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Inulin Particles as a Vaccine Adjuvant for Hepatitis B

Development and in Vitro Evaluation of Their Immunotoxicity

Dissertação no âmbito do Mestrado em Biotecnologia Farmacêutica orientada pela Professora Doutora Olga Maria Fernandes Borges Ribeiro e co-orientada pela Doutora Sandra Cristina Campos de Jesus e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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Candidature thesis for master degree in Pharmaceutical Biotechnology, submitted to the Faculty of Pharmacy of the University of Coimbra

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List of Abbreviations

AHB	Acute HBV
AI-1	Alpha-Inulin 1
AI-2	Alpha-Inulin 2
ALT	Alanine Aminotransferase serum
CHB	Chronic HBV
DC	Dendritic cells
DDA	Deacetylation degree
DI	Delta-Inulin
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EC	Entrapment Capacity
EE	Entrapment Efficacy
EI	Epsilon-Inulin
ELISA	Enzyme-linked immunosorbent assay
ELS	Electrophoretic light scattering
EMA	European medicines agency
FDA	Food and Drug Administration
GI	Gamma-Inulin
HBsAg	Hepatitis B surface antigens
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
IFN therapy	Interferon-based therapy
IL-	Interleukin

INW	Inulin not washed
IW	Inulin washed
LC	Loading Capacity
LE	Loaded Efficacy
LYS	Lysozyme
MYO	Myoglobin
NED	N-1-napthylethylenediamine dihydrochloride
NO	Nitric Oxide
NP	Nanoparticle
OD	Optical density
OI	Omega-Inulin
PBS	Phosphate buffer saline
PDI	Polydispersity index
PINW	PCL-Inulin not washed
PIW	PCL-Inulin washed
PNW	PCL not washed
PW	PCL washed
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization
ZI	Zeta-Inulin

Abstract

Hepatitis B virus (HBV) is an enveloped DNA virus associated with the *Hepadnaviridae* family [1], being highly hepatotrophic and species-specific, meaning that HBV replicates exclusively in human hepatocytes [2]. HBV infection can occur early in life, generally leading to chronic infection if not treated correctly. If HBV infection occurs later, an acute self-resolving infection might take place [5].

To prevent HBV infection, there is currently a prophylactic vaccine, that consists in 3 or 4 doses (depending on the individual geographic region) of the injectable HBV surface antigen (HBsAg) adjuvanted with aluminum salts [3]. Despite the effective vaccine available, HBV prevalence is still a serious global health problem [12], thus, eliminating hepatitis is a major goal in the United Nations 2030 Agenda.

Although the future looks prospective, two main problems constitute a threat to the eradication of HBV, with the first problem being the need to improve vaccination in developing countries, and the second the non-existence of a therapeutic vaccine to be used in chronic patients [45;46;56-58]. A way of trying to solve these problems is the use of nanotechnology to design vaccine adjuvants, since it allows to obtain nanoparticles with different compositions, sizes, and surface properties, which help to increase antigen stability, immunogenicity, and allow targeted delivery [61-63].

Delta inulin isoform (called Advax ™) is a polysaccharide comprised of fructose units, that has been tested in clinical trials with the HBV vaccine. [66]. One of the important qualities of inulin shown in animal studies, was the enhancement in the adaptive immune response, which was confirmed against a wide variety of viral and bacterial antigens, like influenza [84;87], Japanese encephalitis [86;90;112], HIV [91], and others.

Therefore, this master dissertation aims to develop and produce Inulin particles blended or not with other polymers, previously studied for vaccine adjuvant purposes, such as Polycaprolactone (PCL) or Chitosan.

The production method developed and optimized was based on nanoprecipitation techniques and the particles were characterized regarding their physicochemical and immune-toxicological characteristics.

Three main delivery systems were developed: inulin particles, PCL-inulin particles and PCL particles. While PCL-inulin and PCL particles had sizes of 301.26 nm and 275.32 nm

respectively, inulin particles, were much bigger, and out of the nanoscale (1320.86 nm). The size measurement results allowed us to infer that the particles were stable for 42 days, with the exception of Inulin based particles that present a tendency to increase their size. In terms of immunotoxicity, Inulin and PCL-Inulin particles have shown no toxicity in primary murine spleen cells, although in RAW 264.7 cell line, the results were a little different, as PCL-Inulin particles have shown some toxicity in higher concentrations, which was the same observed in PCL particles. Considering the nitric oxide (NO) and cytokine production studies, the particles did not induce NO production when incubated with RAW 264.7 cell line nor GM-CSF production.

Considering the TNF-α, IL-6, IL-10, and IL-12 production by DCs incubated with different particles, the results revealed that the PCL-Inulin at 2 μg/mL does not induce the production of significant cytokine levels. However, at 4 μg/mL concentration, we observed a small induction of the production of the IL-12 and IL-10.

Overall, we reported a successful development and production of Inulin particles blended with Polycaprolactone polymer, which preliminary results suggest that they are suitable for use as antigen delivery systems, with no expectable toxicity associated. However further studies are required to confirm the results obtained for cytokine production with DCs since donor variability was significant. Ultimately, *in vivo* studies in mice using HBV antigens with the developed Inulin and PCL-Inulin particles, should elucidate about the real ability of these formulations as a strategy to improve HBV vaccination.

Keyword: Hepatitis B Virus, HBV Vaccine, Inulin NPs, PCL/inulin NPs.

Resumo

O vírus da Hepatite B (HBV) é um vírus de DNA com envelope associado à família *Hepadnaviridae* [1], o qual é altamente hepatotrófico e espécie-específico, ou seja, replica-se exclusivamente em hepatócitos humanos [2]. A infeção por HBV pode ocorrer no início da vida, o que geralmente leva a uma infeção crónica, caso não seja tratada corretamente. Caso a infeção por HBV ocorra mais tarde, geralmente resulta numa infeção aguda que tem a capacidade de se auto-resolver [5].

Atualmente, para prevenir a infeção pelo HBV existe uma vacina injectável profilática, que consiste em 3 ou 4 doses (dependendo da região geográfica do indivíduo) contendo o antigénio de superfície do HBV (HBsAg) combinado com sais de alumínio. [3]. Apesar da vacina disponível ser eficaz, a prevalência do HBV ainda é um sério problema de saúde pública [12], razão pela qual eliminar a hepatite é uma das principais metas inscrita na Agenda 2030 das Nações Unidas.

Embora o futuro pareça promissor, existem dois problemas que constituem uma ameaça à erradicação do HBV, sendo o primeiro a necessidade de melhorar a vacinação nos países em desenvolvimento e o segundo a inexistência de uma vacina terapêutica para uso em doentes crónicos [45;46;56-58]. Uma forma de tentar resolver estes problemas é o uso da nanotecnologia como ferramenta para o desenvolvimento de adjuvantes de vacinas, uma vez que esta permite "desenhar" as nanopartículas com diversas composições, tamanhos e propriedades de superfície. A inclusão do antigénio em nanopartículas ajuda a aumentar a estabilidade do antigénio, a imunogenicidade e permite a entrega direcionada deste mesmo antigénio a células apresentadoras de antigénio [61-63].

A delta inulina (denominada Advax ™) um polissacarídeo composto por unidades de frutose, tem vindo a ser testada em ensaios clínicos para uso na vacina contra o HBV [66]. Uma das qualidades mais importantes da inulina, observada em estudos com animais, foi o aumento da resposta imune adaptativa, confirmada numa ampla variedade de antigénios virais e bacterianos, como influenza [84;87], encefalite japonesa [86;90;112], HIV [91], entre outros.

Por conseguinte, esta dissertação de mestrado tem como objetivo desenvolver e produzir partículas de inulina combinadas ou não, com um polímero previamente estudado, como a Policaprolactona (PCL) ou Quitosano, para ser usado como adjuvante em vacinas.

O método de produção desenvolvido e otimizado foi baseado em técnicas de nano-precipitação e as partículas foram caracterizadas quanto às suas características físico-químicas e imunotoxicológicas.

Neste contexto três sistemas de entrega foram desenvolvidos: partículas de inulina, partículas de PCL-inulina e partículas de PCL. Enquanto as partículas de PCL-inulina e PCL apresentaram tamanhos médios de 301,26 nm e 275,32 nm respetivamente, as partículas de inulina eram muito maiores, encontravam-se fora da nano-escala (1320,86 nm). Os resultados das determinações de tamanho permitiram-nos inferir que as partículas são estáveis por 42 dias, com exceção das partículas de inulina que apresentaram uma tendência para aumento de tamanho ao longo do tempo. Em termos de imunotoxicidade, as partículas de inulina e PCL-inulina não mostraram toxicidade em culturas de células primárias do baço de murganho, embora na linha celular RAW 264,7 os resultados tenham sido ligeiramente diferentes, uma vez que as partículas de PCL-inulina mostraram alguma toxicidade em concentrações mais elevadas, o que foi também observado nas partículas PCL. Considerando os estudos de avaliação de produção de óxido nítrico (NO) e citocinas, as partículas não induziram a produção de NO quando incubadas com a linha celular RAW 264,7, nem a produção de GM-CSF.

O mesmo foi verificado para o TNF-α, IL-6, IL-10, e IL-12 após incubação das partículas de PCL-inulina a 2 μg/mL com as DCs, onde se observou que não foram induzidos níveis significativos de citocinas. No entanto, a 4 μg/mL pode ver-se uma pequena indução da IL-12 e IL-10.

No geral, relatamos o desenvolvimento e produção de partículas de inulina e policaprolactona, cujos resultados preliminares de diversos ensaios *in vitro* sugerem que são adequados para uso, como sistemas de entrega de antigénios, sem toxicidade esperada associada. No entanto, são necessários mais estudos para confirmar os resultados obtidos para a produção de citocinas com DCs, uma vez que a variabilidade entre dadores foi significativa. Por fim, estudos *in vivo* em murganhos usando o antigénio do HBV com as partículas de inulina e PCL-Inulina desenvolvidas, deverão elucidar-nos em futuras experiências sobre a real capacidade das formulações como estratégia para desenvolver novas vacinas e melhorar a vacinação contra o HBV.

Palavras-Chave: Virus Hepatitis B, Vacina para Hepatite B, NPs de Inulina, NPs de inulina/PCL.

1. Introduction

1.1. Hepatitis B

1.1.1. General Characteristics of Hepatitis B Virus

Hepatitis B virus (HBV) is an enveloped DNA virus that is associated with the *Hepadnaviridae* family [1]. HBV envelope is in the form of a lipid bilayer and surrounds an icosahedral nucleocapsid, where is enclosed a partially double-stranded DNA with a genome size of 3.2 kb (Fig. 1). Expressed in the envelope surface are large, medium and small proteins that are detected by the human immune system as antigens, known as hepatitis B surface antigens (HBsAg).

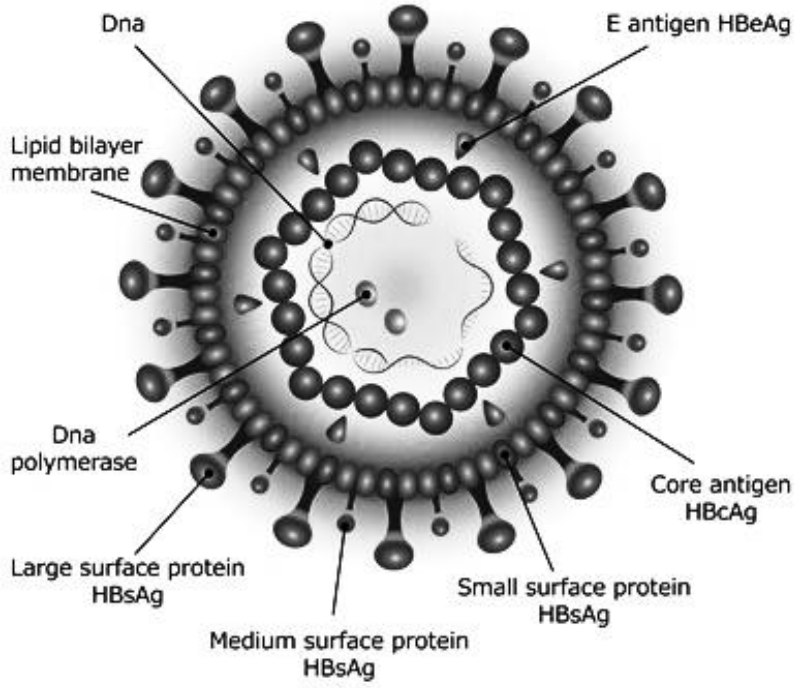


Figure 1 - *HBV constitution. Adapted from [1].*

HBV is highly hepatotrophic and species-specific, meaning that HBV can only replicate exclusively in human hepatocytes [2].

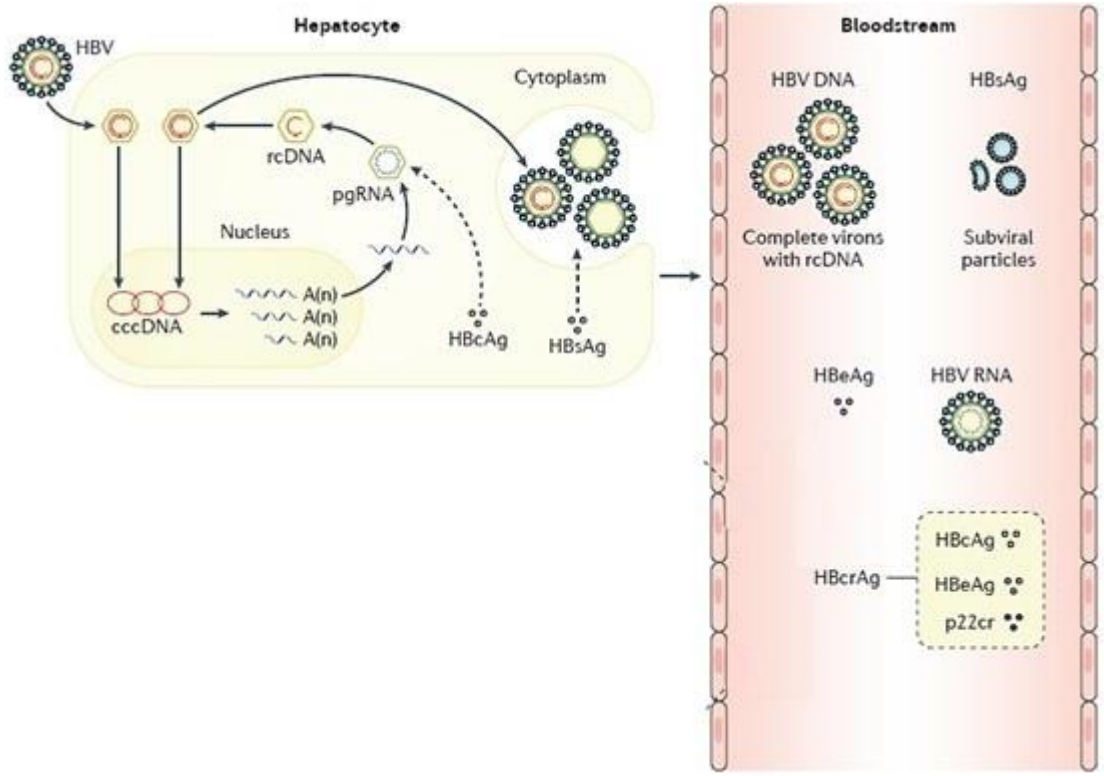


Figure 2 - *HBV replication cycle and Key Viral markers. Adapted from [2].*

Replication of HBV begins with the entry of virus in hepatocytes via the high-affinity receptor sodium taurocholate co-transporting polypeptide as we can see in the upper left diagram presented in Fig. 2. After that, HBV relaxed circular DNA (rcDNA) enters the nucleus and is converted into closed circular DNA (cccDNA) in the form of a mini-chromosome [3].

Then, the transcription products are exported from the nucleus with pre-genome RNA (pgRNA) and are incorporated into replication complexes in the cytoplasm comprising the viral polymerase and core protein. This pgRNA suffers reverse transcription into HBV DNA which can replenish cccDNA or undergo further packaging [3].

HBV DNA with capsid binds to the HBV surface proteins on the endoplasmic reticulum and are translocate to the lumen before exiting the hepatocytes through the secretor pathway. After they exit from hepatocytes, they are mature virus particles. An important fact is that mRNA transcribed from cccDNA, also produces various viral antigens. With the exception of cccDNA all other viral products, like HBV rcDNA, HBV RNA, HBeAg, HBsAg, and HBcAg are easily measurable in the blood. These compounds are represented in the right part of Fig. 2 [3].

1.1.2. HBV World Prevalence and Transmission

Currently, there are ten different genotypes of HBV described in literature. These genotypes go from A to J. and each genotype is distinct and geographical distributed in the world (Fig. 3) [3].

