Sofia dos Santos Carvalho

DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN BASED NANOPARTICLES FOR FUTURE APPLICATIONS AS DELIVERY SYSTEMS

Monografia realizada no âmbito da unidade Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientado pela Professora Dra. Olga Borges, e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Julho de 2015

UNIVERSIDADE DE COIMBRA

Eu, Sofia dos Santos Carvalho, estudante do Mestrado Integrado em Ciências Farmacêuticas, com o nº 2009010814, declaro assumir toda a responsabilidade pelo conteúdo da Monografia apresentada à Faculdade de Farmácia da Universidade de Coimbra, no âmbito da unidade curricular de Estágio Curricular.

Mais declaro que este é um trabalho original e que toda e qualquer afirmação ou expressão, por mim utilizada, está referenciada na Bibliografia desta Monografia, segundo os critérios bibliográficos legalmente estabelecidos, salvaguardando sempre os Direitos de Autor, à exceção das minhas opiniões pessoais.

Coimbra, 10 de Julho de 2015.

_____

O Professor Orientador

(Olga Maria Fernandes Borges Ribeiro)

O Aluno

(Sofia dos Santos Carvalho)

Agradecimentos

Aos meus pais, a quem deixo o maior agradecimento! Que me deram as bases para ser a pessoa que hoje me orgulho ser, e que me irá permitir ser uma profissional com princípios e valores, dignos da classe farmacêutica. Pelo vosso sacrifício, pelo apoio incondicional que me deram desde o início, e pelo amor que nunca me faltou, e me deu força em tantas alturas, o meu maior OBRIGADA!

À minha irmã Ana Luísa, por me obrigar a ser um exemplo para ela, e a dar sempre o melhor de mim, por ser a minha melhor amiga, e a minha parceira de macacadas: manas FiNi!

Aos meus avós, maternos e paternos, pelo carinho, por cuidarem de mim, e por verem sempre o melhor em mim!

A todos os meus tios por viverem esta aventura comigo desde o início! E aos meus primos que atrás de mim seguem nesta vida de estudante!
À restante família!

Às amizades de infância, que me continuaram a apoiar em todo o caminho, provando que o tempo conserva!

Às minhas afilhadas Alex, Andreia e Rita, por me ensinarem o que é cuidar de alguém, e sentir este carinho incondicional. Vão estar sempre no meu coraçãozinho!

Aos meus colegas de curso, e àqueles que já são meus amigos para a vida, a maior recompensa de aqui ter passado.

À UFS por me ter feito sentir em casa, quando estava longe dela, por me ter ensinado o que é trabalhar com paixão, e pela amizade!
A toda a equipa da Farmácia do Fórum, pela disponibilidade, amizade e aprendizagem!

À Prof. Olga Borges por num determinado dia me ter deixado iniciar o estágio no laboratório, e ter iniciado o trabalho que culminou nesta monografia. À Sandra e Edna, por terem sido incansáveis, e nunca terem desistido de mim, o resultado está à vista!

A todos os que, de 2009 a 2015, se cruzaram comigo e fizeram desta a maior aventura de todas, a mais gratificante e recompensadora, e que mais vai deixar saudades. Valeu mais que a pena!

A Coimbra, a cidade que me viu nascer e crescer.

MUITO OBRIGADA!

Index

1. Abstract/Resumo

Abstract

Mucosal vaccination, unlike systemic, is non-invasive, allows self-administration, diminishing associated costs, and have better patient compliance. Alongside, nanocarriers have been studied more intensively and its properties and applicability to vaccine field are promising. Therefore, series of molecules have been tested for this purpose, and there are a few that stood out, for instance chitosan. This can react with a great variety of molecules, and so casein was selected as a promising cross-link to this polymer.

Therefore, we optimize this NP's production and characterized them, and then they were compared to NP's made of chitosan and TPP.

Thus we combine the properties of these two, hoping they can help turning vaccination a safer, more comfortable and specific delivery system.

Resumo

Ao contrário da vacinação per *si*, a vacinação ao nível das mucosas, não é invasiva, permitindo que o doente administre a si próprio, o que reduz custos, e favorece uma melhor *compliance*. Paralelamente, os *nanocarriers* têm sido cada vez mais estudados, sendo as suas propriedades e aplicabilidade, no âmbito da vacinação, é promissora. Assim, inúmeras moléculas têm sido estudadas neste sentido, e algumas destacaram-se, nomeadamente o Quitosano. Este reage com diversas moléculas, tendo sido escolhida a Caseína como *cross-link* deste polímero.

Por conseguinte, otimizou-se a produção de nanopartículas e procedeu-se à sua caracterização, tendo sido ainda comparadas com nanopartículas de Quitosano e TPP.

Deste modo é possível combinar as propriedades das duas moléculas, e tornar a vacinação um *delivery system* mais seguro, confortável e específico.

2. Abbreviations

BALT – Bronchus Associate Lymphoid Tissue

BCA - Biocinchoninic Acid

Chi – Chitosan

Da – Dalton

DD – Deacetylation Degree

GALT – Gut Associate Lymphoid Tissue

GI - Gastrointestinal

IgA - Immunoglobulin A

MALT - Mucosal Associate Lymphoid Tissue

M-Cells: Microfold Cells

NAG - N-Acetyl-D-Glucosamine

NALT – Nasal Associate Lymphoid Tissue

NP – Nanoparticle

PP - Peyer's Patches

TPP – Penta-Triphosphate

α-cas – α-casein

3. Introduction

3.1. Mucosal Vaccination

Generally, when referring about vaccination, invasive injections, trained personnel and low patient compliance pop up.

Mucosal vaccination, unlike systemic, is non-invasive, allows self-administration diminishing associated costs, and have better patient compliance. Nevertheless, the greatest advantage of mucosal vaccine delivery is the ability to neutralize pathogens at the moment they enter the human body (Lubben, Van der *et al.*, 2001).

The mucosal immune system has three main functions: to protect mucus layer against invasion and colonization by microorganisms, prevent uptake of foreign proteins , and avoid potential harmful immune responses against these (Holmgren e Czerkinsky, 2005).

