Anyse Sofia Fernandes Pereira

Pharmacy Bachelor

Synthesis of micro-carriers for bioactive compounds transport and controlled release in colorectal tumors

Thesis submitted to the Faculty of Science and Technology

in partial fulfillment of the requirements for the degree

of Master of Science In Molecular Genetic and Biomedicine

Supervisor: Arturo Álvarez-Bautista

Juri:

President: Doctor Arturo Álvarez-Bautista

Examiner: Doctor Lorenzo Pastrana

Member(s): Doctor Paula Gonçalves

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Anyse Sofia Fernandes Pereira,
FCT/UNL

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After an intensive period of ten months, today is the day: writing this note of thanks is the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

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ABSTRACT

According to the World Cancer Report statistics provided by WHO, colorectal cancer (CRC) is one of the most aggressive and lethal types of cancer, standing on the top five ranking. The therapeutic efficacy of many anti-cancer drugs is limited by their poor penetration into tumor tissue and their adverse effects on healthy cells. Nano/microcarriers carriers stand as good candidates as transport means where bioactives for diagnosis, treatment and/or prophylaxis could be entrapped, or platforms where ligands, receptors or therapeutic agents could be anchored. Inverse microemulsion (IM) method is reported for preparing monodispersed phenetyl isothiocyanate (PEITC) loaded chitosan coated citrus pectin particles, which posteriorly were analyzed with respect to their morphology and pH stimuli responsiveness. Crosslinked unloaded and ibuprofen loaded chitosan:citrus pectin microspheres were prepared by a spray drying method. The parameters studied were the surface morphology and loading capacity. Complementarily, impregnation by supercritical CO2 was performed in spray dried unloaded particles as an alternative encapsulation method, comparing the effects of slow and rapid depressurization in encapsulation efficiency. The particles synthesized by IM presented a regular surface, spheroid shape and nearly uniform particle size distribution. The pH stimuli response was confirmed with particle size increasing or decreasing according to the medium acidification or alkalization. It was found that the size of the particles is directly proportional to the number of days of crosslinking reaction. Spray dried unloaded particles showed a spheroid shape and smooth surface with small concavities; while ibuprofen loaded particles presented a highly irregular surface and shape with a collapsed surface. This fact may be due to ionic interactions between drug and chitosan. The loading of 89% rate was very positive, and proved that this is a great method for encapsulating actives. Encapsulation by impregnation showed less than 1% of encapsulation efficiency, both for slow and rapid depressurization.

Key words: colorectal cancer, polymeric particles, natural extracts, supercritical CO_2, spray drying, inverse microemulsion

RESUMO

De acordo com as estatísticas da OMS (World Cancer Report) o cancro colorectal é um dos mais agressivos e letais, posicionando-se entre os cinco primeiros, com 694.000 só em 2012. A eficácia terapêutica de muitas drogas anticancerígenas é limitada pela fraca penetração no tecido tumoral e pelos efeitos adversos nas células saudáveis. Nano/micropartículas são uma boa alternativa como meio de transporte aonde agentes bioactivos para diagnóstico, tratamento e/ou profilaxia podem ser encapsulados, ou plataformas aonde ligandos, receptores ou agentes terapêuticos possam ser ancorados. O método de microemulsão inversa foi usado para a preparação de partículas monodispersas de pectina cítrica recobertas com quitosano, carregadas com PEITC, que depois foram posteriormente analisadas quanto à sua morfologia e resposta às mudanças de pH. Microesferas reticuladas de quitosano:pectina cítrica foram preparadas por spray drying. Os parâmetros estudados foram a morfologia da superfície e capacidade de encapsulação. Complementarmente, foi feita uma impregnação por CO_2 supercrítico nas partículas vazias produzidas por spray drying, comparando os efeitos da despressurização rápida e lenta na eficiência da encapsulação. As partículas sintetizadas por microemulsão inversa apresentaram uma superfície regular, forma esférica e uma distribuição de tamanho uniforme. A resposta à variação pH foi confirmada com o tamanho da parícula a aumentar e a diminuir consoante com a acidificação ou alcalinização do meio. Foi demonstrado que o tamanho das partículas é directamente proporcional ao número de dias de reacção de cruzamento. As partículas descarregadas do spray drying apresentaram uma forma esferóide com superfície lisa com pequenas concavidades, enquanto que as partículas carregadas mostraram um superfície e forma irregulares com uma superfície colapsada. Isto deve-se a interacções entre a droga e o quitosano. Uma percentagem, muito positiva, de encapsulamento de 89%, foi conseguida provando que esta é uma forma muito eficaz de encapsular activos. A encapsulação por impregnação foi de menos de 1%, tanto para a despressurização lenta como para a rápida.

Palavras-chave: cancro colon-rectal, partículas poliméricas, extractos naturais, CO_2supercrítico, spray drying, microemulsão inversa

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List of abbreviations

CRC – colorectal cancer

CS:CPect – Chitosan/citrus pectin

DLS – Differential light scattering

DSC - Differential scanning calorimetry

EPR - Enhanced Permeability and Retention

GIT – Gastrointestinal tract

IARC - International Agency for Research on Cancer

IBET – Instituto de Biologia Experimental e Tecnológica

IBf – the final amount of ibuprofen

IBi – the initial amount of ibuprofen

IM – Inverse microemulsion

IPO – Instituto Português de Oncologia

IST – Instituto Superior Técnico

ITQB – Instituto de Tecnologia Química e Biológica

NMR - Nuclear magnetic resonance

PCS - correlation spectroscopy

PdI – Polydispersity Index

PEC – polyelectrolyte complex

PEITC – Phenetyl isothiocyanate

QELS - quasi-elastic light scattering

RD – Rapid depressurization

RIP – Ribosome inactivating proteins

SD – Slow depressurization

SD - Spray Drying

SEM (Scanning Electronic Microscopy)

SSCO2 – Supercritical CO2

TEM (Transmition Electronic Microscopy)

TPf – the final amount of powder (chitosan+pectin+ibuprofen)

TPi – The initial amount of powder (chitosan+pectin+ibuprofen)

YP – Yield of production

1. INTRODUCTION

1.1 – HISTORICAL AND SOCIOCULTURAL FRAMEWORK

As the human race evolves in knowledge and technologies, lifestyle and general habits, tends to follow these changes. So there are, for each stage of human history, some aspects that characterize that particular age. For many scholars, 20th century shall be known as the Cancer´s Century. Ever since Hippocrates and his disciples had first described cancer cells agglomerates as *carcinos* and carcinoma (from Greek Karkinos, meaning crab), because of the structural similarity of the cell projections that seems to emerge from tumours as crab´s claws (*Taylor et al, 2015*), to this day, cancer has, certainly, made millions or even billions of victims. According to Sontag (*Sontag, 1978*), cancer was perceived, not like other diseases, but like a malediction that could even represent banishment of the affected person from social interaction. It was known as the disease that "does not knock before it enters", and, unlike other diseases, it did not seem to be dependent from economic conditions of the patients. The very word "cancer" was, for many decades, filled with stigma, and it was, literally a synonym for death. It distinguished itself from other diseases as there was no ultimate and absolute solution, a universal treatment, a cure, as penicillin was for tuberculosis, the disease that astonished the civilization for a long period of time (*Diamandopoulus, 1996*). **Table 1.1** presents a small review of historical society views about that disease, along with some advances in the field of psycho-oncology.

Table 1.1 - Historical perspective about society attitudes with regard to cancer patients, and development of psycho-oncology in USA. Retrieved from: Holland, 2002.

1800s	Prevailing attitude was that cancer equals death
	Diagnosis never revealed to patient
	Fatalistic acceptance of diagnosis by doctor and family
1900-20s	American Cancer Society formed to fight fatalism and educate public that early treatment by surgery could be curative
1930s	National Cancer Institute formed to seek a cure for cancer (1937)
1950s	Combined modalities lead to increased survival in Hodgkin's disease, acute lymphoblastic leukemia (ALL), and childhood tumours
	First studies of psychological response to cancer reported
1960-70s	Debate about telling or not telling diagnosis; change to revealing the diagnosis; federally mandated guidelines for informed consent

	Psychosomatic research in psychiatry led to focus on studies of psychological causes for cancer
1980s	Prevalence studies of psychiatric and psychological sequelae in cancer
	Psycho-oncology units develop in larger cancer centers
	Education of public about lifestyle and cancer prevention; behavioral research in changing habits (eg, smoking), diet, and lifestyle
1990s	Health-related Quality of Life assessment was accepted as an outcome measure in clinical trials
	Intervention studies undertaken to impact quality of life and reduce distress
	Development of psychoneuroimmunology and exploration of its relevance to cancer and risk/ survival
2000	Development of standards for psychosocial care and clinical practice guidelines

At present, cancer patients have rid themselves of that stigma, for the advances of science and technology have demystified the concept that cancer means death. Especially for the last ten years, the scientific research about cancer has seen widespread growth, and each day more and more drugs and treatment option have been arising from that tireless effort (*American Cancer Society, 2016a*). Although that subject has come to occupy a prime position in the field of health related sciences and industries, the challenge is far from being won. Today, cancer is a constant reminder of the impotence of the human being when it comes to control illness and death.

1.2 - COLORECTAL CANCER

According to the statistics provided by GLOBOCAN in their last worldwide cancer report, just in 2012, the most recent year for which international estimates are available, there has been 14 million new cases and 8.2 million of people dying from some form of cancer (*Stimpfel and Virant-Klun, 2016*). Of those, Colorectal cancer (CRC) is one of the most aggressive and deadly cancer types, standing on the top five rank, with 694 000 deaths, just in 2012 (*Ferlay et al, 2014*). It is the third most common type of cancer in men and the second in women, with almost 55% of the cases occurring in most developed countries, although, in terms of incidence/mortality ratio, mortality is higher in less developed countries (*Siegel et al, 2015*). **Figure 1.1** and **Table 1.2** represent the main results of the last World´s Cancer Statisctics report, performed by the International Agency for Research on Cancer (IARC).

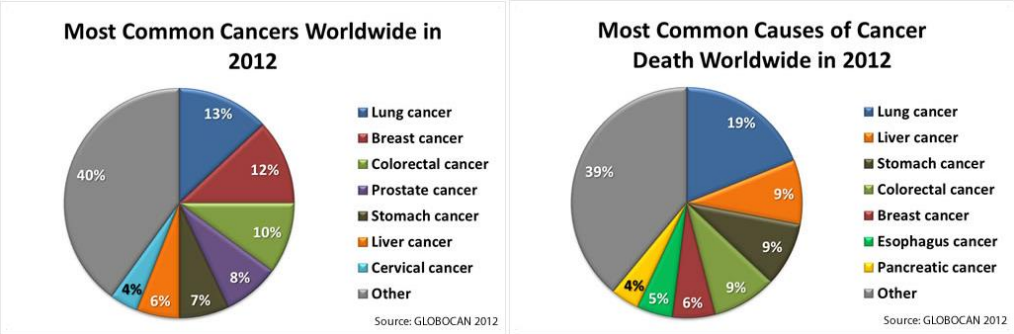

Figure 1.1- Graphic describing cancer incidence (left) and mortality (right) worldwide. Source Globocan (IARC), 2012.

Table 1.2- 2012´s worldwide statistics of incidence and mortality of CRC, according to geography and gender. Source: Globocan (IARC), 2012.

Estimated numbers (thousands)	Men			Women			Both sexes		
	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.
World	746	374	1953	614	320	1590	1361	694	3544
More developed regions	399	175	1164	338	158	966	737	333	2130
Less developed regions	347	198	789	276	163	624	624	361	1414
WHO Africa region (AFRO)	16	11	32	15	11	31	31	22	63
WHO Americas region (PAHO)	125	57	362	121	55	342	246	112	705
WHO East Mediterranean region (EMRO)	18	12	40	15	10	33	33	21	73
WHO Europe region (EURO)	255	120	686	216	108	573	471	228	1258
WHO South-East Asia region (SEARO)	68	48	122	52	37	93	120	85	216
WHO Western Pacific region (WPRO)	264	125	711	195	100	518	460	225	1229
IARC membership (24 countries)	418	187	1181	351	167	976	769	353	2157
United States of America	69	29	214	65	27	199	134	55	413
China	147	79	338	107	60	245	253	139	583
India	37	28	50	27	21	37	64	49	87
European Union (EU-28)	193	83	536	152	69	417	345	152	953

The colon consists of four parts: ascending, transverse, descending and sigmoid colon. It measures about 1.5 meters and it has a retention time ranging from 3 to 10h. It extracts water and salts from solid wastes before they are eliminated from the body. The parts of the colon are located either in the abdominal cavity or behind it, in retro peritoneum. The ascending and descending colon and rectum are retroperitoneal, while transverse colon is intra peritoneal (*Sreelatha and Brahma, 2013*).

The definition of CRC covers all neoplasia that start in any part of the large intestine or rectum, and it can be named according to its location (colon or rectum). CRC is almost always preceded by an adenomatous polyp, that is, a projection of the inner lining towards the lumen. These polyps are, by a large majority, benign tumours, with only a minority developing cancer over time (*Boyle and Lang, 2000a*). To be considered a malignant tumour, it has to penetrate through muscularis mucosae into submocosa (*Aaltonen & Hamilton 2000*). According to *Langman & Boyle (2000*), CRC relies heavily on environmental factors, since the incidence of CRC varies in space and time, "groups of migrants quickly lose the risk associated with their original home community and acquire the patterns of the new community" (*Boyle and Lang,*

2000b). The individual risk of developing CRC is dependent of lifestyle habits and genetic predisposition, and also in increasing age (*National Collaborating Centre for Cancer, 2011*).

