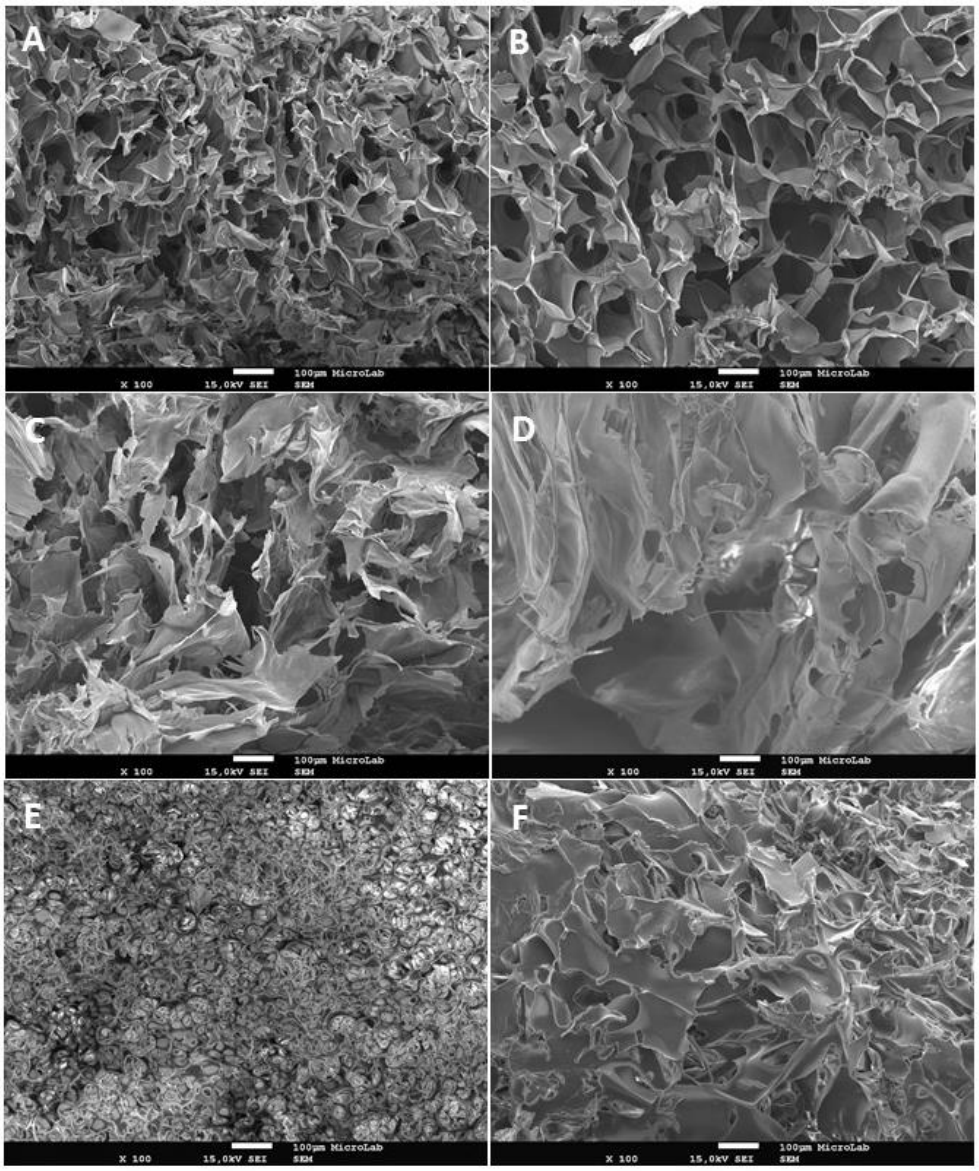

Figure 3.3-SEM images of cross-section of the polymers native scaffolds produced by freeze-drying (A) CHT 2% wt/wt, (B) CHT 3% wt/wt ,(C) XG 2% (wt.%),(D) XG 3% wt/wt,(E) PM 2% wt/wt,(F) PM 3% wt/wt

The addition of MBA and MBA combined with TEMED and APS, created structures with smaller and homogenous pores, in all scaffolds. However, in case of XG scaffolds, the addition of TEMED and APS turned them miscellaneous when compared with the scaffolds prepared only with MBA. The addition of crosslinkers on PM opened the pores, making them bigger and homogenous.

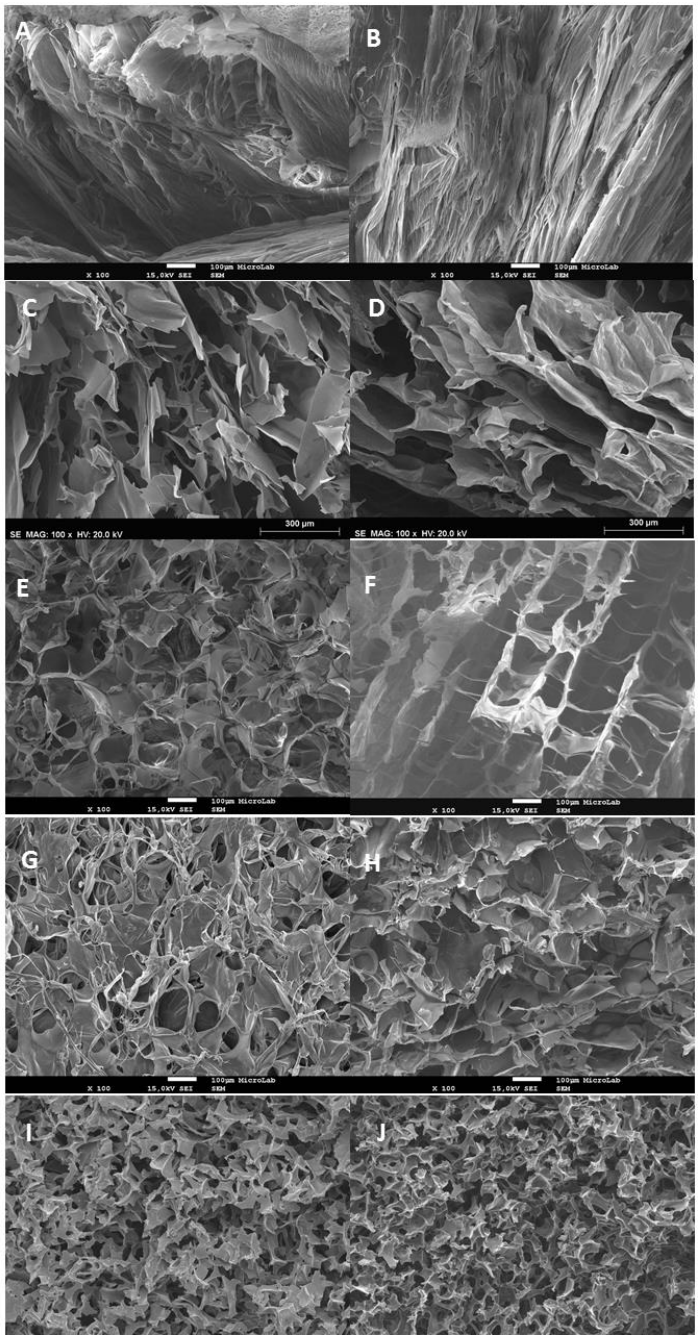

Figure 3.4- SEM images of cross-section of the crosslinked scaffolds produced by freeze-drying (A) CHT with MBA 2% wt/wt, (B) CHT with MBA 3% wt/wt (C) CHT with TEMED and APS 2% wt/wt, (D) CHT with TEMED and APS 3% wt/wt, (E) XG 2% wt/wt with MBA, (F) XG

Figure 3.5 presents the porous structures of scaffolds prepared by freeze-drying of polymer mixtures. The PM and XG mixture (Figure 3.5; (B)) and (Figure 3.5; (F)) present a homogenous pore distribution although the size in (Figure 16, (B))) was much smaller (50μm) and more spherical than in (Figure 3.5, (F))) (700 μm) which were semi-spherical; the scaffolds (Figure 13, (D))) were miscellaneous, and had some smaller and other larger pores with different shapes (150μm). In contrast with XGCHT, here was the PM that got its pores bigger with the addition of the XG in its structure. The addition of XG to PM opened the PM pores when compared with PM Freeze-dry native scaffolds. The mixture of CHT and XG presented a large and semi-spherical pores; the addition, first of MBA and then of TEMED and APS, become the pores larger keeping the homogeneity previous observed. In CHTXG scaffolds, the presence of

CHT led to an enlargement of XG pores.

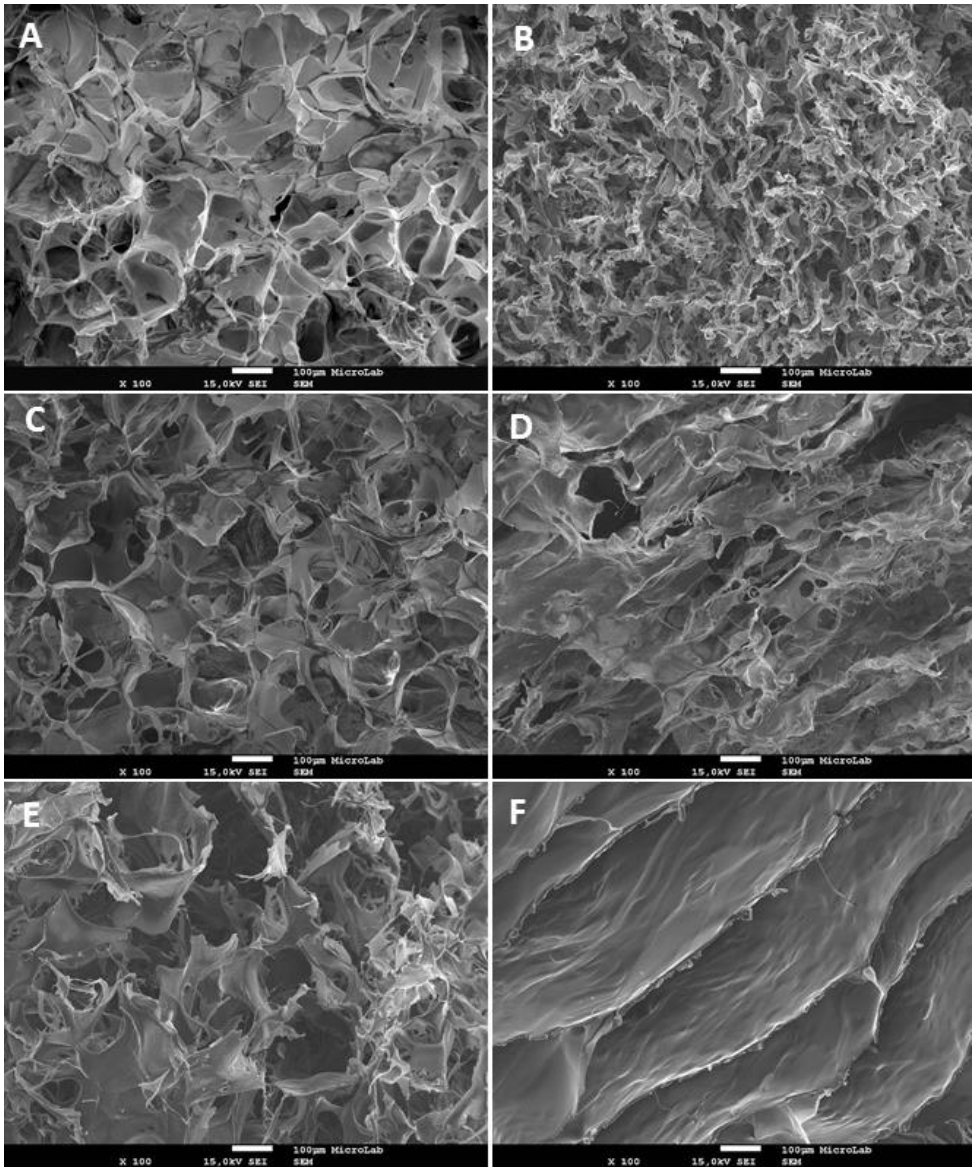

Figure 3.5- SEM images of crossection of polymers mixtures obtain by freeze-drying: (A)CHT+GUM 2% without crosslinker; (B) PM+ GUM 2% without crosslinker; (C) CHT+GUM 2% with MBA; (D) PM+ GUM 2% with MBA; (E) CHT+GUM 2% with MBA, TEMED and APS; (F) PM+GUM 2% with MBA, TEMED and APS

Scaffolds prepared by gelation process followed by $scCO_2$-assisted method presented large pores with a semi-spherical shape. Both structures had a heterogeneous distribution, which suggest a high depressurize time. When compared with freeze-dried ones, $scCO_2$ had bigger pores but also structures that were more compact. This may be due to the gelation process. Since the water-acetone substitution in the solutions result in a higher stiffness of the scaffolds[123].

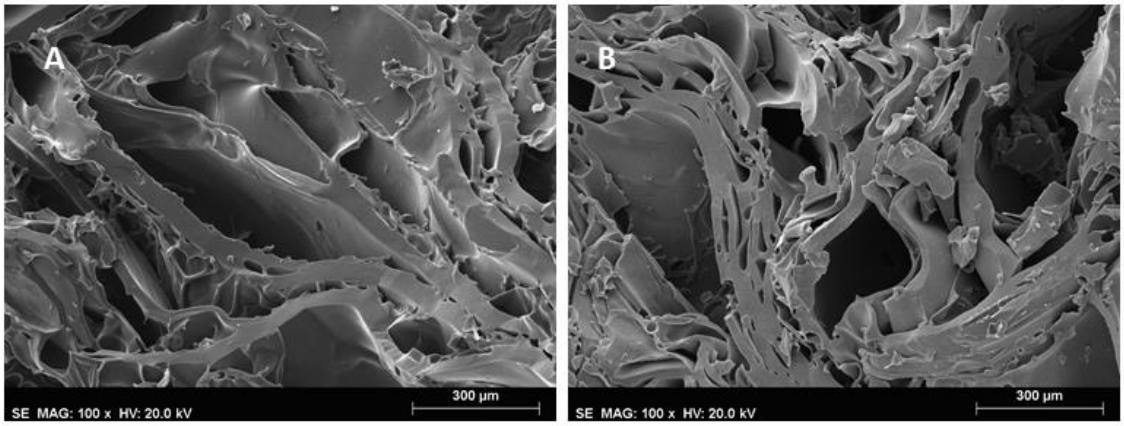

Figure 3.6- SEM images of crossection of ScCO2 assisted scaffolds: (A) TCHT 3% wt/wt and (B) PM 3% wt/wt

Thus, the results suggest that combining: the polymer nature, the concentration of casting solution and the presence or absence of crosslinkers in the casting solution, different porous structures by freeze-drying method can be prepared with a medium pore size diameter between 50 and 100 μm.

Moreover, according to the method of scaffolds preparation, the porous network of the scaffolds can be tuned since using freeze-drying method it is possible to obtain pores with 50-100 μm while in $scCO_2$, the same structure can reach pore sizes around 160 μm.

Thus, for the gum option the best structures are the ones obtained by $scCO_2$-assisted method, since they are the ones closer to the target (150μm). While for the candy option, the scaffolds obtained by freeze-drying method are more suitable, since the target, for this case, was 50 μm.