Most of the pathogens enter the human body through mucosal surfaces, after they been ingested or inhaled. More specifically, depending on the entrance site, uptake is normally performed by cells of the associated lymphoid tissue from the gut (GALT), nasal mucosa (NALT), bronchus (BALT), and others.

The Peyer's patches (PPs) contains lymphocytes, macrophages, dendritic cells and some plasma cells serving as the principal GALT inductive site (Holmgren e Czerkinsky, 2005). In the lumen side of the Peyer's patch, we can find Microfold cells (M-cells), specialized epithelial cells of the MALT, wich are responsible for the uptake of viruses, bacteria, toxins and other particles smaller than 10 μm (Holmgren e Czerkinsky, 2005). When it happens, and after antigen is processed by antigen presenting cells (APC's), the cells leave the Peyer's patch and go to lymph nodes, through lymphatic vessels, where they present antigen to T-cells. Imature antibody-producing cells migrate to specific mucosal sites, mainly the mucosa of origin, so they can differentiate into memory or effector cells (Holmgren e Czerkinsky, 2005). Thus they can reach distant mucosal sites, to clonally expand and mature into IgA plasma cells.

The main challenge of mucosal vaccines is to increase the retention time at the mucosa surface, and target the antigens to M-cells (Lebre *et al.*, 2012) leading to higher immune response, and therefore, IgA production (Lubben, Van der *et al.*, 2001).

The adaptive humoral immune response of mucosa comprehends the production of secretory IgA, which is predominant in human external secretions. these antibodies are the most suited defense for mucosa because of its resistance to proteases, the large amount produced, the ability to inhibit bacterial adhesion, and to neutralize viruses and bacterial toxins (Holmgren e Czerkinsky, 2005).

In oral vaccination, antigens need to be protected against degradation due to the low pH values of the stomach and enzymes of the gastrointestinal (GI) tract, and theirs uptake by PPs must be increased. Therefore, to achieve a good immune response, antigens need to be co-administered with adjuvants or encapsulated in particles. The incorporation of vaccines in particulate systems, avoids degradation and target them to M-cells (Lubben, Van der *et al.*, 2001).

In contrast to oral administration of vaccines, nasal administration have the advantage of going through a smaller distance, not being exposed to low pH values or degrading enzymes, and only remain about 15 minutes in the nasal cavity (Lubben, Van der *et al.*, 2001).

In the last years, the interest in nanocarriers, as an antigen delivery system, has grown dramatically and a great variety of options have shown good results, especially biodegradable polymeric material-based particles.

On this regard, chitosan have shown huge potential as an answer to a large number of problems when it comes to mucoadhesion, immunogenicity, and safety, therefore profitability and effectiveness in mucosa (Lubben, Van der *et al.*, 2001).

On the other hand, protein-based nanoparticles are easily prepared and scaled up during manufacture, and also can interact differently with therapeutic compounds, protecting and targeting them to specific sites of action (Elzoghby, Samy e Elgindy, 2012).

3.2. Chitosan

Chitosan is a cationic polymer and generally presented as a homopolymer. Obtained by deacetylating chitin, by a process rarely complete, and so most commercial products tend to be a copolymer of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (NAG) (Anal *et al.*, 2008).

When referring to chitosan, it covers a large number of polymers, since they differ from each other for its N-deacetylation (40 % to 98 %) degree, and molecular weight (50 kDa to 2000kDa), that's why chitosan molecules can vary, and so its biological and chemical properties. Particle size, density, viscosity, degree of deacetylation, and molecular weight are important parameters of chitosan, influencing pharmaceutical formulations.

It has attracted attention as matrix for a sustained and controlled release system, since it possesses reactive functionalities, and it's easily degraded into non-toxic product by enzymes (Muzzarelli, 1977). These end products are amino sugars which are completely absorbed in the human body (Anal *et al.*, 2008)

Chitosan is insoluble in water and it's a weak base with a p*K*a=6.5, and therefore insoluble in neutral and alkaline pH values. It is partially soluble in acids, so when solubilized in diluted acid, the amine groups of the polymer become protonated resulting in a soluble and positively charged polysaccharide, with a high positive charge density (Anal *et al.*, 2008; Hejazi e Amiji, 2003).

It has a highly number of applications, due to its abundance, and beneficial biological properties, like the fact it's a natural polymer (chitin is the second most abundant polysaccharide in nature, found in the exoskeleton of crustacean, insects and fungi), biodegradable, and biocompatible; has a reasonable cost, it's safe and non-toxic, versatile, and that's why it has attracted so much attention, for its use (Sanford, 1990)

Adjuvants are substances which enhance the immune response elicited by antigens. Some chitosans have been shown to have an immune stimulating activity like increasing the accumulation and activation of macrophages and polymorphonuclear cells (Lubben, Van der *et al.*, 2001)

3.3. Casein

Casein is a protein found in cow milk, and has 4 subtypes, based on their molecular weight: α1, α2, β and k, in the following proportions 4:1:4:1. It can be used in the acid form with low aqueous solubility, or as sodium caseinate, soluble except near the isoelectric point. Food proteins have high nutritional value and functional properties like emulsification, gelation, foaming and water binding capacity (Elzoghby, Abo El-Fotoh e Elgindy, 2011).

Casein-based nanovehicles are good raw materials since they have the advantages of synthetic polymers, and they have good absorbability and low toxicity of the end products degraded, since they are metabolized *in vivo* by digestive enzymes into innocuous peptides (Elzoghby, Samy e Elgindy, 2012).

Casein as a natural product is biodegradable and biocompatible, inexpensive, non-toxic, and highly heat stable, since it has few secondary and tertiary structure, it also binds to ions and small molecules, has good surface-active and stabilizing proprieties (Elzoghby, Samy e Elgindy, 2012). It has a variety of possible drug loading mechanisms, and binding sites, but also the possibility of surface modification allowing specific drug targeting.