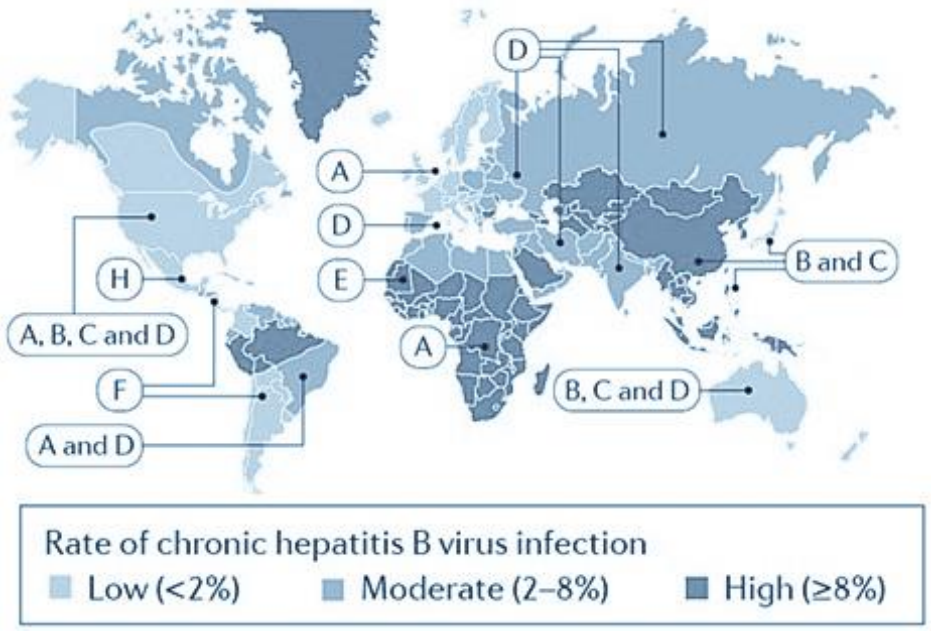


Figure 3 - *Regions with a different population prevalence of chronic hepatitis B infection, categorized as high (>8%), moderate (2–8%), and low (<2%). Adapted from [2].*

Characterization of these different genotypes can be important in epidemiological terms because the genotype reflects a country and allows to track back the transmission patterns [4] and establish the better therapeutic approaches in each case [5].

HBV infection can occur early in life, generally leading to chronic infection. If HBV infection occurs later, commonly what happens is an acute infection that is a self-resolve illness [5].

HBV can be efficiently transmitted from blood or other body fluids. Transmission can occur in a horizontal way or a vertical way [6]. The vertical transmission is characterized for perinatal transmission, which occurs at the birth of a newborn from an infected mother. Perinatal transmission contributes to the majority of HBV cases worldwide [6]. Almost 90% of perinatal infections become chronically infected, while about 20 to 60 % of children with age between 1 to 5 years become chronically infected and 5 % to 10 % of teenagers and adults become chronically infected [7-9].

The horizontal transmission occurs throughout open cuts and scratches, transfusion of blood products from an infected person, breaks in good practices to prevent blood-borne

infections in the health care setting, sexual transmission, injecting-drug, tattooing, body piercing, and scarification procedures without the use of sterilized equipment and needles [5;10].

HBV infection has evolved substantially since 1980 due to the widely used hepatitis B vaccine and demographic changes as a result of population migration [11;12]. However, despite the fact that we already have effective vaccines, as well as disease control measures, including antiviral therapy, the prevalence of HBV is still a serious global health problem. Data from the World Health Organization (WHO) estimates that in 2015, about 257 million individuals (approximately 3.5 % of the global population) have HBV in chronic form, many of whom were newborns without the use of vaccine [12;13]. Asia and sub-Saharan Africa are the regions with greater risk for the development of Hepatocellular carcinoma (HCC) resultant from HBV [11]. Furthermore, the Asia-Pacific region registers one million deaths each year due to HBV complications [11;14].

1.1.3. *Acute and Chronic Hepatitis B infection*

Acute HBV (AHB) remains primarily an adulthood disease [15], short-term illness [16], related with risk practices, such as sexual contact with infected individuals or use of injectable drugs. The incubation period varies from 1 month to 4 months post-infection and clinical presentation varies from asymptomatic in 2/3 of patients to 1/3 with symptoms. Rarely some patients can suffer from fulminant liver failure. Some symptoms, in this case, are relatively common to other diseases, like fever, arthralgia, rash, anorexia, nausea, and others [15].

Diagnosis of AHB can be made in a biochemical way because AHB is characterized by an elevation in the concentration of alanine aminotransferase serum (ALT), which is higher than the aspartate aminotransferase concentration and bilirubin concentrations [15]. How was said, the AHB worst scenario is the fulminant hepatic failure, an unusual complication that only affects 0.5 % of patients, affecting only patients with 8 weeks of jaundice development, which is another symptom of AHB [15].

The introduction of the HBV vaccine dramatically decreased the incidence of AHB. The number of people in the USA infected with AHB had declined from 8036 cases in 2000 to 2.953 in 2014 representing a 62 % decreased in the reported cases [17]. Similar examples can be seen in European Union, where the frequency of AHB in HBV cases reported, decreased from 10.2 % in 2015 to 6 % in 2019 [17;18].

Chronic HBV (CHB) is a long-term illness [16] with different infection patterns varying considerably from country to country, and with time. The biggest number CHB infections

happens early in childhood and chronicity risk is inversely related to the age of infection [19;20]. Most cases of CHB occur in endemic populations resulting from perinatal or childhood transmission. The development of the disease is determined by the interplay between virus replication and the host immune response [15], and can be divided into four phases (Fig. 4):

1st Phase, also known as the Immune tolerant phase, is characterized by high levels of viral replication, with viral HBV serum load up to 1012 IU/mL and detectable HBeAg, but no evidence of liver disease, and normal ALT serum measurement. At this moment T-cell response is weak or undetectable [21]. The duration of this phase varies from 10 to 30 years, being longer in people that acquired HBV in a perinatal way [22].

2nd Phase, also known as Immune clearance phase, is characterized for HBeAg positive signal, variable levels of HBV DNA, and abnormal levels of ALT in serum. In this phase, the immune tolerance is lost, and patients transit to an active hepatitis with hepatic necroinflammation. During this time, the disease activity causes progressive liver damage [10;15].

3rd Phase, known as Low replicative or Immune control phase, is characterized by high levels of anti-HBe, product of seroconversion of HBeAg, low levels of HBV DNA, and normal ALT concentration in serum. In this phase, the activity in the liver is minimal compare to 2nd phase, due to ALT concentration in the serum and there is a high reactivation of T-Cell activity to maintain viral suppression. In some patients can be undetectable HBV DNA in serum even using a polymerase chain reaction [15;23].

Transition from Immune tolerance to Immune clearance phase commonly happens during the second and third decade of life. The transition is characterized by increased HBV-specific T-cell immunity, decreasing HBV DNA titers, increased serum ALT as said back, but can happen that the patient has severe ALT flares result of hepatic decompensation and, rarely, death derived from hepatic failure. A marker used to see the end of the immune clearance phase is the level of HBeAg [10;15].

4th Phase, known as Immune escape phase, is characterized as anti-HBe signal positive, variable concentration of HBV DNA in serum and abnormal level of ALT in serum. During this phase, re-proliferation of HBeAg can happen and patient can have an increase in necroinflammation in the liver.

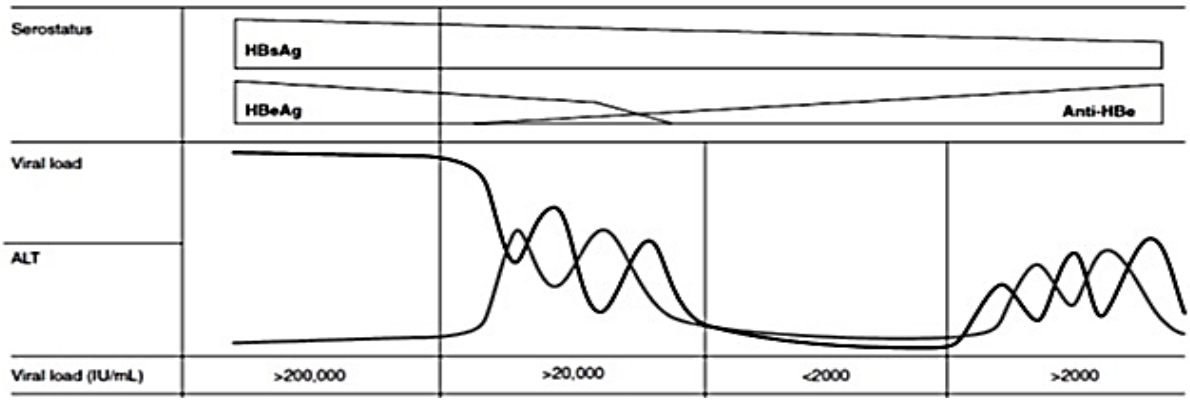


Figure 4 - *Relationship between serology, biochemisty and molecular virology in CHB four phases. Adapted from [3].*

Progression to cirrhosis is very slow in the Immune tolerant phase (studies point that can be less than 1 % a year) while during the Immune clearance and escape phase the progression to cirrhosis may occur at 2-10 % per year. [2]

1.1.3.1. CHB treatment

CHB treatment can be divided into two options. The first is the use of antiviral drugs such nucleotide analogues (NUCs), that have as target reverse transcription during HBV life cycle and can prevent infectious virion release. The second is the use of conventional Interferon Alpha that can stimulate the antiviral immune response in patients [2].

Interferon-based therapy is a therapy comprising Interferon Alpha-2b (IFNα-2b) and Polyethylene glycol – Interferon Alpha-2a (PEG-IFNα-2a). This kind of therapy has immune-modulatory and antiviral properties and finally, the treatment is made by administering these compounds in subcutaneous way once per week. To evaluate the results of therapy, it is analyzed the HBeAg positive signal, if the patient HBV DNA is less than 2,000 IU/mL and if ALT levels are in normal range. The desired outcome is the seroconversion of HBeAg leading to a HBeAg loss.

About 30 % of patient subject to this treatment respond well and if this response continues for about a year, the treatment with this therapy can be extended. Although the extent of treatment can be difficult to tolerate in some patients because of the adverse effects like clinical symptoms of malaise, fever and depression. In addition to this, PEG-IFNα is contraindicated in patients with decompensated cirrhosis, so people with this kind of treatment need to be constantly monitored.

As the response rate is moderate and side effect are frequent, this kind of treatment is only offered to HBeAg-positive patients, infected with HBV genotype A or B, because they are more responsive to IFN therapy, or to young patients that, lack major comorbidities and

have lower serum HBV DNA levels and big ALT levels. Also, this treatment can be indicated to patients that are HBeAg negative, if ALT levels are big and levels of HBV DNA are low [24-28].

NUCs therapy is based on target the reverse transcriptase activity of the HBV polymerase, without significant occurrence of viral resistance [29]. Like this, $CD8^+$ T-Cell function against HBV can be restored after long-term therapy, and also can suppress the HBV replication, expand and give a good life quality to patients [30].

In terms of approved drugs by FDA, exist six drugs approved in his pharm market, that is Lamivudine, Adefovir Dipivoxil, Entecavir, Telbivudine, Tenofovir disoproxil fumarate (TDF), and Tenofovir Alafenamide (TAF) [3]. As by EMA, they have eight drugs approved in his pharm market, that is IFNα-2a and 2b, PEG- IFNα-2a and 2b, Lamivudine, Entecavir, Adefovir and Tenofovir [31]. From all these eight, Entecavir and Tenofovir are used as first-line agents to treat CHBV patients in Portugal, because of their potency and low resistance rates [32].

Entecavir is a nucleoside analogue, is administered as an oral solution, has low side effects, which could be muscle pain, sleepiness, dizziness, general feeling of discomfort, and others [33]. A problem that can happen to Entecavir is if the patient has been already been treated with Lamivudine, then there is a probability of patient have been develop Lamivudine resistant and so the patient can be Entecavir resistant too [34].

Tenofovir are also administered by the oral route, and have some side effects similar to Entecavir, but are the best treatment option to short and medium-term range of 1 to 5 years. This is because NUCs treatment can sustain the suppression of HBV DNA to very low levels, and in some patients, these levels are so low that are undetectable [35;36]. Consequently, the treatment can lead to normal ALT levels in the majority of patients, leading to finish long term treatment with NUCs and to a slowdown in cirrhosis of the liver [37;38]. It is important refer that these advantages not dependent on the genotype of HBV. For the majority of patients, NUCs should be take on long-term treatment, probably lifelong in patients with HBsAg sero-clearance who have more than 50 years or that have cirrhosis [3].

Combining IFN-based therapy with NUCs is possible, and some study results point to an increased rate of HBsAg sero-clearance. One study with 740 treatment-naive patients, combined PEG-IFNα with TDF resulting in a higher rate of HBsAg sero-clearance. Results show that 9.1 % of patients treat with TDF and PEG-IFNα have sero-clearance after 48 weeks, and 2.8 % in the same treatment have sero-clearance after 16 weeks. Otherwise, none of the patients with TDF monotherapy after 120 weeks have sero-clearance and only 2.8 % of

patients with PEG-IFNα monotherapy have sero-clearance after 48 weeks. Most patients that use this kind of combination are infected with HBV genotype A or B [3].

Another study using the same agents but in HBeAg negative with undetectable HBV DNA patients have results a little different from the first study, so the conclusion that we can get is that maybe this combination with these agents can work better only with some genotypes. At present, combination treatment is not made [3].

An important note is that nowadays in some countries, one way of minimizing the vertical transmission, in case of vaccination fail to prevent infection in moms, consists of giving an antiviral agent. Some recent studies have shown that when the mom takes an Antiviral Prophylaxis at 30 or 32 weeks of gestation, the % of children born with HBV is 0 % [3].

1.1.4. Hepatitis B vaccine

In order to better understand HBV vaccines, it is important to understand what types and technologies are behind the development of vaccines, as well as how they came about. Vaccines represent one of the most important advances in science and medicine, helping people around the world in preventing the spread of some infectious diseases [39].

The first vaccine, discovered by Edward Jenner in 1796, was used to combat smallpox, which had provoked the death of 10 % of the population in England and an unknown number around the world. Thanks to mass vaccination, in 1980 smallpox was considered by World Health Organization (WHO) eradicate [40], which leaves high hope for vaccines in control diseases [41]. Beyond this mark, around the years other examples of vaccine began to emerge, as for example: in 1881-1885 Louis Pasteur developed successful vaccines against anthrax and rabies, in the 20th century Albert Calmette and veterinarian Camille Guérin developed a tuberculosis vaccine, between other marks.

Despite that, vaccines have already proved, for the past years, that can eradicate diseases, (locally or worldwide), can control mortality, morbidity, and complications associate with a disease [42], protecting individuals and communities from serious infectious. However, vaccines are often linked to some questions, criticism, lack of confidence, or other questions [43].

Vaccines are rigorously tested and checked by their manufacturers and the health system of the country where they are applied. For a vaccine to be licensed and be commercialized, it needs to be approved by specific regulatory agencies and has passed the clinical trial, constituted by 3 phases with accredited volunteers. The so-called phase IV occurs

only after approval of the commercialization, being the main objective to detect adverse events not registered in the phases before [43].

There exist two types of vaccines, prophylactic vaccines, and therapeutic vaccines, prophylactic vaccines are designed to generate neutralizing antibodies and are used to protect against a future disease. So prophylactic vaccines are given to an individual who has never been in contact with the disease. This type of vaccine corresponds to the majority of approved vaccines by EMA [44;45]. Therapeutic vaccine differs from prophylactic in a way that generates cell-mediated immunity against transformed cells and as the name indicates are used to treat an individual that have currently a disease [44;45]. Vaccines can be comprised of live attenuated virus, inactivated virus, subunits of a specific virus, recombinant proteins, genetic material such as RNA or DNA, among others.

Live attenuated virus vaccines are the oldest, obtained by passing the virus through successions of cell cultures to weaken it, making it impossible to replicate in human cells [46]. Although these vaccines are more immunogenic, because single inoculation with a small dose can induce an immunological response that will confer immunity for futures infections [47], some problems may appear, such as reversion to virulence, more tissue damage between others [48]. In this way, other options available already in the market, as vaccines that use recombinant antigens, are a safer approach comparing to live attenuated virus technology. However, to induce an immune response equal to live attenuated virus, these technology use adjuvants, that confer an immune boost to the vaccine [49].

HBV prophylactic vaccination consists in the individual get the HBV surface antigen (HBsAg) injected via intramuscular with 3 to 4 doses of vaccine (the number of doses depends on the individual region). The first dose is given at birth moment and the other 2 or 3 doses are given in future life [3].

The currently available HBV vaccines are produced from yeast (Saccharomyces cerevisiae), so the vaccine is contraindicated to individuals with a history of hypersensitivity to yeast or any other vaccine components [50], although there has not been any evidence demonstrating that individuals with yeast allergies are at risk for developing an allergic reaction to the HBV vaccine [51].

In the USA pharmaceutical market, there are three vaccines available, all prophylactics. One is single antigen yeast-derived recombinant HBV vaccines called Recombivax HB produced by Merck and Engerix-B produced by SmithKline Beecham Biologicals [52;53] and a combination vaccine, called Twinrix (GlaxoSmithKline), which includes the Engerix-B HBV vaccine and HAVRIX hepatitis A vaccine [54].

An interesting fact about the vaccines for HBV is that the first HBV vaccines were derived from donor plasma, although nowadays this kind of vaccine is not used, derived to side effects and health risks. In almost all countries there are now HBV programs, where all infants receive the HBV Vaccine, being any person in family with or without HBV [55].

Although from background it looks prospective, two main problems constitute a threat to the eradication of HBV. The first problem is the need of improved vaccination in developing countries, and for that researchers focus on the development of more stable vaccines (less susceptible for instance to temperature) and on the development of vaccines for new administration routes (not parenteral routes) [56;57]. In fact, the absence of a cold chain of transportation and storage as well as the absence of trained medical personal to administer the vaccines, poses a major problem to the eradication of HBV in that same countries [58]. The second problem is the non-existence of a therapeutic vaccine to be used in chronic patients since the vaccine that is commercialized is a prophylactic vaccine and so as already said only will protect against future disease, not providing a therapeutic effect if the person has already HBV infection [45;46]. A therapeutic vaccine needs to restore the level of the HBV-specific T Cells population and will give a boost in CD4+ T-Cell response, activating humoral and cytolytic immune response [59;60].

