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Chitosan/dextran sulfate nanoparticles: stability evaluation and assessment of the effect of different acidic media on nanoparticle preparation

Jorge Filipe Rodrigues Pontes

Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob orientação de Prof. Doutora Ana Margarida Moutinho Grenha
e coorientação de Prof. Doutora Clara Maria Henrique Cordeiro

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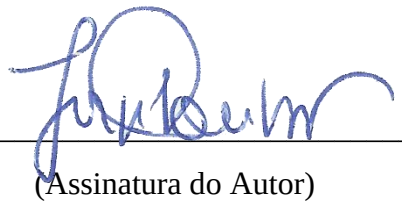
Declaração de autoria de trabalho

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



(Assinatura do Autor)

Every morning we are born again. What we do today is what matters most.

Buddha

Believe you can and you're halfway there.

Theodore Roosevelt

I can't change the direction of the wind, but I can adjust my sails to always reach my destination.

Jimmy Dean

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Contents

Abstract

Nanoparticles present great potential in drug delivery applications, yet there are some issues regarding their stability. In this context, this study was conducted to define the conditions to stabilize polysaccharide (chitosan/dextran sulfate, CS/DS) nanoparticles by a process of freeze-drying, assessing the cryoprotectant capacity of two sugars (sucrose and glucose). Additionally, it was also intended to find if the solubilisation of chitosan in different acids affected nanoparticle preparation and characteristics.

CS/DS nanoparticles were produced by polyelectrolyte complexation and the suspensions adjusted to 1 mg/mL or 2 mg/mL. For the study of stabilisation by freeze-drying, three approaches were conducted: i) after production of nanoparticles, 5% or 10% (w/v) of glucose or sucrose were included in the suspension before freezing, being nanocarriers characterized for size and zeta potential before freeze-drying and immediately after freeze-drying and reconstitution; ii) nanoparticles were produced, and then stored at 4 ºC (no cryoprotectants added); iii) nanoparticles were produced, freeze-dried with cryoprotectants and then stored, in a desiccator, at room temperature, being characterized (size and zeta potential) every 15 days, after the needed reconstitution.

Acetic acid and hydrochloric acid (HCl) at 0.1 and 0.01 M were used to solubilise chitosan. CS dissolved in HCl 0.1 M did not enable the production of nanoscale particles. When the remaining acids were test, nanoparticles had sizes above 500 nm. Furthermore, zeta potential presented an unexpected behaviour. Thus, it was concluded that this study needs optimisation.

The storage of nanoparticle suspensions at 4 ºC resulted in instability after 50 days. Therefore, a freeze-drying approach was established. In general, the choice of cryoprotectant was the most important factor affecting the preservation of nanoparticle physicochemical characteristics. Moreover, results indicated that in short- and long-term periods, glucose presented a more suitable behaviour despite some variations.

Keywords: chitosan, cryoprotectants, dextran sulfate, drug delivery, freeze-drying, glucose, nanoparticles, sucrose, stability

Resumo

As nanopartículas apresentam-se como uma interessante estratégia de veiculação de fármacos. Contudo, a sua estabilidade é uma limitação desenhado este estudo que pretende estabelecer condições de estabilização de nanopartículas polissacarídicas (quitosano/sulfato de dextrano, CS/DS) através de um processo de liofilização, avaliando-se a capacidade crioprotetora de dois açúcares. Adicionalmente, procurou-se entender a influência de diferentes ácidos usados na dissolução do quitosano no tamanho e potencial zeta das nanopartículas produzidas.

Nanopartículas de CS/DS foram preparadas por complexação polieletrolítica e as suspensões ajustadas a concentrações de 1 e 2 mg/mL. Para a abordagem da liofilização, foram delineados 3 ensaios: i) após a produção das nanopartículas, foi adicionado crioprotetor à suspensão antes do congelamento e a sua caracterização foi realizada imediatamente após a liofilização; ii) as nanopartículas foram produzidas e armazenadas a 4 ºC, sem qualquer adição de crioprotetor e iii) as nanopartículas foram produzidas, liofilizadas com crioprotetor, e armazenadas num exsicador, à temperatura ambiente, tendo sido caracterizadas de 15 em 15 dias.

O ácido acético e o ácido clorídrico (HCl) a 0.1 M e 0.01 M foram os ácidos usados para solubilizar o quitosano. Os veículos derivados do uso de quitosano dissolvido em HCl 0.1 M encontraram-se fora da escala nanométrica. Por outro lado, nos restantes, os tamanhos ficaram acima dos 500 nm. Além disso, o potencial zeta demonstrou uma tendência inesperada, pelo que este estudo requer otimização.

O armazenamento de nanosuspensões a 4 ºC resultou em parâmetros de caracterização instáveis, a partir do dia 50. Desta forma, estabeleceu-se um protocolo de liofilização em que, no geral, a escolha do crioprotector foi o fator determinante que afeta a preservação das características fisico-químicas das nanopartículas. Além disso, os resultados sugerem que a glucose possui uma melhor capacidade crioprotetora, a curto e longo prazos, apesar das grandes variações que os dados revelaram.

Palavras-chave: crioprotetores, estabilidade, glucose, liofilização, nanopartículas, quitosano, sacarose, sulfato de dextrano, veiculação de fármacos

Resumo Alargado

A medicina é, hoje em dia, uma área em crescente desenvolvimento tal como as ciências farmacêuticas. Da mesma maneira que o conhecimento se adensa sobre determinadas patologias e outras novas parecem surgir, a forma como se pode abordar o tratamento e o diagnóstico das mesmas evolui até novos patamares. Ainda que a regulação a nível de ensaios clínicos e de introdução de novas moléculas no mercado esteja cada vez mais apertada, o facto é que estes aspetos permitiram que os investigadores focassem as suas atenções em problemáticas mais direcionadas para a melhoria do que já está em utilização. Paralelamente, a investigação de novas moléculas está naturalmente em curso, mas requer um investimento cada vez maior que poderá nunca chegar a ter um retorno. É neste preciso ponto que a nanotecnologia assume um grande destaque, em especial no que às nanopartículas diz respeito.

As nanopartículas fazem parte de uma área da tecnologia farmacêutica que tem vindo a ganhar destaque desde há alguns anos. Desde o grande número de combinações de polímeros aos mais diversos métodos de produção, passando por novos sistemas que permitem direcionar uma determinada molécula para um local de interesse no organismo humano, como os dendrímeros ou os nanotubos/nanopartículas de carbono e pelos veículos de composição inorgânica como sílica e ouro, a sua utilização disseminou-se desde as áreas de diagnóstico até à vertente terapêutica. Não obstante as interessantes propriedades que as caracterizam, as nanopartículas têm graves limitações no que concerne à sua estabilidade. De facto, a suscetibilidade face a condições ambientais e a dificuldade em mantê-las estáveis em ambientes aquosos, levou à conceção do projecto laboratorial que se descreve no presente documento.

O trabalho laboratorial que foi realizado baseou-se na produção de nanopartículas poliméricas de quitosano/sulfato de dextrano (CS/DS) com um rácio de massa de 1:3, ou seja, uma molécula de quitosano para três de sulfato de dextrano. Dada a densidade eletrónica negativa nos grupos sulfato do sulfato de dextrano, as nanopartículas apresentam um potencial zeta negativo. Precisamente por isto é que a reação deste último polímero com o quitosano, carregado positiviamente, se realiza de uma forma muito favorável, levando a uma interação entre grupos de cargas opostas, que resulta nas nanopartículas. A partir deste ponto, foram aplicadas três abordagens de forma a estudar a estabilidade das nanopartículas e propor um método eficaz para a preservação das suas características principais – o tamanho e o potencial zeta.

O método aplicado para a estabilização foi a liofilização, que consiste no congelamento de amostras das nanopartículas poliméricas e posterior remoção da água congelada por

sublimação. Obtiveram-se liofilizados que foram posteriormente reconstituídos, sendo as nanopartículas caracterizadas para determinar se o método é realmente eficaz na manutenção das características destes veículos. Contudo, e porque o processo de congelamento envolve a formação de cristais de gelo que provocam uma disrupção completa destes veículos e posterior agregação, estudou-se a capacidade crioprotetora de dois carbohidratos: a glucose e a sacarose.

Numa primeira abordagem, produziram-se as nanopartículas poliméricas por complexação eletrolítica cujas amostras foram concentradas até se atingirem duas concentrações distintas: 1 mg/mL e 2 mg/mL. Posteriormente, às amostras foi adicionado o carbohidrato que se pretendia estudar – glucose ou sacarose – também a uma concentração definida, 5% ou 10% (m/v), caracterizando os veículos antes de se proceder à sua congelação. Após este último passo, as amostras foram liofilizadas, reconstituídas e caracterizadas logo após a reconstituição.

Numa segunda abordagem, as mesmas nanopartículas foram produzidas e ajustadas a concentrações de 1 mg/mL e 2 mg/mL. Contudo, desta vez, não se procedeu nem à adição do carbohidrato nem à sua congelação, permanecendo em suspensão que foi armazenada a 4 ºC durante 113 dias.

Numa terceira abordagem, nanopartículas de CS/DS foram produzidas, foi-lhes adiconado o crioprotetor e realizada a caracterização antes do congelamento. Posteriormente, foram liofilizadas e armazenadas, à temperatura ambiente, num excicador durante 90 dias. Dado que esta última abordagem envolvia o estudo da estabilidade destes veículos na forma de liofilizados, de 15 em 15 dias, as nanopartículas foram sendo reconstituídas e caracterizadas, em termos de tamanho e potencial zeta, pretendendo-se o estabelecimento de uma relação entre a capacidade crioprotetora do carbohidrato utilizado e o tempo de armazenamento das nanopartículas.

Adicionalmente, foi realizado um outro ensaio que pretendeu analisar a influência de diferentes ácidos como agentes de solubilização do quitosano e de que forma esses mesmos ácidos influenciavam as características fisico-químicas das nanopartículas produzidas. Neste ensaio, foram usados 4 diferentes rácios de massa entre os polímeros (quitosano/sulfato de dextrano) – 2/1, 3/1, 4/1 e 5/1. Como o quitosano está sempre em maior proporção face ao sulfato de dextrano, esperava-se que as nanopartículas resultantes tivessem um potencial zeta positivo.

Dos resultados obtidos no estudo de liofilização, é sugerido que a glucose possui uma melhor capacidade crioprotetora face à sacarose. De facto, apesar dos valores de desvio-padrão

serem elevados, as médias de variação do tamanho foram, gernericamente, mais próximas de 1. Relativamente ao potencial zeta, e devido ao facto dos desvios-padrão serem elevados para os dois carbohidratos, não foi possível concluir quanto à melhor ou pior capacidade crioprotetora da glucose ou da sacarose. É possível assumir que o comportamento dos dois é semelhante.

O estudo em que consistiu esta primeira abordagem de liofilização, foi realizado um desenho fatorial, de modo a tentar entender que fatores são os mais preponderantes na estabilidade das nanopartículas CS/DS. Os resultados sugerem que é a escolha do crioprotetor, neste caso ora a glucose ora a sacarose, que mais influencia a variação nos tamanhos das nanopartículas. Quando se considera as variações nos potenciais zeta destes veículos, é sugerido que tanto a concentração como o tipo de carbohidrato bem como a interação entre ambos, são os fatores preponderantes que influenciam as variações.

O estudo da estabilidade das nanopartículas quando armazenas em meio aquoso e a 4 ºC, indicou que até ao dia 50 as características fisico-químicas das nanopartículas mantiveram-se relativamente estáveis. Contudo, a partir do dia 50 de armazenamento, houve um decréscimo notório do seu tamanho em algumas amostras para além de se notar, noutras, uma agregação substancial. No que concerne ao potencial zeta, este manteve-se aproximadamente estável ao longo dos 113 dias em que decorreu o estudo, mostrando um máximo de 10% de variação nos seus valores.

Por fim fez-se um estudo da estabilidade das nanopartículas após liofilização, tendo-se armazenado os liofilizados à temperatura ambiente e em local tendencialmente seco. Os resultados sugeriram que é a glucose o carbohidrato que melhor mantém as características fisico-químicas das nanopartículas.

No ensaio que envolveu a solubilização de quitosano em diferentes ácidos, os resultados, de uma forma geral, levaram à produção de nanopartículas com tamanho demasiado elevado para uma potencial aplicação em administração de fármacos. Além disso, foi notada uma tendência incomum no que concerne aos resultados do potencial zeta, que decresceu à medida que a quantidade de sulfato de dextrano também diminuiu. De facto, esta tendência incomum vai contra o que se esperava: menor presença de sulfato de dextrano, mais carga positiva, maior potencial zeta. Assim, concluiu-se ser necessário um estudo mais aprofundado para estabelecer uma eventual relação entre o ácido usado como solvente e as características finais das nanopartículas.

Abbreviations

ANOVA – ANalysis Of VAriance

CS – Chitosan

CV – Coefficient of Variation

DDS – Drug Delivery Systems

DS – Dextran Sulfate

EFSA – European Food Safety Authority

EMA – European Medicines Agency

FD – Factorial Design

FDA – Food and Drug Administration

PLA – PolyLactic Acid

PLGA – Poly(Lactic-co-Glycolic) Acid

SD – Standard Deviation

TPP – Tripolyphosphate

WHO – World Health Organization

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1. Introduction

Public health has been, today, one of the most troubling subjects of every government all around the world and a constant concern for the World Health Organization (WHO). Diseases like AIDS, tuberculosis, Ebola virus disease or the most recent Zika virus infections are just the tip of the iceberg in what regards this very complicated matter where the solutions are not as obvious and immediate as desired. In the subject of health, Portugal, in 2014, dedicated 11.9% of its public budget to Health, being the second most expensive subject after the Social Security and Social Actions (1). According to the website PORDATA, which displays a series of statistical data regarding the country, in 2014, there were 138.6 elder people per 100 younger ones and, not only this is worrying, but also the number keeps increasing (2). As time progresses and science improves, it seems natural that life expectancy grows accordingly thus being, at this point, of 85.6 years for women and 82.2 for men. Combining these numbers with the decrease of natality rates, it is logical that government's healthcare annual budget will be more directed to the elderly specifically on a more dedicated medical assistance, an increasing number of nursing homes and, more importantly, the medicine's prices, rather than the younger (3).

As the human body gets older, there are certain types of complications that tend to appear and in order to counteract them, certain molecules are required. It is asked, nowadays, a more close relationship between different scientific subjects in order to provide not only new molecules with fewer side effects but also new approaches on previously studied drugs. One of the most interesting subjects is, probably, the pharmaceutical technology which has a certain influence in the various areas of medicine beginning in the production of drugs and finishing in their administration to the patient. According to Nogueira Prista *et al.*, pharmaceutical technology is the study of the transformation of natural or synthetic products in drugs in order to administer them to living beings with the aim of being prophylactic drugs, curative ones or simply the diagnostic of certain illnesses (4). It is possible to deduce that this discipline is the natural evolution from the concept of Galenic Pharmacy that was used, in the first centuries of the current era, to describe the works of Claudius Galenus in the preparation of various pharmaceutical forms that the population in general still utilizes, such as the antidotes (4,5). It is accepted that when science progresses, its concepts, in many areas, also follow this evolution. The vehicles and the administration routes to which it is possible to deliver the molecule that has the actual therapeutic effect are two great examples of the said evolution. However, these

achievements require investments that, in many occasions, are not possible to conduct by many different reasons.

Since 1960, the pharmaceutical industry in North America has seen a great expenditure in research being, by the year of 1990, 8 millions of dollars (6). It is interesting to see that, by the final decade of the 20th century, the regulation of the industry itself tightened up, increasing to more than 12 years the time necessary to get the approval of a certain drug, which can explain that not everything is viable and the loss of money could be immense (6,7). The general investment in research is however very high, although lower in Europe comparing with United States of America (8), making Europe less competitive (9).

All around the world, society entered in a century of great challenges especially in the pharmaceutical area. Although investments still linger on uncovering new molecules, the greater knowledge on how the human organism works allowed the study of other therapeutic approaches and, specifically, new pharmaceutical forms (6). Today, they range from suspensions and solutions to pills and, interestingly, to the so called drug delivery systems (DDS) that make use of a matrix to deliver a certain drug or drugs to a site of interest. Nanosized DDS are becoming a reality specially due to the fact that the patient's well-being and consequently, public health, are the cores of the nowadays therapeutic approaches providing a safer choice on drug uptake (10–13).