In addition, is an amphiphilic protein, presenting clear hydrophobic and hydrophilic domains (Elzoghby, Abo El-Fotoh e Elgindy, 2011). Its pH-responsive gel swelling behavior makes it a

great release system (Elzoghby, Samy e Elgindy, 2012). However, this protein may act as immunogenic/allergenic (Elzoghby, Abo El-Fotoh e Elgindy, 2011).

3.4. NP Chitosan/Casein

Previous studies showed that chitosan can interact with proteins and form soluble or insoluble complexes, depending on the pH, due to its reactive amino/hydroxyl groups (Anal *et al.*, 2008; Hejazi e Amiji, 2003).

However there are few studies about the interaction between casein and chitosan. It's known that at specific ratios of casein and chitosan and below the casein's isoelectric point (4.6), they have opposite charges and can form soluble nanocomplexes (Anal *et al.*, 2008; Elzoghby, Abo El-Fotoh e Elgindy, 2011). There is also a few studies that characterize chitosan-caseinate NPs (Anal *et al.*, 2008), and this was a starting point for our study, since we used the same method of production, but studying different variables and optimizing the process.

It's well known that chitosan can easily form micro- and nanoparticles with high loading capacity for various antigens, it's a great candidate for mucosal vaccination carrier system, since it enhance systemic and mucosal immune response after nasal delivery (Lubben, Van der *et al.*, 2001).

It was the need for more information that lead us to produce these NPs and characterize them, so we could add something more for the nanoparticle investigation.

Attending the fact that chitosan will behave differently according to its molecular weight and deacetylating degree, we produced different NP with different conditions, and with casein as crosslink. The main objective was to obtain a narrow size distribution, possibly peaked markedly below 500 nm, high and positive zeta potential, to provide both electrostatic stabilization and the possibility of depositing on the surface a negatively charged polyelectrolyte, (Nasti *et al.*, 2009), such as casein.

In the present study, it has been produced chitosan/casein nanoparticles, as a combination of each one's advantages: the mucoadhesive and immunogenic character of the chitosan, along with the targeting of the casein. (Elzoghby, Samy e Elgindy, 2012; Lebre *et al.*, 2012)

Therefore, different ratio of a specific chitosan and casein in the NP were used, and its physical and chemical properties were evaluated, and NP made of chitosan and Penta-Sodium Triphosphate (TPP) were used as control, since they were already been studied.

The main point of this study was to optimize the production conditions of NP made of chitosan and casein to be evaluated in future projects as a mucosal delivery system of recombinant antigens

.

4. Materials and Methods

4.1. Materials

Chitosan (ChitoClear™ - 95 % DD and 8 cP viscosity measured in 1 % solution in 1 % acetic acid) was purchased from Primex BioChemicals AS (Avaldsnes, Norway). Penta-Sodium Triphosphate (TPP) and α-Casein (αcas) from bovine milk were acquired from Sigma-Aldrich Corporation (St Louis, MO, USA). Pierce™ Biocinchoninic acid (BCA) Protein Assay Kit was purchased from Pierce Chemical Company (IL, USA). All other chemicals and reagents were analytical grade.

4.2. Methods

4.2.1. Nanoparticle Production

Two types of chitosan (Chi) based nanoparticles (NP) were produced using the following method. For Chi-TPP NPs production different volumes of 0.16 % w/v TPP solution were added dropwise to 2 mL chitosan solution (0.1 % w/v in 25 mM sodium acetate buffer, pH=5.0) under high speed vortexing for one minute, and further maturation for half hour. The same methodology was applied to Chi-αcas NP using different volumes of a 0.167 % or a 0.4 % α-Casein solution. The different conditions tested are presented on table 1.

To remove non-reacting compounds the resultant suspension was centrifuged for 10 min at 21000 g and the supernatant was discarded. The resultant pellet was re-suspended in 2 ml of deionized water.

Table 1: Conditions tested for Chi-TPP and Chi- αcas NPs production

Formulation	Chi solution (0.1 %)	TPP solution (0.16 %)	Chi:TPP ratio (µg:µg)	Formulation	Chi solution (0.1 %)	αcas solution (0.167 %)	αcas solution (0.4 %)	Chi:αcas ratio (µg:µg)
Chi-TPP 300	2 ml	300 µl	1:0.24	Chi-αcas 300	2 ml	300 µl	-	1:0.25
Chi-TPP 350	2 ml	350 µl	1:0.28	Chi-αcas 350	2 ml	350 µl	-	1:0.29
Chi-TPP 400	2 ml	400 µl	1:0.32	Chi-αcas 400	2 ml	400 µl	-	1:0.33
Chi-TPP 450	2 ml	450 µl	1:0.36	Chi-αcas 450	2 ml	450 µl	-	1:0.38
Chi-TPP 500	2 ml	500 µl	1:0.40	Chi-αcas 500	2 ml	500 µl	-	1:0.42
Chi-TPP 550	2 ml	550 µl	1:0.44	Chi-αcas 550	2 ml	550 µl	-	1:0.46
Chi-TPP 600	2 ml	600 µl	1:0.48	Chi-αcas 600	2 ml	600 µl	-	1:0,50
-	-	-	-	Chi-αcas 300	2 ml	-	300 µl	1:0.60
-	-	-	-	Chi-αcas 600	2 ml	-	600 µl	1:1.20

4.2.2. Nanoparticle Characterization

i. Size and Zeta Potential

Delsa™ Nano C particle analyzer (Beckman Coulter, CA, USA) was used to measure particle size by Dynamic Light Scattering (DLS) and zeta potential by electrophoretic light scattering (ELS). Both analyses were performed at 25 °C and particle size scattered light collected at a 165° angle. Particle suspensions were characterized after production.

ii. Transmittance

Transmittance of freshly prepared NPs (before centrifugation) was measured at 500 nm, relatively to a blank solution, using an UV-Visible 1603 Shimadzu spectrophotometer (Kyoto, Japan).

iii. Chitosan incorporation

Chitosan quantification was performed using a colorimetric determination, based on the ionic interaction between the protonated amino groups of chitosan and sulfonic acid groups on the anionic dye, Cibacron Brilliant Red®. A standard curve was prepared using chitosan, dissolved in a concentration of 0.1 % (w/v) in an acetic solution with good reproducibility and linearity in the range of 8–32 µg/mL (Mendelovits *et al.*, 2012; Muzzarelli, 1998; Wischke e Borchert, 2006).

