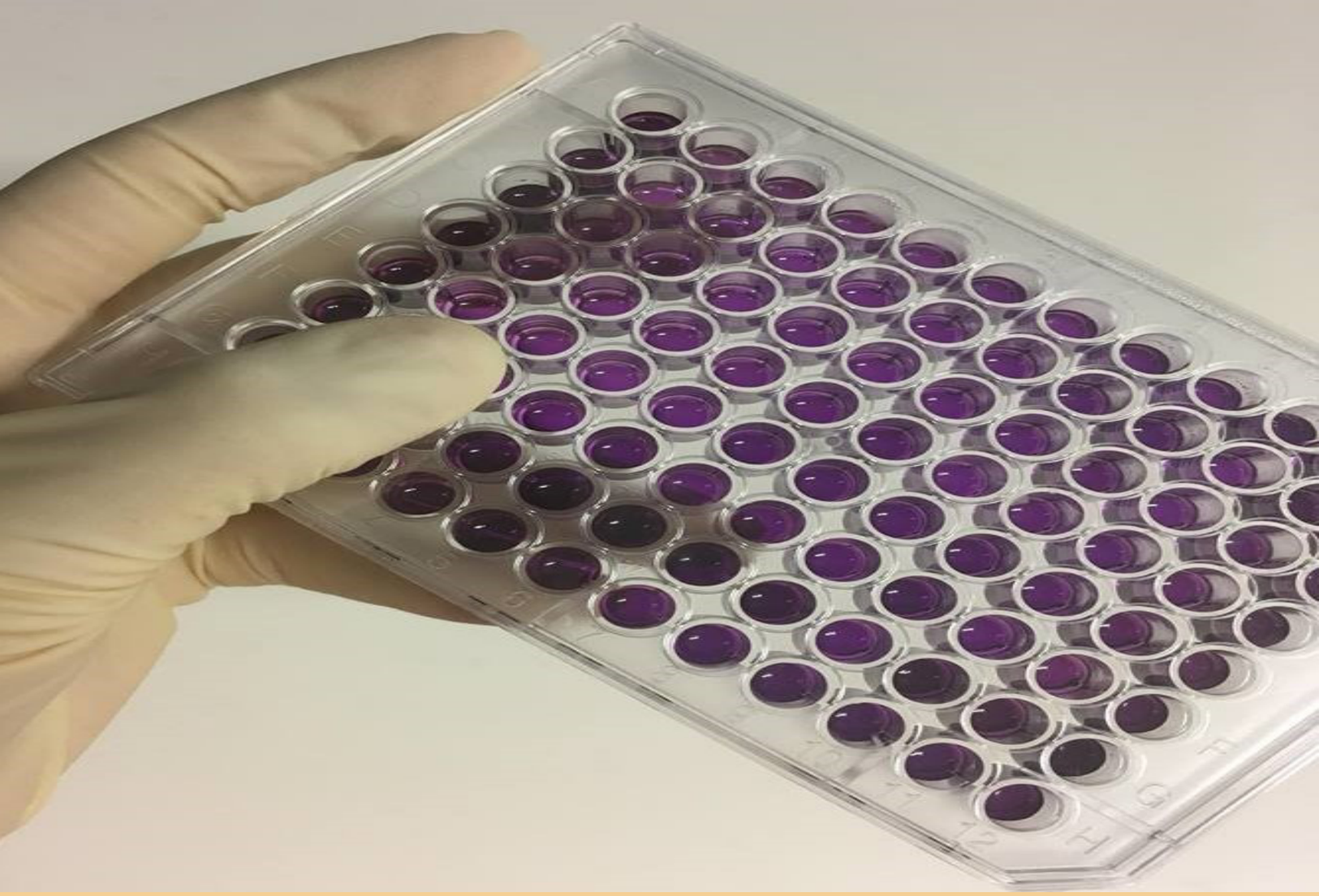

Alana Gabriely Reis Duarte

Immunocytotoxicity of Chitosan Nanoparticles: Effect of Chitosan Deacetylation Degree

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pela Professora Doutora Olga Maria Fernandes Borges Ribeiro, e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Julho 2018

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Universidade de Coimbra

Front page figure caption: image of 96-well plate used in a MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.

Immunocytotoxicity of Chitosan Nanoparticles: Effect of Chitosan Deacetylation Degree

Alana Gabriely Reis Duarte

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

Tese de candidatura ao grau de mestre em Biotecnologia Farmacêutica, apresentada à Faculdade de Farmácia da Universidade de Coimbra

The experimental work presented in this thesis was developed under the scientific supervision of Professor Olga Maria Fernandes Borges Ribeiro from the Pharmaceutical Technology Laboratory of Faculty of Pharmacy, University of Coimbra. This work was funded by ERDF funds through the Operational Programme Competitiveness Factors - COMPETE 2020, and through the Centro 2020 Regional Operational Programme under project CENTRO-01-0145-FEDER-000008: BrainHealth 2020 and national funds by FCT - Foundation for Science and Technology under the project PROSAFE/0001/2016 and strategic project POCI-01-0145-FEDER-007440. Alana Duarte thanks Dr.ª Ana Donato for expertise and assistance in hemocompatibility studies in the Clinical Analysis Laboratory of Faculty of Pharmacy.

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Acknowledgements / Agradecimentos

Com a conclusão desta etapa, gostaria de deixar aqui o meu agradecimento a todos aqueles que de alguma forma contribuíram para a realização deste trabalho.

À minha orientadora, Professora Doutora Olga Maria Fernandes Borges Ribeiro, pela oportunidade de realização deste trabalho no seu laboratório, permitindo-me contactar com o mundo da investigação científica. Obrigada por todo o apoio, orientação, conhecimentos transmitidos e disponibilidade.

À Patrícia Marques, Sandra Jesus, Edna Soares e ao João Panão que sempre se mostraram disponíveis a ajudar-me e ensinar-me ao longo deste ano tendo sido sem dúvida um grande apoio. Obrigada por todos os momentos que partilhámos dentro e fora do laboratório. A vossa amizade, simpatia e boa disposição tornou tudo mais fácil.

À Mariana Colaço, minha colega de mestrado, companheira de laboratório pela disponibilidade e apoio demonstrados. Obrigada pela amizade, boa disposição e pela companhia ao longo deste ano de trabalho.

Às restantes meninas do "Nanolab", Jéssica da Silva e Natália Bernardi, agradeço pela vossa simpatia e pela vossa disponibilidade para os nossos almoços saudáveis.

A todos que trabalham no piso 4 da Faculdade de Farmácia da Universidade de Coimbra e que sempre mostraram disponibilidade para ajudar.

Ao meu namorado Tony, agradeço não só pelo companheirismo, apoio e amor, mas também pela paciência e incentivo que foram sem dúvida muito importantes para mim. Obrigada por tudo.

Aos meus amigos que me apoiaram, incentivaram e com quem partilho tantos momentos. Vocês sabem quem são, obrigada por tudo.

Finalmente, quero agradecer à minha família em especial à minha mãe, ao meu pai e ao meu irmão por toda a educação, carinho e amor. Obrigada por tudo o que me têm proporcionado, por me ajudarem a chegar até aqui e por sempre me terem incentivado para a realização dos meus sonhos.

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Abbreviations

Abs – Absorbance

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

APTT – Activated partial thromboplastin time

BSA – Bovine serum albumin

$Chit_A$ NPs – Chitosan NPs Method A

$Chit_B$ NPs – Chitosan NPs Method B

CMH – Cyanmethemoglobin

CryoSEM – Cryo-scanning electron microscopy

DD – Deacetylation degree

DLS – Dynamic light scattering

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl sulfoxide

dTBH – diluted total blood hemoglobin

ELISA – Enzyme-Linked Immunosorbent Assay

FBS – Fetal bovine serum

Filt – Nanoparticle solvent

HRP – Avidin-Horseradish peroxidase

IL – Interleukin

IL-1β – Interleukin-1β

LPS – Lipopolysaccharide

MTT – Thiazolyl blue tetrazolium bromide

MW – Molecular weight

NC – Negative control

NMR – Nuclear magnetic resonance

NO – Nitric oxide

NPs – Nanoparticles

OD – Optical density

PBMCs – Peripheral blood mononuclear cells

PBS – Phosphate buffer saline

PC – Positive control

PDI – Polydispersity index

PLC – Platelet count

PRP – Platelet-rich plasma

PT – Prothrombin time

RPMI – Roswell Park Memorial Institute 1640

RT – Room temperature

SEC – Size exclusion chromatography

SEM – Scanning electron microscopy

T – Temperature

TNF-α – Tumor necrosis factor alpha

TPP – Tripolyphosphate

ZP – Zeta potential

Abstract

Nanoparticles (NPs) conquered an important role in many areas such as drug delivery. The number of studies that include them has grown in the last years. Nonetheless, the correlation between their properties and their effects on the immune system is poorly understood. Chitosan is a natural polymer that has shown interesting properties as biocompatibility and biodegradability. Considering the application of Chitosan NPs in the drug delivery field, the aim of this work was to study its immunotoxicity as a case study. On the one hand it was tried to clarify some controversial information that was found in the literature related with immunotoxicological properties of the chitosan NPs and on the other as a secondary objective to establish methods for testing immunotoxicity of the NPs that can be adapted to other nanomaterials which is important to their biomedical application and safe design.

This work evaluated the Chitosan NPs toxicity in murine macrophages (RAW 264.7) and in human peripheral blood mononuclear cells (PBMCs). Other parameters important to the immunotoxicological assessment as the hemocompatibility were evaluated and was reported the relevance of the correct characterization of chitosan as their properties as deacetylation degree (DD) and molecular weight (MW).

Chitosan NPs were produced by a coacervation method with different crosslinks: Method A with tripolyphosphate ($Chit_A$ NPs) and Method B with sodium sulphate ($Chit_B$ NPs). The average size of the $Chit_A$ NPs were 102.2 ± 8.7 nm, 133.1 ± 4.6 nm and 269.4 ± 38.5 nm with a chitosan DD of 80 %, 86 % and 93 %, respectively. With the method B, the average size of the Chi_B NPs obtained were 351.7 ± 32.5 nm and 549.6 ± 12.4 nm with a chitosan DD of 80 % and 86 %, respectively. These results showed that the particle size increased with the chitosan DD and MW. Quantification studies showed that almost 100 % of chitosan was incorporated in the NPs. Stability tests were performed, and it was showed that $Chit_A$ NPs are stable at 4 °C and 20 °C at least for 5 weeks. The stability results in cell media showed a size increase after 24 h incubation with DMEM. In the incubation with RPMI the $Chit_A$ NPs 93 % also showed a size increase while the size of $Chit_A$ NPs decreased. Relatively to the immunotoxicological tests, the NPs showed to be more cytotoxic than the polymers in human PBMCs and in murine macrophages. An inhibitory effect of the NPs and polymers in LPS-induced NO production was observed which was more significant with the $Chit_A$ NPs with lower DD (80 %). However, without LPS, none of the concentrations of the NPs and polymers had a stimulatory effect in the NO production. The effect of chitosan in cytokine production was evaluated with two pro-inflammatory cytokines: TNF-α and IL-1β. None of

the Chitosan NPs induced TNF-α, while the polymer with higher DD (93 %) showed to induce this cytokine production in a concentration-dependent manner. Both polymers and NPs were not able to stimulate the production of the IL-1β. The hemocompatibility of chitosan was also evaluated neither the NPs or polymers had hemolytic effect, but the $Chit_A$ NPs 80 % at 1mg/mL affected the plasma coagulation time by the intrinsic pathway. The effect on platelet aggregation wasn't conclusive as during the assay, the interference of the NPs with the method was observed.

These results show that the DD of chitosan and nanoparticle size can affect some immunotoxicological parameters. This work also highlights the importance of selecting appropriate methods and controls to avoid misinterpretations. These results together with further studies will contribute to develop a knowledge base and guidelines to implement the safe-by-design approach for nanobiomaterials, with focus on polymeric drug delivery systems.

Keywords: Chitosan; nanoparticles; polymer; immunotoxicity; safe-by-design.

Resumo

As nanopartículas (NPs) conquistaram um papel importante em muitas áreas, como a entrega de fármacos. O número de estudos que as incluem tem crescido nos últimos anos. No entanto, a correlação entre suas propriedades e seus efeitos sobre o sistema imunitário é pouco compreendida. O quitosano é um polímero natural que tem mostrado propriedades interessantes tais como biocompatibilidade e biodegradabilidade. Considerando a aplicação de NPs de quitosano na área de entrega de fármacos, o objetivo deste trabalho foi estudar a sua imunotoxicidade como um caso de estudo. Por um lado, procurou-se esclarecer algumas informações controversas encontradas na literatura relacionadas às propriedades imunotoxicológicas das NPs de quitosano e, por outro, como objetivo secundário estabelecer métodos para testar a imunotoxicidade das NPs que podem ser adaptados a outros nanomateriais o que é importante para a sua aplicação biomédica e design seguro.

Este trabalho avaliou a toxicidade de NPs de quitosano em macrófagos e murganho (RAW 264.7) e em células mononucleares do sangue periférico humano (PBMCs). Outros parâmetros importantes para a avaliação imunotoxicológica tais como a hemocompatibilidade foram avaliados e foi mostrada a relevância da correta caracterização do quitosano tal como suas propriedades como grau de desacetilação (DD) e peso molecular (PM).

As NPs de quitosano foram produzidas por um método de coacervação com diferentes ligações cruzadas: Método A com tripolifosfato ($Chit_A$ NPs) e Método B com sulfato de sódio ($Chit_B$ NPs). O tamanho médio das NPs de $Chit_A$ foi de 102,2 ± 8,7 nm, 133,1 ± 4,6 nm e 269,4 ± 38,5 nm com um quitosano com DD de 80 %, 86 % e 93 %, respetivamente. Com o método B, o tamanho médio das $Chit_B$ NPs obtidas foi de 351,7 ± 32,5 nm e 549,6 ± 12,4 nm com um quitosano com DD de 80 % e 86 %, respetivamente. Estes resultados mostraram que o tamanho das partículas aumentou com o DD e MW do quitosano. Estudos de quantificação mostraram que quase 100 % do quitosano foi incorporado nas NPs. Testes de estabilidade foram realizados, e foi demonstrado que as $Chit_A$ NPs são estáveis a 4 °C e 20 °C pelo menos por 5 semanas. Os resultados de estabilidade em meios celulares mostraram um aumento de tamanho após 24 h de incubação com DMEM. Na incubação com RPMI, as $Chit_A$ NPs 93 % também apresentaram um aumento de tamanho, enquanto o tamanho das $Chit_A$ NPs diminuiu. Relativamente aos testes imunotoxicológicos, as NPs mostraram ser mais citotóxicas do que os polímeros em PBMCs humanas e em macrófagos de murganho. Observou-se um efeito inibitório das NPs e polímeros na produção de NO

induzida por LPS, que foi mais significativo com as $Chit_A$ NPs com menor DD (80 %). No entanto, sem o LPS, nenhuma das concentrações das NPs e polímeros teve efeito estimulante na produção do NO. O efeito do quitosano na produção de citocinas foi avaliado com duas citocinas pró-inflamatórias: TNF-α e IL-1β. Nenhuma das NPs de quitosano induziu TNF-α, enquanto o polímero com maior DD (93 %) mostrou induzir a produção de citocinas de maneira dependente da concentração. Ambos os polímeros e NPs não foram capazes de estimular a produção da IL-1β. A hemocompatibilidade do quitosano também foi avaliada, nem as NPs ou polímeros tiveram efeito hemolítico, mas as NPs de $Chit_A$ 80 % a 1mg/mL afetaram o tempo de coagulação plasmática pela via intrínseca. O efeito na agregação plaquetária não foi conclusivo, pois durante o ensaio, a interferência das NPs com o método foi observada.

Esses resultados mostram que o DD do quitosano e o tamanho das nanopartículas pode afetar alguns parâmetros imunotoxicológicos. Este trabalho também destaca a importância de selecionar métodos e controlos apropriados para evitar interpretações erradas. Esses resultados, juntamente com outros estudos, contribuirão para o desenvolvimento de uma base de conhecimento e diretrizes para implementar a abordagem safe-by-design para nanobiomateriais, com foco em sistemas de entrega de fármacos poliméricos.

Palavras-chave: Quitosano; nanopartículas; polímero; imunotoxicidade; safe-by-design.

Chapter I
Introduction

1.1. Nanotechnology

Nanotechnology is a multidisciplinary field that can be defined as the management of material properties at nanoscale (size range between 1 nm and 100 nm) by their production and use with novel properties and functions (Samir *et al.*, 2015). It is considered to be an emerging area with a big potential in medical application. (EMA, 2006).

The European Medicines Agency (EMA) defines nanomedicine as a nanotechnology application with the purpose of improving disease diagnosis, treatment and prevention (EMA, 2006). The exploration of engineered nanomaterials has increased over the years in many areas, such as drug delivery of novel drugs or the reformulation of traditional medicines with the improvement of stability, solubility, pharmacokinetics and reduction of immunotoxicity (Dobrovolskaia, 2016).

Nanoparticles (NPs) are defined to have a diameter in the range of 1 nm to 100 nm but commonly a broader scale between 1 nm and 1000 nm is applied in the particles. Nanoparticles can have many physical and chemical properties that can be modulated accordingly with the desired application, such as vaccine adjuvants where immunostimulation is desirable (Dobrovolskaia e McNeil, 2007). Exists many types of nanoparticles such as liposomes, metallic nanoparticles, polymeric micelles, polymeric nanoparticles and others (Bhatia, 2016; Bolhassani *et al.*, 2014).

1.2. Polymeric nanoparticles - Chitosan

Polymeric nanoparticles can be prepared from synthetic or natural polymers and they are the most-common materials to the production of nanoparticle-based drug due to their unique properties such as easy synthetization, low costs, biocompatibility, biodegradability, non-immunogenicity, non-toxicity (Bolhassani *et al.*, 2014; Crucho e Barros, 2017). Cationic polymers such as polyethyleneimine, poly(lactic-co-glycolic acid) also seem to produce more stable complexes and have been object of many studies and among the natural polymers chitosan has attracted attention (Bolhassani *et al.*, 2014; Chopra *et al.*, 2006).