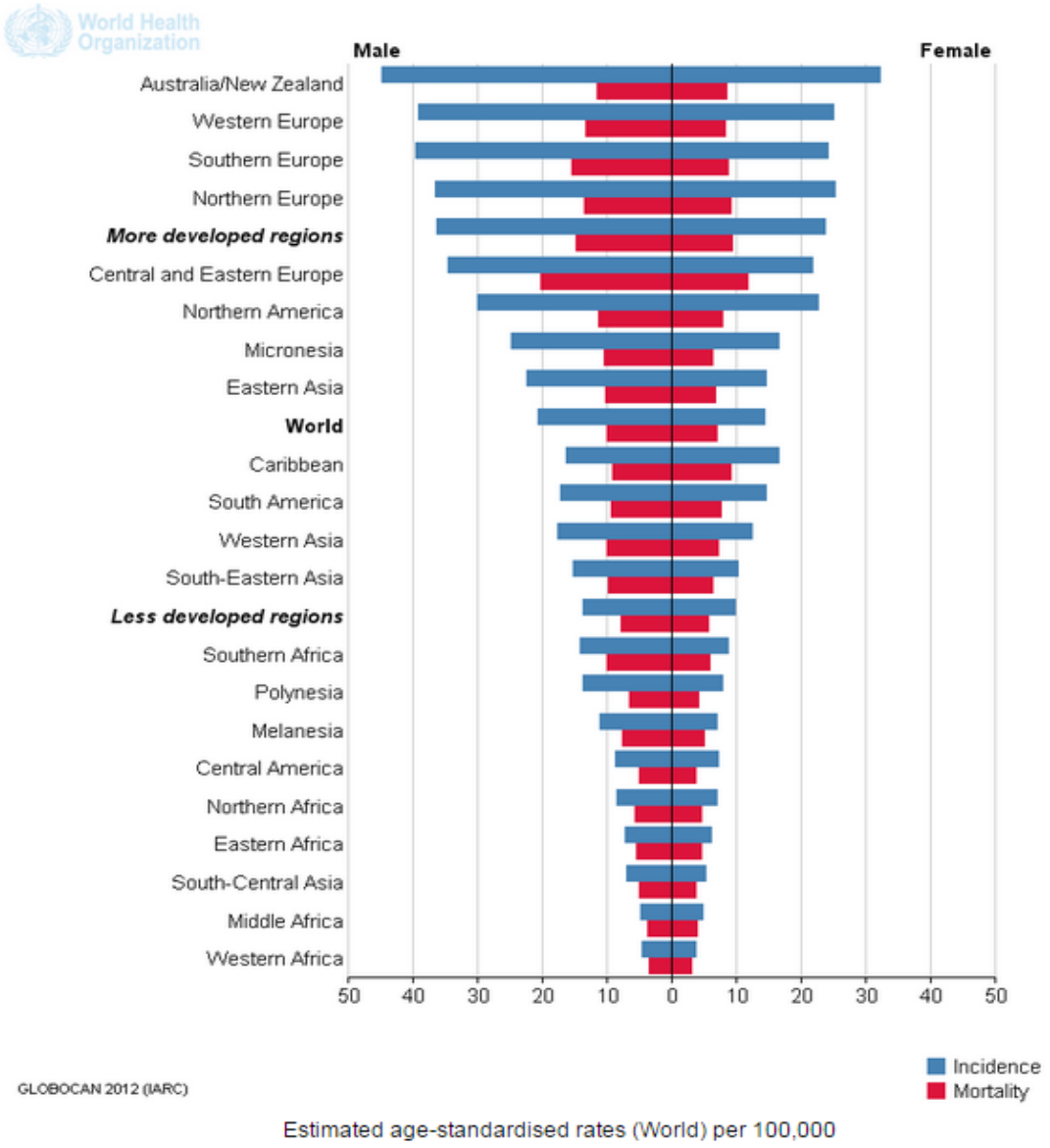

Figure 1.2 - 2012´s Worlwide statistics of incidence and mortality of CRC, according to geography and gender. Source GLOBOCAN 2012 - Colorectal cancer statistics.

As we can see on **Figure 1.2**, although the incidence of CRC is higher in more developed countries, these countries are more successful in treating and defeating CRC. According to the American Cancer Society, surgery still is the first approach most of the time, the type of surgery being dependent on stage, localization, size and other tumour related factors. Other treatment options can be considered, especially when surgery is no longer an option, or when is not possible to remove the entire tumour or all tumour metastasis. These options include ablation, when the tumour is destroyed without removing it, and

embolization that consists in blocking the blood flow to the cancer cell agglomerate (*American Cancer Society, 2016b*). Radiotherapy and chemotherapy are normally used as adjuvant or neo-adjuvant therapy, or in order to induce and/or consolidate remissions (*Ayanian el al, 2003*). It is also very common to combine chemo and radiotherapy as chemotherapy makes cancer cells more sensitive to the radiation; this procedure is called chemoradiation (*Goldberg et al, 2011*). Although chemo and radiotherapy can be very effective, the side effects are, sometimes, nearly as aggressive as the cancer itself. The side effects are not very predictable as they tend to be different in occurrence and in proportion for each person; however some side effects like hair loss, diarrhoea, persistent fatigue, faintness and drowsiness seem to be problems shared by every patient (*National Cancer Institute (2015*). These reactions are due to the fact that those treatments are not very selective to the type of cells that are killed, and, despite the fact that cancer cells are more vulnerable to them, normal cells are also killed during the treatment, as these systems do not distinguish between fast-growing cancer cells and other types of fast-growing cells, such as blood cells, skin cells and the cells inside the stomach (*Angelis, C. D. 2008a*).

1.3 - TARGETED THERAPY

Angelis, compared the experience of many patients with cancer to an ancient Greek mythology story that tells of a titan *Angelis (2008),* Prometheus, that for having stolen fire and handed as a gift to the man kind, was punished by Zeus to be chained alongside to the top of the Mont Caucasus and to have his liver eaten by an eagle (*Angelis, C. D. 2008b*). As Prometheus was immortal his organ regenerates itself constantly, and so the destructive cycle restarted day after day. This myth is an accurate analogy to what happens many times with cancer cells: just when it is thought that they are all destroyed, one remaining group of those starts to grow again. This recurrence relies, most of the times, on the low specificity of the treatment. The introduction of chemo and radiotherapy in cancer treatment resulted in an immediate, significant and undeniable impact towards cancer cure and reduction of cancer related mortality, and the constant research in this field lead to the development of new agents and therapies with new, but non-selective mechanisms of action (*Chabner and Roberts 2005*). Conventional chemotherapy associated toxicity, due to the indiscriminate killing of healthy cells, as well as the development of multidrug resistance due to numerous complex mechanisms, supports the need to find new effective targeted treatments based on the changes in the molecular biology of the tumour cells (*Pérez-Herrero and Fernández-Medarde, 2015*). So it was clear that new approaches would be necessary, if better results were expected, and with the rapid increase in knowledge and technology development in the areas of molecular biology, biotechnology, nanotechnology and other related sciences, we soon came to learn more about the molecular and structural differences between normal and cancer cells, and exploit that knowledge in order to bring to the next level the cancer treatment options, namely by targeting those cells based on specific biomarkers. The aim is now to improve the uptake of the anticancer agent by malignant cells, without

affecting normal tissue, by "focusing on unique features of the tumour microenvironment, such as leaky vasculature, overexpressed cell surface receptors, and intratumoural pH differences, as well as features of the cell uptake process, such as endosomal pH" (*Alexander-Bryant et al, 2013*). The development of molecularly targeted cancer therapeutics such as anti-angiogenic agents, proteasome inhibitors and growth factor receptor inhibitors have contributed to advances in the treatment, by making it possible to selectively target the physiology of cancer cells. (*Pathania et al., 2009*).

1.4 - NANOTECHNOLOGY

The rise of nanotechnology has led to major advances in the field of targeted therapeutics and tumour targeted drug delivery, enabling more effective drug design and development, which has revolutionized cancer therapy.

About half a century after the physicist Richard Feyman´s, considered the father of the nanotechnology, most famous lecture "There's plenty of room at the bottom", in which he prophesied about the rise of small carriers that would be able to mimic biological systems through manipulation and control of small atoms and molecules (*Gazit and Mitraki, 2007*), nanotechnology has developed amazingly and become one of the most promising study fields of the new century, with practical application in many scientific areas. In all, over 4000 calls for proposals were published by the end of 2014, and a total of 1381 nanotechnology based formulations were registered for clinical trials (*Wicki et al., 2015a*). Expressions such as nanotherapy, nanomedicine and nanotheragnosis are now synonymous of innovation and efficiency. The study of artificial or natural biocompatible materials, with low toxicity and low immunogenicity, and advances in cancer research lead to the development carriers that can transport anticancer agents specifically to the cancer cells (*Pala et al, 2014a*). Nano/microparticulate carriers can be made from several organic and inorganic materials such as non-degradable and biodegradable polymers, lipids (liposomes, nanoemulsions, and solid-lipid nanoparticles) self-assembling amphiphilic molecules, dendrimers, metal, and inorganic semiconductor nanocrystals (*Couvreur and Vauthier, 2006*). These small carriers stand as good candidates for transport means where agents of interest can be entrapped for diagnosis, treatment and/or prophylaxis, platforms where ligands and/or receptors could be anchored and used as tools that can be used in hyperthermia, phototherapy and magnetic therapy (*Brigger et al, 2012*). The increasing knowledge of tumour specific characteristics has been a strong allied in the development of nanotechnology for tumour targeting, by bringing to light many aspects in which tumour cells differ from regular healthy cells. This knowledge can be used to choose the right biomaterial, synthetize smart carriers and enhance targeting by functionalization with ligands and engineering particles that can respond to certain specific stimuli. Nanocarriers are used to enhance the bioavailability of drugs by helping to overcome solubility and chemical stability problems. Paclitaxel, is an example of a drug whose poor

pharmacokinetic properties have been improved by the use of a nano-based therapeutic albumin-bound drug (*Dy et al, 2014*). Lipophilic agents can be enveloped inside as a mean to improve internalization and uptake, as the cellular membrane is an assembly of phospholipids and lipoproteins (*Baek and Na, 2013*). Besides the particles can be designed so that they can penetrate the tumour cell agglomerate, and achieve the central core of the tumour (*Minchinton and Tannock, 2006*). Drugs cleaved enzymatically (e.g., siRNA by RNAses in the plasma, proteins by pepsin or trypsin in the stomach) can be protected from biodegradation, and can have their half-life extended (*Wicki et al., 2015b*).

In summary, the high tunability allowing modification of properties of the biomaterial, the ability to target tumour cells, the possibility of particles accumulation in tumour tissue, the ability of improvement of drug bioavailability and half-life, controlled drug release and several methods of triggering drug release and ability of entrapment of different therapeutic agents simultaneously, including imaging agents, are some of the main reasons that make nano/microcarries one of the most promising tools on tumour diagnosis and treatment.

Regarding this matter, two targeting strategies known as passive targeting or smart approach, and active targeting or targeted approach can be mentioned:

- Passive targeting takes advantage of the fact that nanoparticles have shown to have a prolonged blood half-life than conventional drugs, and tend to accumulate at particular sites due to the balance between vascular hemodynamic forces and diffusion mechanisms (*Pala et al, 2014b*). Typically, tumour vessels are highly disorganized and dilated with a high number of pores, resulting in enlarged gap junctions between endothelial cells and compromised lymphatic drainage. The 'improvised' vascularization, creates what is called the Enhanced Permeability and Retention (EPR) effect, and allows migration of macromolecules into the surrounding tumour region and retention of those for prolonged periods. Although EPR has afforded an efficient strategy for anti-cancer drug design by allowing high selectivity, improved therapeutic efficacy and decreased toxicity (*Maeda et al, 2006*), it should be noted that this phenomenon is highly heterogeneous and there is not an established pattern, as it tend to differ from patient to patient and from tumour to tumour (*Jain and Stylianopoulos, 2010*).
- Acording to *Pala et al*, "active drug targeting involves the use of a variety of affinity ligands to direct the binding of nanoparticles to many biological targets, largely represented by antigens that are differentially overexpressed both in the plasma membrane and in diseased tissue" (*Pala et al, 2014c*). Active targeting concept lies on the fact that many proteins and receptors are expressed, overexpressed or mutated differentially by cancer cells, this biological process can be used to

"hit" specific tumour cells. Monoclonal antibodies, specific peptides and proteins, aptamers, and small-molecule drugs have been used as a means to achieve that goal (*Pala et al,2014d*).

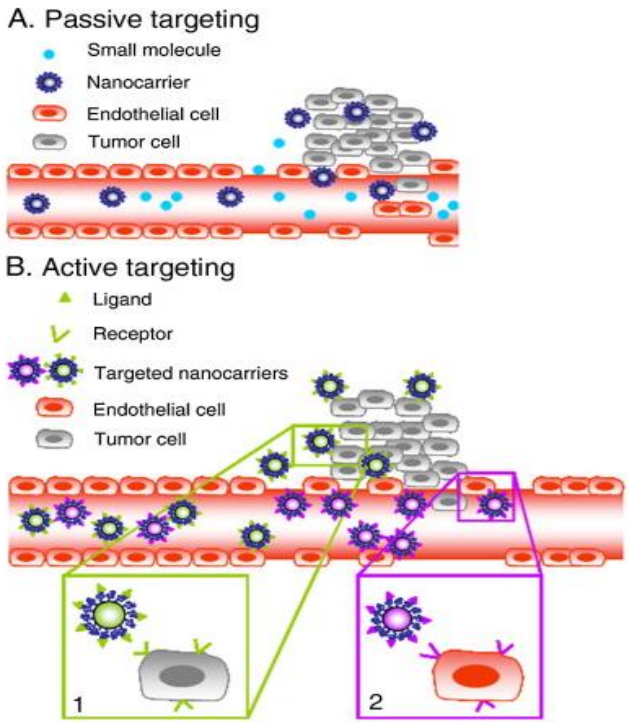

Figure 1.3 - Small illustration comparing the effectiveness of specif drug delivery using passive(A) or active(B) targeting. Retrieved from: Préat el al, 2010.

The first targeted therapy ever used, employed the pharmacological agent imatinib (Gleevec® or Glivec® by Novartis), an anticancer drug, to actively target a form of the enzyme tyrosine kinase, that stimulates uncontrolled cell growth in white blood cells. Imatinib is indicated in the treatment of Chronic Myeloid Leukemia (*Druker et al, 2001; Lugo et al, 1990*).

1.5 – COLORECTAL CANCER TARGETING STRATEGIES

A key aspect to be taken into account concerning colon targeting is the existence of a direct route: the gastrointestinal tract (GIT). The oral administration, besides being the most convenient route to reach the colorectal region, is the most common method of drug delivery, as it is relatively less invasive and, certainly, more accepted among the patients (*Kurtz et al, 2003*). Orally administrated formulations, aiming for the colon, can be designed either for local or systemic delivery. In the local delivery, the site of action and the site of delivery are the same, and in systemic delivery the drug will be absorbed in the colon but will take effect in another organ (*Hua, 2015*). In this case, successful colon-specific drug delivery is dependent on the synergistic interaction between the delivery system and the gut physiology. More

concretely, when using nano-based formulations, the overall performance of a colon-specific delivery system will be influenced by the chemistry and characteristics of the biomaterial, and on the release mechanism (*Yang, 2008*). Because of the proximity between colon and rectum (**Figure 1.4**), it may appear that rectal administration is a more appropriate, direct and logic, approach, to reach cancerous site, however, due to the peristaltic motion, it is difficult to patronize the distribution of the drugs administered by this route, and to reach ascending, transverse, and even upper descending colon, although it could be an option when the tumour localization is near or in the rectum. In this case, rectal formulation such as enemas and suppositories can be exploited (*Fell,1996; Patel et al, 2012a*). In order to avoid the premature release of the active compound in the stomach and small intestine, providing that release upon entry to the colon region, is required a triggering mechanism, built in the delivery system, responsive to the physiological changes particular to the GIT.

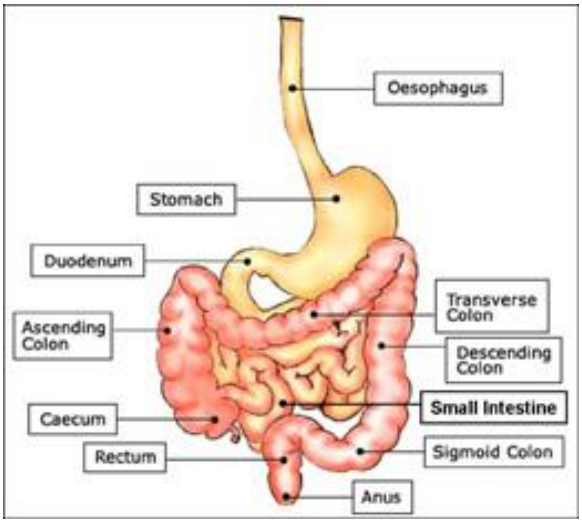

Figure 1.4 - Schematic representation of gastrointestinal tract. Retrieved from: Visionary Health Compounding Chemist. (2011).