Due to the method working range linearity, to quantify chitosan on the different NPs supernatants different volumes were optimized for measure. For all Chi-αcas NPs and for Chi-TPP300 and Chi-TPP350 NPs, 200 µL supernatants were used. For the other Chi-TPP NPs conditions 400 µL were used. These supernatants were mixed with 100 µL of Soerenson Glycin-HCl buffer (pH=2.8) and 1 mL of Cibacron reagent (75 µg/mL). All samples were diluted to a final volume of 5 mL with deionized water.

The mixture was incubated for 20 min and the optical density (OD) measured at 575 nm, relatively to a blank solution, using an UV-Visible 1603 Shimadzu spectrophotometer (Kyoto, Japan). Knowing chitosan concentration in NPs supernatants chitosan incorporation into NPs was calculated as the following equation (eq.1):

(eq.1)

$$\text{Chitosan Incorporation (\%)} = \frac{[\text{Total Chitosan}]-[\text{Supernatant Chitosan}]}{[\text{Total Chitosan}]} \times 100$$

iv. Protein adsorption

For protein adsorption, 500 µl of Chi-TPP NPs were centrifuged for 15 min at 5000 g and then re-suspended in 250 µl of deionized water. Then 250 µl of BSA concentration was added, the mixture was incubated under agitation for 30 min and then centrifuged again for 10 min at 21000 g for supernatants collection.

NP protein adsorption was evaluated by BCA protein assay, a colorimetric method, performed according to manufacturer's instructions. Briefly, 25 µl of supernatants were mixed with 200 µl of the working solution. Incubation for 30 min at 37 °C allowed for the development of a purple color according to protein content. A standard curve was prepared using dilutions of a BSA solution (2000 – 2 µg/mL) and tested simultaneously. Optical density (OD) of the samples was measured at 570 nm on a spectrophotometer plate reader.

v. Stability Test

The stability of Chi-TPP and Chi-αcas NPs was studied in different media, measuring the transmittance at different time points at 37 °C. The NPs were suspended in a proportion of 1:1 (v/v) in the following media: sodium Acetate Buffer (pH = 5), Simulated Gastric Fluid (SGF, pH = 1.2), Simulated Intestinal Fluid (SIF, pH = 6.8), Simulated Nasal Electrolyte Solution (SNES, pH = 6.8), Phosphate Buffered Saline (PBS) (pH = 7.4), RPMI cell culture media (pH = 8).

vi. Transmission electron microscopy (TEM)

Samples of Chi-αcas NPs were placed in a microscopy grid and observed under a FEI-Tecnai G2 Spirit Biotwin, a 20-120 kV transmission electron microscope (TEM) (FEI Company, OR, USA).

5. Results

5.1. Optimization of Chi/TPP NPs production conditions

Chi/TPP NPs were produced by the electrostatic interaction between the positively charged chitosan and the negatively charged TPP.

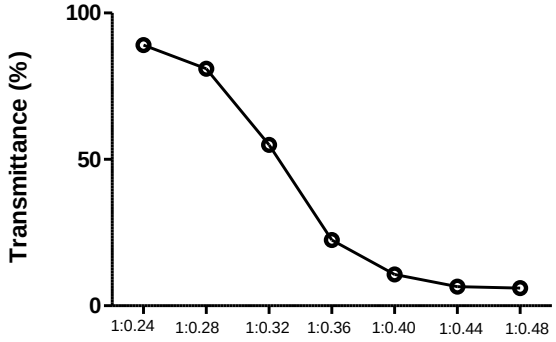


Figure 1: Titration of Chi-TPP NP suspension transmittance at 500 nm according to the NP Chi:TPP ratio (μg:μg) (see table 1 from methods). Results are expressed in percentage. (n=5)

In the first place, in order to optimize the best Chi:TPP ratio that leads to more NP production, transmittance of freshly prepared NP was measured at 500 nm, relatively to a blank solution.

The test ratios were selected by fixating chitosan's concentration, and varying TPP's concentration according to table 1 from methods. The results are presented in figure 1. Increasing the cross-link concentration (decreasing the Chi:TPP ratio), transmittance gets lower indicating a higher NPs production

But from ratio 1:0.36 on the values stabilize, suggesting no more increase in NP production possibly because all available chitosan was already incorporated in the formulation. Therefore, to sustain this observation we evaluated the percentage of chitosan relative to the original solution that is retained in the NPs. Moreover, we tested the protein binding capacity of the different formulations to assess the differences.

Knowing that different TPP concentrations produces different NPs formulations, 3 ratios were picked, with low, intermediate and high amounts of TPP in order to assess the resulting formulation characteristics.

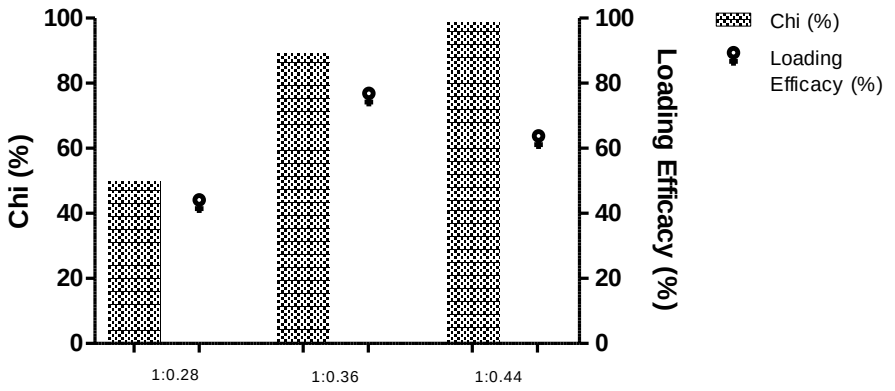
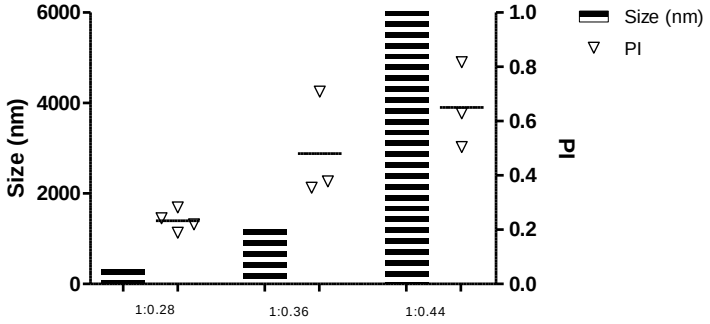