Chitosan is a natural polymer β-(1-4)-linked D-glucosamine (deacetylated unit) and Nacetyl-D-glucosamine (acetylated unit) monomers (Figure 1) derived from the partial deacetylation of chitin, a polysaccharide that is a structural component of the exoskeleton of crustaceans and insects (Nadesh *et al.,* 2013).

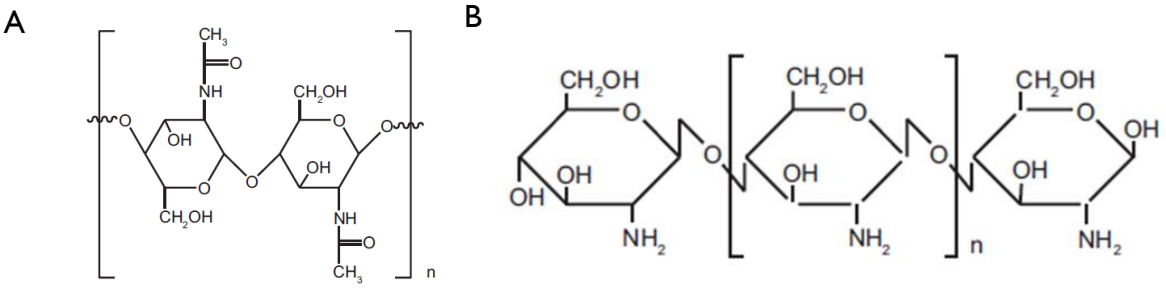


Figure 1: Chemical structure of chitin (A) and chitosan (B) (Adapted from (Islam *et al.*, 2012)).

This polymer is a weak base, being insoluble in neutral and alkaline pH values. In acidic medium, the availability of free amino groups makes chitosan a soluble positive charged polymer (Illum *et al.*, 2001). The term chitosan refers not only to one polymer, but a wide variety of polymers that differ in the deacetylation degree (DD) and molecular weight (MW) that can range between from 40 % to 98 % and from 50 kDa to 2000 kDa, respectively (Hejazi e Amiji, 2003). Deacetylation degree and molecular weight are important to chitosan characterization due to their influence in the properties of formulations based on chitosan (Sinha *et al.*, 2004). Chitosan has shown to be a very versatile material due to its attractive properties as biocompatibility and biodegradability (Kean e Thanou, 2010). In addition, chitosan has also mucoadhesive properties derived from the OH and NH_2 capacity to form hydrogen bonds which can be useful for example, to prolong contact at the site of administration of some drugs (Chopra *et al.*, 2006). Chitosan natural abundance also makes this a reasonable cost polymer (Islam *et al.*, 2012). Therefore, chitosan has been used in many areas, such as agriculture, cosmetics, food processing and in biomedicine, as tissue engineering, vaccines (Wiegand, Winter e Hipler, 2010). Chitosan has also been studied to be applied as a drug delivery vehicle of many drugs, proteins, peptides and nucleic acids to controlled release in the therapy of cancer and other diseases (Rhee *et al.*, 2014; Kim *et al.*, 2006; Chronopoulou *et al.*, 2016) The exploration of chitosan as nanocarrier may allow the drug delivery of novel medicines or the reformulation of traditional medicines with the improvement of stability, pharmacokinetics and immunomodulation (Dobrovolskaia, 2016).

However, its immunotoxicological evaluation is poorly systematized and even some studies reveal contradictory results.

1.3. Importance of immunotoxicological studies

Immunotoxicological studies of nanoparticles (NPs) are extremely important as research has shown the potential of NPs to interact with the immune system by stimulating or suppressing immune responses (Figure 2) (Dobrovolskaia e McNeil, 2007). Although this immunomodulating potential can be desirable, for example, in the NP application as adjuvants to increase vaccine efficacy, the unexpected side effects must be considered as they can lead to unpredictable outcomes (Dobrovolskaia e McNeil, 2007; Jiao, 2014).

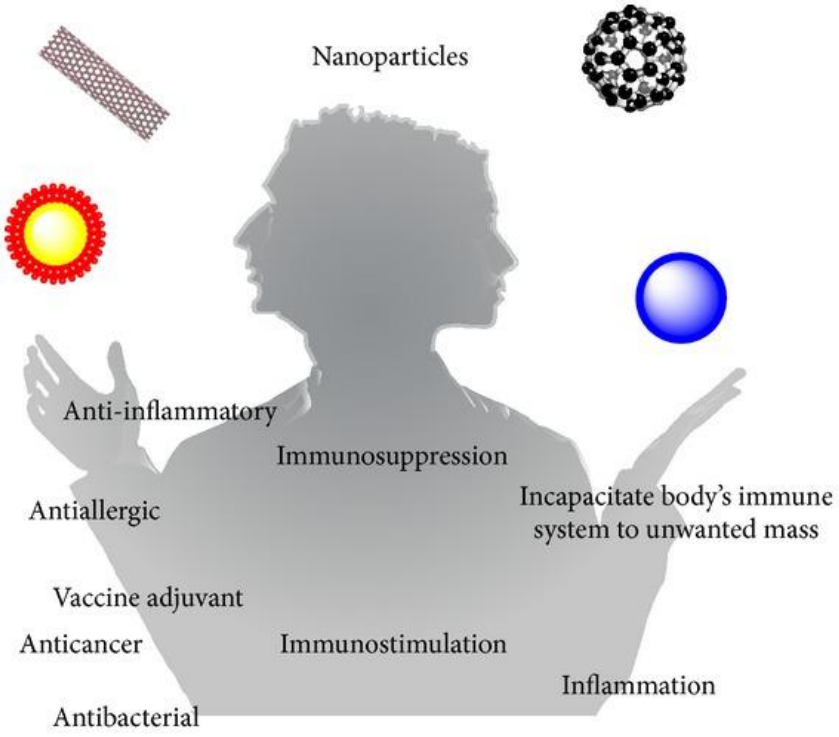


Figure 2: The immunomodulation of NP in nanomedicine applications: immunosuppression vs immunostimulation. (Adapted from (Jiao, 2014)).

Besides that, even with the rigorous toxicological studies applied to medicines, between 10 % and 20 % of drugs were withdrawn from the market between 1969 and 2005 due to immunotoxic effects (Wysowski e Swartz, 2005). As medicines, nanomaterials also need to be subject to an immunotoxicological evaluation to study the interactions with the immune system before regulatory approval (Dobrovolskaia *et al.*, 2008). However, there's a lack of guidelines specifically developed and applied to nanomaterials with standardized and validated immunotoxicological tests that can help to understand their biological effect which is important to their biomedical application and safe design (Hirsch *et al.*, 2010; Jiao, 2014).

1.4. *In vitro* tests for immunotoxicological assessment

To date there is no International guideline providing guidance on toxicity and biocompatibility testing for nanomaterials and, in particular, for nanotechnology-drug release systems. However, there are international guidelines, which guide us for other materials and can serve as guidelines for nanoparticle studies. For instance, the International Standard ISO-10993, "Biological evaluation of medical devices" provides guidance to the risk and biocompatibility assessment of medical devices. According to ISO-10993, the biocompatibility assessment includes the evaluation of *in vitro* cytotoxicity, systemic toxicity, irritation, sensitization, hemocompatibility, implantation, genotoxicity, carcinogenicity and effects on reproduction, including developmental effects. Not all of the tests need to be done, it depends on the purpose of the medical device and other criteria like the device properties or the nature of exposure to the body (Goode, 2016). Some of these biocompatibility tests can also be important to NPs studies, namely the hemocompatibility as many are produced to be administered systemically (Li *et al.*, 2009). While in the circulatory system the NPs will interact with immune cells, blood cells, plasma proteins and can affect normal physiologic processes (Huang *et al.*, 2016). Consequently, tests for assessing the hemolytic activity of NPs, their effects in platelet function (thrombogenicity) and in coagulation can be performed as described in ISO-10993 with the necessary adaptations.

Dobrovolskaia and co-workers highlighted the selection of appropriate and relevant study models, as one of the challenges of *in vitro* assessment of nanoparticle immunotoxicity (Dobrovolskaia e McNeil, 2016).

1.5. Relevant models to study nanoparticle effects in immune system

One of the most relevant questions to study the in vitro immunotoxicity is the choice of the cell line that will be most appropriate to the study and which can better mimic what will happen *in vivo*. Cell lines are often used in research as they offer several advantages, such as their low costs in maintenance, are easy to work, allow an unlimited supply of material and avoid ethical concerns associated with the use of animal and human tissue (Kaur e Dufour, 2012).

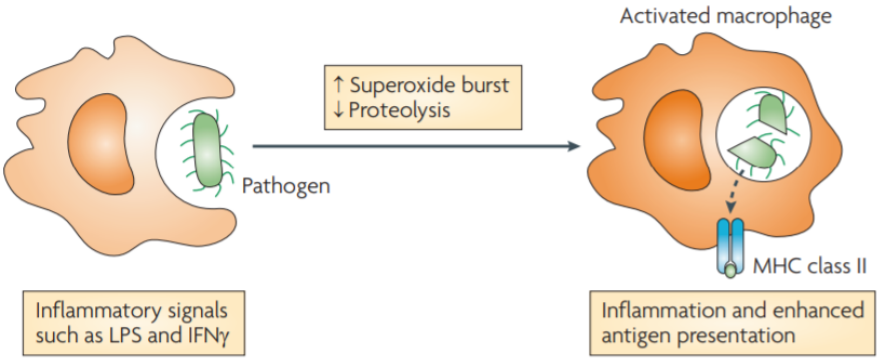


Figure 3: Representation of macrophages activation by inflammatory signals as lipopolysaccharide (LPS), or cytokines, such as interferon-γ (IFN-γ) (Adapted from (Russell *et al.*, 2010).

Macrophages (Figure 3) are mononuclear cells of innate immune system with a long life span (Parihar, Eubank e Doseff, 2010). These cells have phagocytic activity and act also as antigen presenting cells playing an important role in immune response, with the activation of other immune cells of the adaptive immune system (Feng, Zhao e Yu, 2004; Kim *et al.*, 2016).

The RAW 264.7 is a murine macrophage cell line that is frequently used in the research in immunotoxicological assays, to evaluate early possible cytotoxic effects and is one of the cell lines chosen for this study.

However, it is important to use more than one cell type to corroborate the results (Dobrovolskaia e McNeil, 2016). The use of human cells in *in vitro* tests can lead to more complete and relevant information when compared to the use of animal cells (Oostingh *et al.*, 2011).

Peripheral blood mononuclear cells (PBMCs) refer to any blood cell with a round nucleus (i.e. lymphocytes, monocytes, natural killer cells or dendritic cells). PBMCs can be isolated from blood of healthy donors or buffy coats (Figure 4) (Verhoeckx *et al.*, 2015).

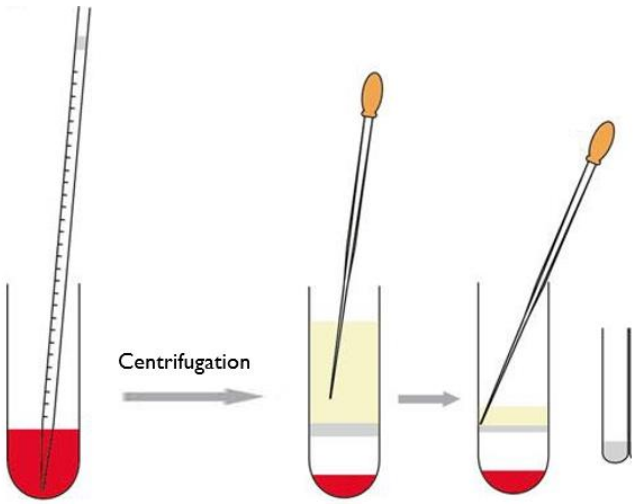


Figure 4: Schematic representation of PBMCs isolation from blood (Adapted from (Verhoeckx *et al.*, 2015)).

PBMCs are an easy and accessible source of different immune cell types. These cells have been widely used and can be a more reliable reflection of the *in vivo* immune response (Verhoeckx *et al.*, 2015; Farace *et al.*, 2016).

The composition of PBMCs can be affected by physiological factors being different between donors. When compared to the use of cell lines, the use of different donors can lead to increased inter-experimental variation. However, the existence of reproducibility in the results with cells from several donors will support the results (Verhoeckx *et al.*, 2015).

1.6. Immunotoxicological studies of Chitosan

Although there are several studies that evaluate the *in vitro* toxicity of chitosan, the effects in the immune system are poorly understood and the results found are, in some cases contradictory. A review of the literature concerning existing immunotoxicity studies of chitosan was performed and the results of some studies are summarized in table 1 and 2. The studies in table 1 used PBMCs and macrophages as study models and in table 2 are summarized studies that used other study models. Information about the molecular weight (MW), deacetylation degree (DD), particle size, zeta potential (ZP), cross-link, LPS (lipopolysaccharide) contamination, the treatment duration, the performed assays and the effects observed was collected. The parameters chosen are considered important as they can interfere with the biological properties of chitosan and with the immunotoxicological evaluation.

Table 1 – Comparison of results in studies with chitosan in PBMCs and macrophages.

Particle	Size (nm)	MW (KDa)	DD	ZP (mV)	Cross link	Concentration/ Dose	LPS	Treatment duration	Model	Assay	Effect	General Conclusion	Reference
Chitosan Nanocapsules	340 ± 30	-	-	-	-	$1x10^{11}$/mL	-	24 h	PBMCs	Anexin V - Flow cytometry	Increased apoptotic T cells, monocytes	Chitosan induced both innate and adaptive immune response	Farace *et al*., 2016
										Edu incorporation	Increased proliferation in PHA+IL-2 stimulated cells		
										Flow Cytometry	Increased monocytes diameter similar to LPS		
										Flow Cytometry	Strongly activation imunne cells (CD25, CD69 - T cells and monocytes)		
										Multiplex ELISA	TNFα, IFN-γ, IL-13, IL-4, IL-12 secretion		
Chitosan NP	373.1	50 - 60 KDa	81.6 %	-	-	54.5/68.1/81.8 µg/mL	Neutralized with polymyxin B	24 h	PBMCs	Trypan blue dye	Increased lymphocyte proliferation	Chitosan NP showed strong immunoactivating properties	Pattani *et al*., 2009
										PCR	Absence of IL-6 induction		
										Griess reaction	Increased Nitric Oxide production		
Chitosan NP	255	-	>75 %	39.5	-	5, 25, 50, 75 and 100 µg	-	24 h	Mouse peritoneal macrophages	MTT	Decreased cell viability	Chitosan NPs decreased viability and had no effect in inducing inflammation	Das *et al*., 2008
OligoChitosan	-	-	-	-	-	40 µg/mL	-	-	RAW 264.7 - murine macrophage	RT-PCR + ELISA	TNF-α and IL-1β production increase	Increased inflammatory cytokines	Feng, Zhao e Yu, 2004

Abbreviations: EdU – 5-ethynyl-2-deoxyuridine; ELISA – Enzyme-linked immunosorbent assay; IFN-γ – Interferon gamma; PCR – Polymerase chain reaction; RAW 264.7 – Murine macrophage cell line; RT PCR – Reverse transcription polymerase chain reaction.

Table 2 – Comparison of results in studies with chitosan using other study models.

Particle	Size (nm)	MW (KDa)	DD	ZP (mV)	Cross link	Concentration/ Dose	LPS	Treatment duration	Model	Assay	Effect	General Conclusion	Reference
Chitosan NP	40-100	40 kDa	_	31.8	Sodium Sulfate	75 and 150 µg/mL	Not detected	_	S-180 cells	Tripan blue dye	90 % S-180 cells remained viable with normal morphology	No alterations	Chakrabarti *et al.*, 2014
Chitosan NP	64.02	220 kDa	95%	30.5	TPP	0-96 µg/mL	_	48 h	LPS-inflamed Caco-2 cell	MTT	No effect in cell viability	Anti-inflammatory effect by suppression of inflammatory processes.	Tu *et al.*, 2016
						0, 6, 12, 24 µg/mL		24 h		LDH	Inhibited the LPS-induced cell damage		
						0, 6, 12, 24 µg/mL		24 h		ELISA	Inhibited LPS-induced cytokine production (TNF-α, MIF, IL-8 and MCP-1)		
						0, 6, 12, 24 µg/mL		6 h		RTqPCR	Down-regulated LPS-induced TLR4 expression		
						0, 6, 12, 24 µg/mL		6 h		WB	Inhibited LPS-induced degradation of IκB-α and translocation of p65		
						0, 6, 12, 24 µg/mL		6 h					
Chitosan NP/Chitosan Solution	376 ± 59	_	93.2 %	21 ± 4	DNA solution in Sodium Sulfate	_	_	48 h	Human dendritic cells	Flow cytometry	Chitosan NPs increased CD80, CD86 and CD83 expression/chitosan solution had no effect	Chitosan NP increased DCs maturation/Chitosan had no effect	Bivas-Benita *et al.*, 2004
Chitosan NP	134.4 ± 2.8 (10-30)	50-190 kDa	92%	_	_	_	_	_	BEAS-2B27	MTT	No effect in viability	No alterations	Muhsin *et al.*, 2014
										ELISA	No effect in IL-8		

Abbreviations: BEAS-2B27 – human bronchial epithelial cells; Caco-2 – human intestinal epithelial cells; LDH – Lactate dehydrogenase; RT-qPCR – Quantitative real-time polymerase chain reaction; S-180 – Murine sarcoma cancer cell line; WB – Western blot.

By analysis of the table I, it is possible to conclude that in the two studies performed in human PBMCs, the chitosan particles tested had immunostimulatory properties. About the studies performed in macrophages (mouse peritoneal macrophages or RAW 264.7) the results were contradictory. In the study published by Ishany Das and co-workers, the macrophages viability was tested after 24 h incubation with the chitosan nanoparticles and was observed a decrease but no inflammation effect was detected (Das *et al.*, 2008) . On the other side, in the tests performed by Feng and co-workers in a RAW 264.7 cell line, it was observed an increase in inflammatory cytokines production (Feng, Zhao e Yu, 2004).