According to *Patel et al (2012)*, triggering mechanisms can be classified as follows:

- SPECIFIC

  1. pH-sensitive natural or synthetic polymers, which will respond to pH variations of the GIT tract, dissolving at the pH value associated with colon environment;

  2. Microflora activated systems, which will be metabolized and/or modified in some way by enzymes produced by GIT microbial.

- NON SPECIFIC - In this kind of delivery system the aim is not to trigger the delivery, but is to take advantage of cancer´s particular characteristics to release the drug, preventing premature release.

  1. Time dependent;

2. Osmotic controlled;
3. Pressure controlled;
4. Swelling systems;
5. Eroding systems;
6. Those using slowed transit in the colon (pellet dosage forms) to release the majority of the drug when trapped in the ascending colon (*Patel et al, 2012*b).

1.5.1 - pH triggered release

In the pharmaceutical industry, pH based delivery systems constitute the majority of colon targeted formulations (*Kumar and Mishra 2008*) and, despite the new drug delivery technologies that are currently available, stimuli responsive polymers still stand as the most popular biomaterial used, due to their associated low cost of production and design/synthesis flexibility (*Yoshida et al., 2013*). The intraluminal pH is rapidly changed from highly acid in the stomach to about 6.5 in the proximal part of small intestine and in distal part of small intestine it is 7.5. The pH is 6.4 in caecum, 5.7 in ascending colon, 6.6 in transverse colon and 7.0 in descending colon (*Philip and Philip, 2010*).

As we know, by changing the pH of the environment, the charge of the solute is also changed. If the pH of the GIT region is such that a particular biomaterial carries no net electric charge, the solute often has minimal solubility (*Boundless – Textbooks, 2016*). Therefore, these types of systems use stimuli responsive polymers or copolymers that show very poor solubility at highly acidic pH environments, such as stomach, but are soluble in the distal gastrointestinal tract, where the pH reaches values close to neutral (*Hales et al., 2015*).

It can also be observed an extra and intracellular alteration of pH as a biologic response to disease. While the intracellular pH of the cells within the healthy tissues and tumours is similar, tumours exhibit a lower extracellular pH than normal tissues, due to the build-up of metabolites such as lactic acid and carbonic acid in the extracellular space. As healthy non-cancerous cells excrete metabolites from the cellular cytoplasm to the interstitial space, surrounding vasculature and lymphatic ducts will eliminate the metabolites from the tissue. In cancerous tissues, insufficient vasculature and lymphatic processes hinder metabolite elimination, leading to their accumulation and resulting in acidic microenvironments (*Préat el al, 2010*). Here, the biomaterial choice plays an important role and makes it possible to engineer nanocarriers that can exploit these pH differences and allow for delivery of the encapsulated active ingredient to specifically selected extracellular or intracellular sites.

pH sensitive polymers can be classified according to their source, as natural or synthetic, and, according to their nature, as anionic and cationic, depending on their charge status at pH 7.4. To be considered as

suitable candidates to be used in drug delivery, they have to be biocompatible/biodegradable and have no toxic or immunogenic by-products.

1.5.1.1 - Chitosan

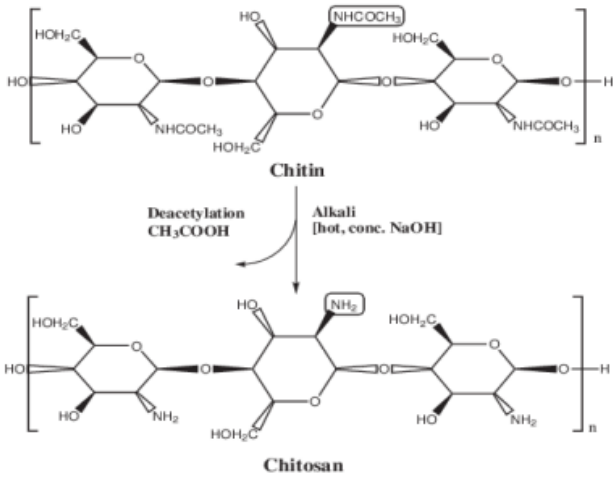

Figure 1.5 - Schematics of chemical reaction of deacetylation of chitin. Retrieved From: Elson Santiago de Alvarenga (2011).

Among natural polymers chitosan in one of the most widely used in colon delivery due to its pH swelling properties. Structurally, chitosan is a straight-chain copolymer composed of D-glucosamine and N-acetyl-D-glucosamine being obtained by the partial deacetylation of chitin, as seen of **Figure 1.5** (*Alvarenga, 2011*).

Chitosan is usually combined with other polymers, by crosslinking, in order to produce stable matrixes and to enhance its solubility in organic fluids (*Pujana el al , 2013*). These matrixes are known as hydrogels, and they are crosslinked macromolecular networks that swell in water or biological fluids. They have become a potential candidate for carriers of bioactive macromolecules, wound dressing and controlled release of drugs (*Kawaguchi, 2000*). Chitosan nanogels have been widely used as bioactive compound delivery system that offer less toxicity, is biodegradable and biocompatible, all of that associated with cost efficiency (*Nagpal et al, 2010*). Blends of chitosan with other polymers have been used as in some cases, by synergistic effects; the blend provides better properties than the pure components. Chitosan, due to its many functional groups, can be easily modified, and some properties, such as swelling behavior, can be tunable by changing the ratio between chitosan and other polymers in the same formulation (*Islam et al., 2012*). **Table 1.3** presents some examples of formulations using chitosan blended with other polymers as pH triggered delivery system.

Table 1.3 - Review of some articles that use chitosan based polymer blends as a pH triggered delivery system.

Polymer blend	Experimental work	Main results	Bibliografy
Chitosan and poly(methacrylic acid)	The effects of pH, ionic strength, and inorganic salt on the swelling behavior of the hydrogel were studied	Hydrogel has excellent pH sensitivity in the range of pH 1.40 to 4.50, pH reversible response between pH 1.80 and 6.80	Chen, S. et al. (2008) Synthesis and swelling properties of pH-sensitive hydrogels based on chitosan and poly(methacrylic acid) semi-interpenetrating polymer network. Journal of Applied Polymer Science, v. 98, n. 4, p. 1720-1726.
Chitosan-alginate	*In vitro* study of swelling behaviour and ninfedipine release, of alginate-chitosan mixed beads and alginate-chitosan coated beads at different pH solutions (1.5, 2.5, 5.0, 6.8, 7.4, and 8.0)	Dependent on the presence of the polyelectrolyte complex between chitosan and alginate; mixed beads and coated beads showed low release at 1.5 pH , and approximately 99% of release at pH 6.8	Dai, Y. et al. (2008), Swelling characteristics and drug delivery properties of nifedipine-loaded pH sensitive alginate–chitosan hydrogel beads. Journal of Biomedical Materials Research Part B: Applied Biomaterials, v. 86B, n. 2, p. 493-500.
Polyacrylamide and chitosan	Determine their swelling and ascorbic acid delivery kinetics at various chitosan concentrations, at acidic, neutral and basic pH.	The results of the swelling study showed that the swelling properties of the network varied with the changes of the pH in the swelling solution, as well as concentration of chitosan.	Martinez-Ruvalcaba (2008), A. Swelling characterization and drug delivery kinetics of polyacrylamide-co-itaconic acid/chitosan hydrogels. expresspolymlett, v. 3, n. 1, p. 25-32.
Chitosan and acrylic acid	Their swelling behavior in different pH buffer solutions	The obtained results show that these hydrogels have good pH sensitivity which can avoid drug release in stomach, and their swelling kinetics in stimulant intestinal environment follow second-order swelling kinetics equation.	Gong, S. et al. (2010) Chitosan-g-PAA hydrogels for colon-specific drug delivery: Preparation, swelling behavior and in vitro degradability. J. Wuhan Univ. Technol.-Mat. Sci. Edit., v. 25, n. 2, p. 248-251.
Carboxymethyl chitosan	pH specific delivery of nateglinide. pH responsive swelling behavior of prepared hydrogels was checked using different pH values (1.2, 6.8 and 7.4)	Low swelling at pH 1.2 (for first 2 h) and quick swelling at pH 6.8 (for next 3 h) followed by linear swelling at pH 7.4 (for next 7 h) with slight increase. In vitro release profile of hydrogels showed biphasic release pattern dependent on swelling behavior	S. Vaghani, S. et al. (2012) Synthesis and Characterization of Carboxymethyl Chitosan Hydrogel: Application as pH-Sensitive Delivery for Nateglinide. Current Drug Delivery, v. 9, n. 6, p. 628-636.

1.5.1.2 - Pectin

Pectin based delivery systems have been also one of the methods of choice regarding pH triggered colon delivery. Pectins are linear polyanionic polymers derived from plant cell walls, that are composed of α-1,4 D-galacturonic acid and 1,2 D-rhamnose with D-galactose and D-arabinose side chains having average molecular weights between 50000 to 150000 (*Chourasia and Jain, 2004a*). The most notable feature of pectins is their gelling activity. Crosslinked polymers of pectin, can form hydrogels that are able to absorb and retain hundreds of times their weight of water and are known as superabsorbents (*Rubinstein et al, 1990*). Also, pectin is resistant to proteases and amylase which are active in the upper GIT (*Sinha and Kumria, 2007a*).

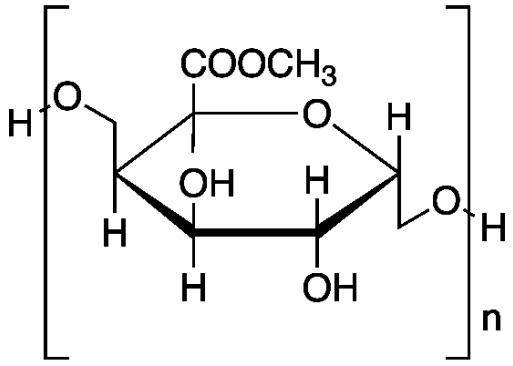

Figure 1.6- Chemical structure of pectin. Retrieved from: Pectin. (2012).

Regarding chemical structure and molecular weight, they can be grouped as low methoxy (LM), high methoxy (HM) and amidated pectins. They have a number of pharmaceutical applications and are presently considered as promising biodegradable carriers for colon-specific drug delivery for systemic action or topical treatment of diseases such as ulcerative colitis, Crohn's disease, and colon carcinomas (*Sriamornsak et al, 2007*). Various techniques have been reported to manufacture the pectin-based drug delivery systems, especially ionotropic gelation and gel coating (*Birch and Schiffman, 2014*). The type and concentration of pectin, the modifications of hydroxyl group, the pH of pectin solution, the temperature and the presence of cations, are some of the factors of which gel´s principal characteristics depends (*Liu et al., 2013*). **Table 1.4** presents some examples of delivery systems that use pectin for its pH sensitive properties.

Table 1.4 - Review of some articles that use pectin based polymer blends as a pH triggered delivery system.

Polymer blend	Experimental work	Main results	Bibliografy
pectin/chitosan	Hydrogels were prepared at different weight ratios (4:1, 7:1, 10:1; pectin/chitosan), loaded with vancomycin hydrochloride.	Water uptake was increased by raising the environmental pH (from 2.0 to 7.4) and the pectin/chitosan weight ratio, while drug availability was increased by raising the environmental pH (from 2.0 to 7.4) and decreased by raising the pectin/chitosan weight ratio.	Bigucci, F. et al. (2009) Pectin-based microspheres for colon-specific delivery of vancomycin. j pharm pharmacol, v. 61, n. 1, p. 41-4.
Pectin and polyacrylonitrile	Synthesis and swelling behavior of a superabsorbent hydrogel	The hydrogel exhibited a pH-responsive swelling-deswelling behavior at pH's 2 and 8. This on-off switching behavior provides the hydrogel with the potential to control delivery of bioactive agents.	Sadeghi, M. (2011) Pectin-Based Biodegradable Hydrogels with Potential Biomedical Applications as Drug Delivery Systems. Journal of Biomaterials and Nanobiotechnology, v. 02, n. 01, p. 36-40.
pectin-Hydroxy propyl methyl cellulose	Evaluation of *in vitro* dissolution characteristics comparatively between pH sensitive polymer coated tablets and natural polymer coated tablets in a various simulated fluids (pH values 1.2, 6, 6.8, 7.2, 5).	The lowered pH condition in diseased state could not trigger the drug release, as it was not the threshold pH of the particular coated polymer.	Newton, A. et al. (2012) K. Pectin-HPMC E15LV Vs pH sensitive polymer coating films for delayed drug delivery to colon: a comparison of two dissolution models to assess colonic targeting performance in-vitro. International Journal of Applied Research in Natural Products, v. 5, n. 3.
lysozyme-pectin	Nanocarrier for the antitumour agent, methotrexate (MTX).	Nanogels were pH-dependent, accelerated release of MTX at a decreasing pH from 7.4 to 5.3. The MTT assay indicated that encapsulated MTX exhibited higher anticancer activity than free MTX	Lin, L. et al. (2015) Construction of pH-sensitive lysozyme/pectin nanogel for tumour methotrexate delivery. Colloids and Surfaces B: Biointerfaces, v. 126, p. 459-466.
Pectin and Eudragit RS30D, Eudragit NE30D or Surelease	The dissolution profiles of pectin coated pellets were investigated in pH of 1.2 (2 h), pH of 7.4 (4 h) and pH of 6.8 in the absence as well as presence of rat cecal contents (18 h).	Coated pellets, prepared out of pectin and Surelease at a ratio of 1:3 at coating level of 35% (w/w), could increase budesonide release statistically in presence of rat cecal content, while they released no drug in pH of 1.2 and 7.4 .	Varshosaz, J. Et al. (2016)N. Pectin Film Coated Pellets for Colon-targeted Delivery of Budesonide: In-vitro/In-vivo Evaluation in Induced Ulcerative Colitis in Rat. Iranian Journal of Pharmaceutical Research, v. 11(3), p. 733-745.

1.5.2 - Microflora triggered release

Although, pH based delivery approach is very common, no reliable and sufficient pH gradient exists between the small and large intestine, since the pH in the small intestine and in the colon are not very different. This could cause an unpredictable and unstandardized site-specificity and release pattern, as dissolution behavior of the polymers used as excipients are, normally, not sensitive to small pH differences (*Chourasia and Jain, 2004b*). The nature of the bacterial population within the GIT seems to be strictly linked to the variety of pHs along this channel. The upper GI tract is sparsely populated with acid-resistant species, such as aerobic lactobacilli and streptococci. They are mainly Gram-positive, facultative aerobic microorganisms.