3.2.2. FTIR-ATR analysis

FTIR analysis is an important method to know which chemical interactions are more predominant in polymers. This technique becomes especially important to evaluate the crosslinking effect of polymers, interactions between polymers and interactions between polymers and impregnated model drugs. FTIR analysis was only performed with scaffolds obtained by freeze-drying method.

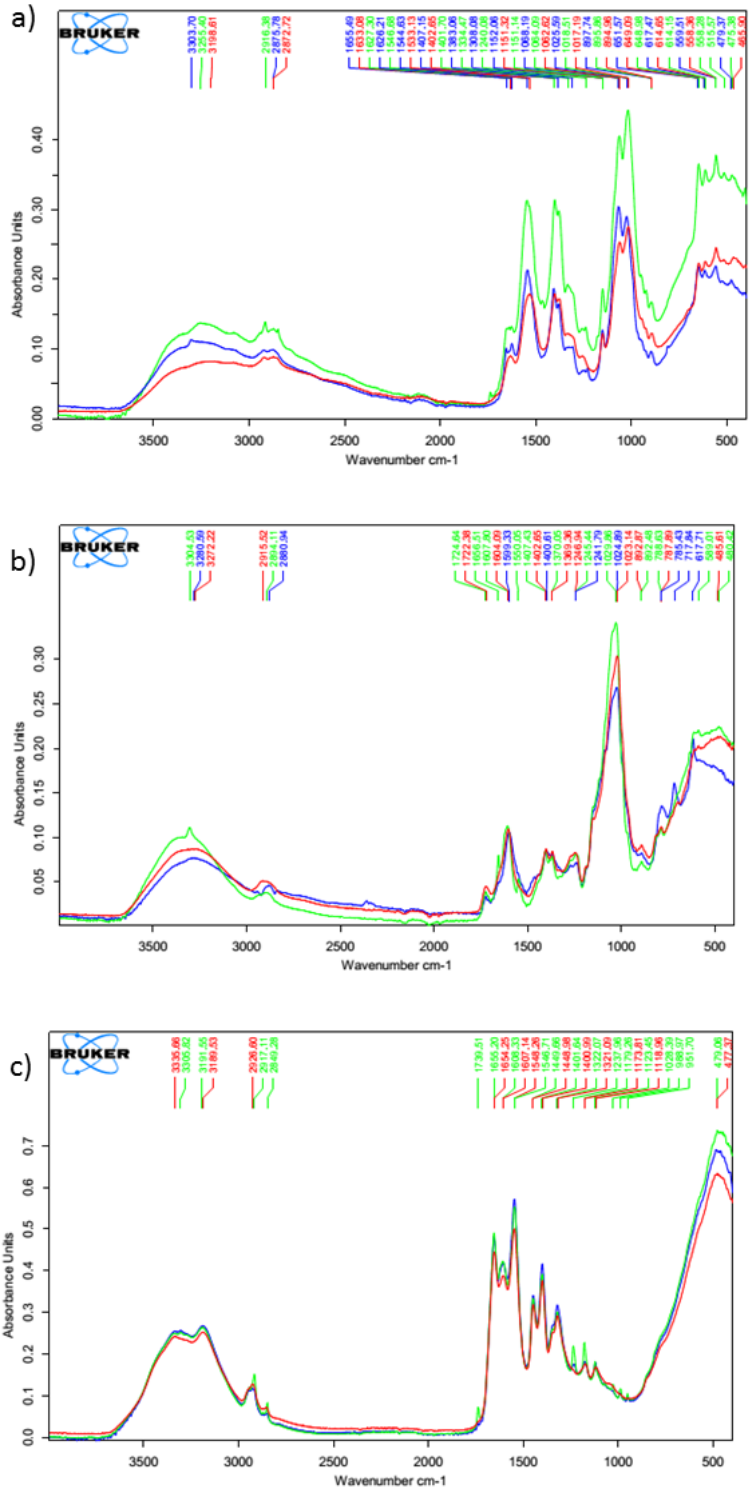

Figure 3.7- FTIR-ATR of a) CHT scaffold vs CHT crosslinked with MBA vs CHT crosslinked with TEMED and APS; b) native XG scaffold vs XG crosslinked with MBA vs XG crosslinked with TEMED and APS c) of native PM scaffold vs PM crosslinked with MBA vs and PM crosslinked with TEMED and APS

For CHT crosslinked with MBA (Figure 3.7; a)) shows a devihation in the –NH stretching vibration pick (3303.70cm^{-1}). This suggest that exist a crosslinking effect between MBA and CHT, in –NH group, making the $–NH_3^+$ group deprotonated to $–NH_2$. It had also influence an alteration in the vibrations of –OH, -CH in the ring (1407.15 cm^{-1}) and –C–O–C– in glycoside

linkage (1068.19cm^{-1}) which reinforces the loss of the charge in the amino group. [124] Similar behaviour was observed for CHT scaffolds with TEMED and APS.

XG crosslinked scaffolds (Figure 3.7, b)) show a deviation in –OH band (3304.53cm^{-1}) which means that the crosslinking occurs in the carboxyl group. It is further supported by the shift in –C=O of pyruvate pick (1607.80cm^{-1}) which is affected by the charge change in the carboxyl group.

PM crosslinking (Figure 3.7 c)) had a deviation in –NH band (1655.48cm^{-1}), which suggests the crosslinking occurs in the amino group.

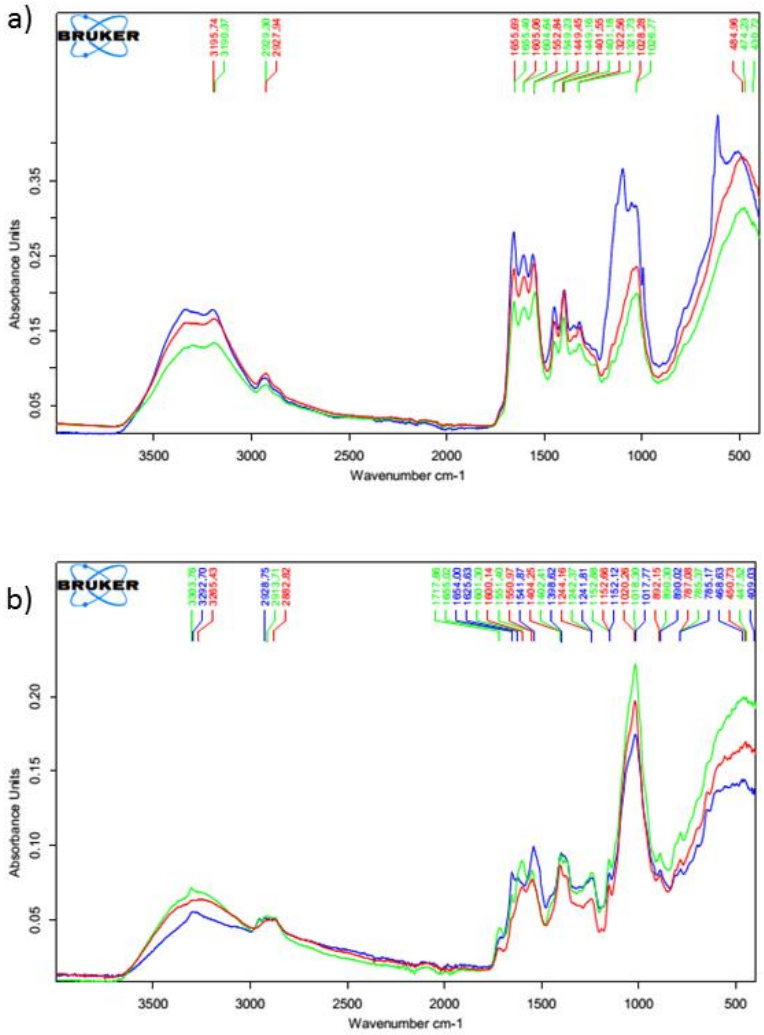

Figure 3.8- FTIR-ATR of a) native XGPM scaffold vs XGPM crosslinked with MBA vs XGPM crosslinked with TEMED and APS and b) native XGCHT scaffold vs XGCHT crosslinked with MBA vs XGCHT crosslinked with TEMED and APS

For the polymer mixtures XGPM and XGCHT. For XGPM (Figure 3.8, a)) it has been shown a clear change in the FTIR-ATR spectra, since the polymers proportion is 1:1, there are interaction between the amino group in PM and the carboxyl in XG. -NH bending and –C-N stretching bands had a clear deviation of the native PM scaffold (1605.06cm^{-1} and 1028.28 cm^{-1}, respectively). While the XG peak most affected was the –OH (3195.74cm^{-1}).

While in XGCHT (Figure 3.8, b)) showed an interaction between the amino group from CHT and the carboxyl group form XG. –NH bend and –C-N stretch had a deviancy of the native

CHT scaffold (1632.04 cm^{-1} and 1019.29 cm^{-1}, respectively). While the XG peaks most affected was the –OH (3201.40 cm^{-1}).

Regarding the impregnated polymers. In the FTIR spectra, which suggests that may exist a chemical interaction. BSA structures show a chemical interaction with the polymeric structures, with an accentuated peak at 3300cm^{-1} for the CHT scaffold (Figure 3.9). This fact may suggest an interaction with the –NH group. Ibu show a similar behaviour. The effect of the model drugs in the PM, XGPM and TXGPM scaffolds are similar, and are represented in Figure 6.4 appendix.

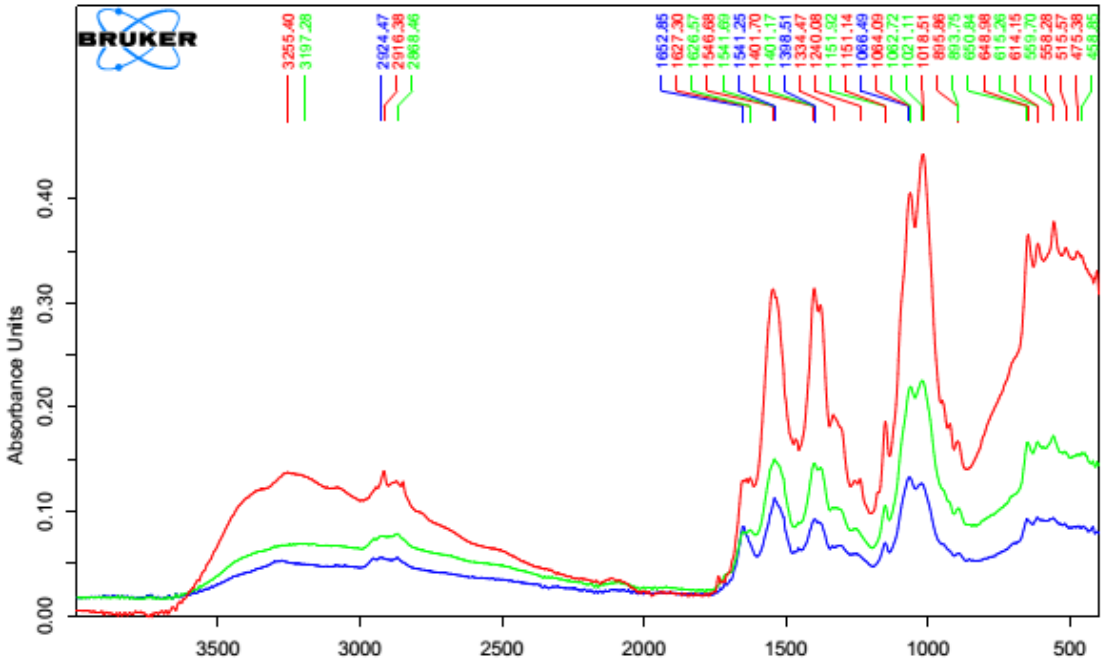

Figure 3.9- FTIR-ATR of TCHT scaffold vs CHT impregnated with Ibu vs CHT impregnated with BSA

Thus, the results suggests that the polymers studied tend to form chemical bonds, which may produce very distinct scaffolds. Moreover, these interactions may affect the drug release behaviour.

3.2.3. Swelling tests

For native CHT and PM scaffolds prepared by freeze-drying method (Figure 3.10), it was observed that the swelling rate decreased with the increase of polymer concentration in scaffold composition. These results are in agreement with those reported in the literature. [118] In case of CHT, it should be considered its hydrophobic nature, consequently, with its increasing concentration, the hydrophilic character of hydrogel will decrease and then the water update is lower. The swelling tests for XG native scaffolds were impossible to be performed due to its unstable behaviour in water as the structures lost their structural integrity.

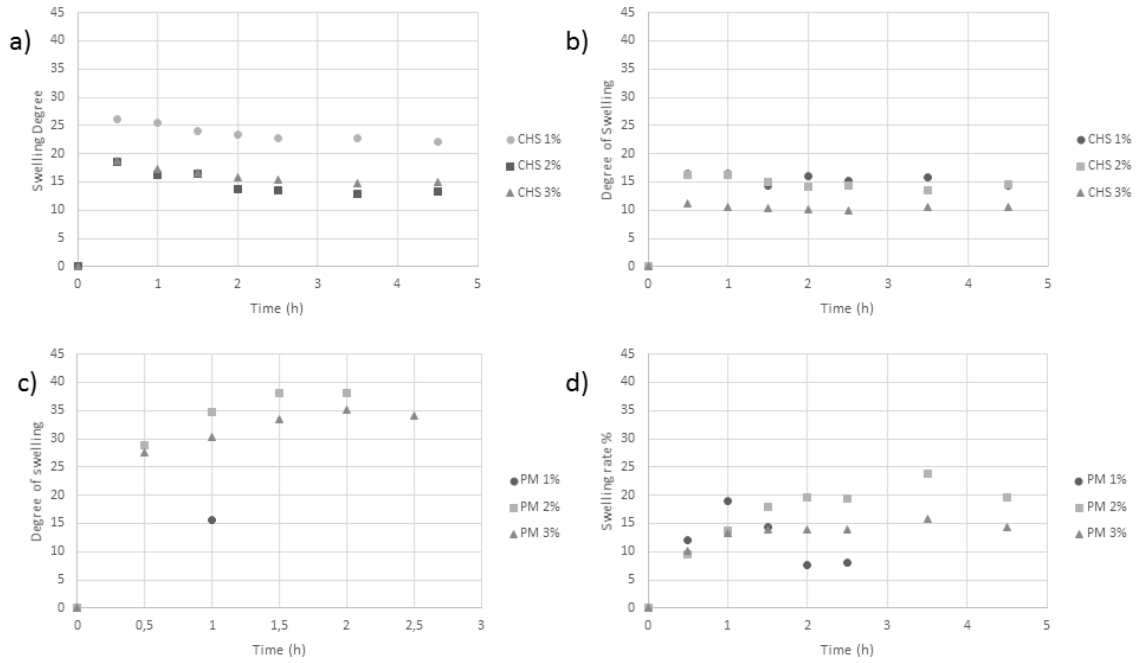

Figure 3.10- Swelling rate of native scaffolds prepared by freeze-drying method: a) CHT pH5.5; b) CHT pH 7.4; c) PM pH 5.5 and d) PM pH 7.4

The amine groups of CHT at acidic pH (Figure 3.10, a)) are protonated, which induce an electrostatic repulsion between the polymer segments. It causes an osmotic pressure in the network. This ionization of the amines may lead to the dissociation bonds, especially the cross-linker, initiator and catalyst and thus to a relaxation of the macromolecular chains. At neutral pH (Figure 3.10, b)) the swelling is reduced up to 15 % to the absence of amine group ionization. At basic pH CHT (Figure 6.5 a), appendix) NH_3^+ groups change back to NH_2 groups, thus no ionic links are formed. [125]

XG native scaffolds have carboxylic groups which are protonated at low pH, thus no ani-on-anion repulsive forces exists, and the swelling rate decrease. At higher pH values, the car-boxylate groups should are deprotonated and the swelling capacity increases. However, due to XG structural instability and fast (less than 30 min) degradation, it was not possible to evaluate its swelling behaviour.[126]

PM and CHT exhibited a similar swelling behaviour. Since at low pH the amine group is protonated (NH_3^+), the swelling rate is 15% higher than at neutral pH. At pH > pKa of amine groups (Figure 6.5 b), appendix), deprotonation will occur, and the excess of negative charges increase the structure disintegration, having the swelling rate achieved 33% before complete fragmentation. The only scaffolds which presented stability were the ones with a PM concentra-tion of 3% wt/wt.