In table 2 are summarized four studies that were performed in other study models. In the first example, Chakrabarti and co-workers tested the effect of chitosan NPs in S-180 cells viability and concluded that 90 % of cells remained viable and also presented a normal morphology (Chakrabarti *et al.*, 2014). Muhsin and co-workers tested chitosan NPs in cell viability and cytokine production of BEAS-2B27 cell line and their results also did not show alterations (Muhsin *et al.*, 2014). On the other hand, in the study published by Tu and co-workers it was observed an anti-inflammatory effect of chitosan NPs tested in caco-2 cells (Tu *et al.*, 2016). The effect of chitosan NPs and a chitosan solution was tested in human dendritic cells by (Bivas-Benita *et al.*, 2004). Their results showed that chitosan NPs increased dendritic cells maturations while the chitosan solution had no effect.

These contradictory studies can result from the lack of some information as the DD and the MW of chitosan that are important to characterize the polymer or as a second hypothesis, the studies described may not have been performed with LPS-free chitosans. The term chitosan refers to many polymers with different DD and MW and these characteristics can influence the results obtained (Wiegand, Winter e Hipler, 2010). Studies have also shown the importance of nanoparticle characterization as the size, zeta potential and other properties can also influence their immunotoxicity and unfortunately this information is not always present in the reports published (Dobrovolskaia e McNeil, 2007). Other aspect that is important to relate is the information about the nanoparticle endotoxin contamination. Endotoxin is present in gram-negative bacteria cell walls and has the potential to induce inflammation even at low concentrations (Dobrovolskaia, 2016). Some materials show to have inflammatory potential, however it can be potentiated by the presence of endotoxins (Dobrovolskaia, 2016). In the reports here analysed, only two of them refers the use of methods to detect and avoid the endotoxin contamination. In fact, the endotoxin contamination is the reason of the failure of 30 % of nanotechnology formulations in the early stages of pre-clinical development (Dobrovolskaia, 2016). Other challenge of *in vitro*

assessment of nanoparticle immunotoxiciy reported by Dobrovolskaia and co-workers is the choice of appropriate positive and negative controls to evaluate the possibility of nanoparticle interference with the assays performed (Dobrovolskaia e McNeil, 2016). The lack of appropriate controls and the assurance that there is no interference in the assays used can also result in controversial results. It is necessary to study the correlation between the physical and chemical properties of nanoparticles and their effect on the immune system. Furthermore, it is important to identify the interferences in the traditional assays and when necessary develop new methods to the immunotoxicological evaluation of nanomaterials.

Considering all these variables and the extensive application of chitosan, namely as a drug delivery vehicle, it was chosen to be the case study of this work to establish methods for testing immune function effects that can be adapted for other nanomaterials.

1.7. Aim of the work

The main objective of this work was to study the effect of the DD of the chitosan on the immunotoxicity properties of the chitosan as raw material or chitosan nanoparticles.

As second objectives:

- To prepare chitosan nanoparticles with the different LPS-free chitosans (chitosan with different DD) and characterize them;
- To study the nanoparticle interferences on the methods established.

In this work, polymers with different deacetylation degree (DD) and molecular weight (MW) were used and two methods were optimized to prepare Chitosan NPs with the different polymers, using tripolyphosphate (TPP) or sodium sulphate as cross-links. Immunotoxicological studies of the Chitosan NPs prepared were performed in a murine macrophage cell line (RAW 264.7) and in human peripheral blood mononuclear cells (PBMCs) to evaluate the immunomodulation potential of these NPs. Several immunotoxicity parameters were studied, using the appropriate controls to clarify the contradictory results in the literature. The effect of physical and chemical parameters as size, DD and MW of Chitosan nanoparticles were evaluated as they can influence chitosan biological properties.

Chapter II
Characterization

2.1. Materials and methods

2.1.1. Materials

Three different chitosans (ChitoClear™) were acquired from Primex BioChemicals AS (Avaldsnes, Norway). Penta-Sodium Triphosphate (TPP) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Sodium sulfate was purchased from *Merck KGaA* (Darmstadt, Germany). Acetic acid was purchased Biochem chemopharma (France). Apyrogenic water was purchased from Labesfal Farma (Coimbra, Portugal). Sartorius™ Vivaspin™ 20 Centrifugal Concentrator MWCO 300 KDa was purchased from Fisher. The other chemicals and reagents used are from normal suppliers of analytical grade.

2.1.2. Chitosan purification

Before nanoparticle production, chitosan was purified (Figure 1) using a method adapted from (Gan e Wang, 2007). Briefly, 1 g of chitosan was dissolved in 10 mL NaOH 1 M and stirred for 3 h at 40 °C to 50 °C. After this, the solution was filtered using a Buchner funnel and washed with 20 mL of ultra-pure/apyrogenic water. The recovered chitosan was dissolved in 200 mL acetic acid solution (1 %) and stirred for 3 h at room temperature (RT). Then, the solution was filtered with a paper filter and the pH of the filtrate was adjusted with approximately 32 mL of a 1 M NaOH solution to pH 8.0. Finally, the precipitate was washed three times, using ultra-pure/apyrogenic water through 30 min centrifugations at 4500 x g and the recovered chitosan was freeze-dried.

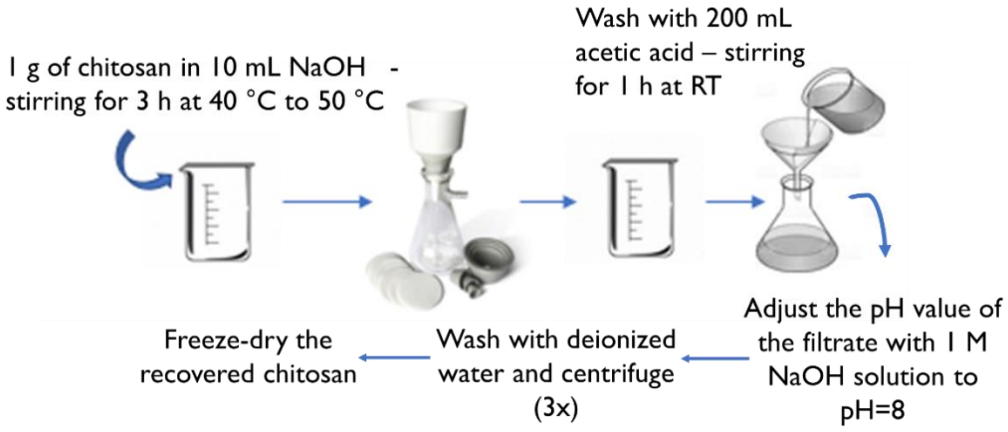


Figure 1: Schematic representation of chitosan purification method.

2.1.3. Chitosan characterization

Deacetylation degree (DD) of the three different chitosans were determined before and after purification by nuclear magnetic resonance (NMR) as previously described in (Lavertu *et al.*, 2003). Chitosan (0.05 w/v) was dissolved in 0.4 % deuterium chloride /deuterium oxide. Chitosan ^{1}H NMR spectra were obtained at 70 °C. The analysis of the obtained results was done with Mnova software. DD % can be calculated by using the peaks of proton at the position 1 of deacetylated (H1D) and acetylated (H1A) monomer, using the following equation:

$$DD\ (\%) = \left(\frac{H1D}{H1D+H1A}\right) \times 100 \quad \text{(Equation 1)}$$

The molecular weight of chitosan was measured by size exclusion chromatography (SEC) (AF2000 MT Chromatography) equipped with a multi-angle laser light scattering (MALLS PN3609) using a refractive index detector (RI PN3150). The experimental setup consisted of a solvent degasser, an isocratic HPLC pump (PN1130), an autosampler (PN 5300) and a channel oven (PN4020). An inline filter (0.1µm) was installed between the pump and the autosampler. A TSKGel G3000 PWXL-CP (Toso Haas, Japan) column of 7.8 mm inside diameter and 30 cm length was used. The mobile phase was a solution of 0.1 M acetate buffer (pH 4.0) in 0.3 M NaCl. Three types of chitosan polymers before and after purification were dissolved in 0.1 M acetic buffer (pH 4.0) containing 0.3 M NaCl to obtain solutions of 1 mg/mL. They were then filtered through 0.22 µm filters and collected in the chromatographic sample vials. For each analysis, 100 µL were injected at a flow rate of 1 mL/min at room temperature (RT). Each sample was measured in triplicate. The interpretation of obtained results was done with NovaFFF software.

2.1.4. Nanoparticle production - method optimization

In order to optimize chitosan NPs production, two methods were extensivelly tested using a range of different chitosan and cross-link concentrations, as described below. In the method A were tested chitosan concentrations between 0.01 % and 0.5 % and tripolyphosphate concentrations between 0.016 % and 0.5 % with purpose to obtain NPs with approximately 100 nm. In the method B were tested concentrations between 0.005 % and 0.2 % and sodium sulfate concentrations between 0.0625 % and 2.5 % with purpose to obtain larger NPs than the ones obtained by the method A.

Method A

Chitosan NPs produced by this method ($Chit_A$ NPs) were prepared by the dropwise addition of 1.75 mL tripolyphosphate (TPP) (0.016 % - 0.5 %) as cross-link to a 10 mL chitosan (0.01 % - 0.5 %) solution in acetic acid 1 % (pH 4.6) during stirring using a high-speed homogenizer (Ystral X120, Ballrechten-Dottingen, DE). After this, the solution of nanoparticles was left under magnetic stirring for maturation for 30 min.

Method B

Chitosan NPs produced by this method ($Chit_B$ NPs) were prepared by the dropwise addition of 5 mL Sodium sulfate (0.0625 % - 2.5 %) as cross-link to a 5 mL chitosan (0.005 % - 0.2 %) in acetate buffer (pH 5.0) during stirring using a high-speed homogenizer (Ystral X120, Ballrechten-Dottingen, DE). After this, the solution of nanoparticles was left under magnetic stirring for maturation for 30 min.

2.1.5. Nanoparticle production - optimized conditions

Method A

Chitosan NPs produced by this method ($Chit_A$NPs) were prepared by the dropwise addition of 1.75 mL tripolyphosphate (TPP) 0.16 % as cross-link to a 10 mL chitosan 0.1 % solution in acetic acid (pH 4.6) during stirring using a high-speed homogenizer (Ystral X120, Ballrechten-Dottingen, DE). After this, the solution of nanoparticles was left under magnetic stirring for maturation for 30 min (Figure 2).

Method B

Chitosan NPs produced by this method ($Chit_B$ NPs) were prepared by the dropwise addition of 5 mL sodium sulfate 0.625 % as cross-link to a 5 mL chitosan 0.1 % in acetate buffer during stirring using a high-speed homogenizer (Ystral X120, Ballrechten-Dottingen, DE). After this, the solution of nanoparticles was left under magnetic stirring for maturation for 30 min (Figure 2).

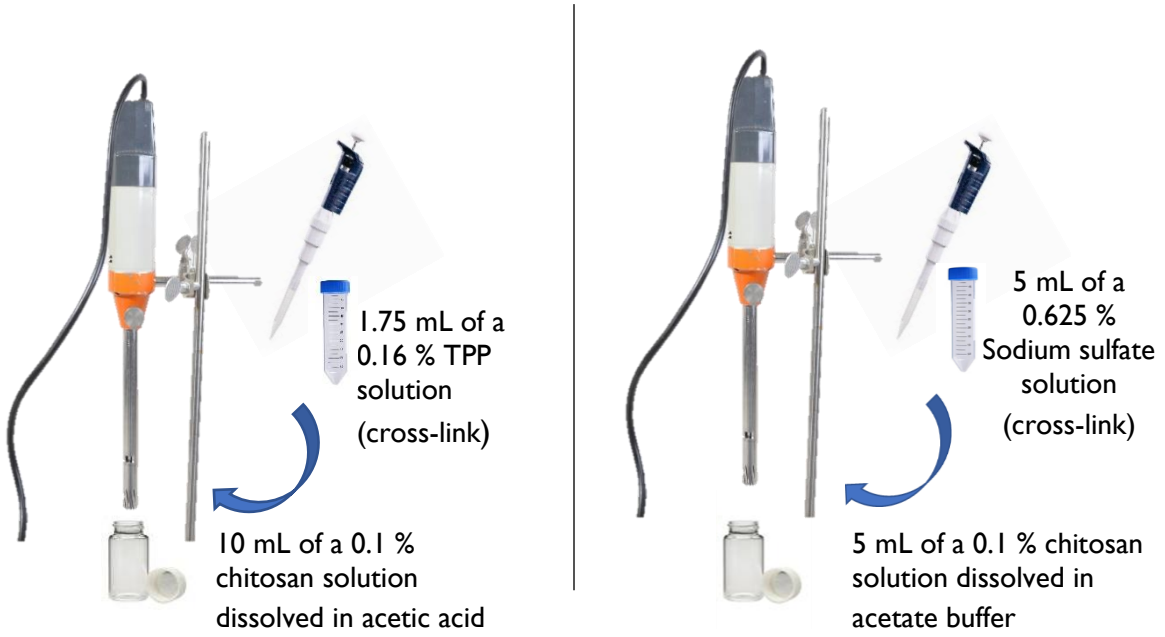


Figure 2: Schematic representation of $Chit_A$ NPs (method A) and $Chit_B$ NPs (method B) production.

2.1.5.1. NPs isolation and concentration

After production, NPs were isolated by centrifugation using Vivaspin 20 centrifugal concentrator (MWCO 300 kDa) at 3000 x g. An exception was the $Chit_A$ NPs prepared with chitosan DD 93 %, that were isolated by centrifugation using eppendorfs at 10000 x g.

Mostly, all the tests were done with the polymers with DD 80 % and 93 % as they have a larger difference in DD which is more interesting to see the different effects related to this parameter. Also, almost all the assays were performed with the $Chit_A$ NPs as it was observed that the $Chit_B$ NPs were so unstable that seemed to be destroyed when incubated in plasma and in the cell media, which would invalidate the tests done.

2.1.6. Nanoparticle characterization

Nanoparticle size was measured by Dynamic Light Scattering (DLS) and their zeta potential by Laser Doppler Micro-electrophoresis. Size measurements were made using the Delsa™Nano C Particle Analyzer (Beckman Coulter) and zeta potential measurements were made using the Zetasizer Nano ZS (Malvern Instruments).

2.1.6.1. Chitosan quantification

In order to quatify the incorporation of chitosan in the $Chit_A$ NPs, the polymer was quantified by the colorimetric method "Cibacron Brilliant Red 3B-A dye" binding (Muzzarelli, 1998). In this method, a 0.1 % chitosan solution was prepared to be used as stock solution to the standard solutions of the calibration curve. A solution of the dye was prepared by dissolving 0.075 g of the powder in 500 mL ultrapure water. Lastly, a 200 mL glycin/HCl buffer solution (pH 2.8) was prepared from 50 mL of a 0.2 M glycin solution and 16.8 mL of a 0.2 M HCl solution. The standard solutions to the calibration curve were prepared by the addition of 100 µL of glycin/HCl buffer and 1 mL of the dye. The chitosan concentrations used for each standard solution are described in the following table.

Table 1: Chitosan concentrations used for each standard solution.

0.0004 %	20 µL sol chit 0.1 % + 3.88 mL of water
0.0008 %	40 µL sol chit 0.1 % + 3.86 mL of water
0.0010 %	50 µL sol chit 0.1 % + 3.85 mL of water
0.0012 %	60 µL sol chit 0.1 % + 3.84 mL of water
0.0014 %	70 µL sol chit 0.1 % + 3.83 mL of water
0.0016 %	80 µL sol chit 0.1 % + 3.82 mL of water
0.0020 %	100 µL sol chit 0.1 % + 3.80 mL of water

The test samples were prepared with the addition of 100 µL of glycin/HCl buffer, 1 mL of the dye solution, 900 µL of ultra-pure water and 3 mL of the supernatants obtained from the nanoparticle concentration. Then, all the samples were left for 20 min in agitation and then the absorbance at 575 nm was read.

To calculate the quantity of chitosan incorporated in the nanoparticles, the test samples absorbance was interpolated in the calibration curve (a). The concentration (%) resultant from this interpolation was then used in the following equation in order to calculate the quantity of chitosan (b) present in the NPs supernatants:

$$b\ (g) = \frac{a \times sample\ volume^{*1}}{100} \quad \text{(Equation 2)}$$

*[1]Volume of the NPs supernatants obtained after NP concentration

Then, the quantity of chitosan (b) calculated in the previous equation was used to calculate the percentage of incorporated chitosan in the NPs:

$$c = 100 - (\frac{b \times 100}{0.01^{*2}}) \quad \text{(Equation 3)}$$

*[2]This value refers to quantity of chitosan (g) used to prepare the nanoparticles.

2.1.6.2. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is a technique useful to study in detail a specimen's surface. A high-energy electron beam scans across the surface of a specimen, usually coated with a thin film of gold or platinum. As result of the interactions between the sample and the electron beam result in different of electron signals emitted. These electronic signals are collected, processed, and transmitted to a monitor to form an image of the specimen's surface (Carter e Shieh, 2015). This method was used to obtain images of the chitosan nanoparticles.

The analysis was performed in LED&MAT (Instituto Pedro Nunes - Coimbra - Portugal) using the microscope ZEISS MERLIN Compact/VPCompact, gemini II, Field emission scanning electron microscope (FDSEM).

2.1.6.3. Cryo-scanning electron microscopy (CryoSEM)

Cryo Scanning Electron Microscopy (CryoSEM) was performed using a high-resolution Scanning Electron Microscope: JEOL JSM 6301F (CEMUP - Materials Centre of the University of Porto, Portugal).

The sample was rapidly cooled (plunging it into sub-cooled nitrogen – slush nitrogen) and transferred under vacuum to the cold stage of the preparation chamber. Then, was fractured, sublimated ('etched') for 120 s at -90 °C, and coated with Au/Pd by sputtering for 46 s. The sample was then transferred into the SEM chamber and was studied at a temperature of -50 °C.

