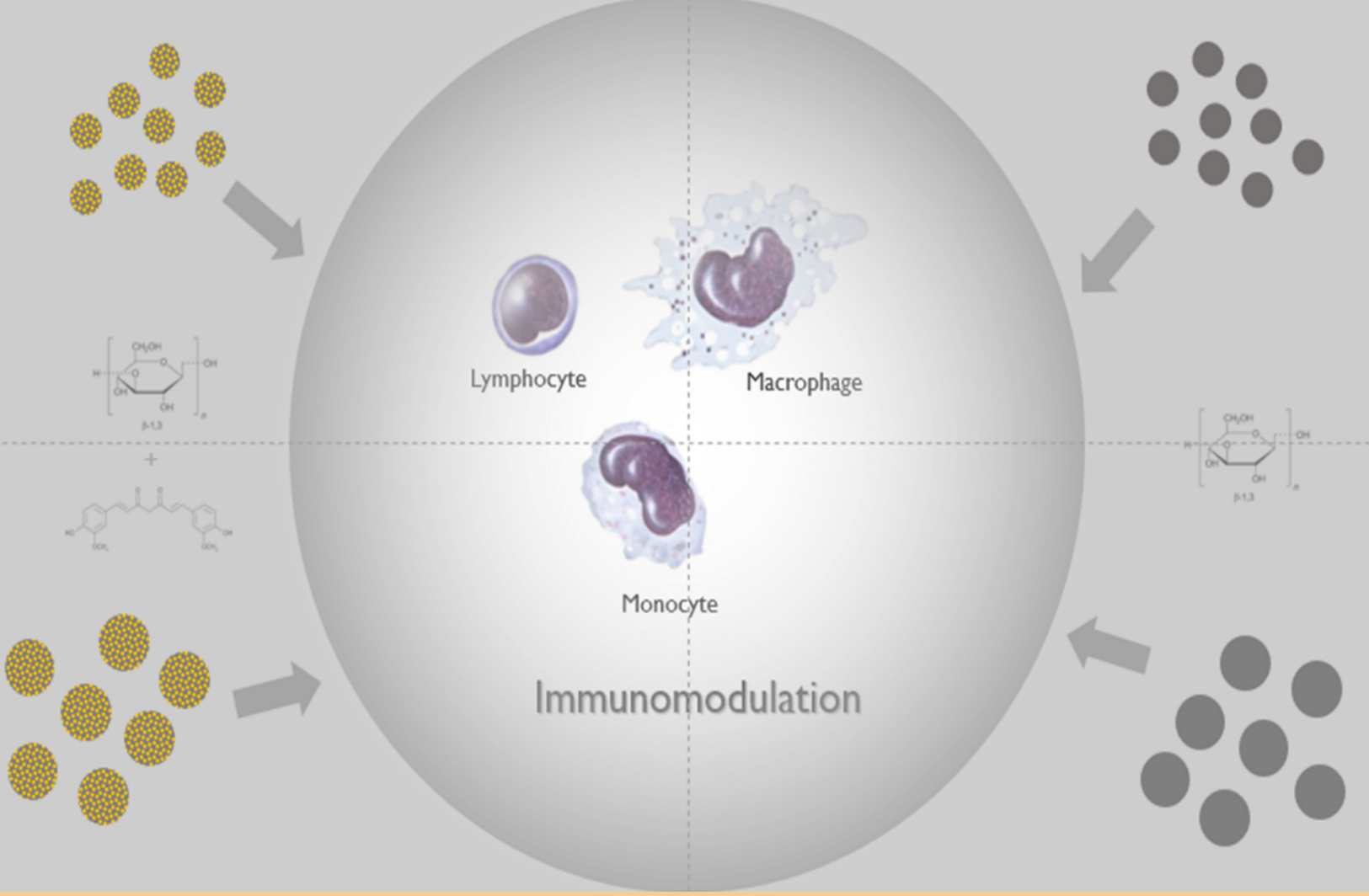

Mariana Raquel Antunes Colaço

Development of Nanosized Glucan Particles with Immunostimulatory Function as a Delivery System for Curcumin

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 illustrating the interaction between curdlan nanoparticles (Glu 100 and Glu 250) and curcumin loaded curdlan nanoparticles (GluCur 100 and GluCur 350) with cells of the immune systems as macrophages, monocytes and lymphocytes. Adapted from: https://www.cancer.gov/images/cdr/live/CDR503952-750.jpg

Development of Nanosized Glucan Particles with Immunostimulatory Function as a Delivery System for Curcumin

Mariana Raquel Antunes Colaço

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 Doctor 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: Brain Health 2020 and national funds by FCT - Foundation for Science and Technology under the project PROSAFE/0001/2016 and strategic projects POCI-01-0145-FEDER-007440. TEM microscopy analyses were performed at IBILI with the expert technical assistance of Mónica Zuzarte, under the project PPBI-POCI-01-0145-FEDER-022122. Mariana Colaço 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

"Se não puder voar, corra.
Se não puder correr, ande.
Se não puder andar, rasteje, mas
continue em frente de qualquer jeito."
Martin Luther King

Em primeiro lugar, quero agradecer à minha orientadora, Professora Doutora Olga Maria Fernandes Borges Ribeiro, por me ter acompanhado neste meu percurso, ainda na licenciatura e ao longo destes 2 anos de mestrado. Obrigado por me ter transmitido o gosto pela investigação científica e por todo o apoio e incentivo dados ao longo do meu caminho.

Não posso deixar de agradecer também o grande apoio de todos os elementos do "Nanolab" em especial à Edna, à Patrícia, à Sandra e ao João, não esquecendo, contudo, muitos outros colegas que também me acompanharam neste trabalho e já não se encontram no laboratório. Todos eles me transmitiram a sua experiência e o seu saber, estando sempre disponíveis para me ajudar no que fosse necessário. Igualmente importante foram os momentos partilhados nas nossas convivências diárias e a amizade que ficou.

Agradeço em especial à Alana, minha colega de mestrado, pelo companheirismo e pela amizade demonstrada em todos os momentos. Nunca me irei esquecer de todo o alento que me deu mesmo nos momentos mais complicados, nem das nossas pausas para o chocolate.

Agradeço ainda a ajuda prestada e a disponibilidade demonstrada por todas as pessoas do piso 4 da Faculdade de Farmácia, especialmente à Maria Mendes não só pela experiência laboratorial compartilhada, mas também por todos os conselhos que me foi dando.

Quero deixar uma palavra de gratidão para todos os meus colegas e amigos, especialmente à Mariana Neves, à Catarina Luz e à Inês Barros por todos os bons momentos que passámos e por estarem sempre presentes tanto nas boas como nas más ocasiões.

Por último, mas não menos importante, gostaria de deixar um agradecimento muito especial aos meus pais, por todo o apoio prestado e por serem um dos grandes pilares da minha vida. Sem o vosso apoio e o vosso esforço nunca teria chegado aqui e espero que estejam orgulhosos do meu percurso.

