UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIAS

Application of experimental design in the preparation of Chitosan/Carrageenan nanoparticles by polyelectrolyte complexation: Study of the effect of salt addition

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Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação da Professora Doutora Ana Margarida Moutinho Grenha e coorientação da Professora Doutora Ana Maria dos Santos Rosa da Costa

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Inês Rocheta Silva

“If you’re not part of the solution, you’re a part of the precipitate.”

“Science never solves a problem without creating ten more.”

— George Bernard Shaw

Acknowledgements

Apesar de toda a dissertação se encontrar escrita em inglês, sinto o dever de usar esta oportunidade para agradecer a todos os que fizeram parte deste meu percurso na minha língua materna.

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Por último, a mim e ao que ainda está por vir.

Abstract

Chitosan (CS) and carrageenan (CRG), two marine-derived polymers, were selected as matrix materials for the preparation of nanoparticles by polyelectrolyte complexation. The effect of polymer concentration in the production of CS/CRG nanoparticles and on the characteristics of the final product (size and zeta potential) was studied. Furthermore, an experimental design was used to evaluate the effect of adding three different salts, NaCl, $CaCl_2$ and Na_2SO_4, to the formulations. From the obtained results, different mathematical models were established, allowing to have a tool to predict the characteristics of the nanoparticles according to the production parameters.

Overall, the results permitted drawing some conclusions, including the fact that the concentration of CRG in the formulations did not impact the size of the nanoparticles and the observation that the inclusion of Na_2SO_4, even at a concentration of 0.006M, which is considered low, results in the production of micro-sized carriers instead of nanoparticles. For NaCl and $CaCl_2$ it was observed that there was an inversion in the pattern of evolution of NP size with variation of CS concentration when the salt concentration increased. Nevertheless, there was a significant lack of fit of the model regarding the size, which indicates that an improvement is necessary to better fit the data. Regarding the zeta potential, and as expected, a general increase was seen with the increase of CS concentration, although some deviations to this normal behaviour were observed that deserve further testing.

The concentrations used for these salts might not have been enough to produce a noticeable effect in the particles' characteristics. As the concentration of the salts were not liable to increase, since they were at the highest concentration possible that guaranteed polymer solubility, other salts could be tested in the future.

Keywords: Carrageenan; Chitosan; Nanoparticles; Polyelectrolyte Complexation; Salts.

Resumo

O quitosano (CS) e a carragenina (CRG), dois polímeros marinhos, foram selecionados como materiais de matriz para a preparação de nanopartículas por complexação polieletrolítica. Foi estudado o efeito da concentração dos polímeros na produção de nanopartículas de CS/CRG e nas características do produto final (tamanho e potencial zeta). Para além disso, foi usado um desenho experimental para avaliar o efeito da adição de três sais diferentes, NaCl, $CaCl_2$ e Na_2SO_4, nas formulações. A partir dos resultados obtidos, foram estabelecidos diferentes modelos matemáticos, permitindo ter uma ferramenta para prever as características das nanopartículas de acordo com os parâmetros de produção.

De uma maneira geral, os resultados permitiram tirar algumas conclusões, incluindo o facto de que a concentração de CRG nas formulações não teve impacto no tamanho das nanopartículas. Também se verificou que a inclusão de Na_2SO_4, mesmo à concentração de 0,006M, considerada baixa, resulta na produção de transportadores na escala micrométrica em vez de nanopartículas. Para o NaCl e o $CaCl_2$ observou-se que, com o aumento da concentração de sal, houve uma inversão no padrão de evolução do tamanho das nanopartículas em função da variação da concentração de CS. No entanto, houve uma falta de ajuste significativa do modelo em relação ao tamanho, o que indica que é necessária uma melhoria do ajuste dos dados. Em relação ao potencial zeta, e como esperado, observou-se um aumento geral com o aumento da concentração de CS, embora tenham sido observados alguns desvios a este comportamento normal que requerem mais estudo.

As concentrações usadas para os sais utilizados podem não ter sido suficientes para produzir um efeito percetível nas características das partículas. Como a concentração dos sais não pode ser aumentada, visto ser a concentração máxima possível que garantia a solubilidade dos polímeros, outros sais poderiam ser testados no futuro.

Palavras-chave: Carragenina; Complexação Polieletrolítica; Nanopartículas; Quitosano; Sais.

Resumo Alargado

Apesar do avanço que tem vindo a acontecer a nível científico, ainda existem muitos problemas a serem enfrentados no ramo da medicina e terapêutica, o que é evidenciado pelos milhões de pessoas que continuam a morrer devido a doenças. Atualmente, as micro e nanotecnologias têm-se unido no sentido de oferecer cada vez mais otimismo no que se refere a avanços no diagnóstico, prevenção e tratamento dos problemas de saúde mais relevantes da atualidade.

As aplicações médicas das nanotecnologias têm impulsionado o desenvolvimento de vários tipos de veículos à escala nano capazes de transportar fármacos, como os lipossomas, as nanopartículas (NPs) e as micelas, os quais se usam como veículos para entrega de fármacos. De acordo com a *Food and Drug Administration* (FDA), dos Estados Unidos da América, a nanotecnologia deve ser considerada para materiais ou produtos com dimensões até 1000 nm manufaturados para exibir propriedades ou fenómenos atribuíveis a tais dimensões. As NPs são interessantes nos campos da medicina e, especificamente, na veiculação de fármacos, devido às suas características únicas que exibem, como a elevada relação superfície/volume e a capacidade de associar substâncias de interesse.

As NPs podem ser classificadas em diferentes grupos com base nas suas propriedades, formas e tamanhos, sendo os mais comumente usados na veiculação de fármacos os grupos de NPs baseados em lípidos e em polímeros. São muitos os polímeros que têm sido usados para preparar NPs, mas os biodegradáveis são os preferidos, uma vez que normalmente têm boa biodisponibilidade, solubilidade, tempo de retenção e potencialmente evidenciam menos toxicidade. O quitosano (CS) e a carragenina (CRG) são dois polímeros de origem marinha que pertencem a esta classe de materiais e que demonstraram ser capazes de formar NPs. O CS é um polissacárido composto por unidades aleatoriamente alternadas de *N*-acetilglucosamina e D-glucosamina, obtido através da desacetilação parcial da quitina, presente no exoesqueleto de crustáceos. A CRG é um polissacárido com grupos sulfato, composto por unidades de galactose e anidrogalactose ligadas por ligações glicosídicas, extraído da alga vermelha.

Existem vários métodos para a preparação de NPs à base de CS, sendo os métodos de gelificação iónica e complexação polieletrolítica os mais comuns. A última é um termo geral usado para descrever a separação de fases em soluções de polieletrólitos com carga oposta, onde a fase densa resultante pode ser um coacervado/precipitado ou uma fase turva. Isso é possível porque o CS, carregado positivamente, tem alto grau de protonação e forma hidrogéis na presença de polianiões específicos. Esse processo leva a ligações cruzadas inter- e intramoleculares mediadas por macromoléculas aniónicas, das quais a CRG é um exemplo. Este comportamento de complexação de misturas aquosas de polieletrólitos com carga oposta pode ser afetado pela variação do pH e adição de sal.

Os complexos polieletrolíticos (PECs) são uma forma segura e ecológica de produzir materiais para libertação de fármacos e a utilização de polímeros como CS e CRG neste processo traz também as vantagens de serem considerados biodegradáveis, abundantes na natureza e terem relativamente baixo custo de produção. Os PECs baseados em CS estão presentes na literatura com diversas aplicações na distribuição de substâncias ativas, mas são usados principalmente na entrega de fármacos a nível das mucosas, na terapêutica anticancerígena, na terapia génica e antirretroviral.

Este trabalho desenvolveu um estudo das caraterísticas de NPs CS/CRG produzidas por complexação polieletrolítica. Aplicou-se um desenho experimental, utilizando uma metodologia de superfície de resposta para verificar os efeitos da inclusão de diferentes sais (NaCl, $CaCl_2$ e Na_2SO_4) como componentes na produção de NPs de CS/CRG por complexação polieletrolítica, bem como os efeitos da variação das concentrações dos polímeros (CS e CRG).

Foram purificados os polímeros CS e CRG comerciais por meio de diálise (96 h), a que se seguiu um passo de liofilização. Os polímeros foram reconstituídos antes da sua utilização, de modo a atingir uma concentração de 2,5 mg/mL, em água ultrapura e HCl pH = 4, respetivamente. Os níveis de pH das soluções foram mantidos entre 3 e 4. Os três sais foram então adicionados conforme necessário, de acordo com os diferentes requisitos de formulação.

Em todos os casos, as NPs foram formadas espontaneamente pela incorporação de 1,5 mL da solução de CRG em 1,5 mL da solução de CS, atingindo um volume total

de 3 mL, sob agitação magnética e à temperatura ambiente, por um período de aproximadamente 10 minutos.

O tamanho e o potencial zeta de todas as NPs que foram produzidas foram medidos por espectroscopia de correlação de fotões e anemometria de laser Doppler. A partir dos resultados obtidos com a adição de cada sal, foram construídos gráficos de superfície 3D (X; Y; Z), mostrando o efeito da concentração dos polímeros e da concentração de sal nas NPs resultantes. Nestes gráficos estão representadas duas variáveis independentes (X e Y), que representam a concentração de CS e CRG, e uma variável dependente (Z) que é o tamanho de partícula ou o potencial zeta.

De modo geral, os resultados obtidos permitiram tirar algumas conclusões, como o facto de que a concentração de CRG nas formulações não ter impacto no tamanho das nanopartículas e a observação de que a inclusão de Na_2SO_4, mesmo na concentração de 0,006M, considerada baixa, resultou na produção de transportadores na escala dos micrómetros, em vez de nanopartículas. Para NaCl e $CaCl_2$, com o aumento da concentração de sal observou-se uma inversão no padrão de evolução do tamanho das nanopartículas em função da variação da concentração de CS. No entanto, houve uma falta de ajuste significativa do modelo em relação ao tamanho, o que indica que é necessária uma melhoria no ajuste aos dados. Em relação ao potencial zeta, e como esperado, observou-se um aumento geral com o aumento da concentração de CS. Observou-se também uma diminuição no potencial zeta com a adição de NaCl e Na_2SO_4, quando comparados com a ausência de sal. As concentrações usadas para os sais utilizados podem não ter sido suficientes para produzir um efeito percetível nas características das partículas. Como a concentração dos sais não poderá ser aumentada, visto ser a concentração máxima possível que garantia a solubilidade dos polímeros, outros sais poderiam ser testados no futuro.

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List of Abbreviations

ANOVA – Analysis of Variance

CRG – Carrageenan

CS – Chitosan

EPR – Enhanced Permeability and Retention

FDA – Food and Drug Administration

GBD – Global Burden of Disease

NP – Nanoparticle

PdI – Polydispersity Index

PEC – Polyelectrolyte complexes

USA – United States of America

WHO – World Health Organization

1 Introduction

1.1 Health and disease

Health and disease have always been some of society's biggest concerns. This has led to a constant need to improve people's quality of life in an attempt to maintain a healthy society. Throughout this process the concepts of health, disease and treatment have suffered a big evolution.

First, we must know what we understand as health, as it can be considered from many different perspectives. From a merely clinical point of view, health could be described as the absence of any disease or impairment. However, it can also be seen as a state that allows individuals to handle all demands of daily life in a competent manner (also in the absence of disease and impairment) or as a personal state of equilibrium established between an individual and the surrounding environment. Adopting only one of these definitions has considerable consequences. Assuming that the first concept is the true one would mean that only medical professionals could judge someone as healthy, ignoring completely how the individual feels about his or her state. When it comes to the second definition, it assumes that someone that has a disease is not capable of functioning well in their community. Lastly, the third theory suggests that someone could be considered healthy as long as they establish an internal equilibrium that allows them to get as much as they can from their life, regardless of manifesting a disease or not (1). Acknowledging the limitations of these theories, the World Health Organization (WHO) Constitution defined health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (2).

Another important subject is understanding disease and the causes behind it. Throughout the centuries there have been many theories about disease causation. The oldest known theories, the demonic theory and the punitive theory, attributed disease to supernatural powers or to a punishment delivered by a divine being. The theory of the four humours by Hippocrates was one of the first theories to separate disease from occult forces, and instead link it to an imbalance in our own organism (3). The miasma theory (4) circulated mainly thought the XVIII and XIX centuries and was also important for introducing the idea that diseases could be airborne. Next, the germ theory (4) by Louis Pasteur and Robert Koch in the XIX century introduced the idea of germs or microbes as

causative agents of disease. This theory proposed standard criteria that should be met before concluding that a disease was caused by a particular bacterium.

The germ theory reinforced the biomedical model, which is the foundation of modern Western medicine (5–7). This model assumes that diseases are the result of deviations in the normal biological variables, disregarding the social, environmental, behavioural and psychological dimensions of health and illness. This view on health and illness is outdated and takes a reductionist approach to this complex phenomenon (8–12). Taking into account the missing dimensions of this model, another one was constructed considering today's definition of health, the biopsychosocial model (13,14). The evolution of these concepts is imperative when it comes to the combat against today's health issues, since now we understand that "disease is always generated, experienced, defined and ameliorated within a social world" (15).

Between 1950 and 2017 there was a rapid progress in life expectancy for both men and women, from 48 to 71 years and from 53 to 76 years, respectively (16). This is linked to new diagnostic technologies and therapeutic capacity as well as to the improvement of sanitation and hygiene (15,17). However, despite the advancement of science there are still many unresolved medical problems to be faced, which is made evident by the millions of people who continue to die from disease.