2.1.6.4. Stability tests

Two tests were performed in order to evaluate the stability of the chitosan nanoparticles after production, as described bellow.

a) Stability at 4 °C and 20 °C

The $Chit_A$ NPs produced with 80 % and 93 % DD chitosan were concentrated and stored at 4 °C and 20 °C during 5 weeks to assess the nanoparticle stability. During this period, 100 µL of the sample were diluted in 900 µL of ultra-pure water and the size and zeta potential were measured as described before at different time points.

b) Stability in DMEM and RPMI

The purpose of this study was to evaluate the stability of $Chit_A$ NPs produced with 80 % and 93 % DD chitosan in Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI) that are the media used in cell studies. The NPs were concentrated and added to DMEM and RPMI at 37 °C. The final concentration of the nanoparticles was 156.25 µg/mL. Size and polidispersity index were measured after 0, 1, 6 and 24 h of incubation in each medium.

2.2. Results and discussion

2.2.1. The purification process did not affect the DD of chitosan

Chitosan DD of the three different chitosans were determined before and after purification by NMR. The chitosans MW after purification was determined by SEC. The results obtained were summarized in table 1.

Table 1: Characterization of chitosan polymers: DD and MW.

	DD (%)		MW (kDa)
Chitosan	**Before purification**	**After purification**	**After purification**
A	78	80	91
B	88	86	87
C	96	93	128

The results showed that the chitosans DD before the purification was 78 %, 88 %, 96 %. After the purification method, the DD was not significantly different, 80 % for chitosan A, 86 % for chitosan B and 93 % for chitosan C. The chitosan MW after the purification was 91 kDa for chitosan A, 87 kDa for chitosan B and 128 kDa for chitosan C.

2.2.2. Nanoparticle size increased with the DD

Two methods were optimized to produce Chitosan NPs with different characteristics. The method A was used to produce Chitosan NPs with a size around 100 nm ($Chit_A$ NPs), while method B was used to prepare the nanoparticles with a size around the 500 nm ($Chit_B$ NPs). In each case, a range of different concentrations of chitosan and cross-link were tested in order to obtain nanoparticles with the desired sizes. Also, the cross-link selected was different in each method: tripolyphosphate was selected for method A and Sodium sulfate was selected for method B. The size and zeta potential results obtained in this optimization process were summarized in table 3 and 4, using the 80 %, 86 % and 93 % DD chitosans.

Table 2: Characterization of chitosan NPs. Particle mean size distribution (nm) and polydispersity index (PDI) before and after concentration and resuspension in ultrapure water. (Mean ± SEM n = 3 to 4).

			Before concentration		After concentration	
	DD (%)	**n**	**Size ± SEM (nm)**	**PDI ± SEM**	**Size ± SEM (nm)**	**PDI ± SEM**
$Chit_A$ NPs	80	4	102.2 ± 8.7	0.27 ± 0.01	127.0 ± 4.5	0.28 ± 0.01
	86	4	133.1 ± 4.6	0.26 ± 0.02	267.5 ± 16.7	0.25 ± 0.03
	93	3	269.4 ± 38.5	0.27 ± 0.02	291.9 ± 52.3	0.18 ± 0.03
$Chit_B$ NPs	80	4	351.7 ± 32.5	0.07 ± 0.02	497.2 ± 37.5	0.16 ± 0.03
	86	4	549.6 ± 12.4	0.11 ± 0.01	784.7 ± 152.4	0.25 ± 0.01

The results showed that chitosans with higher degree of deacetylation (DD) and higher MW form larger particles (Table 2). These results are in agreement with a work reported by Huang and co-workers that also show the effect of DD and MW variations in nanoparticle size. Their results showed that NPs size decreased from 188 nm to 122 nm lowering the MW from 213 kDa to 17 kDa, however in their study the increase in DD did not increased the NPs size, but had a decrease effect (Huang, Khor e Lim, 2004). Therefore, the increase on size observed in our study is, most probably related with the MW and not with DD of the polymer.

Table 3: Zeta potential (mV) of Chitosan NPs after concentration and resuspension in ultrapure water (Mean ± SEM; n = 3 to 4).

	DD (%)	**n**	**Zeta Potential (mV ± SEM)**
$Chit_A$ NPs	80	4	+28.98 ± 1.27
	86	4	+43.10 ± 1.05
	93	3	+9.71 ± 9.77
$Chit_B$ NPs	80	4	+15.55 ± 1.11
	86	4	+24.86 ± 3.58

All the Chitosan NPs produced have a positive zeta potential (Table 3). This was expected because chitosan is a positively charged polymer. The Chitosan NPs prepared with the chitosan with a deacetylation degree of 86 % showed a higher zeta potential when both production methods were used. To evaluate the effect of the concentration method on mean size of the NPs, the graphics with the size distribution were designed and compared.

Chit$_A$ NPs 80 %

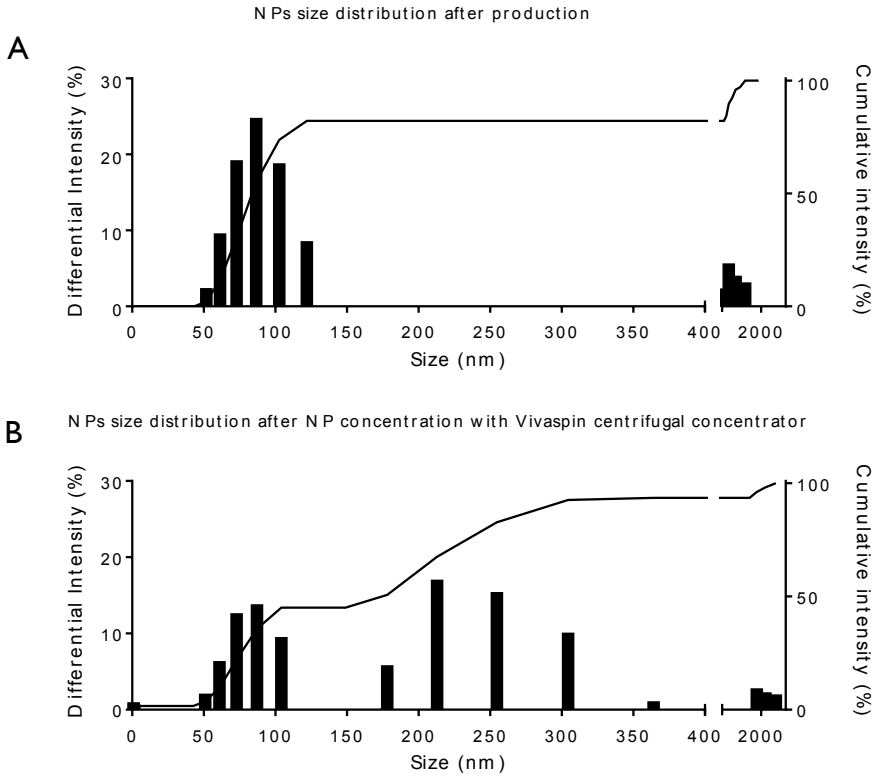


Figure 1: Chit$_A$ NPs 80 % size distribution after production (A) and NP concentration (B).

Chit$_A$ NPS 86 %

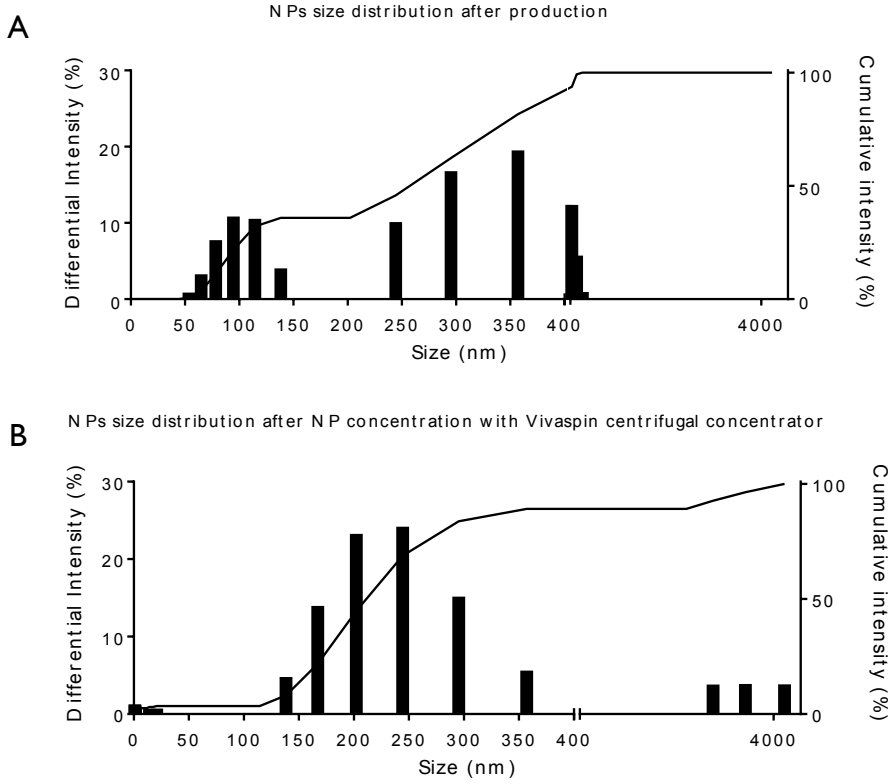


Figure 2: Chit$_A$ NPs 86 % size distribution after production (A) and NP concentration (B).

Chit$_A$ NPs 93 %

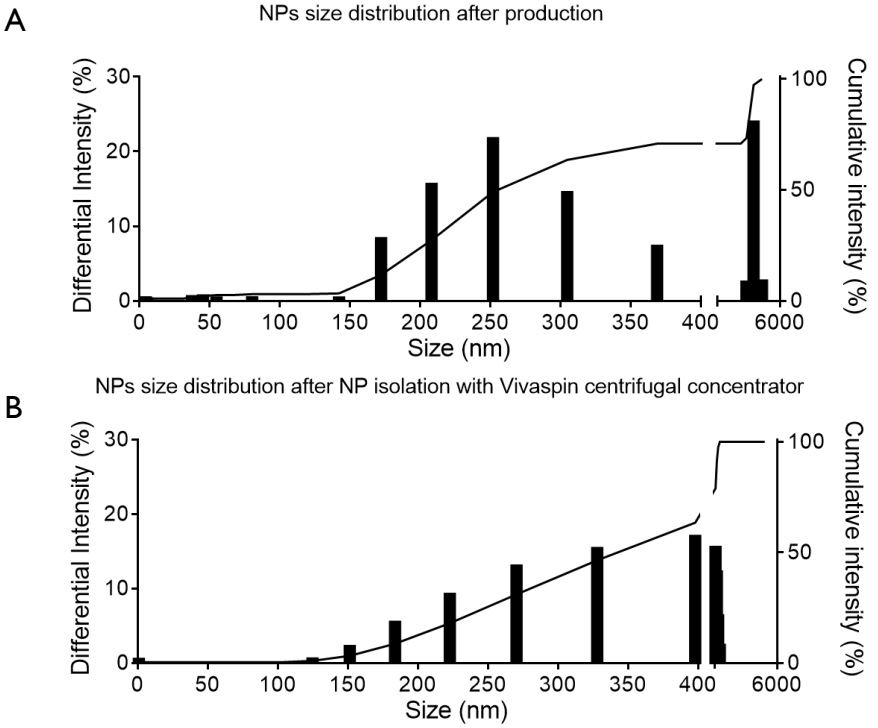


Figure 3: Chit$_A$ NPs 93 % size distribution after production (A) and after NP concentration (B).

Chit$_B$ NPs 80 %

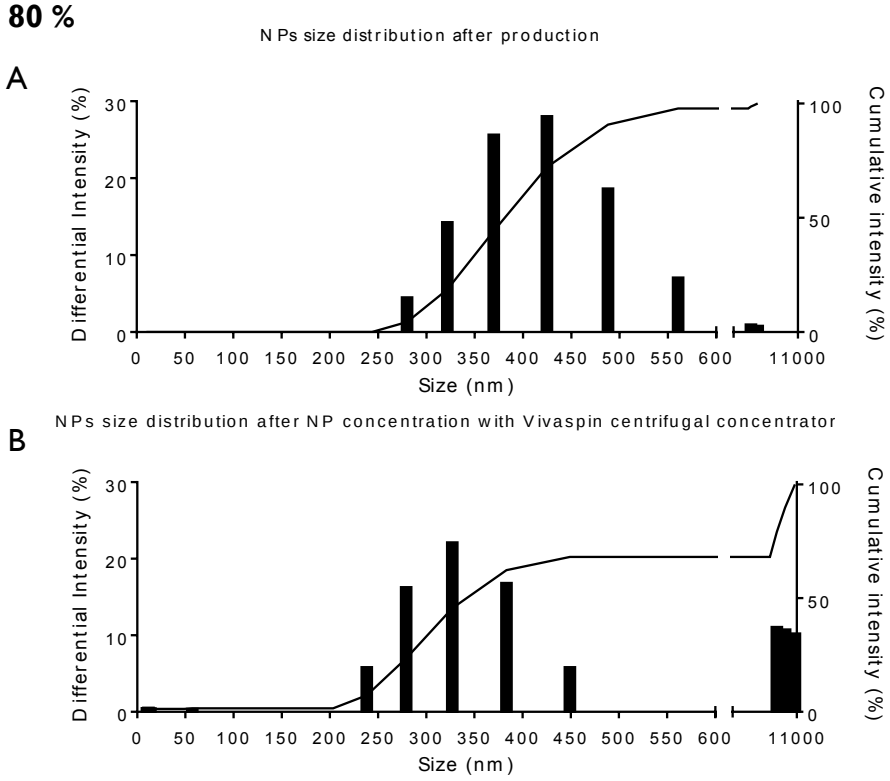


Figure 4: Chit$_B$ NPs 80 % size distribution after production (A) and NP concentration (B).

Chit$_B$ NPs 86 %

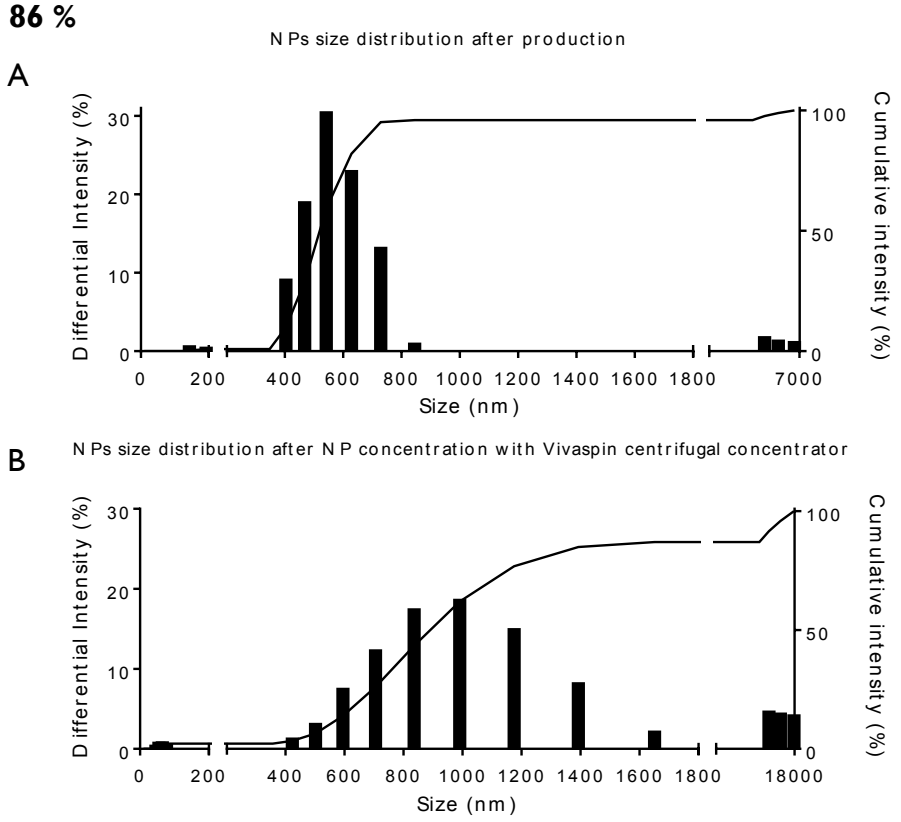


Figure 5: Chit$_B$ NPs 86 % size distribution after production (A) and NP concentration (B).

After the production, the Chit$_A$ NPs 80 % size distribution shown in figure I is mostly intense around 100 nm. After the concentration of the NPs, the size showed to move to the right, remaining the size populations around the 100 nm but appearing some populations of higher sizes (aggregates). The Chit$_A$ NPs 86 % after the production also showed the NPs populations with a size distribution around the 100 nm, however also showed other populations of higher sizes. After the concentration the Chit$_A$ NPs 86 % showed a size distribution with many populations but more concentrated around the 200 nm and 250 nm with also the presence of some aggregates (Figure 2). The Chit$_A$ NPs 93 % presented a greater size distribution after production and after concentration of the NPs (Figure 3). So, the concentration process did not modify the initial size distribution. The NPs size distribution of the NPs produced by the method B (Chit$_B$ NPs) is represented in figure 4 and 5. The Chit$_B$ NPs 80 % show a size distribution mostly around the 300 nm and 400 nm, after the concentration this populations were also present and some populations around the 600 nm that were less significant seemed to have formed some aggregates (Figure 4). The Chit$_B$ NPs 86 % size distribution showed a NPs population mostly concentrated around the 550 nm, after the concentration other NPs populations are shown, with higher sizes indicating

the formation of aggregates (Figure 5). The formation of NPs aggregates seems to be present in all types of NPs, however these aggregates appear to be more significant in the $Chit_B$ NPs. Despite these aggregates, the desirable NPs populations are also presented showing that the NPs medium size is not significantly affected by the NPs concentration process.

2.2.3. Almost 100 % of chitosan is incorporated in the $Chit_A$ NPs

Chitosan was quantified on the supernatant of the NPs by a colorimetric method "Cibacron Brilliant Red 3B-A dye" in order to determine, by an indirect method, the percentage of chitosan that was incorporated in the $Chit_A$ NPs.