A way of solving part of these problems may be the use of Nanotechnology. How? Well, the use of nanotechnology allows to design the particles using a varying of compositions, sizes, and different surface properties, allowing it is use in different applications. Indeed, different types of nanoparticles are recognized in vaccines formulations to increase antigen stability, to increase immunogenicity, to allow targeted delivery and a slow release [61-63].

In both types of vaccines, prophylactic and therapeutic, nanoparticles can be used as either a delivery system or/and immunostimulatory adjuvant, enhancing immunity [64]. Some examples where the use of adjuvants can help is in immunosuppressed people, smokers, obese and people with other risk factors, where it is possible to see their immune response decreased with levels of antibody being not so high. So, in these cases, inject a booster like a polymer that gives an immunostimulatory effect or an adjuvant it is important for that kind of individuals to get the correct level of antibody [65].

The laboratory where this master dissertation has been developed, is dedicated to develop and test polymeric adjuvants for vaccines, having already published several articles with different types of polymers used as adjuvants, such as Chitosan, Polycaprolactone, and Glucan.

One new adjuvant that has been tested in clinical trials to use in HBV vaccine is Advax ™, which is Delta Inulin a crystalline form of Inulin [66].

1.2. Inulin

1.2.1. General Characteristics of Inulin

Inulin [α-D-glucopyranosyl-β-D-fructofuranosyl-(n-1)-D-fructofuranoside)] is a natural polysaccharide, that belongs to fructans class [67]. It has fructose monomers linked by β-D (2→1) glycosidic bonds with β-configuration at anomeric C2, and in its chains, may contain up to 100 or more furanose units linked to single terminal glucose [68] (Fig. 5). These β-configurations make inulin resistant to hydrolysis in human gastrointestinal tract, which make great a polysaccharide to be used [69].

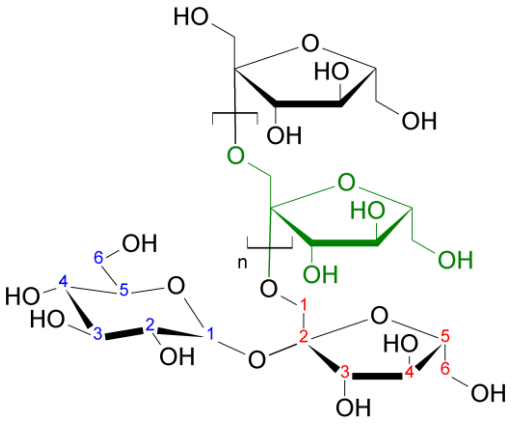


Figure 5 *- Chemical Structure of Inulin. Adapted from [4].*

Historically inulin was first described in 1804 by a German scientist named Valentine Rose, but indirectly it was discovered long before 1804 [70;71]. The first appearance was described by the ancient Greeks and Egyptians, 5000 years ago, as they used inulin due to the use of chicory, one of the sources of inulin, as food and back then they already report a beneficial effect of its consumption. Some years later during Napoleonic Wars, the French people use Chicory drink as a coffee replacement due to the lack of coffee beans [72]. It was only years later, as said above that was describe for the first time inulin not associated with chicory. Although inulin was described by Valentine Rose, who discover inulin in roots of *Inula Helenium*, it was only named "Inulin" in 1818 by Thomson [71;73]. Since that time, several studies, to isolate, identify, characterize, and producing inulin were carried out [72].

In terms of source, inulin can be obtained through natural sources cultivated in different climatic conditions, as it is present in bulbs, tubers, and tuberous roots of many plants, or can be obtained synthetically, prepared from sucrose [72;74;75]. As natural sources described in

literature, we have different types of plants such Chicory roots (*Cichorium intybus*), Jerusalem artichoke (*Helianthus tuberosus*), Dahlia tubers (*Dahlia pinnata*), Dandelion (*Taraxacum officinale*), Asparagus (*Asparagus offcinalis* and *Asparagus* racemosus, Banana, Onion, Garlic, Yacon, and Leek [74-77].

From all of the natural sources, only the Chicory roots, Dahlia tubers, and Jerusalem artichoke, (Fig. 6), were used as a source for the industry, and from all of these three, the most used in industry is chicory roots because it has 65-79 % of inulin in his constitution [78].

***Figure 6** - Main sources of Inulin in industry: 1. Chicory Roots, adapted from [5]; 2. Jerusalem artichoke, adapted from [6]; 3. Dahlia Tubers, adapted from [7].*

Over the years, the industrial processes to extract and purify inulin have been optimized. The main process is hot water extraction from the vegetable source, plus purification steps. One important factor in extraction and purification process is the pH of inulin, because inulin is sensitive to acidic hydrolysis when pH is lower than 6.0. Another reason why inulin pH is so important is that specific applications of inulin require a pH not lower than 6.0, like food, pharmacy, cosmetic [71].

1.2.2. Inulin Isoforms

Inulin, unlike other polysaccharides, have the ability to generate crystalline forms or as it is described and know in literature, isoforms. These isoforms are obtained by a long procedure, involving different profiles of temperature for different amounts of time [68;69].

Until now, seven isoforms were discovered and named, Alpha-Inulin 1 (AI-1), Alpha-Inulin 2 (AI-2), Gamma-Inulin (GI), Delta-Inulin (DI), Zeta-Inulin (ZI), Epsilon-Inulin (EI), and Omega-Inulin (OI), being AI-1 the first isoform that can be obtained and, until now, OI being the last isoform, as shown in table 1. Each isoform has more 6 fructose units than the previous one, starting with AI-1 that have 19 units [68;79].

Table 1 - *Inulin Isoforms Characteristics [68;79].*

Isoform	Frutose units	Critical Temperature (°C)	Melting Point (°C)
AI-1	19 units per chain	17	168.79
AI-2	25 units per chain	32	170.86 ± 0.598
GI	31 units per chain	45	174.282 ± 0.183
DI	37 units per chain	54	177.08 ± 1.095
ZI	43 units per chain	60	180.14 ± 1.626
EI	49 units per chain	64	182.88 ± 0.363
OI	55 units per chain	71	185.53 ± 2.255

These differences in the number of units lead to different characteristics. As a matter of fact, all isoforms present different solubility temperatures in water [68], and most importantly, they present different immune properties [80-93].

For obtaining the most percentage of a determined isoform from raw inulin, we need to begin from the first isoform, AI-1 and escalate [68;79]. As we need to escalate, the procedure to obtain an isoform like EI or OI will take several time.

From these seven isoforms, the most addressed in literature are GI and DI [68]. GI is described as an isoform capable of activating complement, that plays a central role in immune response [94], resulting in an increase of peritoneal neutrophils and lymphocytes [70]. DI is distinct from Alpha Inulin because is the first type of isoform being insoluble at mammalian body temperature, which makes it a good candidate for use as a particulate adjuvant, as it can provide adjuvant activity without solubilizing [71]. DI is a potent safe adjuvant being well-tolerated by immune modulators and is capable of enhancing adaptive immune responses by a non-inflammatory immune pathway [68].

1.2.3. Inulin applications

Inulin is non-toxic and safe for humans and it was approved for use in food and as fiber in the United States of America by the FDA (Food and Drug Administration) in 1992 and receive in same year the GRAS (Generally Recognised As Safe) acronym by the FDA. Because inulin is described in USP, USA pharmacopeia, it is possible to be used in drugs. Therefore,

over the years several applications of inulin have been studied in the pharmaceutical field, with different objectives such as:

1.2.3.1. To evaluate kidney function:

Inulin can be used as a biomarker to evaluate kidney function. It is intravenously injected, to be distributed over the extracellular volume only, not being metabolized or bind to plasma proteins. Then, it is excreted via glomerular filtration, and not reabsorbed in renal tubes. Consequently, it allows to determine the glomerular filtration rate by measuring inulin concentration in urine and plasma. As inulin is filtered out freely, the excretion rate is proportional to the glomerular filtration rate [95-97]. Until now the only side effect of inulin injection was a small hypotension in some patients [74].

1.2.3.2. To enhance calcium absorption:

It is described in the literature that inulin does not influence negatively important ions involved in calcium absorption, like the proper calcium or magnesium; on the contrary, it seems to help to increase these ions [98–102], increasing then calcium absorption, bone mineral density, and calcium uptake. All these qualities derived from the use of inulin in the daily diet, in moderate concentration. In a study carried out by *Marisol Rivera-Huerta et al.* [102], where they used male BALB/cAnNhsd mice with 5 weeks of age and female Hsd:Wistar rats with 8-9 weeks of age, it seemed that the mice and rats with Inulin in diet do not have calcium concentration in blood altered, although calcium concentration in urine was decreased. These results demonstrate a favorable calcium absorption in intestine/colon. Another interesting result in this assay is that bone densitometry was increase, which translates to a benefit for the use of inulin in the diet. Still, the mechanism of action in increasing absorption is unknown and the researchers think it may be related to increased colonic calcium bioavailability.

1.2.3.3. As substitute of fat and sugar:

Inulin use in the daily diet increases the sense of satiety [72;103–105] as demonstrated in *in vivo* assays with animal models, where animals with inulin introduced in daily diet eat a little less [101;102]. In detail *Maria V. Salinas et al.* [101] used male Wistar rats with 3 weeks (21 days old) and fed them with Inulin. Besides demonstrating an increase in calcium absorption, uptake and bone mineral density they showed that the rats with Inulin in the diet ate less than the others, suggesting that inulin provoke satiety what can be good for substitute fats and sugars.

1.2.3.4. As a drug delivery vehicle in colon cancer:

As inulin is only hydrolyzed by inulinases produced in the colon, where exist microorganism communities capable of metabolizing inulin by fermentation, and not in the upper parts of the gastrointestinal tract [72;105;106], inulin can be used as a drug delivery vehicle with target ability to use in colon cancer treatment. An *in vitro* study carried out by *C.A. Schoene et al.* [107], where it was used inulin from Dahlia Tubers, conjugated with doxorubicin in three different human colorectal adenocarcinoma cell lines SW620, Caco-2, and HT29, have shown 76 %, 70 %, and 60 % more cytotoxic for HT29-MTX, SW620, and Caco-2 cells, respectively than free DOX demonstrating, which is more potent and toxic than doxorubicin.

1.2.3.5. As prebiotic:

Inulin help grow some beneficial bacteria communities, that make a reduction in pH which leave to lower survival rate for most of the pathogenic bacteria [74]. A study already cited above [102], besides demonstrating positive effects in calcium absorption and other mechanism related to that, show to that inulin stimulate the increase of bifidobacteria communities in the colon. These bifidobacteria have several functions, some related to colon cancer and protection against opportunistic infections. In terms of relation with colon cancer, it was seen that these bacterial communities when fermenting inulin, produce products such as butyrate and propionate, that are capable of inhibiting the growth of colon tumor cells, histone deacetylases, reduce metastasis in the colon cell line, and protects from genotoxic carcinogens as enhance the expression of the enzyme involved in detoxification [108].

1.2.3.6. Inulin as a vaccine adjuvant:

For some time, inulin has been explored in the vaccine adjuvant area and although the raw inulin form can enhance vaccine efficacy and is non-toxic and safe, its mechanism is still unclear. In the literature it is describe that raw inulin does not change Th1/Th2 immune response and is not able to stimulate dendritic cells (DC) *in vitro* [109]. As said by Doctor Mariusz Skwarczynski in the commentary with the title "Inulin: A new adjuvant with unknown mode of action" published in EBioMedicine [109], "because inulin cannot stimulate DC, a crucial step in adaptive immunity, it is possible that inulin act as a delivery system, by preventing antigen degradation or though improving delivery APC's, without immune-stimulating abilities". However *in vivo* DC maturation experiments have shown that acted as an adjuvant and enhanced the expression of maturation markers on DC Cells. A study carried out by *Fadi Saade et al.* [110] where it was studied the HBsAg antigen with alumn or Advax™ (inulin isoform) in guinea pigs and mice, revealed as one of the conclusions that when Advax™ was injected at the same time as the HBs antigen but into the opposite limb is that the anti-HBs

response is reduced, suggesting that Advax™ act as an immune decoy drawing dendritic cells away from the site of antigen injection. Nevertheless, the real reason for this behavior *in vitro* and *in vivo* is yet unknown [111;112].

Overall, what is more studied and described for adjuvant activity are not raw inulin but inulin isoforms, in particular Gamma and Delta inulin. Below are described *in vitro* and *in vivo* studies reported in the literature concerning the adjuvant activity of these two isoforms.

1.2.3.6.1. Gamma-Inulin

In vitro studies: Studies performed with GI are few in comparison with the other isoform most described, DI. Compiling all studies found, there is one that draws attention, is a study performed with GI, alumn, and Algammulin (alumn combined with GI isoform). In this study, the author uses frozen human plasma to verify which activates more the complement pathway and the conclusion is that GI activates the complement pathway more than alumn or inulin raw, although Algammulin activates the complement pathway in the same proportion [111]. In another study, where it was used the macrophages from Balb/c mice, the researchers discover that component C3 from complement is activated by GI, suggesting the possibility of adjuvant effect of GI can be mediated by C3 fragments, also, they verify T Cell independent and dependent responses, where T Cells have shown to express CR1/2, so it's possible [112].

In vivo studies: Different studies with GI in animals have already been made. One of the studies, where it was studied the adjuvanticity of GI to improve both humoral and cell-mediated responses to a variety of antigens, the researchers inject Balb/c mice firstly with 30-300 hemagglutination (HA) units of live or gamma-irradiated influenza virus (strain A/JAP, H2N2) plus or not with 50 μg of GI and after 2-4 weeks made an intranasal challenge with five or 25 lethal doses of influenza virus strain A/WSN (H1N1). The results of this immunization show that only 3.8 % of the animals with live or gamma-irradiated influenza virus have survived the intranasal challenge and 50 % of animals immunized with the virus plus GI have survival to the same challenge. These show that GI is useful against mucosal infections and that can confer protection against lethal infection in mice (intranasal influenza virus) [89].

Another study that uses GI as an adjuvant in different vaccines, have tested GI as an adjuvant for HBsAg and has use formulations of GI and algammulin plus HBsAg reformulated from the currently available human Hepatitis B Virus (HBV) vaccine containing alum (50 μg per mouse), or HBsAg (1 μg per mouse). Groups of 10 C57Bl/6 mice were immunized two times, one at day 0 and another at day 30. Humoral responses were evaluated at day 44. The study has shown that, when immunized with GI, mice have more IgG2a subtype, specific for

HBsAg, consistent with increased Th1 response. Interestingly when splenocytes from immunized mice were, after, re-stimulated with HBsAg *in vitro*, mice immunized with GI, algammulin, and alum formulations have obtained a cytokine profile with similar levels of HBsAg stimulation [83].

Regarding cancer therapeutic vaccines, one study carried was out to verify the anti-tumor effects of GI in C57BL/6J mice injected intraperitoneally with 10^6 B16 melanoma cells. In this study, GI was given intraperitoneally (10 µg - 5 mg in 0.2 ml saline) 1 to 3 days after the B16 cells. Results have shown an increase in the survival time of the animals injected with GI and the anti-tumor activity was confirmed by the activation of the alternative pathway of complement with depletion of C3, indicating a protective effect, this anti-tumor activity was verified in the lowest dose 5O µg (2.5 mg/kg) [92].

Although GI has great potential to be used as an adjuvant in vaccines unfortunately, GI is susceptible to damage from sterilizing doses of gamma irradiation [113].

1.2.3.6.2. Delta-Inulin

DI adjuvant ability has been widely described in the literature as an adjuvant already developed and patented by Vaxine Pty Ltd [71]. The name for this adjuvant is Advax™ and animal studies have already demonstrated an enhance in the adaptive immune response against a wide variety of viral and bacterial antigens, as influenza [84;87], Japanese encephalitis [86;90;112], HIV [91], and others.

In vitro studies: Advax™ *in vitro* studies have shown several qualities for his application as an adjuvant. One study has stimulated dendritic cells (DC) from mouse bone marrow with Advax™, alumn and lipopolysaccharide for 15 hours to measure the expression of CD40, a factor that indicates DC activation. The result has demonstrated that nor alumn or Advax™ have influenced CD40 expression, in contrast, *in vivo* results have shown expression of CD40 in pDCs, a population of DC. As the author says, the result suggests that Advax™ is capable of activating DC, but as an indirect effect, which is unknown [114].

In the same study, in order to identify the adjuvant effect of Advax™, some biological factors as IL-1β and TNF-α production were examined. The results reveal a non-production of IL-1β and TNF-α. As pointed by the author and from other articles, the non-production of IL-1β can be due to the fact that particulate adjuvants need NLRP3 inflammasome activation [115;116]. So, the author test the adjuvant effect of Advax™ in NLPR3 inflammasome deficient mice, and the results obtained demonstrate an adjuvant effect of Advax™, which indicates that the adjuvant effect of Advax™ is independent of the NLPR3 inflammasome / IL-1β signaling

pathway. The absence of TNF-α production suggests that Advax™ has a decrease adjuvant effect on antibody response [116].

Another study, by other authors, was carried out to see if the incorporation of Advax™ in whole inactivated influenza vaccine (WIV) formulation has an influence on the type and scale of cell-mediated immune response induced. For that, IFN-γ and IL-4 was measured. Advax – WIV formulation was compared with the WIV formulation alone and has demonstrated an increase of IFN-γ and IL-4, which indicates a balanced Th1/Th2 response [117].

In vivo studies: Animal studies using Advax™ with HBsAg, have demonstrated an enhanced in humoral and cellular immunity without inducing local and systemic reactogenicity [112]. In a particular study, mice immunized with HBsAg antigen and Advax™ have shown a proliferation of CD4 and CD8 T-Cell, and IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, and GM-CSF production, being the values higher in comparison with the mice immunized with HBsAg alone or plus aluminum [91; 118]. Also, it showed no excessive inflammatory effects, as there was not any change in sedimentation rate, C-reactive protein, plasma globulins, complement levels or white blood cell counts post-immunization and unlike aluminum, there was not any abnormality in the injection site.