1.1. Encapsulation techniques: Nanotechnology as a tool in therapeutics

Everything can be categorized by having a form and a size. In Nature, it is possible to find animals that are smaller and other ones that are bigger, even in the same species. The same happens with objects, which have a wider range of sizes. Before and in the early days of the scientific revolution, diseases were taught to be a punishment without a real reason for their development. However, that changed when microorganisms were discovered changing the paradigm of medicine (5). The therapeutic fields is also changing, going into more molecular approaches. Nanostructured DDS, represented in Figure 1.1, may be understood as the consequence of that evolution, being in fact believed to entail the future of many treatments (14).

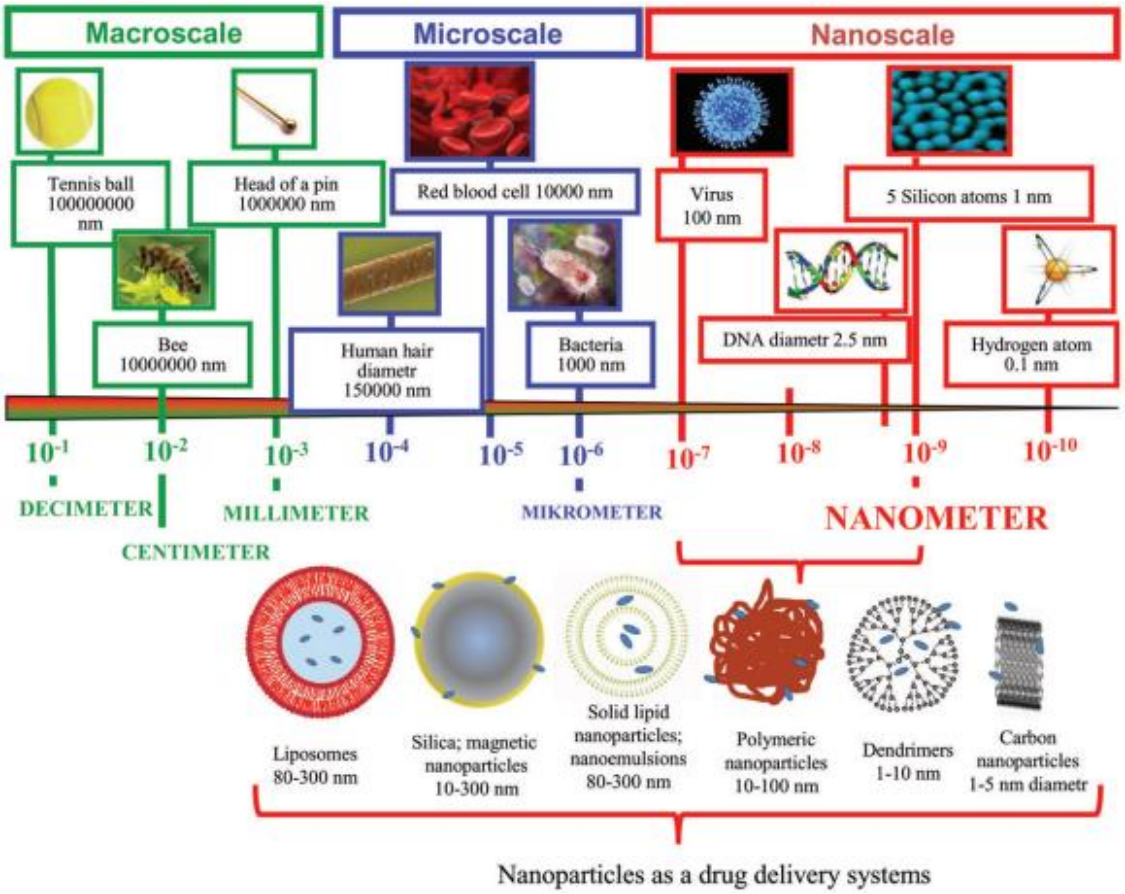


Figure 1.1 – Schematic representation of the different size scales with the nanoscale and the different delivery systems highlighted. Adapted from (14).

In a pharmaceutical approach, DDS are vehicles that can carry one or more drug molecules, to a specific site or sites in the human organism. In the recent years, DDS have grown to get a particularly good spot around the scientific community due to their advantages in medicine, healthcare and the global economy. Improvement of drug bioavailability is one of the key achievements. By preventing the degradation by certain enzymes, ensuring stabilisation or maintaining the therapeutic concentration that is required for the treatment to be successful and even the reduction of side effects, the use of drug carriers brought many advantages (15,16).

DDS are mainly used to provide drug protection and there is a variety of materials that can be used as matrixes to encapsulate drugs, which explains the wide range of these systems that go from liposomes to carbon nanotubes as depicted in Figure 1.1 presented before.

Figure 1.2 depicts a timeline of drugs approved by the Food and Drug Administration (FDA) that use these encapsulating technologies. As the image clearly depicts, even though the molecules comprising some of the formulations were discovered and developed almost thirty years ago, many are still used these days because they are effective and lead to high patient compliance.

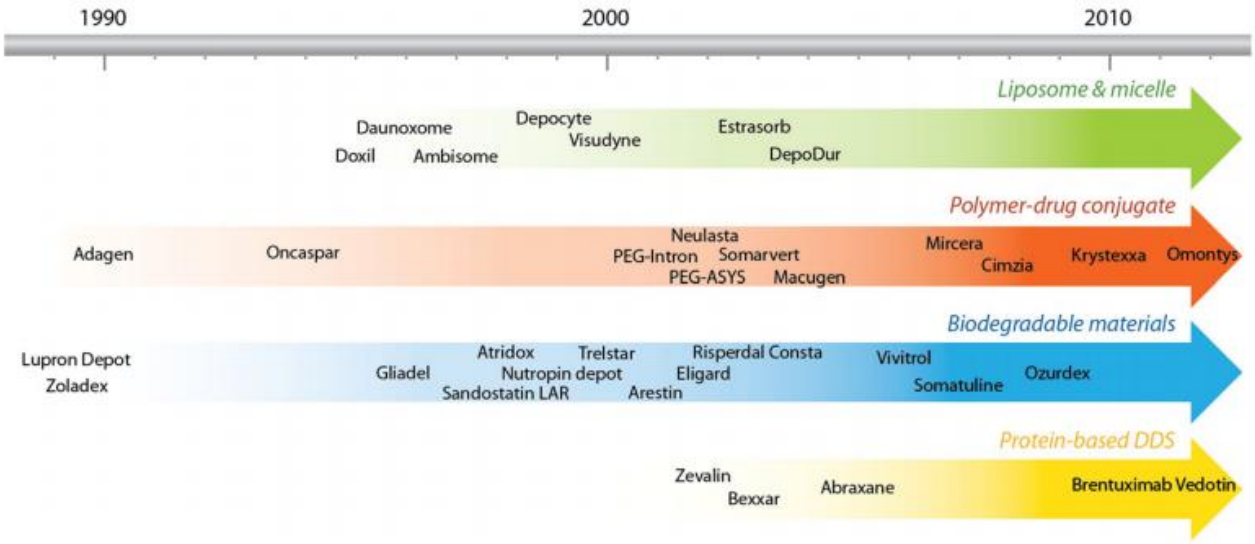


Figure 1.2 – Chronological approval of formulations that make use of the encapsulation techniques by the Food and Drug Administration (FDA). Adapted from (15).

As depicted by a green arrow in Figure 1.2, there are formulations that use liposomes to deliver the active principles. Liposomes are comprised of phospholipids and aside from the drugs presented on the image, they can also be used in other therapeutic approaches (17,18).

Another approach deserving reference is Abraxane®, which makes use of albumin, a common protein in the human organism that helps maintaining the oncotic pressure (19), in order to deliver paclitaxel. Highlighted in the orange and blue arrows, there are other formulations that use these encapsulating technologies to deliver drugs to a tissue or organ of interest.

Nanotechnology is a discipline that is very diverse in the approaches that can be used on drug delivery, which is increasing in importance and utility (20–24). Many works reported the use of nanoparticles as a suitable means for drug targeting using the most diverse administration routes. The oral (25–27), parenteral (28,29) and nasal routes (30,31) are widely reported alongside the pulmonary route that has been studied intensely for future therapeutics (32–35) even though some challenges need to be faced (36,37). Other possible routes such as the ocular (38,39) and vaginal (40,41) have also been subject of study for drug targeting using nanoparticles.

Nanoparticles are vehicles which size ranges between 1 nm and 100 nm, although vehicles that measure up until 1000 nm are also considered nanoparticles by the majority of the scientific community (11,12,14). These nanocarriers are usually characterized by two different properties, size and zeta potential. The first defines the dimension of the nanocarrier and is highly dependent on the production method and the composition of the vehicle. As for the latter, it indicates the surface charge of the nanoparticle and is greatly related to the composition of the nanoparticle, to the molecules that are absorbed to the surface of the nanocarrier if there is any and lastly, the media where the nanoparticle is being characterized. In this regard, the ionic strength and pH are the most important aspects affecting zeta potential (42).

Nanoparticles can also be categorized according to their composition. Metals, lipids and polymers are some of the most used materials to compose the matrix of nanoparticles, with polymers assuming a position of relevance (43–45). In that case, they are called polymeric nanoparticles, being comprised of either synthetic or natural polymers. The most common of the used synthetic polymers are poly(lactic-co-glycolic) acid (PLGA) and polylactic acid (PLA). The high compatibility, good releasing properties and low toxicity of these polymers has enabled their inclusion in several formulations currently approved by the FDA and the European Medicines Agency (EMA) (46,47). Additionally, their favourable properties potentiates their use in several biomedical applications (48–51).

In the case of polymeric nanoparticles which matrix derives from natural materials, for example chitosan (52), alginate (53), hyaluronic acid (54) and others (55,56), they present a

viable approach, even though no pharmaceutical formulation is available in the market making use of nanosystems with these components. According to data provided in the literature, nanoparticles based on natural polymers also present high biocompatibility, low toxicity and good biodegradability (57). Whichever the source of the polymer, the requisites of biocompatibility and biodegradability need to be observed for any biomedical approach.

The knowledge on the different pathological and physiological aspects of the human organism is growing day by day. This makes way for the possibility of using some of the harsh conditions of the living organisms to favour the action of nanocarriers. That is the case of the so called smart polymers (58,59). Nanocarriers comprised of these special polymers are able to respond to different physiological conditions (for example, pH, temperature, etc). Many variables may be taken into account, but the main message is that these polymers enable a controlled and many times targeted release of the encapsulated molecules, thus improving the patient's therapeutics.

In the following sub-sections, the characteristics of chitosan and dextran sulfate will be addressed in detail, as they were the two natural polymers materials used throughout the experimental procedures reported in this manuscript.

1.1.1. Chitosan

Chitosan is obtained by the *N*-deacetylation of chitin, a polymer that can be extracted from various sources (crustacean's shells, exoskeletons of certain invertebrates – ladybugs – and the cell walls of fungi, for example). It is considered the second most abundant polymer on Earth (60), after cellulose (61). However, chitin is not very versatile due to its structure and poor solubility in many solvents. Chitin plays a role in the protection of certain animals in nature, being organized in semi-crystalline microfibrils to provide the said protection (62). The deacetylated form of chitin is chitosan, which structure can be seen on Figure 1.3.

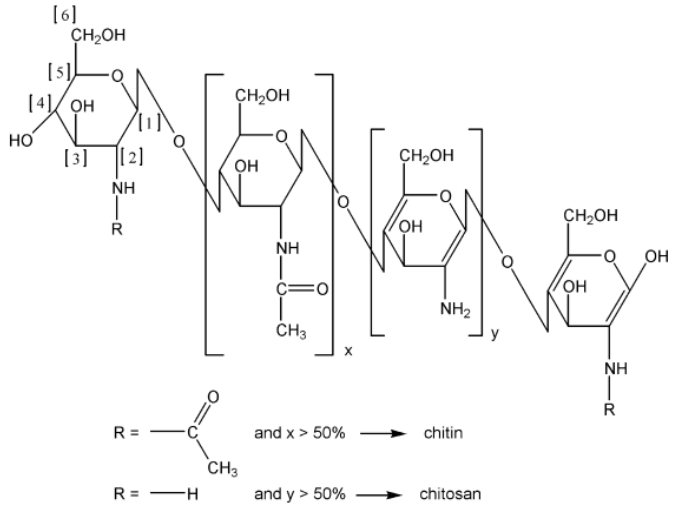


Figure 1.3 – Chemical structure of chitin and chitosan. Adapted from (62).

Chitosan (MW ~30-190 kDa (33,63–65)) is the polymer obtained when deacetylation surpasses 50% and is comprised of β (1-4)-links of D-glucosamine and *N*-acetyl-D-glucosamine monomers that are distributed randomly throughout the chain. This is the only natural polymer exhibiting a cationic character (66). Due to the fact that the amine groups are present throughout the structure of chitosan, the more deacetylated the polymer is, the more susceptible it is to protonation because nitrogen has an unused pair of electrons that can easily interact with electrophilic groups. When chitosan undergoes protonation, which occurs at low pH levels, it acquires a positive charge, thus providing the possibility to interact with negatively charged groups. This ability has been widely explored in drug delivery, with the preparation of nanoparticles by electrostatic interaction, as reported using carrageenan (67), tripolyphosphate (TPP) (34) or dextran sulfate (68) as counterions.

Chitosan is not soluble in water. Instead, and because the amine groups have a logarithmic acidity constant (pK_a) of ~6.5 (62), chitosan dissolves easily in acidic media. The most usual solvent for its dissolution is 1% (v/v) acetic acid. Due to the fact that the polymer is easily protonated, it can also be dissolved in formic and lactic acids and in hydrochloric acid solutions (62,69). In a pH of 7 or higher, as the pH is higher than the pK_a of amine groups, chitosan becomes insoluble, rendering its biologically applications are scarce or non-existent (66).

As mentioned earlier, chitosan presents adequate characteristics regarding biological applications (34,37,63,64,70), including biocompatibility, biodegradability and low toxicity. Its biodegradability is due to a metabolism by lysozyme (71). Moreover, due to its protonation in acidic media, it presents a favourable interaction with mucus (72), which is particularly relevant in the intestine, where there is a high amount of mucus. This is actually one of the reasons justifying its application in specific disease conditions, such as colonic inflammation (27). Nevertheless, the description of chitosan applications in literature is endless, including pulmonary and oral insulin delivery (25,37) as well as the delivery of antibiotics and small genetic fragments (73–75), just to mention some examples.

Finally, chitosan can also be found in a variety of dietary supplements (Lipoforte® and EasySlim® Blocker, just to name a few) for weight loss. However, the European Food Safety Authority (EFSA) considers that there is not enough evidence that supports this indication (76). There are other studies that report its beneficial use on lowering LDL-cholesterol (77,78), a claim that EFSA found to have enough evidence that supports it. However, some problems have also been described for supplements, reporting chitosan interference with certain treatments, for example, anticoagulation therapy with warfarin (79) and anti-epilepsy therapy with valproate (80).

1.1.2. Dextran sulfate

Dextran sulfate was the other polymer used in this experimental work. It is also a natural polymer, obtained from bacteria of the *Leuconostoc* genus. It is comprised of a branched anhydroglucose backbone with attached sulphur groups, the latter believed to be 17% of the total mass of the polymer. This means that, 2.3 sulphur groups are present for each glucosyl residue, thus the polymer being expected to be negatively charged (32,63,68). Its chemical structure can be seen in Figure 1.6.

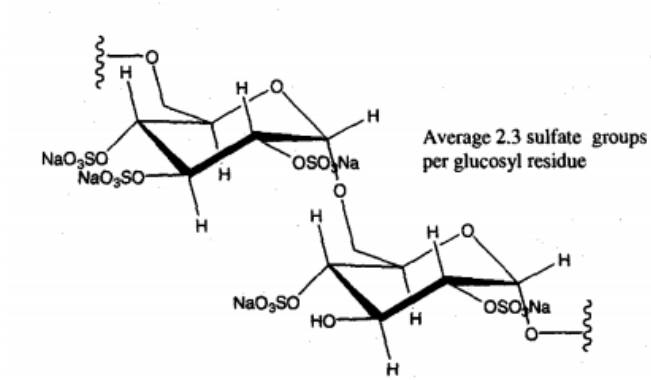


Figure 1.4 – Dextran sulfate chemical structure. Adapted from (68).