Medicine has suffered a lot of changes in the last century. According to a study from Carolina Demography, a department within Carolina Population Center at the University of North Carolina, (Figure 1.1**Erro! A origem da referência não foi encontrada.**), while pneumonia and tuberculosis were the main causes of death in 1900, by 2010 these were exchanged for heart disease and cancer in the United States of America (USA). The Global Burden of Disease (GBD) study, dating from 2017, also has similar data, stating that between 1990 and 2017 early death from enteric infections, respiratory infections and tuberculosis declined, but progress in reducing mortality from some common diseases had stalled or reversed, mainly for non-communicable diseases such as cardiovascular diseases and cancer (16), proving that illness is a never-ending fight. Chronic diseases such as those referred are the epidemics of today, even though infectious diseases such as malaria, tuberculosis and human immunodeficiency virus (HIV) remain major health concerns (16).

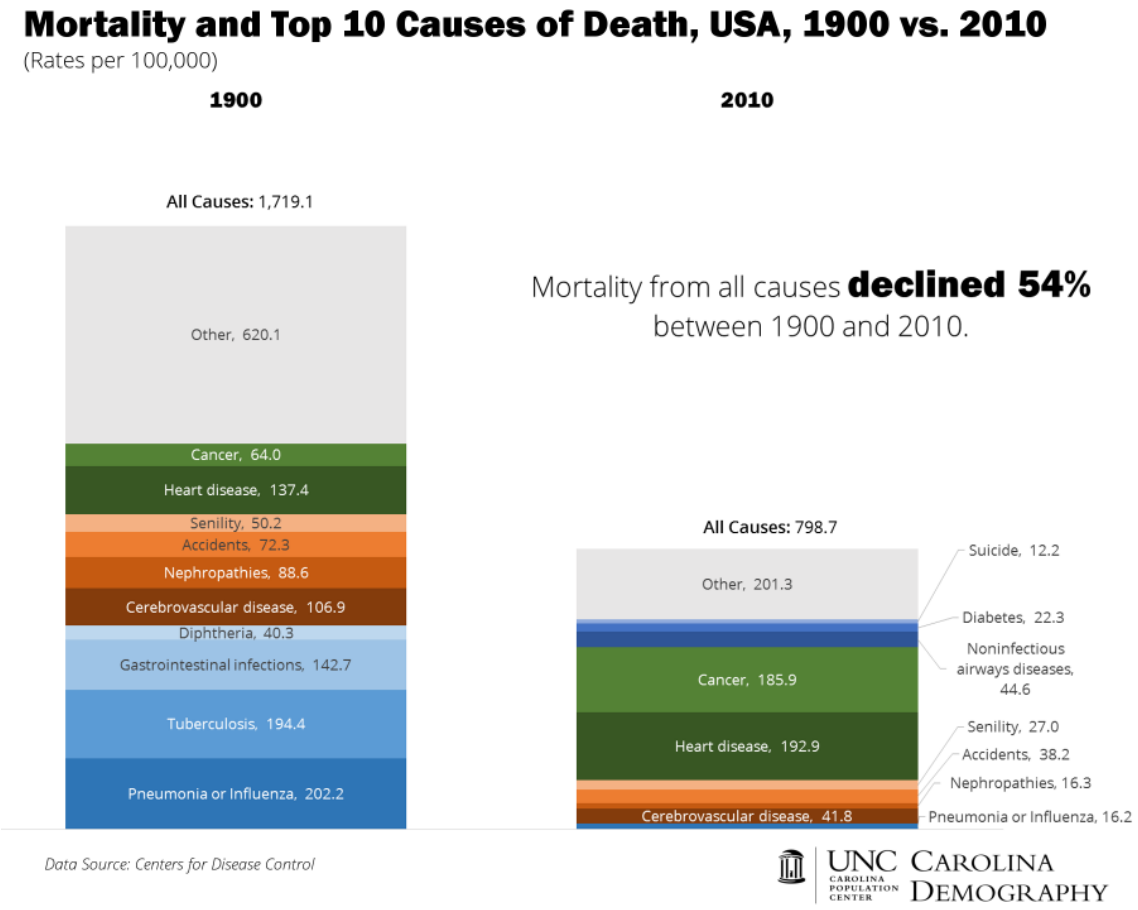

Figure 1.1:Comparison between the top 10 causes of death in the USA in 1900 and 2010 (18).

1.2 Nanotechnology as the future in therapeutics

As diseases become more difficult to treat, the scientific community attempts to develop multiple new technological approaches. If finding new molecules in nature and turning them into tablets was enough effort a few decades ago, this is not the case any longer. Today, micro- and nanotechnologies are coming together to offer increasing optimism when it comes to advances in diagnosis, prevention and treatment of current health problems (19).

Nanotechnology, according to the Food and Drug Association, should be considered for materials or products with dimensions up to 1000 nm engineered to exhibit properties or phenomena attributable to nanometric dimensions (20). These materials and

products can be designed to interact with cells at a molecular level with functional specificity, allowing technology and biological systems to merge in a way that was previously unachievable (21).

Nanotechnology once focused on understanding the link between the intrinsic properties of nanomaterials, namely optical, electrical and magnetic, and their size, shape and surface chemistry as a way to solve technological impasses present in several branches of science. The studies performed in this area had great importance in creating a foundation for engineering nanotechnology-based electronic, computer and biomedical devices. However, potential applications in the biomedical field soon became evident and the focus has since shifted to investigating the interactions of nanomaterials with biological systems. Clarifying the said interactions permits identifying design rules that will allow the successful engineering of target-driven nanoparticulate delivery systems. The evolution of drug-delivery systems, aimed at biomedical applications, can be described by their three generations. In the first generation, developed from the 1050s to the 1970s, nanotechnology was naturally not present. They were instead based on formulations directed essentially to oral and transdermal administration that made controlled release possible. In the 1980s, a second generation appeared, which aimed at creating self-regulating controlled-release drug delivery systems, able to adapt release to internal triggers. It was only in the beginning of the 21st century that this became a real possibility when nanotechnology started to be applied to medicine. In the third generation, research is done in order to design and program NPs to assist therapeutic agents in achieving selective targeting by going through biological barriers, mediating molecular interactions and identifying targeting sites (22,23).

Drug delivery is one of nanotechnology's many medical applications. The paradigm of finding and developing new drugs was replaced in a certain way by the improvement of pre-existing drugs, namely by finding new ways and new routes for their delivery (24). The main goals for research of nanotechnologies in drug delivery are obtaining more specific drug targeting and delivery, thus maximising efficacy, while reducing drug toxicity and increasing safety and biocompatibility. However this field of research is not lacking in challenges, including the search for appropriate carriers, since the design of new materials comprises knowledge on drug incorporation and release, formulation stability and shelf life, biocompatibility, biodistribution and targeting,

functionality and possible adverse effects of residual material after the drug is released (25). The availability of a wide range of materials and the requirement of compatibility with the selected drug, has given impulse to the development of various types of drug nanocarriers (Figure 1.2), such as liposomes, nanoparticles (NPs) and micelles, which are used as drug delivery vehicles (26).

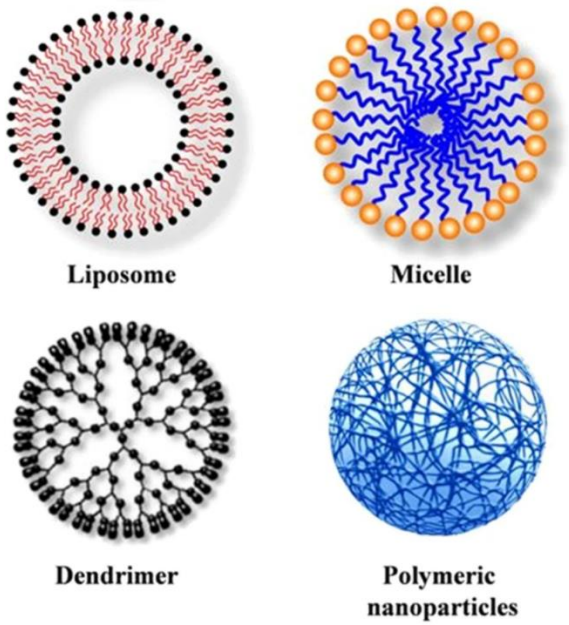

Figure 1.2: Examples of nanomaterial-based drug delivery vehicles (27).

1.3 Nanoparticles as drug delivery systems and their characterisation

NPs are interesting in biomedical applications due to their unique features, such as the large surface-to-volume ratio and the ability to associate compounds of interest. They can be classified according to different aspects, namely their type, morphology, size and chemical properties. These are all factors that will directly influence cellular uptake and impact the pharmacokinetics and pharmacodynamics in nanomedicine applications (28). NPs have a relatively large functional surface which can bind, adsorb and carry different compounds. The engineered NPs may have many different compositions and source materials, so for them to be useful in drug delivery there must be a deep understanding of the pathophysiological basis of disease, since the interaction with cells will vary according to the origin of the materials, namely, whether they are biological components

like lipids or non-biological components like metals, their lipophilicity and size, among other characteristics (25).

NPs are, by definition, disperse systems, being constituted by a disperse phase and a continuous phase. When the size of the dispersed particles ranges from 1 nm to 1 µm they are considered as a colloidal dispersion. NPs fit this definition since a product is thought to involve nanotechnology if its dimension is in the nanoscale (1 – 100 nm) or up to 1 µm as long as the product has properties related to the nanoscale range, as was said before (20). When the size of the dispersed particles is larger, they are considered coarse dispersions, as are examples emulsions, suspensions and aerosols. Within the size range already specified, there is often a wide distribution of sizes of the dispersed particles in the same NP formulation. Therefore, the particle size that is typically assumed for a formulation is an average value that is dependent on the experimental technique used in its measurement. One of the methods used to measure particle size is dynamic light scattering (photon correlation spectroscopy). When a beam of light is passed through a colloidal sol, some of the light may be absorbed, some is scattered and the remainder is transmitted through the sample. Because of the scattered light, the sol appears turbid, which is known as the Tyndall effect. As colloidal particles undergo Brownian motion because of multiple collisions with neighbouring particles, the intensity of the scattered light will fluctuate in time due to the distance between particles constantly changing. Essentially, this method compares scattering intensity at very short time intervals, thus allowing to measure the NPs' average size (29).

When NPs are dispersed in aqueous medium, the materials composing their surface, and their inherent charge, affect the distribution of ions in the proper dispersion, by attracting counterions to the surface and repelling co-ions. As a consequence of the established equilibrium, the NPs will exhibit a determined surface charge. This configures the concept of the electrical double layer, typical of colloidal dispersions, which is depicted in Figure 1.3. The double layer is divided in two parts: the inner part, known as the Stern layer, which includes adsorbed ions, and the diffuse part, where ions are distributed according to electrical forces and thermal motion. Dividing these two layers is the Stern plane. Naturally, the ions in the Stern layer are surrounded by a certain amount of solvent that is in contact with the charged NP surface and accompanies any movements of the particles. This solvating layer is held to the surface and at its edge is the surface of

shear, which marks the boundary of movement between the attached material and the liquid. The potential at the surface of shear, meaning how much energy it takes to move a single point charge to the particle from infinitely far away, is termed the zeta potential and is the charge that is measurable. The zeta potential is also an indicator of the repulsion existing between NPs as a function of their charge. Therefore, the exhibited charge plays a role on the stability of colloidal dispersions, as highly positively or negatively charged particles tend to repel each other, while less charged particles will more easily aggregate and cause flocculation. Usually, colloids with zeta potential values above +30 mV or -30 mV are prone to be stable. Other components pertaining to the colloidal dispersion, namely other excipients that might be used, may disturb the ionic equilibrium. In this context, an increase of the electrolyte concentration of the aqueous medium will lead to compression of the double layer and, consequently, to a decrease in the zeta potential (29,30).

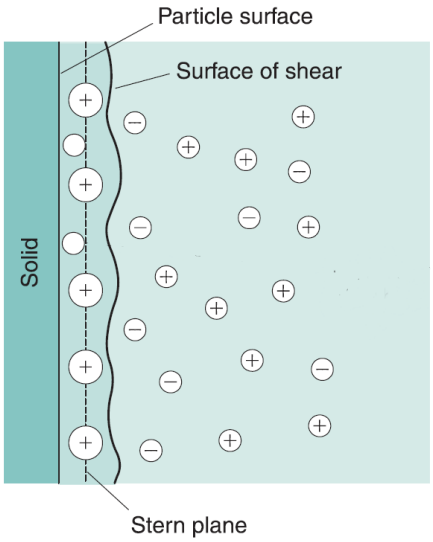

Figure 1.3: Schematic representation of the electrical double layer. Adapted from (29).

It can be often tempting to imagine NPs as simple molecules, but they are anything but that. A NP can be split in up to three possible layers, as shown in Figure 1.4. First, there is the surface layer which can be functionalised, which will greatly affect its stability in aqueous medium. Second, the shell layer, composed of chemically different material from the core material and usually prepared intentionally. And finally, the core, usually used to refer to the NP itself, that dominates its properties of interest (31). However, this

is not mandatory and not all NPs present structured layers, so various possibilities must be taken into account.