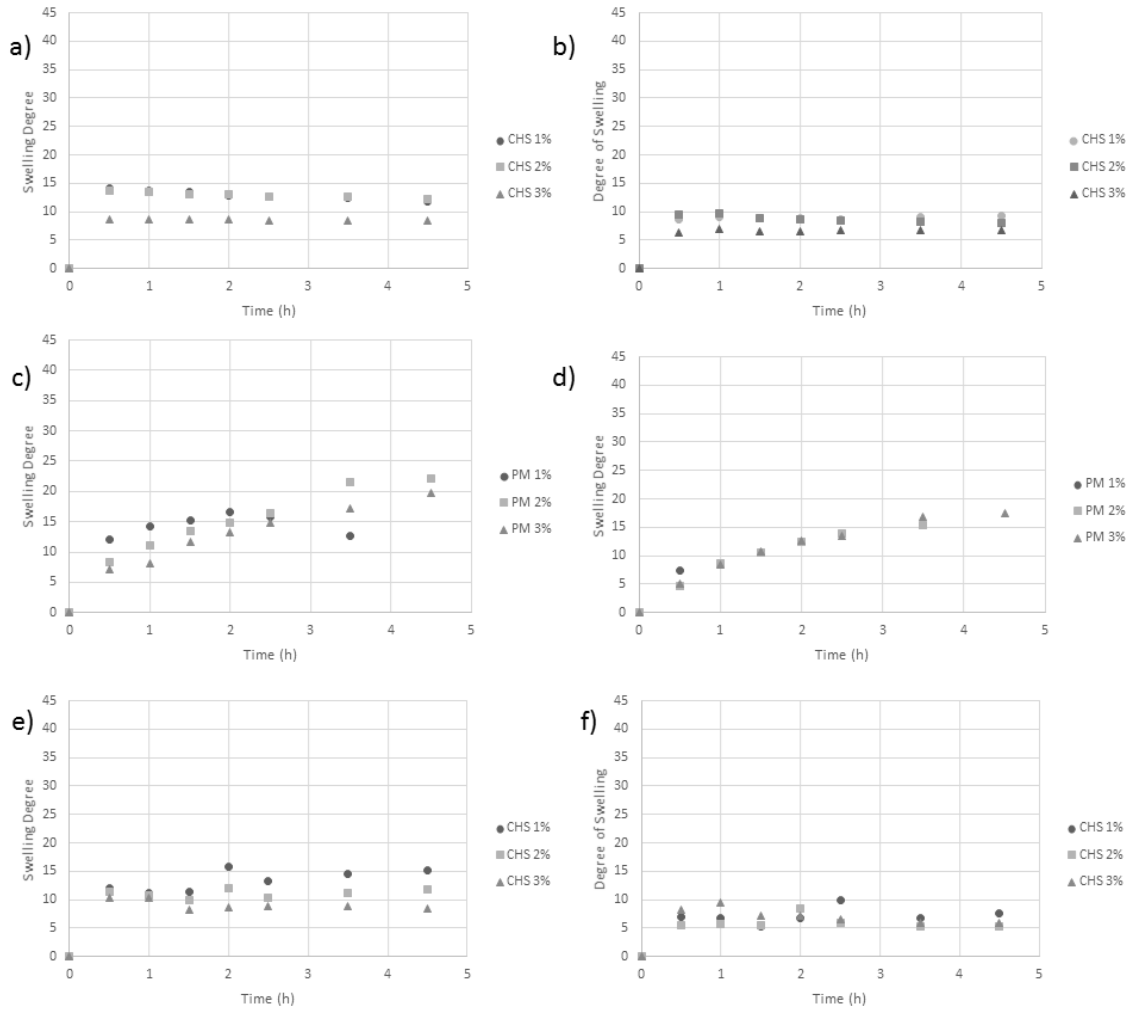

Figure 3.11-Swelling rate of crosslinked scaffolds obtain by freeze-drying: a) CHT with MBA pH5.5; b) CHT with MBA pH 7.4; c) PM with MBA pH 5.5, d) PM pH 7.4 with MBA, e) CHT with TEMED and APS at pH 5.5 and f) CHT with TEMED and APS at pH 7.4

The addition of crosslinker, initiator and catalyst produced structures with lower swelling rate (not surpassing 25%) but with similar behaviour, than native scaffolds. Crosslinked CHT presented a stable swelling behaviour than native ones. This suggests that this structure is suitable to be employed as a gum, since it shows lower swelling values (till 15% in acidic pH medium) and has slower degradation. The only exception PM with TEMED and APS. This can be caused by the crosslinking effect, which makes the osmotic pressure higher with the addition of crosslinkers. PM with TEMED and APS showed high degradability not being able to draw a swelling curve.

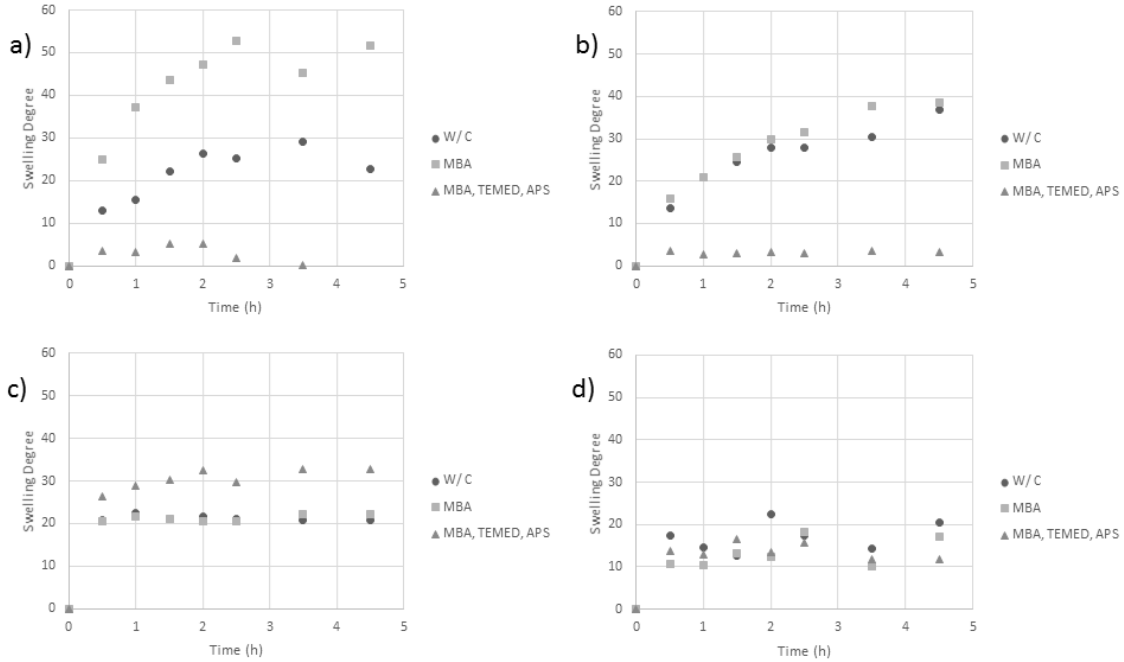

Figure 3.12-Swelling rate of polymers mixtures produced by freeze-drying: a) XGPM 2% wt/wt at pH 5.5, b) XGPM 2% wt/wt at pH 7.4, c) XGCHT 2% wt/wt at pH 5.5 and d) XGCHT 2% wt/wt at pH 7.4

In case of XGPM (Figure 3.12, a) and b)) there was an increase of the swelling rate (about 20%) when compared with native PM scaffolds. This may be due to weak interactions between positively charged amine groups of PM and negatively charge carboxylic groups of XG. This may lead to a loose network, and hence to increased swelling. The addition of cross-linkers in XGPM increased the structures instability. XGCHT scaffolds (Figure 3.12, c) and d)) show the best behaviour when it was added the TEMED and APS (swelling rate of 30% in acidic medium). The value and the behaviour of swelling of TXGCHT turn this structure a suitable option for a gum scaffold.[84]

PM based scaffolds showed better mechanical properties adequate for the use as a degradable candy, being the best options the native based ones, PM and XGPM.

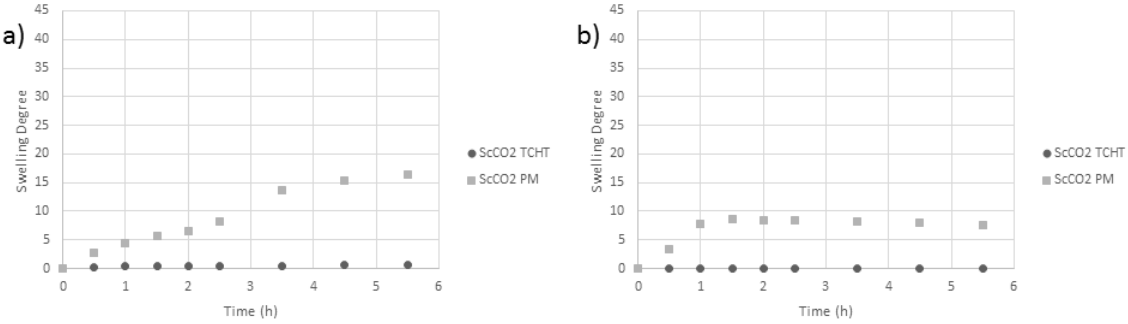

Figure 3.13- Swelling rate of $ScCO_2$-assisted scaffolds with BSA of TCHT 3% wt/wt and PM: 3% wt/wt a) at pH 5.5 and b) at pH 7.4

$ScCO_2$ scaffolds present a similar behaviour to its freeze-dried counterparts; however, since they are more rigid, the swelling rate is significantly lower.[127]

Since the main objective in this work was to produce two types of structures, one degradable (candy) and another not degradable (gum), it was defined that as the candy scaffolds should have a bigger and faster swelling rate, PM and XGPM seemed to be the adequate mate-

rials, while for the gum structures TCHT and TXGCHT could be applied, as the swelling degree at different pH has to be considered but may be smaller. Also, since the buccal pH, when is healthy is neutral and when it is ill, is acidic, the swelling rate has also to be bigger at pH 5 than 7.4.

Thus, the results suggest that the swelling behaviour depends on the physical properties of the polymers, instead of the pore sizes of the structures. In detail, structures with smaller pores (about 50 µm), as PM and XGPM, presented higher swelling rate (between 40 and 60%) while TCHT and TXGCHTstructures, which exhibit larger pores (about 100 µm) showed smaller swelling rate (till 30%).

3.2.4. Mechanical analysis

The mechanical proprieties of the scaffolds were studied using uniaxial compression measurements under dry conditions. The slope of the stress-strain curves, which translate the material stiffness (Table 3), gives the compression modulus (kPa).

The objective of this analysis was to obtain two structures with two distinct mechanical moduli. For a gum the desired scaffold should have low compressive modulus (<5kPa), while for a candy type the objective was to achieve a high compression modulus (>5kPa).

Table 3.1- Compressive Modulus (kPA) of dry scaffolds

	COMPRESSIVE MODULUS (KPA)		
	Without Crosslinker	**With MBA**	**With MBA, TEMED and APS**
		Freeze-drying	
CHT 2%	4,42	2,78	0,43
CHT 3%	10,30	3,24	0,49
XG 2%	0,70	4,36	0,82
XG 3%	5,47	5,89	5,10
PM 2%	0,20	a	0,15
PM 3%	0,20	a	0,06
XGPM 2%	0,18	0,14	0,65
XGCHT 2%	1,90	1,35	1,80
		$ScCO_2$	
CHT 3%	b	b	3,70
PM 3%	28,85	b	b

[a] The structures collapsed.

[b] Structures not selected for these studies.

The mechanical compression values were independent of the casting solution. For the freeze drying process, the most stiff structure was CHT 3% wt/wt that showed a compression of 10.3 kPa, while the most soft was the crosslinked PM 3% wt/wt. Freeze-drying scaffolds presented a lower compression modulus when compared with the ones prepared using $scCO_2$. The $scCO_2$ structure had the higher compression modulus in PM 3% wt/wt with a value of 28.25 kPa.

In general the addition of TEMED and APS did not improve the structures stiffness, however the addition of MBA improved the XG scaffold mechanical properties.

Concerning the scaffolds prepared using $scCO_2$, PM presented the higher compression

level (28.85 kPa) while the PM scaffold obtained by the freeze-drying method only reached 0.2 kPa. This may be due to the crosslinking effect of BSA, as well lower water absorption capacity and the acetone effect in the gelation step. TCHT has a similar behaviour. The gelation process helps to form rigid structures and decreases the water absorption ability.

Compression is inversely proportional to ductility. The objective is to make two different structures, one with ductile structure, low compression (<5 kPa) and lower swelling rate (<30%), and another, the candy with the opposite properties. However, the mechanical compression values are not in agreement with the SEM and swelling characterizations results. In literature, it is mentioned that structures with small pores and consequently low swelling capacity are more stiff. Herein, the opposite was observed. This fact suggests that the polymer nature prevails to the porous network of the structures.

Nevertheless, considering a balance between the values obtained in the swelling, mechanical studies, and SEM analysis, the best scaffolds prepared by freeze-drying for the candy structures were i) PM and ii) XGPM. The swelling rates at acidic pH were larger than 30%; the average porous size was around 50 μm, and the mechanical compression were inconclusive due to the compression values for the freeze-dry scaffolds. To produce the gum structures TCHT and TXGCHT were chosen. The swelling rate at acidic pH was approximately 30%, and the average porous size for these structures is around 100 μm.

3.2.5. Zeta potential tests

Since the pH is an important parameter for the purpose of this work, zeta potential analysis were performed in order to understand the charges of the structures under certain pH environments.

In figure 3.14, zeta potential of CHT, PM, XGPM and XGCHT polymers at pH 5.5 and 7.4 are plotted. The results represented in Figure 3.14 show that the polymeric structures present different zeta potential values according to the pH. Higher zeta potential values are registered for pH 7.4. This is because at 7.4 CHT and PM polymers are deprotonated (without charge). On the other hand, at pH 5 the zeta potential values are more negative, which make sense since at this pH all polymers are protonated due to the excess of H^+ ions presented in the solution.[59] The exception is the XG in which an opposite zeta potential behaviour is observed.