Dextran sulfate (MW ~5-500 kDa (70,81–83)) is also reported as biocompatible and biodegradable (84), thus theoretically complying with mandatory requisites for biomedical applications. Studies regarding the use of dextran sulfate as a component of DDS are scarce, even though it was widely studied in the eighties for its anti-HIV activity, against HIV-1. Its mechanism of action, in this context, involves inhibiting the reverse transcriptase, a viral enzyme responsible for turning the viral RNA in viral DNA that will, ultimately, be part of the host cell DNA resulting in the production of viral particles (82,85,86). Additionally, dextran sulfate has also been shown to activate macrophages by establishing an interaction with the scavenger receptor present in these cells (87). Moreover, regarding the immune system, it is believed that dextran sulfate induces activation of B lymphocytes even though that mechanism is mediated by macrophages (88). Another aspect that is widely mentioned in studies is an anticoagulant capacity similar to heparin, even though it wasn't studied further (68,70,89). Finally, dextran sulfate is used, nowadays, to induce colitis in mouse models (90,91).

Approved formulations containing this polymer include two EMA-approved orphan medicines: i) a medicine for the prevention of graft rejection and its further damage on

pancreatic transplantation by inhibiting the activation of complement system and clotting (92) and ii) a medicine to promote mobilisation of progenitor cells, prior to stem cell transplantation, through a cytokine released by bone marrow that can guide these important cells through blood stream (93).

Dextran sulfate is a very interesting polymer with many characteristics that can be used in drug delivery. However, there is still much work to do and, more importantly, study possible associations with other polymers to produce nanocarriers with a potential use in therapeutics.

1.2. The freeze-drying process

As mentioned previously, nanoparticle's stability is an issue that may compromise the use of these systems. Problems of aggregation that result in the increment of particle's size are the most common limitation (36). Moreover, as many nanoparticle formulations are produced in aqueous environment, their direct storage is not feasible for long periods of time as there is high probability of contamination by microorganisms which, ultimately, may affect the stability of the nanosuspension (94). It is thus important to carefully control the conditions of production and storage of nanocarriers. Considering that stability issues in suspensions may prevent the clinical use of nanoparticles as DDS, the description of stabilisation processes has been increasing.

Freeze-drying, alongside supercritical fluid drying (95), has been described as one of the most adequate techniques to stabilise nanoparticles produced in aqueous environment (96). Water may exist in various states that can be characterized by the organization of its molecules: solid, liquid and gas. This plasticity is one of the cores of the process of freeze-drying. The different states that water can adopt are presented in Figure 1.7, where the specific conditions that need to be settled in order to produce a change in water's state are also referred (97).

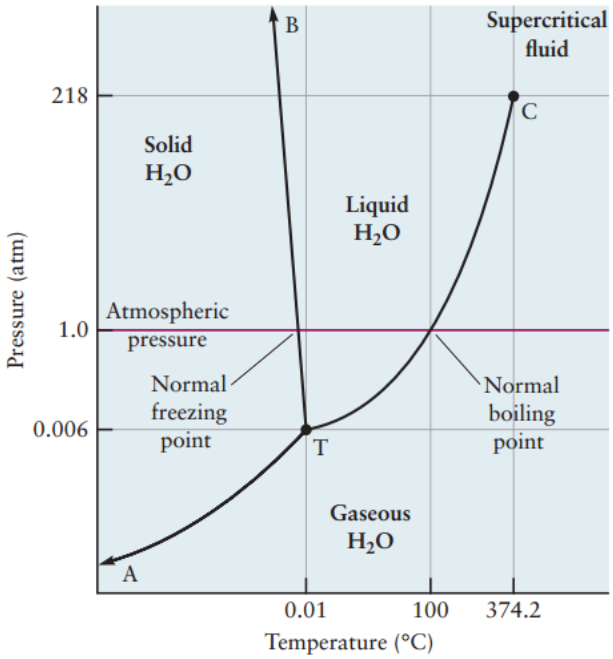


Figure 1.5 – The different molecular states that water can adopt when certain conditions are met. T represents the water's triple point. Adapted from (97).

When temperature is 0.01 ºC (or 273.16 K) and pressure is 0.006 atm (or 6.12 mBar), the so called triple point is met, with water assuming the three states simultaneously. So, in order to ensure the process of sublimation (transformation of water in solid state to the gas state) water needs to meet conditions of temperature and pressure that are below those of the triple point (97). When a frozen suspension of nanoparticles is subject to these conditions, ice sublimates and the remaining residue corresponds uniquely to nanoparticles. Theoretically, the solid state provides better stability than the aqueous analogue (6).

The process of freeze-drying is comprised of three different steps: freezing, primary drying and secondary drying. The freezing step is the first and it has to be considered differently than just the freezing of water, as the colloids act like a solute. When water freezes, molecules tend to form crystals, which will have a more amorphous structure if freezing is rapid or almost instantaneous. Due to this fact, after freeze-drying, the resulting cake will be very porous due to the empty spaces left by the ice crystals once formed thus being, its reconstitution, easier. On the other hand, if freezing is slow, a more organized structure will be formed because ice crystals have time to grow and form a more organized crystalline net, resulting in a more dense cake which reconstitution will be more difficult to achieve (94,98). The first process occurs when liquid nitrogen is used, while the second results from the use of a freezer, which has far higher temperature than liquid nitrogen (99). Whichever is the method selected to freeze the colloidal suspension, ice crystals will be formed. The unoccupied spaces between these crystals will be filled with the solute (nanoparticles in this case) that will be "trapped", resulting in a more concentrated solution. As the solution experiences an even lower temperature, the more concentrated and viscous it gets, reaching a temperature where it finally freezes, named eutectic temperature (T_{eu}). It is in this process that cryoprotectants have a relevant role to play. Cryoprotectants are excipients used to provide protection to a certain product from the stress that it undergoes upon/during freezing (98). Carbohydrates such as sucrose and glucose are of the most used cryoprotectants and have been proposed for the stabilisation of nanoparticles (100–102). These sugars, when added to colloidal suspensions and are subjected to freezing, inhibit the formation of big and fully developed ice crystals by reducing their nucleation and so, as the temperature goes even lower, water will keep freezing and the solution gets even more concentrated and viscous, as said before. At some point, freezing will stop, reaching a new temperature, the glass transition temperature (Tg') where, in this state, the sample is vitrified or glassified. This state is characterized by a solid solution where the solutes

(nanoparticles and sugar) are concentrated (C_g') with unfrozen water. The latter is associated with the solid solution and so, it will only be removed in the third step of the freeze-drying process, if this takes place. The water that managed to freeze, thus forming very small ice crystals, will be removed in the step of primary drying, to be mentioned next (94,96). All these processes are depicted in graphical form, in Figure 1.6.

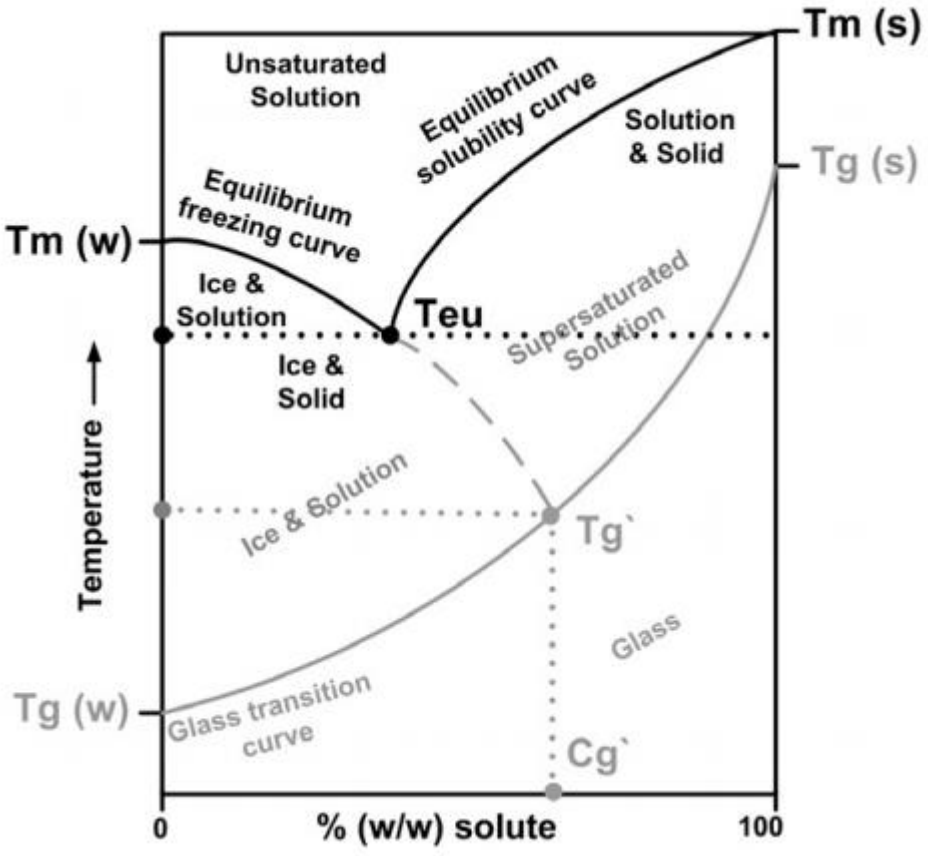


Figure 1.6 – Phase diagram for a solution comprised of water and a solute. T_{eu}' represents the eutectic temperature, T_g' the glass transition temperature and C_g' the maximal freeze concentration. Adapted from (94).

The freezing step, even though it is the first step, represents the most critical phase of freeze-drying. This is because it is when all molecular changes occur and, consequently, the phase where the cryoprotectant capacity of different components can be studied.

The step of primary drying occurs under vacuum. After a first step of low temperatures (between -30 ºC and -50 ºC (94,103,104)), the temperature raises when primary drying starts, in order to allow sublimation of ice and the consequent elimination of water from the samples. From this step, it is originated a rather porous material inside the reservoir where samples were stored. The duration of this phase is directly related to two aspects: the proper formulation and

the vial where the sample is inserted (94). The first aspect is related to the volume of solvent comprising the formulation (naturally, a colloidal suspension that has 1 mL will dry more rapidly than one that has 10 mL). Also, this drying is directly related to the capacity of water extraction of the equipment, so, small samples require less time to dry. The second aspect refers to the format of the vial. If it is large and deep, the drying will take longer time to be effective than if the vial is narrow and has little depth like an Eppendorf tube. When primary drying ends, some solvent will still be retained in the sample (about 15% - 20%), remaining bound to the solid sample (96).

The third step of the process is the secondary drying. This is an optional step and is just intended to dry the solvent that is bound to the formulation, which did not freeze, as explained above. This phase involves more severe vacuum conditions, meaning that the pressure inside the equipment will be lower, while the temperature will be higher to allow the elimination of the remaining water. However, this final step will only remove about 5-10% of the bound solvent and so, final freeze-dried formulations will still be comprised of, at least, 5% of humidity (6). Although optional, performing this final step is beneficial for the formulation due to the fact that, by removing the excess of water that is bound to it, the damaging long-term effects of water will be reduced (105).

As it was mentioned above, the freeze-drying process is one possible way to overcome stability problems of colloidal suspensions. However, and due to the fact that ice crystals (small or large) are formed upon freezing, this could seriously damage the polymeric vehicles that were produced. In fact, if nanoparticles are frozen without a component that protects them from ice crystal formation, the polymeric vehicles will disrupt and naturally aggregate. During freeze-drying, the aggregates will produce a sediment comprised of carriers which size is very far from the nanoscale, an occurrence only noticeable upon reconstitution. The use of cryoprotectants to protect polymeric nanoparticles during freeze-drying, and particularly during the freezing step, has long been studied. Cryoprotectants will fill the spaces between the nanocarriers inhibiting the production of ice crystals or the formation of small-sized crystals. Due to the different chemical structures that different carbohydrates present, the final cakes that result from freeze-drying may also look very different.

Nowadays, there are several studies reporting the use of freeze-drying as a possibility to stabilise nanocarriers with therapeutic value: i) Cerdeira *et al*. refer to freeze-drying of miconazole and itraconazole nanosuspensions (106); ii) Soares *et al.* study the viability of insulin-loaded solid lipid nanoparticles (100); iii) Hafner *et al.* suggest lechitin/chitosan

nanoparticles for the delivery of lipophilic drugs (107). Many other works consider freeze-drying as a valuable technique to overcome nanoparticle's stability problems (51,108,109).

However, some limitations have also been identified. One of them concerns the possibility of particle aggregation during the process of freeze-drying, which is only confirmed upon reconstitution of the system. Another limitation is related with the used equipment and whether it may allow secondary drying. If this is not performed, the amount of humidity associated may, at long-term, lead to microorganism growth, which is further potentiated by the presence of carbohydrates. Finally, another limitation of this process refers to the proper components of the lyophilizates, as some molecules are more susceptible to degradation, such as proteins (110).

Although nanocarrier stability is still an issue, many are the works addressing this aspect. With the advances of medicine and chemistry, it is believed that in some years more formulations containing these carriers will become available. Therefore, ensuring their long-term stability is a relevant matter, worth of research investment.

2. Objectives

This work was established within a wider research line being developed in the Drug Delivery Laboratory regarding the preparation of polysaccharide nanoparticles. The specific aim of the work was to study the stabilisation of chitosan/dextran sulfate (CS/DS) nanoparticles, which were previously developed by the group. In this regard, the technique of freeze-drying was used and the capacity of two different sugars, glucose and sucrose, to work as cryoprotectants in the process was tested. Both the short- and the long-term stability of reconstituted vehicles were evaluated, determining their size and the zeta potential immediately after the process and upon 3 months of storage at room temperature in a desiccator.

Additionally, a study was conducted to verify the effect of different acidic media (acetic acid 1%, hydrochloric acid (HCl) 0.1 M and 0.01 M) to dissolve chitosan on the final characteristics of CS/DS nanoparticles.

3. Materials and Methods

The work presented in this memory refers to the preparation and stabilisation of CS/DS nanoparticles. An important part of the work was based on an experimental design, a very important tool in science that allows researchers to obtain results with statistical significance. That is the part referring to the study of nanoparticle stabilisation by freeze-drying, in which different conditions were approached to produce a dry and stable formulation that permitted the preservation of nanoparticle physicochemical characteristics.

Additionally, a study was also performed to evaluate the effect of different acidic media used to dissolve chitosan, on the final physicochemical characteristics of CS/DS nanoparticles.

3.1. Reagents

Chitosan-base (low molecular weight, deacetylation degree ≥ 75-85% (111)) and dextran sulfate sodium salt from *Leuconostoc spp.* were purchased from Sigma-Aldrich® (Germany). Glucose, sucrose and hydrochloric acid 37% were purchased from VWR Chemicals® (Portugal). Glacial acetic acid was purchased from Fisher Scientific® (United Kingdom). Ultrapure water (Millipore®, Portugal) was used throughout. All other chemicals were reagent grade.

3.2. Preparation of CS/DS nanoparticles

CS/DS nanoparticles were prepared by polyelectrolyte complexation, in which the negatively charged sulfate groups of DS interact with the positive regions of CS (amino groups), leading to the production of nanoparticles as a result of the interactions that are established between these two polymers.

3.2.1. Study of the effect of different acidic media to dissolve chitosan

In order to determine the effect of different acidic media used to dissolve chitosan on nanoparticle characteristics, a study was performed using 1% (v/v) acetic acid and HCl at the concentrations of 0.01 M and 0.1 M. While chitosan solution (1 mg/mL) prepared in acetic acid had a pH of 2.88, those prepared in HCl had 2.26 (0.01 M) and 1.04 (0.1 M), respectively. To

allow meaningful comparisons, nanoparticles were produced at different CS/DS mass ratios (2/1, 3/1, 4/1 and 5/1). A stock solution of DS (1 mg/mL, pH 7.32) was prepared using ultrapure water, which was then diluted to the specific concentrations needed to respect the previously established ratios. All stock solutions were filtered (0.45 µm) before use. CS/DS nanoparticles were also spontaneously formed when 0.8 mL of the DS solution were added to 2 mL of CS solution (1 mg/mL), under magnetic stirring at room temperature, for 10 minutes. Nanoparticle suspensions were then transferred to Eppendorf tubes, onto a 10 µL glycerol layer and isolated by centrifugation at 16000 g, at 15 ºC, for 30 minutes (Thermo Scientific®, Germany). The supernatants were discarded and the pellet was resuspended in 200 µL of ultrapure water.

3.2.2. Freeze-drying study

Considering a previous work performed by the team, it was established that CS/DS nanoparticles of mass ratio 1/3 were those to perform in the present study. CS was dissolved in acetic acid at 1% (v/v) at a concentration of 0.83375 mg/mL. DS was dissolved in ultrapure water at a concentration of 1 mg/mL. All stock solutions were filtered (0.45 µm) before use and their pH determined. CS/DS nanoparticles were spontaneously formed when 0.8 mL of the CS solution were added to 2 mL of DS solution, under magnetic stirring, at room temperature. Stirring was maintained for 10 minutes. After this, the pH of nanosuspensions was determined and these were transferred to Eppendorf tubes, onto a 10 µL glycerol layer and isolated by centrifugation at 16000 g, at 15 ºC, for 30 minutes (Thermo Scientific®, Germany), as described in Figure 3.1A. After the centrifugation step, the supernatants were discarded and the pellet was resuspended in 200 µL of cryoprotectant solution, as depicted in Figure 3.1B.