Table of Contents

List of Abbreviations

APCs	Antigen presenting cells
APTT	Activated partial thromboplastin time
CMH	Cyanmethemoglobin
CO_2	Carbon dioxide
COX	Cyclooxygenase
CR	Complement receptor
Cryo-SEM	Cryo-scanning electron microscopy
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dTBH	diluted Total Blood Hemoglobin
ELISA	Enzyme-linked immunosorbent assay
ELS	Electrophoretic Light Scattering
FBS	Fetal bovine serum
Glu 100	Nanoparticles of glucan with 100 nm
Glu 250	Nanoparticles of glucan around 250 nm
GluCur 100	Nanoparticles of glucan with encapsulated curcumin with 100 nm
GluCur 350	Nanoparticles of glucan with encapsulated curcumin around 350 nm
Glu Polymer	Glucan Polymer
GSH	Glutathione peroxidase
HCl	Hydrogen chloride
IL-	Interleukin
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
INR	International normalized ratio
LPS	Lipopolysaccharide
LOX	Lipoxygenase
MCP	Monocyte chemoattractant protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide

NC	Negative control
NK cells	Natural killer cells
NO	Nitric oxide
NPs	Nanoparticles
OD	Optical density
PAMPS	Pathogen-associated molecules patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PC	Positive Control
PDI	Polydispersity index
PFH	Plasma free hemoglobin
PLGA	Poly(lactic-co-glycolic acid)
ppp	Platelet-poor plasma
PRRs	Pattern recognition receptors
PT	Prothrombin time
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
RT	Room temperature
SEM	Standard error of the mean
SEM (disambiguation)	Scanning electron microscopy
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
VC	Vehicle control (filtrate)
ZP	Zeta potential

Abstract

Curcumin is a natural compound isolated from the rhizome of the plant *Curcuma longa*. It is a hydrophobic diphenol known for its diverse pharmacological activities including antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic properties. However, despite its advantages, curcumin does not have a good clinical efficacy due to its low aqueous solubility, its poor bioavailability and rapid systemic elimination. A promising approach to solve this problem and enhance curcumin's clinical relevance is to encapsulate it using a drug delivery system that is dispersible in aqueous media. In this regard, nanoparticles (NPs) have gained importance as drug delivery systems since they improve the therapeutic impact of the drug by enhancing its retention time and allowing a controlled release or even providing better targeting and tissue penetration. More recently, biodegradable polymeric nanoparticles have grown interest since they enable a greater stability in body fluids, they present low toxicity and they can act as immunostimulants or adjuvants. Curdlan is a neutral polymer consisting of β-1,3-linked glucose residues. It is extracted from *Alcaligenes faecalis* and it has many applications based on its physicochemical properties and in its relevance in the medical field. In the literature glucan NPs have been addressed in different medical fields like for cancer treatment or as gene delivery system. Taking this into account, this work aims to develop a method to prepare curcumin loaded glucan NPs, characterize them and evaluate its immunotoxicity *in vitro*.

The work herein describes the production of two glucan-based delivery systems with 2 different sizes to encapsulate curcumin. These systems were produced through a nanoprecipitation technique. The suspensions of NPs were characterized, having obtained formulations with relatively low polydispersity index and one system with a size of 100 nm and the other around 300 nm. Both NPs, exhibited round shape morphology and neutral zeta potentials, presenting minor variations between each batch. It was also observed a greater stability at 4 °C for at least 42 days. These characteristics lead us to considerate both 100 and 300 nm polymeric NPs ready to start the studies with cells. The effect of the size of the NPs on biological properties was evaluated. Therefore, the immunotoxicity and immunomodulatory effects were tested *in vitro* on a murine macrophage cell line (RAW 264.7) and in human peripheral blood mononuclear cells (PBMCs), two appropriate cell models to evaluate the interaction of NPs with cells of the immune system. Moreover, it was evaluated the compatibility of the formulations with blood components. The cell viability studies performed in the two cell types showed that Glu 100 and GluCur 100 NPs present a higher toxicity compared with Glu 250 and GluCur 350 NPs, being this toxicity associated

with the particles and not curcumin. This higher toxicity of the smaller particles was also observed in the hemolysis assay. However, all types of formulations showed a great compatibility in time coagulation assay, by not altering prothrombin time and activated partial thromboplastin time. In relation to the immunomodulatory effects, Glu 250 NPs presented a higher immunomodulatory capacity by producing NO and the cytokines TNF-α and IL-1β and by inducing the proliferation of PBMCs. Contrary, with the encapsulation of curcumin it was observed the inhibition of NO and the cytokines TNF-α and IL-1β, which evidences curcumin anti-inflammatory property.

To conclude, in this work we reported the successful production of curcumin encapsulated glucan NPs as a strategy to improve curcumin low bioavailability. Further studies are now required to access in more detail more immunological effects of these delivery systems in order to safely use it in *in vivo* studies.

Keywords: Curcumin; anti-inflammatory; polymeric delivery systems; nanoparticles; glucan; immunotoxicity.

Resumo

A curcumina é um composto natural isolado do rizoma da planta *Curcuma longa*. É um difenol hidrofóbico conhecido pelas suas diversas propriedades farmacológicas, incluindo efeitos antioxidantes, anti-inflamatórios, antimicrobianos e anti-carcinogénicos. No entanto, apesar destas vantagens a curcumina não apresenta uma boa eficácia clínica devido à sua baixa solubilidade em água, reduzida biodisponibilidade e rápida eliminação sistémica. Uma abordagem promissora para resolver esse problema e aumentar a relevância clínica deste composto é encapsulá-lo utilizando um sistema de entrega de fármacos que seja dispersível em meio aquoso. Nesse sentido, as nanopartículas (NPs) ganharam relevância como sistemas de entrega, uma vez que são capazes de melhorar o impacto terapêutico do fármaco ao aumentar o seu tempo de retenção, permitem uma entrega deste composto de forma controlada e ainda proporcionam um melhor direcionamento para alvos selecionados e uma melhor penetração tecidual. Mais recentemente, as nanopartículas poliméricas biodegradáveis têm sido alvo de um crescente interesse, uma vez que permitem uma maior estabilidade nos fluidos biológicos, apresentam uma baixa toxicidade e podem atuar como imunoestimulantes ou adjuvantes. Curdlan é um polímero neutro constituído por resíduos de glicose com ligações β-1,3. É extraído de *Alcaligenes faecalis* e tem associado diversas aplicações baseadas nas suas propriedades físico-químicas e na sua relevância no campo da medicina. Na literatura, as NPs de glucano têm sido abordadas em diferentes áreas da medicina, como para o tratamento do cancro ou como sistema de entrega de genes. Tendo isso em consideração, este trabalho tem como objetivo desenvolver um método para preparar NPs de glucano com curcumina encapsulada, caracterizar esse sistema e avaliar a sua imunotoxicidade *in vitro*.

O trabalho aqui apresentado descreve a produção de dois sistemas de entrega com dois tamanhos diferentes à base de glucano, para encapsular a curcumina. Estes sistemas foram produzidos através de uma técnica de nanoprecipitação. As suspensões de NPs foram caracterizadas, obtendo-se formulações com índice de polidispersão relativamente baixo e um sistema com tamanho de 100 nm e o outro por volta dos 300 nm. Ambos os sistemas de NPs exibiram uma morfologia em forma redonda e potenciais zeta neutros, apresentando pequenas variações entre cada lote. Também foi observado uma maior estabilidade a 4 °C por pelo menos 42 dias. Estas características levaram-nos a considerar as NPs poliméricas de 100 e 300 nm prontas para iniciar os estudos com células. O efeito do tamanho das NPs nas propriedades biológicas foi avaliado. Para isso, a imunotoxicidade e os efeitos

imunomodulatórios foram testados *in vitro* numa linha celular de macrófagos de murganho (RAW 264.7) e em células mononucleares do sangue periférico humano (PBMCs), dois modelos celulares apropriados para avaliar a interação de NPs com células do sistema imunitário. Além disso, também foi avaliado a compatibilidade das formulações com componentes sanguíneos. Os estudos de viabilidade celular realizados nos dois tipos de células mostraram que as NPs Glu 100 e GluCur 100 apresentam maior toxicidade quando comparadas às NPs Glu 250 e GluCur 350, sendo esta toxicidade associada às partículas e não à curcumina. Esta maior toxicidade das partículas de menor dimensão também foi observada no ensaio de hemólise. No entanto, todos os tipos de formulações apresentaram grande compatibilidade no ensaio dos tempos de coagulação, não alterando o tempo de protrombina e o tempo de tromboplastina parcial ativada. Em relação aos efeitos imunomoduladores, as NPs Glu 250 apresentaram uma maior capacidade imunomoduladora ao levarem à produção de NO e das citocinas TNF-α e IL-1β e também ao induzirem a proliferação de PBMCs. Ao contrário, com o encapsulamento da curcumina, observou-se a inibição da produção de NO e das citocinas TNF-α e IL-1β, evidenciando assim as propriedades anti-inflamatórias da curcumina.