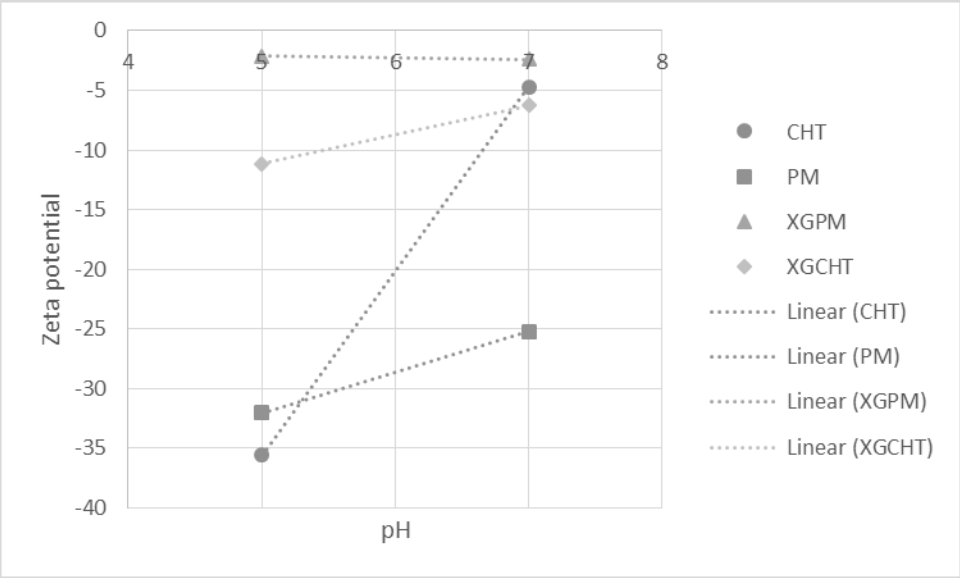

Figure 3.14-Zeta potential analysis of the selected scaffolds

3.2.6. Degradation studies

Degradation studies were performed for PM, TCHT, XGPM and TXGCHT structures, the ones that revealed the suitable chemical, physical and morphological characteristics to be applied as gum or candy oral devices. For the candy option it was envisaged a faster degradation. The samples were incubated in a lysozyme solution. The mass loss after 120 days of scaffolds incubation in lysozyme solution is included in Figure 3.15.

Data show (a)) that PM 3% wt/wt and XGPM 2% wt/wt scaffolds revealed a faster degradation, achieving their total degradation after 5 days and 9 days, respectively. The TCHT (b)) is less degradable, since the addition of MBA, TEMED and APS also improved the biostability. When CHT is mixed with XG the biostability of the structure decreases. This structure gets stable after 89 days of the study.

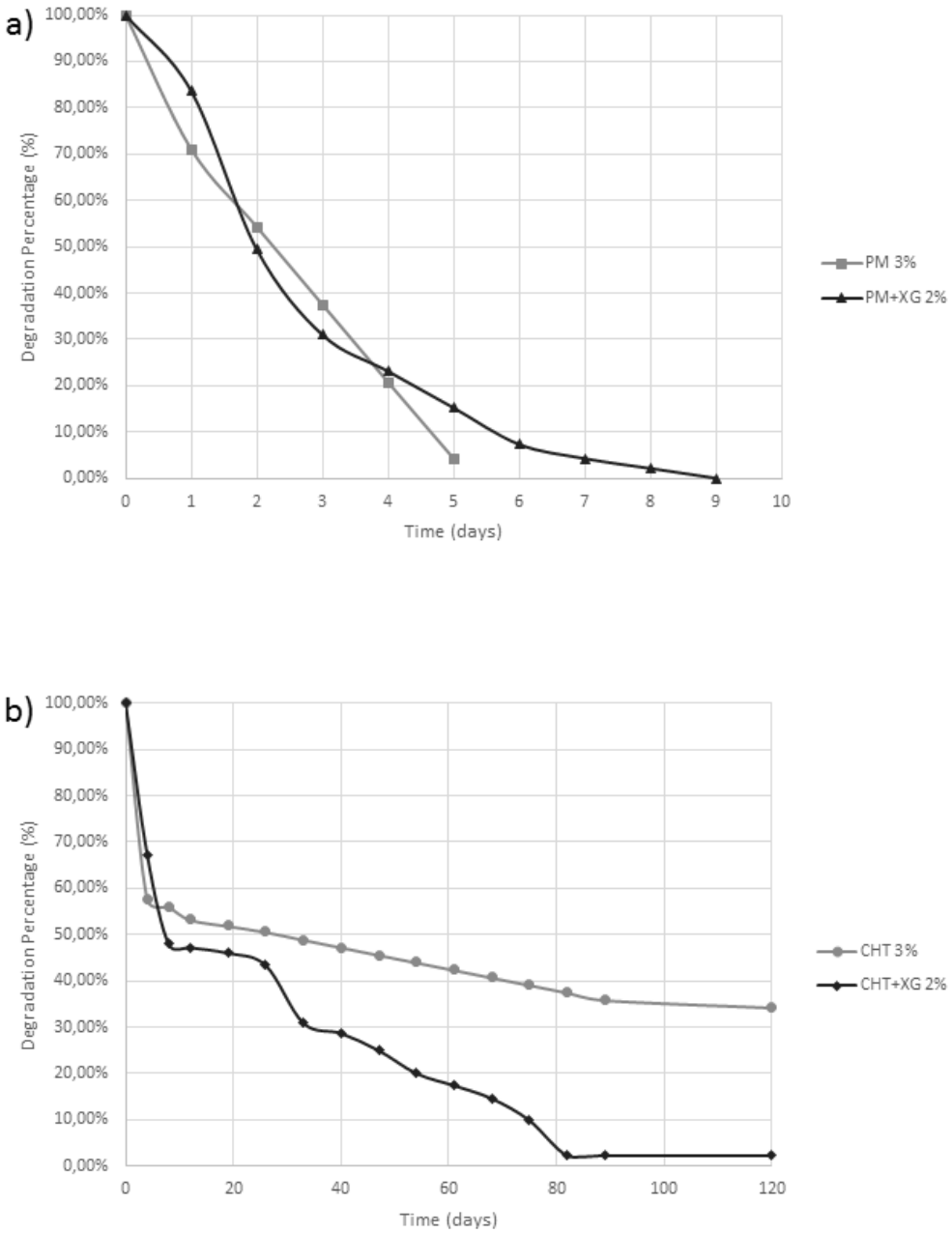

Figure 3.15- Degradation studies a) Candy option (PM 3% wt/wt and XGPM2% wt/wt) and b) Gum option (TCHT 3% (wt.%) and TXGXHT 2% wt/wt)

The results are in concordance with the objective of this thesis. While PM 3% wt/wt and XGPM 2% wt/wt present a higher degradation, which is a good option for a candy, TCHT 3% wt/wt and TXGCHT 2% wt/wt present a much more retard degradation, being more suitable for a gum type structure.

In addition, the degradation behaviour is in agreement with the results obtained in the swelling studies and SEM analysis. Since PM 3% wt/wt and XGPM 2% wt/wt have higher swelling rate (>30%) and small pores (50μm), the degradation was faster (till 9 days).

3.2.7. Biocompatibility

The polymer was soluble in cell culture media at the different concentrations tested, form-

ing a viscous solution for the highest polymer concentrations. Above 1.0 mg/mL it became very sticky appearing to have some adhesive properties. After 24 hours in contact with the polymer solutions, the human dermal fibroblasts presented normal morphology and dead cell were not present in the supernatant. Nevertheless, above 1 mg/mL it seems that the cells did not proliferate very much, comparing to the control and the lower concentrations. This could be due to the high viscosity of the polymer solution that can interfere in the transference of oxygen and nutrients to the cells (Figure 3.16).

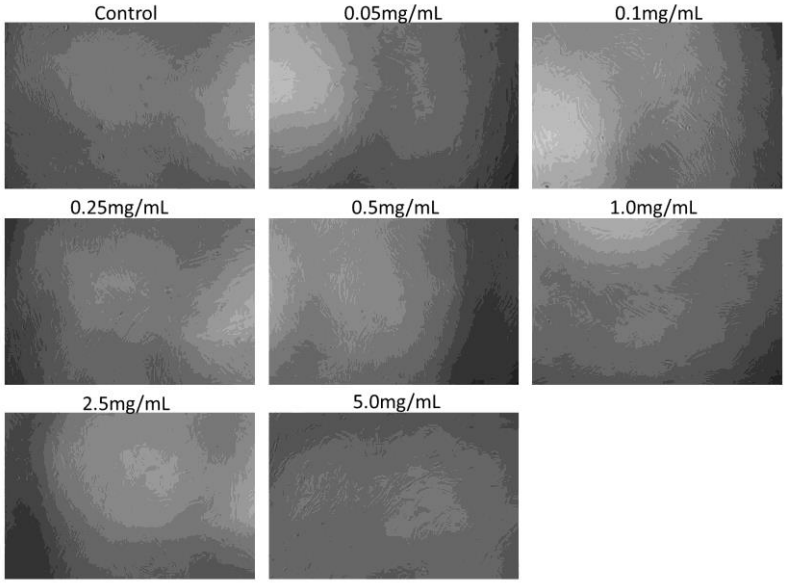

Figure 3.16-Bright-field micrographs of human dermal fibroblasts after 24 hours in contact with different concentrations of PM polymer.

In line with the results obtained by microscopy, the ATP measurements revealed a small difference in the percentage of metabolic active cells when the fibroblasts were incubated with PM polymer comparing to the control. Nevertheless, it was only statistically different when hu-

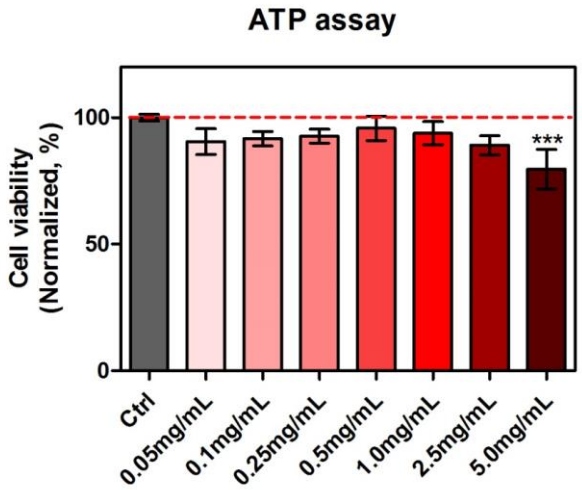

Figure 3.17- Cell viability after 24 hours in contact with PM at different concentrations. Data is presented as the average of three replicates with standard deviation. ***p<0.01, one-way ANOVA and Bonferroni's post-hoc test.

man dermal fibroblasts were cultured with 5.0 mg/mL of PM (Figure3.17).

PM polymer is a non-cytotoxic material to human dermal fibroblasts.

3.3. Release studies

In order to evaluate the performance of the scaffolds as drug delivery systems, it was crucial to investigate the pharmacokinetics release profile under different physiological conditions as well as their responsiveness to pH and temperature external stimuli. According to the morphological characterization and mechanical properties of the scaffolds previously discussed, PM 3 % wt/wt, XGPM 2% wt/wt (as candy option) an TCHT 3% wt/wt, TXGCHT 2%wt/wt (as gum option) were selected to investigate the release profiles of a small drug ibuprofen (Ibu) and a model protein, bovine serum albumin (BSA).

3.3.1. Release of model drugs

The release profiles of BSA and Ibu at pH 5.5 and 7.4 were investigated using the four chosen structures above produced by freeze-drying; while the two by $scCO_2$ were only studied using BSA, due to Ibu solubility in $scCO_2$. Ibu solubility in CO_2 is due to the fact that CO_2 interacts specifically and non-specifically with ibuprofen molecule.[128]

For PM scaffolds (Figure 3.18, a)). At pH 5.5, PM is protonated, while BSA is deprotonated, this result in an interaction between the polymer and the protein, which delays BSA release. At pH 7.4, PM is uncharged, so it does not contribute with attractive or repulsive forces, BSA still is deprotonated, making the attractive forces weak, and the release is more accentuated since BSA retention is less assured. A similar profile can be extrapolated to TCHT release of BSA (Figure 3.18, b)). This behaviour was observed for freeze-dried TCHT and PM scaffolds impregnated with BSA and also for the ones impregnated with Ibu (Figures 6.6, appendix).

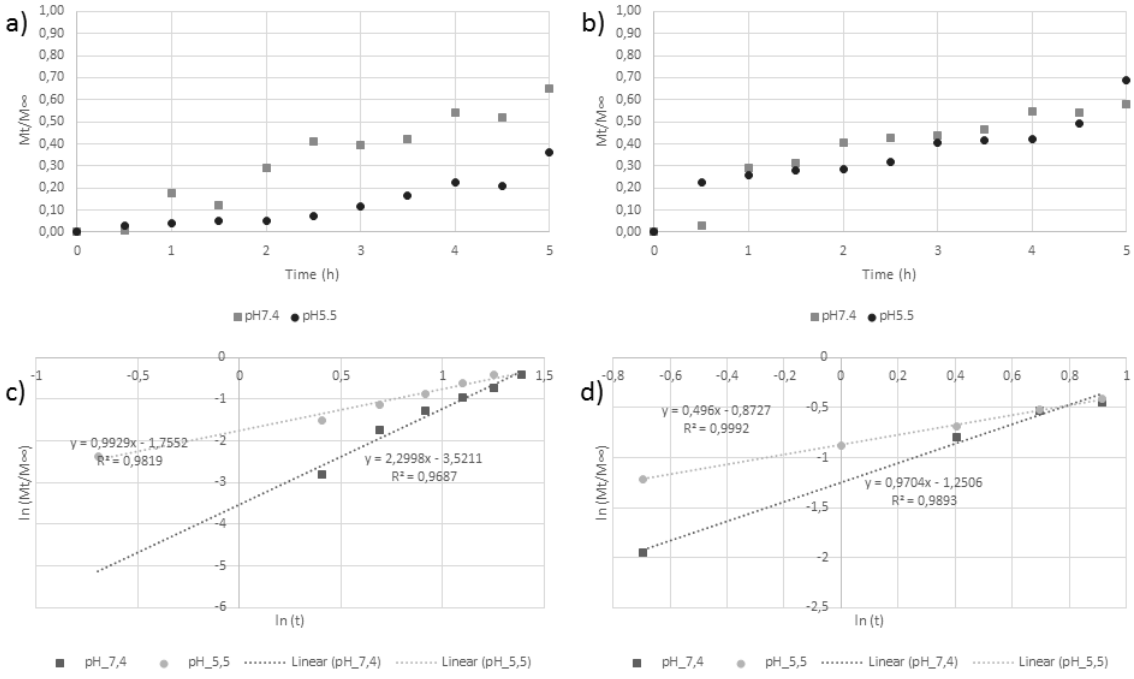

Figure 3.18- BSA release profile of freeze-drying obtain scaffolds a) PM 3% wt/wt, b) TCHT 3% wt/wt and mathematical modulation of the best method (power law) c) PM and d) TCHT

The polymer mixtures of XGPM and TXGCHT have similar behaviour (Figure 3.19). At acidic pH, both XG and PM are protonated (Figure 3.19, a)), which influenced XG being uncharged and PM positively charge. The release profile is higher at acidic pH, which may be due

to the crosslinking effect between the polymers. The interaction between the charged groups may be weaker at an acidic medium, which leads to a looser network. Also, the BSA deprotonation at pH 5.5 might influence. In this case, the protein is deformed, and the looser network may help BSA diffusing out of the scaffold. The other possibility is the easiest broking of the interaction between BSA and the structure at acidic pH than at neutral one. In addition, the higher degradation observed for the structure at acidic pH, also shown in the swelling studies, may facilitate the faster release of the protein. XG, according to literature, behaves only as a drug release retardant.[129] However, this behaviour was not observed as the addition of XG to the others polymers increased the release in the acidic medium, which may be due to more degradability of XG.