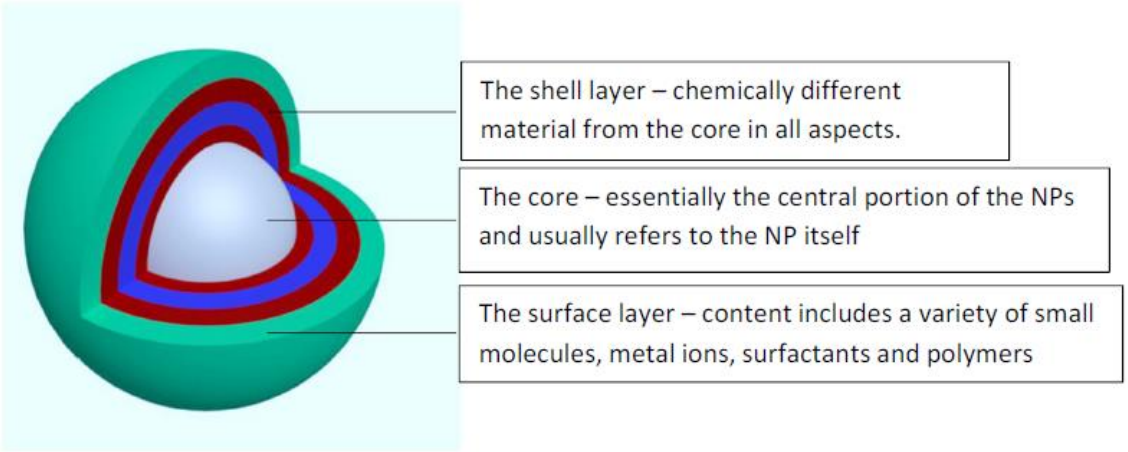

Figure 1.4: Schematic of NP component-structured layers (32).

1.4 Polymeric nanoparticles

Based on their chemical and physical properties, some of the best-known classes of NPs are carbon-based NPs, ceramic NPs, semiconductor NPs, metal NPs, lipid-based NPs and polymeric NPs, being the last three the most studied when it comes to applications in drug delivery and therapeutics (32,33).

Polymeric NPs are those explored in the present study and, therefore, will be described in more detail. This type of carriers provides versatility through the use of polymers with different chemical composition, hydrophilic/lipophilic character, charge and physical structure, among other properties. As a result, the prepared NPs could be formulated to deliver a range of drugs and be adaptable to many clinical applications. Moreover, in some cases it can be possible to have the ability to control the degradation and disassembly of polymeric NPs, which grants the ability to control the temporal aspects of drug delivery across a wider range of time than that provided by other types of NPs (34).

The design of polymeric NPs depends on the therapeutic application, target site and route of administration. Even though intravenous injection is the main route of administration for this type of NPs, there are less invasive ways, such as

dermal/transdermal, pulmonary, oral and other routes of mucosal delivery. Awareness of the different barriers and challenges in every route of administration is imperative for the proper design of nanocarriers. These barriers can be external, such as skin and mucus, *en route*, as renal and hepatic clearance, destabilisation, aggregation and opsonisation, and at the cellular level, which includes cellular uptake, entrapment during endocytosis, degradation in the cytoplasm, difficult translocation to cellular organelles and clearance by exocytosis, as is summarised in Figure 1.5 (35).

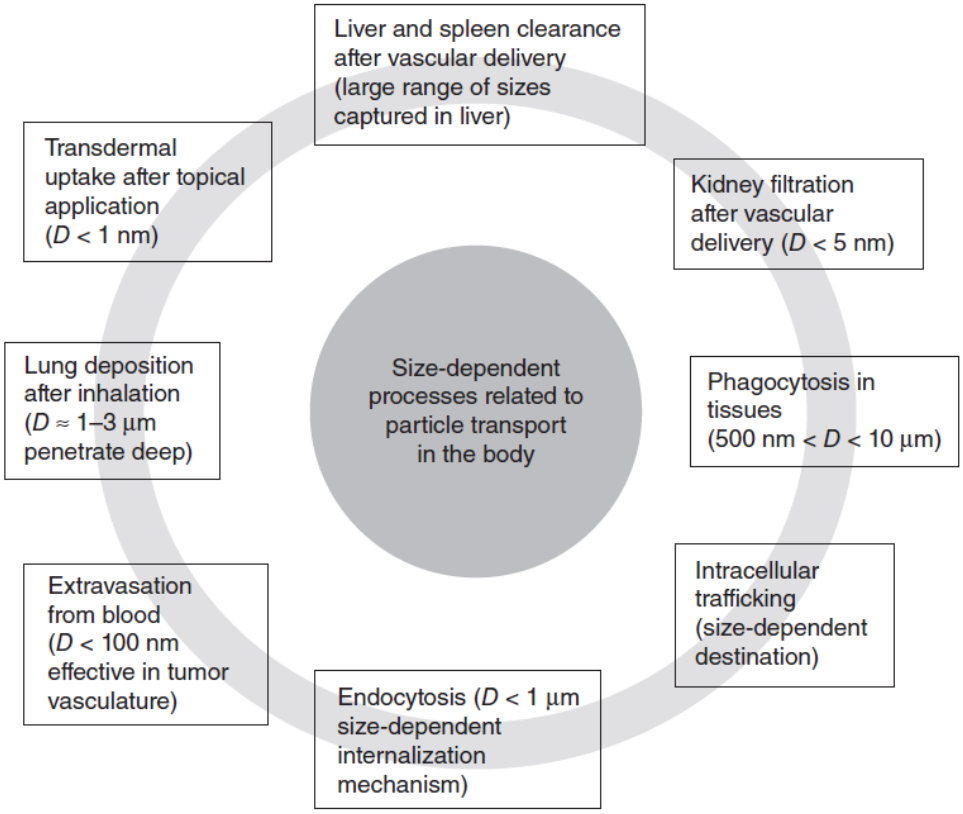

Figure 1.5: Biological barriers present in the body that can cause variability in nanoparticle transport (28).

1.4.1 Materials composing polymeric nanoparticles

Initially, polymeric NPs were based on non-biodegradable polymers, so they needed to be designed in a way that ensured that the particles had a rapid and efficient clearance though urine or faeces, as to not accumulate or distribute in tissues at a toxic level. Nevertheless, and as expected, chronic toxicity and inflammatory reactions were observed with use, fostering a shift of the focus to biodegradable polymers. At first,

synthetic polyester-based polymers were the most used, but natural polymers such as chitosan, alginate, gelatine and albumin have then emerged as potential possibilities, given the reduced concerns on toxicity and the increased biocompatibility associated to these materials (28). Although attention has been given to the toxicology and health implications of NPs, the environmental behaviour of these nanocarriers has been less discussed. NPs can impact the environment in four ways: i) direct effect on biota, ii) changes in the availability of toxins or nutrients, iii) indirect effects on ecosystems and iv) changes of the environmental microstructures. Understanding the interactions between NPs and the environment is crucial to estimate the potential impact of NPs and ensure their environmentally sustainable production and use (31,36).

Following in this section, the specific properties of chitosan and carrageenan will be detailed, as these are the natural polymers that were selected to produce the NPs explored in the present work.

Chitosan

Chitosan (CS) is a derivative of chitin, which is the second most abundant natural polymer in the planet, after cellulose. Chitin is a naturally occurring polysaccharide consisting of repeating units of β-(1-4)-*N*-acetylglucosamine found in crustacean shells, insect exoskeletons, fungal cell walls, microfauna and plankton (37). Depending on its source, chitin occurs as two allomorphs: α-chitin and, the rarer, β-chitin (38). In crustacean shells, chitin is found as a part of an intricate system with proteins and calcium carbonate. Thus, industrial processes for its extraction consist of three main steps: deproteinization of the raw materials with the addition of an alkaline solution, demineralisation by the treatment with an acidic solution and discoloration of the final product with an alkaline solution once again. These processes may vary according to the chitin source due to the diversity of their structures (38,39). Chitin has poor solubility in water and organic solvents when compared to CS because of its rigid structure and strong intra and inter molecular hydrogen bonds, which limits its use (40,41).

Its deacetylated derivative, CS, is a polymer formed of repeating units of *N*-acetylglucosamine and *D*-glucosamine and is the most used in commercial applications. Generally, about 20% of the repeating units are acetylated, with the remaining 80%

deacetylated, but these percentages may vary according to the source of chitin and processing methods (37). The chemical structure of both chitin and CS is represented below in Figure 1.6.

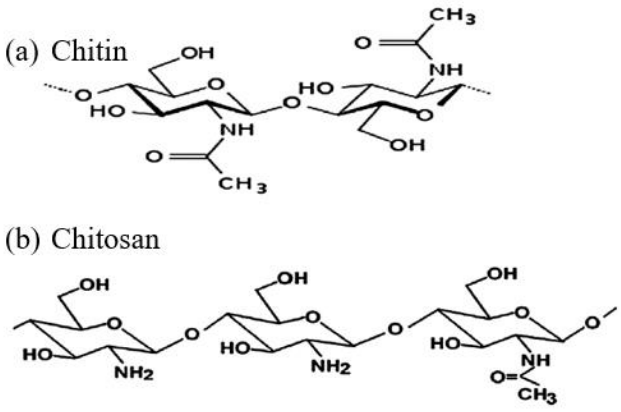

Figure 1.6: Chemical structure of chitin and chitosan (38).

CS can be produced in a variety of ways including a thermochemical deacetylation method (40), an isolation method from chitosan raw materials (42) and a bioconversion method (43). The most common method to produce commercial CS is the thermochemical deacetylation of chitin. In this method, acetamide linkages go through *N*-deacetylation under strenuous alkaline conditions at a high temperature, removing about 75% of acetyl groups. More than 80% deacetylation cannot be achieved without depolymerisation and shortening of the polymer chains (37). In this procedure, conditions like the starting material's quality, particle size, reactants mixture ratio, additives and agitation rate have a determinant impact in the final product. Consequently, notable variability can be noticed. Among the physicochemical characteristics of CS, degree of deacetylation and molecular weight are considered the main characteristics influencing its properties, which emphasizes the need for standardized methods. After the deacetylation process, CS is left with diverse functional groups: the amino moieties, capable of ionisation, and the remaining acetamide groups, prone to hydrophobic associations (44).

In its solid state, CS is generally organized in highly ordered crystallites contained in amorphous regions. There are two main CS crystalline polymorphs, the "tendon chitosan" and the "annealed" polymorphs. In both polymorphs the crystal is formed by

two antiparallel CS molecules with a two-fold helix conformation stabilized by hydrogen bonds. The difference between them is that the "tendon chitosan" polymorph is a hydrated form, meaning that there are water molecules between crystal cells stabilising the structure by multiple hydrogen bonds, while the "annealed" polymorph is an anhydrous crystal form (44).

As referred previously, the degree of deacetylation, meaning the proportion of deacetylated and acetylated groups, is very important and distinguishes between chitin and CS. The extent and distribution of the chitin deacetylation reaction relates to several changes in the main properties of the molecule. One of the most notorious changes is that, as the amino groups can be ionized, CS becomes polycationic in acidic media and the only cationic natural polymer. Intra- and intermolecular electrostatic repulsion due to protonation are enhanced at low pH, generating higher solubility and chain expansion, which allows it to interact with multiple types of molecules. Diluted inorganic acids, like hydrochloric acid, are good solvents for CS. Apart from solubility, the degree of deacetylation determines properties such as swelling in water, susceptibility to biodegradation, bioactivity and biocompatibility (39,44).

Molecular weight is another characteristic of great importance, having particular influence on the viscoelastic properties of solutions and hydrated colloidal forms. In CS, the molecular weight can vary due to the depolymerisation that happens during the deacetylation process and during chitin's extraction procedures, as well as a result of constant synthesis and degradation of chitin in living tissues (44).

CS is known for its remarkable properties, such as the mucoadhesion capacity and permeation enhancement, *in situ* gelling ability, transfection enhancement and inhibition of efflux pump (45). The polymer also exhibits other biological properties such as antibacterial (46–50), antifungal (51–55), antitumoral (56–58) and antioxidant activity (59–61). These make CS interesting for several applications that go far beyond drug delivery (62,63) and include tissue engineering and wound healing (64–67), water treatment (68–71) and obesity treatment (72–75). Drug delivery is, however, one of the most relevant fields of application and CS has been proposed to incorporate the matrix of many drug delivery systems, as nanoparticles, microspheres, hydrogels and films, among others (41,76). Different formulations typically find different applications,

varying from the delivery of genes or proteins, including vaccines, to that of poorly soluble drugs (77,78).

Biodegradation is also a very important property of drug delivery systems. CS, as a hydrophilic polymer, must have a suitable molecular weight for renal clearance in the case systemic absorption takes place. CS is thought to be degraded in vertebrates mostly by lysozyme and by bacterial enzymes in the colon. However, its biodegradation mechanism *in vivo* is still not fully understood. Both rate and extent of biodegradability in living organisms seem to be dependent on the degree of deacetylation of CS, lower deacetylation degree resulting in faster rate of degradation, and on the polymer's molecular weight, lower molecular weight leading to increased degradation (79,80). When it comes to biodistribution, it will be related to all aspects of the CS formulation, from molecular weight and degree of deacetylation to size and charge, in the case of using drug carriers, as well as the route of administration. When CS is delivered intravenously, studies reported that it appeared to accumulate mostly in the liver and stomach (81–85). Even though CS uptake into the blood stream in oral administration studies is not commonly investigated, its systemic absorption and distribution may be closely related to its molecular weight. In this regard, the absorption of CS polymers by the gastrointestinal tract is not expected and they are unlikely to show distribution, whereas CS oligosaccharides may be absorbed to some extent (80,86). As for toxicity, CS is widely regarded as a non-toxic, biologically compatible polymer. However, there is mixed information about this aspect, with some *in vivo* studies reporting low toxicity (87,88) but an *in vitro* study testing the toxicity of CS-based NPs in a zebrafish embryo model reporting the opposite (89), indicating the need for more safety studies.