Table 4: Percentage of chitosan incorporated in the $Chit_A$ NPs (Mean ± SEM; n = 5).

$Chit_A$ NPs	**Incorporated chitosan (%)**
80 %	99.55 ± 0.09
86 %	99.40 ± 0.18
93 %	98.46 ± 0.04

The results presented in table 4 show that almost all the chitosan was incorporated in the $Chit_A$ NPs as the percentage obtained was around 99 % for the three types of chitosans studied.

The SEM and CryoSEM were used to obtain images of the chitosan nanoparticles to evaluate their shape and morphology.

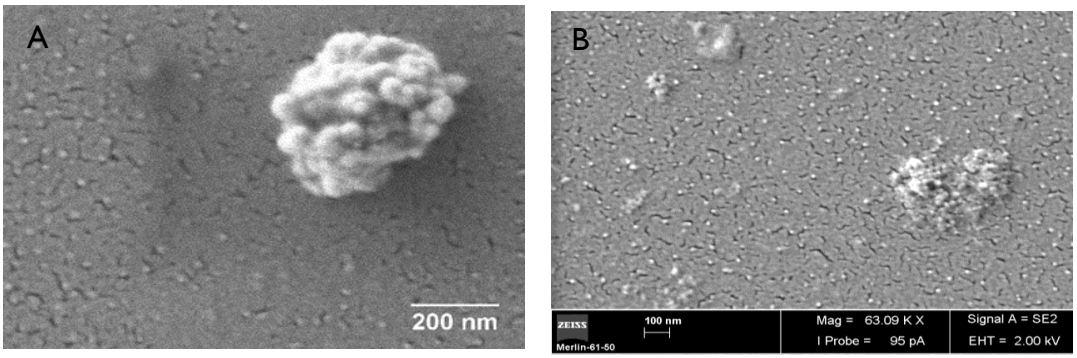


Figure 6: Scanning electron microscopy (SEM) images of the chitosan nanoparticles. (A) $Chit_A$ NPs 80 % (B) $Chit_B$ NPs 80 %.

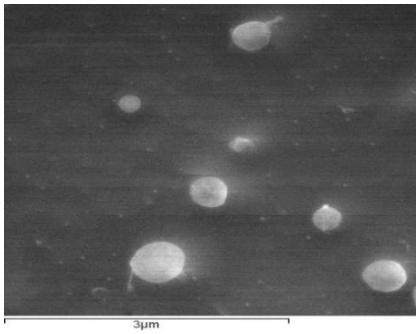


Figure 7: Cryo-scanning electron microscopy (CryoSEM) images of the $Chit_A$ NPs 80 %.

The images showed round shape $Chit_A$ NPs 80 % of several sizes, which is in accordance with the size distribution graphics that showed the existance of NPs populations with different sizes, and with the NPs size results obtained by DLS (Figure 6 and 7). The $Chit_B$ NPs were analysed only by SEM (Figure 6 B) and the images also show round shape and polydispersity.

2.2.4. $Chit_A$ NPs showed to be stable when stored at 4 °C and 20 °C but showed some aggregation in cell media

Two stability tests were performed in order to evaluate the stability of the chitosan nanoparticles regarding the storage temperature and the dispersion media.

In the first test, the $Chit_A$ NPs 80 % and 93 % stability was studied when stored at 4 °C and 20 °C during 5 weeks.

The initial size and zeta potential of $Chit_A$ NPs 80 % in water was 89.3 nm and +23.9 mV respectively and for $Chit_A$ NPs 93 % was 403.2 nm and +28.3 mV respectively.

The results showed that $Chit_A$ NPs 80 % are stable at 4 °C and 20 °C since did not show many differences in the size between week 0 and week 5 (Figure 8 A-B). $Chit_A$ NPs 93 % also did not show many differences in the size during the 5 weeks as the size was around 400 - 500 nm, despite the different storage temperatures (Figure 8 C-D).

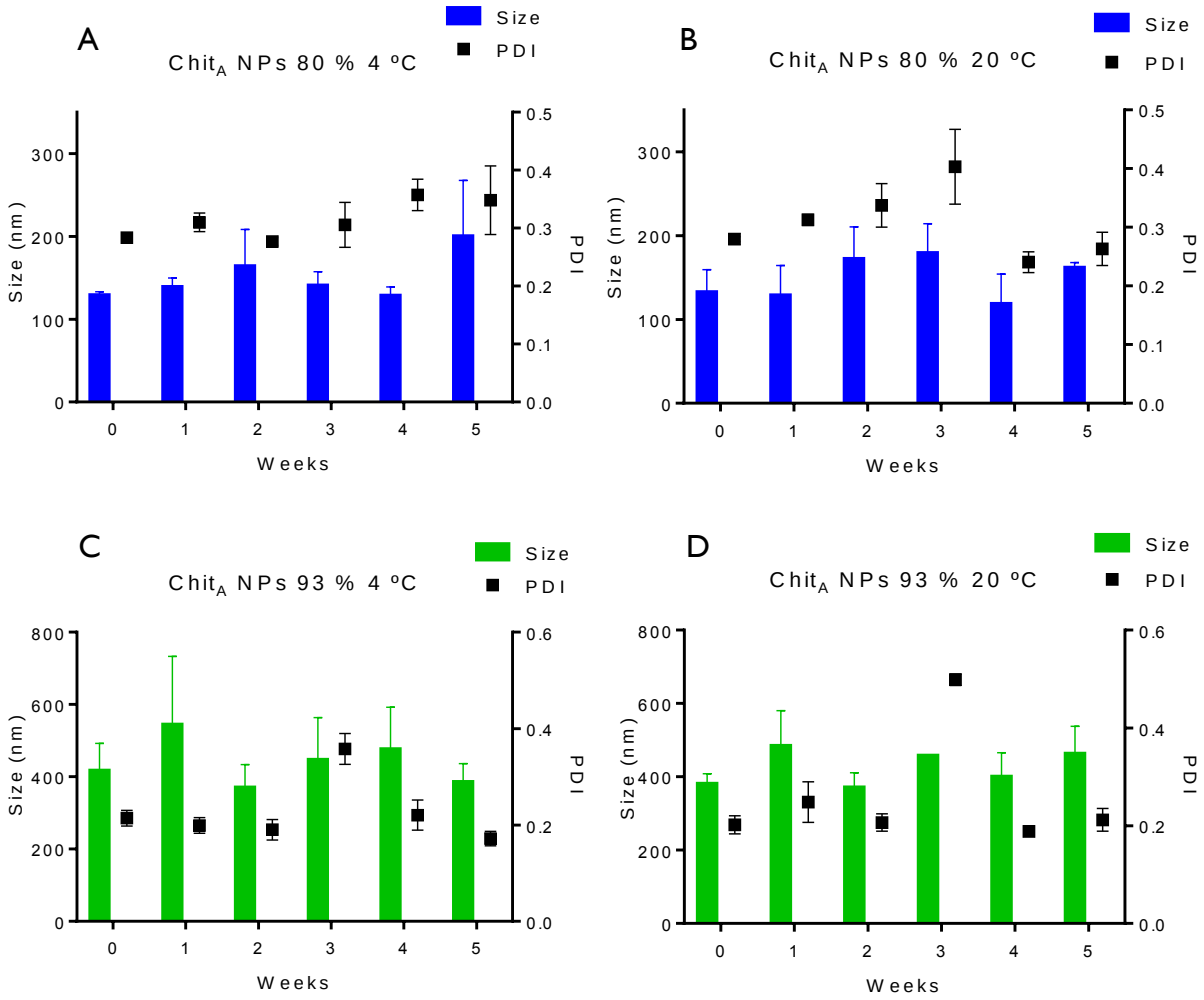


Figure 8: Size and polidispersity index (PDI) of Chit$_A$ NPs 80 % (A-B) and Chit$_A$ NPs 93 % (C-D) stored at 4 °C and 20 °C for 5 weeks (n = 3; Mean ± SEM).

The Chit$_A$ NPs zeta potential was also measured for 5 weeks at the two conditions. The results are summarized in table 5.

Table 5: Zeta potential (ZP) of Chit$_A$ NPs. Measurements during 5 weeks of Chit$_A$ NPs 80 % and 93 % stored at 4 °C and 20 °C (n = 3; Mean ± SEM).

		Weeks						
		0	**Storage T (°C)**	**1**	**2**	**3**	**4**	**5**
Chit$_A$ NPs 80 %	**ZP (mV) ± SEM**	23.9 ± 4.2	**4**	+12.8 ± 5.1	+16.4 ± 4.9	+21.5 ± 8.5	+17.9 ± 7.0	+21.4 ± 10.1
			20	+15.7 ± 4.4	+18.2 ± 11.4	+17.1 ± 7.7	+12.9 ± 7.8	+14.8 ± 11.5
Chit$_A$ NPs 93 %		28.3 ± 6.9	**4**	+22.2 ± 7.3	+31.9 ± 7.7	+36.8 ± 4.8	+36.8 ± 8.9	+30.4 ± 10.3
			20	+26.8 ± 8.7	+41.4 ± 4.4	+37.2 ± 6.8	+33.9 ± 6.5	+45.5 ± 0.2

Zeta potential was positive for both $Chit_A$ NPs as expected because chitosan is a positively charged polymer (Table 5). $Chit_A$ NPs 80 % at 4 °C did not show many alterations in zeta potential, however at 20 °C the zeta potential suffered a higher variation over the weeks. The $Chit_A$ NPs 93 % at 4 °C showed an increase in zeta potential from +14 mV to +30 mV. At 20 °C, the $Chit_A$ NPs 93 % zeta potential did not show many diferences over time.

In the second test, the objective was to study the NPs behavior in cell culture concerning their size and zeta potential. So, the DMEM and RPMI, culture medium used in our cell studies have been chosen. The $Chit_A$ NPs were added to DMEM and RPMI at 37 °C in a concentration of 156.25 µg/mL and the size and polydispersity index were measured after 0, 1, 6 and 24 h of incubation in each medium.

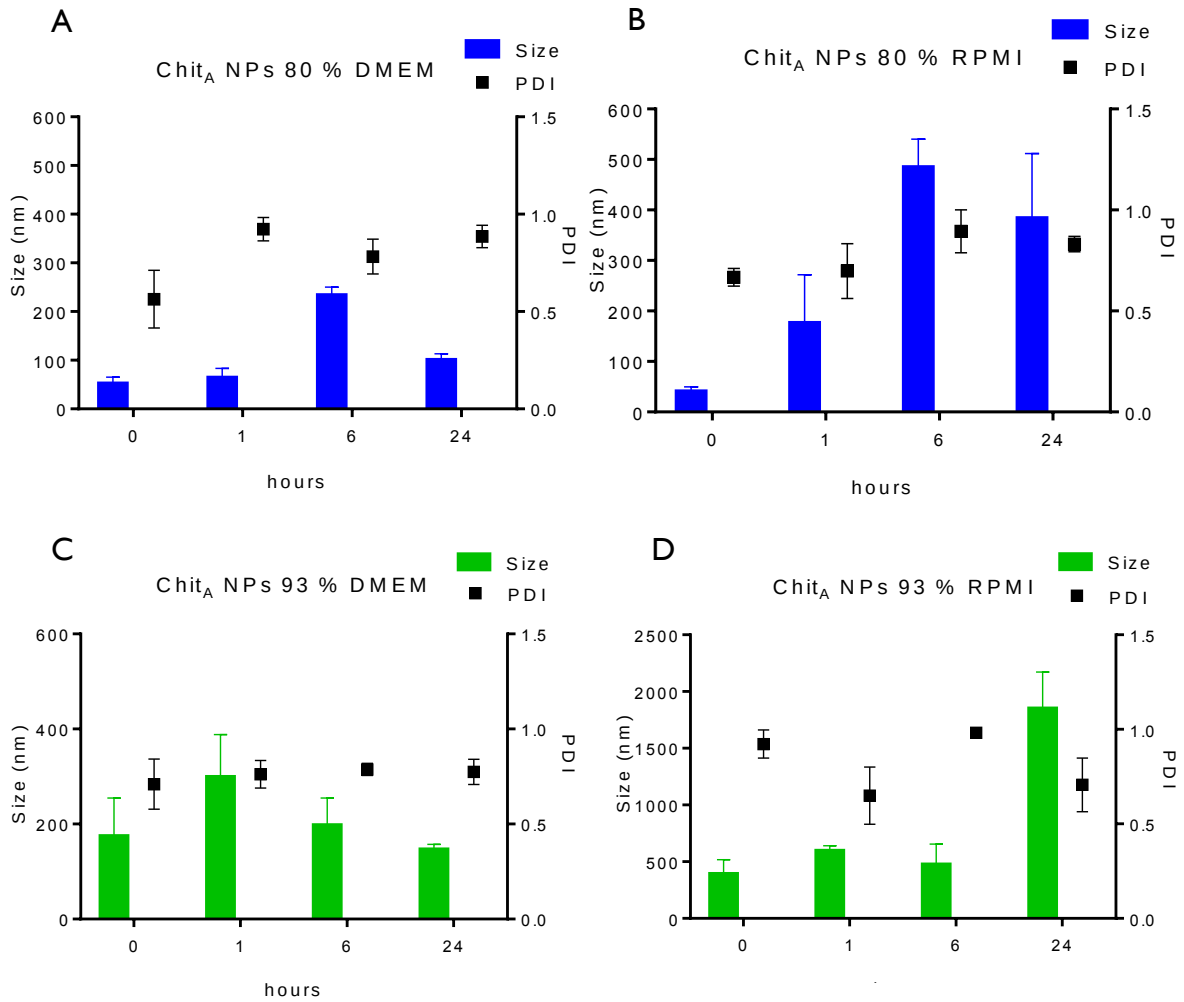


Figure 9: Size and polydispersity index (PDI) of $Chit_A$ NPs 80 % (A-B) and 93 % (C-D) in DMEM and RPMI media. Measurements after 0, 1, 6 and 24 h of incubation at 37 °C (n = 3; Mean ± SEM).

Both types of $Chit_A$ NPs showed an increase in size after the 24 h incubation with RPMI (Figure 9 B; 9 D). Relatively to the NPs in DMEM, the $Chit_A$ NPs 80 % also showed an increase in size after the 24 h incubation, but the $Chit_A$ NPs 93 % had a size decrease (Figure 9).

Table 6: Zeta potential (ZP) of $Chit_A$ NPs. Measurements of $Chit_A$ NPs (A-B) and 93 % (C-D) in DMEM and RPMI 1640 media. Measurements after 0 h of incubation at 37 °C (n = 3; Mean ± SEM).

		t = 0 h (water)	**t = 0 h (DMEM)**	**t = 0 h (RPMI)**
$Chit_A$ NPs 80 %	**ZP (mV) ± SEM**	+22.6 ± 2.8	-2.5 ± 1.7	-1.6 ± 0.9
$Chit_A$ NPs 93 %		+44.7 ± 3.1	-4.7 ± 0.2	-4.4 ± 2.7

The initial zeta potential for both chitosan NPs was positive, however when incubated in DMEM or RPMI an inversion of the charge was observed (Table 6). As the $Chit_A$ NPs are positively charged and the cell media contain proteins that are negatively charged this charge inversion can result from the adsorption of media proteins to the NPs surface or is because of the pH of the media that is neutral. This charge inversions were also reported with studies with other NPs as described by Schollbach and co-workers that studied gold nanoparticles decorated with oligo(ethylene glycol) thiols that had negative charge but when incubated with positive proteins showed a charge inversion staying with positive charge resulting from the protein adsorption (Schollbach *et al.*, 2014).

The polymers and NPs characterized in this chapter were used in the tests described in the chapter III.

Chapter III
Immunotoxicological assays

3.1. Materials and Methods

3.1.1. Materials

Three different chitosans (ChitoClear™) were acquired from Primex BioChemicals AS (Avaldsnes, Norway). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and Penta-Sodium Triphosphate (TPP) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Sodium sulfate was purchased from Merck KGaA (Darmstadt, Germany). Acetic acid was purchased Biochem chemopharma (France). Apyrogenic water was purchased from Labesfal Farma (Portugal). Sartorius™ Vivaspin™ 20 Centrifugal Concentrator MWCO 300 KDa was purchased from Fisher. RAW 264.7 Cell Line murine # 91062702-1VL was acquired from Sigma. The other chemicals and reagents used are from normal suppliers of analytical grade.

3.1.2. Nanoparticle production

Method A

Chitosan NPs produced by this method ($Chit_A$NPs) were prepared by the dropwise addition of 1.75 mL tripolyphosphate (TPP) 0.16 % as cross-link to a 10 mL chitosan 0.1 % solution in acetic acid (pH 4.6) during stirring using a high-speed homogenizer (Ystral X120, Ballrechten-Dottingen, DE). After this, the solution of nanoparticles was left under magnetic stirring for maturation for 30 min.

Method B

Chitosan NPs produced by this method ($Chit_B$ NPs) were prepared by the dropwise addition of 5 mL sodium sulfate 0.625 % as cross-link to a 5 mL chitosan 0.1 % in acetate buffer during stirring using a high-speed homogenizer (Ystral X120, Ballrechten-Dottingen, DE). After this, the solution of nanoparticles was left under magnetic stirring for maturation for 30 min.

3.1.2.1. NPs isolation and concentration

After production, NPs were isolated by centrifugation using Vivaspin 20 centrifugal concentrator (MWCO 300 kDa) at 3000 x g. An exception was the $Chit_A$ NPs prepared with chitosan DD 93 %, that were isolated by centrifugation using eppendorfs at 10000 x g.

3.1.3. *In vitro* studies with Raw 264.7 cell line

A murine RAW 264.7 macrophage cell line was used to evaluate the cytotoxicity of the nanoparticles. These cells were cultured at 37 °C and 5 % CO_2, in DMEM with 10 % heat inactivated fetal bovine serum (FBS) supplemented with 1 % Penicillin/Streptomycin, 10 mM HEPES and 3.7 g/L Sodium Bicarbonate. Subcultures were performed detaching the cells by scraping.