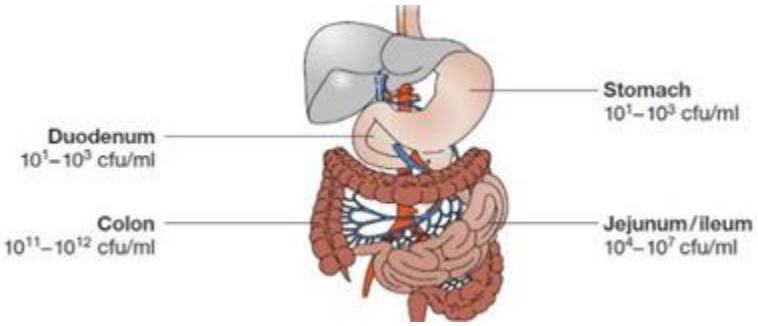

Figure 1.7- Bacterial density estimative along GIT. Retrieved from: Patel et al., 2012.

Bacterial density increases in the distal small intestine, and in the large intestine it rises to an estimated 10^{11} to 10^{12} bacteria per gram of colonic content (**Figure 1.7**), which contributes to 60% of fecal mass (*Patel et al., 2012c*) The colon is the site of the most abundant microflora in the GI tract. It consists predominantly of anaerobic or facultative anaerobic bacteria, such as *Bacteroides*, Bifidobacteria, *Lactobacillus*, and Enterococci (*Scheline, 1973*). These microbes are so active that *Bocci* declared that colonic microflora metabolic activity is equal to the one of a virtual organ, calling it a "organ within an organ" or "the neglected organ", stating that the intestinal microbiome has a metabolic activity that is both adaptable and renewable (*Bocci, 1992*), and *O'Hara et al (2006)* referred to it as the "forgotten organ" (*O'hara and Shanahan, 2006*).

The colonic bacterial flora fulfil their energy requirement for the cellular function by fermentation of various substrates that are left indigested in the small intestine (*Rubinstein, 1990*).

These substrates include di, tri and polysaccharides. To do so, these bacteria produce a wide range of enzymes, such as β-glucuronidase, β-xylosidase, α-arabinosidase, β-galactosidase, nitroreductase, azoreductase, deaminase, urea hydroxylase etc. (*Kinget, 1998*). Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages and on hydrolysis give the constituent monosaccharides or oligosaccharides (*Chourasia and Jain, 2004c*). Besides the capacity of polysaccharides to act as substrates for the bacterial inhabitants of the colon, their properties, such as swelling, film forming and their biocompatability, biodegradability

supports their use as colon specific delivery agents (*Sinha and Kumria, 2007b*). The release of the entrapped bioactive agent is triggered by hydrolyzation of glycosidic linkages, and, consequentely, depolymerization of those polysaccharides on arrival in the colon region. The main bacterial *genera* responsible for this biodegradation are *lactobacilli, bacteroides* and *bifidobacterium* (*Patel et al., 2012d*). Polysaccharides have been applied as controlled release coatings, matrices, macromolecular carriers, and biodegradable carriers, as they retain their integrity and prevent the release of drug during its passage through the GIT (*Kosaraju, 2005*).Furthermore, they can be chemically modified with relative ease, are very stable, safe, nontoxic, hydrophilic and gel forming. These microflora activated delivery systems can be based on drug-saccharide conjugates, or biopolymeric matrixes or coating.

Because they are natural polysaccharides, chitosan and pectin have been evaluated for their potential as colon specific drug delivery in several forms such as capsules, matrices, hydrogels and microspheres. Chitosan, an aminopolysaccharide of animal origin, has shown to suffer enzymatic hydrolytic cleavage of the β-(1-4) saccharide linkage (*Hirano, 1989*). The increased water solubility of chitosan and its amine functionality has encouraged its use as a biomaterial polymer conjugate. Due to his high solubility at acidic pH values, it usually requires an adequate coating, which would protect it against the acidity of the stomach. As the formulation reaches the intestine, the pH increases and the enteric layer, which is normally soluble at neutral to alkaline pH, dissolves releasing the chitosan coated core. This core is rapidly degraded by enzymatic activity, and the payload is released. (*Tominaga et al. 2001; Li, 1992; Tozaki et al, 1996*). Chitosan formulations have been increasingly applied in CSDS synthesis due to its many advantageous properties: pH swelling capacity, solubility, cationic charge, colonic microflora degradation, mucoadhesiveness (*Sogias et al, 2008*), bactericide and fungicide capacity, and, additionally, chitosan itself has some anticancer properties (*Gibot et al, 2015*).

Unlike chitosan, pectins are non-starch, linear polysaccharides extracted from plant cell walls, which remain intact in the stomach and small intestine, but are degraded by the bacterial inhabitants of the human colon. Pectin is completely fermented by microflora, low esterified pectin being fermented faster than high esterified pectin. In spite of that, according to Saito and colleagues, a small part of pectin can be degraded at acidic stomach environment via side chain hydrolysis, and at pH 5 to 6 (conditions of small intestine) via β-elimination of main chain or de-esterification. (*Saito et al, 2005*) That is why it is usually required to coat pectin based delivery systems, in order to protect the drug (*Ashford et al, 1993*; *Amol et al, 2007*).

1.6 - PLANT DERIVED BIOACTIVE PRODUCTS

The use and manipulation of plants and its derivatives for therapeutic and/or prophylactic purposes, is something that has been following the evolution of human race itself, practically since the first man on earth (*Petrovska, 2012*). It is proven by undeniable historical evidences ranging from archaeological

findings in the Neanderthals tombs of over 60.000 years, to several 4000 years old Sumerian clay tablets, which have the name of various plants and herbal extracts and its correspondent use for each disease (*Kong JM et al, 2003*). This proves that the search of cure through plants is a very ancient practice and it can be found in practically all cultures and societies. However, there are some problems associated with the direct use of unprocessed plants or herbal extracts for therapeutic ends. Those issues can include dosage related toxicity, or toxicity related with interactions between two or more active principles, due to the fact that a pure vegetal extract often has more than one active principle, their stability and bioavailability after administration (*Wang et al, 2012a*), and also, as many industrialized drugs, the low specificity delivery problem. All of this, associated with each individual metabolism and genetic particularities, results in drastic data variation.

As time goes by, knowledge about medicine, biology, biochemistry and health technologies, have allowed the isolation of active principles from herbal extracts and, through study of the excipients and proper administration techniques that should be applied for each case, it was possible to overcome a big part of the existing problems.

However, the use of plant sources applied to the improvement of health conditions was never left behind, and new branches of science such as nutraceuticals, ethnobotany and ethnopharmacology, are areas that try to bring together the most recent discoveries of science and, sometimes, millennial knowledge, about the healing powers attributed to the plants, present in various cultures.

A number of active ingredients, and even unrefined natural extracts, have shown to have cytotoxic and/or antiproliferative effects on a range of cancer cells, *in vitro*, *in vivo* and *ex vivo*. Despite there is still a great deal of research to be done concerning the use of extracts in clinics and pharmaceutical industry, it is safe to say that at the present time there is in place a set of information and conditions in the areas of biotechnology, molecular biology, botany, pharmacognosy, and other sciences, that make up a broad spectrum of knowledge in this field. Among those herbal medicines, there has been a range of clinical studies testing the effect of some of those as anticancer agents, and some of them have proven to have shown at least some kind of antiproliferative or cytotoxic activity. These include curcumin from tumeric, genistein from soybean, tea polyphenols from green tea, resveratrol from grapes, sulforaphane from broccoli, isothiocyanates from cruciferous vegetables, silymarin from milk thistle, diallyl sulfide from garlic, lycopene from tomato, rosmarinic acid from rosemary, apigenin from parsley, and gingerol from gingers, Diindolylmethane/Indole-3-carbinol from brassica vegetables, cyanidins from grapes, crocetin from saffron, among others (*Wang et al., 2012b*). Each one of these bioactives has a specific mechanism of action, which are responsible for preventing or delaying the progression of the colon cancer. Increase detoxification by inhibition of cyclooxygenases, DNA methylation, modification of PI3 kinase pathway, and tumour angiogenesis inhibition, modification of microbial population, and regulation of inflammation,

etc., are some of most common mechanisms (*Williams et al., 2015a*). Below, some examples of plant derived products, that have proven to have great potential as chemopreventive, antiproliferative and/or cytotoxic agents, are considered:

- **Curcumin -** Curcumin (diferuloylmethane) is the major pigment of a popular Indian spice turmeric, Curcuma longa L., a member of the ginger family, which possesses both anti-inflammatory and antioxidant properties (*Agrawal et al., 2014; Surh, 2002*).There are several studies that demonstrate curcumin´s anticancer activity, with many of them including studies referring specifically CRC cell lines. The great key factor is that it has the ability to induce apoptosis in cancer cells without cytotoxic effects on healthy cells, which is very attractive to cancer research scientists (*Williams et al., 2015b*). One of the mechanisms by which cancer cell death was observed, was through curcumin's ability to reduce pro-caspase-3 levels, polymerase-1 cleavage and chromatin condensation, in a time- and dosage-dependent manner (*Watson et al, 2010*), but many other apoptosis inducing pathways have been suggested and proved (*Bachmeier et al, 2007; Aggarwal and Shishodia, 2004; Beevers et al, 2009*).
- **Phenetyl Isothiocyanate -** Phenethyl isothiocyanate (PEITC), one of the major compounds from dietary cruciferous vegetables, has been found to have antitumour properties and therefore could generate special interest for the development of chemopreventive and/or chemotherapeutic. (*Phenethyl isothiocyanate. n.d*). It is a breakdown product of the gluconasturtiin, and it is belived to reduce carcinogen activation by P450 enzymes (*Plate, 2006*). Although the mechanism of action is unclear, phenethyl Isothiocyanate (PEITC) was shown to induce apoptosis in tumour cells, possibly mediated through its metabolic intermediates, reactive oxygen species (ROS). PEITC also is able to have an inhibitory effect on the some important factors such as SOS-1, PKC, ERK1/2 and Rho A. PEITC also affected Ras, FAK, PI3K or inhibited GRB2, NF-κB, iNOS and COX-2 for causing the inhibition of cell proliferation in CRC cell lines (*Lai et al., 2010*).
- **Momordica Charantia (bitter melon) -** Momordica charantia L. Is an endemic plant in many country of Asia, South America and Africa, commonly known as bitter melon , and has been present in the traditional medicine of these countries since long ago, being used in a wide range of therapeutic applications, from curing infections and wound healing, to the treatment of diabetes and cancerous conditions (*Fang et al, 2012a*). All the parts of the plant are used, from the leafs to the root. Although some components of this plant have been identified as potential cancerous agents, the proteins Alpha-mormocharin (α-MMC) and Mormodica Anti-HIV (MAP30) have been the most studied and have shown to have anti-proliferative effect *in vivo* and *in vitro* in several types of cancer, including those deemed most difficult to treat. These proteins were tested

against pulmonary, colon, pancreatic, liver, epidermal, breast and melanoma cancer (*Fang et al, 2012b; Meng et a., 2014).* α-MMC e MAP30 are categorized as type 1 Ribosome Inactivating Proteins (RIP). They are constituted by a polypeptide chain of approximately 30 Kda, that exert their toxic effect by a linkage to the major subunit of ribosomal 60s. They act as a N-glicosidase, cleaving specifically A4324 adenin in rRNA 28S ribosomal subunit, as a result, factor 2, which is indispensable to elongation, is prevented from binding, blocking translation (*Yao et al., 2014*).

2. AIMS AND OBJECTIVES

This work was developed as part of a large ongoing project - iNOVA4Health-Advancing Precision Medicine - that involves several research institutions, including IBET and ITQB and Portuguese Institute of Oncology (IPO).

This dissertation has, as primary goal, the engineering of smart carriers, through which it would be possible to evaluate and improve the cellular uptake of bioactive agents: This carriers would be able to deliver bioactive agents directly and specifically to CRC cell, by responding to certain external stimuli as pH and charge, and by degradation of the carrier by glycolytic enzymes produced by colonic microflora. They could be used as an adjuvant or neoadjuvant orally administrated cancer therapy.

The strategy used is the synthesis and characterization of different microcarriers based in chitosan and citrus pectin, both multilayer and complexed polymers, in order to define the best synthesis method and formulation, as well as determining the best encapsulation method.

- One of the particles is multilayered, constituted by chitosan and citrus pectin. Chitosan, for its properties, like muchoadherence, as colon is a mucosa. This propriety helps particle to cling to the colon walls, resisting better to the peristaltic movement. Chitosan positive charge is also strategic, as it will electrostatically attract the particle to the cancer cell agglomerate, as cancer cells are negatively charged, and its swelling at acidic pH will help particle degradation when it arrives to the caecum and ascending colon. The particle will start to be degraded by bacterial enzymes as soon as it achieves colon region. Citrus pectin in the particle will be recognized by Galectin-3 receptors. All of this will make the payload release to occur just in cancer site.
- The other particle is a PEC complex, blending chitosan and citrus pectin. In this system, what is being valued, is the mucoadherent properties and the enzymatic lyses that occurs in both biomaterials. In this case, chitosan quantity is higher than citrus pectin quantity, in the particle structure, rendering the overall charge of the particle positive, therefore, making the particle electrostatically attracted to CRC cells. Citrus pectin, which is also present at the particle surface, will be recognized by galectin-3 receptors.