Another study that uses Advax™ as an adjuvant, has studied the use of Advax™ adjuvant platform to enhance SARS-CoV-2 vaccine protection while avoiding the risk of lung eosinophilic immunopathology. In this *in vivo* study, BALB/c mice were immunized intramuscularly two times with 3 weeks apart, using a recombinant spike protein or inactivated whole-virus vaccine adjuvanted or not with alum, CpG, Advax- 1, or Advax-2. Advax-1 formulation is a sterile suspension in bicarbonate buffer with a concentration of delta inulin at 50 mg/ml, and Advax-2 being the Advax-1 plus 10 μg CpG per 1 mg of delta inulin [119].

Results have shown an enhance in humoral immunity when was use Advax™ formulations were used in comparison with other groups- Advax-1 formulation was capable of enhancing IgG1 response at 2 weeks post-boost, maintaining the enhanced antibodies up to 1-year post-immunization. Advax-2 formulation was capable of increasing IgG1, IgG2a, IgG2b, and IgG3 response, keeping the enhanced response up to 1-year post-immunization. In this case, if we compare the result obtained by Advax-2 and CpG adjuvant, we see that both have increase IgG2a, IgG2b, and IgG3 response, so it is possible that Advax-2 produce a broad range of antibody isotypes thanks to CpG, since Advax-2 formulation has CpG embedded. Another interesting result is that Advax™ formulations have spike protein-specific IgM responses

peaked at 2 weeks post-immunization that prevailed for 1-year post-immunization, suggesting generation IgM positive B cells. Also Advax-1 group have an increase on spike protein-specific CD4 and CD8 T-cell proliferation [119].

Furthermore, in this study, it was shown that Advax™ formulations were capable of protecting mice from a lethal challenge with SARS Cov-2 and that these mice have a reduced eosinophilic immunopathology in lung compare to mice immunized with the spike protein and alum. This demonstrates that Advax™ adjuvant platform is capable of enhancing SARS-CoV-2 vaccine protection while avoiding the risk of lung eosinophilic immunopathology [119]. All these qualities distinguish Advax™ from others adjuvant classes for example emulsions, TLR agonists, and others.

If we visit the website *clinicaltrails.gov* and search for Inulin, it will be shown 245 trials that use Inulin, from which 158 trials are completed. The great majority of these, analyze the use of Inulin as a nutritional supplement, in kidney function or in cancer. Nevertheless, 5 trials use Inulin, or more precisely Advax™, as an adjuvant for influenza virus, HBV, Covid-19 vaccine and as an adjuvant for an anti-venom [120].

The clinical trial that uses Advax™ as adjuvant against chronic HBV is a phase 1 trial, NCT number: NCT01951677, with 240 participants, in each participant has receive one of the treatments listed in Table 2.

Table 2 - *Treatment in clinical trial NCT number: NCT01951677 [121].*

Treatment	Details
HBsAg + standard alum adjuvant	Standard hepatitis B vaccine antigen + Alum
HBsAg + Advax-1™	Standard hepatitis B vaccine antigen + Delta inulin adjuvant
HBsAg + Advax-2™	Standard hepatitis B vaccine antigen + Supermix
HBsAg + Advax-3™	------
preS HBsAg + alum adjuvant	preS hepatitis B surface antigen + alum adjuvant
preS HBsAg + Advax-1™	preS hepatitis B surface antigen + Delta inulin adjuvant
preS HBsAg + Advax-2™	preS hepatitis B surface antigen + Supermix
preS HBsAg + Advax-3™	------
High dose preS HBsAg + alum adjuvant	preS hepatitis B surface antigen + alum adjuvant
High dose preS HBsAg + Advax-1™	preS hepatitis B surface antigen + Delta inulin adjuvant
High dose preS HBsAg + Advax-2™	preS hepatitis B surface antigen + Supermix
High dose preS HBsAg + Advax-3™	------

The main aim of the trial is to see the safety and the adverse effects of the formulations, and the secondary aim is to verify the titers of antibodies against HBsAg, HBsAg-specific T cell responses, and efficacy of treatment (seroconversion and seroprotection rates). Although the study begins in July of 2013 and finishes in May 2019, no result is available on clinicaltrials.gov platform or has been published in article, also no more details are available on the platform [121].

In the clinical trial that use Advax™ as an adjuvant in Jack Jumper Ant (JJA) Venom immunotherapy, NCT number NCT03066986, the author have the aim to compare responses to in-hospital sting challenges and JJA venom specific IgE and IgG4 responses to semi-rush JJA Venom Immunotherapy (JJA VIT) at doses of 25 and 50 mcg of JJA venom, with and without Advax™, to reduce the costs of therapy, reduce systemic allergic reactions of JJA VIT, and use low doses of the venom. Although this clinical trial is on run, no results or further information has been posted on the platform [122].

For the clinical trial that uses Advax™ in influenza virus, NCT number NCT02335164, the aim of the author is the use of a new vaccine formulation against pandemic avian influenza, identified as the H5N1 strain of influenza virus. In this trial have been recruited 270 participants, each one has received one of the treatments listed in Table 3. The treatments describe as Advax 1 ™ or Advax 2 ™ are different adjuvant formulations of DI [123].

Table 3 - *Treatment in clinical trial NCT number: NCT02335164 [123].*

Treatment	Details
Recombinant Influenza Hemagglutinin (H5)	45 µg of H5, intramuscular injection, 2 doses
H5 + Advax 1™	45 µg of H5, 20 mg of Advax 1 ™, intramuscular injection, 2 doses
H5 + Advax 2™	45 µg of H5, 20 mg of Advax 2 ™, intramuscular injection, 2 doses
H5 + Advax 1™	15 µg of H5, 20 mg of Advax 1 ™, intramuscular injection, 2 doses
H5 + Advax 2™	15 µg of H5, 20 mg of Advax 2 ™, intramuscular injection, 2 doses
H5 + Advax 1™	5 µg of H5, 20 mg of Advax 1 ™, intramuscular injection, 2 doses
H5 + Advax 2™	5 µg of H5, 20 mg of Advax 2 ™, intramuscular injection, 2 doses
H5 + Advax 2™	2,5 µg of H5, 20 mg of Advax 2 ™, intramuscular injection, 2 doses
H5	15 µg of H5, intramuscular injection, 2 doses

As the first clinical trial, this one has no results on the platform or in publish article although having the status complete and have begun on January 9, 2015, and finish on May 7, 2019.

Finally, the 2 clinical trials that remain to be mentioned uses Advax™ as an adjuvant for the Covid-19 vaccine. The 2 trials use Covax-19™, a vaccine constituted by SARS Cov-2 recombinant spike protein with Advax™ adjuvant.

The first clinical trial, NCT number NCT04428073, was first posted on June 11 of 2020, and has the status of "Not yet recruiting". The trial aims at evaluating the safety and the immune responses of a therapeutic vaccine, Covax-19™ in SARS-CoV-2 infected patients in a time frame of 26 weeks. These patients are Covid-19 confirmed with mild or no symptoms.

The trial aims to recruit 32 participants, with each one receiving 1 mL of a low dose of Covax-19™ at week 0 and 2 or 1 mL of a High dose of Covax-19™ at week 0 and 2.

In terms of outcomes, the author wants to evaluate the immunogenicity of Covax-19™ after 6 weeks by measuring $CD8^+$ T cells immune response, verify Virologic response after vaccination using RT-PCR in respiratory tract specimens at weeks 0, 1, 2, 3, and 4, and finally analyze clinical outcome and progression after vaccinations. Unfortunately, no further information has been posted on the platform [124].

The second clinical trial, NCT number NCT04453852, aims to test Covax-19™. As outcomes the author wants to see the incidence of adverse events 1-week post-immunization, SARS Cov-2 antibody titers 2 weeks post second immunization, and frequency of SARS Cov-2 spike specific T cells 1-3 weeks post second immunization. As treatments, the patients have received Covax-19™ or saline control solution, intramuscularly. Although the trial has the status complete, as some other trials already indicated, no information has been posted in the platform or in a published article [125].

Because all trials described here have the status of not finished or finished but without results, conclusions cannot be taken as exist a lack of results [123]. Currently, Advax ™ as an adjuvant for use in prophylactic HBV vaccines was not yet approved although many advantages have been described.

1.3. Aim of the master dissertation

As previously mentioned, the laboratory where this master dissertation was carried out, already has a long history in terms of the development of particles as adjuvants, with the use of diverse polymers (ex PCL, PLA, etc.) and polysaccharides (glucan, chitosan, etc). The particles already developed by the laboratory have demonstrated promising results by the subcutaneous route [126-129], which makes it interesting to include in those particles a second polysaccharide like the Inulin and test if a synergistic effect may exist.

Therefore, this Master dissertation aims to develop and produce Inulin particles blended or not with a previously studied polymer, such as Polycaprolactone or Chitosan, to characterize the particles physicochemically, and to characterize its immunotoxicity. The outputs of the work herein comprised will constitute the first steps towards the development of a new adjuvant for a HBV vaccine.

2. Development of a method to produce Inulin NPs and their physicochemical characterization

2.1. Materials and Methods

2.1.1. Materials

Pluronic® F 68 Prill was purchased from BASF (Ludwigshafen, Germany). Inulin from chicory, Polycaprolactone (average Mw cs. 60 000), Bovine Serum Albumine (BSA), Lysozyme (LYS) and Myoglobin (MYO) were obtained from Sigma-Aldrich Corporation (St. Louis, USA). Chitosan (ChitoClear™—degree of deacetylation 95 %; viscosity 8 cP (1 % solution)) was obtained from Primex Bio-Chemicals AS (Avaldsnes, Norway). Other chemicals were purchased from normal suppliers.

2.1.2. Development of a method to produce different inulin nanoparticles

2.1.2.1. Development of the method to produce Inulin NPs

The technique to produce Inulin NPs is based on the solubilization of 20 mg of Pluronic® in a scintillation bottle with 2 mL of acetone. Next, under magnetic stirring, 1 mL of Inulin (5 mg/mL) was added dropwise. This inulin solution was previously warmed at 80 °C for 1 hour. Then, the scintillation bottle was kept for 2 days at 27 °C in an orbital stirring. Over that period, the scintillation bottle was kept slightly open to evaporate acetone. After 2 days, the 1 mL remaining in the scintillation bottle, was transferred to a centrifugation tube and corresponds to INW (Inulin not washed) NPs. Alternatively, NPs were centrifuged at 15000*g for 10 min, the supernatant was removed, and the IW (Inulin washed) NPs were resuspended in 1 mL of MilliQ water. NPs.

This optimized protocol was established after testing different conditions described in table 4.

Table 4 - *Overall summary of the conditions tested to produce INW particles. The blue color represents the optimized condition for the production of these particles.*

Inulin [5 mg/mL] (mL)	Acetone (mL)	Tween 80 (mg)	Pluronic (mg)
2	2	2	0
1	2	20	0
1	2	0	10
1	2	0	20

2.1.2.2. Development of the method to produce PCL-Inulin NPs

The technique used to produce Inulin + PCL NPs was similar to the Inulin NPs technique with a noteworthy difference. The acetone solution (2 mL) where the inulin was added dropwise, contained 1.25 mg/mL of PCL. As above, the particles obtained after acetone evaporation were called PINW (PCL-Inulin not washed) NPs, while the ones after the wash procedure were called PIW (PCL-Inulin washed) NPs.

This optimized protocol was established after testing the conditions present in the table 5.

Table 5 - *Overall summary of the conditions tested to produce PINW particles. The blue color represents the optimized condition for the production of these particles.*

Inulin [5 mg/mL] (mL)	Acetone + Polycaprolactone [1.25 mg/mL] (mL)	Acetone + Polycaprolactone [2.5 mg/mL] (mL)	Tween 80 (mg)	Pluronic (mg)
2	2	0	2	0
2	0	2	2	0
1	2	0	20	0
1	0	2	20	0
1	2	0	0	10
1	0	2	0	10
1	2	0	0	20

2.1.2.3. Development of the method to produce PCL NPs

PCL NPs were obtained using the same technique as for PCL-inulin NPs, with the difference that the inulin solution was replaced by MilliQ water. The particles obtained after acetone evaporation are call PNW (PCL not washed) NPs and after the wash procedure PW (PCL washed) NPs.

This optimized protocol was established after testing the conditions present in the table 6.

Table 6 - *Overall summary of the conditions tested to produce PNW particles. The blue color represents the optimized condition for the production of these particles.*

MilliQ water (mL)	Acetone (mL)	Acetone + Polycaprolactone [1.25 mg/mL] (mL)	Acetone + Polycaprolactone [2.5 mg/mL] (mL)	Tween 80 (mg)	Pluronic (mg)
2	0	2	0	2	0
2	0	0	2	2	0
1	0	2	0	20	0
1	0	0	2	20	0
1	0	2	0	0	10
1	0	0	2	0	10
1	0	2	0	0	20

2.1.3. Physicochemical characterization of the NPs

2.1.3.1. Size and zeta potential

To measure the particle size and zeta potential, 1 mL of a diluted NP suspension was used (100 µL of NPs + 900 µL of Milli-Q water). Particle size was measured by dynamic light scattering (DLS) and zeta potential by electrophoretic light scattering (ELS). Both measurements were performed at 25 °C and scattered light was collected at a 165 °angle.

2.1.3.2. Stability in DMEM cell culture media

Size and Zeta potential of NP samples prepared by adding 100 µL of NP suspension in 900 µL of DMEM medium were measured to assess its stability in cell culture conditions.

2.1.3.3. Stability in pyrogen-free water

Size and Zeta potential of two lots of IW NPs, PIW NPs and PW NPs were measured 3 times, over a period of 42 days to assess its stability.

2.1.3.4. Protein *adsorption studies*

To determine the formulations ability to adsorb proteins at their surface, INW, PINW, and PNW NPs were suspended in Milli-Q water, to obtain a concentration equal to 750 µg/mL and were incubated with protein aqueous solutions of Bovine serum albumin (BSA) in three different concentrations (250, 750, and 1500 µg/mL). Mixtures were put under rotational agitation at room temperature for 1 h in 2 mL centrifugation tubes. After that, the centrifugation tubes were centrifuged at 21000*g for 20 min at 20 °C. The supernatants were collected and used to determine the amount of protein not bound to the NPs.

To determine this amount of protein not bound the DC™ protein assay was made. A calibration curve of BSA, protein was prepared in triplicate in concentrations ranging between 0 and 1000 μg/mL, in a 96-wells plate, as for the samples, 5 μL of the supernatant of each sample was platted. Next 25 μL of a reagent previously prepared by mixing reagent A and reagent S in a proportion of 1:0.02 mL was added to each well, followed by 200 μL of reagent B. The plate was then left to incubate at 20°C for 60 min, and the absorbance values were measured at 750 nm using a microplate reader. The percent values of Loading Efficacy (LE) and Loading Capacity (LC) were calculated by the following equations:

$$LE = \frac{Total\ amount\ of\ protein - Non\ bound\ protein}{Total\ amount\ of\ protein} * 100$$

$$LC = \frac{Total\ amount\ of\ protein - Non\ bound\ protein}{Total\ amount\ of\ particle} * 100$$

2.1.3.5. Protein *entrapment* studies

BSA, LYS and MYO incorporation into INW, PINW, and PNW NPs during the NPs production technique was also assessed. At the end, similarly to protein adsorption studies, the protein content not entrapped in the NPs was measured by an indirect way in the supernatants. In this assay, the DC™ protein assay, was used according to the manufacturer's protocol. The protein entrapment was tested at three different protein concentrations, 1.25, 2.5 and 5 mg/mL. Briefly, the protein solutions were mixed with the aqueous solution that was added dropwise to the acetone solution, during the production method. After the 2 days incubation period and acetone evaporation, the formed NPs were transferred to centrifuge tubes and centrifuged at 15000g for 10 min at 20 °C to collect supernatants and measure protein content.

For the DC™ protein assay, the procedure was the same as indicated back, with the difference that a calibration curve of BSA, LYS and MYO protein was prepared in triplicate in concentrations ranging between 0 and 2000 μg/mL. The percent values of Entrapment Efficacy (EE) and Entrapment Capacity (EC) were calculated by the following equations:

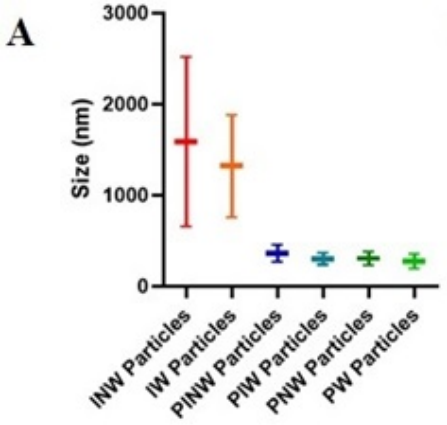
$$EE = \frac{Total\ amount\ of\ protein - Non\ bound\ protein}{Total\ amount\ of\ protein} * 100$$

$$EC = \frac{Total\ amount\ of\ protein - Non\ bound\ protein}{Total\ amount\ of\ particle} * 100$$

2.2. Results and Discussion

2.2.1. Inulin and PCL based particles physicochemical characterization

Inulin and PCL based particles were successfully obtained with a nanoprecipitation technique followed by the evaporation of the organic solvent over 48 h at 27 °C. Three different formulations were developed – Inulin particles, PCL-Inulin particles and PCL particles – and were tested in their original supernatant (not washed), and after the supernatant being replaced by Milli-Q water (washed). The formulation with bigger particle size and dispersion obtained were without doubt inulin particles (INW and IW particles), with a size of 1586.29 nm and 1320.86 nm respectively, Fig. 7A and B. Their size, in the range of micrometers, is significantly higher than the remaining particles comprising PCL.