3.1.3.1. Nanoparticle cytotoxicity - Raw 264.7 cells

Cytotoxicity assays were done 24 h after seeding 100 µL RAW 264.7 cells in a 96-well plate at a density of 2 x 10^5 cells/mL. 100 µL of medium was removed, and then new medium was added before the NPs addition. Serial dilutions were prepared in DMEM for a nanoparticle concentration in the well ranging between 312.5 µg/mL and 5000 µg/mL.

After 24 h incubation, the MTT cell viability assay was performed with the addition of 20 µL MTT solution (5 mg/mL in phosphate buffer saline pH 7.4) to each well and incubated for 1h30 at 37 °C and 5 % CO_2. After the incubation time, the supernatant was removed and 200 µL dimethyl sulfoxide (DMSO) at 37 °C were added. The absorbance was measured at 540 nm with wavelength corrector set at 630 nm using a microplate reader.

The relative cell viability (%) was calculated using the following equation:

$$Cell\ viability\ (\%) = \frac{\text{OD sample (540nm)} - \text{OD sample (630nm)}}{\text{OD control (540nm)} - \text{OD control (630nm)}} \times 100 \quad \text{(Equation 1)}$$

The inhibitory concentration for 50 % of cell viability (IC 50) was calculated by plotting the log concentration of the NPs versus inhibition percentage of cell viability and extrapolating the value from a non-linear regression.

3.1.3.2. Nitric Oxide (NO) production

A murine RAW 264.7 macrophage cell line was used to evaluate the NO production of the cells in the presence of the nanoparticles prepared in sterile and apyrogenic conditions. NO production assays were done 24 h after seeding 500 µL RAW 264.7 cells in a 48-well plate at a density of 4.5 x 10^5 cells/mL. Then, 500 µL of the medium were removed, and then new medium was added before LPS-free Chitosan NPs and polymers addition at 39.06 µg/mL, 78.13 µg/mL and 156.25 µg/mL. LPS 1 µg/mL was used as a positive control. After a 24 h incubation, 100 µL of supernatants were transferred to a 96-well plate. A standard curve

was prepared from a 1 mg/mL $NaNO_2$ stock solution at the concentrations described in the following table.

Table 1: Standards concentrations prepared from $NaNO_2$ stock solution.

$NaNO_2$ Concentration (µM)	80	70	60	50	30	20	10	5	2.5	1.25	1	0.625

A volume of 100 µL of Griess reagent (1 % sulphanilamide in 2.5 % phosphoric acid and 0.1 % naphylethylenediamine dihydrochloride in 2.5 % phosphoric acid mixed in equal volumes accordingly with the volume needed) was added to each well in a 96-well plate and then incubated for 10 min at room temperature and protected from light. The absorbance at 550 nm was measured with a microplate reader. The concentration of Nitric oxide produced was calculated using the $NaNO_2$ standard curve.

3.1.3.3. Cytokine quantification

RAW 264.7 cells (4.5 x 10^5 cells/mL) were incubated with Chitosan NPs and polymers for 24 h in a 48-well plate. Three different concentrations were used (39.06 µg/mL, 78.13 µg/mL and 156.25 µg/mL). After 24 h, the supernatants were removed and stored at -80 °C until TNF-α and IL-1β quantification by Enzyme-Linked Immunosorbent Assay (ELISA) technique using the PeproTech ELISA Development kit for the respective cytokine.

The capture antibody was diluted with PBS to the respective concentration accordingly to the cytokine quantified. Then, 100 µL were added to each ELISA plate well and left to incubate overnight at room temperature. The next step was to aspirate the wells and wash the plate four times using 300 µL of wash buffer (0.05 % Tween-20 in PBS) per well. After removing the residual wash buffer, 300 µL of block buffer (1 % bovine serum albumin (BSA) in PBS) was added to each well and incubated for 1 h at room temperature and the plate was aspirated and washed again four times. This step of aspiration and wash was always done after the incubation periods. Then, the standard was diluted and 100 µL of the standard or sample were added to the plate and incubated for at least 2 h. After this time, 100 µL of the diluted detection antibody were added to the plate and incubated for 2 h. After the 2 h, 100 µL the diluted avidin- Horseradish peroxidase (HRP) conjugate was added and incubated for 30 min at room temperature. Finally, 100 µL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) liquid substrate was added to the plate, incubated at room

temperature and color development was measured at 405 nm with wavelength corrector set at 630 nm using a microplate reader.

3.1.4. *In vitro* studies with human peripheral blood mononuclear cells (PBMCs)

Buffy coats from healthy donors for PBMCs isolation were kindly given by IPST IP (Coimbra, PT). Blood was diluted (1:5) in physiological serum 0.9 %. 2.5 mL of lymphoprep™ were added to 15 mL tubes. 7.5 mL of the diluted sample were added to lymphoprep™ tubes previously prepared creating a layer, without mixing. The tubes were centrifuged for 20 min at 1190 x g and 20 °C. After this step, a ring of mononuclear cells was formed. The liquid above the ring was removed and then two rings of mononuclear cells were removed and added to a 15 mL tube. The rings removed were diluted in PBS (pH 7.4) at 37 °C, homogenized and centrifuged for 10 min at 487 x g at 20 °C. The supernatants were rejected, and the wash was repeated two times. After the last wash, the supernatant was rejected, and the cells were resuspended in 6 mL RPMI 1640 (2 mM L-glutamine, 1 % penicillin/streptomycin and 20 Mm Hepes).

3.1.4.1. *In vitro* nanoparticle cytotoxicity - PBMCs

Cytotoxicity assays were done after a 24 h incubation of 100 µL PBMCs in a 96-well plate at a density of 5 x 10^6 cells/mL. Serial dilutions were prepared in RPMI 1640 for a final nanoparticle or polymer concentration ranging between 2.44 µg/mL and 5000 µg/mL. After 24 h incubation, an MTT cytotoxicity assay was performed with the addition of 20 µL MTT solution (5 mg/mL in PBS pH 7.4) to the samples and incubated for four hours at 37 °C. After the incubation time, the plates were centrifuged at 800 x g for 25 min, 200 µL of medium was removed and 100 µL DMSO at 37 °C were added. The absorbance was measured at 540 nm and 630 nm using a microplate reader.

The relative viability (%) was calculated using the following equation:

$$Cell\ viability\ (\%) = \frac{\text{OD sample (540nm)} - \text{OD sample (630nm)}}{\text{OD control (540nm)} - \text{OD control (630nm)}} \times 100 \quad \text{(Equation 2)}$$

The inhibitory concentration for 50 % of cell viability (IC 50) was calculated by plotting the log concentration of the NPs versus inhibition percentage of cell viability and extrapolating the value from a non-linear regression.

3.1.5. *In vitro* studies with human whole blood

3.1.5.1. Hemolysis assay

Human whole blood samples were obtained from volunteer healthy donors in tubes with the anticoagulant heparin. The whole blood was diluted with PBS to adjust the hemoglobin concentration to 10 mg/mL. PBS was used as a negative control and Triton X-100 as a positive control. To each sample was added 700 µL of PBS, 100 µL of the diluted whole blood and the respective NPs. The final concentrations of the samples were 0.1 mg/mL, 1 mg/mL and 2 mg/mL. Other samples with the NPs or polymer and 800 µL of PBS but without blood were prepared to be used as control to evaluate the possible NPs interferences with the assay. Then, the samples were homogenized and incubated during 3 h at 37 °C. During the incubation time, the samples were shaken every 30 min. After this, all the samples prepared before were centrifuged at 800 x g for 15 min. The percentage of hemolysis was calculated by the following equation.

$$\boldsymbol{Hemolysis}\ (\%) = \frac{(\text{sample Abs} - \text{negative control Abs})}{(\text{positive control Abs} - \text{negative control Abs})} \times 100\ \%$$ (Equation 3)

Diluted total blood hemoglobin (dTBH) was prepared with 400 µL of diluted whole blood and 5 mL of cyanmethemoglobin (CMH). 100 µL of the supernatants of all the samples and 200 µL of PBS control and dTBH were added to a 96-well plate and after this 100 µL of CMH was added to all the wells which only 100 µL were added. Finally, the 96-well plate was read in a microplate reader at an absorbance of 540 nm.

3.1.5.2. Platelet aggregation

To evaluate the effects of the different Chitosan NPs and polymers on platelet aggregation, platelet count (PLC) was determined by HMX Beckman Coulter. Platelet-rich plasma (PRP) was obtained from human blood from healthy donors collected in tubes with sodium citrate by centrifugation at 200 x g for 8 min, incubated with 0.1 mg/mL and 2 mg/mL of Chitosan NPs for 15 min at 37 °C and the platelet count was performed. PBS and 100 µg/mL collagen were used as negative and positive control, respectively. The percentage of platelet aggregation (% platelet aggregation) was calculated by the following equation.

$$\boldsymbol{Platelet\ aggregation}\ (\%) = \frac{(\text{PLC negative control} - \text{PLC sample})}{\text{PLC negative control}} \times 100\ \%$$ (Equation 4)

3.1.5.3. Coagulation time

Human blood samples were obtained from volunteer healthy donors. The two pathways of blood coagulation activated partial thromboplastin time (APTT) and prothrombin time (PT) were separately tested. The blood was collected using sodium citrate as anticoagulant and the plasma was obtained by centrifugation of the blood at 2500 x g for 10 min. Chitosan NPs and polymers were incubated at 0.1 mg/mL and 1mg/mL concentration with plasma for 30 min, mixed by different reagents for testing coagulation time and then analyzed by Biomerieux Option 4 Plus. The reagents used were BIO-TP LI for determination of PT and BIO-CK for APTT.

3.1.6. Statistical Analysis

Results are expressed as mean values ± standard error of the mean (SEM). Data analysis and determination of significance ($p<0.05$) were determined using GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA).

3.2. Results and discussion

3.2.1. Chitosan NPs showed to be more toxic than the respective polymers in RAW 264.7 cell line

In vitro toxicity studies give an indication about the cytotoxic profile that may be observed *in vivo*. MTT is a rapid and precise assay that measures the metabolic activity of living cells. Viable cells, metabolic active, convert 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to insoluble crystals of formazan. Their dissolution in DMSO generates a purple signal, measured by colorimetry (Patravale, Dandekar e Jain, 2012) in a spectrophotometer. Therefore, the MTT assay was used to evaluate the toxicity of Chitosan NPs and polymers in a murine macrophage cell line (RAW 264.7) and the results obtained were presented in Figure I.

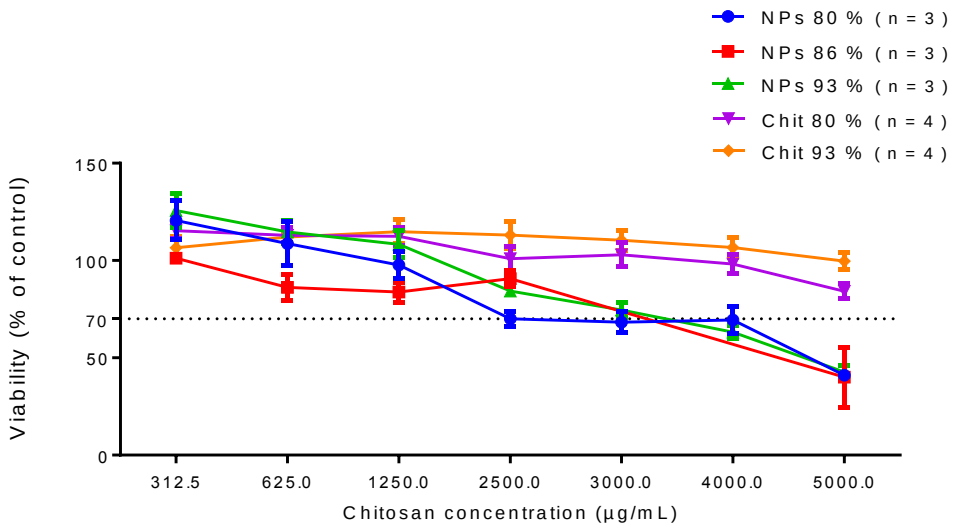


Figure 1: *In vitro* cytotoxicity assay MTT, performed in RAW 264.7 macrophages (cell passage number 45 to 60) after 24 h of incubation with Chitosan NPs, polymers (Mean ± SEM, n = 3 to 4). Comparison of three types of $Chit_A$ NPs and polymers with different DD (80 %, 86 % and 93 %) and MW.

The results in murine macrophages showed that $Chit_A$ NPs have a similar cytotoxic profile despite the differences in deacetylation degree and molecular weight. All the $Chit_A$ NPs presented toxic effects only at the highest concentration tested (5000 µg/mL), with the cells viability lower than 70 % (Figure 1). $Chit_A$ NPs 80 % induced a decrease in cell viability, with 50 % decrease in cell viability (IC 50) at approximately 4614 µg/mL, for the $Chit_B$ NPs 86 %

the IC 50 is 4465 µg/mL and 4563 µg/mL for the $Chit_B$ NPs 93 % which show their similar cytotoxicity.

The polymers also showed a similar cytotoxicity despite different deacetylation degrees and molecular weights. However, no toxicity was detected in macrophages when incubated with the polymers at the same NPs concentrations tested. The Chitosan NPs showed to be more cytotoxic than the polymer (Figure 1). Solvent controls were done to eliminate the possibility that it can contribute to the cytotoxic effects observed and no cytotoxic effects on the cells were detected. Huang and co-workers also studied the effect of MW and DD of Chitosan NPs and polymers in A549 cells. This study described that cytotoxicity is not significantly reduced by lowering the polymer MW to 10 kDa. On the other hand, decreasing the DD of the polymer from 88 % to 61 % was found to attenuate the NPs cytotoxicity (Huang, Khor e Lim, 2004). We did not include in our study a chitosan with a low DD and so this relation was not observed in this work as the three types of $Chit_A$ NPs showed similar cytotoxicity (Figure 1). Concerning the influence of the MW of the polymer, the results showed in the figure 1 are in agreement with S. Omar Zaki and co-workers that observed that the cytotoxicity of chitosan NPs in mouse hematopoietic stem cells is not influenced by the chitosan MW (Sarah *et al.*, 2015).

3.2.2. Chitosan NPs and polymers showed an inhibitory effect in NO production in RAW 264.7 cell line

Nitric oxide (NO) is an important inflammatory mediator released by macrophages during inflammation, being one of the main cytostatic, cytotoxic, and pro-apoptotic mechanisms of the immune response (Boscá *et al.*, 2005).

NO production by RAW 264.7 cell line was measured using the Greiss reaction method after the incubation with Chitosan NPs and the polymers prepared in endotoxin-free and sterile conditions to eliminate possible contaminations that could interfere with the assay. The results are showed in Figure 2.

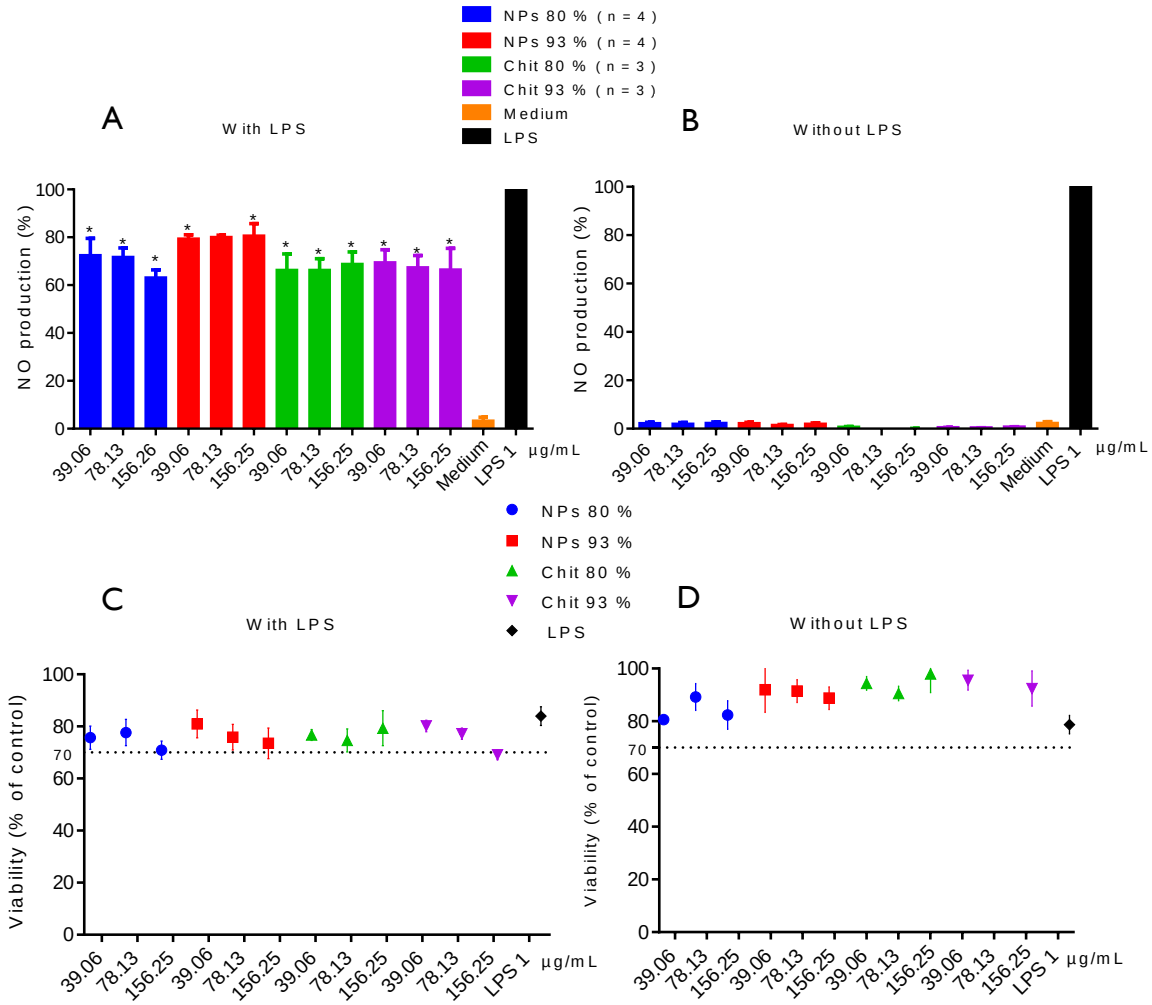


Figure 2: NO production by RAW 264.7 cell line (cell passage n° 8 to 19) after 24 h in culture with $Chit_A$ NPs 80 %, $Chit_A$ NPs 93 % and the polymers prepared in endotoxin-free and sterile conditions, stimulated with LPS (A) or without LPS (B). Cell viability (MTT assay) results for $Chit_A$ NPs and the polymers performed after NO assay. (C) Assay performed in the presence of LPS (D) Assay performed in the absence of LPS (Mean ± SEM; n = 3 to 4). (*p<0.05 compared to LPS control).