Despite being a versatile compound, CS-based pharmaceutical products are hardly found. This is mainly due to its variability, as previously discussed, but also to its hygroscopic nature, as these factors can lead to CS degradation. The polymer's stability can be influenced by intrinsic parameters, such as purity level, molecular weight, deacetylation degree and moisture content, as well as external factors, like environmental conditions, humidity, temperature, processing and sterilisation. As increasing attention was drawn to CS, several strategies have been proposed to improve the long-term stability of CS-based products by preventing polymer chain damage. These strategies go from the addition of stabilising agents (mannitol, sorbitol, glycerol), to complexation with non-

ionic polymers creating CS blends and modification of CS structure via covalent (dialdehydes and genipin) and ionic (carrageenan, hyaluronic acid, sodium alginate) crosslinking (90).

Carrageenan

Seaweeds are abundant, renewable marine biomass which are found worldwide. Many seaweeds produce hydrocolloids associated with the cell wall and intercellular spaces. Members of the red algae, *Rhodophyta*, produce galactans, such as carrageenans (CRG) and agars, and members of the brown algae, *Phaeophyceae*, produce urinates, like alginates. CRG can be extracted from *Rodophyta* and from *Carrageenophytes* and represents one of the major texturizing ingredients used in the food industry, being generally regarded as safe (GRAS) after being used for food applications for decades. Its original source was from the red seaweed *Chondrus crispus*, which is only used in limited quantities nowadays. The most used commercial CRG are extracted from *Kappaphycus alvarezii* and *Eucheuma denticulatum* (91).

Among all the commonly used hydrocolloids, CRG can be difficult to characterize due to different red seaweed species having different types and compositions of CRG (92). In any way, it is established and widely accepted the existence of three main types of commercial CRG composed of D-galactose residues linked alternately in 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units, which vary in their degree of sulfation: 1) kappa-carrageenan (κ-CRG), with one sulfate group per disaccharide repeating unit; 2) iota-carrageenan (ι-CRG) having two sulfate groups per disaccharide repeating unit and; 3) lambda-carrageenan (λ-CRG) having three sulfate groups per disaccharide repeating unit. Generally, seaweeds do not produce these idealized and pure carrageenans, but more likely a range of hybrid structures and/or precursors. Several other carrageenans, with a different pattern of repeating units exist, including, xi (ξ), theta (θ), beta (β), mu (μ) and nu (ν), as seen in Figure 1.7 (91,93–95).

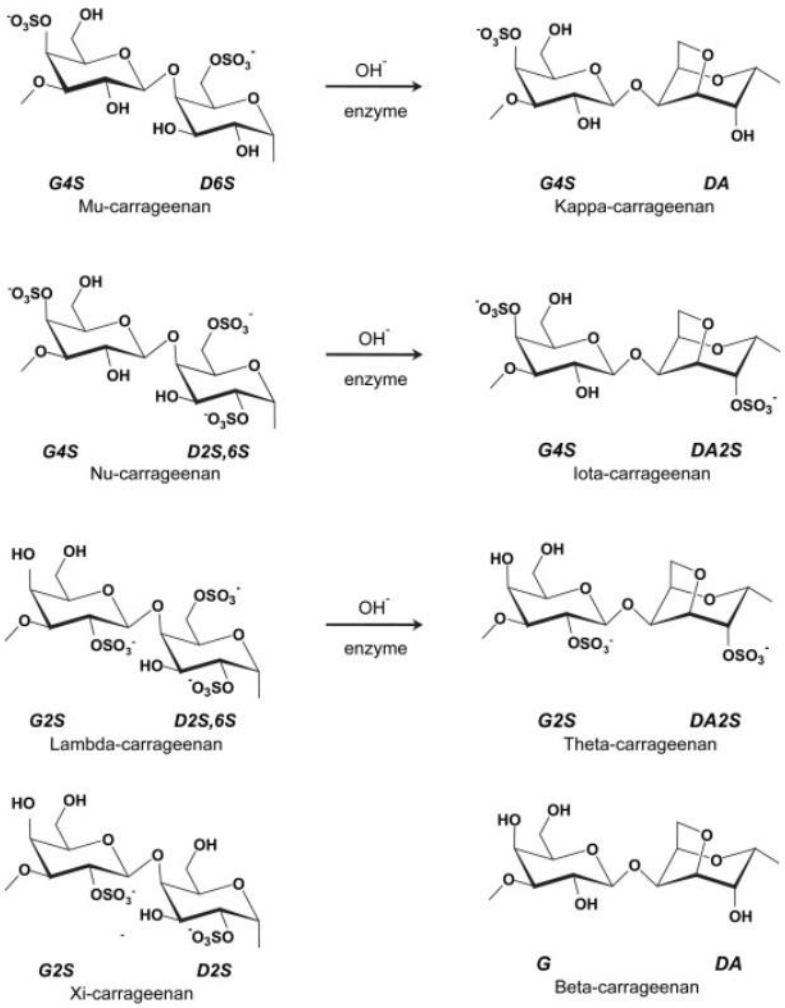

Figure 1.7: Chemical structures of different types of carrageenans (96).

The production of CRG traditionally involves high quantities of chemicals, water and energy consumption, as well as high waste generation throughout the entire process, which is cost ineffective and not environmental-friendly. Therefore, green extraction methods such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE) and pressurized solvent extraction (PSE) have been considered as a way to help reducing the use of chemicals and improve the extraction yield and quality of seaweed-derived polymers (92).

Apart from applications in the food industry due to thickening, stabilising and gelling properties, CRG is also used in the pharmaceutical, cosmetics, printing and textile industries (97). Thanks to its gelling properties, it has been an attractive candidate for the

preparation of hydrogels to be used in biomedical areas like tissue engineering, wound dressing and drug delivery (98,99). When it comes do drug delivery, CRG can form an unique bilayer matrix for drug release when in aqueous environment, which allows to control the dissolution and diffusion of the loaded drug. Another useful property is the high capacity for water absorption, improving drug dissolution and consequently increasing the oral bioavailability of poorly water-soluble drugs (100). Preparation of nanoparticles NPs comprising CRG and CS have also been reported as advantageous drug delivery systems (101,102). CRG also has other interesting biological activities such as antioxidant, immunomodulatory, anticoagulant, antithrombotic, antiviral and antitumoral effects. Even though CRG is generally regarded as a relatively non-toxic and non-irritating substance when used in non-parenteral pharmaceutical formulations, it has also been shown to induce inflammatory responses in laboratory animals used to study anti-inflammatory drugs. Therefore, its long term safety is a major concern and requires further studies contextualised with the intended routes of administration (94,103,104).

1.4.2 Nanoparticle preparation methods

Different methods can be employed for the production of NPs, but they are mostly divided into two main approaches: the top-down approach and the bottom-up approach. In the methods included in the top-down class, a destructive approach is used. It starts with larger molecules, which are broken down or decomposed into smaller units and then converted into suitable NPs. Opposed to this principle is the bottom-up class, in which NPs are formed/constructed from basic units or monomers. These approaches are further divided according to the operation, reaction conditions and adopted protocols (32,33).

As discussed before, CS is one of the most studied and promising natural polymers used in the production of polymeric NPs. As a result, varied methods have been developed for this purpose, such as emulsification and cross-linking, emulsion droplet coalescence, emulsion solvent diffusion, reverse micellar method, ionic gelation and polyelectrolyte complexation, which are all part of the bottom-up approach (105).

Methods involving emulsification appeared first, but typically involved the application of harsh cross-linking agents to harden the formed droplets, which caused obvious toxicity and compromised drug integrity, decreasing their interest (105).

Ionic gelation and polyelectrolyte complexation both rely on CS's capacity to form hydrogels in the presence of specific polyanions, as a consequence of the inter- and intramolecular cross-linkages mediated by the anionic molecules. However, a simple factor distinguishes these techniques. When CS gelation is induced by small anionic molecules, such as phosphate, the technique is called ionic gelation. On the contrary, when it is induced by anionic macromolecules, such as CRG, it is referred as polyelectrolyte complexation (105). Polyelectrolyte complexes (PEC) are formed when the solutions of two polyelectrolytes of opposite charges are mixed, as occurs with CS and CRG. The phase separation in this mixture leads to a "dense phase", referred to as a coacervate or precipitate (depending on its appearance and water content), and a supernatant phase dilute in polymer. Coacervates have a higher water content than precipitates, which are solid structures, and look like a viscous liquid. Between the two extremes, cloudy phases with different degrees of turbidity can be observed (106). Although the electrostatic interactions between the complementary ionic groups of the polyelectrolytes are the main driving force for PEC formation, hydrogen bonds and hydrophobic interactions also contribute to the complexation. The final structure, as represented in , having hydrophobic and hydrophilic regions, makes PECs a particular class of physically cross-linked hydrogels that are sensitive to pH and other environmental factors, such as temperature and ionic strength (107).

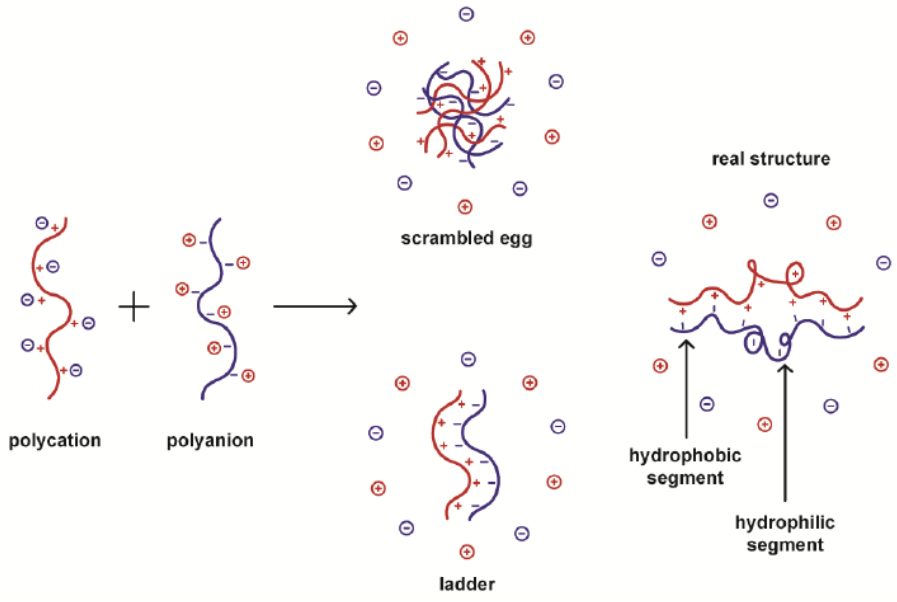

Figure 1.8: Schematic representation of the structure of polyelectrolyte complexes (107).

When CS-based PEC particles are formed, charge neutralisation tends to occur, leading to the formation of aggregates. Therefore, to obtain NPs two factors are extremely important, as shown in Figure 1.9. First, the polyelectrolyte solutions must be diluted and second, one of the polyions has to be in the appropriate excess so that the charge ratio is different from one (107).

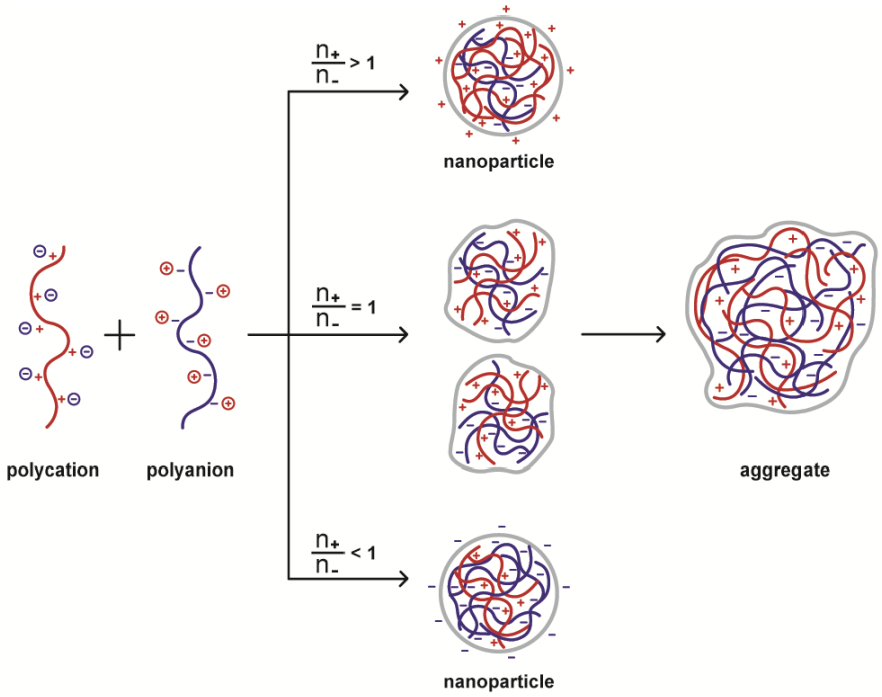

Figure 1.9: Effect of the polyelectrolytes charge ratio on the size and charge of the formed PECs (107).

The inclusion of salt in the reaction media also plays an important role in the formation of PECs, as salts will reduce the interaction between polyelectrolytes, inducing a rearrangement and leading to the reduction of aggregation owing to a less stiff and more-coiled structure of the polymers (108,109).

As PECs show promise as drug delivery systems, drug incorporation and its release are vital steps. Active substances can be incorporated into PEC NPs in four ways: 1) by entrapment of the drug (present in solution) at the time of complex precipitation; 2) by adsorption of the drug (present in solution) to the already prepared PEC when they come into contact; 3) by chemically binding the drug either to the polyanion or polycation and 4) by using the drug as the polyanion or the polycation itself, which is only possible for ionizable drugs (108). As for the mechanisms of drug release, the NPs deliver the

active substance to the target site by: swelling of the polymeric NPs followed by release through diffusion; rupture or degradation of the NP at site via enzymatic reactions, releasing the drug from the entrapped core; or dissociation of the drug from the NP (110).