B

	Size		
Particles	Mean (nm)	SD (nm)	PDI
INW	1586.29	± 930.69	0.53
IW	1320.86	± 564.01	0.33
PINW	360.14	± 92.68	0.23
PIW	301.26	± 64.25	0.12
PNW	305.15	± 73.14	0.20
PW	275.32	± 77.92	0.12

Figure 7 - *(**A**) Overall summary of size of Inulin, PCL-Inulin and PCL Particles washed or not washed. Results are the mean ± SD, n=19 (19 independent experiments, each in triplicate). (**B**) Mean and Standard Deviation (SD) of size of Inulin, PCL-Inulin and PCL Particles washed or not washed, n=19 (19 independent experiments, each in triplicate).*

Remarkably, PINW and PNW particles present a difference close to 55 nm (360.14 nm vs 305.15 nm) that can be attributed to the presence of inulin in the PCL NPs. PCL particles are formed instantaneously when the acetone is dropped into the aqueous solution. Since the aqueous solution contains inulin, inulin may be trapped within PCL particles, or it may precipitate afterwards, around the preformed PCL particles, either way increasing the particle final size.

Comparing each particle not washed with the respective washed particle we can verify that in the Inulin case there is a decrease in size after the washing procedure, while in PCL-Inulin and PCL case, washed or not washed particles have similar size, Fig. 7B.

These results were compared with the results of similar particles developed by our laboratory group and with results reported from other research groups. For instance, comparing the size of INW and IW particles with the size reported for the delta inulin isoform

particle (microparticles contained in Advax™ licensed adjuvant), it is possible to observe that they are in the same size range since INW, and IW display a size of 1586.29 nm and 1320.86 nm respectively and Advax™ display a size between [1000 - 2000] nm [113]. Although PINW and PIW particles had Inulin as well, they do not are comprehended in the delta inulin microparticle range, possibly due to the fact that PCL precipitation occurs before, not allowing inulin to precipitate freely, and entrapping it within the PCL nanoparticle.

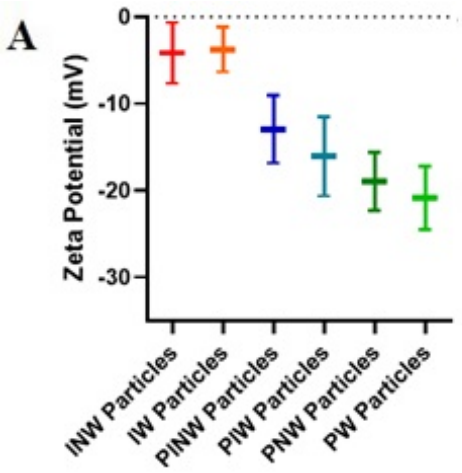
On its turn, PINW and PIW particles (360.14 nm and 301.26 nm) have a similar size to Carboplatin loaded PCL NPs (311.6 nm), a PCL particle with an anti-neoplastic drug describe in [130]. PNW and PW NPs have a similar size to PCl-Chitosan NPs (318 nm) and PCL NPs (268 nm) described by Vásquez Marcano et al, and by Singh et al [131;132]. Other authors present slightly smaller sizes for PCL based NPs [133;134]. Paclitaxel loaded PCL and PCL NPs presented a size of 215.6 nm, and 201.7 nm, respectively. Note that each PCL particle pointed here is different, since each was obtained by a different production technique, with or not a different surfactant/emulsifier, and with PCL that has different molecular weight.

Regarding zeta potential, Inulin particles (INW and IW particles) present a neutral surface charge (-4.15 mV and -3.76 mV respectively), while PCL-Inulin and PCL particles have a more negative surface charge (Fig.8A). Comparing the particles developed with the particles already referred back, it is possible to see that like the PCL particles, also the PINW and PIW particles have a slightly negative zeta potential as the zeta is approximately 15 mV [132;133;134]. Now for the PNW and PW particles developed, zeta potential is a little more negative (- 18.96 mV and -20.86 mV, respectively), which is strange since polycaprolactone alone is a neutral polymer. This negative charge can be hypothesized to be influenced by the nonionic surfactant, Pluronic® F68 [135]. An example where the surfactant influenced the zeta potential can be observed in [136], where the author has tested different surfactants (different types of Pluronic® surfactant and different concentrations of PVA surfactant) with a single polymer (PLGA) and has got particles with different zeta potentials, some more negative than others, which lead him to conclude that depending on the surfactant and its concentration, we get more or less negative zeta potential.

Among the reports that found negative zeta potential for PCL particles, we can compare our formulations with the Gatifloxacin loaded PCL NPs described by *Aida Maaz et al.* [137], which are PCL NPs obtained through a nanoprecipitation technique. However, unlike our developed particles the surfactant used was PVA. Comparing the particles, we can say that we have a less negative zeta potential since the PW particles zeta potential is -20.86 mV and

the PCL NPs described have -30.1 mV, which can be due to the fact that were used different surfactant.

A



B

	Zeta Potential	
Particles	Mean (mV)	SD (mV)
INW	-4.15	± 3.52
IW	-3.76	± 2.58
PINW	-12.94	± 3.90
PIW	-16.06	± 4.53
PNW	-18.96	± 3.36
PW	-20.86	± 3.64

Figure 8 - *(**A**) Overall summary of zeta potential of Inulin, PCL-Inulin and PCL Particles washed or not washed. Results are the mean ± SD, n=23 (23 independent experiments, each in triplicate). (**B**) Mean and Standard Deviation (SD) of zeta potential of Inulin, PCL-Inulin and PCL Particles washed or not washed, n=19 (19 independent experiments, each in triplicate).*

In terms of stability, it was analyzed the stability of the IW, PIW, and PW particles in pyrogen-free water at 4 °C for 42 days, since the *in vitro* assays performed with these particles (Chapter 3) were not done on the particles production day. So, to see if the particles remain unchanged during the set of *in vitro* assays is necessary to avoid misleading results, that may not be related to the particles but with their degradation. Results presented in Fig. 9 show that IW particles are the only ones that have some variability in size over time, more specifically they show a tendency to increase. As for the zeta potential, it is possible to observe that the zeta potential of the 3 formulations remains stable over 42 days.

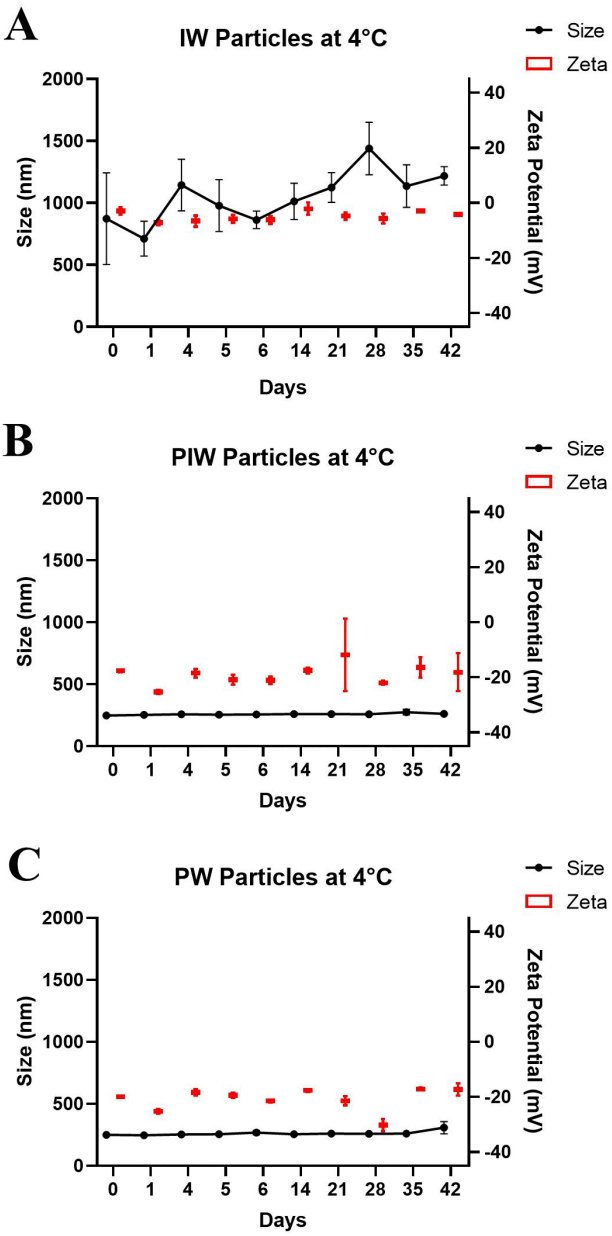


Figure 9 - *Stability test of IW Particles (A), PIW Particles (B), PW Particles (C) performed at 4 °C, presenting in each graph the mean size and the zeta potential over time. Results are the mean ± SD, n=2 (two independent experiments with two batch).*

2.2.2. Protein loading ability of Inulin and PCL based particles

In order to evaluate the ability of the developed particles as protein delivery vehicles, protein adsorption studies and protein entrapment studies were performed. For the protein adsorption studies, BSA was used as the model protein and several ratios particle:protein were tested. Results after 1 h incubation of the protein with the particles showed that all particles presented a relatively low loading efficacy for the tested ratios, never exceeding 40 % as we can see in the figure below, Fig. 10A.

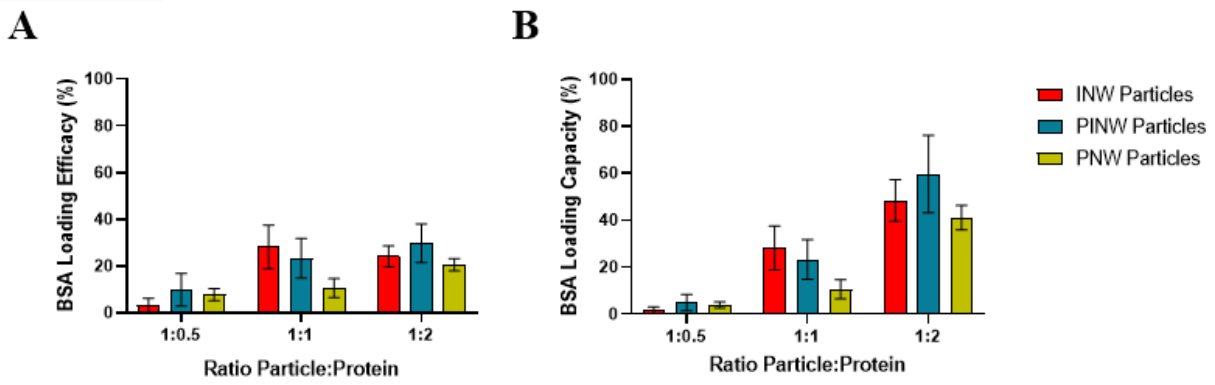


Figure 10 - *(**A**) Results of the loading efficacy (LE %) obtained for Inulin, Inulin + PCL and PCL NPs using bovine serum albumin (BSA). Proteins were adsorbed to the particle surface for 1 h at 20 °C. Data are expressed as mean ± SEM, n=3 (three independent experiments, each in quadruplicate). (**B**) Results of the loading capacity (LC %) obtained for Inulin, Inulin + PCL and PCL NPs using bovine serum albumin (BSA). Proteins were adsorbed to the particle surface using three NPs: Protein ratios at 20 °C and evaluated after 1 h of agitation. Data are expressed as mean ± SEM, n=3 (three independent experiments, each in quadruplicate).*

Despite the low loading efficacy percentages, it is important to note that this value is specific to the tested ratios (1:0.5, 1:1 and 1:2, w/w) which are high. Consequently, when we calculate the loading capacity, Fig.10B, results show that for the ratio 1:2 (particle:protein), the PINW particle is able to adsorb protein equivalent to approximately 60% of its weight.

Comparing PINW and PNW particles with PCL and Chitosan–PCL NPs, described by *Sandra Jesus et al.* [138], we can see that the developed particles cannot get a loading efficacy similar to the described particles even in the higher ratio. The loading efficacy for BSA in [138] is a little higher than 50 % when they use a ratio of 1:1.6 and 1:1.5 NPs:Protein respectively. For the PINW and PNW particles, loading efficacy is below 40 % at all ratios tested. Nevertheless, when we compare the loading capacity described by *Sandra Jesus et al.* [138], with 1:2 ratio (48.42 % for INW, 59.62 % for PINW and 41.11 % for PNW particles), we conclude, that the developed particles have a loading capacity similar to the PCL NPs (Chitosan-PCL particles have a loading capacity higher than 100 %).

From the results, it is possible to see to, that the increase on the NP:Protein ratio increases the loading efficacy, so when we increase the ratio from 1:0.5 to 1:2 ratio (NP:BSA

Protein) the protein adsorption increases. At first sight this was not expected, because theoretically, the smaller the amount of protein, the higher will be loading efficacy, as we have less protein to adsorb to the particle surface. However, as the developed particles had a neutral surface charge (INW particles) or a slightly negative surface charge (PINW and PNW particles), we can hypothesize that a high concentration of protein is necessary to increase the interactions with the particles leading to its adsorption. In this hypothetic situation, protein might be adsorbed based on hydrophobic interactions, rather than electrostatic ones [139;140].

Moving to protein entrapment studies, 3 different proteins were tested by adding them during the particles production process. To note, the production yields of Inulin, PCL-Inulin and PCL particles are different due to the different amount of starting polymers used. Consequently, adding equal amounts of protein to different formulations results in different particle:protein ratios.

Figure 11A shows the results for myoglobin (MYO) and lysozyme (LYS) entrapment, since for BSA entrapment, no positive results were found. It was observed an entrapment efficacy higher than 60 % for almost all conditions tested with MYO. On its turn, LYS studies showed lower entrapment efficacy. After normalizing these results to the amount of particles used (entrapment capacity), Fig.11B, it is possible to observe that PNW particles were the ones achieving better results. For the ratio 1:2 (particles:protein, w/w) the PNW particle show an ability to entrap almost 1.5 times its weight in MYO. However, PNW particles were the only ones tested using the ratio 1:2, which hinders a proper comparison between formulations.

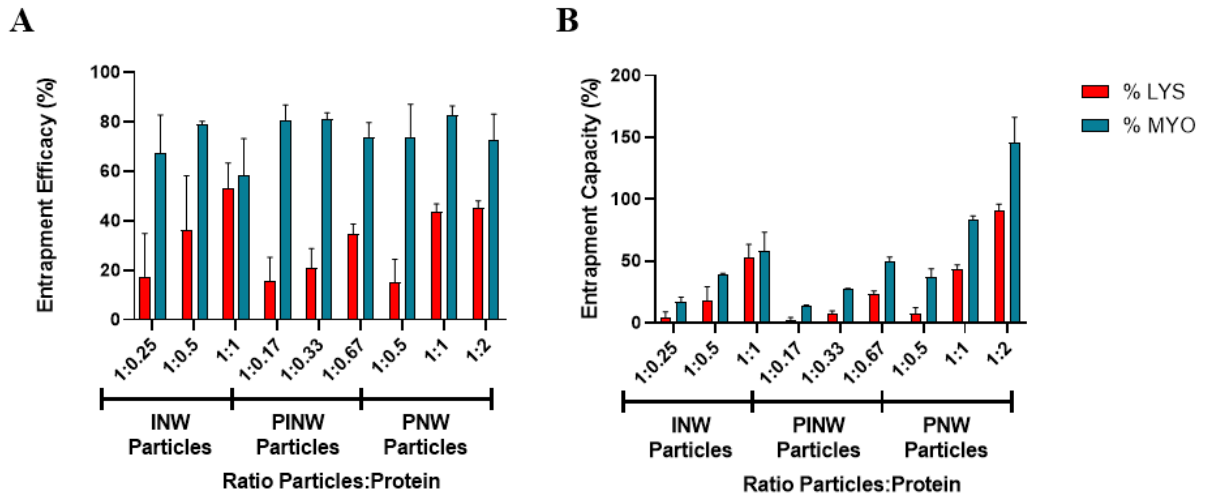


Figure 11 - *(**A**) Results of the entrapment efficacy (EE %) obtained for IW, PIW and PW NPs using Lysozyme and Myoglobin. Proteins were entrapped in the particle using three NPs: Protein ratios at 27 °C and evaluated after NP formation. Data are expressed as mean ± SEM, n=3 (three independent experiments, each in triplicate). (**B**) Results of the entrapment efficacy (EC %) obtained for IW, PIW and PW NPs using Lysozyme and Myoglobin. Proteins were entrapped in the particle using three NP's: Protein ratios at 27°C and evaluated after NP formation. Data are expressed as mean ± SEM, n=3 (three independent experiments, each in triplicate).*

The developed inulin and PCL based particles were then used in *in vitro* assays (Chapter 3) in order to access it is toxicity and immunomodulatory properties.

Note that another approach to develop another inulin particle blended with chitosan polymer was performed, however, results were not so successful and further studies were not carried on. Nevertheless, the tested production method and optimization protocols are presented in Supplemental Information.

3. *In vitro* assays to evaluate NPs toxicity and biological activity

3.1. Materials and Methods

3.1.1. Materials

Raw 264.7 were purchased to ATCC (Manassas, VA, USA), RPMI 1640 medium and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma- Aldrich Corporation (St.Louis, USA). Murine IL-6, IL-10, IL12, TNF-α and GM-CSF ELISA kits were acquired from PeproTech (Rocky Hill, NJ, USA). Other chemicals were purchased from normal suppliers.

3.1.2. Cell viability assay using spleen cells isolated from $CD1^+$ mice

3.1.2.1. Primary cell culture isolation

Splenocytes were obtained from spleens of $CD1^+$ mice after sacrifice.