Ibu also shows a faster release at acidic pH than at neutral pH independently of the polymeric matrices under study. This may be due to the stronger influence of the morphological structural effects of the constructs and their swelling behaviour rather than the influence of Ibu charge and interactions with the structure.

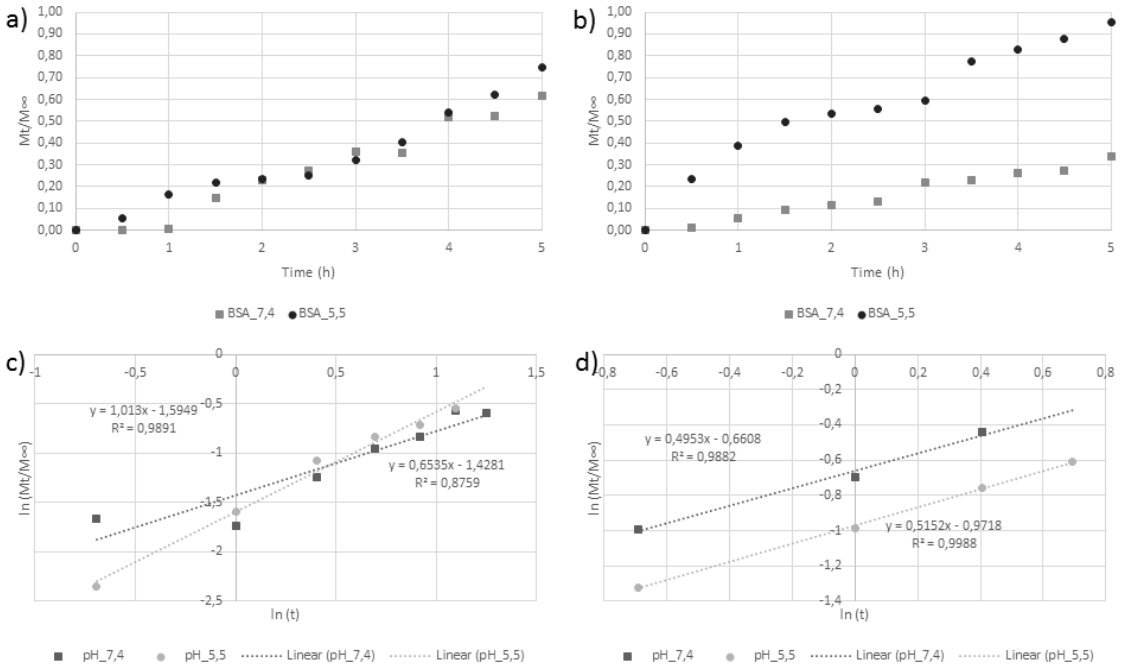

Figure 3.19-BSA release profile of freeze-drying obtain scaffolds a) XGPM 2% wt/wt, b) TXGCHT 2% wt/wt and mathematical modulation of the best method c) XGPM and d) TXGCHT

At the pHs under study, both Ibu and BSA are deprotonated (pKa≈4.8; pI≈4.5-4.7, respectively) and thus, both should follow the same trend of charge interactions with the polymers. Ibu is a small model drug and thus it should diffuse more slowly across the networks. However this was only observed for TXGCHT scaffolds[118]. The remaining structures presented a higher release of Ibu, which may be a consequence of pores heterogeneity that slow down the BSA release.

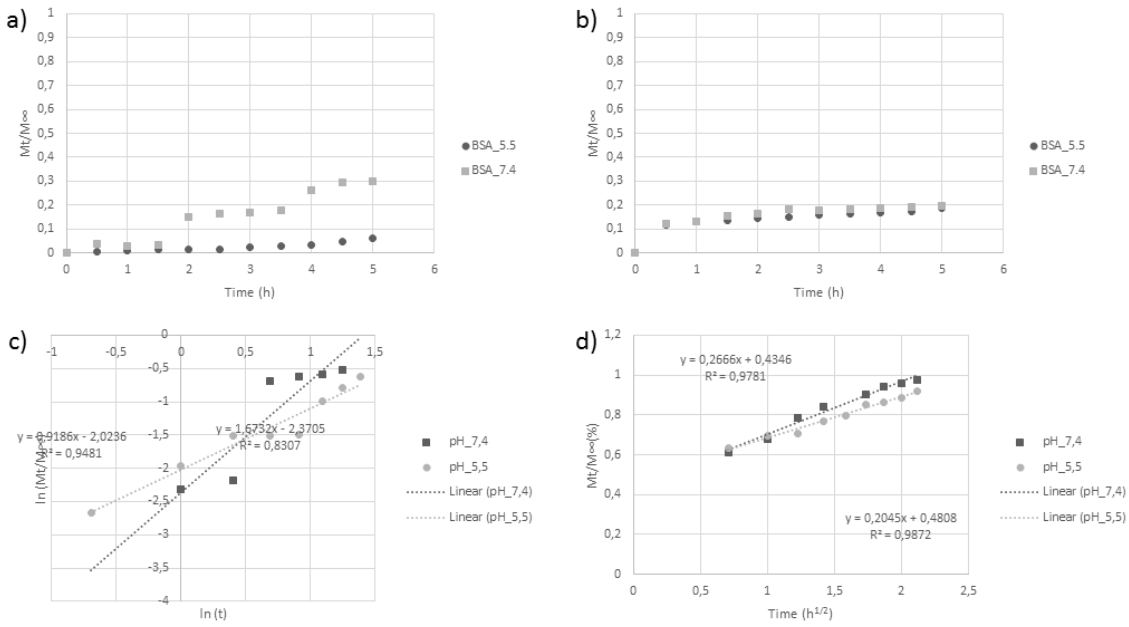

Figure 3.20- BSA releasing profile of scCO2-assisted scaffolds at pH 5.5 and 7.4: a) PM 3% wt/wt and b) TCHT 3% wt/wt and mathematical modulation c) PM with power law and d) TCHT Higushi model

The BSA release profiles observed were similar for scaffolds produced by $scCO_2$-assisted method (Figure 3.20) and by freeze-drying. However, the quantity of BSA release by this method is considerably low (not surpassing 30%) when compared with the freeze-drying PM and TCHT impregnated with BSA, which had a release of protein around 70%. This may be due to the effect of the gelation step, which may lead to a higher crosslinking degree, than the scaffolds obtained by only freeze-drying. The decrease in the protein release of $scCO_2$ obtain scaffolds can be related with the decrease of the swelling rate. Since the release profile is also dependable of the swelling profile of the structure. ScCO2 obtain scaffolds had a 30% decrease in swelling when compared with the freeze-drying obtain scaffolds.

The objective was that the scaffolds had a controlled release of the model drugs. Since they were two distinct structures, the drug release in the candy option should be faster than the gum. However, the difference between the release rate was not noticeable for the different structures. All tested scaffolds showed a linear controlled drug release, without reveal a release pick (burst), which is an ideal scenario for any drug release profile.

The other main factor was the quantity of drug released. The desired value was 50 mg/g, which is the amount used in oral commercial drugs. This value was obtained for the freeze-dried scaffolds, since the total amount of drug impregnated structures was 80 mg/g, and in all scaffolds there was an accumulative release of at least 70%. The same was not possible to accomplish with the scCO2-assisted scaffolds. With these structures the amounts released did not surpass 30%.

Considering that the release rate should also be higher at acidic pH, the best options were XGPM 2% wt/wt for the candy option and TXGCHT 2% wt/wt for the gum, since both PM and TCHT showed faster release at neutral pH.

3.3.2. Modelling of drugs release

The modulation figures of Ibu release are in the Figures 6.6 and 6.7 in the appendix. The

values of the fitted parameters are summarized in Table 3.2, which describe the drug release profile relationship for each loaded active ingredient. TCHT scaffolds obtain by gelation process assisted by $scCO_2$ only had Higuchi model fitting. To apply the power law, $M_t/M_\infty<0.6$ and in this case all the values are above, making TCHT obtain by gelation assisted by $scCO_2$ release model inconclusive.

The other mathematical model applied was the Power law, which is the best fitting for the drug release of these structures. For PM load with Ibu and other swellable polymeric systems, the exponent parameter for cylinders were between 0.45 and 0.89, which indicates both diffusion controlled drug release, and swelling- controlled drug release.

The ones with n lower than 0.45, as is the case of Ibu released from TCHT at acidic pH, show indications of diffusion- controlled drug release. While n is higher than 0.89, indicates a swelling- controlled drug release, which is the case of Ibu released from XGPM.

These modulations gave a good idea how the drug release behaviour occurs. However, other option that was not developed was the study of drug release considering the structure degradation. This is given by Hopfenberg model, which considers the effects of structure format, suggesting different equations for spherical or cylindrical shapes, and for the case with matrix degradation.

Table 3.2- Modelation values for Higushi and the Power law

		Higuchi		*Power law(Mt/M∞<0,6)*		
		k	r^2	k	n	r^2
PM_Ibu	pH_7,4	0,542	0,9939	0,399117	0,5271	0,9715
	pH_5,5	0,4602	0,9688	0,300202	0,7466	0,9974
PM_BSA	pH_7,4	0,7371	0,9901	0,029567	2,2998	0,9687
	pH_5,5	0,776	0,9829	0,172873	0,9929	0,9819
XGPM_Ibu	pH_7,4	0,5666	0,9824	0,178726	0,9275	0,965
	pH_5,5	0,5973	0,8577	0,254488	0,8853	0,9989
XGPM_BSA	pH_7,4	0,5516	0,985	0,239764	0,6535	0,8759
	pH_5,5	0,4514	0,9292	0,202929	1,013	0,9891
TCHT_Ibu	pH_7,4	0,3824	0,9835	0,410122	0,6934	0,9988
	pH_5,5	0,565	0,9936	0,553773	0,3735	0,9986
TCHT_BSA	pH_7,4	0,4556	0,996	0,286333	0,9704	0,9893
	pH_5,5	0,4644	0,9854	0,417822	0,496	0,9992
TXGCHT_Ibu	pH_7,4	0,4564	0,9833	0,470622	0,6084	0,999
	pH_5,5	0,4794	0,9916	0,425624	0,4832	0,963
TXGCHT_BSA	pH_7,4	0,409	0,9791	0,516438	0,4953	0,9882
	pH_5,5	0,496	0,9901	0,378401	0,5152	0,9988
ScCO2 PM_BSA	pH_7,4	0,7742	0,9137	0,093434	1,6732	0,8307
	pH_5,5	0,4256	0,8321	0,132179	0,9186	0,9481
ScCO2 CHT_BSA	pH_7,4	0,2666	0,9781	a	a	a
	pH_5,5	0,2045	0,9872	a	a	a

[a] The power law model was not applicable to the release curve of $ScCO_2$-assisted TCHT.

The drug release profile of the scaffolds show that the structures depend both of the diffusion effect of the drug and the swelling rate of the scaffold. This can be supported by the mathematical modulation, which showed an abnormal distribution. This suggests that the diffusion effect as well as the structure swelling have a determinant role in the release profile. The swelling effect and pores distribution may justify the higher release of both Ibu and BSA on the XG (XGPM and TXGCHT) based scaffolds.

4. Conclusion

The aim of this project was the design of pH sensitive porous devices, with controlled morphological and mechanical proprieties for oral drug delivery applications. There were developed a set of polymeric scaffolds by: (1) freeze-drying method and by (2) gelation process followed by $scCO_2$-assisted method.

The variation of the scaffolds compositions combined with the method of structures processing, allowed tuning the morphological and mechanical properties of the porous scaffolds. Scaffolds prepared with CHT, XG, PM, crosslinked first with MBA, and after with TEMED and APS by freeze-drying method led to smaller porous (till 100μm) and low mechanical compression, while scaffolds produced with PM, XGPM, TCHT and TXGCHT by gelation process followed by $scCO_2$ drying exhibited larger pores (160 μm) and tended to be rigid.

The best scaffolds candidates for a candy structure to treat oral diseases were native PM and XGPM scaffolds because the medium pore size in these structures was 50 μm, the swelling rate higher than 30%, and had high degradation rate (till 9 days).

Regarding the best scaffolds for the gum format, the selected structures were TCHT and TXGCHT. The medium pore size in these structures was 100 μm, the swelling rate lower than 30%, and had low degradation rate (more than 120 days).

The release profile showed to be dependent of both the charge of the medium and swelling effects of the structures. The best matches were scaffolds mixtures with XG, for the candy option XGPM, and for the gum option TXGCHT. The best match in drug release were the XG based scaffolds. For XGPM in the acidic medium, the drug released after 5 h were 80 mg/g of structure. Moreover, for the candy option, after 2.5 h the amount of drug released was 60 mg/g of structure.

Overall, the structures produced by freeze-drying method are more suitable for the purpose of this work, since structures prepared by gelation process followed by $scCO_2$-assisted method presented a weak pH effect.

As future work, the operation conditions of gelation and $scCO_2$-assisted methods should be optimized, for a better tuning of porous network of the scaffolds; (2) specific model drugs, to treat oral diseases, should be impregnated into the scaffolds for further evaluation of release

profile, and (3) biocompatibility tests, regarding crosslinked structures, must be performed in order to evaluate biological compatibility of these structures.

5. References

[1] J. a Maertens, “History of the development of azole derivatives.,” *Clin. Microbiol. Infect.*, vol. 10 Suppl 1, pp. 1–10, 2004.

[2] D. W. Denning and W. W. Hope, “Therapy for fungal diseases: Opportunities and priorities,” *Trends Microbiol.*, vol. 18, no. 5, pp. 195–204, 2010.

[3] V. C. H., L. A., V. J., V. Ph., C. ssement W., D. C. R., and M. R., “Toxicological profile and safety evaluation of antifungal azole derivatives,” *Mycoses*, vol. 32, no. SUPPL. 1, pp. 60–66, 1989.

[4] C. N. FDA Recommendations, “Draft Guidance on Amphotericin B,” vol. 4, no. 2009, pp. 2009–2011, 2014.

[5] N. Takakura, H. Wakabayashi, H. Ishibashi, S. Teraguchi, Y. Tamura, H. Yamaguchi, and S. Abe, “Oral Lactoferrin Treatment of Experimental Oral Candidiasis in Mice,” vol. 47, no. 8, pp. 2619–2623, 2003.

[6] R. López-Martínez, “Candidosis, a new challenge,” *Clin. Dermatol.*, vol. 28, no. 2, pp. 178–184, 2010.

[7] W. T. Godbey, K. K. Wu, and A. G. Mikos, “Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability,” *Biomaterials*, vol. 22, no. 5, pp. 471–480, 2001.

[8] C. L. GEBHART and A. V. KABANOV, “Perspectives on polymeric gene delivery,” *J. Bioact. Compat. Polym.*, vol. 18, no. 2, pp. 147–166, 2003.

[9] D. Schmaljohann, “Thermo- and pH-responsive polymers in drug delivery,” *Adv. Drug Deliv. Rev.*, vol. 58, no. 15, pp. 1655–1670, 2006.