This work has been carried out at:

- ITQB (Instituto de Tecnologia Química e Biológica) from UNL (Universidade Nova de Lisboa
  - Polielectrocomplexation of chitosan and pectin;
  - Particle synthesis by Inverse Microemulsion (IM);
  - DLS (Dynamic Light Scattering)
  - DSC (Differential Scanning Calorimetry)
  - NMR (Nuclear Magnetic Ressonance)
- IBET (Instituto de Biologia Experimental e Tecnológica)
  - Particle synthesis by Spray Drying (SD)
  - Impregnation
- IST (Instituto Superior Técnico)
  - SEM (Scanning Electronic Microscopy)
  - TEM (Transmition Electronic Microscopy)

3. EXPERIMENTAL SECTION

3.1 - MATERIALS AND REAGENTS

- Chitosan - low molecular weight chitosan, with a molecular weight of 190-310 kDa, and 75-85% deacetylated, from Sigma-Aldrich; CAS Number 9012-76-4. All chitosan solutions were prepared in acetic acid solution (5%).
- Pectin from citrus peel - Modified citrus pectin (MCP), with ≥74.0% of galacturonic acid at dry basis and ≤10% of impurity (moisture); CAS Number 9000-69-5 All MCP solutions were prepared with MilliQ® water.
- Calcium chloride ($CaCl_2$) - from Kemira, imported and packed by José M. Vaz Pereira, Lda., 80+0,5% of purity; CAS number 10043-52-7. All $CaCl_2$ solutions were prepared with MilliQ® water.
- Acetic acid - CH_3CO_2H –96% from Sigma-Aldrich; CAS Number 64-19-7.
- Cyclohexane - C_6H_{12} –99+% from Alfa Aesar; CAS number 110-82-7.
- 1-hexanol - $C_6H_{14}O$ –99% from from Alfa Aesar; CAS number 111-27-3.
- Acetone (CH_3COCH_3) – ≥ 99,5% from Sigma Aldrich, CAS number 67-64-1.
- Phenethyl isothiocyanate (PEITC) –99% from Sigma Aldrich; CAS number 2257-09-2
- 5-Fluorouracil - 5-FU ($C_4H_3FN_2O_2$) –99% from from Sigma Aldrich; CAS number 51-21-8
- MilliQ® apparatus - Mini Spray Dryer B-290
- Ibuprofen Sodium - ≥ 98% from Sigma Aldrich, CAS number 1568-27-1

Instrumental

- Spray Drying apparatus – Mini Spray Dryer B-290 from Buchi
- Impregnation apparatus - artisanal apparatus for the supercritical impregnation was adapted from (Varona, Rodríguez-Rojo, Martín, Cocero, & Duarte, 2011) and is schematically represented in **Figure 10**.
- Spectrophotometer – ThermoSpectronic, Genesys 10uv
- Scanning Electronic Microscopy apparatus (SEM) – FEG-SEM da marca JEOL, modelo JSM7001 (Japan)
- Dynamic Light Scattering apparatus (DLS) - Zetasizer Nano Series, Malvern Instruments Ltd, UK
- Centrifuge 1 - Avanti J-26XP Beckman Coulter

3.2 - SYNTHESIS OF CHITOSAN AND CITRUS PECTIN PARTICLES BY SPRAY DRYING METHOD

3.2.1 – Introduction

The aim of this synthesis was to build through bottom up approach, a microparticle resulting from a chitosan and citrus pectin polyelectrolyte complexation (**Figure 3.2**), by spray drying. The basic steps in the microencapsulation through this method, involves the preparation of a stable and homogenized solution to be processed and subsequently atomized, and the dehydration of the resulting particles. In the case here presented, chitosan being in higher amount will confer an overall positive charge to the particle, contributing that to the electrostatic attraction between particle and CRC cells. The particle will start to be degraded by bacterial enzymes as soon as it achieves colon region. Citrus pectin in the particle will be recognized by Galectin-3 receptors. All of this will make the payload release to be just in cancer site, minimizing, thus, the uptake of the bioactive agent by healthy cells. This method is considered "green", as it does not requires the use of organic solvents.

3.2.2 - Experimental procedure

The synthesis of chitosan:citrus pectin (CS:CPect) polielectrocomplex (PEC) was performed according to procedure previously published (*Grabnar and Kristl, 2010*), with some modifications. CS:CPect polymer blend (CS:CPect ratio = 2:1) was prepared in a two-step procedure based on the ionotropic pre-gelation of the pectin solution (2.13 mg/mL), with $CaCl_2$ (0.806 mg/mL) ($CaCl_2$:CPect ratio = 1:10), in order to lead the formation of macromolecular aggregates shown at **Figure 3.1** ("egg-box" cavity).

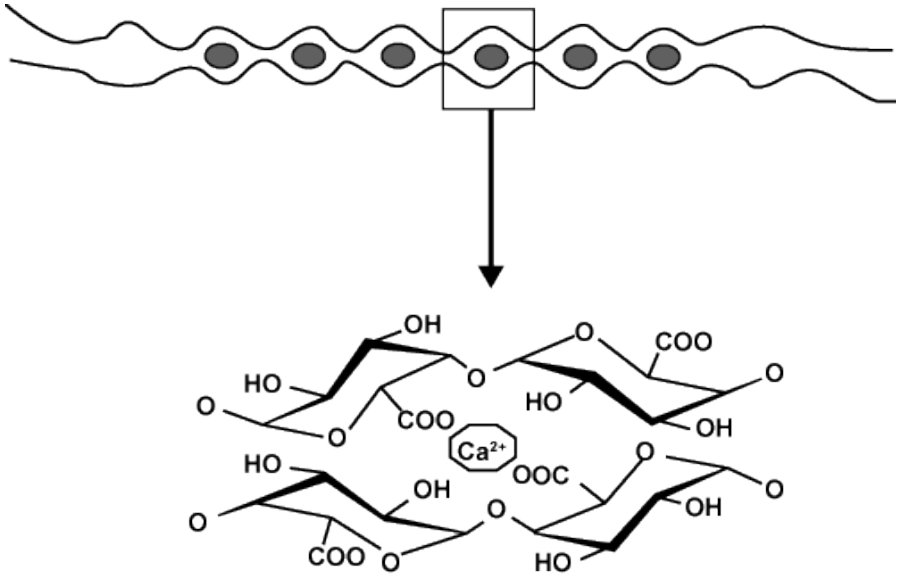

Figure 3.1 - Pectin has the ability to cross-link with divalent ions such as calcium ions. Pectin, being hydrophilic, forms a gel upon swelling. When calcium chloride concentration increases, the pectin's ability to swell is reduced (Maestrelli et al., 2008).

$CaCl_2$ solution (10 mL) was injected under gentle stirring into a beaker containing 40 mL of pectin solution (2.625mg/mL) and stirred for 10 min to provide a pre-gel. Afterwards, that pre-gel, was slowly injected through a capillary micropipette into the 220 mL chitosan solution (0.938 mg/mL), under vigorous stirring, and the resulting solution was left under stirring for 24 hours. For loading studies, 20 mL of Ibuprofen solution and Phenetyl Isothiocyanate (PEITC), by using 20 mL of Ibruprofen solution in acetone (3 mg/mL) and by adding that solution to the pectin solution before the addition of $CaCl_2$. This solution was passed through Mini Spray Dryer, setting the maximum inlet temperature to 150 ºC, the compressed air spray gas at 25. The spray dispersion was maintained at a rate of 4–8 mL/min.

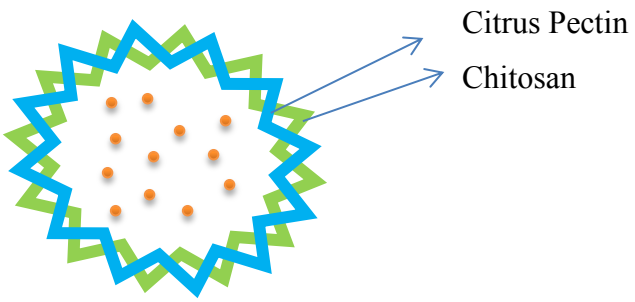

Figure 3.2 - Representation of PEC resulting particle with the encapsulated bioactive agent.

3.2.3 - Particle characterization

Chitosan and citrus pectin PEC particles were subjected to DSC study, to determine the size and to evaluate the pH responsiveness, through suspending about 0.5 mg of dry particles in 1.5 mL of MilliQ water that was treated with 1M NaOH or 1M CH_3CO_2H, in order to regulate pH to 3.66 , 5.2 , 7.1 , 8.2 , 10.22. SEM imaging was also performed as a means of measuring dry particle diameter, and to analyze particle morphology.

3.2.4 - Yield

Dried microparticles were accurately weighed, and considering the total amount of drug and polymers used for preparing the feed solution, the yield of production (YP) was calculated, as a percentage, using the following equation:

$$YP = \frac{MC}{CS + CPec + IB}\ x\ 100$$

where MC stands for the amount of produced microspheres and (CS + CPec + IB) for the sum of the amounts of two types of polymer and ibuprofen solubilized for the preparation of microparticles.

3.2.5 - Encapsulation efficiency determination

To determine the exact amount of ibuprofen encapsulated into CS:CPec matrix, the total amount of particles obtained were suspended in 20 mL etanol, so that the non-encapsulated ibuprofen could be solubilized. Later, that solution was centrifuged at 13.000 rpm for 20 mn, and the supernatant was removed, filtered, and a sample of 2 mL was withdrawn from this solution, to determine the ibuprofen particles content. Another ibuprofen solution was prepared at the concentration of 0.1 mg/mL and diluted with etanol (1:3, 1:4, 1:6, 1:10, 1:15, 1:20, 1:40) in order to prepare a calibration curve with ibuprofen solutions with known concentrations. All solutions were assayed spectrophotometrically at 264 nm.

3.3 - SYNTHESIS OF CHITOSAN COATED CITRUS PECTIN PARTICLES BY INVERSE MICROEMULSION METHOD

3.3.1 – Introduction

A microemulsion is defined as a thermodynamically stable isotropic dispersion of two immiscible liquids, since the domain of either or both liquids has been stabilized by an interfacial film of surface-active molecules (*Tartaj et al, 2003*). The surfactant molecules limit the nucleation, growth, and agglomeration of the particles. In inverse microemulsion, it constitutes one barrier that separates the continuous phase from the disperse phase, in other words, the oil phase from the aqueous phase. (*Bagwe et al, 2001*). Due to the very small size (nanometer range) of the core, the particles formed usually have a very small size, sometimes less than 15 nm and a very narrow size distribution. However, the particle size can be controlled by controlling the size of the core and the duration of the reaction (*Munshi and Maitra, 1997*).

The aim of this synthesis was to develop a multilayer particle (**Figure 3.3**) that would be able to achieve specifically CRC cells with minimum damage to normal cells. The external layer would be made of chitosan; this will make the particle to be electrostatically attracted to CRC cells, placing the particle near cancer cells region. Simultaneously, microflora enzymes will start to degrade the chitosan layer, revealing the inner core: citrus pectin. Citrus pectin, as in the particle synthesized above, will be recognized by galectin-3 receptors, making the particle to "anchor" in CRC cell´s surface. After particle degradation, the payload will be delivered directly at the cancer site.

3.3.2 - Experimental procedure

Chitosan coated particles were prepared using the IM methodology. Primarily, a 4mg/mL chitosan solution was prepared by dissolving it in acetic acid; once it was completely dissolved, 2 mL of hexanol and 10 mL of cyclohexan were added to 5 mL of chitosan solution, under stirring. Then Triton X-100 was added to that solution, until the solution became clear, to form the microemulsion. The second solution was obtained by dissolving pectin into MilliQ water (3.33 mg/mL), followed by the addition of 0,5mL of $CaCl_2$ (3 mg/mL), under stirring. Then, same quantity was added the of n-hexanol and cyclohexan that was

added to the chitosan solution. Triton X-100 was also added drop-by-drop into this solution until it became translucent. After having both solutions, pectin microemulsion was added drop-by-drop to the chitosan microemulsion under vigorous stirring.

This last mixture was kept under stirring for 10 days at room temperature. A sample of the solution was taken by the third, sixth, seventh and tenth day, washed with anhydrous ethanol, centrifuged at 13000 rpm, and the pellet was re-suspend in MilliQ filtered water.

3.3.3 - Particle characterization

Chitosan and citrus pectin PEC particles were subjected to DLS studies, in order to determine the size and to evaluate the pH responsiveness, through suspending about 0.5 mg of dry particles in 1.5mL of MilliQ water that was treated with 1M NaOH or 1M CH_3CO_2H, in order to regulate pH. This characterization was performed at various pH valued, and with samples from the 3^{th}, 6^{th} and 10^{th} day of reaction. SEM imaging was also performed as a means of measuring dry particle diameter, and to analyze particle morphology.

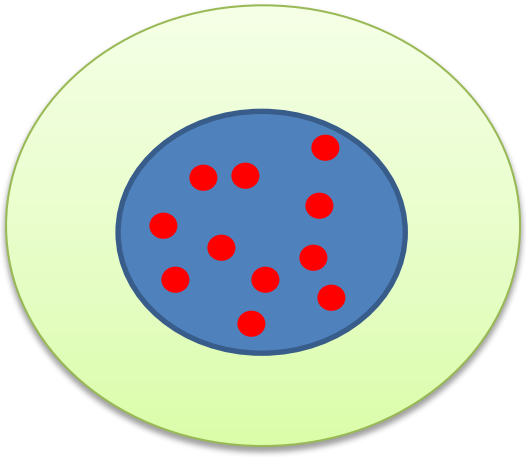

Figure 3.3 - Representation of multilayered, synthetized by reverse microemulsion method, particle with the encapsulated bioactive agent.

3.4 - ENCAPSULATION OF PHENETYL ISOTHYOCYAONATE BY SUPERCRITICAL CO_2 IMPREGNATION

3.4.1 - Introduction

The impregnation by supercritical CO_2 ($SCCO_2$) method allows incorporation of the active compound into previously synthesized particles of carrier material. It has been demonstrated that the supercritical impregnation technique allows a highly accurate and reproducible drug loading of large arrays of microcontainers (*Marizza et al, 2014*). At high pressure, CO_2 can be dissolved in most polymers, resulting in a considerable swelling of the polymer. The increase in the free volume of CO_2 swollen polymers

improves the diffusion rate and penetration of solute molecules into the polymeric matrix. It consists in the deposition of a soluble substance, the PEITC, in a super-critical fluid into the polymer matrix upon depressurization. In this way, even a solute that has low affinity for the polymer matrix can be trapped within a polymer matrix (*Varona et al, 2011a, Kikic e Vecchione, 2003*). As essential oils are highly soluble in $SCCO_2$ (*Varona et al, 2011b)* its believed that once $SCCO_2$ is released into the cell that contains PEITC, it will dissolve in the extract, forming a PEITC/CO_2 mixture. That mixture will pass through the polymeric microparticles, with the $SCCO_2$ making the particles swell, so the PEITC can, through diffusion, go into the carriers. To "lock" PEITC inside the particle, depressurization is required, and it can be done slowly or rapidly.

This method is also considered "green" as it does not involve the use of any organic solvents.

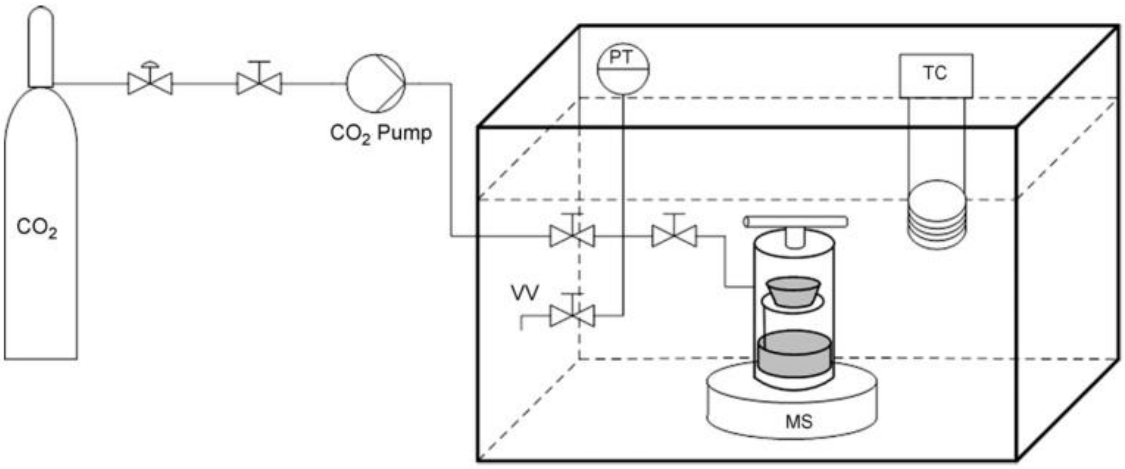

Figure 3.4 - Scheme of an impregnation apparatus. MS: magnetic stirrer, VV: vent valve, PT: high pressure transducer, TC: temperature controller. Retrieved in: Varona et al, 2011.