1.4.3 Application of PECs in drug delivery

The formation of PECs is a safe and environmentally friendly way of manufacturing materials for drug delivery applications. The employment of polymers, such as CS and CRG, in this process also gives it the advantages of being generally regarded as safe and biodegradable, as discussed before, but also abundant in nature with a relatively low production cost. Most of them also have hydrophilic groups which could form non-covalent bonds with biological tissues, making bioadhesion possible (111).

CS-based PECs have been reported for many applications as drug delivery systems. They are mainly used in mucosal drug delivery, cancer therapy, gene delivery and anti-HIV therapy (111,112).

Mucosal surfaces, like ocular, respiratory tract and reproductive tract are susceptible to infection by pathogenic microorganisms. Therefore, these sites have been used as therapeutic targets and have established the basis of many of the developed nanotechnology-based therapies. Moreover, as mucosal surfaces are frequently the primary site of infection, the administration of vaccines onto these surfaces is being increasingly proposed. Mucosal vaccination is expected to induce mucosal immune responses in addition to the conventional systemic response, which could entail a more efficient approach to protect these areas against infection (111). Therefore, many studies have directed the efforts to making these approaches possible (113–115). In our research group, PECs were described in applications as varied as oral vaccination and respiratory delivery of proteins. When it comes to vaccines with interest in oral administration, NPs composed of CS and a synthesized locus bean gum sulfate derivative (LBGS) were proposed as oral immunoadjuvants(116). In this study a significant adjuvant effect was seen when OVA-loaded CS/LBGS NPs were used, possible due to the mucoadhesive properties of CS and the capacity of LBGS to target the intestinal M cells. Regarding the delivery of proteins through respiratory routes, our group has described applications both in pulmonary and nasal delivery. For the effect, PECs based either on aminated-

pullulan/CRG and sulphated-pullulan/CS (117) or on CS/CRG/tripolyphosphate (118) were proposed as protein carriers for pulmonary and nasal transmucosal delivery. Finally, other groups have also relied on PECs to propose oral insulin delivery (119).

PECs have also been described in the context of cancer therapy. In this regard, depending on their composition, PECs may enable the creation of more specific treatments, mainly due to the Enhanced Permeability and Retention (EPR) effect exhibited by tumours. This effect permits an interplay between characteristics of materials and those of tumour tissues, resulting in potential benefits from the use of smart drug delivery systems that are stimuli-responsive (120–123).

When it comes to the field of gene delivery, efforts are being made to develop non-viral gene delivery vectors, as they are safer than viral vectors and can reduce inflammation and the inherent immune response that is induced (124). As for anti-HIV therapy, nanotechnology-based systems for antiretroviral drug delivery have been showing improved results, especially because nanoparticulate-based carriers could allow drugs to reach latent virus reservoirs and promote the absorption of antiviral drugs, improving their bioavailability (125–127).

2 Objectives

This work was stablished within a wider line of research concerning the production of polysaccharide NPs and the parameters that affect the NPs' final characteristics.

The specific goal of the work was to study the effect of polymer concentration and of the addition of different salts in the production of chitosan/carrageenan (CS/CRG) NPs. A response surface methodology was used to evaluate its effects on the size and zeta potential of the obtained particles. From the obtained results, different mathematical models were established, allowing to have a tool to predict the characteristics of the nanoparticles according to the production parameters.

3 Materials and methods

3.1 Materials

Chitosan (CS) (low molecular weight, deacetylation degree 75%–85%), was purchased from Aldrich Chemistry® (Germany). κ-carrageenan (CRG) was obtained from Fluka Analytical® (Germany) and acetic acid (glacial) from Merck® (Germany). NaCl, $CaCl_2$ and Na_2SO_4 were purchased from Sigma-Aldrich® (Germany). Hydrochloric acid (HCl) 37% obtained from VWR Chemicals® (France) and ultrapure water (Millipore®, Portugal) were used throughout.

3.2 Screening nanoparticle preparation conditions - Preliminary study

CS was dissolved in 1% (v/v) acetic acid and CRG was dissolved in ultrapure water at 60 ºC, to obtain solutions of 1 mg/mL (w/v) and 1.25 mg/mL (w/v), respectively. The resulting pH of each solution assumed a mean value of 3.2 for CS and 7.2 for CRG (pH meter PHS-25CW). NPs were prepared to reach final theoretical CS/CRG mass ratios of 2/1, 3/1, 4/1, 5/1 and 6/1. The carriers were spontaneously formed by polyelectrolyte complexation, upon incorporation of 0.8 mL of CRG solution into 2 mL of a CS solution at the concentration of 1 mg/mL, under magnetic stirring at room temperature. A schematic representation of the procedure is depicted in Figure 3.1. To reach the specified mass ratios and thus obtain different NP formulations, the concentration of CRG was varied between 0.42 mg/mL and 1.25 mg/mL.

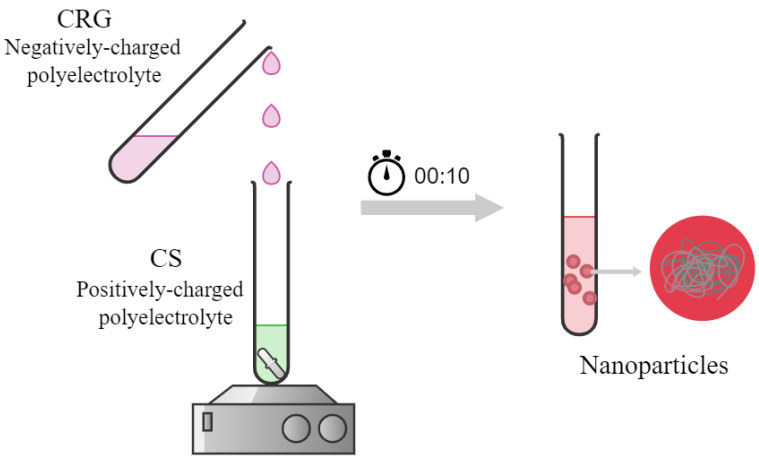

Figure 3.1: Schematic representation of the Polyelectrolyte Complexation method.

3.3 Experimental design

A response surface methodology was applied to verify the effects of the inclusion of different salts (NaCl, $CaCl_2$ and Na_2SO_4) as components in the production of CS/CRG NP by polyelectrolyte complexation, as well as the effects of varying the polymer (CS and CRG) concentrations. A central composite design (CCD) was performed using the software Design Expert 6.0 (Stat-Ease Inc., MN, USA). The established experiments thus involved three independent variables: concentration of CS, concentration of CRG and concentration of salt. The levels of the different factors are described in Table 3.1.

Table 3.1: Final concentrations of the polymers and salts used through the study for the production of the NPs.

CS (mg/mL)	CRG (mg/mL)	NaCl (M)	$CaCl_2$ (M)	Na_2SO_4 (M)
0.020	0.060	-	-	-
0.635	0.655	0.500	0.015	0.003
1.250	1.250	1.000	0.030	0.006

A total of 20 experiments with six center points were generated from the statistical design. The experiments were divided in 3 blocks, where blocks 1 and 2 had each 6 formulations that were prepared and measured on the same day, and where block 3 had 8 formulations and was generally prepared the day after block 1 and 2. The conditions of each experiment are detailed in the annexes section, in Tables 7.1, 7.2 and 7.3.

The responses were analysed by using a second order polynomial model:

$Y_k = \beta_{k0} + \sum_{i=1}^{n} \beta_{ki} x_i + \sum_{i=1}^{n} \beta_{kii} {x_i}^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{kij} x_i x_j$ where Y_k is the response variable, Y_1 is the particle size (nm), Y_2 is the zeta potential (mV); x_i are the independent variables, x_1 is the chitosan concentration (mg/mL), x_2 is the carrageenan concentration (mg/mL), x_3 is the salt concentration (M); β_{k0} is the model intercept coefficient; β_{ki}, β_{kii} and β_{kij} are interaction coefficients of linear, quadratic and the second order terms, respectively.

3.4 Preparation of nanoparticles for the study of the effect of salt inclusion

3.4.1 Polymer dialysis

CS was dissolved in HCl pH = 3 ($[H^+] = 10^{-3}$ M) and CRG was dissolved in ultrapure water at 60 ºC, to obtain solutions of 2.5 mg/mL (w/v). Dialysis tubing from Sigma Aldrich® (Germany) with 9 mm average flat width was used for the dialysis of both polymer solutions. The dialysis lasted 96 hours and was made against HCl pH = 4 and ultrapure water for CS and CRG, respectively. The pH of the solutions before the dialysis were 2.4 for CS and 6.7 for CRG (pH meter PHS-25CW). At the end of the dialysis process the pH values were 5.2 for CS and 3.6 for CRG. The resulting solutions were frozen at -80 ºC and then freeze-dried under the following conditions: pressure of (3.9 - 4.9) x 10^{-5} atm (or 0.04-0.05 mBar) and 72 h of primary drying starting at -49 ºC (Labconco® FreeZone 6 Liter Benchtop Freeze Dry System freeze dryer, Labconco®, USA).

3.4.2 Formulation

After freeze-drying, CS and CRG powders were reconstituted in ultrapure water and HCl pH = 4 ($[H^+] = 10^{-4}$ M), respectively, to obtain a concentration of 2.5 mg/mL. The resulting pH of each stock solution assumed a mean value of 5.2 for CS and 2.7 for CRG. The pH of the CS solution was then adjusted to a mean value of 3.8 using HCl 0.75M.

The three salts NaCl, $CaCl_2$ and Na_2SO_4 were included in the formulation of NPs to study their effect on physicochemical characteristics (size and zeta potential). As can be observed in Table 3.1., each salt was tested at two different concentrations, one corresponding to the maximum that could be solubilised in the established conditions, thus the maximum concentration, and the other corresponding to the half of that. CS/CRG NPs without any salt addition were also prepared.

In all cases, the NPs were spontaneously formed upon incorporation of 1.5 mL of CRG solution into 1.5 mL of a CS solution under magnetic stirring at room temperature, for a period of approximately 10 minutes, as represented in Figure 3.1 above. A total volume of 3 mL was therefore obtained for each formulation that was prepared. In order

to easily incorporate the specified salt concentration in each formulation, that specified concentration was previously added to each of the polymeric solutions used for NP assembly (CS and CRG). Moreover, the dilution of the polymer stock solutions to the desired concentrations in each formulation, was performed using HCl solution of pH 4 already added of the specific concentration of salt. In brief, each polymeric stock solution, namely CS and CRG, was initially divided in three parts, as is shown in Figure 3.2. To the first portion no salt was added, to the second portion a medium concentration of salt was added and to the third portion the highest concentration of salt used in the study was added.

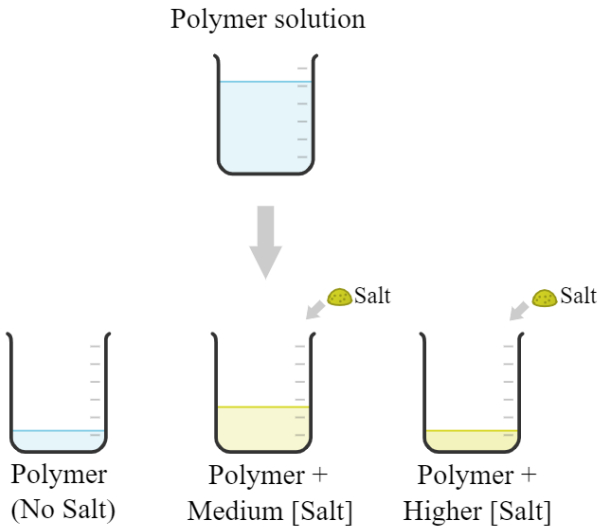

Figure 3.2: Schematic representation of the preparation of the CS+Salt and CRG+Salt solutions.

After that, the polymeric solutions were diluted to the required concentrations using HCl (pH = 4), adjusted to a final volume of 1.5 mL, having already the specified concentration of salt for the formulation under preparation, as depicted in Figure 3.3. The specific volumes used in the preparation of all solutions involved in the production of the NPs are described in the annexes section, in Table 7.1, Table 7.2 and Table 7.3. In the end of this process, a solution of CS at the specified concentration for a determined experiment (V1) and another one of CRG, also at the specified concentration for the same experiment (V2), were obtained.

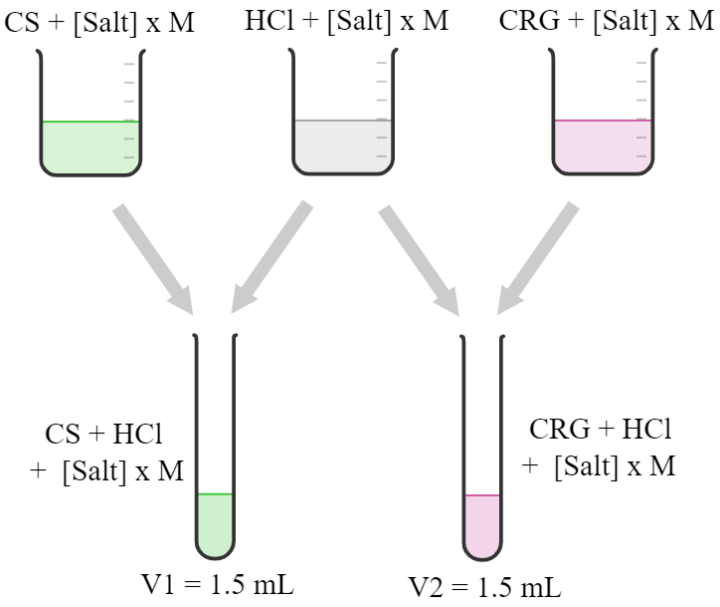

Figure 3.3: Schematic representation of the preparation of the solutions used in the production of the NPs. [Salt] x M meaning that the same concentration of salt, in molar, was used in every solution.