[10] R. J. Whitley, “Herpes Simplex Virus Infection,” vol. 13, no. 1, pp. 6–11, 2002.

[11] E. P. Acosta and J. Balfour H.H., “Acyclovir for treatment of postherpetic neuralgia: Efficacy and pharmacokinetics,” *Antimicrob. Agents Chemother.*, vol. 45, no. 10, pp. 2771–2774, 2001.

[12] R. J. Whitley and B. Roizman, “Herpes simplex virus infections,” vol. 357, pp. 1513–1518, 2001.

[13] R. J. Whitley, D. W. Kimberlin, B. Roizman, R. J. Whitley, and D. W. Kimberlin, “Herpes Simplex Virus,” *Oxford Journals*, vol. 26, no. 3, pp. 541–553, 2012.

[14] K. L. Davis, L. J. Thal, E. R. Gamzu, C. S. Davis, R. F. Woolson, S. I. Gracon, D. A. Drachman, L. S. Schneider, P. J. Whitehouse, T. M. Hoover, J. C. Morris, C. H. Kawas, D. S. Knopman, N. L. Earl, V. Kumar, and R. S. Doody, “The New England Journal of Medicine Downloaded from nejm.org at UNIVERSITY OF WATERLOO on June 24,

2014. For personal use only. No other uses without permission. Copyright © 1992 Massachusetts Medical Society. All rights reserved.," *N. Engl. J. Med.*, vol. 327, pp. 1253–1259, 1992.

[15] D. Criteria, P. Factors, M. Treatment, and P. Indicators, "The First International Consensus on Mucous Membrane Pemphigoid," vol. 138, 2002.

[16] C. Scully and S. Porter, "Oral mucosal disease: Recurrent aphthous stomatitis," *Br. J. Oral Maxillofac. Surg.*, vol. 46, no. 3, pp. 198–206, 2008.

[17] J.-D. Fine, G. R. Neisis, and S. I. Katz, "Immunofluorescence and Immunoelectron Microscopic Studies in Cicatricial Pemphigoid." 1984.

[18] S. Silverman, M. Gorsky, A. Liu, and S. Francisco, "Oral mucous membrane pemphigoid."

[19] R. J. H. Smith, R. B. Sessions, and S. F. Bean, "Benign mucous membrane pemphigoid," *Ann. Otol. Rhinol. Laryngol.*, vol. 91, no. 2 I, pp. 142–144, 1982.

[20] R. S. Rogers, H. O. Perry, S. F. Bean, and R. E. Jordon, "Immunopathology of Cicatricial Pemphigoid: Studies of Complement Deposition," *J. Invest. Dermatol.*, vol. 68, no. 1, pp. 39–43, 1977.

[21] R. S. Rogers, P. J. Sheridan, and R. E. Jordon, "Desquamative gingivitis. Clinical, histopathologic, and immunopathologic investigations," *Oral Surgery, Oral Med. Oral Pathol.*, vol. 42, no. 3, pp. 316–327, 1976.

[22] A. . Fallis, "FOLHETO INFORMATIVO INFARMED: Fluconazol," *J. Chem. Inf. Model.*, vol. 53, no. 9, pp. 1689–1699, 2013.

[23] S. Saurabh and P. K. Sahoo, "Lysozyme : an important defence molecule of fish innate immune system," pp. 223–239, 2008.

[24] T. D. Rees and W. H. Binnie, "Recurrent Aphthous Stomatitis," *Dermatol. Clin.*, vol. 14, no. 2, pp. 243–256, 1996.

[25] S.-B. WOO and S. T. SONIS, "Recurrent Aphthous Ulcers: a Review of Diagnosis and Treatment," *J. Am. Dent. Assoc.*, vol. 127, no. 8, pp. 1202–1213, 1996.

[26] R. Landesberg, M. Fallon, and R. Insel, "Alterations of T helper/inducer and T suppressor/inducer cells in patients with recurrent aphthous ulcers," *Oral Surgery, Oral Med. Oral Pathol.*, vol. 69, no. 2, pp. 205–208, 1990.

[27] S. A. Hospital, "Drug-induced mouth disorders," no. October 1994, pp. 10–18, 1995.

[28] R. Barrons, "Treatment strategies for recurrent oral aphthous ulcers," *Am J Heal. Pharm—Vol 58*, no. FEBRUARY 2001, 2001.

[29] L. P. Samaranayake, W. Keung Leung, and L. Jin, "Oral mucosal fungal infections," *Periodontol. 2000*, vol. 49, pp. 39–59, 2009.

[30] R. López-Martínez, "Candidosis, a new challenge," *Clin. Dermatol.*, vol. 28, no. 2, pp. 178–184, 2010.

[31] G. Sandri, S. Rossi, F. Ferrari, M. C. Bonferoni, C. Muzzarelli, and C. Caramella, "Assessment of chitosan derivatives as buccal and vaginal penetration enhancers," *Eur. J. Pharm. Sci.*, vol. 21, no. 2–3, pp. 351–359, 2004.

[32] D. Patel, A. W. Smith, N. Grist, P. Barnett, and J. D. Smart, "An in vitro mucosal model predictive of bioadhesive agents in the oral cavity," *J. Control. Release*, vol. 61, no. 1–2, pp. 175–183, 1999.

[33] S. Senel, M. J. Kremer, S. Kaş, P. W. Wertz, a a Hincal, and C. a Squier, "Enhancing effect of chitosan on peptide drug delivery across buccal mucosa.," *Biomaterials*, vol. 21, no. 20, pp. 2067–71, 2000.

[34] F. Veuillez, Y. N. Kalia, Y. Jacques, J. Deshusses, and P. Buri, "Factors and strategies for improving buccal absorption of peptides," *Eur. J. Pharm. Biopharm.*, vol. 51, no. 2, pp. 93–109, 2001.

[35] J. Hao and P. W. S. Heng, "Buccal delivery systems.," *Drug Dev. Ind. Pharm.*, vol. 29, no. 8, pp. 821–32, 2003.

[36] V. Dodane, M. Amin Khan, and J. R. Merwin, “Effect of chitosan on epithelial permeability and structure,” *Int. J. Pharm.*, vol. 182, no. 1, pp. 21–32, 1999.

[37] P. Tengamnuay, A. Sahamethapat, A. Sailasuta, and A. K. Mitra, “Chitosans as nasal absorption enhancers of peptides: Comparison between free amine chitosans and soluble salts,” *Int. J. Pharm.*, vol. 197, no. 1–2, pp. 53–67, 2000.

[38] A. Bernkop-Schnürch, “Chitosan and its derivatives: Potential excipients for peroral peptide delivery systems,” *Int. J. Pharm.*, vol. 194, no. 1, pp. 1–13, 2000.

[39] H. Maeda, “A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs,” *Cancer Res.*, vol. 46, no. December, p. 6387, 1986.

[40] F. Boureau, H. Schneid, N. Zeghari, R. Wall, and P. Bourgeois, “The IPSO study: ibuprofen, paracetamol study in osteoarthritis. A randomised comparative clinical study comparing the efficacy and safety of ibuprofen and paracetamol analgesic treatment of osteoarthritis of the knee or hip.,” *Ann. Rheum. Dis.*, vol. 63, no. 9, pp. 1028–34, 2004.

[41] K. D. Rainsford, “Ibuprofen: pharmacology, efficacy and safety,” *Inflammopharmacology*, vol. 17, no. 6, pp. 275–342, 2009.

[42] K. A. Majorek, P. J. Porebski, A. Dayal, M. D. Zimmerman, K. Jablonska, A. J. Stewart, M. Chruszcz, and W. Minor, “Structural and immunologic characterization of bovine, horse, and rabbit serum albumins,” *Mol. Immunol.*, vol. 52, no. 3–4, pp. 174–182, 2012.

[43] J. S. Stamler, O. Jaraki, J. Osborne, D. I. Simon, J. Keaney, J. Vita, D. Singel, C. R. Valeri, and J. Loscalzo, “Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 89, no. 16, pp. 7674–7677, 1992.

[44] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, and P. E. Bourne, “The Protein Data Bank.,” *Nucleic Acids Res.*, vol. 28, no. 1, pp. 235–242, 2000.

[45] T. Sen, K. K. Haldar, and A. Patra, “Au nanoparticle-based surface energy transfer probe for conformational changes of BSA protein,” *J. Phys. Chem. C*, vol. 112, no. 46, pp. 17945–17951, 2008.

[46] R. Langer and N. Peppas, “Chemical and Physical Structure of Polymers as Carriers for Controlled Release of Bioactive Agents: A Review,” *J. Macromol. Sci. Part C*, vol. 23, no. 1, pp. 61–126, 2006.

[47] J. Folkman and D. M. Long, “the Use of Silicone Rubber As a Carrier for Prolonged Drug Therapy.,” *J. Surg. Res.*, vol. 4, no. 3, pp. 139–142, 1964.

[48] R. Langer and J. Folkman, “Polymers for the sustained release of proteins and other macromolecules.,” *Nature*, vol. 263, no. 5580, pp. 797–800, 1976.

[49] S. J. Desai, P. Singh, A. P. Simonelli, and W. I. Higuchi, “Investigation of Factors Influencing Release of Solid Drug Dispersed in Inert Matrices III,” *J. Pharm. Sci.*, vol. 55, no. 11, pp. 1230–1234, 1966.

[50] R. Duncan, “The dawning era of polymer therapeutics.,” *Nat. Rev. Drug Discov.*, vol. 2, no. 5, pp. 347–60, 2003.

[51] J. Kopecek and H. Bazilova, “Poly[N-(2-hydroxypropyl)methacrylamide]-I. Radical polymerization and copolymerization,” *Eur. Polym. J.*, vol. 9, no. 1, pp. 7–14, 1973.

[52] V. Alakhov, E. Klinski, S. Li, G. Pietrzynski, A. Venne, E. Batrakova, T. Bronitch, and A. Kabanov, “Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials,” *Colloids Surfaces B Biointerfaces*, vol. 16, no. 1–4, pp. 113–134, 1999.

[53] T. R. Bhardwaj, M. Kanwar, R. Lal, and a Gupta, “Natural gums and modified natural gums as sustained-release carriers.,” *Drug Dev. Ind. Pharm.*, vol. 26, no. 10, pp. 1025–38, 2000.

[54] A. M. Avachat, R. R. Dash, and S. N. Shrotriya, “Recent Investigations of Plant Based Natural Gums , Mucilages and Resins in Novel Drug Delivery Systems,” *Indian J. Pharm. Eduation Res.*, vol. 45, no. 1, pp. 86–99, 2011.

[55] V. D. Prajapati, G. K. Jani, N. G. Moradiya, and N. P. Randeria, *Pharmaceutical applications of various natural gums, mucilages and their modified forms*, vol. 92, no. 2. Elsevier Ltd., 2013.

[56] A. Lohani, G. Singh, S. S. Bhattacharya, and A. Verma, "Interpenetrating polymer networks as innovative drug delivery systems.," *J. Drug Deliv.*, vol. 2014, no. Figure 1, p. 583612, 2014.

[57] A. Di Martino, M. Sittinger, and M. V. Risbud, "Chitosan: A versatile biopolymer for orthopaedic tissue-engineering," *Biomaterials*, vol. 26, no. 30, pp. 5983–5990, 2005.

[58] T. Barroso, R. Viveiros, T. Casimiro, and A. Aguiar-Ricardo, "Development of dual-responsive chitosan-collagen scaffolds for pulsatile release of bioactive molecules," *J. Supercrit. Fluids*, vol. 94, pp. 102–112, 2014.

[59] P. He, S. S. Davis, and L. Illum, "In vitro evaluation of the mucoadhesive properties of chitosan microspheres," *Int. J. Pharm.*, vol. 166, no. 1, pp. 75–88, 1998.

[60] L. Illum, "Chitosan and its use as a pharmaceutical excipient, Pharmaveutical Research, Vol. 15, No. 9." 1998.

[61] & W. M. A. Carter, S.M., "from the SAGE Social Science Collections . Reserved . Downloaded All from Rights," *Small Gr. Res.*, vol. 29 no. 5, pp. 583–601, 1998.

[62] F. Bugamelli, M. A. Raggi, I. Orienti, and V. Zecchi, "Controlled insulin release from chitosan microparticles," *Arch. Pharm. (Weinheim).*, vol. 331, no. 4, pp. 133–138, 1998.

[63] C. M. Lehr, J. A. Bouwstra, E. H. Schacht, and H. E. Junginger, "In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers," *Int. J. Pharm.*, vol. 78, no. 1–3, pp. 43–48, 1992.

[64] M. Dash, F. Chiellini, R. M. Ottenbrite, and E. Chiellini, "Chitosan - A versatile semi-synthetic polymer in biomedical applications," *Prog. Polym. Sci.*, vol. 36, no. 8, pp. 981–1014, 2011.

[65] J. D. Smart, I. W. Kellaway, and H. E. C. Worthington, "An in-vitro investigation of mucosa-adhesive materials for use in controlled drug delivery," *J. Pharm. Pharmacol.*, vol. 36, no. 5, pp. 295–299, 1984.

[66] P. I. Lee, "Polymers for controlled drug delivery," *J. Control. Release*, vol. 29, no. 1–2, pp. 202–203, 1994.

[67] J. R. Robinson and G. M. Mlynek, "Bioadhesive and phase-change polymers for ocular drug delivery," *Adv. Drug Deliv. Rev.*, vol. 16, no. 1, pp. 45–50, 1995.

[68] B. P. a. Peppas N. A., "Surface, Inerfacial and Molecular Aspects of Polymer Bioadhesion on Soft Tissues," *J. Control. Release*, vol. 2, pp. 257–275, 1985.

[69] S. S. Illum, Lisbeth; Farraj, Nidal F.; Davis, "Chitosan as a Novel Nasal Delivery System for Peptide Drugs; Pharmaceutical Research, Vol. 11, No. 8." 1994.

[70] W. Cheng and L. Y. Lim, "Peroral delivery of peptide drugs," *Am. Pharm. Rev.*, vol. 11, no. 3, pp. 329–338, 2008.

[71] T. J. Aspden, L. Illum, and Skaugrud, "Chitosan as a nasal delivery system: Evaluation of insulin absorption enhancement and effect on nasal membrane integrity using rat models," *Eur. J. Pharm. Sci.*, vol. 4, no. 1, pp. 23–31, 1996.

[72] H. Santos, F. Veiga, M. E. Pina, and J. J. Sousa, "Compaction, compression and drug release properties of diclofenac sodium and ibuprofen pellets comprising xanthan gum as a sustained release agent," *Int. J. Pharm.*, vol. 295, no. 1–2, pp. 15–27, 2005.

[73] N. Garti, "Hydrocolloids As Emulsifying Agents for Oil-in-Water Emulsions," *J. Dispers. Sci. Technol.*, vol. 20, no. 1–2, pp. 327–355, 1999.

[74] S. Rosalam and R. England, "Review of xanthan gum production from unmodified starches by Xanthomonas comprestris sp.," *Enzyme Microb. Technol.*, vol. 39, no. 2, pp. 197–207, 2006.

[75] O. N. El-Gazayerly, "Release of pentoxifylline from xanthan gum matrix tablets.," *Drug Dev. Ind. Pharm.*, vol. 29, no. 2, pp. 241–6, 2003.