3.4.2 - Experimental Procedure

The previously prepared particles (synthesized by spray drying described at 3.2) were used in this procedure. In this procedure, the polymer is exposed to the solute solubilized in carbon dioxide for a predetermined period, followed by controlled depressurization of the system. When the system is depressurized, the carbon dioxide molecules leave the polymer matrix while the solute molecules remain trapped inside. This operation was carried out in an artisanal reactor. **Figure 3.4** depicts a schematic of the high pressure setup used in the impregnation experiments, according to the procedure described at Varona and colleagues (*Varona et al, 2011c).* This discontinuous apparatus consists of a high pressure stainless steel impregnation cell (22 cm_3 of internal volume), a high pressure CO_2 liquid pump, a

temperature controlled bath, a magnetic stirring plate and a pressure transducer. The cell contains the PEITC (300µL) at the bottom and the CS:CPect particles to be impregnated (300 mg) were wrapped into a small piece of filter paper which was then stapled to avoid particle loss in a stainless steel mesh elevated from the bottom of the cell by a support. The impregnation cell was immersed in the water bath until heated to the defined temperature (35ºC). When the set point temperature was achieved, the on/off valve was gradually opened and the impregnation cell was slowly filled with CO_2 at the pressure of 100bar. In those conditions, PEITC was mixed with $SCCO_2$ while homogenization was achieved by magnetic stirring. The impregnation time was 24h. At the end of the experiments the $SCCO_2$ was released through an on/off purge valve. Two different depressurization methods were tested: rapid depressurization (RD) – with total depressurization time: approximately 2 min (depressurization ~ 50bar/min) - and slow depressurization (SD) – with depressurization rate of 2–2.5 bar/min.

3.4.3 – Characterization

- **Derivatization and quantification of loading capacity**

The amount of essential oil impregnated in CS:CPect particles was determined by derivatization, using the 1,2-benzenedithiole-based cyclocondensation assay, followed by chromatographic analysis. 100 mg of impregnated particles were dissolved in 30 mL of acetic acid, under stirring during 10 minutes. The entire derivatization procedure was developed according to the protocol described at **Anex 1**, with small changes, namely, by adapting the stock solution preparation that was described in the original protocol, which was settled for phenyl isothiocyanate (PITC) instead of PEITC, making the necessary changes. The sample utilized was prepared by dissolving 100 mg of impregnated particles in 30 mL of acetic acid, keeping the particles under stirring in that solution for 10 minutes, followed by a 20 minutes 6000 rpm centrifugation.

Both the calibration curve and the samples were prepared according to the protocol, and the absorbance was later read. The samples used in RD and SD were added, each one to one previously prepared solution of 900 µL of 100 mM of phosphate buffer and 900 µL of methanol, and, subsequently, 100 µL de 80 mM 1,2-Benzeneditiol were added to initiate the reaction. To make the calibration curve, 6 samples were prepared, including a blank, to PEITC concentrations of 512, 256, 128, 64, 32 and 0 µM. The samples were diluted as follows: 1:1, 1:50, 1:100, 1:200. The derivatization reaction was allowed to occur for 2h hours at the temperature of 65ºC. Finally, the supernatant was removed and used to quantify the PEITC through reading the absorbance (365 nm) of the product between of 1,2-Benzeneditiol and PEITC.

4. MAIN RESULTS AND DISCUSSION

4.1 – SYNTHESIS OF CHITOSAN AND CITRUS PECTIN PARTICLES BY SPRAY DRYING METHOD

4.1.1 – Particle morphology and size

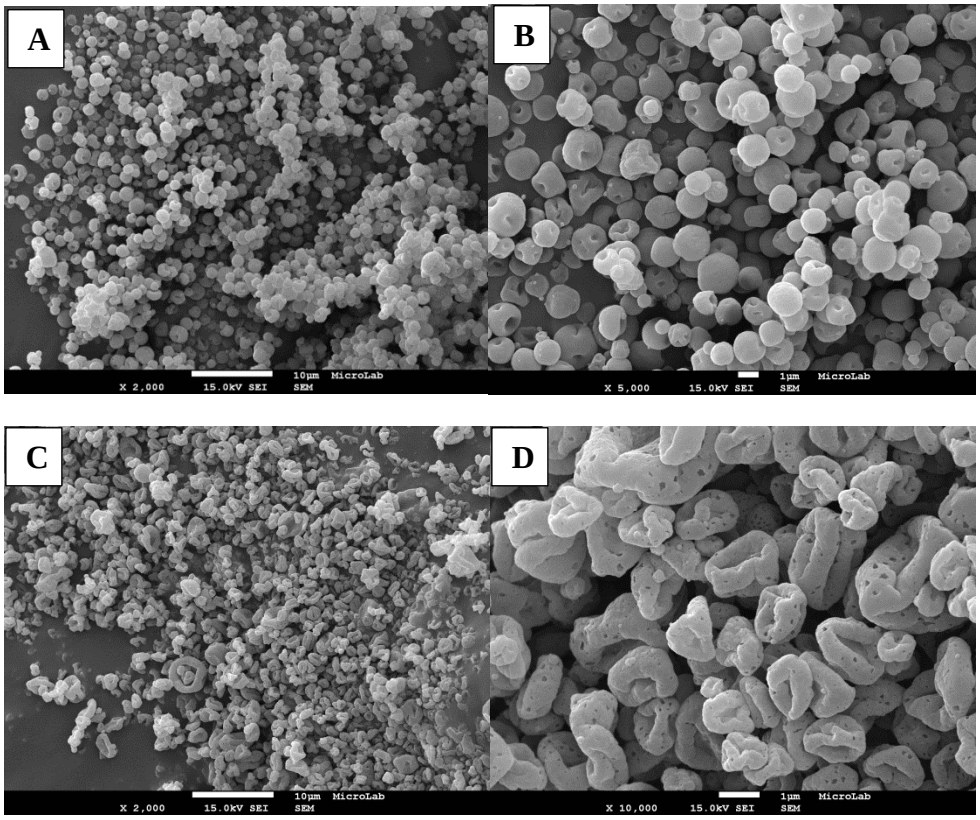

Figure 4.1 - SEM images of CS:CPect microparticles prepared by spray drying technique at A) 2,000 x and B) 5,000 x C) 2000 x D) 10000 x magnification. A and B are unloaded particles, C and D are loaded with ibuprofen.

Scanning electron microscopy was used to visualize the diameter, structural and surface morphology of the spray-dried particles. **Figure 4.1** presents the images of the pulverized particles obtained by electronic microscopy. Spray-dried unloaded particles showed similar characteristics: particles had spherical shape and heterogeneous size, presenting a smooth surface, but with some concavities. One factor that could be involved in particle deformation is the inlet temperature. Wenjie Liu and colleagues found that the drying temperature played an important role in the morphology of spray-dried chitosan based microparticles (*Liu et al, 2010a*). There is a direct correlation between high inlet temperatures (and consequently high outlet temperatures) and particle deformation. Inlet temperatures from 160 to 180ºC "resulted in cap-shaped microparticles". Maa and coworkers explained this phenomenon by focusing on the drying rates differences. High inlet temperature leads to a high drying rate, resulting in a quickly formed dry crust.

When the internal vapor pressure surpasses the range of pressure that the crust can resist, the semidried droplets collapse and forms dimples (*Maa el at, 1997*).

In contrast, loaded particles presented a highly irregular surface and shape, although the particle distribution was still positive. Ibuprofen loaded CS:CPec microspheres presented a collapsed surface, as shown in **Figure 4.1** (C e D imagens). According to the literature, the concentration of the solution to be sprayed has an important role in particle´s shape and overall morphology (*Liu et al., 2010a*) The solutions prepared to obtain both unloaded and loaded particles were exactly alike, except for the addition of ibuprofen in the second case, making, thus, that solution more concentrated compared to the first one. This fact can explain the morphology of the ibuprofen loaded particles.

Another plausible explanation is a possible interaction between the polymers and the drug. Liu and coworkers also showed that ibuprofen can form complexes with chitosan, by positioning close to (and in front of) the amino groups of the low molecular weight chitosan, involving ionic interaction between the ammonium groups of chitosan and the carboxylate anion of ibuprofen (*Liu et al, 2011b*). The CS:CPec PEC formation happens with the interaction between the ionized amino groups of chitosan (NH^{3+}) and the ionized carboxyl acid groups (COO^{-}) of pectin (*Coimbra et al, 2011*). It is believed that electrostatic interaction between the positively charged amino groups at C-2 of the chitosan pyranose ring and negatively charged carboxyl groups at C-5 of the pectin pyranose ring allow the formation of PEC, with a network structure, from the chitosan and pectin (*Rashidova et al., 2004*). With both citrus pectin and ibuprofen tending to form complexes with chitosan by ionic interaction with the same ionic group, it is possible that the simultaneous presence of these two components has led to a change in the conformation of the original PEC´s structure, which had an impact on the particle´s shape and morphology.

4.1.2 - Yield

The yields of spray-dried powders were quantified according to the previously used amount of polymer, or in the case of use of drug powders, the total amount of pulverized material. It is undeniable that the yield depends directly on the quantity of total amount of powder, in this case CS+CPec+IB (*Maury et al, 2005*). As when we can see in the equation:

$$YP = \frac{371mg}{206.4mg + 105mg + 60mg}\ x\ 100\%$$

$$\eta = 30.9\%$$

When 371.4 mg of total powder quantity were used, the obtained powder weighted 114.9mg, thus the yield was 30.9%, however when that quantity is increased to 1500 mg, the yield raises to 43.6%.

4.1.3 – Encapsulation efficiency

According to the concentration of ibuprofen in the withdrawal sample, the encapsulation efficiency was very high. The obtained absorbance was compared with a series of absorbance values form solutions of known ibuprofen concentrations (**Figure 4.2**).

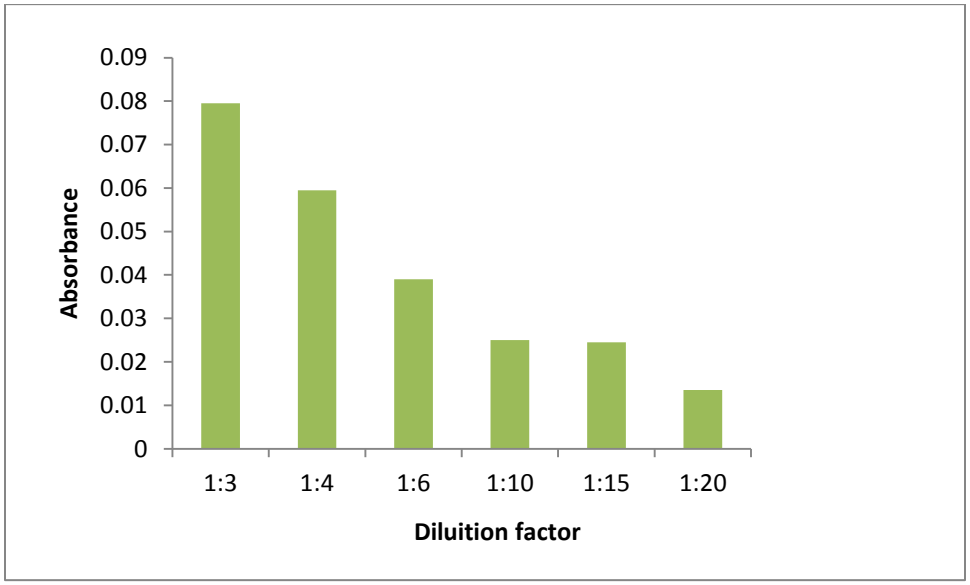

Figure 4.2 - Graphic representation of the concentration of the diluted initial solution of ibuprofen (0.1 mg/mL) and the correspondent absorbance, obtained through spectrometry, for each one of the dilutions.

Posteriorly the achieved values were used to build a calibration curve and to determine the equation of the line shown at **Figure 4.3**.

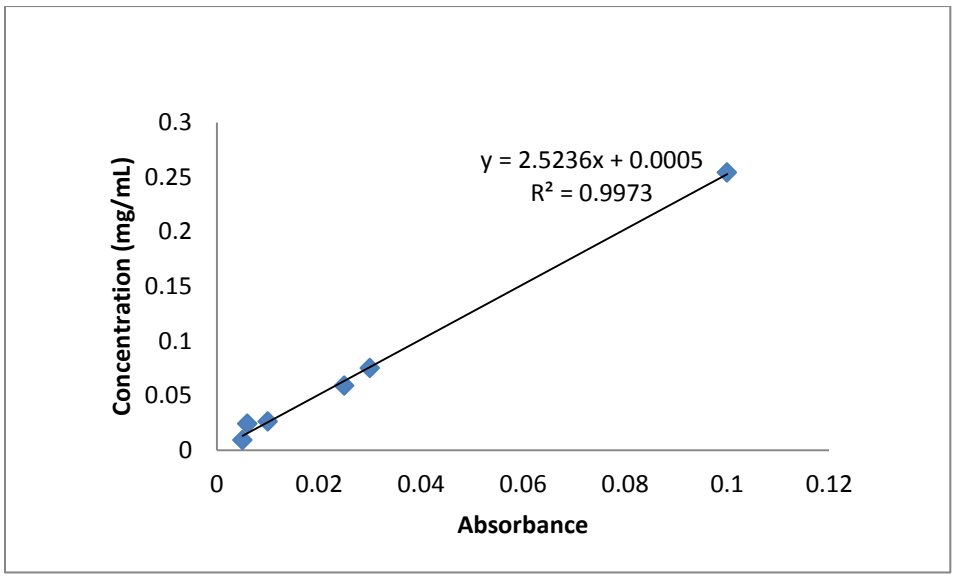

Figure 4.3 - Equation of the line of the absorbance readings presented at Figure 12.

The obtained absorbance of the sample retrieved from the particle suspension supernatant was 0,130, which, when correlated with the other absorbance and concentration values using the Beer-Lambert law:

$$Abs = \varepsilon . \ell . C + \mathscr{b}$$

,lead to a concentration of 0.207 mg/mL. ε and $\mathscr{b}$ were replaced according with the data provided for the equation of the line, as following:

$$0.130 = 2.5236.1.C + 0.0005$$

The C (concentration) found was 0.0513 mg/mL, which when multiplied for the value of dilution (40mL), gives us 2.052 mg of non-encapsulated ibuprofen in the final powder.

To obtain the real encapsulation efficiency, it was necessary to adjust that result to the yield. The total ibuprofen amount present in the solution to be spray dried was 60mg for 371.4 mg of powder (chitosan+pectin+ibuprofen). The final weight of the obtained particles was 114.9 mg (after spray drying), so it was necessary to calculate how much ibuprofen would be present in that powder. It was used the following equation:

$$IBf = \frac{IBi \cdot TPf}{TPi}$$

IB_i being the ibuprofen present in the solution to be spray dried, IB_f being the final ibuprofen quantity in the recollected spray dried product, and TPf and TPi being, respectively, the final and the initial total amount of powder. The resulting value is 18.562 mg of ibuprofen.