In order to evaluate if the NPs and polymers had an inhibitory effect in NO production, was used LPS as a positive stimulus to induce the NO production by the cells. The results showed an inhibitory effect of Chitosan NPs and polymers tested in LPS-induced NO production at all concentrations used when compared to the LPS control. With the $Chit_A$ NPs 93 % the decrease is not significant. This decrease in the NO production was more accentuated with $Chit_A$ NPs 80 % at 156.25 µg/mL (Figure 2 A). With the aim of evaluating whether one of the concentrations tested of the polymers or the chitosan nanoparticles would be able to induce the production of NO, a second battery of tests were done in the

absence of LPS and the results were presented in figure 2B. None of the concentrations tested of Chitosan NPs and polymers induced NO production (Figure 2 B). Then, in both battery of tests (presence and absence of LPS), after collecting the supernatant to quantify the NO, a MTT test was performed to evaluate the metabolic activity of the cells during the NO assay. It was possible to conclude that the polymers and nanoparticles were used in non-cytotoxic concentrations (Figure 2 C; 2 D). Moreover, it is possible to observe that cells stimulated with LPS showed a lower metabolic activity (Figure 2 A) than cells stimulated with only the polymer and the nanoparticles (Figure 2 B). However, in both cases the cell viability was higher than 70 % which is important to conclude that the inhibitory effect of the LPS-induced NO production is really a result of the NPs and polymers tested (Figure 2 A), and not a false result where this effect could result from the cell death if the concentrations used were cytotoxic. The solvent, Chitosan NPs and polymers were tested without cells to evaluate possible interferences on the method and no interference occurred.

These results are in agreement with a report published by Yoon and co-workers in which Chitosan oligosaccharide also inhibited NO production in LPS-stimulated murine macrophages (RAW 264.7) after 6 h and 12 h of incubation (Yoon *et al.*, 2007). In contrast, other group tested the effect of Chitosan nanoparticles in PBMCs after 24 h incubation and their results showed a dose-dependent increase in NO production at concentrations above 68.18 μg/mL of chitosan NPs (Pattani *et al.*, 2009). Luzardo-Alvarez and co-workers studied the effect of Chitosan microspheres in murine macrophages and had no effect in NO production (Luzardo-Alvarez *et al.*, 2005). It is difficult to evaluate that contradictory information found in literature because the reports did not inform if a LPS-free polymers were used or not and it is well known that the presence of LPS in the raw material generate false positives. In fact, among the studies cited, only the report of Pattani and co-workers stated a method to eliminate endotoxins which consisted in the *in vitro* culture of the cells with polymyxin B before the NPs addition to avoid false positives (Pattani *et al.*, 2009). The characterization of the polymer and NPs, parameters as MW, DD and size, are also important and can influence the results, and this information is not present in all studies or is incomplete.

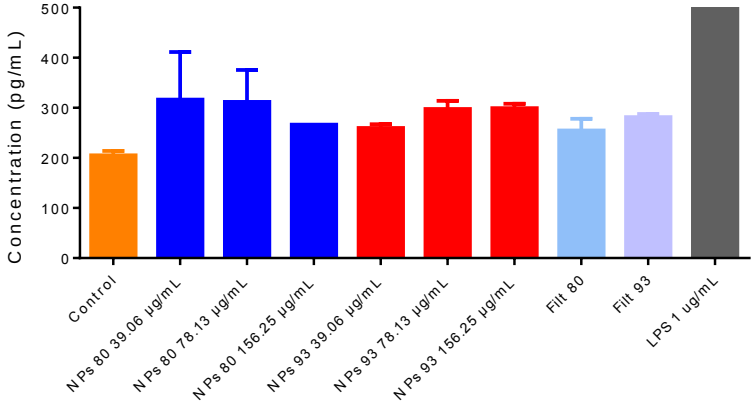
3.2.3. Chitosan with a DD 93 % induced the TNF-α production in RAW 264.7 cell line

The cytokines are signaling molecules that play an important role as they can regulate several processes (Duque e Descoteaux, 2014). Tumor necrosis factor alfa (TNF-α) is one of the

pro-inflammatory cytokines released when macrophages are activated (Parihar, Eubank e Doseff, 2010). This cytokine induces vasodilation and loss of vascular permeability allowing the infiltration of other immune cells (Duque e Descoteaux, 2014).

In the present work the production of this cytokine was quantificated by ELISA in the supernatants of RAW 264.7 cells incubated with $Chit_A$ NPs and polymers endotoxin-free for 24 h. Three different concentrations of the formulations were used (39.06 µg/mL, 78.13 µg/mL and 156.25 µg/mL).

A



B

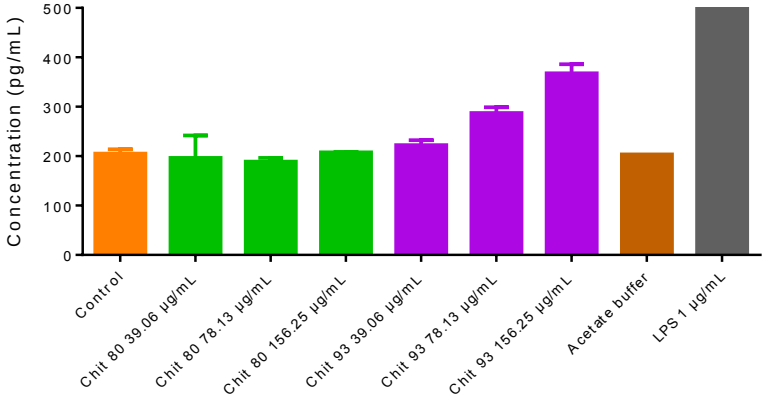


Figure 3: TNF-α cytokine production. RAW 264.7 cells were incubated with endotoxin-free Chitosan NPs and polymers for 24 h. (A) Comparison of Chitosan NPs 80 % and 93 %. (B) Comparison of polymers. LPS 1µg/mL were used as positive controls (n = 2; Mean ± SEM).

The results in figure 3A showed that the $Chit_A$ NPs 80 % and the $Chit_A$ NPs 93 % did not stimulate the TNF-α production when compared with the control. The TNF-α concentration found was similar to the respective solvent controls and to the control (cells and medium) (Figure 4 A). A similar result was found for the polymer with DD 80 %. On the other hand,

although with low values, the polymer with DD 93 % showed a concentration-dependent increase in TNF-α production (Figure 3 B). In fact, the value found for the higher concentration tested (Chit 93 %) is almost twice the value found for the control.

The production of TNF-α has been evaluated by others, for insctance Feng and co-workers reported that chitosan increased TNF-α production by macrophages after 18 h of incubation (Feng, Zhao e Yu, 2004). On the other hand, Jue Tu and co-workers studied CACO-2 cells stimulated with LPS and reported an anti-inflammatory effect of Chitosan NPs, decreasing pro-inflammatory cytokines as TNF-α. In another report, chitosan NPs were used at a range of concentrations between 6 µg/mL and 24 µg/mL and they also observed a higher effect at 12 µg/mL than with higher concentrations and suggested that the aggregation of Chitosan NPs at high concentrations could be the reason of the decrease of anti-inflammatory effects observed (Tu *et al.*, 2016). These studies did not inform if the NPs used are endotoxin-free, which can lead to these contradictory results. The $Chit_A$ NPs used in this assay (Figure 3) are endotoxin-free and the results showed are in agreement with Jue Tu and co-workers report, however the opposite results reported by Feng and co-workers may be a false positive as they don't mention the use endotoxin free NPs and they did not mention controls, such as the solvent control.

3.2.4. Both polymers and NPs were not able to stimulate the production of IL-1β in RAW 264.7 cell line

Cytokines are key modulators of inflammation (Turner *et al.*, 2014). Interleukin-1β (IL-1β) is a pro-inflammatory cytokine from the IL-1 familly produced by immune cells as macrophages. As TNF-α, this cytokine is also released at the early stages of the immune response (Duque e Descoteaux, 2014).

The production of this cytokine was quantificated by ELISA in the supernatants of RAW 264.7 cells incubated with $Chit_A$ NPs and polymers endotoxin-free for 24 h. The same, as mention before for the TNF- α, concentrations were used (39.06 µg/mL, 78.13 µg/mL and 156.25 µg/mL).

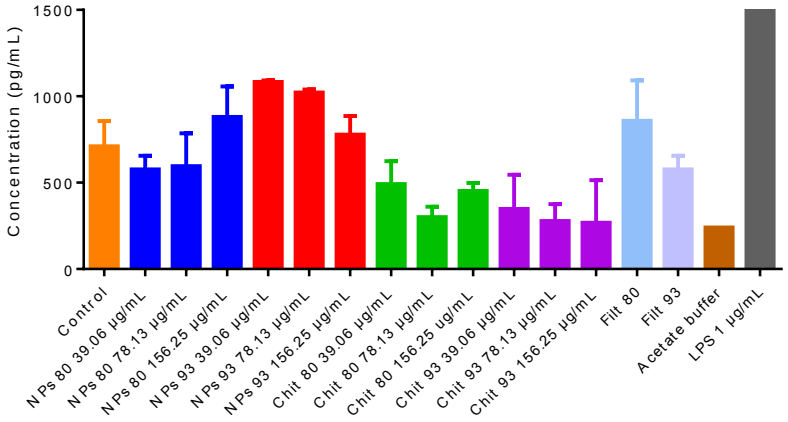


Figure 4: IL-1β cytokine production. RAW 264.7 cells were incubated with Chitosan NPs and polymers for 24 h. Comparison of Chitosan NPs 80 % and 93 % and polymers. LPS at a concentration of 1 µg/mL was used as positive controls (n = 2; Mean ± SEM).

The results in figure 4 showed that both polymers and NPs were not able to stimulate the production of the IL-1β. In some cases, a tendency to show higher values was observed, however the number of assays performed did not allow us to draw a reliable conclusion (Figure 4). In fact, recently, a very exhaustive study was done in our laboratory with chitosan NPs, using a different chitosan polymer (with a different DD) and with dendritic cells. In this study, we concluded that chitosan and chitosan NPs were not able to stimulate the production of the IL-1β (data not published). In fact, the generation of the IL-1β was observed only in cells that received a first stimulus with CpGODN. It is widely described in literature that for IL-1β production by the inflammasome NLRP3 in macrophages a co-stimulation is needed (Lopez-Castejon e Brough, 2011). In the present work, the absence of a first stimulation may explain the IL-1β low concentrations with values similar to the control group (Figure 4). However, we can also find some contradictory results, for instance Feng and co-workers reported the production of IL-1β in macrophages induced by Oligochitosan with a DD higher than 85 % but did not refer the use of chitosan endotoxin-free nor the previous stimulation which can lead to misunderstanding (Feng, Zhao e Yu, 2004).

3.2.5. Chitosan NPs showed to be more toxic than the polymers in PBMCs

Human peripheral blood mononuclear cells (PBMCs) are isolated from peripheral blood and correspond to any blood cell with a round nucleus such as lymphocytes, monocytes and others. PBMCs are an easy accessible source of human immune cells, as the cells are isolated

from full blood or buffy coats (Kleiveland C.R., 2015). Peripheral blood is the place where exposure to chemicals occurs which makes PBMCs an important tool to assess the particle effects in immune system (Pourahmad e Salimi, 2015).

In the present study the PBMCs were incubated for 24 h with Chitosan NPs and polymers at a range of concentrations between 2.44 µg/mL and 5000 µg/mL. MTT viability assay was performed to evaluate the effect of Chitosan NPs and polymers on cell metabolic activity.

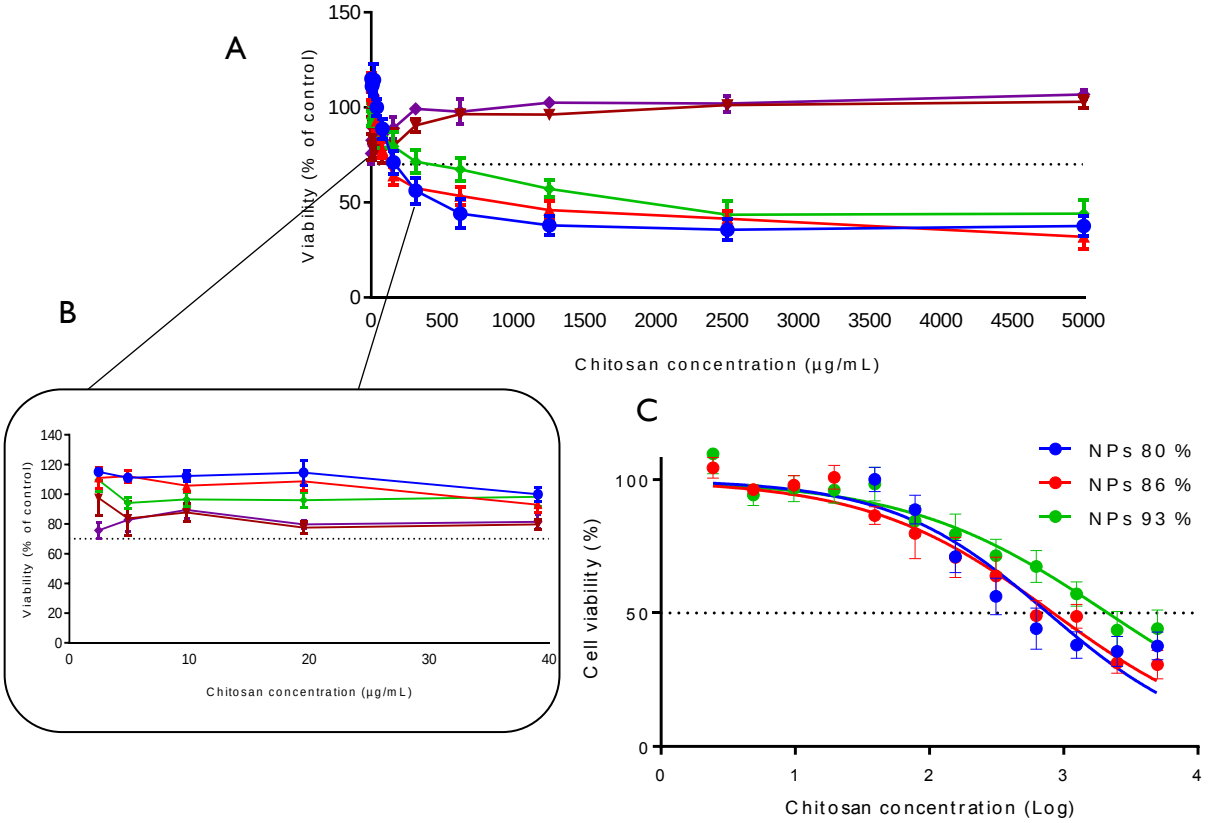


Figure 5: Cytotoxicity assay (MTT), performed in PBMCs after 24 h incubation with NPs and polymers (Mean ± SEM, n = 4 to 7). Comparison of three types of $Chit_A$ NPs with different DD (80 %, 86 % and 93 %) and MW and the polymers (Chit 80 % and Chit 93%) (A-B). Concentration-response curve of $Chit_A$ NPs 80 %, 86 % and 93 % (C).

The cytotoxicity results in human peripheral blood mononuclear cells (PBMCs) are showed in figure 5. The three types of Chitosan NPs showed a viability decrease at concentrations above 156.25 µg/mL. On the contrary, the respective polymers, also tested in this assay, showed a cell viability above 70 % for all concentrations tested. This result showed that Chitosan NPs were more toxic than the respective polymers to human PBMCs. An effect already observed for murine macrophages (Figure 1).

The $Chit_A$ NPs 80 % and the $Chit_A$ NPs 86 % apparently stimulated the cells proliferation, as the viability results at lower concentrations are around the 120 % which may indicate the activation of the metabolic cell activity (Figure 5 B).

The $Chit_A$ NPs 80 % showed a tendence to be more toxic than the other $Chit_A$ NPs tested. $Chit_A$ NPs 80 % showed to induce an accentuated decrease in cell viability, with 50 % decrease in cell viability (IC 50) at approximately 767.8 µg/mL. On its turn, $Chit_A$ NPs 86 % presented a decrease in cell viability, being the IC 50 predicted at 905.5 µg/mL and for $Chit_A$ NPs 93 % showed a 50 % decrease in cell viability at 2130 µg/mL (Figure 5 C).

Several controls were made in order to eliminate false positives or false negatives, like the supernatant of the nanoparticles, the particles itself without cells and was possible to conclude that any cytotoxicity observed, mainly with nanoparticles, represented a true value since all the controls presented values close to zero (no interference was detected).

S. Omar Zaki and co-workers showed an increase of NP size with higher MW. They also reported that cytotoxic effects on hematopoietic stem cells of chitosan NPs were indirectly influenced by molecular weight because it affects the particle zeta potential and size. Then, with low MW, they obtained smaller NPs that showed to be more cytotoxic. Our results also showed that the $Chit_A$ NPs with lower MW resulted in NPs with lower size and showed higher cytotoxicity which is in agreement with the study of S. Omar Zaki and co-workers. (Sarah *et al.*, 2015).