Figure 2: Titration of chi incorporation (left axis) and adsorbed casein (right axis) in Chi-TPP NP according to the NP Chi:TPP ratio (μg:μg) (see table 1 from methods). Results are expressed in percentage. (% chi, n=5) (% Loading Efficacy, n=2)

The amount of incorporated chitosan was measured using Cibacron method, the casein loading on particles was calculated with BCA protein assay and results are presented in figure 2. We have seen before bigger concentrations of TPP result in more NP produced, and now we confirmed that is related to a higher percentage of chitosan used from the initial solution. In order to use all chitosan and produce the maximum NP possible, higher amounts of TPP must be used as cross-link. The loading efficacy on its turn, increases from the first to the second ratio tested, meaning more NPs are adsorbing more casein, however, there is no difference from the second to the third. Considering the transmittance results to explain the differences in the loading efficacy we can see that from 1:0.36 to 1:0.44 the decreasing in transmittance is smaller than from 1:0.28 to 1:0.36. Therefore, the differences in the number of NPs are not enough to increase the loading efficacy of casein. These smaller differences in the number of NPs are also sustained by the percentage of chitosan that only increases about 10 % from the 1:0.36 to 1:0.44 ratio, while from the 1:0.28 to 1:036 the increase is around 40 %.

A)



B)

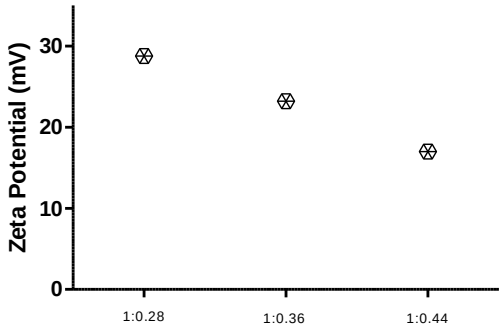


Figure 3: A) Titration of mean diameter (mm) and PI according to the NP Chi:TPP ratio (μg:μg) (see table 1 from methods). (n=1); B) Titration of Chi-TPP NP Zeta Potential (mV) according to the NP Chi:TPP ratio (μg:μg) (see table 1 from methods). (n=1)

Finally we characterized the formulations regarding its size and zeta potential and the results are presented in figure 3. Although the 1:1.028 Chi:TPP ratio is the one generating formulations with lower NPs concentration, it is the formulation with best size results. In fact, sizes are low (around 327 nm) and the polydispersity index (PI) is around 0.2, meaning sizes are homogeneous. On the other hand, the formulation with higher transmittance and more chitosan incorporated possibly presents aggregates that explain the high sizes verified (6000nm), and its high PI indicating lack of size homogeneity in the formulation.

Considering that TPP's charge is negative, it's reasonable to expect that by decreasing Chi:TPP ratio, and so increasing TPP's concentration, zeta potential decreases since it counteracts with the positive charge of chitosan. Indeed, with lower amounts of cross-link, zeta potential is +29 mV and with higher TPP concentrations it decreases to +17 mV.

5.2. Optimization of Chi/Casein NPs production conditions

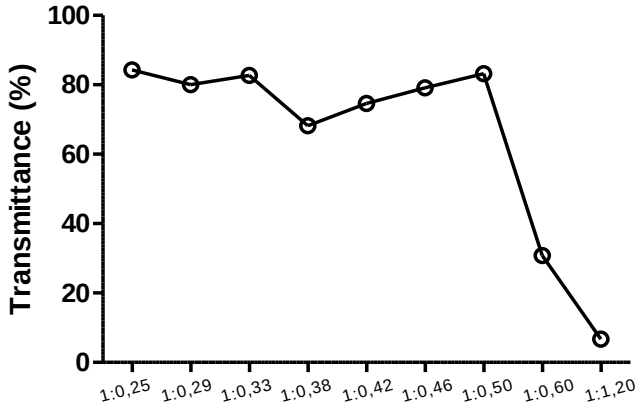


Figure 4: Titration of Chi-αcas NP suspension transmittance at 500 nm (right axis) according to the NP Chi:αcas ratio (µg:µg) (see table 1 from methods). Results are expressed in percentage. (n=1)

Similar studies as described for Chi:TPP NPs were performed on Chi:casein NPs. Once again, in order to optimize the best Chi:casein ratio that leads more NP production, transmittance of freshly prepared NP was measured at 500 nm, relatively to a blank solution.

Chitosan concentration was fixed, and casein concentration varied through production according to table 1 from methods. Transmittance of freshly prepared was then measured at 500 nm, relatively to a blank solution, and this was the result.

In figure 4 we can see that in the beginning as long as ratio diminishes with the higher amounts of casein, transmittance maintains stable around 80 % until the 1:0.50 ratio. From this ratio on transmittance diminishes markedly, possibly meaning more NPs are being produced.