Knowing that were 18.563mg of ibuprofen in the powder and from that quantity, 2.052mg were non-encapsulated, it was possible to postulate that there was 89% of encapsulation of ibuprofen.

This rate of encapsulation is very good and shows that there is a substantial entrapment of the drug between the polymeric matrixes, as stated in the literature (*Rabindra et al., 2012*). It states as a very good method of simultaneously synthetizing the particle and encapsulating the bioactive compound, having the advantage of being relatively easy to perform, cost effective and it dispenses the use of organic solvents.

4.2 - SYNTHESIS OF CHITOSAN COATED CITRUS PECTIN BY PARTICLES INVERSE MICROEMULSION METHOD

4.2.1 – Particle morphology and size through SEM

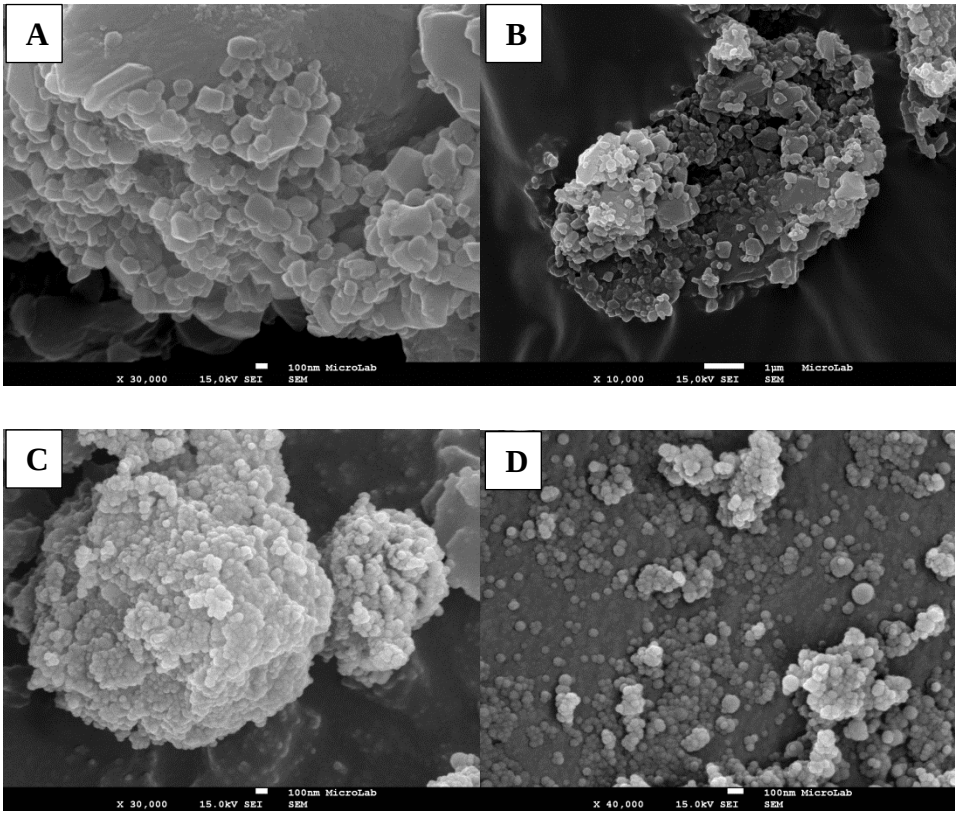

Figure 4.4 - SEM images of CS:CPec particles, A and B being unloaded particles and C and D being PEITC loaded particles.

The morphology of the CS:CPec particles obtained, as shown by the SEM images in **Figure 4.4**, indicates that the chitosan particles have a regular surface, spheroid shape, with size ranging from 150 to 300 nm (image A and B) and 50 to 150 nm (image C and D), and have nearly uniform particle size distribution,

which is very important for delivery. The molecule of calcium chloride has one calcium ion (+2) and two chloride ions (-1), which means that the overall charge for the molecule neutral. Calcium chloride salts can also form crystals based on these same ionic properties. Positive calcium ions can orient themselves so that they are close to the negative chloride ions in another molecule (*Albert, K. S., 1928*). This allows for the formation of solid crystal structures, shown on images A and B. In PEITC loaded particles, a lower concentration of $CaCl_2$ was used, and, shown on the **Figure 4.4**, the crystal formation is practically inexistent.

It can also be observed that the reaction time affects the particle size and size distribution: loaded particles are smaller and less uniform than the unloaded ones, as they were stirring for only six days, and unloaded particles were left stirring for seven days. That translated into a moderate increase of the particle diameter. This fact is more explored with DLS analysis.

Due to the electron beam of the microscope, some small CS:CPec particles fused given rise to larger ones.

4.2.2 – DLS size measurement and pH responsiveness assays

DLS, also known as photon correlation spectroscopy, is one of the most used methods used to determine the size of particles. Particle size can be determined by measuring the random changes in the intensity of light scattered from a suspension or solution. This technique is commonly known as dynamic light scattering (DLS), but it can also be designated correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS). DLS was used both to measure particle size and the size alteration resulting from pH variation, as both polymers are pH sensitive.

Table 4.1- Particle size measurement by DLS after 3 days of reaction, in different pH solutions, and the average, median and standard deviation of the most intense peak. The sizes are presented in manometers. N = 4.

		pH1.7						**pH7.2**						**pH13.5**			
		Size1	**Size2**	**Peak Intensity %**				**Size1**	**Size2**	**Peak Intensity %**				**Size1**	**Size2**	**Peak Intensity %**	
				size1	size2					size1	size2					size1	size2
Measures	1º	137.1	123.7	56.9	43.1	**Measures**	1º	782.5	123.7	92.6	7.4	**Measures**	1º	1204	0	100	0
	2º	153.9	0	55	45		2º	694.5	0	100	0		2º	1308	0	100	0
	3º	400.8	0	55.1	44.9		3º	618.1	0	100	0		3º	1253	0	100	0
	4º	592.1	144.3	58.5	41.5		4º	1123	144.3	92.1	7.9		4º	1155	0	100	0
	5º	348.4	158.5	58	42		5º	1084	158.5	89.9	10.1		5º	1761	4987	74	19.8
Average		326.5				**Average**		860.4				**Average**		1336			
Median		348.4				**Median**		782.5				**Median**		1253			
Standard Deviation		188.6				**Standard Deviation**		229.8				**Standard Deviation**		244.2			

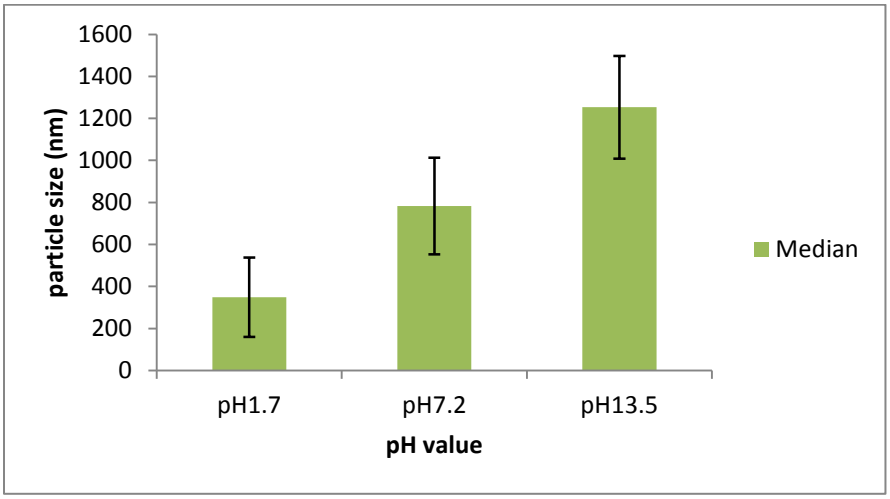

Figure 4.5- Bar chart representing the first peak median of the particle size for each pH value. pH value affects directly the size of the particles. The error bar represents the standard deviation.

With the sample collected after 3 days of stirring, were prepared three suspensions at pH 1.7, 7.2, 13.5, and the resultant size variation is represented on the **Figure 4.5**. A concentration of 0.25mg/mL was used for each of the preparation. As it can be observed in **Table 4.1** the particle size is not very constant along the measurements, especially the median and standard deviation data shows that there is a large discrepancy between each measurement, and more than one peak appeared (% of peak intensity). So it can be assumed that 3 days of crosslinking reaction are not enough to obtain a uniform particle size distribution. The literature stated that the particles respond to pH differences by swelling at lower pH values and shrinking at low pH values, as the chitosan outer layer tends to swell and/or degrade in simulated acidic conditions. The acidic pH represents an increase of the diameter, due to intramolecular electrostatic repulsions. (*Makhlof et al.,2011*). However, that fact could was not clear with the data presented at **Table 4.1.**

It is also interesting that the size of the particles at pH 1.7 is initially very small and, along increases with time. The continuous increase of the diameter as a function of time that was observed indicates aggregation of the system. According to the literature, chitosan based particles exhibit charge cancellation at neutral to alkaline pH values. The absence of repulsive electrostatic forces affects both intra and intermolecular interactions. This causes the system to lose its colloidal stability, causing the uncharged particles to start to aggregate. Since the pKa value of the chitosan is close to neutral pH, particles tend to spontaneously aggregate as a result of the decreased degree of protonation of chitosan in neutral to basic pHs (*López-León et al, 2005*).

Table 4.2 - Particle size of CS:CPec particles after 6 days of crosslinking reaction in three different pH solutions. It can be observed that the peak intensity was 100% in every

	PdI	pH 3.01	Peak Intensity %	PdI	pH 5.5	Peak Intensity %	PdI	pH 7.4	Peak Intensity %
1 measurement	1	536	100	0.432	450.5	100	0.363	761.9	100
2 measurement	1	577	100	0.372	888.6	100	0.445	763.4	100
3 measurement	1	545	100	0.252	1058	100	0.418	1024	100

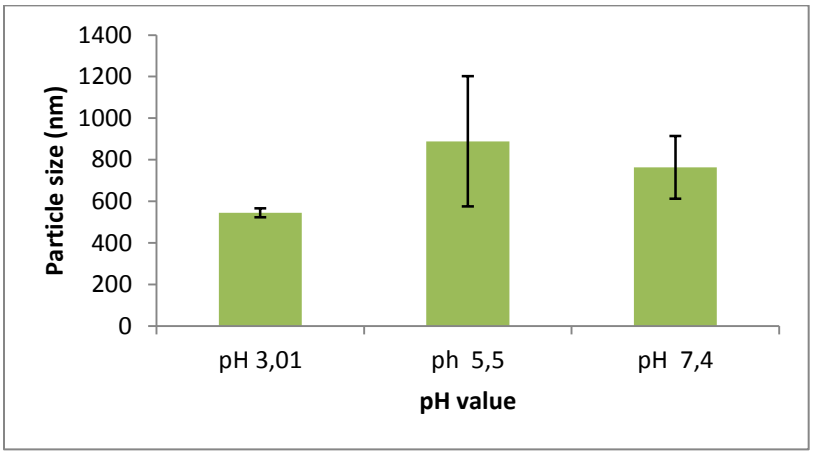

Figure 4.6 - Bar chart representing the median of particle size at 6 days of crosslinking reaction, at different pH values, with standard deviation represented as error bar. N=4.

The data referring to the particles whose reaction was stopped at the sixth day, confirms that the size of the particles becomes more uniformly distributed. A new parameter has been new parameter: PdI (polydispersity index), in order to help the better interpretation of the results. PdI is used to describe the degree of "non-uniformity" of a distribution. For a perfectly uniform sample, the PdI would be 0.0.

In view of the first measurement results in **Table 4.2** and **Figure 4.6**, it can be agreed that the particle diameter increased from 450.5 nm at pH 5.5 to 536 nm at pH 3.0, as it was expected. At pH 5.5 the particles aggregate over time, probably because of the approximation of the pKa value. However, the PdI values indicates that particle size is stable at pH 5.5 and at pH 7. At pH 7, as expected, aggregation of the particles is present, with the aggregates presenting similar sizes. The particle size shown at pH 3.3 is also stable, even though the PdI is the highest. That high PdI is certainly due to the partial dissolution of chitosan coat, what would make the particle to start to have irregular sizes. That phenomenon is described by Sako and co-workers which stated that "cationic polymers with amino groups have higher water

solubility at acidic pH than at neutral pH" (*Sako et al, 2013*). So, it can be concluded that the swelling behavior of chitosan has a limit. When the pH value is very low (~3 and below) chitosan, instead of swelling, becomes soluble in water (**Figure 4.7**), smaller particles are observed, because the particles are partially or completely devoid of the chitosan coat. Even for the particles whose size was measured after 3 days of crosslinking reaction, it can be seen that the diameter of those that were at pH 1.7 was very small in the first two measurements and increased with time.

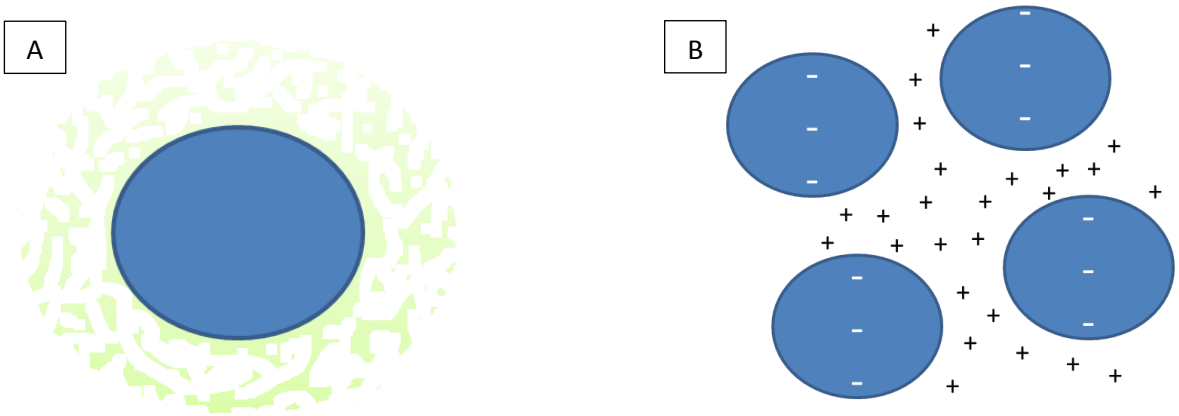

Figure 4.7 - The first image (A) represents the particle at very acidic environment. The chitosan layer swelled and started to dissolve. The second image (B) shows the exposed negatively charged citrus pectin core, in an acidic solution.

Pectin pKa is approximately 3.5. Pectin hydrogels are typically spreadable, with rigidity increasing as pH falls below pectin pKa (*Dixon, 2008)*. Below the pKa, less dissociation of H^+ results in greater hydrogen bonding of the pectin chains, thus originating a stiffer hydrogel (*Moreira et al., 2014*). This may explain the instability of the particle size along the measurements at pH 1.7.

To prove that the particles do lose the chitosan layer in highly acidic environment, a new DLS experiment, with particles being suspended in 4 different pH solutions, was performed.