Two control experiments were performed: one consisted in simply producing nanoparticles, resuspending in water and storing at 4 ºC, performing a monitorisation of size and zeta potential along time; the other comprised the production of nanoparticles, resuspension in water and freeze-drying (in absence of cryoprotectants).

3.3. Evaluation of cryoprotectants for nanoparticle stabilisation during freeze-drying

After production of the nanoparticles as described above (section 3.2.2.), the dispersion of nanoparticles upon resuspension to produce nanoparticle concentrations of either 1 mg/mL or 2 mg/mL (see Annexes A1.1 and A1.2). Sucrose and glucose were tested separately as

cryoprotectants, being used at the concentrations of 5% and 10% (w/v). Nanoparticle suspensions and the cryoprotectant solutions were mixed to obtain a final volume of 1 mL, which was frozen at -80 ºC and then freeze-dried under the following conditions: pressure of (3.9 to 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). After freeze-drying, nanoparticles were reconstituted in the same volume of ultrapure water as that initially comprised the nanoparticle suspension submitted to freeze-drying (1 mL), and their size and zeta potential determined.

The same freeze-drying approach was performed with the control experiment mentioned earlier (freeze-drying of nanoparticle suspension in absence of cryoprotectant).

Additionally, freeze-dried samples were also stored at room temperature in a desiccator for 90 days. With intervals of 15 days (day 1 corresponded to the end of freeze-drying), samples were reconstituted with ultrapure water (1 mL) and size and zeta potential characterised.

3.4. Characterisation of nanoparticles

3.4.1. Physicochemical properties

From every sample prepared, before and after freeze-drying, a 20 µL aliquot was extracted and diluted in 1 mL of ultrapure water. Afterwards, this suspension was placed on an electrophoretic cell and the size and zeta potential analysed by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), at 25ºC.

3.4.2. Nanoparticle production yield

The nanoparticle production yield was determined by gravimetry. For this purpose, nanoparticles were produced according to the procedure described previously, and after centrifugation, the nanoparticle suspension was freeze-dried over 72 h, using a Labconco® freeze dryer (Labconco® FreeZone 6 Liter Benchtop Freeze Dry System freeze dryer, Labconco®, USA). The production yield was determined in three different assays ($n \geq 10$) and was calculated as follows:

$$PY\ (\%) = \frac{nanoparticle\ weight}{total\ solids\ wieght} \times 100$$

where “nanoparticle weight” represents the weight of said vehicles after freeze-drying and “total solids weight” is the total amount of solids used to produce nanoparticles which are, in this particular case of unloaded nanoparticles, chitosan and dextran sulfate.

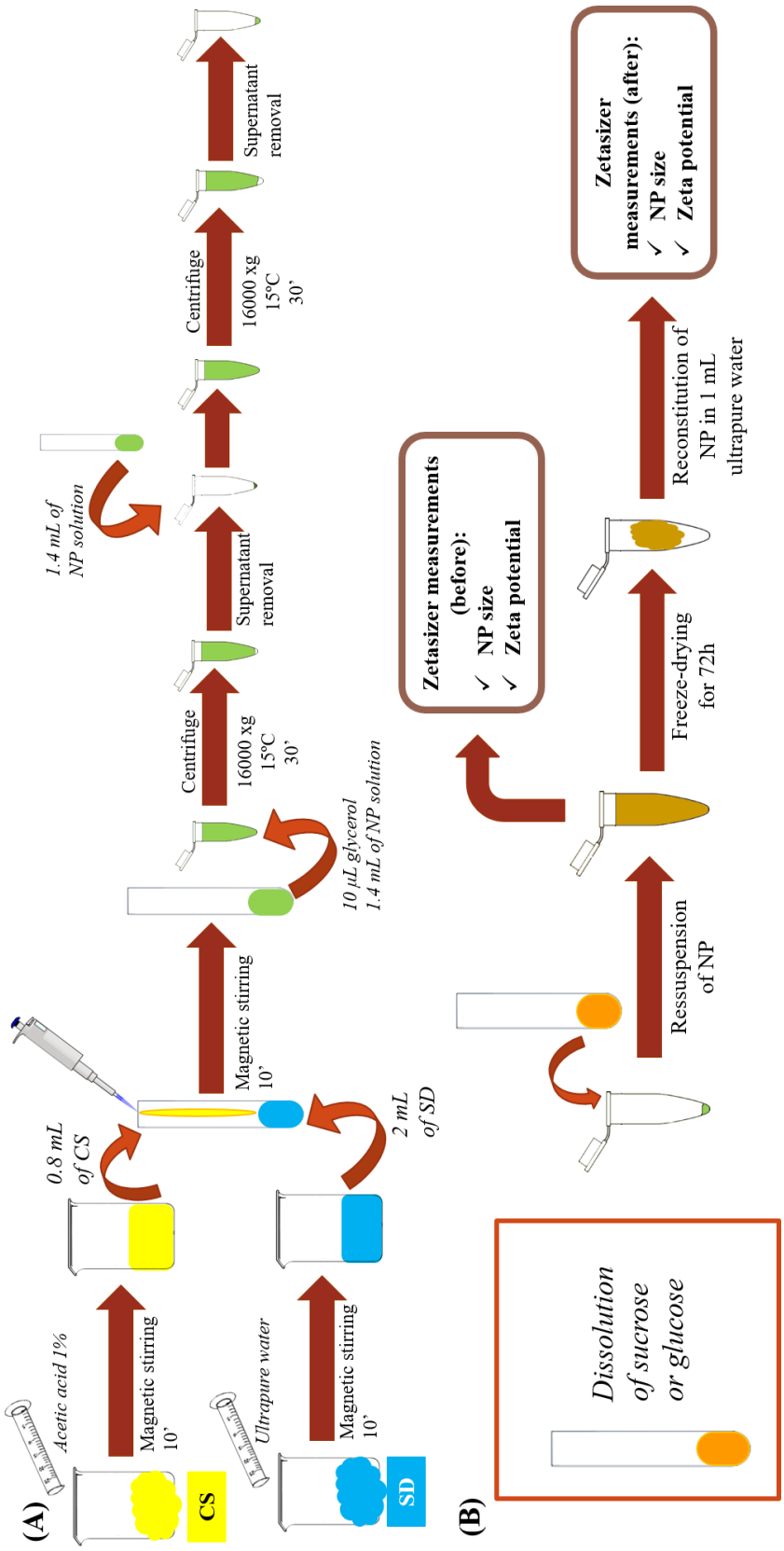


Figure 3.1 – Schematic representation of (A) nanoparticle production including polymer solubilisation and (B) the addition of the cryoprotectant solution to nanoparticle pellets, resuspensions step, freezing at -80 ºC, freeze-drying and reconstitution in 1 mL of ultrapure water for further analysis.

3.5. Statistical methodology

The following section describes the statistical methodologies used throughout this work.

The approach of evaluating nanoparticle stabilisation by means of freeze-drying and immediate reconstitution and characterisation was performed according to a statistical technique named Factorial Design (FD). This methodology is widely used in manipulative experiments where it is necessary to study the joint effect of the factors on a response. This is an experimental strategy in which factors are varied together, instead of one at a time (112). The objective of this factorial experiment was to investigate the influence of three factors (X_1, X_2, X_3) on the response variable (Y) that consists in nanoparticle stability after freeze-drying. Therefore, a 2^3 experimental design (8 treatments) was used, where 3 is the number of factors in the design and 2 refers to the number of levels for each factor. The 2^3 design is particularly useful in the initial phase of the experimental work, when many factors are investigated.

The FD was planned with equal number of observations within each treatment, which is designated as a balanced design. The response of a 2^3 experimental design can be written as

$$Y = X_1 + X_2 + X_3 + (X_1 * X_2) + (X_1 * X_3) + (X_2 * X_3) + (X_1 * X_2 * X_3),$$

where Y is the design response measured during the experiment, X_1, X_2, X_3 are the main factors, and the remaining terms are the 2-order interactions and the 3-order interaction. The linear model associated is the following,

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 (X_1 * X_2) + \beta_5 (X_1 * X_3) + \beta_6 (X_2 * X_3) + \beta_7 (X_1 * X_2 * X_3) + \varepsilon$$

where β_0 is the intercept, β_i, i = 1…7 are the linear model coefficients, and ε is the error component.

Furthermore, after adjusting a FD, the basic assumptions to be checked are that the errors are independent, normally distributed and have constant variance (homogeneity). A graphical approach (Q-Q plot) is used for the inspection of the normality, where the values should appear in to the straight line, and the Levene's test is used to test the equality of variances of the samples. In practice, inspection of the residuals (errors' estimates) is an important issue, since violations of the model adequacy and basic assumptions can be detected.

Besides the underlying assumptions, a careful analysis was performed for unusual and influential data. These observations can be detected through the inspection of the standardized and studentized residuals, and also by measuring influence with Cook's statistic (112,113).

In experiments involving 2^k factorial designs, it is important to examine the magnitude and direction of the factor effects to determine which are more important for the case study.

The pareto plot allows to compare the effects of each one of the factors against each other. The absolute values of the model parameters are plotted from the largest to the smallest magnitude. These coefficients are the exact coefficients from the linear model. The R function paretoPlot() from package pid (114) was used.

The statistical analyses, in section 4.3.3, were performed with SPSS (version 24.0 for windows) and with R software (115). Statistical significance is reached whenever *p*_value is less than $\alpha = 5\%$, the level of significance used in all the performed analysis.

4. Results and Discussion

In the following chapter, the results obtained through the experimental procedures aforementioned are presented and discussed.

CS/DS nanoparticles were successfully obtained by a polyelectrolyte complexation process, using conditions previously established by the research group. The formation of nanoparticles was confirmed by the Tyndall effect occurring after the mixture of the two polymeric solutions, where by emitting a light through the suspension enabled the observation of slight turbidity.

4.1. Effect of different acidic media to dissolve chitosan on CS/DS nanoparticle characteristics

Chitosan-based nanoparticles have long been proposed in the field of drug delivery. When chitosan base is used, as in this study, the most common approach is to perform its solubilisation in acetic acid, although other acids could be used. On the other side, when a salt of chitosan is used, the most reported molecule is chitosan hydrochloride. In this manner, it was decided to test HCl as a second possibility to solubilise chitosan base. The objective of the study was to verify if a different acid could translate into different physicochemical characteristics of nanoparticles.

The tested solvents were: 1% (v/v) acetic acid, HCl 0.1 M, 0.01 M and 0.001 M. However, because the amine groups have a pK_a of ~6.5 (62), resulting in an acidity constant (K_a) of ~3.16×10^{-7}, and the pH of 0.001 M HCl solution is ~3, the acid is not strong enough, in this case, to protonate the polymer and provide the solubilisation (62,69). Therefore, under these conditions, chitosan remains undissolved, as can be observed in Figure 4.1, preventing its use to produce nanoparticles.

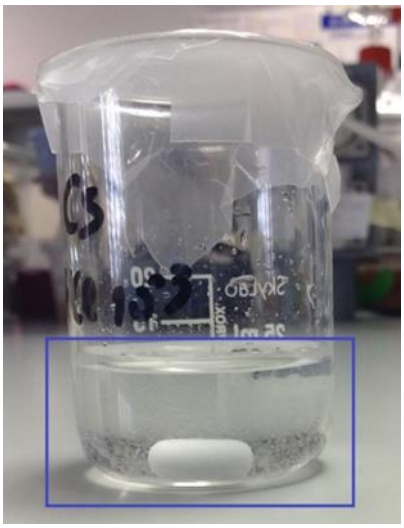


Figure 4.1 – Dispersion of chitosan after tentative solubilisation in hydrochloric acid (HCl) 0.001 M. Personal photograph in use.

Three different media were, thus, finally used to solubilise chitosan and, subsequently, to prepare CS/DS nanoparticles. These were produced at mass ratios of 2/1, 3/1, 4/1 and 5/1.

Acetic acid at 1% and HCl 0.01 M produced nanocarriers, although with sizes greater than 500 nm (data not shown), which in many times considered the limit of size adequate for drug delivery applications, namely if mucosal delivery is envisaged (116,117). The use of HCl 0.1 M demonstrated to not be feasible for the purpose of nanoparticle production, although the polymer efficiently dissolved chitosan. In fact, the sizes obtained for the nanoparticles were very high (> 1250 nm), and far from the nanometre range. Zeta potential was positive in all formulations but demonstrated unexpected behaviour, although it was consistent when any of the three acids were applied. In this regard, it was observed that, for a constant, amount of CS present in each formulation, the zeta potential became less positive with the decrease of DS (data not shown).

Charge ratios of the produced CS/DS nanoparticles were calculated. The theoretical mass ratios of 2/1, 3/1, 4/1 and 5/1 mean (in theory) that there will be present 2, 3, 4 and 5 more chitosan mass comparing with dextran sulfate, respectively. The supplier reports per glucosyl residue of dextran sulfate 2.3 sulphur groups (118), while for chitosan, 1 deacetylated residue equals to one positive charge (when protonated). The used chitosan has 80% deacetylation (a mean of the interval mentioned in section 3.1) which means that, considering the monomeric polymer weight ratio of 169 g/mol (119) and the mass contained in the formulation, there are present ~9.47 x 10^{-6} positive charges in the media. As for dextran sulfate,

considering 2.3 negative charges per glucosyl residue, the monomeric polymer weight of 484 g/mol (68) and also depending on mass weighted to produce the stock solution and further dilutions, there are present ~4.75 x 10^{-6} negative charges for a CS/DS ratio of 2/1, ~3.17 x 10^{-6} negative charges for a CS/DS ratio of 3/1, ~2.37 x 10^{-6} negative charges for a CS/DS ratio of 4/1 and ~1.90 x 10^{-6} negative charges for a CS/DS ratio of 5/1. Therefore, the (+/-) charge ratio for CS/DS nanoparticles 2/1 (w/w), 3/1 (w/w), 4/1 (w/w) and 5/1 (w/w) are, respectively, 1.99, 2.99, 3.99 and 4.98. This indicates a clear predominance of positively charged groups in all cases, which is coincident with the overall positive zeta potential that was obtained (between +15 and 52 mV). However, it also reinforces that nanoparticles of mass ratio 5/1 should have a higher positive charge than 2/1, which was not observed in any case.

Due to time constraints, it was not possible to further replicate and widen the assay. It is however considered that this would be helpful to make the results more consistent and provide some explanations, apart from permitting drawing conclusions about the real effect (or absence of it) of using different acidic media to dissolve chitosan. In further studies, a new experiment could be designed with not only a higher number of samples but also with optimized conditions so that objective conclusions could be drawn.

4.2. Production of CS/DS nanoparticles for the freeze-drying stability study

Nanoparticle's stability is a major concern due to their particular characteristics already discussed in previous sections. Moreover, the proper conditions of preparation and the handling of nanoparticles are yet other aspects requiring attention. Due to their rather particular characteristics, nanoparticles are very susceptible to external weather and to the laboratory environment. Atmospheric conditions are very difficult to control in the academic environment, as there is no such a controlled infrastructure, causing variations on humidity and temperature that can affect nanoparticle preparation. In spite of the use of air-conditioning, the conditions cannot always be maintained unaltered.

As referred in the methodology, CS/DS nanoparticles were prepared in a previous work of the team and the formulation corresponding to CS/DS mass ratio of 1/3 was the one selected to perform the present work of studying the effect of cryoprotectants in nanoparticle freeze-drying. Size and zeta potential were the properties used to characterise and evaluate CS/DS nanoparticles throughout the work. Size consists of a measurement of the hydrodynamic diameter of the nanoparticle, while zeta potential entails the measurement of its surface charge.