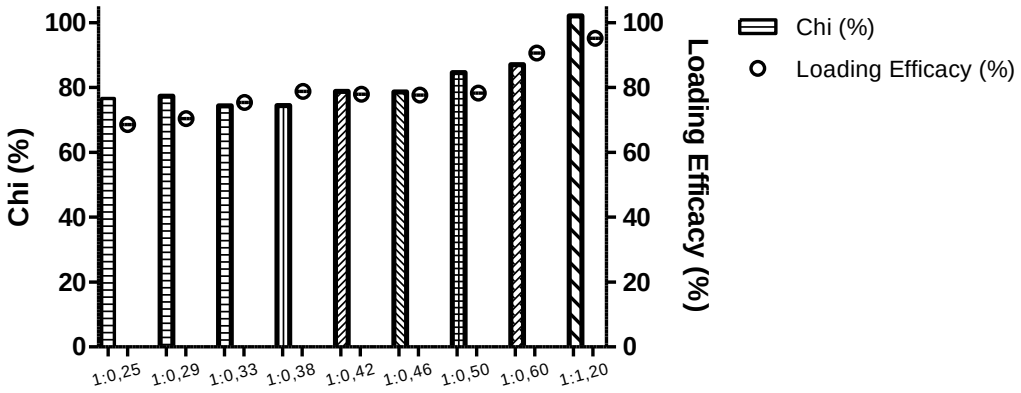


Figure 5: Titration of chi incorporation (left axis) and adsorbed casein (right axis) in Chi-αcas NP according to the NP Chi:αcas ratio (µg:µg) (see table 1 from methods). Results are expressed in percentage. (% chi, n=1) (% Loading Efficacy, n=3)

In figure 5 was evaluated not only how much chitosan was incorporated in NPs production relatively to the initial solution, but also how much casein was used for the NPs production. In fact, in this experiment casein was the cross-link essential for NPs generation.

Despite the great differences in transmittance for the 2 lower Chi:casein ratios compared to the others, the percentage of chitosan incorporated in all systems presented less visible changes. In fact, only the last ratio tested (1:1.20) presented a higher increase in chitosan incorporation reaching the maximum value, this is all the chitosan that was efficiently used for the NPs production. Regarding the casein loading efficacy, and keeping in mind it acts as the cross-link enabling NPs formation, results presented in figure 4 (right Y axis) were expected. A progressive increase in casein loading was achieved when increasing its concentration. However, we can assume that the increase in chitosan incorporation along with the increase in casein concentration (decreasing Chi:casein ratios) and the increase in casein incorporation, are responsible for the differences seen in the transmittance of the 2 lower ratios.

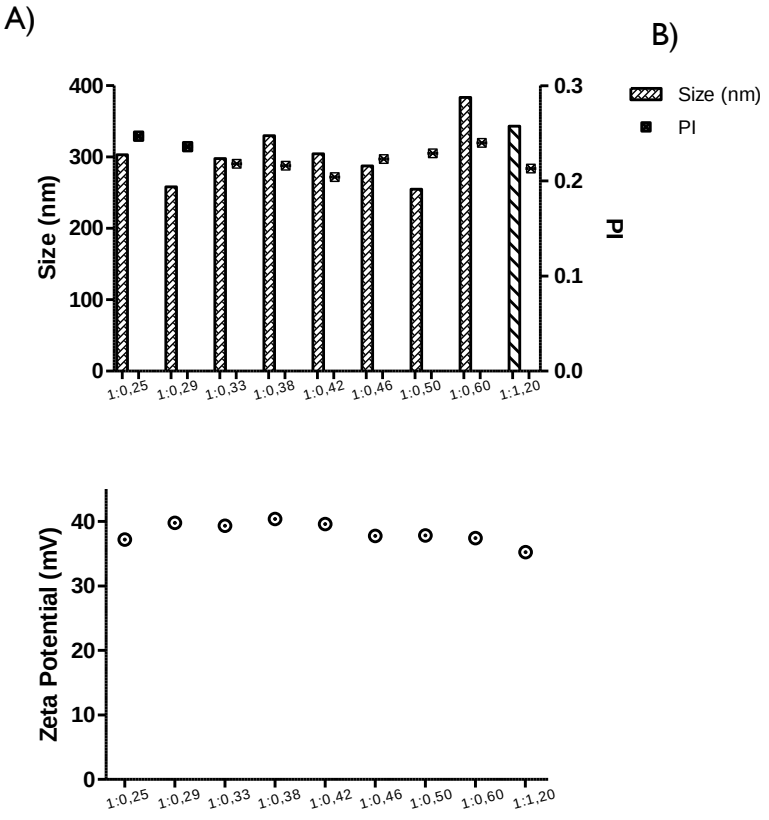


Figure 6: A) Titration of mean diameter (nm) and PI according to the NP Chi:αcas ratio (µg:µg) (see table 1 from methods). (n=1); B) Figure: Titration of Chi-αcas NP Zeta Potential (mV) according to the NP Chi:αcas ratio (µg:µg) (see table 1 from methods). Data (Mean ± SD) represent 3 measurements performed in one illustrative batch for each ratio tested.

As well as for Chi:TPP, we characterized the formulation regarding its size and zeta potential. Figure 6 shows the results: NPs have a more acceptable size (around 300 nm) and its PI show that they are all homogeneous regarding its size.

In comparison to TPP, casein presents a negative charge in the production media and it counteracts with the positive charge of chitosan. However, zeta potential values remain constant when increasing the casein concentration, and are highly positive, which contributes to their stability.