Para concluir, neste trabalho descrevemos a produção bem-sucedida de NPs de glucano com curcumina encapsulada como uma estratégia para melhorar a sua reduzida biodisponibilidade. Mais estudos são agora necessários para verificar mais detalhadamente os efeitos imunológicos destes sistemas de entrega, a fim de poder usá-los com segurança em estudos *in vivo*.

Palavras-chave: Curcumina; anti-inflamatório; sistemas de entrega poliméricos; nanopartículas; glucano; imunotoxicidade.

Chapter 1

Introduction

1. Introduction

1.1 Potential health benefits of polyphenols

Polyphenols are natural compounds that belong to the group of phytochemicals (Sa e Banerjee, 2010). Their chemical structure is known for having more than one phenolic hydroxyl group and depending on the number of phenolic rings, their characteristics differs. These micronutrients can be find in the human diet by consuming fruits, vegetables, red wine, olive oil, etc. The main reason for studying polyphenols is due to their role in prevention of several diseases mainly because of their anti-inflammatory and antioxidant properties (Basnet e Skalko-basnet, 2011). More recently, it was also associated cardioprotective effects by inhibiting the LDL oxidation, anti-carcinogenic and immunomodulating properties (Pandey e Rizvi, 2009). Despite this, it is important to do a pharmacological screen to find, among all polyphenols, those who are more biologically active and have more beneficial effects for humans. A good polyphenol example is genistein that was reported by Lamartiniere and colleagues to reduce the incidence of prostate tumors when administered in the diet of mice (Mentor-Marcel *et al.*, 2001). Quercetin is another polyphenol that is known for its anti- atherosclerotic activity because when injected in mice it caused a reduction of oxidative stress and foam cell accumulation (Kawabata, Mukai e Ishisaka, 2015). Resveratrol, which can be find in grapes is an important polyphenol that is associated with a reduce risk of cardiovascular diseases due to the moderate consumption of red wine (Stephan *et al.*, 2017).

In our work, we decided to use curcumin, a promising polyphenol that has been attributed numerous applications, for example as a medicinal herb in Asian countries, as a spice or colouring agent in food or can even be used in cosmetics (Hewlings, 2017). Besides these applications, it has also been associated with biomedical applications.

1.2 Curcumin, a plant-derived polyphenol

Curcumin is known to have a variety of targets and because of that it is associated with a wide spectrum of biological actions summarized on Figure 1.1. These include anti-inflammatory, antioxidant, anti-carcinogenic, anti-atherosclerotic, neuroprotective and immunomodulating properties (Merrell *et al.*, 2009) (Sa e Banerjee, 2010).

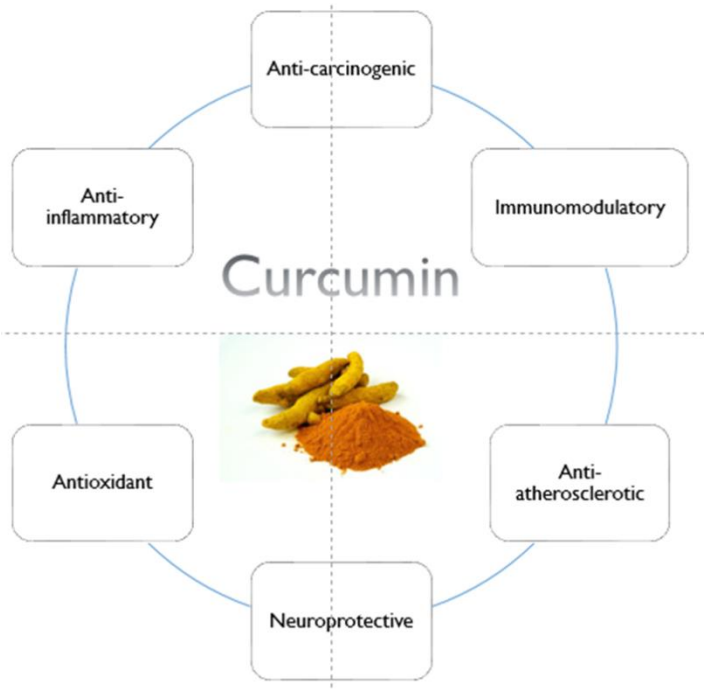

Figure 1.1 – Different biological activities of curcumin.

The majority of biological activities of curcumin are associated with its anti-inflammatory property due to its capability to control a variety of molecular targets related to the process of inflammation like enzymes, cytokines or transcription factors (Rai *et al.*, 2015). Some targets worth noticing are the down-regulation of inflammatory enzymes such as lipoxygenase 5 (LOX-5) and cyclooxygenase 2 (COX-2), which are involved in the production of prostaglandin through arachidonic acid and also inhibition of inducible nitric oxide synthase (iNOS) responsible for the production of NO, an inflammatory mediator. It was reported that the decreasing of COX-2 and iNOS expression was because of the suppression of a transcription factor known to be involved in processes like tumorigenesis or cellular proliferation, NF-kβ (Jurenka, 2009). Curcumin is also capable of inhibiting pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interleukines IL-1β, IL-1, IL-2, IL-6, IL-8, IL-12 and monocyte chemoattractant protein (MCP) (Košťálová *et al.*, 2013). There are some clinical studies that have already demonstrated these effects (Basnet e Skalko-basnet, 2011).

Several studies have been focused in the powerful antioxidant effect of curcumin (Lim *et al.*, 2001) (Merrell *et al.*, 2009). This effect is important to fight oxidative stress that is when an excessive reactive oxygen species are produced and the mechanisms of internal defense as antioxidants cannot eliminate them. This pathology is associated with chronic inflammations

namely cancer. Some reports demonstrated an inhibition of ROS by modulating the activity of antioxidant enzymes as catalase, superoxide dismutase (SOD) or glutathione peroxidase (GSH), by decreasing lipid peroxidation and for being an efficient scavenger of peroxyl radicals (Wojcik *et al.*, 2018). By decreasing the level of ROS, curcumin plays an essential role in stopping the cancer development. However, other reports argue that curcumin has also a pro-oxidant effect and it is capable of increasing the level of ROS (Aggeli *et al.*, 2013). It was clarified that when using lower concentrations of curcumin this acted as an antioxidant and reduce the levels of ROS acting as a chemopreventive agent (López-Iµzaro, 2008). On the contrary, when using higher concentrations of curcumin this acted as a pro-oxidant, leading to an increase of levels of ROS that is capable of inducing cell apoptosis and act as a chemotherapeutic agent (López-Iµzaro, 2008) (Li *et al.*, 2014).

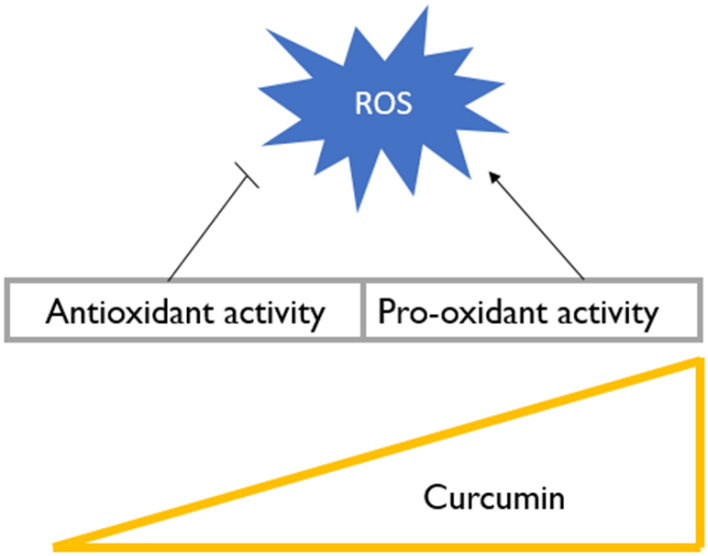

Figure 1.2 – The dual effect of curcumin in the cellular level of ROS: antioxidant or pro-oxidant depending on the quantity of curcumin applied (Adapted from (López-Iµzaro, 2008)).