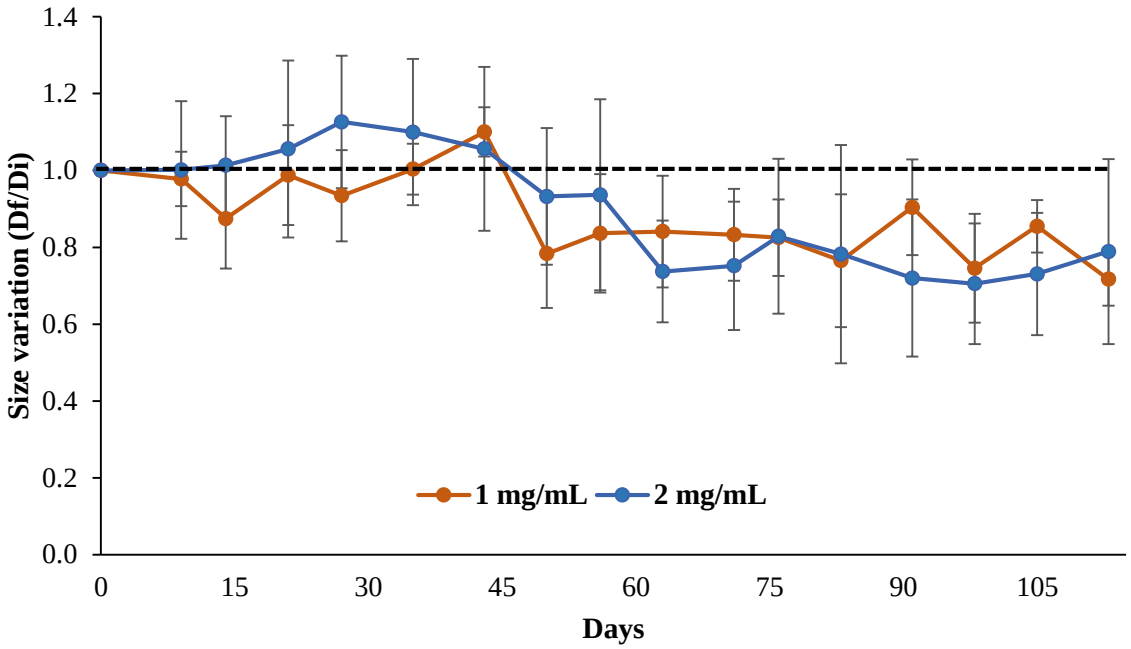
Considering the selected CS/DS mass ratio of 1/3, there will be three molecules of dextran sulfate per each chitosan molecule. Therefore, the net charge of these nanoparticles is expected to be negative, although that depends on the charge density of the polymers. The physicochemical characteristics of freshly prepared CS/DS nanoparticles (1/3, w/w) were determined to be 270 ± 50 nm and -44.8 ± 4.5 mV, which can be considered adequate for drug delivery purposes, from a general point of view (116).

Although most works devoted to drug delivery categorise nanoparticle formulations according to polymeric mass ratios, charge ratios seem to have a greater importance in nanocarriers formed by electrostatic interaction. As referred in section 4.1, the mass of each monomer of the polymers involved in the process was converted to moles of charge. Therefore, and considering both the polymer masses in the formulation, the monomeric polymer weight and the polymeric mass ratio (see methodology), chitosan has ~3.95 x 10^{-6} positive charges, while dextran sulfate has ~9.50 x 10^{-6} negative charges. For a CS/DS mass ratio of 1/3, the (+/-) and (-/+) ratios are, respectively, 0.33 and 3.01, indicating a higher abundance of negatively charged groups. This is in line with the registered zeta potential around -45 mV. Another work describing the preparation of CS/DS nanoparticles reported results that are in line with those described herein (120). In that case, for a CS/DS mass ratio of 1/2 a (-/+) ratio of 2.24 was indicated, while a mass ratio of 1/4 resulted in a charge ratio of 4.48. This is merely indicative, as the number of charges will be strictly dependent on the type of chitosan. The (-/+) ratio indicated for the nanoparticles of the present work (3.01) perfectly fits the trend reported in the mentioned study.

4.3. Evaluation of the stability of CS/DS nanoparticles

4.3.1. Evaluation of the stability of an aqueous suspension of CS/DS nanoparticles at 4 ºC

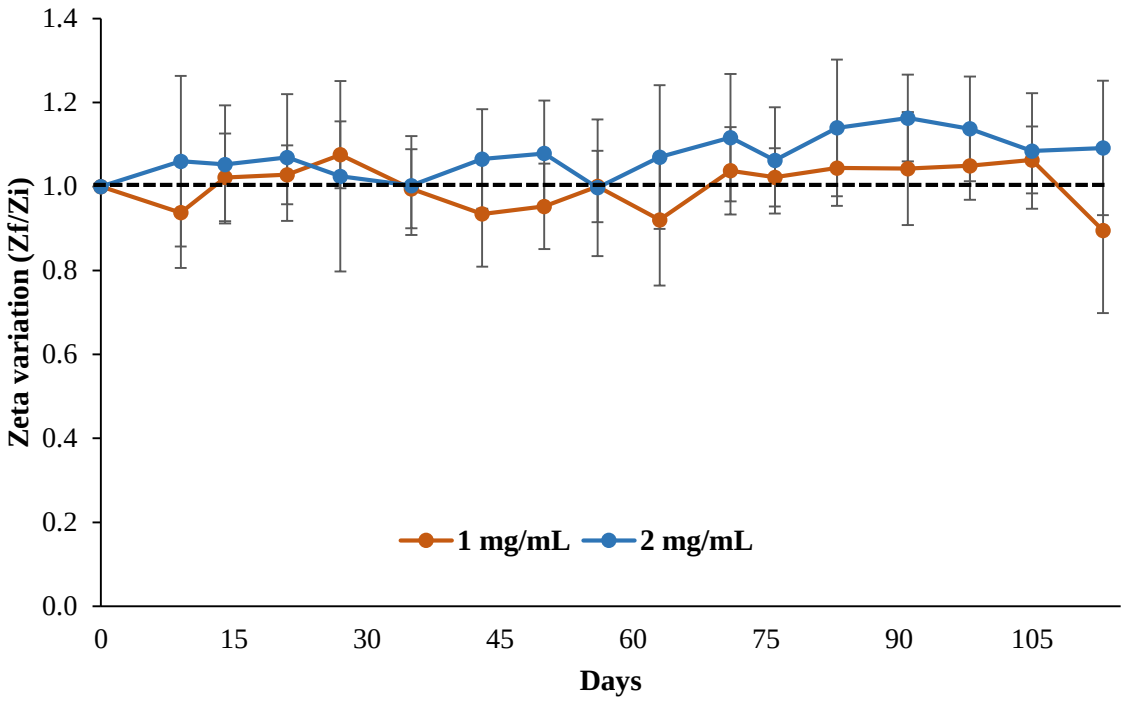
Nanoparticles are usually produced in aqueous environment and, as referred in the introduction, this raises a concern on their long-term storage, as aggregation is frequently mentioned to occur (98,121,122). In this work, an assay was conducted to evaluate the evolution of nanoparticle physicochemical characteristics (size and zeta potential) along time, upon storage of the aqueous suspension of nanoparticles at 4 ºC (nanoparticles were simply resuspended in water). The study lasted 113 days and the results are presented in Graphics 4.1 (size) and 4.2 (zeta potential).



Graphic 4.1 – CS/DS nanoparticle size variation along time (113 days) upon storage as aqueous suspensions at 4 ºC (D_i: initial diameter; D_f: final diameter). Data represent mean ± SD (n = 10).

As depicted in Graphic 4.1, at both concentrations nanoparticles maintained their size relatively stable in the first 43 days, as size variation was around 1. Approximately from day 50 on, a certain decrease of size was observed independently of the concentration, remaining consistent until the end of the assay. These variations were of approximately 20-30%, but they are devoid of statistical significance, due to considerably high standard deviations.

Concerning the results of zeta potential (Graphic 4.4), the general observation is that the parameter remained approximately stable, with 10% maximum variation between the beginning and end of the assay.



Graphic 4.2 – CS/DS nanoparticle zeta potential variation along time (113 days) upon storage as aqueous suspensions at 4 ºC (Z_i: initial zeta potential; Z_f: final zeta potential). Data represent mean ± SD (n = 10).

The ability to maintain the stability of physicochemical characteristics of aqueous suspensions of polysaccharide-based nanoparticles stored at 4 ºC has been referred in several occasions (33,67,103,123,124). However, this does not prevent the need to find other strategies for the long-term storage of nanoparticles, as contamination by microorganisms remains a potential problem not addressed in the study above and there are also the concerns about transportations costs, which are higher for liquids. Therefore, approaching strategies for the production of dry formulations of nanoparticles is a real need, which will be addressed in the next section.

4.3.2. Effect of cryoprotectants in the physicochemical characteristics of the CS/DS nanoparticles

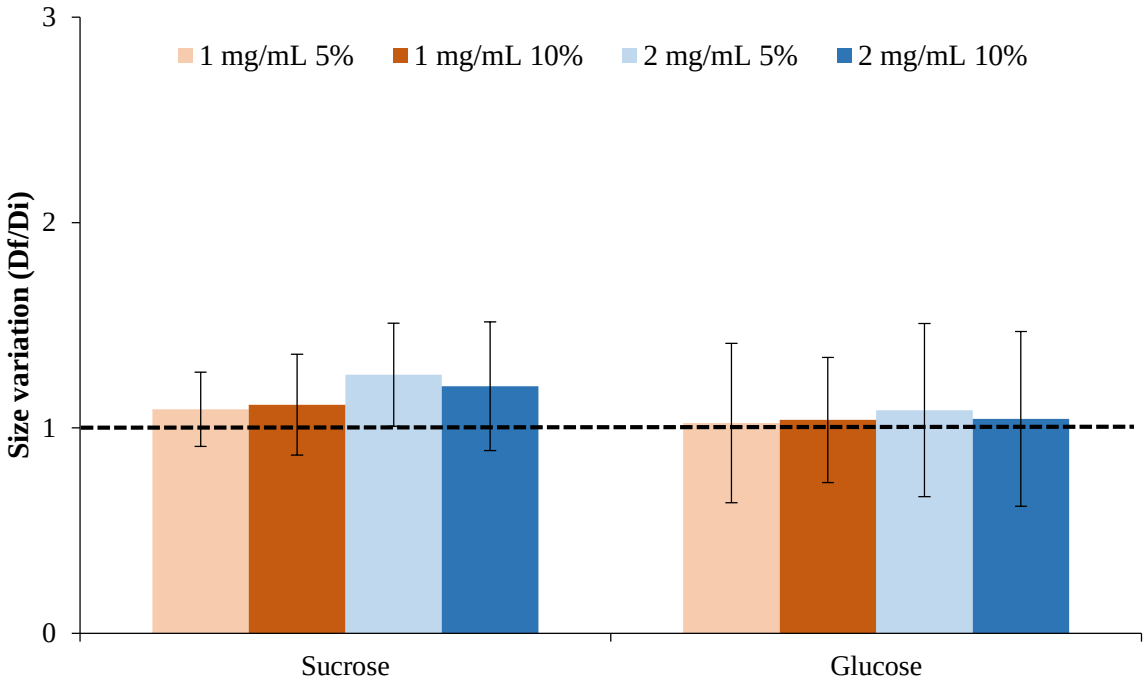
Freeze-drying has been frequently described as adequate methodology for the objective of improving nanoparticle stability and obtaining solid-state nanoparticle formulations. However, colloidal carriers may undergo different stresses during freeze-drying, which can alter their characteristics. Cryoprotectants are, thus, frequently used to overcome that

limitation. In this context, glucose and sucrose were evaluated in this study as cryoprotectants for the stabilisation of CS/DS nanoparticles during freeze-drying.

A control assay was conducted in which CS/DS nanoparticles were resuspended in ultrapure water, then frozen and submitted to freeze-drying in the absence of cryoprotectants. After the reconstitution with ultrapure water, sediment was visible on the bottom of the tube. This is possibly due to the lack of cryoprotectant, which did not prevent the growth of ice crystals, causing nanoparticle disruption and aggregation, as explained in the introduction (36). These observations constituted a baseline for the assay in which carbohydrates (glucose and sucrose) were used as cryoprotectants. After freeze-drying with cryoprotectants and before any reconstitution was performed, the aspect of the cakes was registered, being depicted in Figure 4.2.

Figure 4.2 – Nanoparticle cakes obtained after freeze-drying. From left to right: the first three were samples freeze-dried with sucrose and the other two, with glucose. Personal photograph in use.

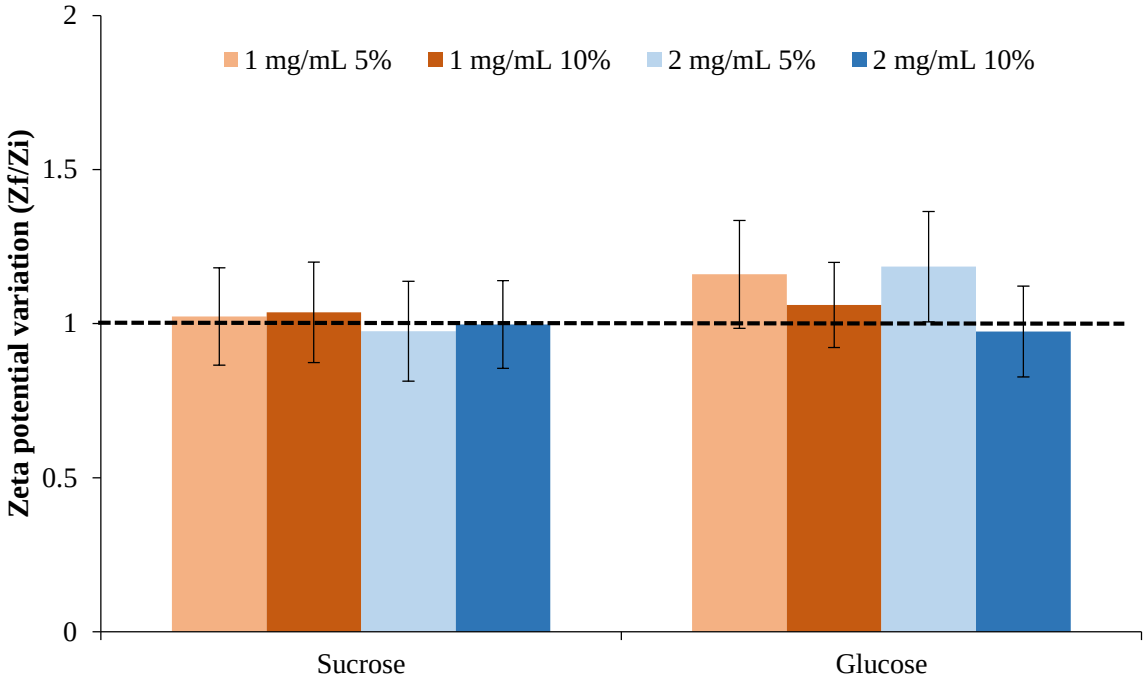
The figure demonstrates clear differences in the formed cakes depending on the use of glucose or sucrose. Sucrose produced a cake with a cotton candy-like texture, while glucose cakes resemble a transparent caramel texture. These differences certainly result from different chemical structures of the carbohydrates. Sucrose cakes were thus more porous, favouring reconstitution. In turn, cakes resulting from glucose had harder reconstitution, but it was completed with success. As mentioned on section 3.3, the freeze-drying equipment was only able to perform a primary drying, theoretically eliminating approximately 80% of the total water contained in the samples. Therefore, in order to further stabilise the samples before reconstitution and subsequent characterisation, they were put into a desiccator for at least 72 h. The results of size variation of CS/DS nanoparticles are shown in Graphic 4.3.



Graphic 4.3 – Size variation of different concentrations of CS/DS nanoparticles upon freeze-drying, as a function of the used cryoprotectant and its concentration (D_i: initial diameter; D_f: final diameter). Data represent mean ± SD (n = 16).

Keeping in mind that an ideal variation would correspond to 1, meaning that reconstituted nanoparticles maintained the initial characteristics, the results suggest a better performance of glucose as a cryoprotectant, when size is the focused characteristic. In that case, a maximum variation of 1.1 ± 0.4 was obtained at nanoparticle concentration of 2 mg/mL and 5% (w/v) glucose. Nevertheless, the standard deviations were considerably high, thus preventing more concrete conclusions. For sucrose, the maximum variation was registered again for 2 mg/mL and 5% (w/v) being of 1.3 ± 0.3. Additionally, considering the results as a whole, although no significant differences were appreciated, the concentration of 1 mg/mL is suggested to be more adequate to undergo the process under stable conditions, as the final nanoparticle sizes remain closer to those determined initially.

Results concerning the zeta potential variation of CS/DS nanoparticles are shown in Graphic 4.4.



Graphic 4.4 – Zeta potential variation of different concentrations of CS/DS nanoparticles upon freeze-drying, as a function of the used cryoprotectant and its concentration (Z_i: initial zeta potential; Z_f: final zeta potential). Data represent mean ± SD (n = 16).