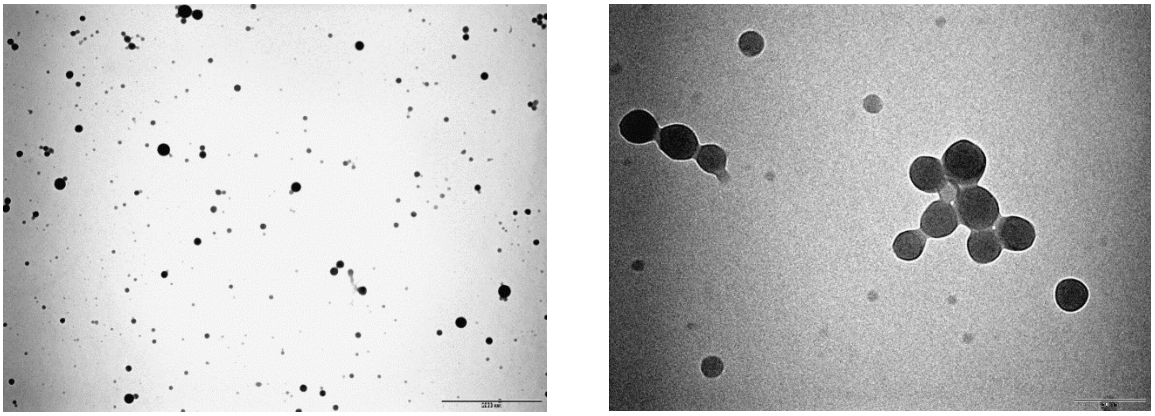


Figure 7: TEM of NP Chi:αcas (using a 2000 nm and 200 nm scale, respectively)

Figure 7 confirms the values presented for NP Chi:αcas size, using TEM technique.

5.3. Differences between Chi:cas NPs and Chi:TPP NPs

As said before, Chi:TPP NPs are well known, and the characteristics found in them matching with other studies, like size rounding 300 nm to400 nm and a positive surface charge ranging from + 54 mV to + 25 mV (Fernández-Urrusuno *et al.*, 1999).

When talking about NP Chi:αcas, we reached the same NP's size as reported from Anal and co-workers (Anal *et al.*, 2008), with values around 200 nm to 300 nm when using 0.1 % chitosan concentration. But since the aim of this study was to get more information about this delivery system, more parameters were evaluated, like loading efficacy, and NP stability capacity in different media.

Comparing Chi:αcas NPs with Chi:TPP NPs the former had smaller sizes with an homogenous distribution for all ratios tested, while Chi:TPP NPs presented bigger sizes increasing with the increasing concentration of cross-link and a high polydispersity.

Apparently Chi:cas NP present good characteristics for vaccine-field applications.

5.4. Stability Test

Stability studies in different media were performed with both NPs in order to evaluate their behavior which can limit future applications. Indeed, by testing the NPs stability in simulated biological fluids we will determine for example if the NPs constitute a promisor delivery system for the oral (simulated gastric fluid) or nasal (simulated nasal fluid) routes. Moreover, testing its stability in cell culture media (RPMI) we can assess if further in vitro studies with cell cultures are feasible

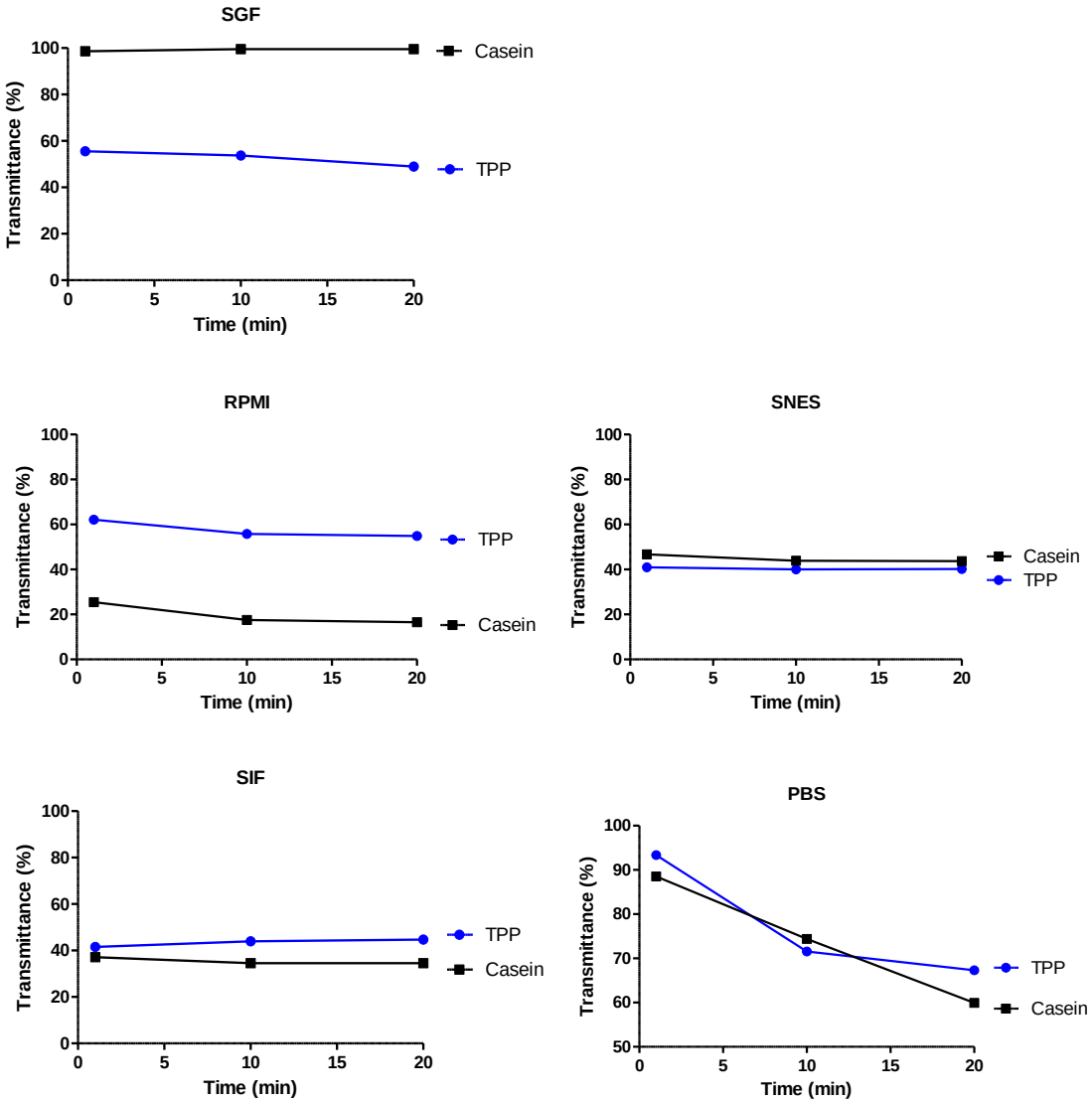


Figure 7: Figure: Stability assessment of 1:1.20 Chi-αcas NP and 1:0.24 Chi-TPP NP in different media, at different time points. Measurements of the transmittance at 500 nm at each condition are expressed in percentage. (n=2 and n=3, respectively)

Considering figure 7, in acetate buffer (the production media7), SIF and SNES, both NPs are stable since they keep, approximately, the same transmittance through time, and they round about 40 % both Chi-αcas NP and Chi-TPP NP.