Briefly, spleen from $CD1^+$ mice were put in a Petri dish where previously was added 5 mL of RPMI 1640 medium. After that, with the help of two needles, splenocytes were removed from the spleen and homogenized with a pipette before transferring the suspension to a tube. The tube was left for 5 min at rest, and then the supernatant was transferred to a new tube and centrifuge at 1100 rpm for 10 min. After this time remove the supernatant and resuspend the pellet with 5 mL of RPMI 1640 and repeat the centrifugation and finally resuspend the pellet in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 1% (v/v) PenStrep, and 20 mM HEPES buffer.

3.1.2.2. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

In order to access cell viability, it was used the MTT tetrazolium reduction assay in which MTT is reduced to formazan, a product with purple color meaning viable cells with active metabolism. The decrease of the purple color means that cells are metabolic less active and not necessarily that cells are dead [141]. To do this assay, cell concentration was adjusted to a final concentration of 1×10^7 cells/mL using RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 1 % (v/v) PenStrep, and 20 mM HEPES buffer. In a 96-well plate, 100 µL of nanoparticles (INW and PINW) at a concentration of 1250 µg/mL till 19.53 µg/mL, and 100 µL of cell suspension was added and incubated for 24 h in a humidified atmosphere at 37 °C, 5 % CO_2. After 24 h, 20 µL of a filtered MTT solution was added, previously prepared at 5 mg/mL in PBS with pH=7.4. The plate was further incubated for 4 h in a humidified atmosphere at 37 °C, 5 % CO_2. Then, the plate was centrifuged at 800*g for 25 min. Carefully, 180 µL of supernatant was removed for each well and 200 µL of DMSO was added and incubated for 30 min. Finally, after total dissolution of the formazan crystals, the

optic density was read at 540 nm and 630 nm in a microplate reader. Cell viability was calculated with the following equation:

$$Cell\ Viability\ (\%) = \frac{\text{OD sample (540 nm)} - \text{OD sample (630 nm)}}{\text{OD control (540 nm)} - \text{OD control (630 nm)}} * 100$$

3.1.3. In vitro studies using RAW 264.7 macrophage cell line

3.1.3.1. RAW 264.7 cell culture

The murine macrophage cell line, more precisely RAW 264.7, was cultured in DMEM medium supplemented with 10 % inactivated Fetal Bovine Serum, 3.7 g/L sodium bicarbonate, 10 mM Hepes and 1 % penicillin/streptomycin. These cells are adherent cells and were maintained in 75 cm^2 flasks with 20 mL of growth medium in a humidified atmosphere at 37 °C with 5 % CO_2. Subcultures were performed by detaching the cells mechanically after being grown at a confluence of approximately 70 % to 80 % and by diluting 1:4 every 2 days.

3.1.3.2. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cell viability assay was performed in 96-well plates and cells were plated at a density of 2 × 10^5 cells/mL and incubated for 24 h in a humidified atmosphere at 37 °C, 5 % CO_2, before adding the samples. Then the medium was removed and 100 µL of fresh medium was added, as well as 100 µL of serial dilutions of INW, IW, PINW, PIW, PNW and PW NPs in DMEM medium. The plate was incubated in a humidified atmosphere at 37 °C, 5 % CO_2 for 22 h. After 22 h, 20 µL of a filtered MTT solution previously prepared at 5 mg/mL in PBS with pH=7.4, was added and the plate was incubated for 1 h and 30 min in a humidified atmosphere at 37 °C, 5 % CO_2. Finally, the cell culture medium was aspirated and 200 µL of DMSO was added to each well. The plate was read at an optic density of 540 nm and 630 nm in a microplate reader. The equation used to obtain cell viability (%) was:

$$Cell\ Viability\ (\%) = \frac{\text{OD sample (540 nm)} - \text{OD sample (630 nm)}}{\text{OD control (540 nm)} - \text{OD control (630 nm)}} * 100$$

3.1.3.3. Nitric Oxide Production assay

In order to evaluate nitric oxide production, it was used the Griess reagent system, which is based on a chemical diazotization reaction. This reaction uses reagent A (Sulfanilamide) and reagent B (N-1-napthylethylenediamine dihydrochloride (NED)) under acidic conditions. These two reagents originate Griess Reagent that indirectly detects nitric oxide through its oxidized nitrite form, leading to the formation of a purple/magenta color [142]. In brief, cell concentration was adjusted to 4.5×10^5 cells/mL using DMEM. In a 48-well plate, 500 µL of cell suspension was added and incubated for 24 h in a humidified atmosphere at 37 °C, 5 % CO_2. After 24 h, the supernatant was removed and 250 µL of DMEM without phenol red and 250 µL of the formulations were added. The formulations used for this assay were IW, PIW and PW lyophilized in which each formulation was tested under 4 different concentrations: 1.25 µg/mL, 2.5 µg/mL, 20 µg/mL and 40 µg/mL. Also was used LPS as positive control at a concentration of 1 µg/mL.

After 24 h, to evaluate the nitric oxide production, 100 µL of supernatant from each well was transferred to a 96-well plate, along with a calibration curve. Next Griess reagent was prepared (combining equal volume of Reagent A and B) and 100 µL were added to each well. The plate was incubated for 10 min in dark at room temperature. Finally, the optic density was read at 540 nm.

To evaluate the cell viability, the MTT assay was performed right after the supernatant was collected. After all supernatants was aspirated, fresh DMEM without serum (200 µL) was added to each well, plus 20 µL of the filtered MTT solution (5 mg/mL in PBS with pH=7.4). Then, the plate was incubated for 1 h and 30 min, the cell culture medium was aspirated, and the formazan crystals dissolved with DMSO for colorimetric reading as described before.

3.1.3.4. GM-CSF production by RAW 264.7 cells after inulin NP stimulation

GM-CSF cytokine levels were also evaluated on the supernatants resulting from the NO assay using the ELISA technique according to the instructions of the Kit manufacturer. In short, high binding 96 well-plate were coated with 100 µL of capture antibody and left incubate overnight at room temperature. Next, the plate was washed 5 times with wash buffer and left incubate with block for 1 hour. After the plate was washed, and 100 µL of standard cytokines and 100 µL of each sample were added, leaving the plate to incubate at room temperature for 2 h. It is important to refer that each sample used, is a pool of one condition done in triplicate in one assay. Two hours late the plate was washed again and 100 µL of avidin-HRP conjugate was added and left to incubate for 30 min. Finally, the plates were washed and 100 µL of

substrate solution was added and left incubate at room temperature for color development. The absorbance was measure at 405 nm and 630 nm and the concentrations of cytokines were extrapolated from absorbance values, using the calibration curve.

3.1.4. *In vitro* studies using human dendritic cells

3.1.4.1. Dendritic Cells (DC's) cell culture

The monocyte-derived dendritic cells were cultured in RPMI medium supplemented with 10 % FBS, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mM MEM NEAA, 1 mM sodium pyruvate. These cells were obtained from human monocytes isolated from peripheral blood of healthy donors that differentiated after maturation with IL-4 and GM-CSF stimulus.

3.1.4.2. Dendritic Cells Incubation with NPs

In order to access the production of IL-6, IL-10, IL-12 and TNF-α, by PIW NPs, cell concentration was adjusted to a final concentration of 3×10^5 cells/mL using RPMI medium supplemented with 10 % FBS, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mM MEM NEAA, 1 mM sodium pyruvate. In a 24 well plate, 500 µL of cell suspension was added. Next 250 µL of freeze-dried PIW particles, previously resuspended in pyrogen-free water with polymyxin b (10 µg/mL) at concentration 2 µg/mL and 4 µg/mL were added, plus 250 µL of supplemented RPMI. Also, it was added in a separate well, 250 µL of LPS (positive control), at concentration of 20 ng/mL, in another separate well was added 250 µL of Pol.B at concentration of 10 µg/mL, and in another well was added only 250 µL of RPMI (negative control). The plate was left to incubate for 24 hours. After 24 hours, the plate was centrifuged at 400*g for 5 min and the supernatants were collected to quantify by ELISA technique.

3.1.4.3. IL-6, IL-10, IL-12 and TNF-α quantification by ELISA technique

The Elisa technique was performed as described before for GMC-SF.

3.2. Results and Discussion

In vitro cytoxicity can be used to predict the toxic profile of particles *in vivo*. One of the most used methods is the MTT tetrazolium reduction assay, which indirectly determines cell viability, measuring the metabolic activity instead of the real number of viable cells. The spleen plays an important role in immune response. Spleen cells are constituted from different cells such as T and B lymphocytes, dendritic cells (DCs) or macrophages [143].

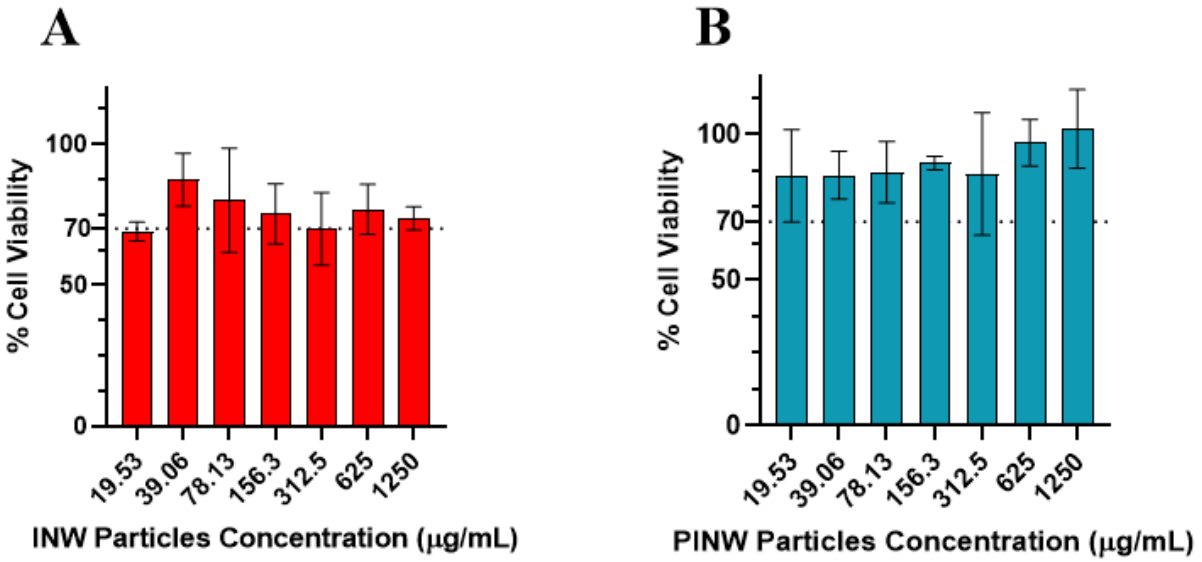


Figure 12 - *Cytotoxicity evaluation using MTT tetrazolium reduction assay, after all INW (Inulin not washed) and PINW (PCL-Inulin not washed) NPs were in contact for 24 h with splenocytes: (**A**) Cell Viability of splenocytes incubated with different concentrations of INW Particles (mean ± SEM, n=3, three independent experiments, each in triplicate), (**B**) Cell Viability of Splenocytes incubated with different concentrations of PINW Particles (mean ± SEM, n=2, two independent experiments, each in triplicate).*

As we can see in the following figures, Fig. 12 A and B, where was determined the cell viability of splenocytes after being incubated with INW and PINW NPs respectively, the decrease of cell viability is minimum, once all bars stay a little higher or around 70 % (minimum considered for cell viability).

Comparing the INW particles with the PINW particles, we can conclude that all particle concentrations tested are not cytotoxic. However, it was expected that higher concentrations induced more cytotoxicity, which did not happen and can indicate that the concentrations that induce cytotoxicity would be very high.

If we compare the results obtained for PINW particles with the results reported by *Sandra Jesus et al.* [139] regarding the cell viability of splenocytes when incubated with PCL particles, we can see that the PINW particles are less cytotoxic. The reported PCL particles at 150 µg/mL have low cell viability, of approximately 20 % [139]. The high cellular viability until 1250 µg/mL concentration in the case of PINW particles can be due to the inclusion of the inulin into the nanoparticle, that decreased the cytotoxicity induced by the PCL polymer. It is

important to mention that the PCL particles studied by Sandra Jesus and his co-workers [139], were particles obtained through a different nanoprecipitation method, which can lead to a different behavior when incubated with cells. Also, these particles have a size and zeta potential of 201.7 nm and −1.4 mV, respectively which were lower than the size and zeta potential observe for the PINW, 360.14 nm, and -12.94 mV.

Following these results with splenocytes, we decided to move to a cell line, because these primary cells obtained from mice have a high variability since are not adherent cells which makes the assay more difficult to be done. So, we initiate the MTT tetrazolium assay in Macrophages cell line, RAW 264.7.

As we see in the following figure, Fig.13D, INW NPs do not cause much cytotoxicity in comparison with PINW NPs or PNW NPs, with these last two types of nanoparticles having more cytotoxicity at higher concentrations, as expected. If we compare INW NPs cell viability with INW NPs supernatant and raw Inulin, Fig.13A, we have always cell viability, which corroborate with what we visualized in the cell viability of splenocytes Fig.12a.

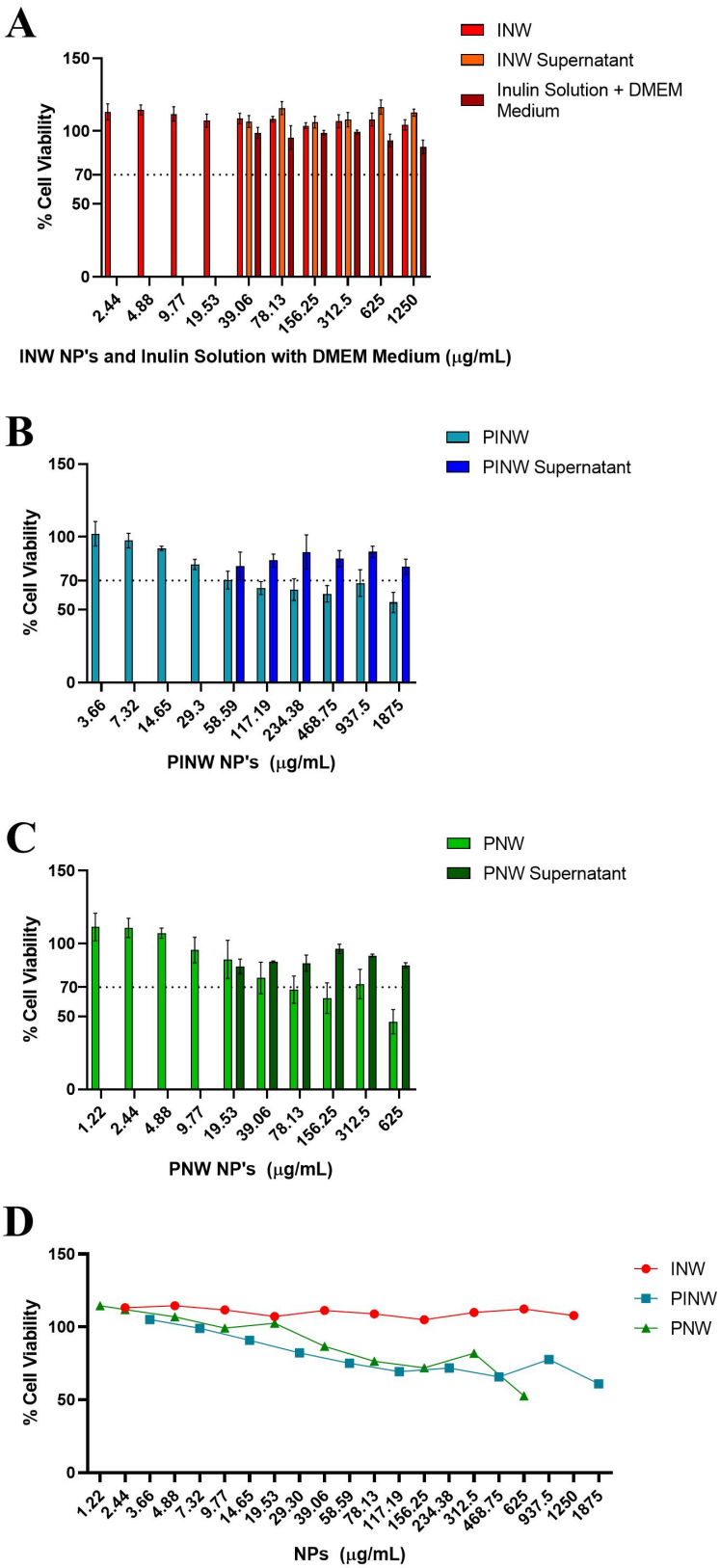


Figure 13 - *(**A**) Cell viability evaluation using MTT tetrazolium reduction assay, after incubation of the cells with INW (Inulin not washed) NPs, INW NPs supernatant and Inulin Solution. Incubation with RAW 264.7 cells was kept for 24 h. Concentrations of INW NPs Supernatant in the graph represent the corresponding concentration of NPs dilutions. Results of INW NPs and inulin solution are expressed as mean ± SEM, n=4 (four independent experiments, each in triplicate), INW NPs supernatant are expressed as mean ± SEM, n=3 (three independent experiments, each in triplicate). (**B**) Cell viability evaluation using MTT tetrazolium reduction assay, after*

*incubation of the cells with PINW (PCL-Inulin not washed) NPs and PINW NPs supernatants. Incubation with RAW 264.7 cells was kept for 24 h. Concentrations of PINW NPs Supernatant in the graph represent the corresponding concentration of NPs dilutions. Results of PINW NPs are expressed as mean ± SEM, n=5 (five independent experiments, each in triplicate), PINW NPs supernatant are expressed as mean ± SEM, n=4 (four independent experiments, each in triplicate). (**C**) Cell viability evaluation using MTT tetrazolium reduction assay, after incubation of the cells with PNW (PCL not washed) NPs and PNW NPs supernatants. Incubation with RAW 264.7 cells was kept for 24 h. Concentrations of PNW NPs Supernatant in the graph represent the corresponding concentration of NPs dilutions. Results of PNW NPs are expressed as mean ± SEM, n=5 (five independent experiments, each in triplicate), PNW NPs supernatant are expressed as mean ± SEM, n=4 (four independent experiments, each in triplicate). (D) Cell viability evaluation using MTT tetrazolium reduction assay, after incubation of the cells with INW (Inulin not washed) NPs, PINW (PCL-Inulin not washed) NPs and PNW (PCL not washed) NPs. Incubation with RAW 264.7 cells was kept for 24 h. Results of INW NPs and inulin solution are expressed as mean ± SEM, n=4 (four independent experiments, each in triplicate), PINW NPs are expressed as mean ± SEM, n=5 (five independent experiments, each in triplicate), PNW NPs are expressed as mean ± SEM, n=5 (five independent experiments, each in triplicate).*

Moving forward, if we compare each supernatant with the respective particle, (Fig. 13B and Fig. 13C) we observed that probably, what is cytotoxic is the particle and not the surrounding solution since supernatant of the particles were not cytotoxic. The assessment of the particle supernatant was done because all assays performed with not washed particles showed low cell viability at high concentrations. Therefore, to investigate if compounds, present in the supernatants of the particles, influences the result of the evaluation of the cytotoxicity of the particles, the supernatants were evaluated as well.