3.5 Nanoparticle characterisation

The size and zeta potential of all the nanoparticles that were produced through the processes described above were measured by photon correlation spectroscopy and laser Doppler anemometry, respectively, at 25 ºC, using a Malvern Zetasizer® Nano-ZS (Malvern Instruments, UK). For the particle size analysis, regular cuvettes were used. As for the zeta potential measurement, folded capillary Zeta Cells were used. Each sample was diluted to an appropriate concentration using ultrapure water.

3.6 Statistical analysis

For the analysis of results of the characteristics of NPs obtained in the preliminary assays of NP production, a t test was performed using GraphPad Prism 6, and differences were considered significant at a level of $p < 0.05$. The best fitting models were determined through multiple linear regressions using the backward elimination method in order to remove non-significant factors and interactions from the initial response surface model step by step. In each following step, the least significant variable in the model was

removed, when possible, until all remaining variables had individual p-values < 0.05 (128). The criteria for eliminating a variable from the full regression equation was based on the coefficient of determination (R^2 value), standard error (SE) estimate, and the significance of the F-test and derived p-values. The lack-of-fit and significance of the effects of each factor was determined by analysis of variance. 3D plots were performed using the software Design Expert 6.0 (Stat-Ease Inc., MN, USA) for each salt and studied parameter.

4 Results and discussion

4.1 Screening of the conditions to prepare CS/CRG nanoparticles

In order to acknowledge and practice the process of preparation of polymeric NPs by polyelectrolyte complexation, a preliminary screening of conditions of polymer concentrations was performed and the resulting product observed and characterised. Following conditions exhaustively described in the literature and also frequently used in the laboratory (102), CS was dissolved in acetic acid and CRG in water, and NPs prepared as described in the methodology section. This permitted rehearsing and optimising the conditions to prepare the said NPs.

CS/CRG NPs were successfully obtained by polyelectrolyte complexation at CS/CRG mass ratios corresponding to 2/1, 3/1, 4/1, 5/1 and 6/1. The Tyndall effect was mainly noticed in the formulations 2/1, 3/1 and 4/1, indicating the formation of NPs. These observations were somewhat expected, because the mentioned ratios correspond to those involving higher amounts of polymer, thus facilitating the occurrence of electrostatic interactions that result in the formation of NPs. The highest turbidity was in fact seen in the formulation corresponding to CS/CRG = 2/1, which has the highest amount of polymers.

Table 4.1 shows the results of the physicochemical characteristics (size, polydispersity index (PdI) and zeta potential) of the produced NPs.

Table 4.1:Particle size, PdI and zeta potential of CS/CRG NPs (mean ± SD; n ≥ 4). Note: These data were previously shown in the final project for UC Projeto.

CS/CRG (w/w)	Size (nm)	PdI	Zeta Potential (mV)
2/1	919 ± 69	0.589 ± 0.057	+66 ± 1
3/1	823 ± 63	0.534 ± 0.074	+69 ± 4
4/1	705 ± 43	0.411 ± 0.037	+70 ± 1
5/1	648 ± 51	0.381 ± 0.132	+67 ± 3
6/1	618 ± 83	0.377 ± 0.054	+65 ± 7

The first observation of relevance is that the presence of a higher proportion of CS in the formulations resulted in a general decrease of particle size ($p < 0.05$). The reduction

totalizes about 300 nm, as CS/CRG = 2/1 NPs registered a size of 919 nm and CS/CRG = 6/1 reached 618 nm ($p < 0.05$). The increase in size of the NPs can be related to the incorporation of increasing amounts of CRG from CS/CRG = 6/1 to 2/1, since CRG is a large polymer and, thus, higher amounts will lead to larger particles. The decrease in size was accompanied by a decrease in the PdI, which indicates a more homogenous formulation. When it comes to zeta potential, all formulations laid around +65 - +70 mV.

Similar results were described in other studies of the research group involving CS/CRG NPs (101,102). However, despite the observed trend of NP size behaviour, nominal results were considerably different. Considering just CS/CRG mass ratios of 4/1 to 6/1, which were those described in other works, significantly larger NPs were obtained in the present work, of 600-700 nm, comparing with the described 430 – 580 nm (101,102). This variation can be ascribed to the use of polymers with different characteristics (molecular weight, deacetylation degree of CS) and/or providers, as even inter-batch variability is known to occur in natural materials. The zeta potential reported in the referred works was also highly positive.

In another line of the work performed with the CS/CRG NPs reported in the present work, the effect of adjusting the pH of the polymer solutions prior to the preparation of the NPs was tested, as it is known that NPs produced by polyelectrolyte complexation are naturally sensitive to pH variations (107). The pH value of the polymeric solutions was adjusted to 4 and it was observed that the adjustment enabled the production of NPs with smaller particle size ($p < 0.05$), lower PdI ($p < 0.05$) and decreased zeta potential ($p < 0.05$). These results were part of a work developed in the context of the course “Projeto” of the Integrated Master in Pharmaceutical Sciences.

4.2 Evaluation of the effect of polymer concentration and salt inclusion in the CS/CRG nanoparticles

The literature indicates that the addition of salts in the formulation has a possible effect on the production of PECs (108,109). Therefore, studying this parameter was deemed appealing and adequate in order to complement previous research that was conducted in the research group, dealing with the conditions providing the formulation of

CS/CRG NPs. Thus, studying the effect of salt addition on the production of CS/CRG, along with the influence of the concentration of the used polymers, became the main goal of the present work.

Preliminary studies and the analysis of bibliography related with the use of polysaccharides in nanotechnology, indicated the need to purify the polymers selected as NP matrix materials. In this case, it is typical to observe the presence of proteins and other components which elimination is granted with the purification of the polymer. Additionally, a previous study confirmed that pH variation affects NP formulation and, therefore, it was decided to maintain the pH of the polymeric solution at 4, which enables the ionisation of CS and does not limit the solubilisation of CRG. Purified CS and CRG were thus used in the production of the NPs and the pH of the intervening solutions was kept at 4 in all cases. The main objective of the study was to verify if the addition of salt in the formulation, along with pre-determined concentrations of the polymers, would translate into different physicochemical characteristics of NPs. For this purpose, the salts NaCl, $CaCl_2$ and Na_2SO_4 were tested. This selection permitted having one salt with monovalent ions (NaCl) and two salts with divalent cation ($CaCl_2$) and anion (Na_2SO_4). The association of the salt was performed directly in the polymeric solutions before the formation of NPs, as was detailed in the methods section. For all three salts, two different concentrations were tested. These correspond to the maximum concentration that could be dissolved in the polymeric solutions and based on that, a half-concentration was calculated and used. Naturally, the solubility of the salts is not the same and, thus, different salt concentrations were used in each case. For each salt, several NP formulations were prepared, according to the conditions (volumes, concentrations) that were specified in the experimental design. Those conditions are detailed in Tables 7.1 to 7.3 of the annexes. The physicochemical characteristics (size, zeta potential) of the individual formulations are described in Table 7.4 of the annexes section. From the individual results obtained upon the addition of each salt, 3D surface plots (X;Y;Z) were constructed, showing the effect of the polymer concentration and the salt concentration of the resulting NPs. These graphics have two independent variables (X and Y), which represent the concentration of CS and CRG, and one dependent variable (Z) which is either the particle size or the zeta potential.

4.2.1 Effect on size

The effect of the polymer concentrations and of the inclusion of salts, at different concentrations, on the size of NPs was studied. CS/CRG NPs were formulated in the absence of salt, showing a size that varied between 132 nm to 937 nm throughout the study. In such NPs, particle sizes generally increased with the increase of the amount of CS. However, when CS concentration was kept constant and CRG varied, the sizes suffered no significant alterations, which suggests that CRG did not affect the parameter.

The results obtained upon inclusion of salts are displayed below in several sequential figures, divided as a function of the salt. Each figure shows the observations for the condition of associating either the lower concentration of salt (figures "a") or the higher concentration of salt (figures "b"). The overall analysis of the results and their statistical significance indicated that the established mathematical model was significant in all cases ($p < 0.05$). However, it was also transversally observed for each salt, a significance of the lack of fit of the model ($p < 0.05$). This may be a consequence of the inadequacy of the polynomial model for the obtained data or it can also be caused by the existence of important terms that are at present unknown and were not included in the model.

Figure 4.1 shows the results corresponding to the study of the inclusion of NaCl. The most visible and transversal observation is that, independently of the salt concentration that is added, the concentration of CRG has no effect on the size, as for a fixed concentration of CS and while varying CRG concentration, the NPs size remained practically unaltered. This was not reported in previous works (101,102) or in the results obtained in the research made for the course "Projeto" of the Integrated Master in Pharmaceutical Sciences. However, many parameters were changed in this study, mainly with the addition of salts, use of HCl as medium in all solutions and a different range of polymer concentrations. The concentration of CS was thus observed to rule the effect of size variation. In Figure 4.1a, which displays the results obtained upon inclusion of NaCl 0.5 M, it is observed that NPs' size varied within 190 nm and 520 nm, approximately, with larger NPs being obtained for higher concentrations of CS. However, the analysis of the whole range of concentrations indicates a certain inversion of the trend and, while for

lower concentrations, the increase in CS concentration led to a decrease in size, at higher CS concentrations, the size increased again.

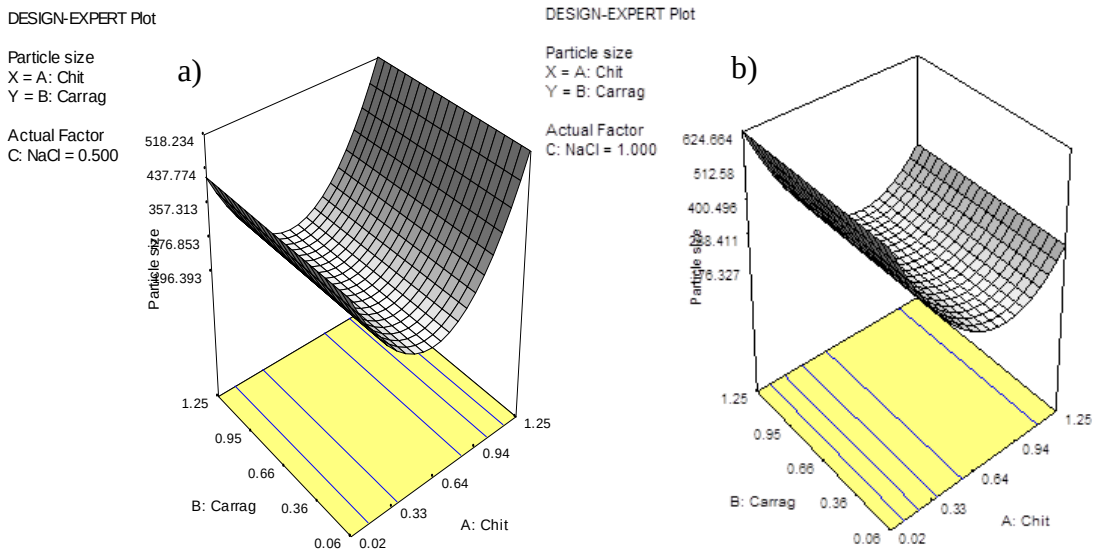

Figure 4.1: Particle size (nm) as a function of polymer (Chit: chitosan, Carrag: carrageenan) concentration and NaCl inclusion. a) Particle size with NaCl 0.5 M; b) Particle size with NaCl 1 M.

Upon the inclusion of NaCl 1 M in the formulation (Figure 4.1b) an opposite effect was seen regarding the evolution of NP size with the variation of CS amount. Under these conditions, the NPs' size varied within 176 nm 625 nm, and larger NPs were obtained for lower concentrations of CS. A certain inversion of the trend of size evolution was also seen in this case. It is of interest to note that the trend observed for the lower concentration of salt is similar to that observed in the absence of salt (data not shown), while the inclusion of the higher amount led to opposite observations.

The addition of $CaCl_2$ to NP formulations let to a rather different size behaviour, as shown below in Figure 4.2. While CRG showed again no contribution to the size of the NPs, the inclusion of a low concentration of $CaCl_2$ (0.015 M, Figure 4.2a) resulted in a linear increase of the NPs' size with the increase of CS concentration, varying between 390 nm and 640 nm, approximately. This effect is quite similar to that observed in the absence of salt (data not shown).