In PBS, the transmittance decreases significantly as a function of time. Those values most likely corresponds NP physical instability, observed immediately after the first contact with PBS. Following this first moment, the particles tend to reorganizing themselves differently and new particles appeared justifying the decrease of transmittance values.

In SGF, Chi-αcas NP, were not physical stable and the polymers dissolve. This phenomenon is observed throughout transmittance measurement that became near 100 %. On the other hand, Chi-TPP NP looks more stable and presenting a transmittance value near 50 %.

In the cell culture medium RPMI, Chi-TPP NP look stable, but Chi-αcas NP present low transmittance, meaning they may form different and more particles indicating that both particles would be appropriate to be used in further in vitro studies using RPMI.

In terms of future application, state that in RPMI, Chi-αcas NPs present better values and stability. In Acetate Buffer, SIF and SNES, none of them outstands, since they behave similarly, but in SGF, Chi-TPP NP seems to be the delivery system more stable with a good transmittance.

6. Conclusion

It's no news that science is evolving every day, and it happens because of the challenges that our body imposes us. So efforts need to be made to find the most adequate answer, fight illness.

That's why not only the API is important, but also its delivery system: it doesn't mean a thing, if it can't get there.

Nanoparticles showed promising results and therefore the next challenge is to find the best compounds that can promote targeting and so API goes to where it's supposed, and adverse events get diminished.

In mucosal vaccination, chitosan proprieties are already well known, so we synthetized NP made of this polymer and we used casein as a cross-link. Since there isn't much information, it was thought that this study could be an addition to the nanoparticle world.

Therefore, we optimize this NP's production and characterized them, and then compared them to NP made of chitosan and TPP.

We can accomplish that these NP have good physical and chemical properties, and since they are stable in RPMI further in vitro studies with cell cultures are feasible, so eventually we could go forward with other studies.

Since the starting point was mucosal vaccination, its size, surface charge, and loading capacity looked promising, and its good stability in SNES medium, confirmed that this could be a delivery system appropriate for nasal vaccination.

7. References

ANAL, Anil Kumar *et al.* - Preparation and characterization of nanoparticles formed by chitosan-caseinate interactions. **Colloids and Surfaces B: Biointerfaces**. . ISSN 09277765. 64:1 (2008) 104–110. doi: 10.1016/j.colsurfb.2008.01.010.

ELZOGHBY, Ahmed O.; ABO EL-FOTOH, Wael S.; ELGINDY, Nazik A. - Casein-based formulations as promising controlled release drug delivery systems. **Journal of Controlled Release**. . ISSN 01683659. 153:3 (2011) 206–216. doi: 10.1016/j.jconrel.2011.02.010.

ELZOGHBY, Ahmed O.; SAMY, Wael M.; ELGINDY, Nazik A. - Protein-based nanocarriers as promising drug and gene delivery systems. **Journal of Controlled Release**. . ISSN 01683659. 161:1 (2012) 38–49. doi: 10.1016/j.jconrel.2012.04.036.

FERNÁNDEZ-URRUSUNO, Rocío *et al.* - Enhancement of nasal absorption of insulin using chitosan nanoparticles. **Pharmaceutical Research**. . ISSN 07248741. 16:10 (1999) 1576–1581. doi: 10.1023/A:1018908705446.

HEJAZI, Radi; AMIJI, Mansoor - Chitosan-based gastrointestinal delivery systems. 89:2003) 151–165.

HOLMGREN, Jan; CZERKINSKY, Cecil - Mucosal immunity and vaccines. **Nature medicine**. . ISSN 1078-8956. 11:4 Suppl (2005) S45–S53. doi: 10.1038/nm1213.

LEBRE, Filipa *et al.* - Chitosan-Based Nanoparticles as a Hepatitis B Antigen Delivery System. **Methods in Enzymology**. . ISSN 00766879. 509:2012) 127–142. doi: 10.1016/B978-0-12-391858-1.00007-1.

LUBBEN, I. M. VAN DER *et al.* - Chitosan for mucosal vaccination. **Advanced Drug Delivery Reviews**. . ISSN 0169409X. 52:2 (2001) 139–144. doi: 10.1016/S0169-409X(01)00197-1.

MENDELOVITS, Asaf *et al.* - Improved colorimetric determination of chitosan concentrations by dye binding. **Applied Spectroscopy**. . ISSN 00037028. 66:8 (2012) 979–982. doi: 10.1366/12-06591.

MUZZARELLI, R. - **Chitin**. [S.l.] : Pergamon Press, 1977

MUZZARELLI, R. A - Colorimetric determination of chitosan. **Analytical biochemistry**. . ISSN 0003-2697. 260:2 (1998) 255–257. doi: 10.1006/abio.1998.2705.

NASTI, Alessandro *et al.* - Chitosan/TPP and chitosan/TPP-hyaluronic acid nanoparticles: Systematic optimisation of the preparative process and preliminary biological evaluation. **Pharmaceutical Research**. . ISSN 07248741. 26:8 (2009) 1918–1930. doi: 10.1007/s11095-009-9908-0.

SANFORD, PA - Chitosan and alginate: new forms of commercial interest. 31:1990) 628–9.

WISCHKE, Christian; BORCHERT, Hans Hubert - Increased sensitivity of chitosan determination by a dye binding method. **Carbohydrate Research**. . ISSN 00086215. 341:18 (2006) 2978–2979. doi: 10.1016/j.carres.2006.10.012.