3.2.6. Chitosan NPs and polymer did not show hemolytic activity in human whole blood

Hemolysis is the rupture of red blood cells (RBCs) and the release of their contents into the surroundings which can lead to anemia, jaundice and renal failure (Dobrovolskaia *et al.*, 2008). In case of systemic administration, the nanoparticles can get in contact with RBCs, therefore it's important to evaluate their effect on these blood elements. However, this evaluation is also important if other administration routes are used to study the biocompatibility.

To evaluate the hemolytic activity of Chitosan NPs and polymers in human blood, three different concentrations were used 0.1 mg/mL, 1 mg/mL and 2 mg/mL. The lower concentration chosen was based in the viability results obtained before and the other concentrations chosen are 10 and 20 times higher (Figure 6).

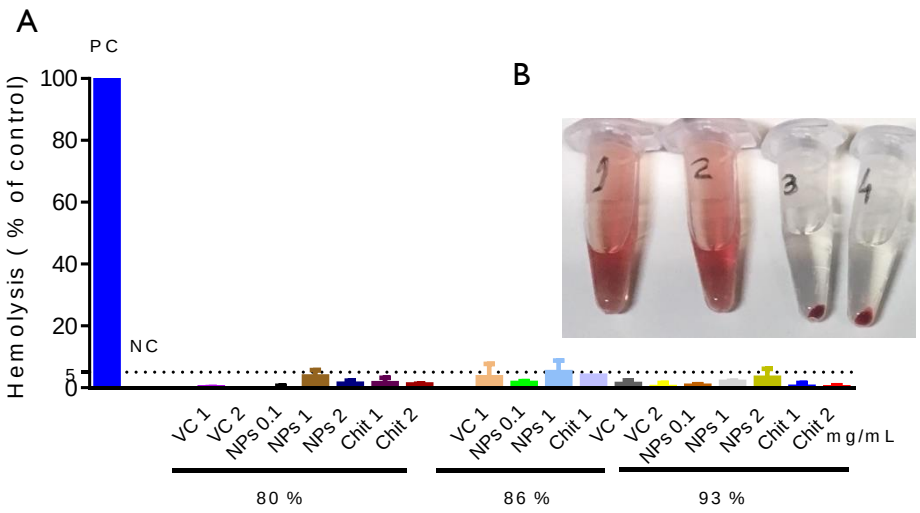


Figure 6: (A) Hemolytic activity of Chitosan NPs and polymers in human blood after 3 h incubation at 37 °C. PBS and Triton-X-100 were respectively used as negative and positive control (Mean ± SEM; n = 2 to 5). (B) Representation of 100 % hemolysis in positive control (1 and 2) and the absence of hemolysis in the negative control (3 and 4).

The results in figure 6 showed that none of Chitosan NPs and polymers caused hemolysis in human blood as the percentage of hemolysis was less than 5 % accordingly to ASTM E2524-08 standard (cit. por Choi *et al.*, 2011). The control of NPs solvent was done and no hemolysis effect was detected as well. Controls with the NPs but without blood were prepared to evaluate the possible NPs interferences with the assay and no interferences were detected. Nadesh and co-workers also showed that chitosan NPs had no effect on hemolysis (Nadesh *et al.*, 2013). In contrast, Muniz de Lima and co-workers described that chitosan NPs produced with a chitosan with DD higher than 75 % induced hemolysis but did not describe the MW of the chitosan used in the assay nor performed important controls which can lead to false positive results such as the solvent controls or the incubation of the NPs without blood to evaluate the NPs interference (Lima *et al.*, 2015). The absence of information about the chitosan characterization and the lack of some controls can be the reason of these contradictory results. Besides that, the NPs used in these reports were also very different as in the study where they observed the induction of hemolysis the NPs tested had 10 nm and in the other NPs with 140 nm were used (Lima *et al.*, 2015; Nadesh *et al.*, 2013).

3.2.7. Chitosan NPs interfered with the platelet aggregation method in human plasma

Platelets play an important role not only in hemostasis but also in immune and inflammatory responses (Jenne e Kubes, 2015). Homeostatic imbalance as result of platelet function alterations affect primary hemostasis and can result in thrombotic or haemorrhagic disorders (Laloy *et al.*, 2014). Then, it is important to study the Chitosan NPs interactions with platelets function.

Platelet aggregation was evaluated by the determination of platelet count after exposure of platelet-rich plasma (PRP) with 0.1 mg/mL and 2 mg/mL of chitosan NPs for 15 min. Platelet aggregation is considered significant to results above 20 % (Rodriguez *et al.*, 2015).

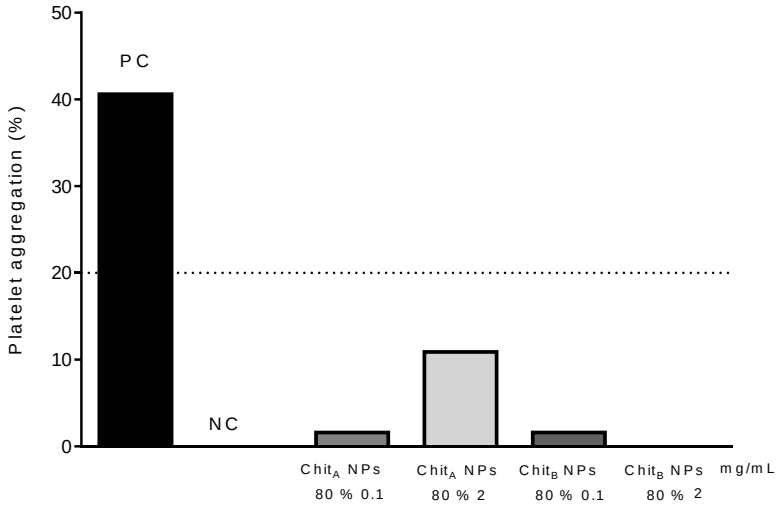


Figure 7: Effect of Chitosan NPs on platelet aggregation – preliminary assessment. Platelet aggregation was detected by incubating PRP with 0.1 mg/mL and 2 mg/mL of chitosan NPs for 15 min. PBS and 100 µg/mL collagen were used as negative control (NC) and positive control (PC), respectively. 20 % of platelet aggregation was defined as the assay threshold (dash line).

The preliminary results showed that none of the Chitosan NPs caused significant platelet aggregation. Collagen was used as positive control and induced about 40.6 % of platelet aggregation and PBS was used as negative control (Figure 7). However, possible interferences of the solvents and Chitosan NPs in platelet counting were evaluated in PRP (plasma rich in platelets) and in PFP (free-platelet plasma) in order to confirm or refute these results.

Table 1: Effect of Chitosan NPs in platelet counting and different controls. Platelet aggregation was detected by incubating platelet-rich plasma (PRP) or platelet-free plasma (PFP) with different concentrations of Chitosan NPs and the respective solvents for 15 min. PBS and 0.1 mg/mL collagen were used as negative and positive controls, respectively.

	Platelet count ($x10^9$/L)
PRP + negative control (PBS)	66
PFP + negative control (PBS)	1
PFP + $Chit_A$ NPs 80 % 2 mg/mL	17
PFP + $Chit_B$ NPs 80 % 2 mg/mL	3
PRP + positive control (collagen)	57
PRP + solvent	59
PRP + $Chit_A$ NPs 80 % 2 mg/mL	57
PRP + $Chit_B$ NPs 80 % 2 mg/mL	65
PRP + $Chit_A$ NPs 80 % 0.1 mg/mL	63
PRP + $Chit_B$ NPs 80 % 0.1 mg/mL	63

In fact, the controls showed that $Chit_A$ NPs 80 % interfered with the platelet counting because when no platelets were present, the platelet count was 17 x 10^9/L. In the case of$TChit_B$ NPs 80 % no interference was detected (Table 1).

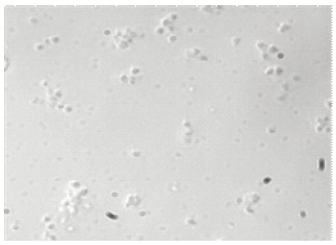
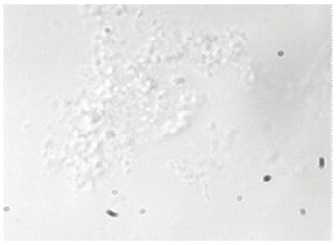
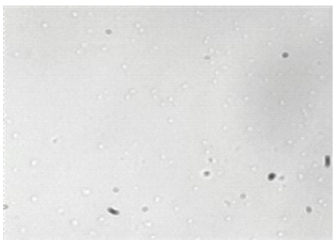
PRP	**PFP + $Chit_A$ NPs 80 %**	**PFP + $Chit_B$ NPs 80 %**
		

Figure 8: Representative images of PRP or PFP incubated with $Chit_A$ NPs 80 % or $Chit_B$ NPs.

Furthermore, with the purpose of evaluating the cause of the interferences observed, NPs size after addition to PFP was measured (Table 2).

Table 2: Characterization of Chitosan NPs. Particles mean size distribution (nm) before and after addition to PFP.

	Size (nm)	
	Before addition to PFP	**After addition to PFP**
$Chit_A$ NPs 80 % (2 mg/mL)	126	524
$Chit_B$ NPs 80 % (2 mg/mL)	455	28

The addition of 2 mg/mL of $Chit_A$ NPs 80 % to PFP caused an increase in the mean NPs size from 127 nm to 524 nm (Table 2) and when observed in the microscope was possible to see the aggregation of chitosan NPs (Figure 8). On the other hand, the addition of 2 mg/mL of $Chit_B$ NPs 80 % to PFP caused the destruction of the NPs, as can be seen by the alteration of NP mean size from 455 nm to 28 nm (Table 2) and by the microscope images (Figure 8).

These results showed that Chitosan NPs interfered with the method used and it also reinforces the importance of the use of appropriate controls to avoid false positives or false negatives. To overcome this interference, new studies need to be performed using a new principle.

3.2.8. $Chit_A$ NPs 80 % affected plasma coagulation time by the intrinsic pathway in human plasma

The plasma coagulation cascade is responsible for blood clotting and consists in a series of protein interactions (Laloy *et al.*, 2014).

To evaluate the effect of $Chit_A$ NPs and polymers on plasma coagulation time after incubation for 30 min, two concentrations were used (0.1 mg/mL and 1 mg/mL). In this assay the two pathways of blood coagulation, activated partial thromboplastin time (APTT) and prothrombin time (PT) were separately tested (Figure 9).

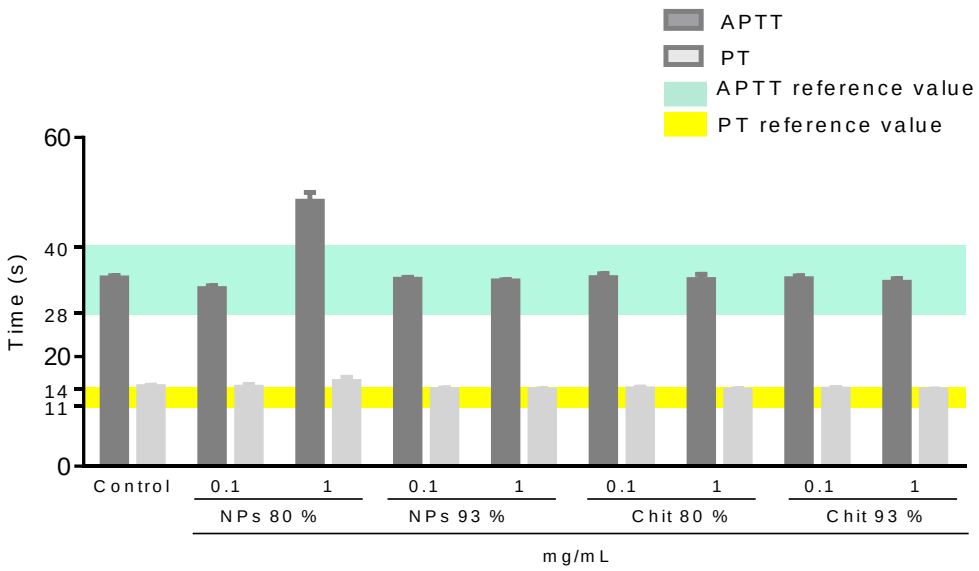


Figure 9: Effect of $Chit_A$ NPs and polymers at 0.1 mg/mL and 1 mg/mL on plasma coagulation time after incubation for 30 min. The two pathaways, APTT and PT were separately tested. APTT reference range of values is 20 s to 40 s and for PT is 11s to 14 s (Mean ± SEM; n = 3; Three independent experiments each in duplicate).

The results showed that $Chit_A$ NPs and polymers at 0.1 mg/mL had no effect on plasma coagulation for the two pathways. However, 1 mg/mL $Chit_A$ NPs 80 % prolonged APTT (intrinsic pathaway), while no effect was observed with $Chit_A$ NPs 93 % and polymers 80 % and 93 % at the same concentration (Figure 9). The solvent controls were done and did not show to affect any pathaway.

The fact that $Chit_A$ NPs 80 % prolonged APTT can result from the affinity of nanoparticles for plasma clotting factors that are involved in the instrinsic pathway (XII, XI, IX, VIII) adsorving them (Palta, Saroa e Palta, 2014). With the factors adsorption, the coagulation is delayed. Nadesh and co-workers also had similar results as they observed that chitosan nanoparticles produced with a size around 140 nm did not induce any clot formation up to 60 s for APTT. They used plasma with 0.9 % saline solution as negative control (Nadesh *et al.*, 2013). In opposition, as shown in figure 9, only the PT showed to be prolonged, while the APTT was not affected.

Chapter IV
Concluding remarks and Future perspectives

4. Concluding remarks and future perspectives

Materials with a size in nanoscale have several new properties and their industrial use creates new opportunities, but they also present new risks and uncertainties. Emergent production and use of nanomaterials result in an increasing number of workers and consumers exposed to nanomaterials. Among several possible applications, nanoparticles have been intensely investigated as drug delivery systems. The studies reported on literature normally describe the method of preparation of the particles, method of encapsulation of the drug into nanoparticles, efficiency of drug encapsulation and tests that prove the efficacy of the drug encapsulated. Rarely the studies reported immunotoxicity results and usually, the only indicator of toxicity provided is cell viability in target cells. The response of the immune system cells to the presence of these nanoparticles is less frequently evaluated. For instance, a frequent approach to develop an oral therapy for diabetes is through the encapsulation of the insulin. If the nanoparticles are able to stimulate the immune system, then it can produce an immune response against insulin. Since, in this case, there is no interest in the immune system being activated, to encapsulate insulin must be chosen one polymer that has reduced capacity to stimulate the immune system. On the other hand, the application of nanoparticles as vaccine adjuvants requires the activation of the immune system, so a polymer with a high capacity of immune system stimulation would be desirable. This consists in the safe-by-design approach. Therefore, a set of tests that evaluate the interaction of the nanoparticles with cells of the immune system is urgent needed.

The immunotoxicological evaluation of nanoparticles (NPs), even without loading any drug is important as NPs can interact with the immune system and the knowledge of how it occurs can be useful in other studies.

Chitosans with different deacetylation degree (DD) and molecular weight (MW) were used in the production of $Chit_A$ NPs and $Chit_B$ NPs to evaluate the effect of these characteristics in the immunotoxicity of chitosan.

The Chitosan NPs produced showed different sizes that were influenced by the DD and MW. The use of polymers with higher DD and MW resulted in larger NPs.

The Chitosan NPs produced were also used in a battery of immunotoxicological tests described in chapter III. In a first set of experiments, the effects of the $Chit_A$ NPs and the respective polymers were assessed in RAW 264.7 cells and human PBMCs viability. The NPs showed to be more toxic than the respective polymers. An inhibitory effect of the NPs and polymers in LPS-induced NO production was observed which was more significant with the

$Chit_A$ NPs with lower DD (80 %). On the other hand, without LPS, none concentration of Chitosan NPs and polymers had a stimulatory effect in the NO production. The effect of chitosan in cytokine production was evaluated with two pro-inflammatory cytokines: TNF-α and IL-1β. Neither type of $Chit_A$ NP induced TNF-α, while the polymer with higher DD (93 %) showed to increase this cytokine production in a dose-dependent manner. Both polymers and NPs were not able to stimulate the production of IL-1β. The hemocompatibility of chitosan was also evaluated and the NPs did not show hemolytic activity NPs but the $Chit_A$ NPs 80 % affected the plasma coagulation time by the intrinsic pathway.

The need to adapt some methods that are used in conventional formulations to the NPs was clearly showed, with the platelet aggregation test, where the NPs interference was observed.

The results presented in this work, show that the DD and MW of chitosan can affect some immunotoxicological parameters such as the toxicity, the cytokine production and the hemocompatibility. The parameters are not affected in the same way. The Chi_A NPs seem to have a higher influence in these parameters, namely cytotoxicity and coagulation.

Furthermore, it was also highlighted that the nanoparticle interference with the assays is a challenge, and that in many published reports there is a lack of important controls, which can lead to some conflicting results. It is essential the use of adequate controls to validate if an assay is appropriate to each nanoparticle formulation, if the solvents are interfering with the supposed nanoparticles effect, if the NPs are contaminated with endotoxins and ultimately to avoid false positive and negative results. Moreover, the characterization of the NPs and respective polymers is also important.

In fact, chitosan has been object of many studies and it versatility and interesting properties makes this a polymer with huge potential in many fields, namely in biomedicine. However, its immunotoxicological are poorly understood and even some studies reveal contradictory results and not providing all the information needed to understand the differences reported. The chitosan DD can range between 40 % to 98 % and 50 kDa to 2000 kDa of the MW. The polymers used in this work have a very similar DD, which may have made it difficult to observe different effects. A chitosan with lower DD should also be studied to evaluate better the differences between the polymers. Furthermore, other assays could be done to complement this immunotoxicological studies as for example, the evaluation of chitosan effect in reactive oxygen species or in cell proliferation. Also, a next step would be the *in vivo* tests to complement the *in vitro* results.

Further studies with other polymers would help to create knowledge to stablish guidelines specifically applied to nanomaterials with standardized and validated immunotoxicological tests to understand their biological effect which is important to their biomedical application and safe-by-design of new nanomedicines.

Chapter V
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