Comparing sucrose with glucose, the former is suggested to have better performance towards the maintenance of zeta potential, with a maximum variation of 3% (1 mg/mL nanoparticles, 10% sucrose). In turn, zeta potential variations after freeze-drying when 5% glucose was used with 1 mg/mL and 2 mg/mL of nanoparticles reached 16-18%, which is certainly less desirable than what was observed with sucrose. However, again the standard deviations are high, preventing conclusions of whether glucose or sucrose has a better performance than the other one. Therefore, it can be said that both cryoprotectants have similar behaviour.

4.3.3. Measuring the factors' influence on the freeze-drying assays

A well-designed experiment is very important because of the results and conclusions possibly drawn from it. The FD is more efficient than one factor at a time experiments and is a useful approach when interactions between factors may be present, in order to avoid misleading conclusions.

The analysis of factors considered for CS/DS nanoparticles' stability (Y) was based on the FD described in section 3.5. This approach investigates if the size and zeta potential variations (Y) are affected by three factors: the type of cryoprotectant (X_1), the cryoprotectant concentrations (X_2) and the nanoparticle concentrations (X_3). Each factor has two levels: X_1 = (glucose, sucrose), X_2 = (5%, 10%) and X_3 = (1 mg/mL, 2 mg/mL). As already mentioned in section 3.5, a balanced FD was planned with 16 observations per treatment, obtaining a total of 128 observations, represented on the contingency table (Table 4.1) shown below.

Table 4.1 – Number of observations per treatment for the 2^3 experimental design. ("Cryoprotector": glucose or sucrose; "Cryo_Conc": cryoprotectant concentration of 5% or 10%, w/v; "NP_Conc: nanoparticle concentration of 1 mg/mL or 2 mg/mL).

		Cryo_Conc			
		5%		**10%**	
		NP_Conc		**NP_Conc**	
		1 mg/mL	**2 mg/mL**	**1 mg/mL**	**2 mg/mL**
Cryoprotector	**Glucose**	16	16	16	16
	Sucrose	16	16	16	16

4.3.3.1. Case study 1: Size variation

This study examined the effect of the aforementioned factors on the variation of CS/DS nanoparticle size. A FD was performed and the underlying assumptions were inspected. Moderate departures from normality are tolerated in the case of a balanced FD, as seen by the examination of the Q-Q plot (Figure 4.3). As suggested by Montgomery *et al*, to assess this normality, "the central observations are more important than the ones present on the extremes" (112).

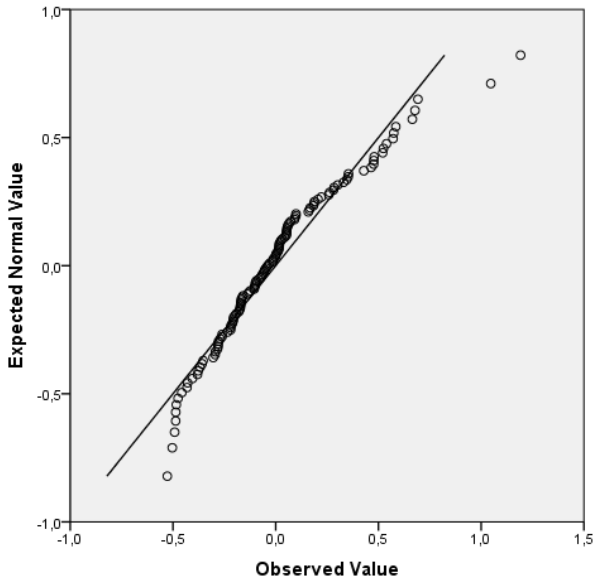


Figure 4.3 – Normal Q-Q plot for residuals for the size factorial design (FD).

The test of homogeneity of variances (Levene's test) is robust against normality assumption (112,125). Moreover, if the assumption of the homogeneity of variance fails, the test statistic is only slightly affected in the balanced design (112), as in this case (*p*_value = 0.041). Inspection of the standardized and studentized residuals and Cook's distance was performed to discover outliers and influential observations (see Annexes B1.1 and B1.2). These are problematic because they can influence the results of the analysis and because their presence may be a sign that the model fails to capture important characteristics of the data (112).

The pareto plot from the linear adjustment is seen in Figure 4.4.

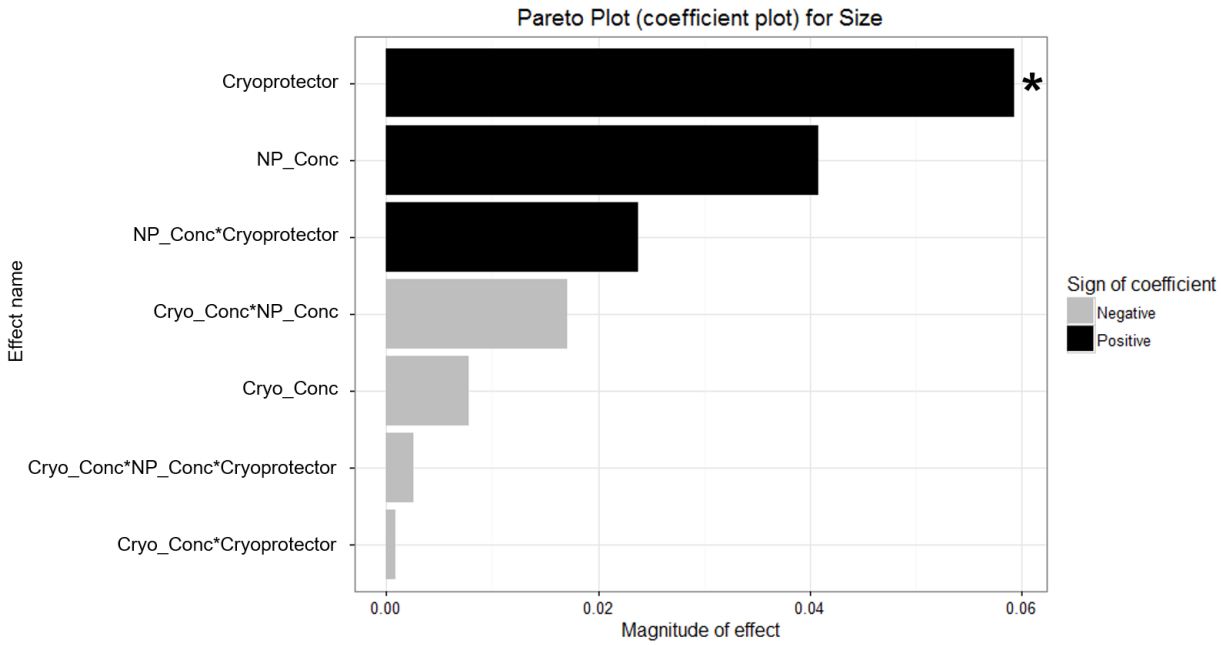


Figure 4.4 – Pareto plot for the size variation (* means *p*_value < 0.05).

The pareto plot shows that the factor Cryoprotector is significant and has a positive increasing effect (0.059) on size variation. The next factors decrease in magnitude: NP_Conc (0.041), NP_Conc*Cryoprotector (0.024) and so on. From these factors, only the cryoprotector revealed to be statistically significant (*p*_value = 0.042), meaning that it is the sole responsible for the observed size variation (see Annex B1.3). Furthermore, an analysis between the two cryoprotectors is performed. Descriptive analysis for each cryoprotector is in Table 4.2.

Table 4.2 – Some of the descriptive statistics for each cryoprotector.

	Cryoprotector	N	Mean	Std. Deviation
RatioSize	**Glucose**	64	1,04820	,379182
	Sucrose	64	1,16694	,255210

Additionally, as it can be seen in Table 4.2, the mean of results obtained for glucose is closer to the ideal ratio of 1 comparing with sucrose. Nevertheless, the comparison between both cryoprotectants should be performed by means of the coefficient of variation (CV), since they have different means. The CV is an important statistical dispersion measure that analyses variation of the data round its mean, and can be calculated through the formula

$$\text{CV}\ (\%) = \frac{\text{SD}}{\text{mean}} \times 100$$

where "SD" is the standard deviation. For glucose, the CV is 36.2% and for sucrose is 21.9%, meaning that the variability for both is lower (in general, CV < 50%) and the mean can be considered representative for both samples. Even though sucrose presents lower CV than glucose, the results suggest that the latter is more adequate to undergo the process under stable conditions, as the final nanoparticle sizes remain closer to the ideal ratio of 1.

4.3.3.2. Case study 2: Zeta potential variation

The approach applied to the study of size variation was further applied on zeta potential. A FD was performed and the underlying assumptions were also inspected. The goodness of the fit can be observed in the Q-Q plot of the residuals represented in Figure 4.5, and through the Levene's test (*p*_value = 0.899).

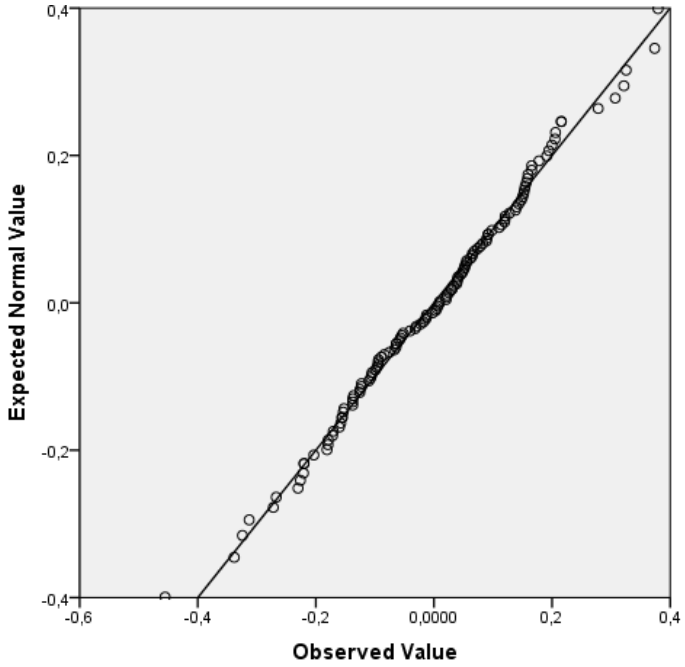


Figure 4.5 – Normal Q-Q plot for residuals for the zeta potential factorial design (FD).

Inspection of the standardized and studentized residuals and Cook's distance was performed to discover outliers and influential observations (see Annex C1.1 and C1.2).

The pareto plot from the linear adjustment is seen in Figure 4.6.

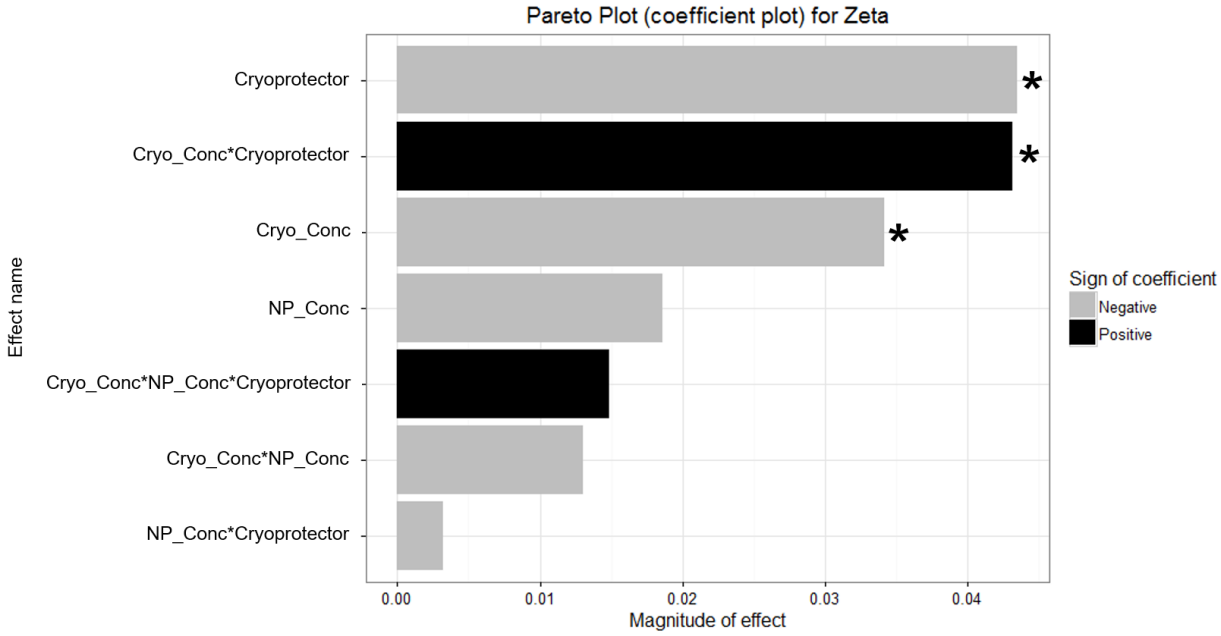


Figure 4.6 – Pareto plot for zeta potential variation (* means *p*_value < 0.05).

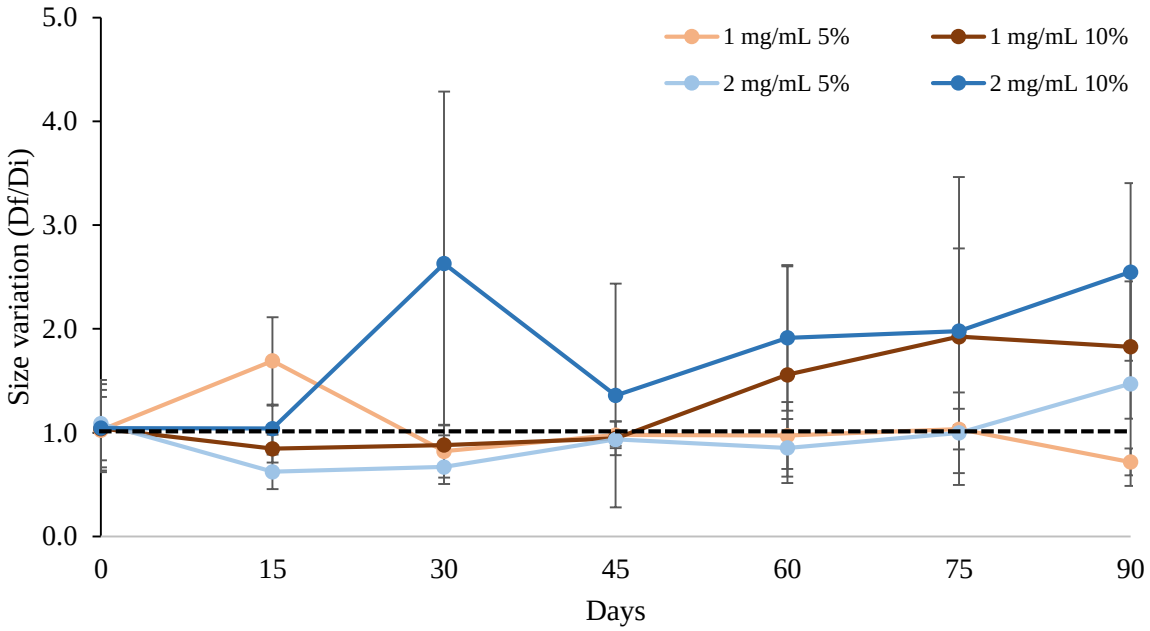
The pareto plot shows that the factors Cryoprotector, Cryo_Conc and their combination Cryoprotector*Cryo_Conc are significant. The main factors have a reducing effect on the outcome, - 0.044 and -0.034, respectively, and the two-factor interaction has a positive increasing effect (0.043) on the outcome (zeta potential variation).

From FD model, the *p*_values = 0.002, 0.016 and 0.003 were obtained for the factors Cryoptoyector, Cryo_Conc and their combination Cryoprotector*Cryo_Conc, respectively, as presented in the ANOVA table (Annex C1.3).

Moreover, a 2^2 FD was performed and the results are shown in Annex C1.4 and C1.5.

4.3.4. Evaluation of the stability of freeze-dried CS/DS nanoparticles during storage at room temperature

The previous study demonstrated the ability of glucose and sucrose to act as cryoprotectants of CS/DS nanoparticles. However, the reconstitution of cakes was performed in fresh freeze-dried samples in all cases. Nevertheless, it was deemed adequate to also verify the stability of these cakes along time, that is, to store freeze-dried nanoparticles for 90 days in a desiccator (room temperature) and perform the reconstitution each 15 days, after which nanoparticle size and zeta potential were characterized. The obtained results for size variation are displayed in Graphics 4.5 (glucose) and 4.6 (sucrose).