Moreover, to confirm once more if the effects were related to the particles, washed particles (IW, PIW, and PW particles), were lyophilized, resuspended in DMEM medium and incubated with RAW 264.7 cell line (Figure 14). In order to verify if the freeze-dried particles maintain the size after resuspending, a new assay to measure the size was performed. The size evaluation after resuspension of the freeze-dried particles demonstrated that the PIW and PW particles had a slightly larger size after resuspended, 394.57 nm and 347.73 nm respectively, compared to the size that was initially described, 301.26 nm and 275.32 nm. IW particles on its turn, had a size of 1050 nm after resuspension, comparable to its size before freeze-dry (1320.86 nm).

Concerning IW particles (Fig. 14A) the cell viability was tested in range of concentrations from 1.22 µg/mL to 2500 µg/mL. The results showed that the particles are not cytotoxic, even in the concentration of 1250 µg/mL. Only the higher concentration (2500 µg/mL) seems to start showing some cytotoxicity (< 70 %), which correlates with the results obtained and presented in Fig. 13A. By the contrary, it was confirmed that PIW and PW nanoparticles were cytotoxic in higher concentrations (from 9 µg/mL to 2500 µg/mL for PIW particles, and 39.06 µg/mL to 2500 µg/mL for PW particles) since the cell viability was lower than 70 %, Fig. 14B and Fig. 14C, what almost correlates with the results obtained in Fig. 13B and 13C respectively.

With this specific result, we proved that the particles PINW and PNW have a cytotoxic effect at higher concentrations and that cytotoxicity is not related with the external phase of the particle suspension (supernatant).

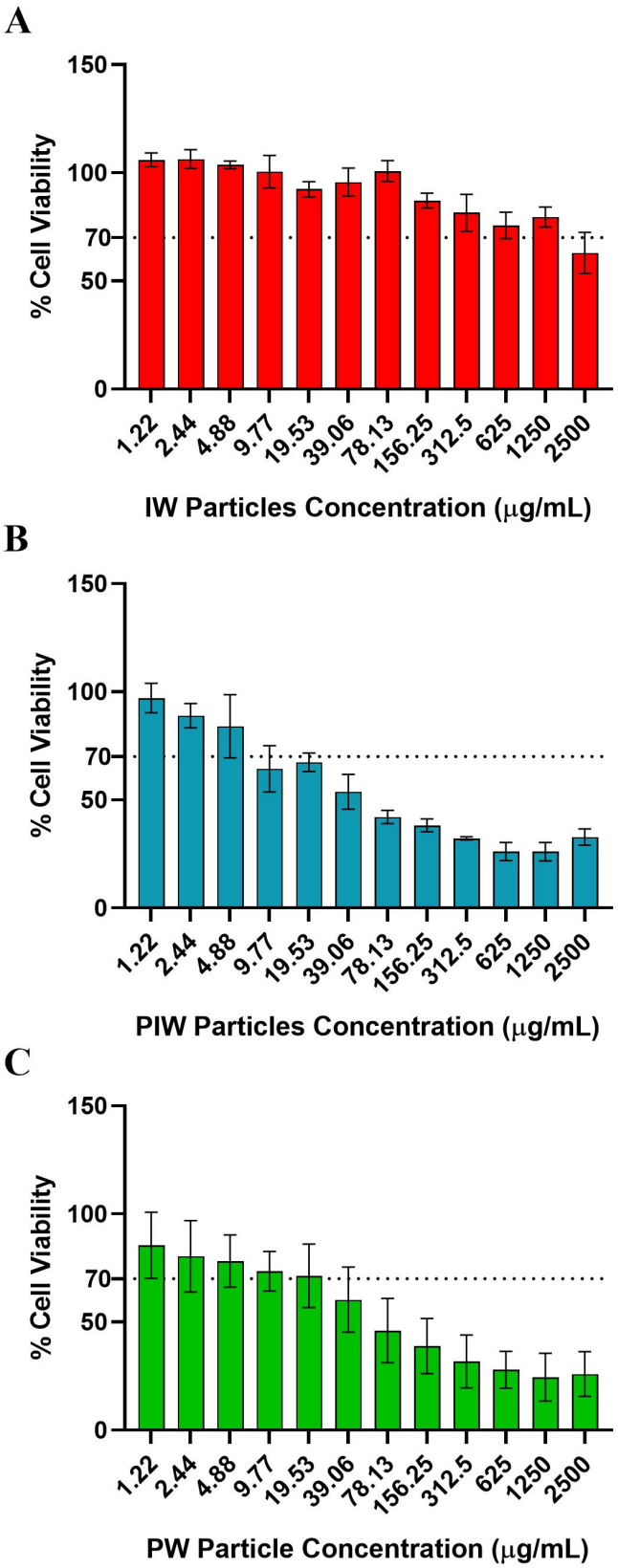


Figure 14 - *(**A**) Cell viability evaluation using MTT tetrazolium reduction assay, after incubation of the cells with IW (Inulin washed) particles. Incubation with RAW 264.7 cells was kept for 24 h. Results of IW particles are expressed as mean ± SEM, n=4 (four independent experiments, each in triplicate). (**B**) Cell viability evaluation using MTT tetrazolium reduction assay, after incubation of the cells with PIW (PCL-Inulin washed) particles. Incubation with RAW 264.7 cells was kept for 24 h. Results of PIW particles are expressed as mean ± SEM, n=4 (four independent experiments, each in triplicate). (**C**) Cell viability evaluation using MTT tetrazolium reduction*

assay, after incubation of the cells with PW (PCL washed) particles. Incubation with RAW 264.7 cells was kept for 24 h. Results of PW particles are expressed as mean ± SEM, n=3 (three independent experiments, each in triplicate).

Comparing these results with results obtained for different particles previously developed in the lab [144;145;146], it is possible to say that compared to glucan particles [144] and chitosan particles [145], inulin (INW and IW) particles are less cytotoxic for RAW 264.7 since even in highest concentrations exist cell viability (112.6 % and 63 % respectively). However, comparing with the particles described by Da Silva and co-workers [146], we see that although the INW and IW particles are less cytotoxic than the PLA_B nanoparticle, the same cannot be said for the PLA_A nanoparticle, since this particle have always 100 % of cell viability, and our particles have 70 % of cell viability, in the highest concentrations.

For the diverse formulations containing PCL and PCL/inulin particles prepared within the scope of this project, if we compare cell viability results of PINW, PIW, PNW and PW particles with the results from chitosan and glucan particles referred back [144;145], we see that our formulations present a higher cell toxicity.

A reason for this higher toxicity, can be related with composition or with the size of the particles. The inclusion of the PCL polymer, a hydrophobic polymer, to form inulin/PCL particles together with the fact that they are particles with size around 100 nm, both characteristics contribute for the biologic effect observed. Normally, the smaller the NP, the more cellular damages can be induced, as the lower NP size is more likely to enter the cell [147]. This statement can be visualized easily in our results, as for INW and IW particles, the bigger particles with a size >1000 nm, no cytotoxicity is verified in all concentrations tested. On the other hand, the lower size particles (PINW, PIW, PNW and PW particles) a little bigger than 100 nm, induced cytotoxicity in higher concentrations.

After the MTT assay, in a way of study the effects of IW, PIW, and PW particles on the possibility of inducing inflammation, it was performed a NO release assay. NO is an important molecule in host defense response against various pathogens, although over-production of NO can lead to the development of chronic inflammation. As said in the methods part, NO assay is based on Griess reaction which allows us to indirectly determine NO production. Along with the NO study, an MTT assay was made to verify the cell viability under the assay conditions. The particles used for these assays, were freeze-dried IW, PIW, and PW particles, resuspended in pyrogen-free water, to assure the absence of endotoxins in the assay that could give rise to false positive results.

As we see in Fig. 15A, all formulations tested, at concentrations that showed no toxicity to cells (Figure 15B), didn´t induced NO. The values are even lower than the negative control

(medium) or, as expected, is much lower than the one induced by the positive control (LPS), which leads us to conclude that the IW, PIW, and PW particles do not induce NO production.

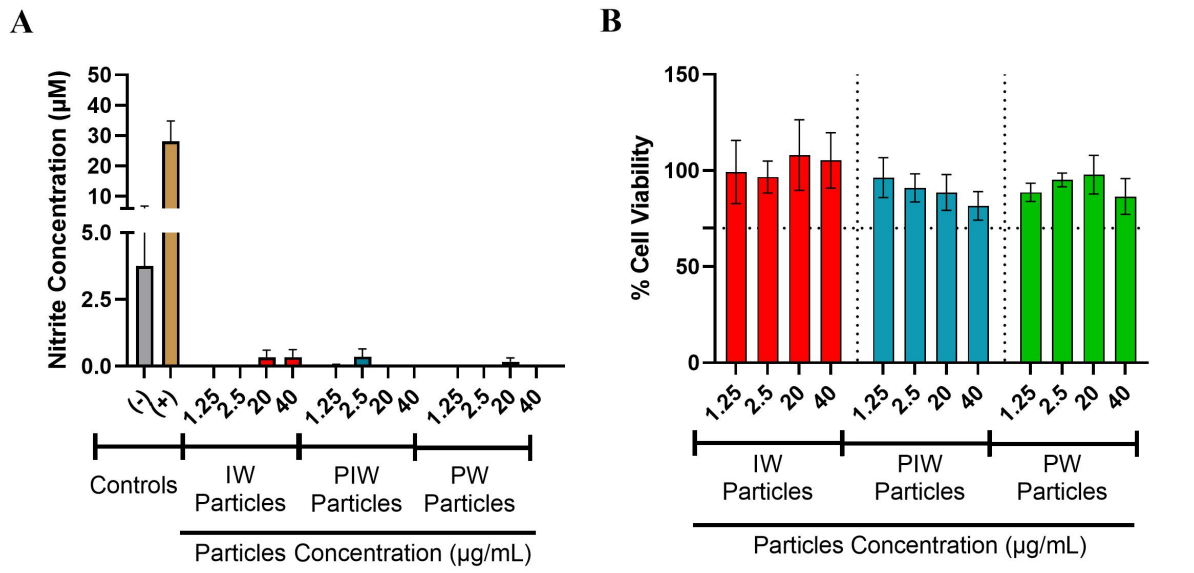


Figure 15 - *(**A**) Effect of IW, PIW and PW Particles on NO production by stimulated RAW 264.7 cells through Griess reaction after incubating the formulations for 24 h. Both results are the mean ± SEM, n=3 (three independent experiments, each in triplicate). (**B**) Cell viability evaluation using MTT tetrazolium reduction assay, after incubation of the cells with IW, PIW and PW particles. Incubation with RAW 264.7 cells was kept for 24 h. Results of IW particles are expressed as mean ± SEM, n=3 (three independent experiments, each in triplicate).*

Then, in same assay, it was also decided to evaluate other "signals" released from cells in contact with formulations. The GM-CSF cytokine levels were evaluated from the supernatants resulting from the NO assay. The ELISA evaluation of GM-CSF levels, does not reveal any production of this cytokine, leading to the conclusion that nor the IW, PIW, or PW particles can induce GM-CSF production from macrophages. An important remark is that although the LPS had induced NO production in RAW 264.7, the same cannot be said for GM-CSF. LPS, was included as the positive control in the GM-CSF production assay, however, did not worked. In the literature several reports state that LPS induces GM-CSF in RAW 264.7 cell line when using 1 µg/mL and 24 h of incubation [148;149]. In the work herein described, although we used the same experimental conditions, no production was achieved, which weakens the strength of the achieved conclusions.

Note that all the three independent assays were made without Polymyxin B (Pol. B), which is used as an antibiotic to ensure that endotoxins (present in Gram-negative bacteria cell walls) were not available in the particles, and also to ensure that no NO was induced by pro-inflammatory cytokines involved [144]. The reason for this decision, was because we made an assay previous with only higher concentrations of particles with and without Pol. B, and the results have shown similar values between solutions with or without Pol. B.

Cytokines are important immunomodulators in cell response against microbes, bacteria, or other agents. Depending on which cytokine is secreted a different immune response can be obtained [150-152]. In order to evaluate if PIW particles induce pro-inflammatory cytokine production in human dendritic cells (DCs), TNF-α, IL-6, and IL-12 levels were evaluated in the cells supernatants after 24 h incubation with the particles, by ELISA technique. TNF-α is an inflammatory mediator produced as a response against bacteria, microbes, and other agents and is released from macrophages or activated T Cells [153;154]. IL-6 has an important role in immune response since can it induce differentiation of B cells activated into antibody-producing cells, induce T-cell differentiation, induce the increase of VEGF (vascular permeability), and more [155]. IL-12 is a pro-inflammatory cytokine, product of activated inflammatory cells such as DCs, macrophages, and others, and is required to develop Th1 cell response and stimulate IFN-γ and TNF-α from T cells and NK cells [156].

In another way to see if PIW particles had anti-inflammatory properties, IL-10 levels on DCs supernatants were also evaluated with the ELISA technique.

An important observation that needs to be made is that the cells used were primary cells isolated from human blood. So, variability between assays existed, which can justify the levels of cytokines presented in Fig.16, where is presented the results of 3 independent experiments. Also, it is important to refer that incubation of the cells with the PIW particles were performed using two different particle concentrations, previously mixed with Pol.B. For control, Pol.B was also tested alone. LPS was used as the positive control.

As is possible to see in Fig.16, LPS, the positive control, has stimulated DCs to produce TNF-α, IL-6, IL-10, and IL-12. Two of the three donors have presented high levels of all cytokines. The third donor has only shown high IL-10 production. This can be justified due to the individual variability.

Also, when Pol.B was used alone to stimulate the cells, the results have shown no relevant induction of any cytokine, which means that we can use Pol.B to eliminate any endotoxin present in PIW particles, without the risk of influencing the result.

Comparing the amount of each cytokine produced by cells, particularly, between cells incubated with the particles and with the LPS, we can say that the particles at 2 μg/mL have not induced any significant amount of IL-12, IL-6, and TNF-α. However, in one donor, the IL-10 concentration achieved with the stimulation of 2 μg/mL of PIW particles, was similar to the one achieved with LPS. As for PIW particles at 4 μg/mL, again for one donor, it is possible to see the levels of IL-12 and IL-10 similar to the ones generated with LPS.

Now, if we compare the obtained results with other results found in the literature, for the TNF-α case, we can say that PIW particles induced for one donor similar results to the Chitosan NPs reported (80 % DDA, 127 nm, 29 mV and 93 % DD, 292 nm, 20 mV) [134]. Since in both cases, a TNF- α concentration lower than 500 pg/mL was induced [134]. On the other hand, the PIW particles on the other two donors have not induced TNF- α production which relates with PCL_1 (170 nm, -7.3 mV), PCL_2 (170.4 nm, -0.05 mV), PCL plus Chitosan (266.1 nm, 15.2 mV) and PCL plus Glucan NPs (183.2 nm, -0.12 mV) reported by *Sandra Jesus et al.* [157].

For the IL-6, the PIW particles have induced a little more concentration than the Chitosan NPs (80 % and 93 % DDA), being more than 100 pg/mL [134]. As for the NPs described by Sandra Jesus and his co-workers [157], it is possible to reach, the same conclusion since PIW particles, have a concentration higher than 100 pg/mL for both tested concentrations and the NPs described by *Sandra Jesus et al.* [157], have almost no induction of cytokines (except the PCL_2 NPs with Pol.B). However, when we compare the PIW particles at 2 and 4 μg/mL concentrations with the PCL_2 NPs plus Pol.B [157], we see a similarity, since it looks that both induce a cytokine concentration higher than 100 pg/mL, which can indicate that possibly the PCL in PIW particle can be inducing the IL-6. However, note that both the levels for TNF-α and IL-6 (Figure 16) are always lower than the levels found for cells with LPS.