[76] X. Mu, M. J. Tobyn, and J. N. Staniforth, “Influence of Physiological Variables on the In Vitro Drug-Release Behavior of a Polysaccharide Matrix Controlled-Release System,” *Analyzer*, vol. 29, no. 1, pp. 19–29, 2003.

[77] J. N. Staniforth and A. R. Baichwal, “TIMERx: novel polysaccharide composites for controlled/programmed release of drugs in the gastrointestinal tract.,” *Expert Opin. Drug Deliv.*, vol. 2, no. 3, pp. 587–95, 2005.

[78] M. M. Talukdar, I. Vinckier, P. Moldenaers, and R. Kinget, “Rheological characterization of xanthan gum and hydroxypropylmethyl cellulose with respect to controlled-release drug delivery.,” *J. Pharm. Sci.*, vol. 85, no. 5, pp. 537–40, 1996.

[79] H. Omidian, S. Fesharaki, and K. Park, “Controlled Release in Oral Drug Delivery,” 2011.

[80] M. J. Caulfield, X. Hao, G. G. Qiao, and D. H. Solomon, “Degradation on polyacrylamides. Part II. Polyacrylamide gels,” *Polymer (Guildf).*, vol. 44, no. 14, pp. 3817–3826, 2003.

[81] M. J. Caulfield, X. Hao, G. G. Qiao, and D. H. Solomon, “Degradation on polyacrylamides. Part I. Linear polyacrylamide,” *Polymer (Guildf).*, vol. 44, no. 5, pp. 1331–1337, 2003.

[82] S. H. Ou, M. C. Wu, T. C. Chou, and C. C. Liu, “Polyacrylamide gels with electrostatic functional groups for the molecular imprinting of lysozyme,” vol. 504, pp. 163–166, 2004.

[83] P. Guo, Y. Yuan, and F. Chi, “Biomimetic alginate/polyacrylamide porous scaffold supports human mesenchymal stem cell proliferation and chondrogenesis,” *Mater. Sci. Eng. C*, vol. 42, pp. 622–628, 2014.

[84] a Thakur, S. Monga, and R. K. Wanchoo, “Sorption and Drug Release Studies from Semi-interpenetrating Polymer Networks of Chitosan and Xanthan Gum,” vol. 28, no. 1, pp. 105–115, 2014.

[85] L. Yin, L. Fei, F. Cui, C. Tang, and C. Yin, “Superporous hydrogels containing poly(acrylic acid-co-acrylamide)/O-carboxymethyl chitosan interpenetrating polymer networks,” *Biomaterials*, vol. 28, no. 6, pp. 1258–1266, 2007.

[86] M. Pulat, A. S. Kahraman, N. Tan, and M. Gümüşderelioğlu, “Sequential antibiotic and growth factor releasing chitosan-PAAm semi-IPN hydrogel as a novel wound dressing.,” *J. Biomater. Sci. Polym. Ed.*, vol. 24, no. 7, pp. 807–19, 2013.

[87] L. Qian and H. Zhang, “Controlled freezing and freeze drying : a versatile route for porous and micro- / nano-structured materials †,” no. July 2010, pp. 172–184, 2011.

[88] X.-Z. Zhang, P. Jo Lewis, and C.-C. Chu, “Fabrication and characterization of a smart drug delivery system: microsphere in hydrogel.,” *Biomaterials*, vol. 26, no. 16, pp. 3299–309, 2005.

[89] P. Mukhopadhyay, K. Sarkar, S. Bhattacharya, A. Bhattacharyya, R. Mishra, and P. P. Kundu, “pH sensitive N-succinyl chitosan grafted polyacrylamide hydrogel for oral insulin delivery,” *Carbohydr. Polym.*, vol. 112, pp. 627–637, 2014.

[90] L. Rey and J. C. May, *Freeze Drying / Lyophilization of Pharmaceutical and Biological Products*, vol. 206. 2010.

[91] H. W. Kang, Y. Tabata, and Y. Ikada, “Fabrication of porous gelatin scaffolds for tissue engineering.,” *Biomaterials*, vol. 20, no. 14, pp. 1339–1344, 1999.

[92] M. G. Haugh, D. Ph, C. M. Murphy, B. Sc, F. J. O. Brien, and D. Ph, “Novel Freeze-Drying Methods to Produce a Range of Collagen – Glycosaminoglycan Scaffolds with Tailored Mean Pore Sizes,” vol. 16, no. 5, 2010.

[93] V. Koot, J. Bergmeijer, and J. Molenaar, “Lyophylized Dura Patch Repair of Congenital Diaphragmatica Hernia: Occurrence of Relapses,” pp. 667–668, 1992.

[94] X. Tang and M. J. Pikal, “Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice,” *Pharm. Res.*, vol. 21, no. 2, pp. 191–200, 2004.

[95] Y. V. Griko, S. Y. Venyaminov, and P. L. Privalov, “Heat and cold denaturation of phosphoglycerate kinase (interaction of domains),” *FEBS Lett.*, vol. 244, no. 2, pp. 276–

278, 1989.

[96] Privalov P.L., “Cold denaturation of proteins,” *Crit. Rev. Biochem. Mol. Biol*, vol. 25, no. 4, pp. 281–305, 1990.

[97] V. R. Patel and M. M. Amiji, “Preparation and characterization of freeze-dried chitosan-poly(ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach,” *Pharmaceutical Research*, vol. 13, no. 4. pp. 588–593, 1996.

[98] B. S. Chang, B. S. Kendrick, and J. F. Carpenter, “Surface-induced denaturation of proteins during freezing and its inhibition by surfactants,” *J. Pharm. Sci.*, vol. 85, no. 12, pp. 1325–1330, 1996.

[99] J. F. Carpenter, S. J. Prestrelski, and T. Arakawa, “Separation of Freezing- and Dring-Induced Denaturation of Lyophilized Proteins Using Stress-Specific Stabilization; Archives of Biochemistry and Biophysics.” pp. 456–464, 1993.

[100] E. Ozdemir, A. Sendemir-urkmez, and O. Yesil-celiktas, “The Journal of Supercritical Fluids Supercritical CO 2 processing of a chitosan-based scaffold : Can implantation of osteoblastic cells be enhanced ?,” *J. Supercrit. Fluids*, vol. 75, pp. 120–127, 2013.

[101] H. Tai, M. L. Mather, D. Howard, W. Wang, L. J. White, J. a. Crowe, S. P. Morgan, A. Chandra, D. J. Williams, S. M. Howdle, and K. M. Shakesheff, “Control of pore size and structure of tissue engineering scaffolds produced by supercritical fluid processing,” *Eur. Cells Mater.*, vol. 14, pp. 64–76, 2007.

[102] S. M. Lee, J. H. Han, K. Y. Kim, Y. J. Ahn, and J. W. Lee, “Sang Myung Lee, Jae Ho Han, Kyung Yl Kim, Young Joon Ahn and Jae Wook Lee*,” vol. 18, no. 2, pp. 83–90, 2010.

[103] S. P. Nalawade, F. Picchioni, and L. P. B. M. Janssen, “Chapter 1 Supercritical carbon dioxide as a green solvent for processing polymers,” pp. 1–16.

[104] J. R. Royer, Y. J. Gay, J. M. Desimone, and S. A. Khan, “High-pressure rheology of polystyrene melts plasticized with CO2: Experimental measurement and predictive scaling relationships,” *J. Polym. Sci. Part B Polym. Phys.*, vol. 38, no. 23, pp. 3168–3180, 2000.

[105] J. R. Royer, Y. J. Gay, M. Adam, J. M. Desimone, and S. a Khan, “Polymer melt rheology with high-pressure CO 2 using a novel magnetically levitated sphere rheometer,” *Polymer (Guildf).*, vol. 43, pp. 2375–2383, 2002.

[106] D. L. Tomasko, H. B. Li, D. H. Liu, X. M. Han, M. J. Wingert, L. J. Lee, and K. W. Koelling, “A review of CO(2) applications in the processing of polymers,” *Ind. Eng. Chem. Res.*, vol. 42, no. 25, pp. 6431–6456, 2003.

[107] T. Sarbu, T. Styranec, and E. J. Beckman, “Non-fluorous polymers with very high solubility in supercritical CO2 down to low pressures,” *Nature*, vol. 405, no. 6783, pp. 165–168, 2000.

[108] E. J. Beckman, “A challenge for green chemistry: designing molecules that readily dissolve in carbon dioxide.,” *Chem. Commun. (Camb).*, no. 17, pp. 1885–1888, 2004.

[109] O. R. Davies, A. L. Lewis, M. J. Whitaker, H. Tai, K. M. Shakesheff, and S. M. Howdle, “Applications of supercritical CO2 in the fabrication of polymer systems for drug delivery and tissue engineering,” *Adv. Drug Deliv. Rev.*, vol. 60, no. 3, pp. 373–387, 2008.

[110] O. R. Davies, A. L. Lewis, M. J. Whitaker, H. Tai, K. M. Shakesheff, and S. M. Howdle, “Applications of supercritical CO2 in the fabrication of polymer systems for drug delivery and tissue engineering,” *Adv. Drug Deliv. Rev.*, vol. 60, no. 3, pp. 373–387, 2008.

[111] C. Gualandi, L. J. White, L. Chen, R. A. Gross, K. M. Shakesheff, S. M. Howdle, and M. Scandola, “Acta Biomaterialia Scaffold for tissue engineering fabricated by non-isothermal supercritical carbon dioxide foaming of a highly crystalline polyester,” *Acta Biomater.*, vol. 6, no. 1, pp. 130–136, 2010.

[112] T. Barroso, M. Temtem, T. Casimiro, and A. Aguiar-Ricardo, “Development of pH-responsive poly(methylmethacrylate-co-methacrylic acid) membranes using scCO2 technology. Application to protein permeation,” *J. Supercrit. Fluids*, vol. 51, pp. 57–66, 2009.

[113] M. Temtem, T. Barroso, T. Casimiro, J. F. Mano, and A. Aguiar-Ricardo, “Dual stimuli responsive poly(N-isopropylacrylamide) coated chitosan scaffolds for controlled release prepared from a non residue technology,” *J. Supercrit. Fluids*, vol. 66, pp. 398–404, 2012.

[114] A. Figueiras, A. a C. C. Pais, and F. J. B. Veiga, “A comprehensive development strategy in buccal drug delivery.,” *AAPS PharmSciTech*, vol. 11, no. 4, pp. 1703–12, 2010.

[115] E. Wenk, H. P. Merkle, and L. Meinel, “Silk fibroin as a vehicle for drug delivery applications,” *J. Control. Release*, vol. 150, no. 2, pp. 128–141, 2011.

[116] Y. Luo and Q. Wang, “Recent development of chitosan-based polyelectrolyte complexes with natural polysaccharides for drug delivery,” *Int. J. Biol. Macromol.*, vol. 64, pp. 353–367, 2014.

[117] M. M. Talukdar and R. Kinget, “Swelling and Drug-Release Behavior of Xanthan Gum Matrix Tablets,” *Int. J. Pharm.*, vol. 120, pp. 63–72, 1995.

[118] T. Fernandes, A. Aguiar-Ricardo, and A. C. Roque, “Functional monolithic platforms for antibody purification; PHD Dicertation,” no. January, 2014.

[119] T. Barroso, R. Viveiros, T. Casimiro, and A. Aguiar-Ricardo, “Development of dual-responsive chitosan-collagen scaffolds for pulsatile release of bioactive molecules,” *J. Supercrit. Fluids*, vol. 94, pp. 102–112, 2014.

[120] J. Siepmann, “Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC),” *Adv. Drug Deliv. Rev.*, vol. 48, no. 2–3, pp. 139–157, 2001.

[121] J. Siepmann and F. Siepmann, “Mathematical modeling of drug delivery,” *Int. J. Pharm.*, vol. 364, no. 2, pp. 328–343, 2008.

[122] E. Physics and I. N. Feld, “A numerical experiment on pore size , pore connectivity , water retention , permeability , and solute transport using network models,” no. March, pp. 99–105, 2000.

[123] S. Vossoughi, “Profile modification using in situ gelation technology - a review,” *J. Pet. Sci. Eng.*, vol. 26, no. 1–4, pp. 199–209, 2000.

[124] A. Pawlak and M. Mucha, “Thermogravimetric and FTIR studies of chitosan blends,” vol. 396, pp. 153–166, 2003.

[125] M. N. Khalid, F. Agnely, N. Yagoubi, J. L. Grossiord, and G. Couarraze, “Water state characterization, swelling behavior, thermal and mechanical properties of chitosan based networks,” *Eur. J. Pharm. Sci.*, vol. 15, no. 5, pp. 425–432, 2002.

[126] P. S. Gils, D. Ray, and P. K. Sahoo, “Characteristics of xanthan gum-based biodegradable superporous hydrogel,” *Int. J. Biol. Macromol.*, vol. 45, no. 4, pp. 364–371, 2009.

[127] R. Magaraphan, W. Lilayuthalert, A. Sirivat, and J. W. Schwank, “Preparation, structure, properties and thermal behavior of rigid-rod polyimide/montmorillonite nanocomposites,” *Compos. Sci. Technol.*, vol. 61, no. 9, pp. 1253–1264, 2001.

[128] M. Muntó, N. Ventosa, S. Sala, and J. Veciana, “Solubility behaviors of ibuprofen and naproxen drugs in liquid ‘CO2-organic solvent’ mixtures,” *J. Supercrit. Fluids*, vol. 47, no. 2, pp. 147–153, 2008.

[129] M. M. Talukdar, A. Michoel, P. Rombaut, and R. Kinget, “Comparative study on xanthan gum and hydroxypropylmethyl cellulose as matrices for controlled-release drug delivery I. Compaction and in vitro drug release behaviour,” *Int. J. Pharm.*, vol. 129, no. 1–2, pp. 233–241, 1996.

6. Appendix

I. Scaffolds production

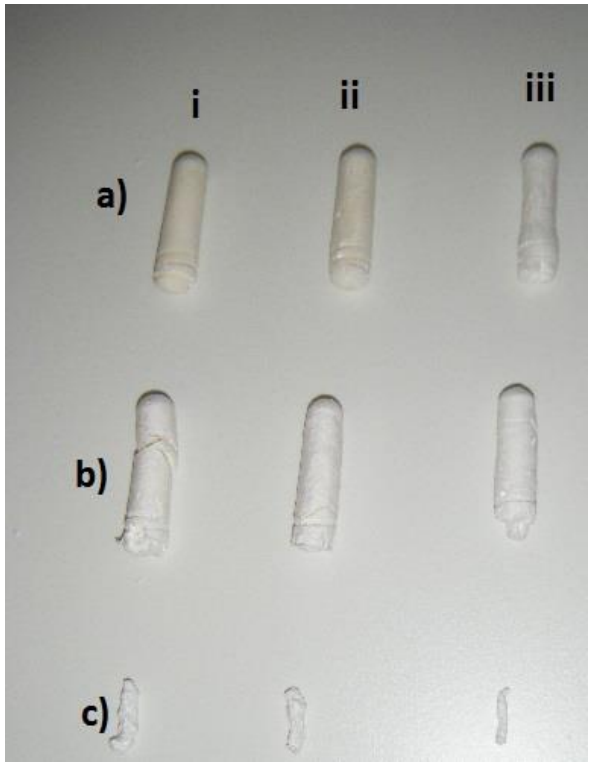

Figure 6.1-Freeze-dry scaffolds crosslinked with MBA; a) CHT scaffolds; b) XG scaffolds and c) PM scaffolds.