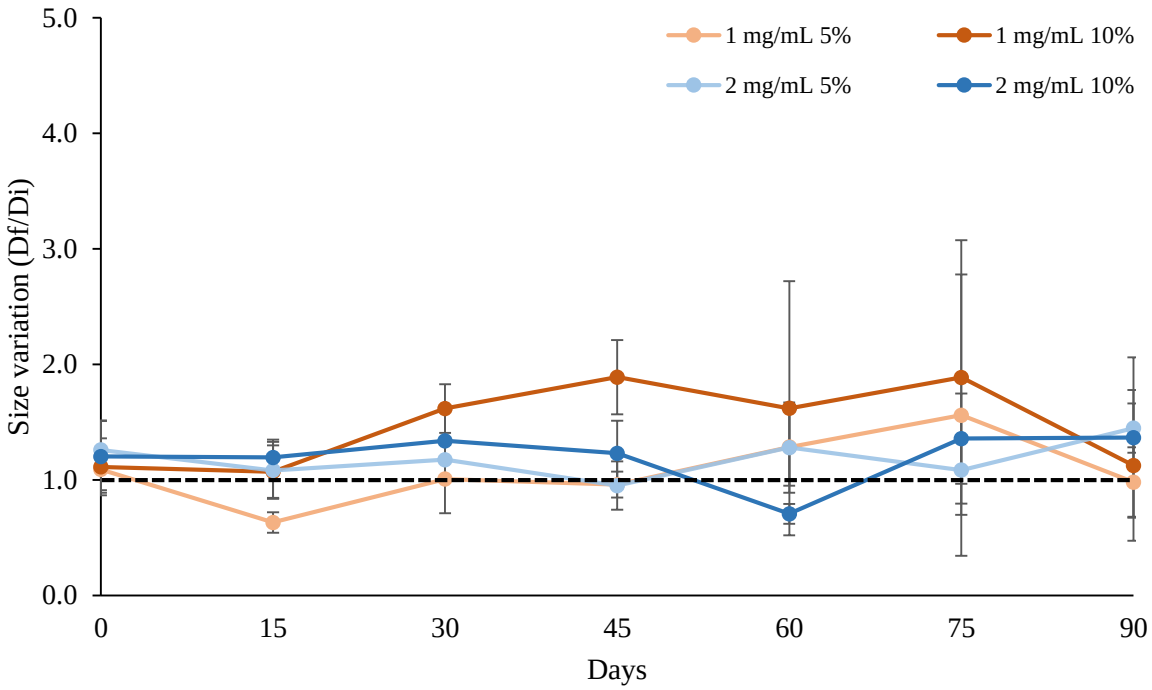


Graphic 4.5 – Size variation along time (90 days) of different concentrations of CS/DS nanoparticles freeze-dried with glucose, upon storage of the cake at room temperature (D_i: initial diameter; D_f: final diameter). Data represent mean ± SD ($n \geq 4$).

Although some variations occurred along the assay, the general information deserving a mention is that the concentration of 5% is apparently more adequate to ensure the stability of nanoparticle's size, as the measured variation remained closer to 1. Nevertheless, even at these conditions, a variation of 30-50% was registered in nanoparticle size, after 90 days, contrasting with only 3-5% at days 60 or 75. This suggests that possibly a period of 60 or 75 days is the maximum admissible for nanoparticle storage in these conditions. The use of 10% glucose,

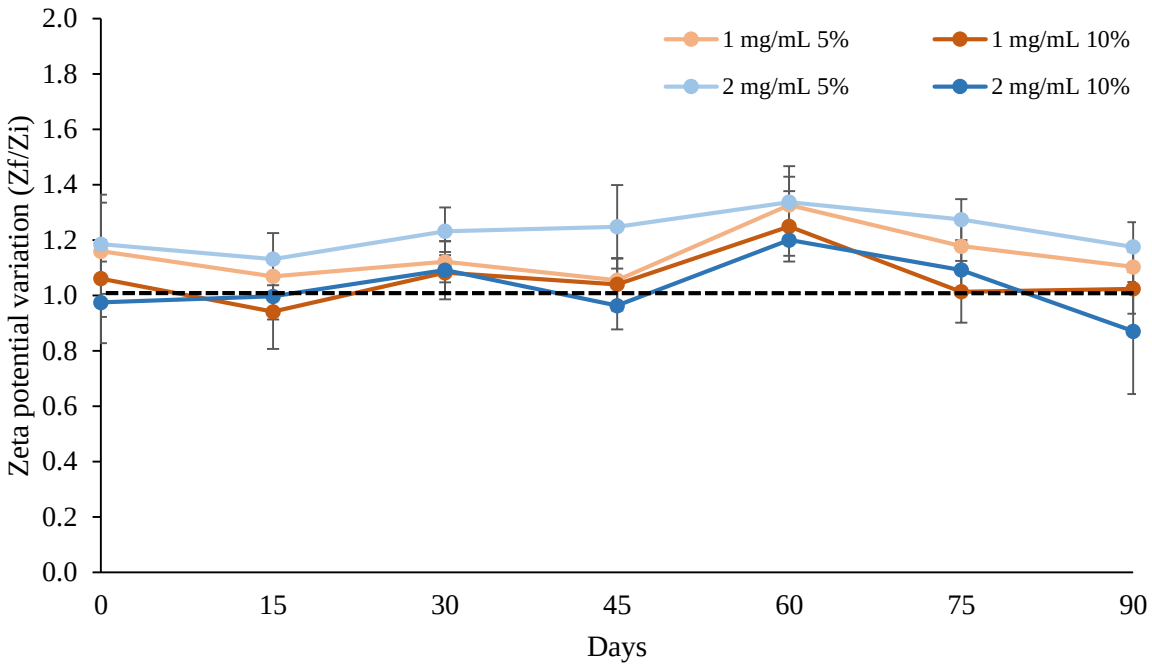
particularly when nanoparticles are at 2 mg/mL results in strong increase of nanoparticle size (variation of 2.6 ± 1.9, upon 90 days).

In turn, nanoparticles formulated with sucrose resulted in more severe variations, observed for the majority of tested conditions. As displayed in Graphic 4.6, and especially if particularly comparing the final size of nanoparticles with that registered initially, size variations range between 2% (1 mg/mL, 5% (w/v) sucrose) and 38% (2 mg/mL, 5% (w/v) sucrose). However, as a general view, having 2 mg/mL and sucrose concentration of 5% is possibly the best option to preserve nanoparticle size. As also observed for glucose, a maximum storage of 75 days is perhaps more adequate (size variation of 8%).



Graphic 4.6 – Size variation along time (90 days) of different concentrations of CS/DS nanoparticles freeze-dried with sucrose, upon storage of the cake at room temperature (D_i: initial diameter; D_f: final diameter). Data represent mean ± SD (n = 5).

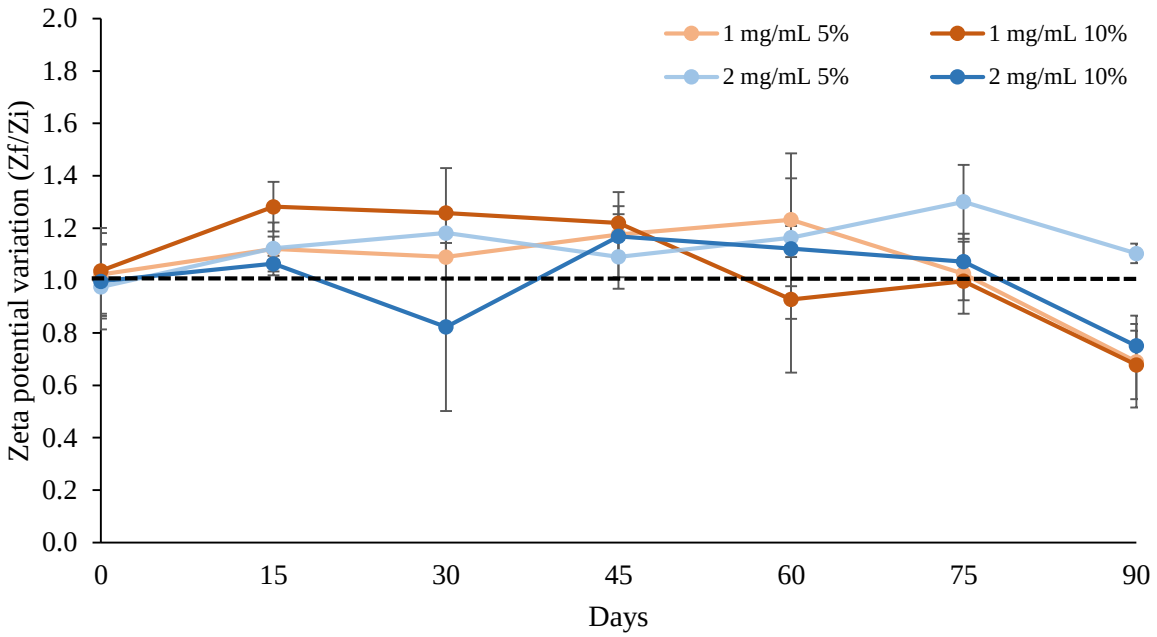
The long-term effect on zeta potential was also characterised and results are displayed in Graphics 4.7 (glucose) and 4.8 (sucrose).



Graphic 4.7 – Zeta potential variation along time (90 days) of different concentrations of CS/DS nanoparticles freeze-dried with glucose upon storage as a cake at room temperature (Z_i: initial diameter; Z_f: final diameter). Data represent mean ± SD (n = 5).

When glucose was used as cryoprotectant, the concentration of 10% (w/v) was the one showing better ability to preserve the zeta potential, independently of the used concentration of nanoparticles (Graphic 4.7). In these conditions, and focusing on the end of the study, the zeta potential variation varies between 2% and 13%. Nevertheless, when 5% glucose was used the variations did not go beyond 17% (after 90 days).

In sucrose, the variations did not go beyond 25% in the whole study (Graphic 4.8). There is a general decrease of zeta potential registered on day 90 (25-30% comparing with initial values), although at day 75 several conditions provide maximal zeta potential variations of 7%, which is considered acceptable.



Graphic 4.8 – Zeta potential variation along time (90 days) of different concentrations of CS/DS nanoparticles freeze-dried with sucrose upon storage of the cake at room temperature (Z_i: initial zeta potential; Z_f: final zeta potential). Data represent mean ± SD (n = 5).

Considering the results as a whole, it is suggested that glucose has a better performance regarding the long-term preservation of the cakes and the inherent characteristics of nanoparticles. Nevertheless, it should be assumed that results are quite variable, inclusive when different storage periods are considered, which does not allow rigorous conclusions. In spite of that, assuming a relatively better performance of glucose, this would be in line with the observations resulting from the freeze-drying study presented above, where it was also suggested that glucose has a better contribution for the maintenance of nanoparticle characteristics when fresh freeze-dried samples were analysed.

During the performance of this assay, temperature and humidity of the laboratory were controlled. Nanoparticle cakes were stored in a desiccator with silica, placed in a laboratory equipped with air-conditioning. The control of temperature and relative humidity are very important in stability studies and general storage of pharmaceutical formulations. EMA has a guideline on this matter, setting that long-term stability studies should be performed at room temperature (25 ± 2 ºC) and 60% relative humidity (± 5%) (126). Even though the proposed document is directed to large-scale trials, it can be adapted to the laboratory setting.

During the 90 days, at least one measurement of temperature and relative humidity was performed using a thermo-hygrometer. The mean temperature, determined from the daily data,

was 23.3 ± 1.4 ºC and the mean relative humidity was 49.4 ± 9.7%. Only the temperature is, thus, in relative accordance with the guideline. The registered relative humidity was lower, probably due to the fact that the assay was conducted between August and October, a time when weather is dry. Additionally, as air conditioning was continuously turned on, the relative humidity of the laboratory decreased.

5. Conclusion and future lines of work

From the laboratory work that was performed, it was verified that polyelectrolyte complexation is a valid method to produce nanoparticle based on chitosan and dextran sulfate, as these displayed adequate characteristics of size and zeta potential regarding a drug delivery approach. The production of nanoparticles was possible using different acidic media to dissolve chitosan (acetic acid and hydrochloric acid), although the concentration of the acid is a parameter requiring attention. The produced nanoparticles, which had predominant chitosan content, displayed sizes above 500 nm, thus requiring further optimization to enable the production of more adequate nanocarriers for drug delivery purposes (size below 500 nm). Moreover, an unexpected tendency was detected regarding zeta potential. Overall, further studies need to be performed to enable objective conclusions regarding the influence of acidic media used to dissolve chitosan, on the final characteristic of nanoparticles.

When stored as aqueous suspension at 4 ºC, CS/DS nanoparticles only keep stable physicochemical characteristics for 50 days, after which important variations are detected. A freeze-drying study was performed to verify the ability of the technique to stabilise nanoparticles, testing the capacity of sucrose and glucose as cryoprotectants. Although with some variations in their behaviour, both carbohydrates were capable of maintaining nanoparticle characteristics (size and zeta potential) relatively stable. Additionally, based on the results of the statistical analysis, the choice of cryoprotectant is a decisive factor for the differences detected in CS/DS nanoparticle size variation. As for the zeta potential, not only the cryoprotector, but also its concentration and the combination of both factors affect significantly this parameter. Nevertheless, the overall analysis has led to the conclusion that glucose has a better behaviour on preserving nanoparticle characteristics.

A step forward in this work would be the association of a therapeutic molecule of interest and the evaluation of the freeze-drying process on the drug carrier. Not only physicochemical characteristics would be examined, but also the effect of the freeze-drying process on drug encapsulation and release.

The freeze-drying approach could be also extended to other polysaccharide-based nanocarriers, in order to establish a general protocol permitting the stabilisation of this kind of nanoparticles, which are attracting much attention in drug delivery.

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7. Annexes

ANNEX LIST

Annex A: Flowcharts detailing the steps required for the production of nanosuspensions

Annex A1.1 – Flowchart for the preparation of 1 mg/mL nanoparticle suspensions.

Annex A1.2 – Flowchart for the preparation of 2 mg/mL nanoparticle suspensions.

Annex B: Statistical study for the response variable "Size ratio"

Annex B1.1 – The standardized dispersion plot from which two outliers were detected, #23 (2.278) and #90 (2.091).

Annex B1.2 – The Cook's distance dispersion plot where two influential points can be detected. Since there is not any Cook's distance bigger than 1, there are no influential observations among the data.

Annex B1.3 – Analysis of variance table for size ratio. "Cryo_Conc" refers to the concentration of carbohydrate used on the freeze-drying process – 5% or 10%; "NP_Conc" refers to the concentration of nanoparticles on the sample that was submitted to freeze-drying – 1 or 2 mg/mL; "Cryoprotector" refers to the carbohydrate used on the process – glucose or sucrose.

Annex C: Statistical study for the response variable "Zeta potential ratio"

Annex C1.1 – The standardized dispersion plot.

Annex C1.2 – The Cook's distance dispersion plot where the values are within the values considered for cut-off.