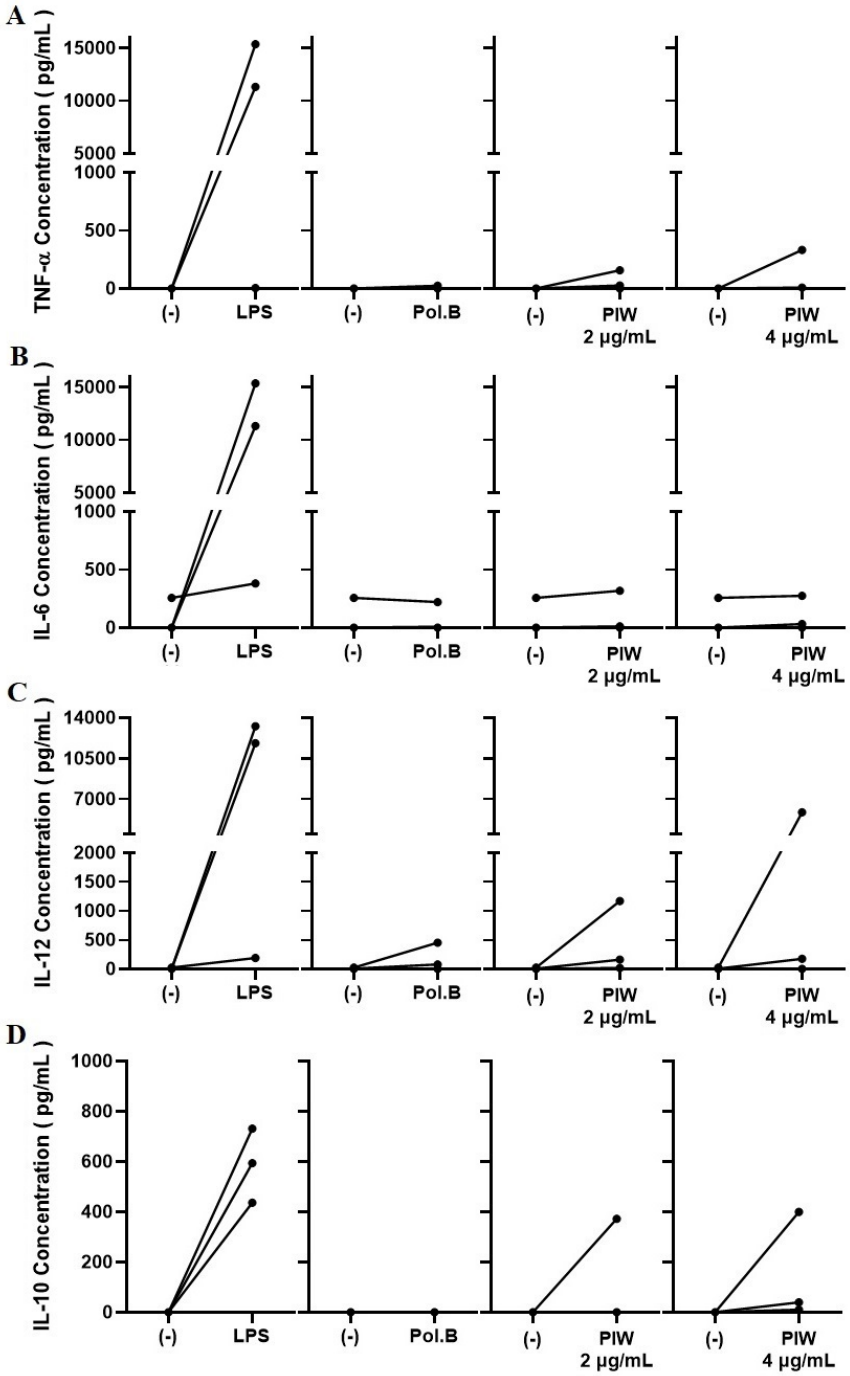


Figure 16 - *Effect of PIW NPs on TNF-α, IL-6, IL-12 and IL-10 production in Dendritic Cells: (**A**) The concentration of cytokine TNF-α represented in pg/mL, (**B**) The concentration of cytokine IL-6 represented in pg/mL, (**C**) The concentration of cytokine IL-12 represented in pg/mL, (**D**) The concentration of cytokine IL-10 represented in pg/mL. These cytokines were measured using commercially available ELISA kits. LPS 0.025 ng/mL was used as a positive control and as expected induced elevated concentrations of this cytokine. Supernatants were harvested after 24 h of incubation with the formulations. Results represent the mean, n=3 (three independent experiments).*

4. Concluding remarks and future perspectives

It is a matter of fact that nanotechnology is a valuable tool, permitting us to study structures in the nanoscale range and develop innovative medicines, more effective and more target precise.

At the end of this dissertation, is possible to conclude, that we reach the established goals, as we developed and produced Inulin based particles blended or not with a second polymer, in this case, PCL. Unfortunately, although we have also tested the blend with chitosan, several challenges particularly related to inulin quantification in the blend chitosan NPs, inhibited us from testing the formulations further. Consequently, we decided to abandon these particles, leaving of course the door open for return at this in the future.

To reach the main goal, different procedures were studied and used as described in the methods section. The first major challenge encountered was to obtain a particle made with Inulin alone and a second delivery system constituted by inulin and PCL, since we could not find in the literature any method already described.

As far as these particles were characterized, we can say that the INW and IW are not nanoparticles but yet microparticles, since the size is higher than 1000 nm (1586.29 nm and 1320.86 nm respectively). However, the particles obtained by mixing inulin with PCL (PINW and PIW) were smaller and their sizes were 360.14 nm and 301.26 nm, respectively. As observed in scientific literature, it is common to consider it nanoparticles. In terms of zeta potential, it is possible to say that only the inulin particles have a neutral zeta potential, with the rest of the particles (PINW, PIW, PNW and PW NPs) presenting a negative charge, not exceed the -20 mV.

Yet, as already explained in the discussion the observed negative zeta potential of the particles probably can be explained by the presence of the surfactant (Pluronic® F68) used during the preparation of the particles and is not the result of the presence of the inulin or the PCL into the particles.

Following to the evaluation of the stability, we have concluded that PIW and PW particles remain stable since the size of the particles did not change during the time period of the assay, while IW particles present variability over time, with a tendency to increase its size, or forming agglomerates.

As already highlighted, immunotoxicity tests were done in primary cells obtained from $CD1^+$ mice (splenocytes) and in a macrophage cell line, RAW 264.7. Both types of cells represent cells of the immune system, being, therefore, suitable models to study the

immunotoxicity. The cell (splenocytes and RAW 264.7) viability was assessed when cells were incubated with INW and IW particles. It was observed that these particles are not toxic for cells as compared to the other developed particles. The higher concentrations of the PINW and PNW particles showed some toxicity in the RAW 264.7 cell line, and this effect was proved to be from the particles and not from the particles supernatant.

To unravel some immunomodulation effects, different assays were performed, such as the analysis of the NO production and GM-CSF production by RAW 264.7 cells and the production of cytokines as TNF-α, IL-6, IL-10, and IL-12 on DCs. Overall, none of the particle formulations have induced NO production, neither induced GM-CSF. As for the production of cytokines by DCs, upon stimulation with PIW particles, the PIW particles at 2 μg/mL have shown no significant induction of cytokines, since all cytokine concentrations were similar to the negative control. However, at 4 μg/mL, the PIW particle induces a small concentration of IL-12 and IL-10 in one donor. This result per si has no significance, and such variability between donors must attenuated by performing further assays.

Overall, with the developed work we reached a reproducible production method for the preparation of PCL-Inulin particles by a nanoprecipitation method. These particles present cytocompatibility in a considerable range of concentrations 3.66 μg/mL to 58.59 μg/mL and 19.53 μg/mL to 1250 μg/mL in raw 264.7 cells and splenocytes, respectively, and also are not expected to induce pro-inflammatory effects as demonstrated by the lack of cytokine induction on DCs. Consequently, they constitute a promising candidate to be further studied as a vaccine adjuvant, for instance for the hepatitis B antigen.

Unfortunately, despite the time-consuming work performed in an attempt to produce chitosan-inulin particles, several setbacks did not allow us to successfully complete this task. Nonetheless, the preliminary work performed, was reported in the supplementary material and can be the starting point for further work at the lab.

In terms of future perspectives for PCL-inulin based particles, it would be interesting to make more assays in DCs with PIW, in a way to mitigate the results obtained; study the use of HBsAg, HBV antigen, in the developed Inulin particles, as well as to verify their behavior *in vivo* with or not HBsAg antigen.

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5.2. Image References

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6. Supplemental Information

6.1. Development of the method to produce Chitosan-Inulin NPs

Purified 93 % DDA Chitosan was obtained with a routine technique used by the group and previously described [160]. Briefly, 1 g of chitosan was dissolved in 10 mL of NaOH solution with a concentration of 1 M, using magnetic stirring, for 3 h at 40 - 50 °C. Next, the suspension was vacuum filtered, the insoluble chitosan present on the filter was recovered by adding 200 mL of acetic acid solution 1 %, using magnetic stirring for 1 h, at room temperature. After that the solution was filtered into a 500 mL beaker, the pH was adjusted to pH=8 and the precipitated chitosan was centrifuge for 30 min at 4500*g. The supernatant was discarded, and the pellet washed by adding 20-30 mL of Milli-Q water. The washing step was repeated 3 times, and then the chitosan powder was recovered after a freeze-dry procedure.

Chitosan NPs were obtained, by a routine technique used by the group. 93 % DDA chitosan at 0.1 % was dissolved in 1 % of acetic acid and left overnight at room temperature in tube rotator. After this time the pH of chitosan solution was adjusted to a pH [4.6;4.8], and 10 mL of this adjusted chitosan solution was added to a scintillation bottle where next was added dropwise 1750 µL of tripolyphosphate aqueous solution at 0.16 % (weight/volume) using a high-speed homogenizer at 25000 rpm. After 1 min under high-speed homogenization the vial was left under magnetic stirring for 30 min and then was centrifuged at 10000*g for 15 min at 20 °C. Then the supernatant was removed, the pellet was washed by adding 1 mL of MilliQ water and was centrifuge again at 7000*g for 15 min at 20 °C. After centrifugation supernatant was again removed, and the pellet was resuspended in MilliQ water until 1 mL of the volume was obtained. These Chitosan NPs were store at 4 °C.

To form Chitosan-Inulin NPs, a certain volume of Inulin according to the test condition reported in Table 7, at a concentration of 1 mg/mL, previously heated at 80 °C, was added to 50 µL of Chitosan NPs under vortex agitation, and then the NPs were incubated in a tube rotator for 1 hour. Finally, they were centrifuged at 15000*g for 10 min, the supernatant was removed, and the pellet was resuspended in 1 mL of MilliQ water.

Table 7 - *Overall summary of the conditions tested to produce Chitosan-Inulin particles for 1 hour, 3 hour and overnight incubation.*

Inulin [1 mg/mL] (µL)	Chitosan NPs (µL)	H_2O MilliQ (µL)	Inulin final Concentration (µg/mL)
375	50	75	750
250	50	200	500
125	50	325	250
62.5	50	387.5	125
0	50	450	0

Another condition tested was trying to form chitosan particles with inulin, adding the Inulin to when forming Chitosan particles was forming. The protocol used is a modified version from the previous one as Inulin at a concentration of 2000 µg/ml and 570 µg/ml was added to the 0.16 % TPP solution, which was subsequently added dropwise as described in the protocol above.

To determine how much inulin was adsorbed in the Chitosan NPs a modified version of the technique reported at [161] was used. It consists in the use of a solution with 1.52 g of Vanillin to 200 mL of Sulfuric acid 98%. In a 96-well plate, a calibration curve of Inulin solution was prepared in triplicate in concentrations ranging between 0 and 1000 µg/mL. 100 µL of each sample was added to the well, in triplicate and 100 µL of Vanillin solution were added after this the plate was left to incubate in a dark environment for 1hour and the absorbance values were measured at 492 nm using a microplate reader. The percent values of Loading Efficacy (LE) were calculated by the following equation:

$$LE = \frac{Total\ amount\ of\ Inulin - Non\ bound\ Inulin}{Total\ amount\ of\ Inulin} * 100$$

6.2. Chitosan and Chitosan-Inulin particles Physicochemical characterization results and discussion

Since we did not find any method in the literature describing the production of chitosan-inulin particles, intense work was necessary to discover a suitable method. Initially, an attempt to make Chitosan-Inulin particles was made adapting an optimized protocol of chitosan nanoparticles available at the lab [134]. Inulin was added to the chitosan nanoparticle production method by mixing Inulin with crosslink (TPP) solution. Unfortunately, the results showed that the concentration of Inulin retained in the Chitosan particle during formation was none or very low.

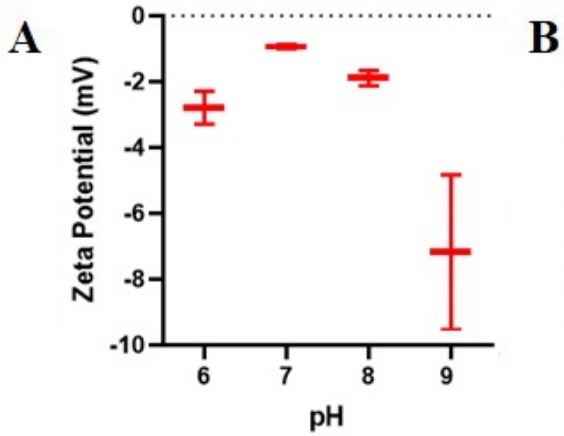
So, it was decided that trying to adsorb Inulin to the surface of Chitosan NPs could be better as Chitosan is a positive polymer and Inulin is a neutral polysaccharide, then we test different volumes of Inulin each one with different times of incubation, 1 hour, 3 hours and overnight as we can see in Table 8. After incubation time it was made the modified colorimetric technique. The results indicate a variability between days and incubation time, indicating that we do not have always the same percentage adsorption of Inulin as is possible to see in Table 8.

Table 8 - *Results of the loading efficacy (LE %) obtained for Chitosan NP's (NP's at a concentration of 10 mg/mL, used only 50 µL) using Inulin. Inulin was adsorbed to the particle surface using three different times of incubation: 1 hour, 3 hour and Overnight. Data are expressed as mean ± SD, n=5 (five independent experiments, each in triplicate) for 1 hour, n=2 (two independent experiments with different batch's, each in triplicate) for 3 hour of incubation and n=1 (one experiments with two batch's, each in triplicate) for overnight.*

	Loading Efficacy (%)		
Free Inulin concentration if not bind to the particles (µg/mL)	1 hour of incubation	3 hours of incubation	Overnight incubation
750	83.99 ± 3.58	44.40 ± 8.17	----
500	43.46 ± 28.56	38.31 ± 28.39	16.04 ± 22.68
250	67.45 ± 30.20	75.47 ± 11.25	74.97 ± 31.42
125	100 ± 0	100 ± 0	61.64 ± 54.25
0	0	0	0

These results were not expected, because chitosan is a positive polymer and Inulin is a negative polysaccharide, so it was expected that all or almost all, Inulin become adsorbed to the surface of chitosan particles.

In order to improve the results, it was hypothesized that since chitosan has a positive charge and Inulin at pH=12 is much more negative, it would be possible to obtain more adsorbed Inulin at that pH. Note that before we elaborate this hypothesis, we have made a titration where we have tested inulin particles with pH ranging from [6-9], and the result, Fig. 17, have shown that in the higher pH tested, pH close to 9, we have got the more negative zeta potential, leading us to hypothesize that maybe in pH 12 we can get the highest negative value of zeta potential.



B

	Zeta Potential	
pH	Mean (mV)	SD (mV)
6	-2.79	± 0.50
7	-0.94	± 0.09
8	-1.89	± 0.24
9	-7.18	± 2.33

Figure 17 - *(**A**) Overall summary of zeta potential of Inulin Particles washed at pH close to 6, 7, 8, 9. Results are the mean ± SD, n=4 (4 independent experiments, each in triplicate). (**B**) Mean and Standard Deviation (SD) of zeta potential of Inulin Particles washed at pH close to 6, 7, 8, 9, n=4 (4 independent experiments, each in triplicate).*

To confirm this hypothesis, we add in a vial 1mL of Chitosan particles, and dropwise under magnetic stirring, it was added 0.5 mL of Inulin [2.5 mg/mL] at pH=12 and kept for 1 h in incubation. As we can see with values present in Table 9, when we mix Inulin at pH=12 with

Chitosan particles we obtain microparticles, much larger than the original chitosan NPs. If we do the same procedure without the pH=12, the Chitosan-Inulin NPs present similar size and zeta potential as bare chitosan NPs. Regarding zeta potential, when we use Inulin at pH close to 12, we have a negative zeta potential and when we use Inulin that is not at pH close to 12, we have a positive zeta potential. Importantly, the observed negative zeta potential is likely to be resultant from the inversion of charge on chitosan due to the extreme basic environment. A previous study from our laboratory has shown that chitosan NPs when at pH=12 acquire negative charge and form large agglomerates [138].

Importantly, the technique used to determine the amount of adsorbed Inulin, is a colorimetric technique described in the literature [161], that we found to be not reliable. To evaluate its reliability, we tested wells with a known inulin concentration, and results originated values under that concentration. Consequently, being aware of this problem we could not rely on more results using this technique.

Table 9 - *Mean of size, PDI, Zeta Potential and MOB, in Chitosan with/without Inulin [2.5 mg/mL] at pH=12 and without pH=12. In blue we have Chitosan – Inulin at pH=12.*

	Size (nm)	PDI (nm)	Zeta Potential (mV)
Chitosan Particles	286.6	0.26	41.03
Chitosan with Inulin in a concentration 2.5 mg/ml with pH=12	2650	0.76	-18.7
Chitosan without Inulin in a concentration 2.5 mg/ml without pH=12	274.7	0.3	30.6

Despite the efforts made to produce chitosan-inulin NPs, the lack of an effective way to quantify inulin in the particles, it was decided not to proceed for *in vitro* studies with these formulations.