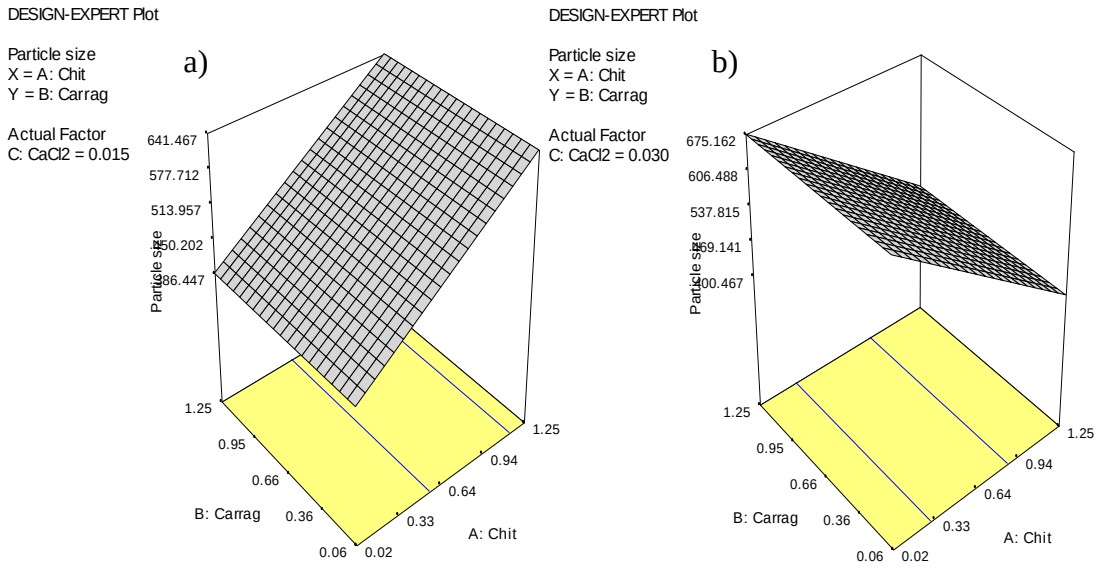

Figure 4.2:Particle size (nm) as a function of polymer (Chit: chitosan, Carrag: carrageenan) concentration and $CaCl_2$ inclusion. a) Particle size with $CaCl_2$ 0.015 M; b) Particle size with $CaCl_2$ 0.030 M.

Curiously, the addition of $CaCl_2$ 0.030M in the formulation (Figure 4.2b) resulted in an opposite effect. Under these conditions, the NPs' size varied between 400 nm and 680 nm, and size decreased with the increase of CS concentration. This effect could be a result of the capacity of the salt to increase the intermolecular links established in the polymeric chains, leading to a certain crosslinking effect and, thus, decreasing the particle size. However, in that case, there is a concentration-limiting effect ruling the observation.

Finally, the inclusion of Na_2SO_4 had a somewhat different result. In fact, in this case the evolution of the size followed a similar pattern with the inclusion of any of the tested salt concentrations (Figure 4.3), which was also similar to that observed in the absence of salt (data not shown). In all cases, the NPs' size decreased with the increase of CS concentration at lower CS concentrations, but increased with the increase of CS concentration when higher concentrations were tested. The greater difference in the behaviour refers to the nominal sizes that were obtained. In the absence of salt, maximum size of 937 nm was observed. The lower salt concentration resulted in NPs within 460 nm and 1 μm roughly, but the higher salt concentration did not permit obtaining NPs, as the registered sizes were above 930 nm in all cases. One possible justification for the similar trend of size evolution through the different conditions, would be the lower concentrations of salt that were used (up to 0.006 M). However, if that was the case, it

would also be expected that the obtained size ranges did not vary as much, contrary to what is seen. Despite these observations, the concentration of CRG had again no effect in the NPs size.

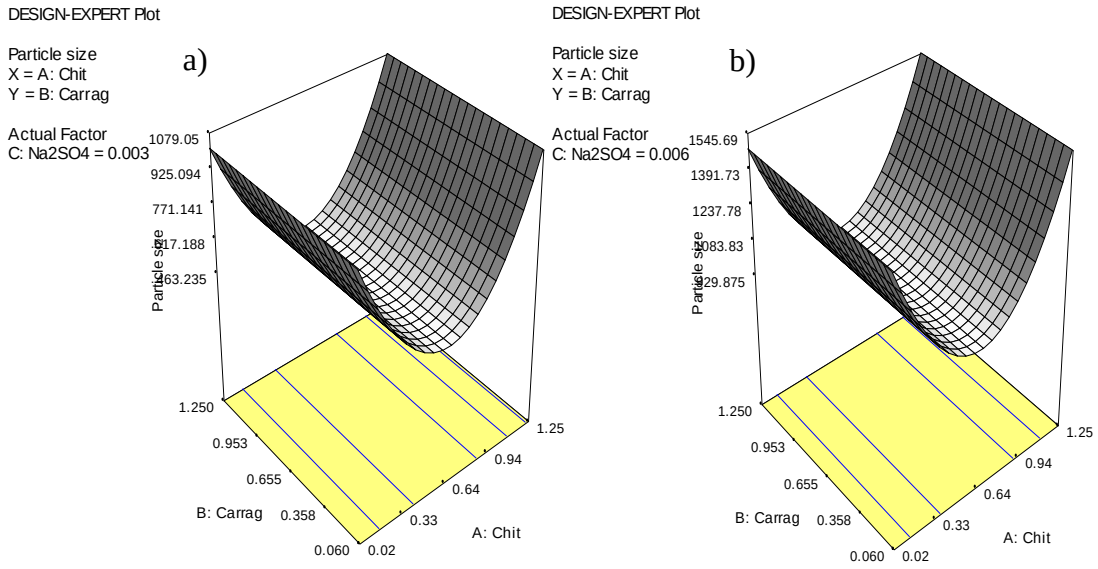

Figure 4.3: Particle size (nm) as a function of polymer (Chit: chitosan, Carrag: carrageenan) concentration and Na_2SO_4 inclusion. a) Particle size with Na_2SO_4 0.003 M; b) Particle size with Na_2SO_4 0.006 M

Several important findings can be retained from this study, being 1) the absence of effect of CRG concentration on NP size, which is clearly ruled by CS concentration, 2) the observation that the inclusion of Na_2SO_4 hampers the formation of nanosized carriers, even at very low concentration such as 0.006M, 3) for salts resulting in NPs production, there was an inversion in the evolution of NP size with variation of CS concentration when the concentration of both NaCl and $CaCl_2$ in the formulations increased.

4.2.2 Zeta potential

In this section, the effect of the variables on the zeta potential of NPs was studied. CS/CRG NPs formulated in the absence of salt showed a zeta potential that varied between -15 mV and +41 mV throughout the study. In these NPs, the zeta potential increased with the increase of CS concentration, which is expected, owing to CS amino

groups that are positively charged. The impact of CRG in the zeta potential was only clearly noticeable when its concentration was much higher than that of CS, in which case the zeta potential assumed a negative value, which is in accordance with CRG negatively charged sulphate groups. On the contrary, when CS and CRG had the same concentration or CS had higher concentration than CRG, the zeta potential was always positive. Taking into account that: (1) CS was considered to have 0.8 positive charges per monomer, corresponding to a mean degree of deacetylation of 80%, and a molecular weight of 199 g/mol and, (2) CRG was considered to have 1 negative charge per dimer and to be in the form of sodium salt, with a molecular weight of 408 g/mol, we can conclude that CS has 4.03×10^{-3} charges/g, while CRG has 2.45×10^{-3} charges/g. Therefore, it is logical that CS has higher impact on zeta potential than CRG, as a consequence of its higher charge density.

The results of the addition of each salt are presented below, in several successive figures. Similarly to the section above, each figure shows the observations for the condition of associating either the lower concentration of salt (figures "a") or the maximum concentration of salt (figures "b"). The overall analysis of the results and their statistical significance indicated that the established mathematical model was once again significant in all cases ($p < 0.05$). In the study of the addition of Na_2SO_4, it was observed a significant lack of fit of the model($p < 0.05$), which may be a consequence of the inadequacy of the polynomial model for the obtained data, as referred previously.

Figure 4.4 shows the results corresponding to the inclusion of NaCl in the formulations. It is observed in a) that, with NaCl 0.5 M, zeta potential values vary between -20 mV and +15 mV, approximately, which shows some decrease when compared to the values obtained in the absence of salt, regarding the positive range. This can be explained by the fact that the addition of salt increases the electrolyte concentration of the aqueous medium, leading to compression of the double layer which causes the zeta potential to be lower (29). As for the trend in evolution of the zeta potential, it increases with the increase of CS amount, which makes sense since CS contributes with positive charges from its amino groups. Continuing with this line of thinking, a decrease of zeta potential would be expected with the increase of CRG, since this polymer contributes with negative charges from its sulphate groups. Nevertheless, this was not observed.

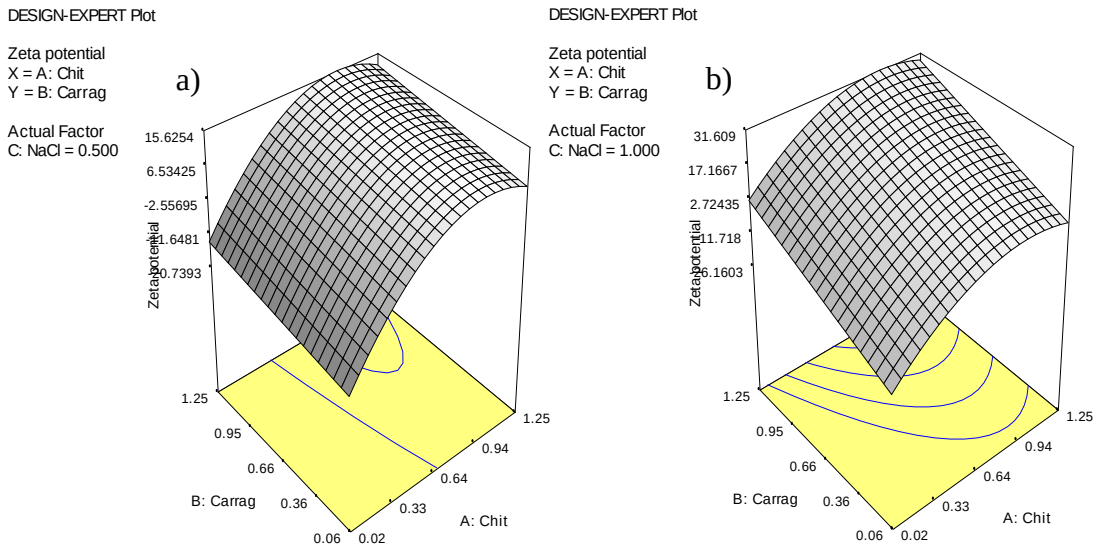

Figure 4.4: Zeta potential (mV) as a function of polymer (Chit: chitosan, Carrag: carrageenan) concentration and NaCl inclusion. a) Zeta potential with NaCl 0.5 M; b) Zeta potential with NaCl 1 M.

The inclusion of NaCl 1 M in the formulation (Figure 4.4b) results in zeta potential values varying within -26 mV and +32 mV. This also shows some decrease when compared to the absence of salt, but not as noticeable as with lower salt concentration. Interestingly, the pattern of evolution of the zeta potential was very similar upon the inclusion of both salt concentrations. However, the increase in the concentration of the salt, led to more accentuated profiles. That is, higher zeta potential was observed when higher amount of CS was present, but the inclusion of more salt, permitted reaching higher values (+32 mV of maximum value comparing with +15 mV when half concentration of salt was present). Moreover, for a determined concentration of CS, the increase in CRG led to an unexpected increase in zeta potential, as commented before. This behaviour was even more visible when higher amount of salt was included, as if the presence of the salt somehow impeded or limited the interaction of CS amino groups with the sulphate groups of CRG, which typically results in charge neutralisation. Interestingly, this zeta potential effect was not observed in the absence of salt, where the concentration of CRG practically did not affect the zeta potential of NPs (data not shown).

When the study was performed using $CaCl_2$ (Figure 4.5), it was concluded that the salt had no effect on the results. A somewhat similar trend of zeta potential evolution was seen comparing with NaCl, as this increased with the increase of CS concentration,

varying within –10 mV and +45 mV. However, the whole profile is exactly the same independently of the salt concentration (Figure 4.5b) and similar to that observed in the absence of salt. It could be the case that the concentration of salt used in the study is not enough to promote a change in the electrical layer that leads to changes in zeta potential. However, it is noticeable that the higher concentration had significant impact in the NPs size, thus suggesting that the salt had some kind of effect on NP formation, although not visible in the charge parameter.

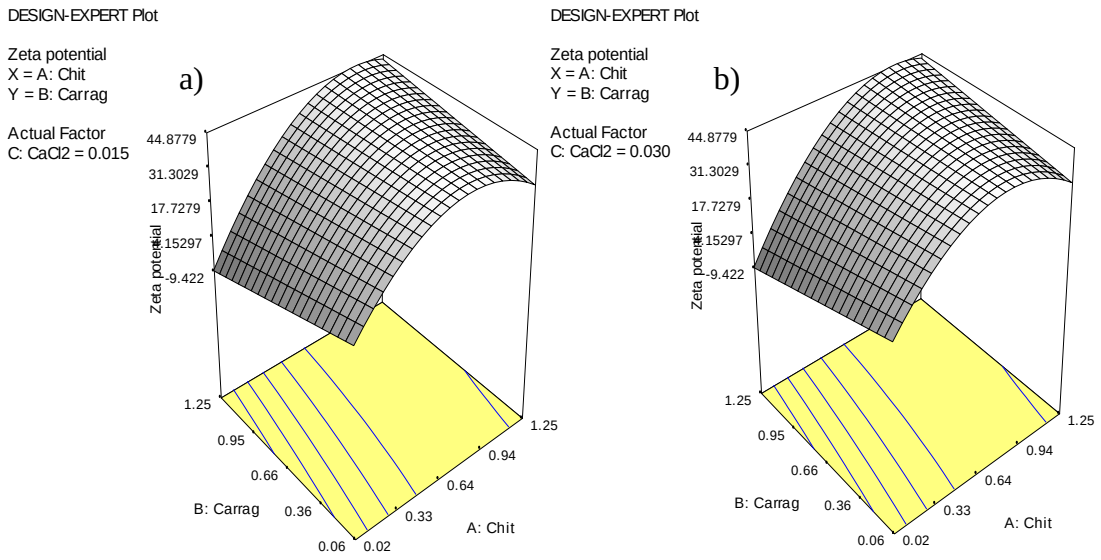

Figure 4.5: Zeta potential (mV) as a function of polymer (Chit: chitosan, Carrag: carrageenan) concentration and $CaCl_2$ inclusion. a) Zeta potential with $CaCl_2$ 0.015 M; b) Zeta potential with $CaCl_2$ 0.030 M.