Table 4.3 - Particle size measurement by DLS after 10 days of reaction, in four different pH solutions, and the average, median and standard deviation. All peak intensities were 100%. The particle size is presented in nanometers. N = 4.

	pH 3.07	PDI	pH5.0	PDI	pH 7,6	PDI	pH 10.4	PDI
1 run	1348	1	2136	0.432	712.4	591	615.7	0.71
2 run	1425	1	3709	0.37	1294	1	458.7	0.27
3 run	1949	1	3993	0.252	2860	0.467	712.4	1
	pH 3.07		**pH 5.0**		**pH 7.6**		**pH 10.4**	
Average	1574		3279.3		1622.1		595.6	
Standard	327		1000.1		1110.7		128	

Deviation				

The effect of pH on particle size is once again noticeable in the 10 day particle crosslinking reaction DLS measurements, presented on **Table 4.3.** Taking as reference the first measurement of the particle size at pH 7, it can be seen that the particle size decreases at higher pH (10.4) from 712.4 nm to 615.7 nm, due to the shrinkage of the chitosan layer, and increases at lower pH (5.0) from 712.4 nm to 2136 nm, due to the swelling of the chitosan layer. The effect of really low pH (3.07) in particle size was also very clear: there is a shift in size comparing to the size verified at pH 5.0. The size changes from 2136 nm (pH 5.0) to 1348 nm (pH 3.07), which is clearly due to the dissolution of part of the chitosan layer. The PdI value also confirms the instability in size and shape at pH 3.07.

In order to explore the stability of the particle size and the reversibility of the chitosan layer dissolution in highly acidic environment, the particles were kept in the respective solutions for 24h and their size was analyzed again through DLS. The results are shown in **Table 4.4.** The PdI in every measurement is 1, so the stability of the particle size is lost, for being that long in solution. Yet, by looking upon the size of the particles in each pH solution, the results are as expected and in accordance with the ones achieved in the first measurements (**Table 4.3**). The particles in pH 3.07 are much smaller, so it can be assumed

Table 4.4 - Particle size measurement by DLS after 10 days of reaction, presented on Table 6, after being 24h in suspension in the respective pH solutions. The only exception goes to the particles at pH 10.4. To the solution that had pH 3 was added NaOH in order rapidly raise the pH to 14. The particle size is presented in nanometers. N = 4.

	PDI	**pH 3**	**PDI**	**pH 7.6**	**PDI**	**pH 11**	**PDI**	**pH 14**
1 run	1	548.2	1	825	1	1314	1	120.1
2 run	1	712.4	1	1859	1	1134	1	53.45
3 run	1	255	1	2292	1	958.6	1	4.253
	pH 3		**pH 7.6**		**pH 11**		**pH 10.4**	
Average	505.2		1658.667		1135.533333		59.2677	
Standard Deviation	231.7119764		753.739		177.7049615		58.142	

that the chitosan layer eventually dissolved completely or almost, leaving just the citrus pectin core. In order to investigate the reversibility of the chitosan layer dissolution, the pH 3.07 solution was basified to the pH value of 10.4, by adding NaOH. In this way, the particles present in the highly acidic pH solution, suffered a drastic change in the environmental pH, as it was abruptly changed to a very alkaline value. The results presented in the **Table 4.4** show that the chitosan layer not only does not reconstitute itself around the citrus pectin core, but also that the particle size diminishes drastically to very low diameters, leading

the particle to nearly disappear. This phenomenon is due to the fact that "anionic polymers with carboxyl groups have higher water solubility at basic pH than at acidic pH" as reported by Sako and colleagues in 2013. That is to say that the pectin core - which will incorporate the payload - also becomes soluble.

4.3 - ENCAPSULATION OF PEITC BY SUPERCRITICAL CO_2 IMPREGNATION

4.3.1 – Derivatization and quantification of loading capacity

According to the values presented above, the results point to an encapsulation capacity lower than 1% for both SD and RD. Due to the very negligible values obtained, only the undiluted sample was used for calculation. After replacing the values at the equation of the line (**Figure 4.8**), the PEITC concentration obtained in SD was 0.02187 g/L (**Table 4.5**). When that value is divided by the PEITC in the acetic acid solution (3.3 g/L), the encapsulation % is 0.66. That means that for each 99,34g of polymer, there is 0.66 g of PEITC. When it comes to RD, the concentration obtained was 0.00838 g/L (**Table 4.6**).

Table 4.5 - Presentation of the concentration and absorbance results, in duplicate, regarding the slow depressurization (SD) for each sample´s dilution. The average, standard deviation and variation coefficient were calculated for each sample. The results used in loading studies were the ones of the 1/1 dilution, and the conversion to uL was also presented above. N = 1.

	SD						
Dilution	**1st Abs.**	**2st Abs.**	**1st Concentration(µM)**	**2ost Concentration(µM)**	**Average (µM)**	**SD (µM)**	**CV (%)**
1/1	0.29	0.28	137.05	130.86	133.95	4.38	3
1/50	-0.05	-0.04	-23.43	-1076.19	-549.81	744.41	**-135**
1/100	0.011	0.06	4.19	2514.29	1259.24	1774.91	**141**
1/200	0.02	0.03	9.90	2457.14	1233.52	1730.46	140

	mol/L	**g/L**	**G**	**cm3**	**uL**
1/1	$1.339 \cdot 10^{-06}$	0.02187	$6.56 \cdot 10^{-04}$	$5.996 \cdot 10^{-07}$	$6 \cdot 10^{-13}$

Table 4.6 - Presentation of the concentration and absorbance results, in duplicate, regarding the rapid depressurization (RD) for each sample´s dilution. The average, standard deviation and variation coefficient were calculated for each sample. The results used in loading studies were the ones of the 1/1 dilution, and the conversion to uL was also presented above. N = 1.

	RD						
Dilution	**1st Abs**	**1st Abs**	**1º Concentration(µM)**	**2º Concentration(µM)**	**Average(µM)**	**SD (µM)**	**CV (%)**
1*1	0.12	0.11	53.71	48.95	51.33	3.37	7
1*50	-0.03	-0.03	-861.91	-861.91	-861.91	0.00	0
1*100	-0.02	-0.03	-1247.62	-1676.19	-1461.96	303.05	-21
1*200	-0.01	-0.03	-1352.38	-3066.67	-2209.52	1212.18	-55

	mol/L	**g/L**	**G**	**cm3**	**uL**
1*1	$5.133 \cdot 10^{-05}$	$8.38 \cdot 10^{-03}$	$2.51 \cdot 10^{-04}$	$2.3 \cdot 10^{-07}$	$2.3 \cdot 10^{-13}$

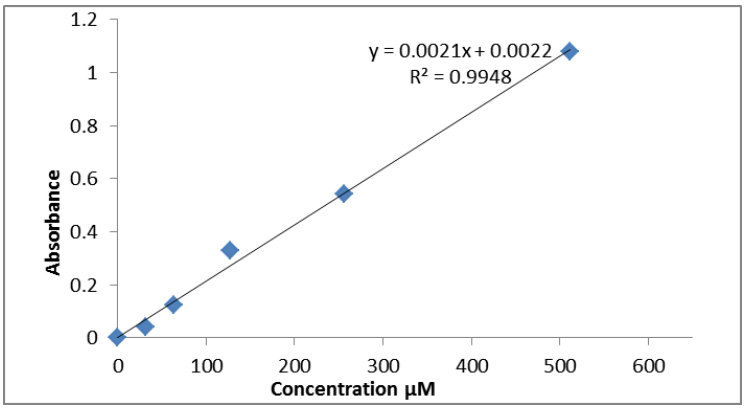

Figure 4.8 - Calibration curve and the respective equation of the line used to determine the PEITC concentration for SD and RD.

From the results obtained, it is clear that the impregnation method has a very poor efficiency in PEITC encapsulation into CS:CPec particles. One of the possibilities that could justify these unpromising results is the chosen method of PEITC release. While in spray drying encapsulation, the loading capacity was calculated through the calculus of the % of non-encapsulated active principle; in the impregnation it was necessary to calculate the % of the encapsulated active. So, it was necessary to add one step in which the release of the payload happened. To release the payload, the particles were destroyed by placing them into a 10% acetic acid solution, the PEITC released being diluted, then, in that solution. It is probable that the acetic acid reacted with the derivatization reagents, considering that when the sample (PEITC released in 10% acetic acid) was added to the derivatization solution, it became opaque. The low rate of impregnation may be explained also by stronger interactions of the drug for the solvent (carbon dioxide) than with the polymeric matrix. According to Diankov and co-workers, the interactions between the solute and the

carbon dioxide molecules increase at higher densities (or higher temperatures) and can diminish the bonding forces between the solute and the matrix (*Diankov et al, 2007*).

Therefore it is necessary to optimize not only the impregnation method, but also the downstream characterization step, focusing on the payload release method. Nevertheless, for the results presented, the SD method was more efficient than the RD. These results contradict the ones of Yokozaki et workers, that shows otherwise: the high depressurization rate results in low impregnation rates - although Yokozaki work is not entirely comparable as the depressurization rate in the present work is much higher than the ones described in literature (*Yokozaki et al, 2015*). It is thought that the extremely high depressurization rate lead to the precipitation and deposition of PEITC, making the solute to be laying on the particle surface instead of being into the particles. In the SD process the solute probably was dragged along with the CO_2.

4. Conclusion and Future Work

The main goal of this study, which was to achieve *in vivo* release phase and to compare the uptake of some encapsulated natural extracts, was not achieved. However, some important conclusions were made during this project regarding the preparation and characterization of the synthesized particles. Two synthesis methods and three encapsulation methods were tested: inverse microemulsion and spray drying technique were used to synthetize the particles and during the synthesis process, the encapsulation also took place. Additionally, impregnation by supercritical CO_2 was also used as an encapsulation method. Inverse microemulsion method is a very good synthesis method, according to the results. Through this method it is possible to control the size, by changing the time of reaction, and to have mostly monodisperse particles. These particles can respond effectively to external stimuli, namely pH, due to the presence of ionizable groups capable of reacting with surrounding medium. It was not possible to calculate the encapsulation or liberation rate, due to the difficulty of destroying the particles with methods that would not affect or react with the payload. The weak point of this method is the demand for organic solvents for its development. This weak point is overcome with the second synthesis method: the spray drying.

Spray-drying technology offers the potential to incorporate a range of excipients into the formulation to be spray-dried, and provides a good control over particle size, particle morphology and microsphere density. It is a very good and effective method, easy and quick to perform, as the solution to be spray dried is prepared in about one hour, and the particles can be obtained in the same day, although, is this case, the particles were obtained on the following day. The non-use of organic solvents in a tremendous asset, it is an alternative, cost-effective and, at the same time, safe and environmentally sound particle production method. These green methodologies help to reduce the margin of error, as they assure, in *in vitro* studies, that the positive results (in this case, the CRC cells death) are due to the designed mechanisms, and not because of some vestigial organic solvent that may have been not entirely withdrawn. The particles obtained through this method were also stimuli responsive and highly monodisperse.

The last encapsulation method is the impregnation by CO_2. This methodology did not present a good result and it requires optimization both of the synthesis and the downstream encapsulation rate estimate. In this work, the determination of that rate was performed by derivatization, that later showed to be very inaccurate due to the solvent in which the PEITC was dissolved being incompatible with derivatization reagents. Another method that can be tested to proceed to this calculation is the HPLC analysis. It can be also experimented different methods of destroying the particles and/or release the payload, through other solvents.

These particles are meant to be ingested, after being entrapped inside a Eudragit® L100 or Eudragit® S100 blended capsule, as means of treating CRC. *Khan* studied different Eudragit® L100/Eudragit S100 combinations in vitro, and observed that the release profile could be changed by changing the ratio between these two polymers, leading to a considerable flexibility in choosing the delivery site within the intestinal region, better than Eudragit® L100 or Eudragit® S100 alone (*Khan et al., 2000; Tirpude and Puranik, 2011*). Eudragit coated formulations stand as the most commonly used synthetic polymers. Many colon specific delivery systems (CSDS) have one layer of Eudragit® to protect the payload from acid degradation in the stomach, and another to perform colon specific release. In addition, these particles could be administrated through rectal route throughout the use of enemas or suppositories, in case of localized rectal carcinomas.

5. BIBLIOGRAPHY

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6. Annexes

6.1 - DETERMINATION OF ISOTHIOCYANATES (ITCS) CONTENT

Adapted from Zhang et al 1992

Method: 1,2-Benzenedithiole-Based Cyclocondensation Assay

Principle of the assay: utilizes 1,2-benzenedithiol as the vicinal dithiol reagent and measures the reaction product, 1,3-benzodithiole-2-thione, spectroscopically (λmax = 365 nm) . The method can be used to measure 1 nmol or less of pure isothiocyanates or in crude mixtures.

Experimental procedure

1) In 7 mL screw-top glass vials add:
    - 900 µL of 100 mM potassium phosphate buffer
    - 900 µL of methanol
    - 100 µL of sample (Blank, ITC Standard (PITC/ PEITC) or extract)
    - 100 µL of 80 mM 1,2-Benzenedithiole (the reaction initiate after 1,2-Benzenedithiole addition)

2) Heat the vials at 65ºC during 2 hours in a water bath
3) Cool at room temperature.
4) Read the absorbance at 365 nm.

Solutions Preparation

a) Potassium phosphate buffer (100 mM; pH 8.5)

Prepare two stock solutions:

Stock A (1M of KH_2PO_4): weight 6.8 g and add 50 mL of H_20dd
Stock B (1M of K_2HPO_4): weight 8.71 g and add 50 mL of H_20dd

- To a 200 mL of H_20dd add: 1,5 mL of Stock A and 23,5 mL of Stock B
- Homogenize the solution and adjust the pH to 8.5 with a solution of KOH
- In a 250 mL volumetric flask adjust the volume with H_20dd.

b) 1,2-Benzenedithiole (BDT; 80 mM; ref: 270865-500mg Sigma)

- Stored at 4ºC (Lab 7.18A)
- Count the number of glass vials that you will use: for each vial is added 100 µL of BDT
- For example, for 5 mL of BDT 80 mM, weight 56.896 mg and add 5 mL of MeOH (MW =142. 24 g/mol)
- Note: the melting point of BDT is very low (22-24ºC). Be fast to avoid BDT melting.

c) Phenethyl Isothiocyanate (sigma)

- Stored at room temperature on the shelf of NutraBrass in lab 7.04
- Prepare a stock solution of 0.1 M of phenyl ITC: 12.2 µL of ITC + 987.8 µL of MeOH
- Dilute the stock solution (0.1M) for a 1000 µM phenyl ITC solution: 10 µL of ITC (0.1M) + 990 µL of MeOH
- Use the 1000 µM phenyl ITC solution to make a standard curve: 0, 40, 80, 160, 320, 640 µM

Standard Curve:

- 640 µM: 360 µL MeOH + 640 µL 1000 µM phenyl ITC solution
- 320 µM: 500 µL MeOH + 500 µL 640 µM
- 160 µM: 500 µL MeOH + 500 µL 320 µM
- 80 µM: 500 µL MeOH + 500 µL 160 µM
- 40 µM: 500 µL MeOH + 500 µL 80 µM
- 0 µM: only MeOH