Furthermore, curcumin can modulate cellular apoptosis or induce cell cycle arrest and these characteristics, together with the anti-inflammatory and antioxidant effects, make this compound a potent anti-carcinogenic agent. The later effect is particularly due to the down-regulation of genes involved in tumor growth and survival such as bcl-2, Bcl-xL and c-myc and up-regulate genes involved in apoptosis such as bax, Bcl-xs and p53. The p53 is a really important tumor suppressor gene that suppresses the proliferation of cells with damaged or altered DNA and curcumin induces a massive expression of this gene predominantly at G2 phase promoting G2 cell arrest and then apoptosis (Sa e Banerjee, 2010).

A neuroprotective effect is associated with curcumin, namely in Alzheimer's disease by decreasing the levels of amyloid and by suppressing the oxidative damage and inflammation in the brain (Lim *et al.*, 2001). Moreover, curcumin is known to reduce certain marks linked to atherosclerosis as the proliferation of vascular smooth muscle cells (VSMCs) (Shishodia, Sethi e Aggarwal, 2005).

In terms of immunomodulating effects, curcumin can activate cells as macrophages, natural killer (NK) cells and T and B cells, and it was even reported to minimize T-cell apoptosis induced by tumors (Sa e Banerjee, 2010).

Despite these advantages, curcumin is reported to have a limited therapeutic efficacy when administered *in vivo*.

1.3 Drawbacks of curcumin as a pharmacological compound

Curcumin presents a good toxicity profile, being considered quite tolerated by humans. This has been shown in clinical studies, where daily doses of 8 g were administered and no significant side effects were detected. Besides, this compound was already approved by FDA as a safe curcuminoid (Hewlings, 2017). Despite all of the safety profile and applications of curcumin, in clinical studies the pharmacologic application is quite limited. Several reports attributed this fact to curcumin's reduced bioavailability. For example, a clinical trial with 15 patients was done, where they received daily oral curcumin doses between 450 mg and 3600 mg, and they concluded that with the highest dose was only detected low levels of the drug and its metabolites in plasma in the order of 0.63 ng/mL (Basnet e Skalko-basnet, 2011). The reasons for this low bioavailability are curcumin's poor water solubility, easily degradable at physiological pH, poor absorption and the fact that curcumin is quickly metabolized and suffers elimination through feces (Jurenka, 2009). The chemical structure of the compound can explain some disadvantages. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also known for diferuloylmethane, is then a low molecular weight polyphenol (M.W 368.37) derived from the rhizome of *Curcuma longa* being responsible for its yellow colour (Ciftci, Tanyildizi e Godekmerdan, 2010). Commercial curcumin is constituted by three curcuminoids being the main component diferuloylmethane and in fewer quantities demethoxycurcumin and bisdemethoxycurcumin (Rai *et al.*, 2015). Curcumin is a bis-α, β-unsaturated β-diketone, which means it has in its composition two polyphenolic rings with a methoxy ether at the ortho position. In acidic environments and in physiological conditions (proximity to neutrality) curcumin possesses principally a bis-keto

form that acts as a donor of H-atoms while in alkaline conditions, curcumin possesses an enol form that acts as an electron donor (Basnet e Skalko-basnet, 2011). In terms of stability, curcumin has shown to decompose very rapidly in basic conditions changing its yellow colour to a dark red. Nevertheless, at acidic conditions the stability of this polyphenol is better (Wang *et al.*, 1997). Curcumin is also known for its hydrophobicity, which means this curcuminoid is practically insoluble in water, however it presents a good solubility in acetone, ethanol and dimethyl sulfoxide (DMSO) (Basnet e Skalko-basnet, 2011). In order to overcome the difficulties caused by curcumin's chemical structure a variety of solutions have been tried and described.

1.4 Approaches to surpass the poor bioavailability of curcumin

Some attempts were done in order to improve the potential activity of curcumin like the addition of adjuvants that are able to decrease the metabolic pathways that lead to rapid curcumin's excretion. One of those examples is piperine, a constituent of pepper, that, when it was administered orally in humans, combined with curcumin, increased the bioavailability of this last compound in 2000 % (Anand *et al.*, 2007). Another method that is currently in vogue, besides the use of other agents to try to provoke a synergistic effect with curcumin, is the use of delivery systems since they are able to protect curcumin from being rapidly metabolized, increase curcumin's bioavailability and provide longer circulation. Curcumin was already encapsulated in a variety of carriers namely polymeric nanoparticles (Huong *et al.*, 2011), micelles (Wang *et al.*, 2012), liposomes (Li, Braiteh e Kurzrock, 2005) or even biodegradable microspheres (Zhang *et al.*, 2013). Of those we are going to focus in polymeric nanoencapsulation, presenting reasons to why this solution may be better than polymeric microencapsulation.

1.5 Drug delivery systems – Microencapsulation and Nanoencapsulation which one presents a better option?

In drug delivery systems, both micro and nanoencapsulation have been used to provide a control release of drugs, improving its bioavailability and therefore increasing its biological activity but also in other applications namely in food industry (Kuang *et al.*, 2010). Therefore, both techniques have similar advantages such as they act as a shell, protecting the core material from enzymatic degradation, they have the ability to release the drug at a specific

target in a controlled way, which means a smaller quantity may be needed to achieve the same biological effect, they can disguise the flavour of the entrapped drug and they are able to prolong the circulation in plasma of the material (Estevinho, 2013). The difference between microparticles and nanoparticles is the size, where microparticles have a size between 1 to 1000 μm, while nanoparticles in the majority of scientific reports are considered with a size between 10 to 1000 nm (Suganya e Anuradha, 2017). However, accordingly to National Nanotechnology Initiative (NMI), it should only be considered nanoparticles, material with a size up to 100 nm (Koo, Rubinstein e Onyuksel, 2005). Overall, it seems that nanosystems present a greater potential compared with microsystems due to the high surface ratio and because of their smaller size they are more efficiently absorbed by cells, presenting a higher cellular uptake (Gelperina *et al.*, 2005). In a report from Klein and co-workers, authors compared particles of chitosan with an enzyme encapsulated into microparticles and nanoparticles of 410 nm and they observed that the NPs showed the enzyme with the highest activity, being considered the system with more advantages (Klein *et al.*, 2012). Consequently, our main objective is to design and develop a method to prepare a curcumin nanoparticulate delivery system. Since curcumin is highly hydrophobic it would beneficiate from the encapsulation. To conclude the nanotechnology has diverse applications in disease diagnosis and treatment, where nanomedicines are distinguished by their unique properties. Of those nanomedicines, polymeric nanoparticles assume an important role because of their ability to act as biological mimetics, sensors or even as a diagnostic agent (Moghimi, 2005).

1.5.1 Polymeric Nanoparticle Systems

Polymeric Nanoparticles are made from natural or synthetic polymers and present a wide range of advantages when used as a drug nanocarrier. These systems offer low toxicity, high loading capacity being able to encapsulate both hydrophilic as hydrophobic substances, improved stability of the drug by protecting it from degradation and by modifying its clearance, they are able to administer the formulation by various routes of administration, including orally and present potential target sites including solid tumors due to the EPR effect (Pawar *et al.*, 2016). Several reports have described different polymeric nanoparticles to encapsulate curcumin. For example, Shaikh and co-workers reported a particle using PLGA to encapsulate curcumin and authors observed that when administered in rats this formulation improved at least 9-fold oral bioavailability compared to curcumin co-

administered with piperine, which was a big achievement since piperine is already used to enhance absorption of curcumin (Shaikh *et al.*, 2009). Another promising delivery system is a polymeric particle called "nanocurcumin" with less than 100 nm, which presented in human pancreatic cell lines a therapeutic efficacy comparable to free curcumin and it also showed a capability to easily dispersed in aqueous medium (Bisht *et al.*, 2007). Moreover, Mathew and colleagues produced a PLGA nanoparticle coupled with Tet-1 peptide to target neurons with curcumin encapsulated, presenting a size ranging from 150-200 nm and proved that this system was efficient in treating Alzheimer's disease by reducing amyloid aggregates and by exerting curcumin's antioxidant activity (Mathew *et al.*, 2012). Despite of all of this attention to nanoencapsulation, it was also described reports of curcumin encapsulated in microcapsules, usually related with stabilizing curcumin for food application (Wang *et al.*, 2012) (Ariyarathna e Karunaratne, 2016).