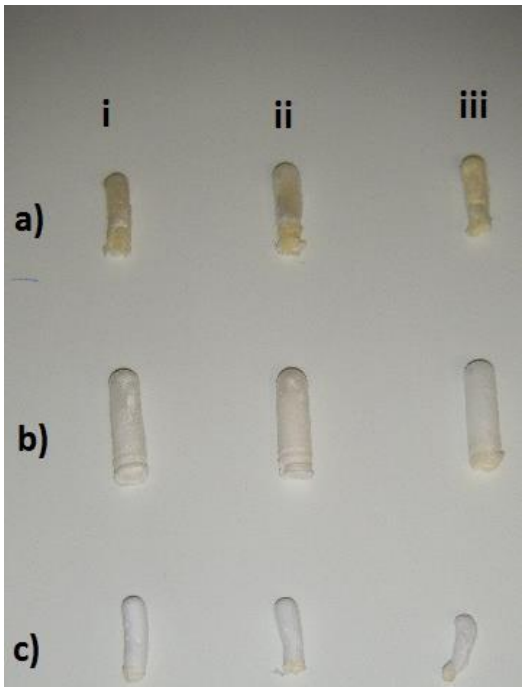

Figure 6.2- Freeze-dry scaffolds crosslinked with TEMED and APS; a) CHT scaffolds; b) XG scaffolds and c) PM scaffolds. i) 3% wt/wt polymer; ii) 2% wt/wt polymer and iii) 1% wt/wt polymer

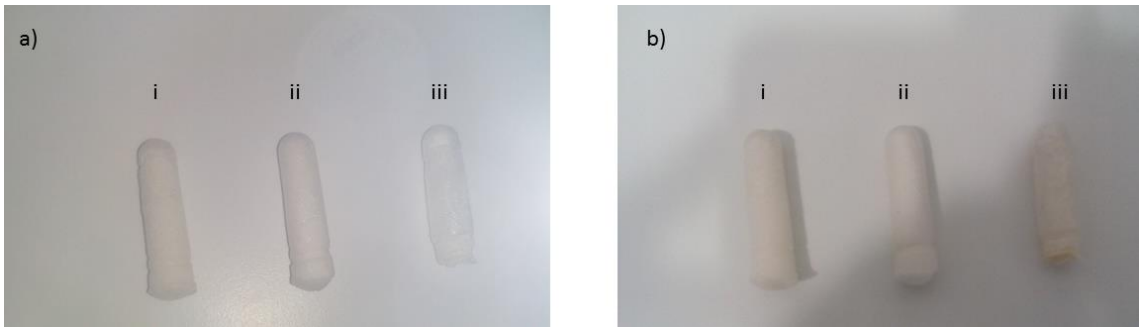

Figure 6.3- Freeze-dry scaffolds of polymers mixtures; a) XGPM 2% wt/wt scaffolds; b) XGCHT 2% wt/wt scaffolds i) native scaffolds, ii) crossliked with MBA and iii) crosslinked with TEMED and APS

II. FTIR-ATR analysis

The FTIR-ATR analysis was only performed on scaffolds processed by freeze-drying. The impregnation on scaffolds were prepared only by the best characterized option.

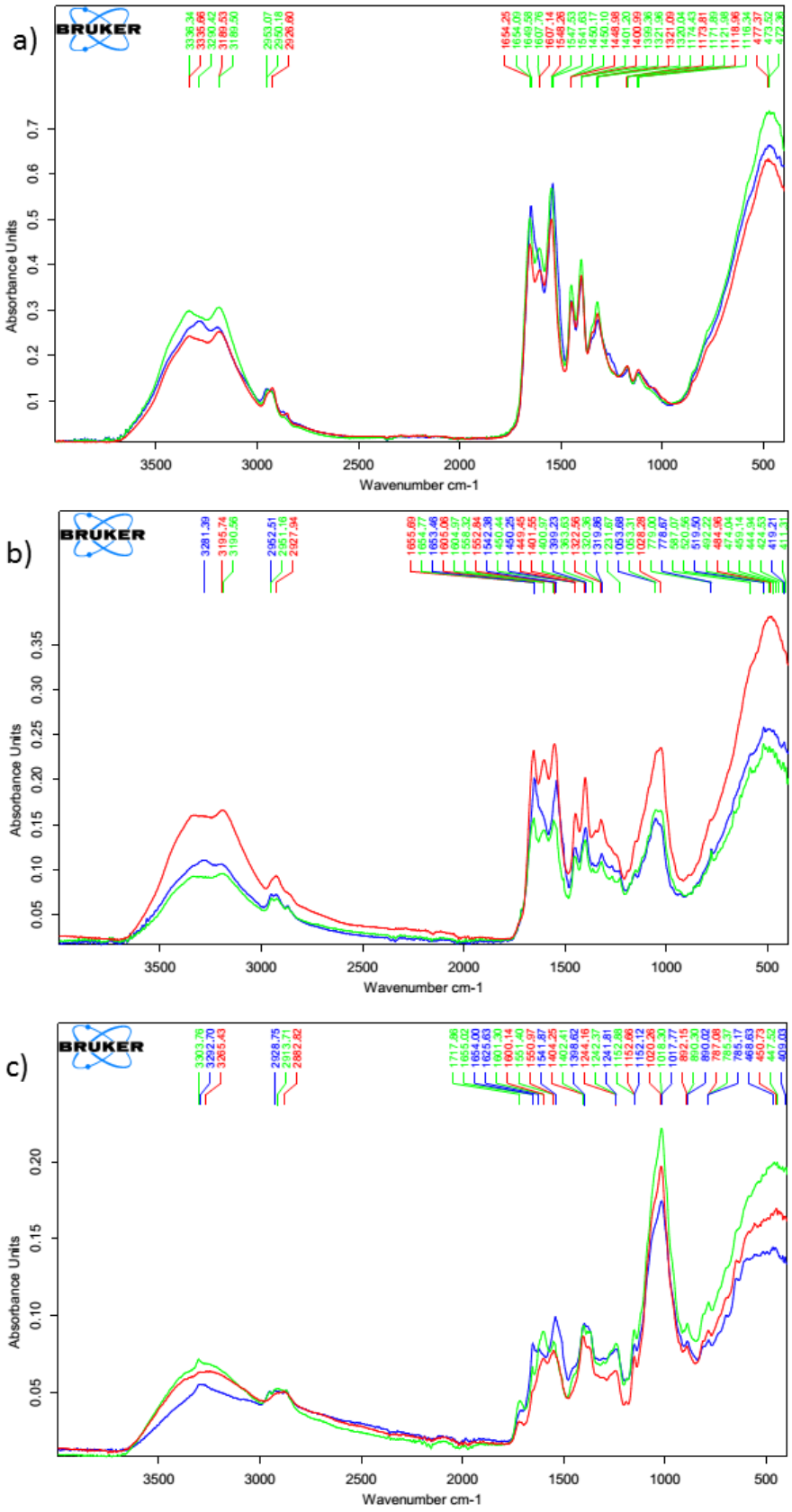

Figure 6.4- FTIR-ATR analysis of scaffolds impregnated with BSA and Ibu a)PM b)XGPM c)TXGCHT

III. Swelling analysis

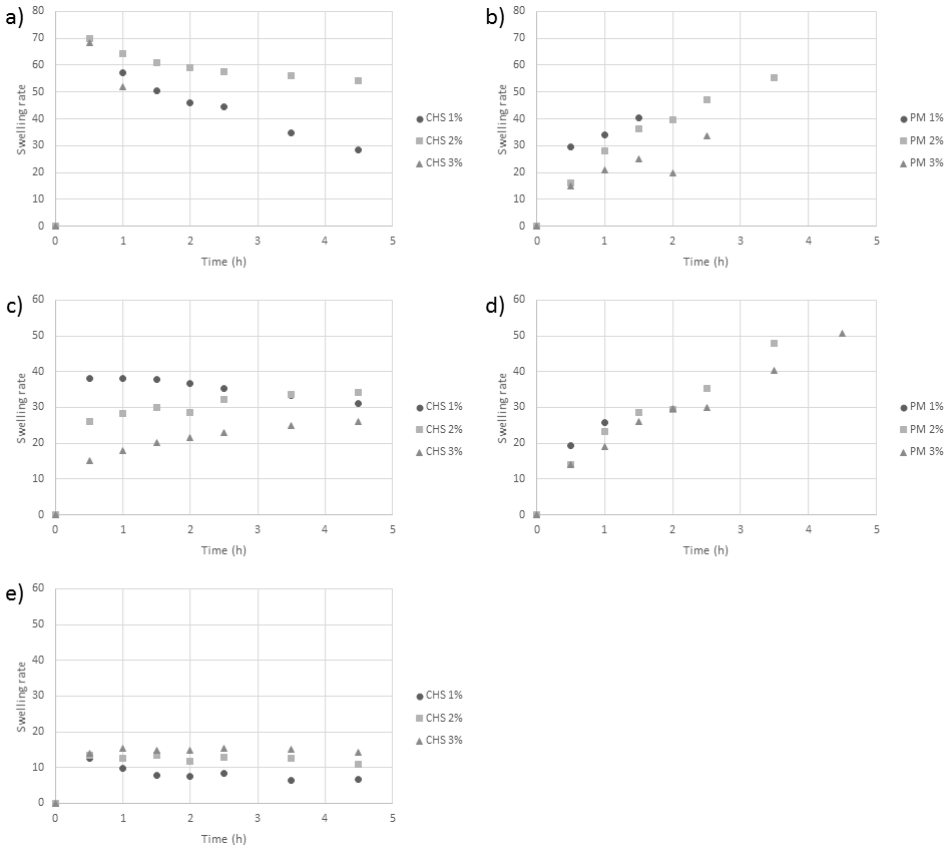

Figure 6.5- Swelling rate of scaffolds obtain by freeze-drying method at pH 9: a) CHT native scaffolds; b) PM native scaffolds; c) CHT crosslinked with MBA d) PM crosslinked with MBA and e) CHT crosslinked with TEMED and APS

IV. Release studies

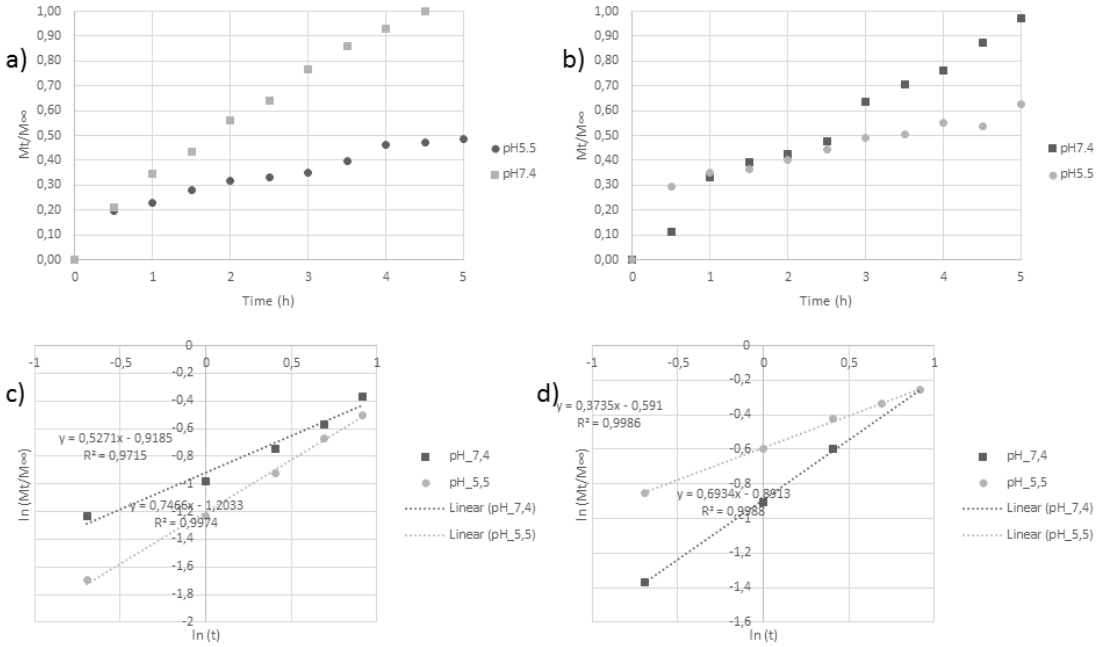

Figure 6.6- Ibu release profile of freeze-drying obtain scaffolds a) PM 3% wt/wt, b) TCHT 3% wt/wt and mathematical modulation of the best method (power law) c) PM and d) TCHT

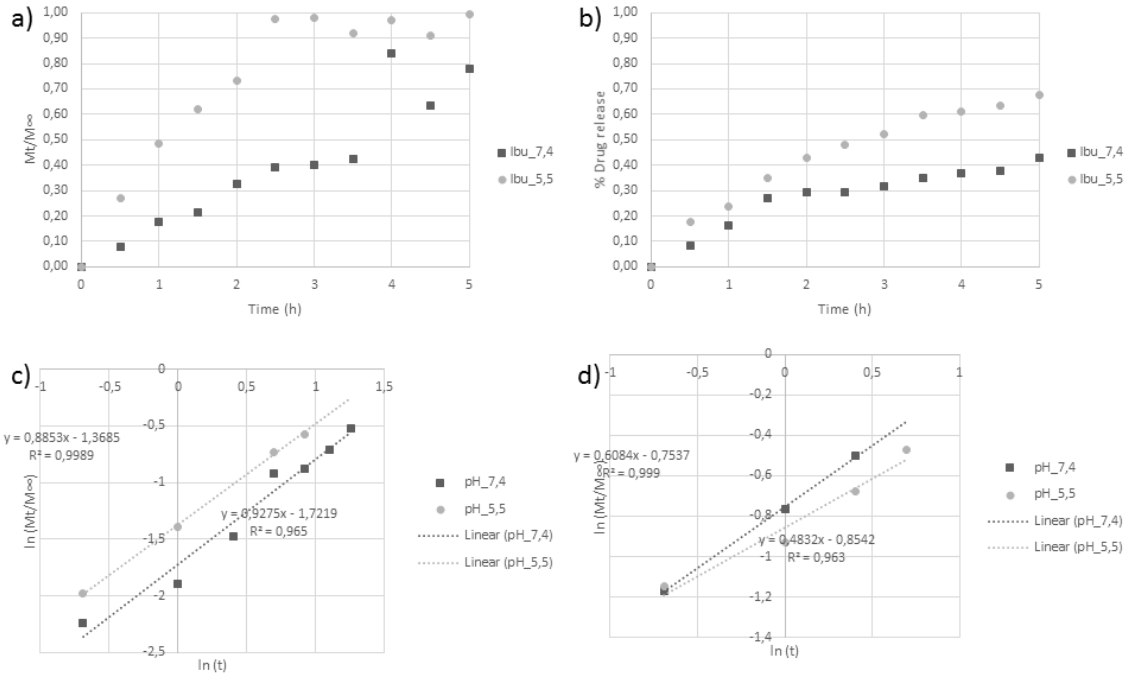

Figure 6.7- Ibu release profile of freeze-drying obtain scaffolds a) XGPM 2% wt/wt, b) TXGCHT 2% wt/wt and mathematical modulation of the best method (power law) c) XGPM and d) TXGCHT