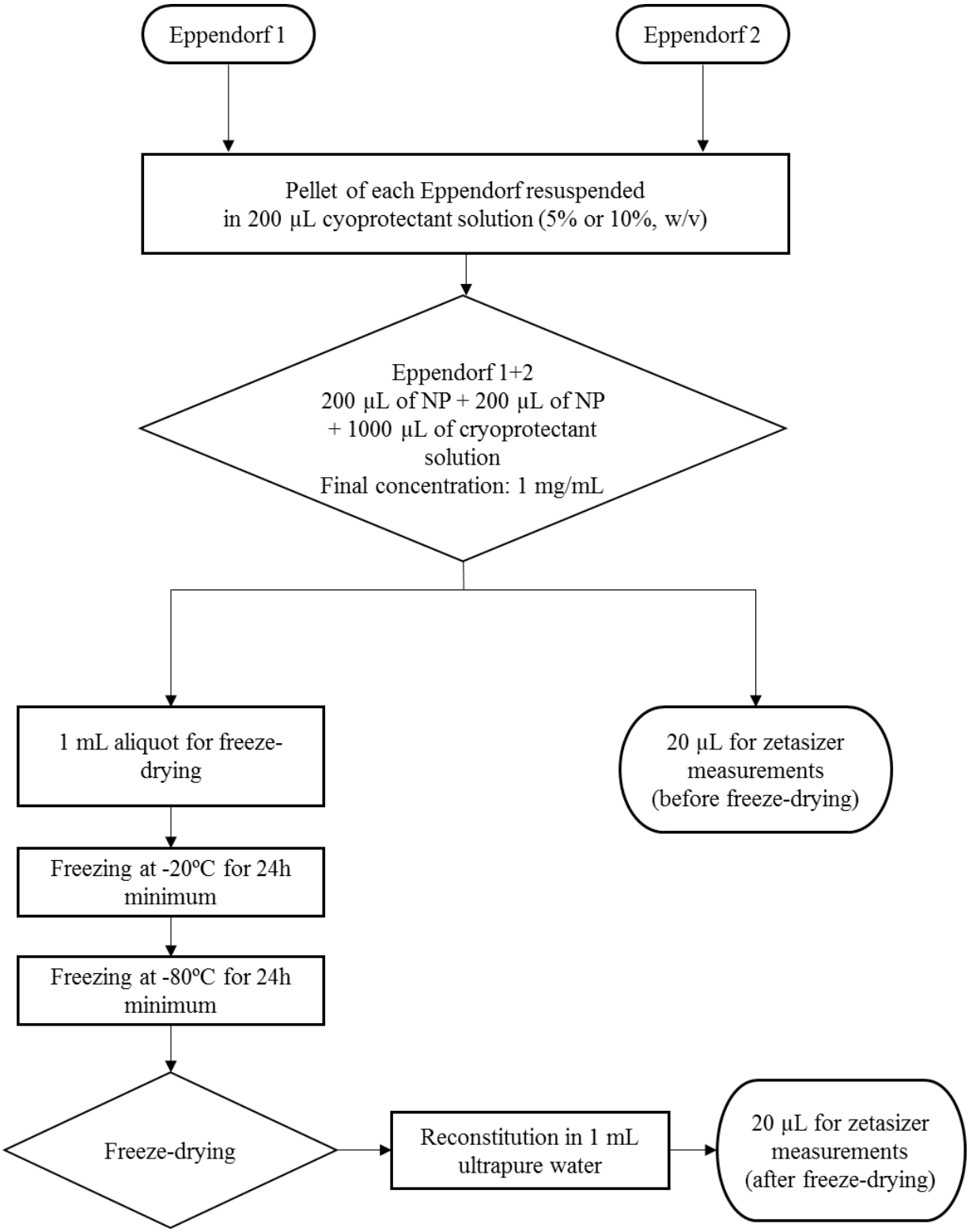
Annex C1.1 - Analysis of variance table for size ratio. "Cryo_Conc" refers to the concentration of carbohydrate used on the freeze-drying process – 5% or 10%; "NP_Conc" refers to the concentration of nanoparticles on the sample that was submitted to freeze-drying – 1 or 2 mg/mL; "Cryoprotector" refers to the carbohydrate used on the process – glucose or sucrose.

Annex C1.4 – The number of observations per treatment for the 2^2 factorial design.

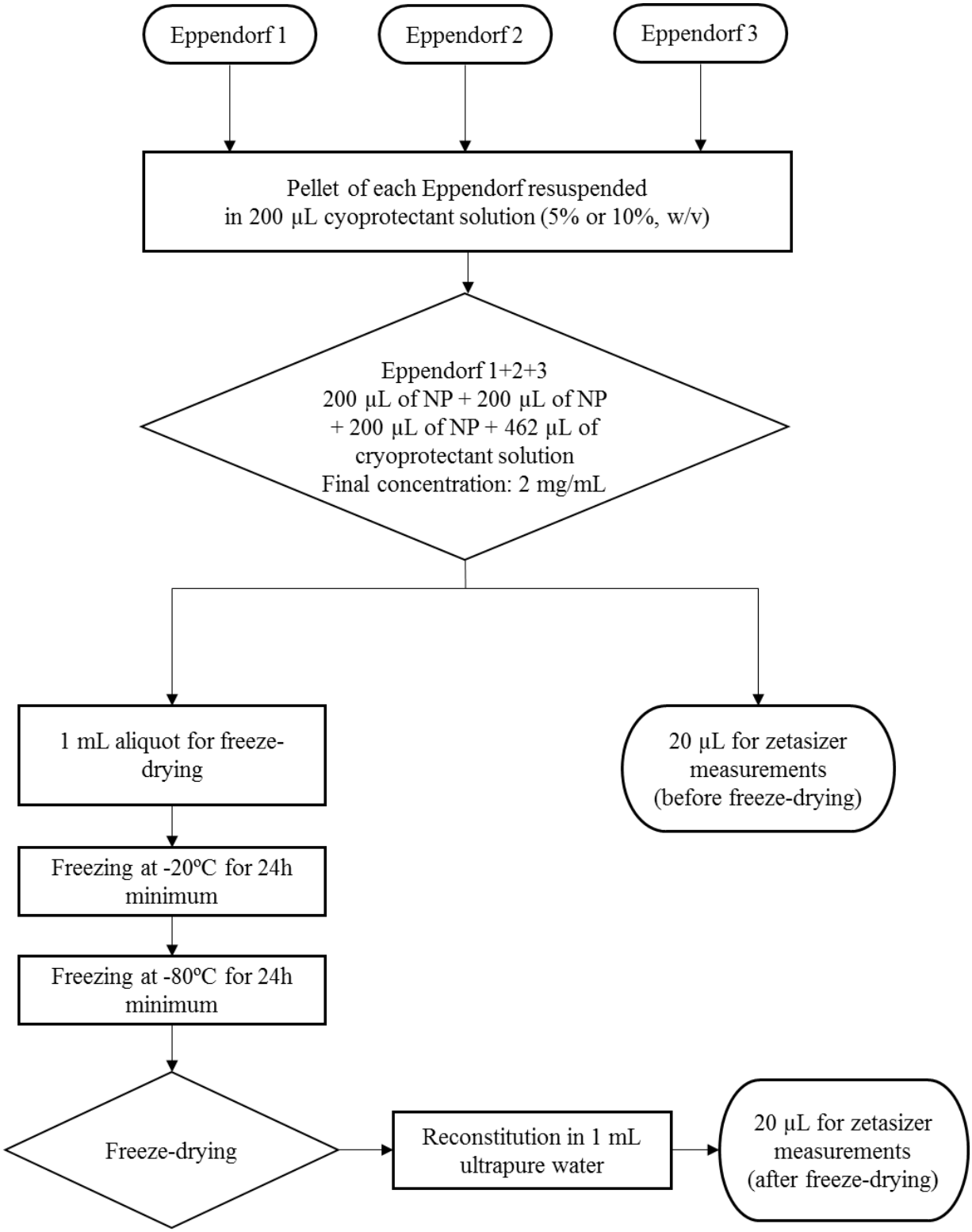
Annex C1.5 – Analysis of variance table for size ratio. "Cryo_Conc" refers to the concentration of carbohydrate used on the freeze-drying process – 5% or 10%; "Cryoprotector" refers to the carbohydrate used on the process – glucose or sucrose.

Annex A: Flowcharts detailing the steps required for the production of nanosuspensions

Annex A1.1 – Flowchart for the preparation of 1 mg/mL nanoparticle suspensions.

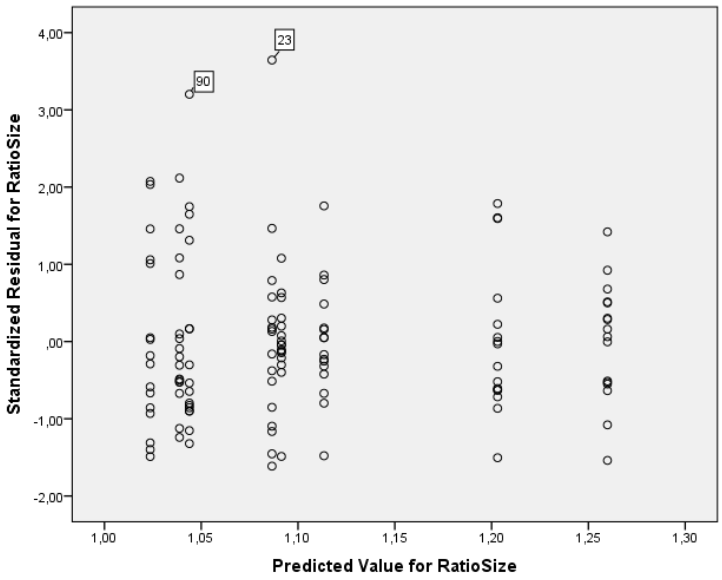


Annex A1.2 – Flowchart for the preparation of 2 mg/mL nanoparticle suspensions.

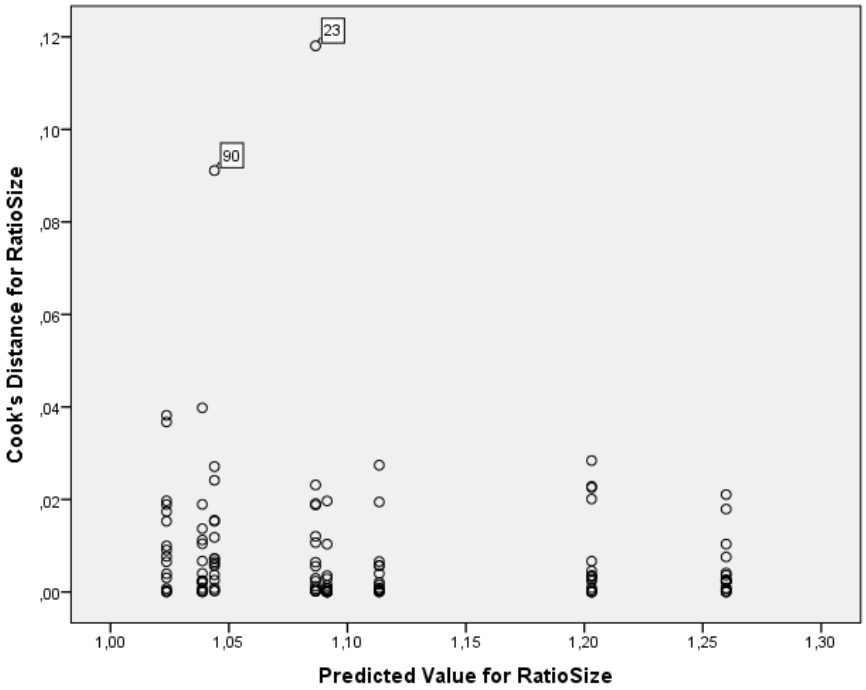


Annex B: Statistical study for the response variable "Size ratio"

Annex B1.1 – The standardized dispersion plot from which two outliers were detected, #23 (2.278) and #90 (2.091).



Annex B1.2 – The Cook's distance dispersion plot where two influential points can be detected. Since there is not any Cook's distance bigger than 1, there are no influential observations among the data.



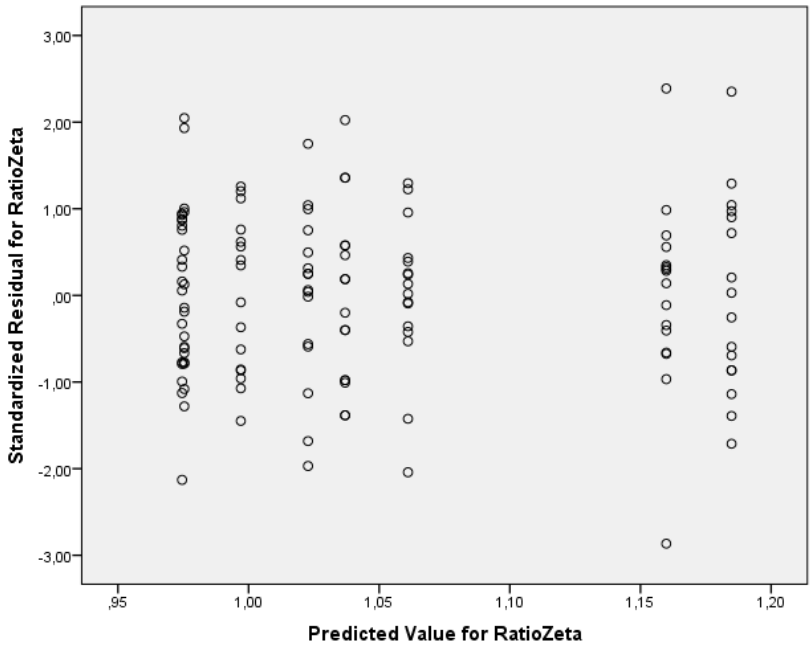
Annex B1.3 – Analysis of variance table for size ratio. "Cryo_Conc" refers to the concentration of carbohydrate used on the freeze-drying process – 5% or 10%; "NP_Conc" refers to the concentration of nanoparticles on the sample that was submitted to freeze-drying – 1 or 2 mg/mL; "Cryoprotector" refers to the carbohydrate used on the process – glucose or sucrose.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Cryo_Conc	,008	1	,008	,073	,788
NP_Conc	,213	1	,213	1,990	,161
Cryoprotetor	,451	1	,451	4,220	,042*
Cryo_Conc * NP_Conc	,037	1	,037	,349	,556
Cryo_Conc * Cryoprotetor	,000	1	,000	,001	,975
NP_Conc * Cryoprotetor	,072	1	,072	,676	,413
Cryo_Conc * NP_Conc * Cryoprotetor	,001	1	,001	,008	,928
Error	12,830	120	,107		

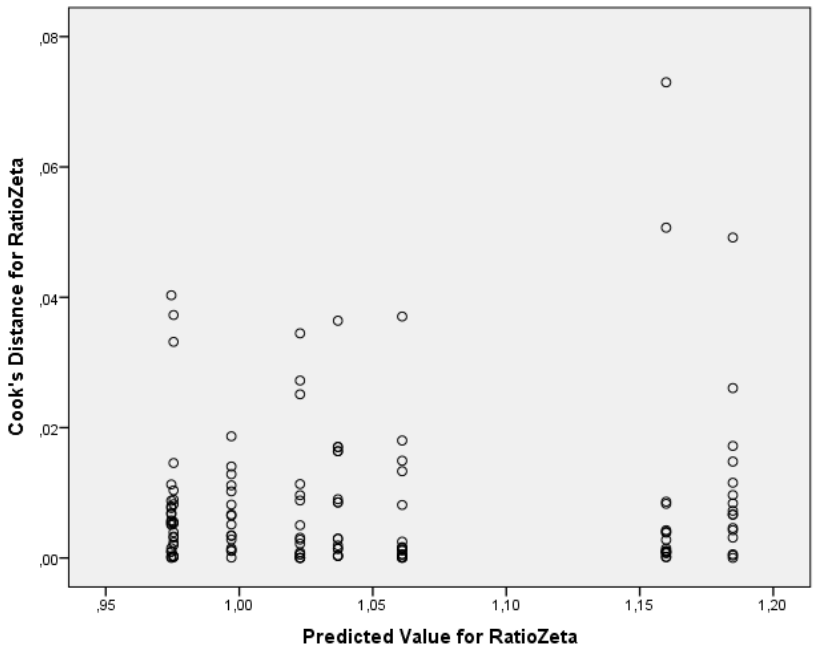
*statistically significant at 5%

Annex C: Statistical study for the response variable "Zeta potential ratio"

Annex C1.1 – The standardized dispersion plot.



Annex C1.2 – The Cook's distance dispersion plot where the values are within the values considered for cut-off.



Annex C1.3 - Analysis of variance table for size ratio. "Cryo_Conc" refers to the concentration of carbohydrate used on the freeze-drying process – 5% or 10%; "NP_Conc" refers to the concentration of nanoparticles on the sample that was submitted to freeze-drying – 1 or 2 mg/mL; "Cryoprotector" refers to the carbohydrate used on the process – glucose or sucrose.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Cryo_Conc	.149	1	.149	5.920	.016*
Cryoprotetor	.242	1	.242	9.603	.002*
NP_Conc	.044	1	.044	1.754	.188
Cryo_Conc * Cryoprotetor	.238	1	.238	9.429	.003*
Cryo_Conc * NP_Conc	.022	1	.022	.857	.357
Cryoprotetor * NP_Conc	.001	1	.001	.053	.819
Cryo_Conc * Cryoprotetor * NP_Conc	.028	1	.028	1.120	.292
Error	3.030	120	.025		

*statistically significant at 5%

Annex C1.4 – The number of observations per treatment for the 2^2 factorial design.

		Cryo_Conc	
		5%	**10%**
Cryoprotector	**Glucose**	32	32
	Sucrose	32	32

Annex C1.5 – Analysis of variance table for size ratio. "Cryo_Conc" refers to the concentration of carbohydrate used on the freeze-drying process – 5% or 10%; "Cryoprotector" refers to the carbohydrate used on the process – glucose or sucrose.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Cryo_Conc	.149	1	.149	5.930	.016
Cryoprotetor	.242	1	.242	9.620	.002
Cryo_Conc * Cryoprotetor	.238	1	.238	9.445	.003
Error	3.125	124	.025		