From all of this, it could be concluded that the choice of polymer is a determinant step since the main goal is to find a material that does not interfere in a bad way with the drug that we want to encapsulate and also choose a potential polymer that interferes with the immune system in the way that we planned. For that to happen it is important to have a clear understanding of the effect of the polymer as well as the polymer-based nanocarrier on the immune system in order to implement the safe-by-design concept on the development of a curcumin delivery system.

1.6 How can polymeric nanoparticles interact with our immune system?

The immune system plays a critical role in keeping the homeostasis in human body. It exercises its function through innate and adaptive immunity. Innate immunity is also called non-specific immune response because it is a first line of defense that eliminates the pathogen in a non-specific way (Jiao *et al.*, 2014). The phagocytic cells, as macrophages or neutrophils, mast cells or even dendritic cells have receptors that can recognise predetermined patterns (PRRs) known as pathogen-associated molecules patterns (PAMPS) like carbohydrates, proteins and lipids that are unique to certain organisms. The PAMPS are not produced by humans, however they can be recognized by human cells and after their recognition leads to phagocytose of the pathogen and the release of cytokines to eliminate this foreign material (Akramien *et al.*, 2007). On the contrary, if the pathogen persists in the human body, the adaptive immunity is activated. The latter is known as an acquired immune response and it is the second line of defense. This type of immunity is considered to be

specific of the pathogen because in this case we have phagocytic cells that act as antigen presenting cells (APCs) that are then going to present the antigens to the two types of specialized cells, T and B lymphocytes. Each type of lymphocyte displays a different response: B cells present an antibody response, where they activate the production of antibodies that are going to bind to the pathogen, blocking its harmful action while T cells present a cell-mediated immune response, where they directly or indirectly through the communication with other cells like macrophages destroy the pathogen (Crotty, 2015). If the same pathogen enters again in the human body, the receptors of phagocytic cells will recognise it and the immune response will be much quicker. This happens due to the immunological memory that was created (Kobayashi, Inohara e Hernandez, 2002). With this in account, it is easy to understand that when nanoparticles are applied *in vivo* they can behave in two ways. On one side, they can act as being part of the human body, which means they are recognised as self and are not able to cause an immune response or on the other side, nanoparticles are recognised as foreign materials, causing an immune response that could be of immunosuppression or immunostimulation (Jiao *et al.*, 2014). This immunomodulating property may have desirable or undesirable effects for example in immunostimulation is desirable if the NPs can act as a vaccine adjuvant but it can also be undesirable if they cause hypersensitivity reactions or an extreme inflammation (Zolnik *et al.*, 2010). Clawson and co-workers reported the production of PLGA NPs with a peptide encapsulated, HP91, where it was observed that HP91 was more potent in initiating the immune response that the free drug, being considered a better option for vaccines (Clawson *et al.*, 2011). Therefore, NPs must be seen as having two faces and it should be taken seriously before administration in clinical application.

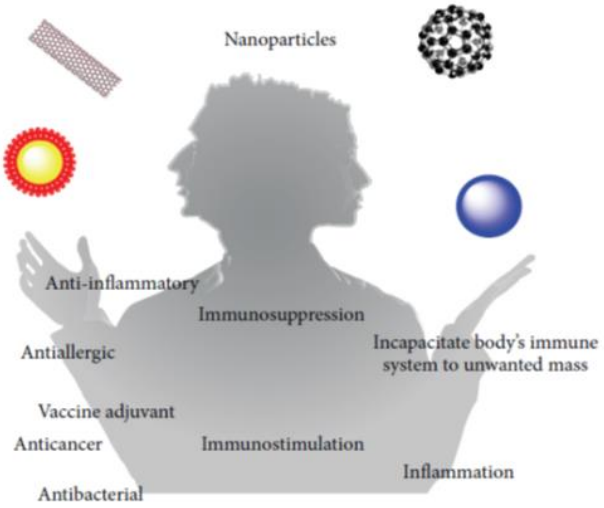

Figure 1.3 – The immunomodulation properties of NPs, when administered *in vivo*. These particles can have a double face since they may cause beneficial effects but it can also bring downsides (Adapted from (Jiao *et al.*, 2014)).

Besides the interaction of NPs with the immune system it is also important to consider the size and morphology of the NPs and the degree of biodegradability of the polymer because when choosing a non-biodegradable one, this can cause an undesirable toxicity (Sundar, Kundu e Kundu, 2010). Because of that, a material widely used is the polysaccharides. Polysaccharides are known natural polymers that commonly present a good biocompatibility, being non-toxic and biodegradable and in some cases are considered biologically active (Malaekeh-Nikouei e Salarbashi, 2018). The polymer that we chose to work was glucan specifically 1,3-β-Glucan because it is one of the most known polysaccharides for its immunomodulatory effect (Chan, Chan e Sze, 2009).

1.7 Curdlan as a type of 1,3-β-Glucan biologically active

Curdlan is a linear neutral polymer composed only by 1,3-β-glucosidic linkages and it is produced by *Agrobacterium* formerly known as *Alcaligenes faecalis* (McIntosh, Stone e Stanisich, 2005). This β-Glucan that can have as many as 12000 glucose units, is water-insoluble because of the extensive intra and intermolecular hydrogen bonds, however it is quickly solubilized in dimethyl sulfoxide (DMSO), formic acid and in alkaline solutions as in NaOH (Cai e Zhang, 2017). It is important when looking at the literature to have in account the diversity of β-Glucans that exist in nature, like in mushrooms or in cell wall of fungus and

bacteria. It is said that depending on the structural complexity of the β-Glucan as its length and branching structure, the biological properties associated change being believed that with higher complexity it is achieved higher immunomodulatory properties (Chan, Chan e Sze, 2009). However, curdlan can be considered the exception to the rule because it presents one of the simplest chemical structures among β-Glucans (unlike for example β-Glucans from mushrooms that have the same backbone of 1,3-β-glycosidic chain but also 1,6-β-linked branches), but it has associated a greater pharmacological activity (Kim *et al.*, 2011).

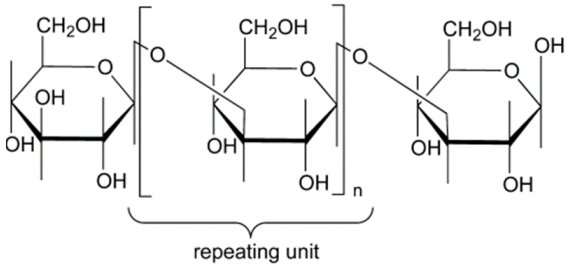

Figure 1.4 – Chemical structure of curdlan, representing its 1,3-β-glucosidic linkages (Adapted from (Zhang e Edgar, 2014)).