The inclusion of Na_2SO_4 generally resulted in carriers of very large size, in many cases in the micrometric range, especially when the higher salt concentration was used. Nevertheless, the obtained carriers were characterised regarding both size, as detailed above, and zeta potential. The results obtained for this salt (Figure 4.6.) were very similar to those obtained for $CaCl_2$ when it comes to the evolution of zeta potential with the variation of CS and CRG. However, in this case the zeta potential decreased with the increase of concentration of salt and reached values of –20 mV, -25 mV, which is clearly above that registered after inclusion of CaCl2. The increase in the salt concentration led to a slight intensification of the negative charge, from -20 mV to -25 mV, which correspond to the maximal values. Additionally, the addition of salt also induced lower charge than that determined in the absence of salt (around -15 mV).

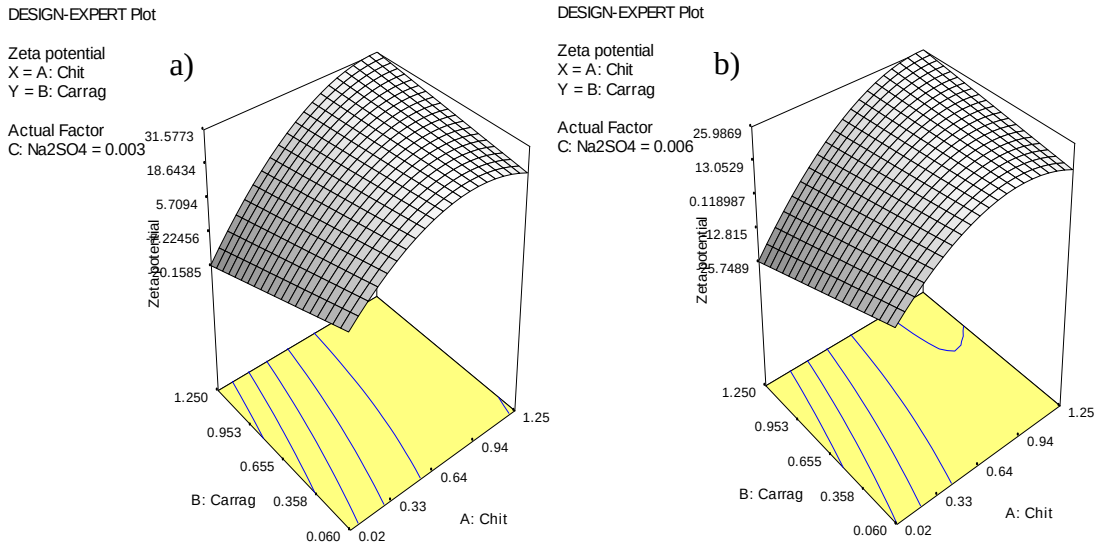

Figure 4.6: Zeta potential (mV) as a function of polymer (Chit: chitosan, Carrag: carrageenan) concentration and Na_2SO_4 inclusion. a) Zeta potential with Na_2SO_4 0.003 M; b) Zeta potential with Na_2SO_4 0.006 M

Differences in zeta potential, when compared to values obtained in the absence of salt, were more easily seen with the addition of NaCl than with the addition of the other two salts. The concentrations used for $CaCl_2$ and Na_2SO_4 in this study might not have been enough to affect the complexation of the polyelectrolytes and originate a pronounced difference. As the concentration of the salts were not liable to increase, since they were at the highest concentration possible that guaranteed polymer solubility, other salts could be tested in the future.

5 Conclusion

In this work, NPs composed of CS and CRG were produced and characterized to study the effect of polymer concentration in NP formation. Moreover, three salts, NaCl, $CaCl_2$ and Na_2SO_4, were further added to the formulations in order to study the effect of their addition and varying concentrations.

It was found that, in the absence of salt, larger NPs were formed when higher amounts of CS were present in the formulations. However, the opposite was seen once NaCl and $CaCl_2$ were added, with NPs being larger at lower concentrations of CS. As for Na_2SO_4, its inclusion hindered the formation of nano-sized carries in many formulations, resulting in particles with as large as 1500 nm. It was also observed that the concentration of CRG has no effect on NPs size, which appears to be ruled by CS concentration. As expected, the zeta potential generally increased with the increase of CS amount, owing to its positively charged amino groups. However, when $CaCl_2$ was present in the formulation, no differences were seen between the NPs formed in the absence and presence of the salt. In the presence of NaCl, lower zeta potential was observed overall when comparing with NPs formed without salt. A similar effect was seen with the addition of Na_2SO_4, even if the obtained carriers could not be categorised as NPs in many cases.

Even though the obtained results permitted the establishment of significant mathematical models, allowing to have a tool to predict the characteristics of the NPs according to the production parameters, the lack of fit in most cases was also significant, which indicates the need to improve the model. It is thus considered that optimisations in the study design would favour more consistent results, which opens possibilities for future research.

6 References

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7 Annexes

7.1 Design of the experiment: blocks of experiments and specific conditions

Table 7.1: Volumes and concentrations of all components used in the preparation of CS/CRG/NaCl nanoparticles.

		[CS] (mg/mL)	[CRG] (mg/mL)	[NaCl] (M)	V_{CS} mL	V_{HCl} (mL)	V_1 (mL)	V_{CRG} (mL)	V_{HCl} (mL)	V_2 (mL)	V_{total} (mL)
Block 1	1	0.02	1.25	1	0.024	1.476	1.50	1.500	0.000	1.50	3.00
	2	0.635	0.655	0.5	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	3	0.635	0.655	0.5	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	4	0.02	0.06	0	0.024	1.476	1.50	0.072	1.428	1.50	3.00
	5	1.25	0.06	1	1.500	0.000	1.50	0.072	1.428	1.50	3.00
	6	1.25	1.25	0	1.500	0.000	1.50	1.500	0.000	1.50	3.00
Block 2	7	0.635	0.655	0.5	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	8	1.25	1.25	1	1.500	0.000	1.50	1.500	0.000	1.50	3.00
	9	0.02	0.06	1	0.024	1.476	1.50	0.072	1.428	1.50	3.00
	10	0.635	0.655	0.5	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	11	1.25	0.06	0	1.500	0.000	1.50	0.072	1.428	1.50	3.00
	12	0.02	1.25	0	0.024	1.476	1.50	1.500	0.000	1.50	3.00
Block 3	13	1.25	0.655	0.5	1.500	0.000	1.50	0.786	0.714	1.50	3.00
	14	0.635	0.655	0.5	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	15	0.635	0.655	1	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	16	0.02	0.655	0.5	0.024	1.476	1.50	0.786	0.714	1.50	3.00
	17	0.635	0.06	0.5	0.762	0.738	1.50	0.072	1.428	1.50	3.00
	18	0.635	1.25	0.5	0.762	0.738	1.50	1.500	0.000	1.50	3.00
	19	0.635	0.655	0.5	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	20	0.635	0.655	0	0.762	0.738	1.50	0.786	0.714	1.50	3.00

Table 7.2: Volumes and concentrations of all components used in the preparation of CS/CRG/$CaCl_2$ nanoparticles.

		[CS] (mg/mL)	[CRG] (mg/mL)	[$CaCl_2$] (M)	V_{CS} mL	V_{HCl} (mL)	V_1 (mL)	V_{CRG} (mL)	V_{HCl} (mL)	V_2 (mL)	V_{total} (mL)
Block 1	1	1.25	0.06	0.03	1.500	0.000	1.50	0.072	1.428	1.50	3.00
	2	0.635	0.655	0.015	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	3	1.25	1.25	0	1.500	0.000	1.50	1.500	0.000	1.50	3.00
	4	0.635	0.655	0.015	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	5	0.02	1.25	0.03	0.024	1.476	1.50	1.500	0.000	1.50	3.00
	6	0.02	0.06	0	0.024	1.476	1.50	0.072	1.428	1.50	3.00
Block 2	7	0.02	1.25	0	0.024	1.476	1.50	1.500	0.000	1.50	3.00
	8	0.635	0.655	0.015	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	9	1.25	0.06	0	1.500	0.000	1.50	0.072	1.428	1.50	3.00
	10	1.25	1.25	0.03	1.500	0.000	1.50	1.500	0.000	1.50	3.00
	11	0.635	0.655	0.015	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	12	0.02	0.06	0.03	0.024	1.476	1.50	0.072	1.428	1.50	3.00
Block 3	13	0.635	0.655	0	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	14	0.635	0.655	0.015	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	15	0.635	0.655	0.015	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	16	0.635	1.25	0.015	0.762	0.738	1.50	1.500	0.000	1.50	3.00
	17	0.635	0.655	0.03	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	18	0.02	0.655	0.015	0.024	1.476	1.50	0.786	0.714	1.50	3.00
	19	0.635	0.06	0.015	0.762	0.738	1.50	0.072	1.428	1.50	3.00
	20	1.25	0.655	0.015	1.500	0.000	1.50	0.786	0.714	1.50	3.00

Table 7.3: Volumes and concentrations of all components used in the preparation of CS/CRG/Na_2SO_4 nanoparticles.

		[CS] (mg/mL)	[CRG] (mg/mL)	[Na_2SO_4] (M)	V_{CS} mL	V_{HCl} (mL)	V_1 (mL)	V_{CRG} (mL)	V_{HCl} (mL)	V_2 (mL)	V_{total} (mL)
Block 1	1	0.635	0.655	0.003	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	2	1.25	1.25	0	1.500	0.000	1.50	1.500	0.000	1.50	3.00
	3	1.25	0.06	0.006	1.500	0.000	1.50	0.072	1.428	1.50	3.00
	4	0.02	1.25	0.006	0.024	1.476	1.50	1.500	0.000	1.50	3.00
	5	0.635	0.655	0.003	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	6	0.02	0.06	0	0.024	1.476	1.50	0.072	1.428	1.50	3.00
Block 2	7	0.635	0.655	0.003	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	8	1.25	1.25	0.006	1.500	0.000	1.50	1.500	0.000	1.50	3.00
	9	1.25	0.06	0	1.500	0.000	1.50	0.072	1.428	1.50	3.00
	10	0.635	0.655	0.003	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	11	0.02	1.25	0	0.024	1.476	1.50	1.500	0.000	1.50	3.00
	12	0.02	0.06	0.006	0.024	1.476	1.50	0.072	1.428	1.50	3.00
Block 3	13	1.25	0.655	0.003	1.500	0.000	1.50	0.786	0.714	1.50	3.00
	14	0.635	0.655	0	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	15	0.635	0.06	0.003	0.762	0.738	1.50	0.072	1.428	1.50	3.00
	16	0.635	0.655	0.003	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	17	0.635	0.655	0.003	0.762	0.738	1.50	0.786	0.714	1.50	3.00
	18	0.635	1.25	0.003	0.762	0.738	1.50	1.500	0.000	1.50	3.00
	19	0.02	0.655	0.003	0.024	1.476	1.50	0.786	0.714	1.50	3.00
	20	0.635	0.655	0.006	0.762	0.738	1.50	0.786	0.714	1.50	3.00

7.2 Physicochemical characteristics of CS/CRG nanoparticles containing salt

Table 7.4: Values of size (nm), polydispersity index (PdI) and Zeta Potential (mV) obtained for each CS/CRG/Salt test (n=1).

		CS/CRG/NaCl			CS/CRG/$CaCl_2$			CS/CRG/Na_2SO_4		
		Size (nm)	PdI	Zeta (mV)	Size (nm)	PdI	Zeta (mV)	Size (nm)	PdI	Zeta (mV)
Block 1	1	552	0.202	-1	563	0.850	+18	400	0.309	+22
	2	137	0.290	+5	417	0.354	+20	937	0.467	+41
	3	172	0.264	+7	937	0.497	+41	1763	0.379	+23
	4	132	0.269	+10	314	0.282	+42	1713	0.707	-20
	5	375	0.457	-14	1237	0.774	-4	458	0.322	+23
	6	937	0.707	+41	132	0.355	+10	132	0.252	+10
Block 2	7	138	0.251	+16	183	0.307	-15	367	0.255	+24
	8	227	0.563	+28	483	0.516	+44	1380	0.821	+16
	9	235	0.319	-21	933	0.805	+35	933	0.505	+35
	10	138	0.271	+9	592	0.478	+44	407	0.232	+22
	11	933	0.551	+35	445	0.310	+44	183	0.263	-15
	12	183	0.191	-15	292	0.389	+17	1851	0.918	0
Block 3	13	151	0.294	+10	448	0.292	+35	519	0.461	+32
	14	160	0.265	+3	366	0.253	+42	448	0.396	+35
	15	567	0.174	+25	404	0.409	+42	864	0.603	+21
	16	924	0.631	-8	430	0.401	+40	465	0.310	+22
	17	304	0.424	+11	322	0.350	+39	449	0.271	+24
	18	136	0.171	+5	568	0.547	-6	550	0.365	+20
	19	192	0.289	+11	469	0.497	+35	1290	0.744	-9
	20	448	0.260	+35	457	0.437	+37	403	0.385	+8

*The concentrations of the polymers are those presented in Tables 7.1 to 7.3, in each corresponding block of experiments.