It is associated to curdlan a variety of applications and one that it is highlighted is its unusual gelling property. This is because depending on the temperature, curdlan can form two types of gels, if the temperature is high (above 80 °C) it can form a high-set irreversible gel, while in lower temperatures it forms a low-set reversible gel. Curdlan is therefore widely used in food industry due to that characteristic (Zhan, Lin e Zhang, 2012). One example was reported by Wang and co-workers that by using curdlan improved the firmness and the texture of noodles (Wang *et al.*, 2010). More recently, it was also discovered biomedical applications namely in the field of immunology as an anti-carcinogenic (Zhou *et al.*, 2017) and also in wound healing (Kim *et al.*, 2012). Besides, curdlan is also known for being a biological response modifier, which means it is able to enhance or restore the immune system (McIntosh, Stone e Stanisich, 2005). This ability of curdlan is due to the fact that this polymer behaves as a PAMP, which means that when it enters in the human body it is recognised by receptors of the innate immune system. The receptors for β-Glucans incorporation are mainly through toll-like receptor 2 (TLR-2), complement receptor 3 (CR3) and dectin-1. Dectin-1 is a transmembrane signalling receptor lectin-like that contains a carbohydrate recognition domain that is able to recognise 1,3-β-Glucans. It is essentially expressed in macrophages, neutrophils and dendritic cells (Akramien *et al.*, 2007). After the binding of the ligand to this receptor, a series of reactions occurs to try to eliminate the foreign material. It

can induce phagocytosis with the consequent production of cytokines as IL-2, IL-6, IL-10, IL-12 and TNF-α, it can also release arachidonate acid that is known as capable of initiating inflammation and it can triggers the formation of reactive oxygen species (Sun e Zhao, 2007). Toll-like receptors when they identify β-Glucans facilitate the activation of the adaptive immune system while CR3 is a transmembrane glycoprotein that it is able to activate factors as NF-kβ (Akramien *et al.*, 2007). In short, all these receptors came together in what is thought to be the right mechanism for the internalization of curdlan, which is the polymer is captured by macrophages through dectin-1 and toll-like receptor 2 and then the large molecule is fragmented in small β-Glucans that are released and taken up by the circulating dendritic cells through CR3. In this way we can have the stimulation of both innate and adaptive immune response (Chan, Chan e Sze, 2009). Apart from these applications, it was also reported the efficiency of using this polymer as a drug delivery system since it allows a sustained, controlled release of a drug from its gel particles (McIntosh, Stone e Stanisich, 2005). Kanke and colleagues reported the preparation of curdlan tablets with theophylline as the encapsulating drug and it was observed a constant drug release rate overt the first 8 h (Kanke *et al.*, 1991). Huong and co-workers described the preparation of 1,3-β-Glucan NPs to encapsulate curcumin, having presented anti-carcinogenic effects (Huong *et al.*, 2011). Tesz and colleagues used glucan particles to deliver a small interfering RNA to phagocytes without the need of adding an additional ligand to target this type of cells, since glucan is preferably uptake by macrophages (Tesz *et al.*, 2011). In addition of using curdlan as it is, nowadays there is an interest in modifying it to make it more functional like in Zhou and co-workers, where they used carboxymethyl curdlan as an hydrophilic carrier to doxorubicin, an anti-carcinogenic drug (Zhou *et al.*, 2017). Because of this potential, curdlan seems a good polymer to encapsulate curcumin and enhance its therapeutic efficacy. However, before analysing the potential pharmacological activity of the delivery system it is essential to perform a traditional toxicological assessment.

1.8 Pre-clinical immunotoxicity studies assessment of polymeric delivery systems

Nanoparticle delivery systems have associated a variety of unique properties and this can lead to an unexpected toxicity profile. For that reason, it is essential during the pre-clinical development of a potential pharmaceutical drug to exert an extensive risk assessment to protect not only human health but also the environment (Engin e Hayes, 2018). These adverse effects may be caused by the physicochemical characteristics of the systems like the

particle size, surface charge, the tendency to agglomerate, the NPs chemistry and their shape, which in turn can lead to a bad interaction with the immune system as immunogenicity or hypersensitivity (Lanone e Boczkowski, 2006). From the possible tests to evaluate the potential immunotoxicity, it is important to highlight the compatibility with blood components through hemolysis and thrombogenicity, where it can be evaluated the plasma coagulation time and the effects on the functionality of the immune system through complement activation or cytokine induction (Dobrovolskaia, 2016). Generally, it is also recommended to perform the cytotoxicity studies in more than one cell line, using also a primary cell type to confirm the "safe-by-design" concept (Engin e Hayes, 2018). When encapsulating a drug in these delivery systems is important to have in account that usually it exists a potential to reduce the drug immunotoxicity, but in some cases we may have an exaggeration of the profile of drug's immunotoxicity due to the alteration of its biodistribution (Dobrovolskaia, 2016).

According to Dobrovolskaia, 2016, some details to take in consideration, before performing these tests and for not having any kind of false results, are:

-1) when producing the NPs to be careful and do it in an aseptic environment and using pyrogen-free water to try to minimize the endotoxin contamination.

- 2) to plan everything including the appropriate controls to avoid wrong conclusions.

From all of this, we can conclude that the *in vitro* assays are critical to identify a good candidate with the most desirable properties to be in the future translated to the clinic (Engin e Hayes, 2018).

1.9 Aim of the thesis

Nanotechnology will have a very large impact in the area of pharmaceuticals. Understanding the potential of nanoparticle delivery systems in improving the therapeutic impact of drugs is an important ability to apply for having better and safer medicines. Understanding how the size, shape, or composition of nanoparticles has influence on efficacy and safety evaluation results of these nanosystems is essential for a safe-by-design approach on the development of new medicines.

Therefore, this work has 3 main goals:

- To develop a method to prepare curcumin loaded glucan nanoparticles with 2 different sizes.
- To characterize (physical and chemical properties) the nanoparticles.
- To test *in vitro* the unloaded and curcumin loaded glucan nanoparticles, namely to perform immunotoxicity tests and to study the effect of nanoparticle size and composition (loaded and unloaded) on the various immunological parameters.

Chapter 2

Development of a method to produce Glu NPs and characterization of the different nanoparticles

2.1 Materials and Methods

2.1.1 Materials

Curdlan (P-Curdl) was purchased from Megazyme and Tween 80® from Riedel de Haen™. Curcumin was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Vivaspin 20 centrifugal concentrator (MWCO 300KD) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All the other chemicals were from analytical grade and obtained from usual suppliers.

2.1.2 Development of the method to produce Glu NPs with 100 nm

The technique to produce 100 nm Glu NPs was based on a precipitation/coacervation method. First, glucan was solubilized in a basic solution (NaOH) and then was precipitated by the addition of an acetic acid solution after a sufficient decrease of the pH. In order to obtained the optimized particles, several experimental conditions were tested. Solutions of acetic acid ranging between 0.1 % to 8 % were added, dropwise, under magnetic stirring, to a solution of glucan varying between 0.001 % to 0.1 % in 0.1 % or 2 % sodium hydroxide and with the addition of the surfactant tween 80® or not. The addition of acetic acid was interrupted at different pH namely pH 11, 7, 6 and 5, where the mixture was kept under magnetic stirring during half an hour. The different variations to optimize the production of the NPs are summarized in table 2.1, where it is emphasized the adopted condition (8 % of acetic acid; 0.025 % of glucan solubilized in 2% of NaOH and 1% tween 80®). To encapsulate curcumin within these glucan nanoparticles, a similar method of production was done, replacing the solution of 8 % acetic acid by the solution of 0.1 mg/mL curcumin in 8 % acetic acid and 1 % tween 80®. The optimized conditions are represented in figure 2.1.

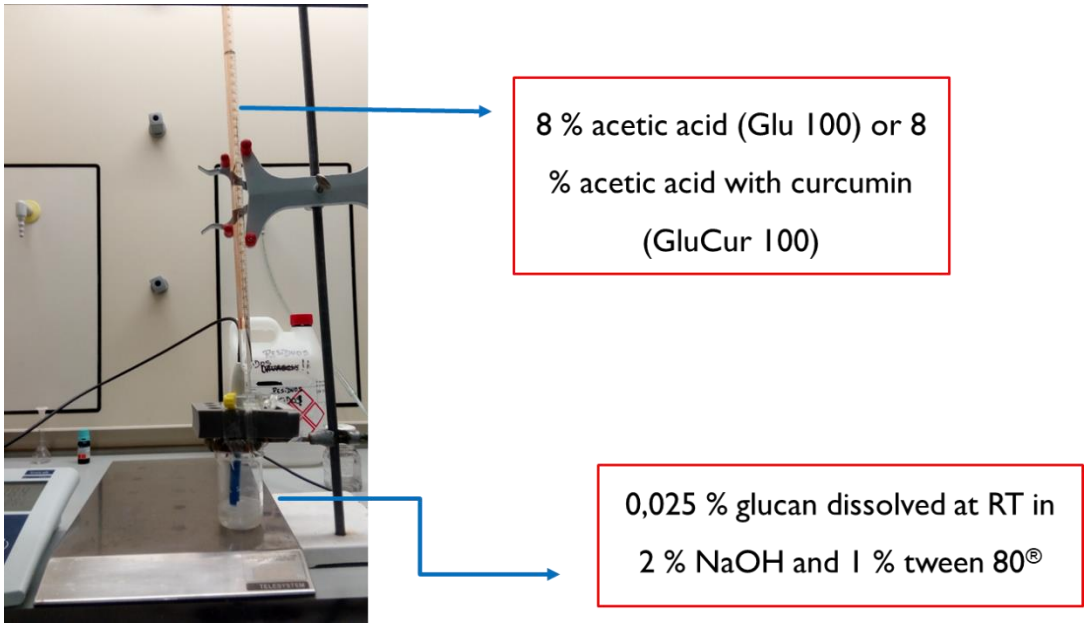

Figure 2.1 – Representation of the production method of 100 nm Glu NPs, both curcumin loaded (GluCur 100) and unloaded (Glu 100).

2.1.2.1 Nanoparticles Concentration

The suspension of nanoparticles was concentrated and washed with ultrapure water (1 mL formulation: 9 mL water) to remove the original medium, using Vivaspin 20 centrifugal concentrator (MWCO 300KD) at 3000 *g*.

2.1.3 Development of the method to produce Glu NPs around 300 nm

The 300 nm Glu NPs were prepared by a similar method as described for Glu 100 and GluCur 100 NPs, which only differs in the concentrations of the solutions used. Shortly, a solution of acetic acid ranging between 0.5 % to 4 % was added, dropwise, under magnetic stirring to a solution of glucan with a concentration varying between 0.025 % to 0.1 % in 0.1 %, 1 % or 2 % sodium hydroxide and with the addition of the surfactant tween 80® or not. This addition of acetic acid was done without interruption from the initial pH of the solution until pH 5. The suspension of nanoparticles was left 1 h under magnetic stirring to achieve particle maturation. The different variations to optimize the production of the NPs are summarized in table 2.2, where it is highlighted the final concentrations used (4 % acetic acid; 0.1 % of glucan solubilized in 2 % of NaOH and 0.1 % of tween 80®). To encapsulate curcumin within these nanoparticles, a similar method was applied with slightly adjustments.

The solution of 4 % acetic acid was added dropwise to a glucan basic solution until reaching pH 11 and then was replaced by 850 µL of a solution of 0.1 mg/mL curcumin in 8 % acetic acid and 1 % tween 80® (final pH 5) to a solution of 0.1 % glucan in 2 % sodium hydroxide and 0.2 % tween 80®. The suspension of nanoparticles was left 1 h under magnetic stirring to achieve particle maturation. The optimized conditions are represented in figure 2.2.

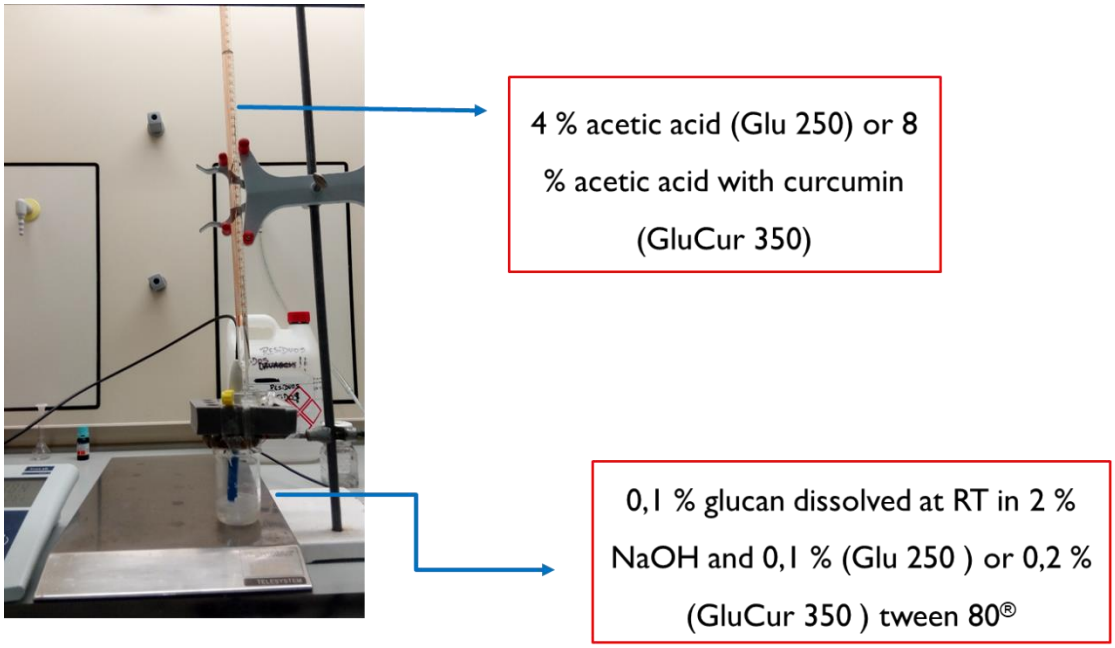

Figure 2.2 – Representation of the production method of 300 nm Glu NPs, both curcumin loaded (GluCur 350) and unloaded (Glu 250).

2.1.3.1 Nanoparticles Concentration

The suspension of nanoparticles was concentrated and washed with ultrapure water (1 mL formulation: 9 mL water) to remove their original medium, using Vivaspin 20 centrifugal concentrator (MWCO 300KD) at 3000 *g*.

2.1.4 Characterization of the Glu NPs

2.1.4.1 Size and zeta potential of Glu NPs

Delsa™ Nano C particle analyzer (Beckman Coulter, Brea, CA, USA) was used to measure the particle size by Dynamic light scattering (DLS), and the zeta potential was measured using Zetasizer Nano ZS (Malvern Instruments, Ltd., Worcestershire, UK) through electrophoretic light scattering (ELS).

2.1.4.2 Transmission electron microscopy (TEM) imaging of Glu NPs

TEM is a powerful technique that allows the use of energetic electrons to provide high-resolution and two-dimensional images (Wang, 2000). Because of this, it is a good tool to study the morphology of Glu NPs after being concentrated and washed. TEM was performed using a JEOL JEM 1400, 120 kV (JEOL, Peabody, MA, USA). For sample preparation, Glu 100 and GluCur 100 NPs were diluted in ultrapure water and then a drop was dried out on a mesh grid, before observation.

2.1.4.3 Cryo Scanning Electron Microscopy (Cryo-SEM) of Glu NPs

Cryo-SEM is another technique to evaluate the morphology of nanoparticles through a focused beam of high-energy electrons. In this method the material was frozen, which presents a great advantage because vulnerable structures are better preserved (Sriamornsak *et al.*, 2008). The SEM was performed using a High resolution Scanning Electron Microscope with JEOL JSM 6301F. For sample preparation, Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs were rapidly cooled using slush nitrogen and transferred under vacuum to a preparation chamber, where the particles were sublimated for 120 s at -90 °C, and coated with Au/Pd by sputtering for 46 s. After that, the particles were visualized at a SEM chamber at -150° C.

2.1.4.4 (1-3)-β-Glucan Quantification

The fluorescence dye-binding microassay for glucan quantification was used to measure the quantity of glucan that was being incorporated into the nanoparticles. The principle of this method is that the aniline blue dye when in solution with 1,3-β-Glucan forms a complex that exhibits fluorescence. This microassay was described elsewhere by Shedletzky et al (Ko e Lin, 2004). Briefly, 30 µL of 6 N NaOH was added to the samples (300 µL) in eppendorfs and then were left incubating at 80 °C for 30 min. After that, the samples were immediately transferred to an ice bath. A volume of 630 µL of a dye mix (40 mL of 0.1 % aniline blue in water, 21 mL of 1 N HCl, and 59 mL of 1 M glycine/NaOH buffer, pH 9.5) was mixed into each eppendorf using vortex, which was then incubated at 50 °C during 30 min to form the complex between 1,3-β-Glucan and aniline blue. To decolorize the unbound fluorescent dye, the samples were incubated during 30 min at room temperature. The fluorescence was measured at an emission wavelength of 502 nm with a slit width of 20 nm and an excitation wavelength of 398 nm with a slit width of 20 nm. None of the samples had to be diluted

because the fluorescence intensity was within the calibration range. To create the calibration curve, a series of diluted glucan concentrations were prepared and diluted in 1 N NaOH to a final volume of 300 µL that were then subject to the dye reaction. The concentrations of the curdlan solutions used to prepare the calibration curve were 0, 4, 8, 12, 16, 20 and 24 µg/mL.

2.1.4.5 *In vitro* quantification of curcumin

After both types of curcumin loaded glucan NPs (GluCur 100 and GluCur 350) were concentrated, the filtrate was used to quantify the curcumin that was not present in the nanoparticles. For that, a calibration curve was prepared using known quantities of curcumin dissolved in the solvent of NPs (acetic acid + NaOH) and the samples were read at a maximum wavelength of 469 nm. After drawing the calibration curve, the quantity of curcumin was determined.

2.1.4.6 Stability tests

- **2.1.4.6.1 Long-term stability at two different temperatures (4 °C and 20 °C)**

After all types of NPs (Glu 100, GluCur 100, Glu 250 and GluCur 350) were concentrated and washed with ultrapure water, an individual batch of each one was maintained at different temperatures, 4 °C and 20 °C. The study was conducted for 6 weeks, in which the size and PDI was measured using Delsa™ Nano C particle analyzer (Beckman Coulter, Brea, CA, USA) and zeta potential through Zetasizer Nano ZS (Malvern Instruments, Ltd., Worcestershire, UK). For each measurement, the NPs were diluted in ultrapure water (50 µL of NPs + 950 µL of water).

- **2.1.4.6.2 Stability in the different culture media: DMEM and RPMI at 37 °C**

The NPs stability was evaluated in the cell culture media to simulate possible events during *in vitro* studies with cells. For that, it was chosen a non-cytotoxic NPs concentration to prepare the NPs suspensions with DMEM and RPMI. The measurements of size, PDI through Delsa™ Nano C particle analyzer (Beckman Coulter, Brea, CA, USA) and zeta potential through Zetasizer Nano ZS (Malvern Instruments, Ltd., Worcestershire, UK) at different time points

were made before adding both culture media (DMEM and RPMI), immediately after adding the culture media and after 1, 6, 24, 48 and 72 h of being at 37 °C with culture media.

2.2 Results and Discussion

2.2.1 Optimization of the production method of both Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs

This work had as a main objective to produce 100 nm Glu NPs using the polymer curdlan. Since we did not find any reliable method described in the literature, an intense work was necessary to discover the method that led to the production of the nanoparticles with the characteristics described. However, some studies found in literature have inspired the way to find the method. For instance, Sathiyanarayanan Anusuya and co-workers demonstrated a nanoprecipitation method with acetic acid to produce glucan nanoparticles using a polymer isolated from the cell wall of *Pythium aphanidermatum* (Anusuya e Sathiyabama, 2014). Inspired on that study, the theory behind the production of our NPs was first to dissolve the polymer in sodium hydroxide and then precipitate it using acetic acid since glucan is insoluble in acidic environments (Cai e Zhang, 2017). At first, we decided to test a variety of glucan concentrations, in which it was kept the concentration of NaOH and acetic acid and it was observed a tendency to create bigger particles when using higher concentrations of the polymer. Despite that, all conditions produced nanoparticles with a size very far from the objective (conditions 1 to 3 in table 2.1). Next, we realized that if we kept the same concentration of NaOH and acetic acid, a large volume of acid would be needed to reach the defined pH of 5, since NaOH is a strong base. Consequently, the particles would be very diluted because of the large volume. Because of that, to obtain concentrated suspensions of the particles we tested more concentrated acetic acid solutions to decrease the pH of the glucan solution and a tendency to get smaller particles was observed (conditions 4 to 6 in table 2.1). Besides these variations we also tried the addition of a surfactant tween 80®, which turned out to be essential to achieve the required size and also to provide a better reproducibility between batches (overall comparison between conditions 1 to 4 without surfactant and conditions 5 to 7 with surfactant in table 2.1). The optimized condition was observed when dissolving 0.025 % of glucan in 2 % of NaOH with 1 % of tween 80® and then precipitate it with 8 % of acetic acid (condition represented in blue in table 2.1).

The next step was the encapsulation of curcumin into the NPs. As curcumin is a compound that it is easily degraded in extreme basic environments (Ashokkumar, Unichem e Mishra, 2015) it could not be added to the NaOH solution containing glucan and the only other option was adding it to the acetic acid solution. First, the basic glucan solution was partially neutralized with the 8 % acetic acid solution and after reaching pH 11 (in the solution), the new acetic acid solution containing the curcumin was added. The pH 11 was chosen because is close to the turning point (in only one or two drops the solution passed from pH 11 to 7) and then we could be sure that the stability of curcumin was not affected. The same concentrations of glucan, NaOH, acetic acid and tween 80® were used. The obtained size for these particles (GluCur 100) were similar to its blank nanoparticles.

Table 2.1 – Overall summary of the conditions tested to produce Glu 100 NPs. The grey color represents the influence in the size of the particles by increasing the % of polymer used. The blue color represents the optimized condition for the production of these particles.

Number of conditions	Glucan (%)	NaOH (%)	Acetic Acid (%)	Tween (%)	Size (nm)
1	0.001	2	1	-	1668
2	0.025	2	1	-	1765
3	0.05	2	1	-	2585
4	0.1	2	1	-	1736
5	0.05	2	4	1	847
6	0.025	2	8	1	131.8
7	0.025	0.1	0.1	0.5	1012

Another important goal that has been achieved was the development of the preparation method to obtain the NPs around 300 nm. With that in mind, we started to remove the addition of the surfactant and decreasing the concentration of acetic acid although, by varying two different parameters, it was obtained very large particles (conditions 12 to 14 in table 2.2). Thereafter, we only studied the impact on size varying one factor like the decrease in the quantity of surfactant and apparently, that did not have effect on the size of the NPs that continued to be in the range of 100 nm (conditions 1 to 4 in table 2.2). Afterwards, we varied the concentration of acetic acid (2.5 %; 2 %; 1.5 %; 1 %) until this was in the same concentration of NaOH and kept the other variables namely the % of surfactant used but that did not have any effect on the size of the NPs, unlike Glu 100 NPs where we observed that increasing the concentration of acetic acid caused a decreased in the size of the nanoparticles (conditions 8 to 11 in table 2.2). Therefore, another variable was studied namely the increase of the glucan concentration. In this case, tween 80® was added and nanoparticles around 300 nm were obtained (condition represented in blue in table 2.2). To

summarize, the optimized preparation protocol consisted in dissolving 0.1 % of glucan in 2% of NaOH with 0.1 % of tween 80® and then precipitate it with 4 % of acetic acid. For the encapsulation of the curcumin, a slightly modification was done. As in the case of Glu 100 and GluCur 100 NPs, first it was added a solution of acetic acid until reaching pH 11 and then it was dropped the acetic acid solution with curcumin. The same concentrations of glucan, NaOH and acetic acid were used but not the surfactant tween 80® because it was observed more aggregates compared with its blank NPs and because of that it was used 0.2 % of the surfactant instead. The obtained size for these particles (GluCur 350) was similar to its blank nanoparticles.

Table 2.2 – Overall summary of the conditions tested to produce Glu 250 NPs. The grey color represents the influence in the size of the particles by decreasing the concentration of the surfactant, the purple color by decreasing the quantity of acetic acid, maintaining the concentration of surfactant and the green color by decreasing the quantity of acetic acid without the use of a surfactant. The blue color represents the optimized condition for the production of these particles.

Number of conditions	Glucan (%)	NaOH (%)	Acetic Acid (%)	Tween (%)	Size (nm)
1	0.05	2	4	1	123.8
2	0.05	2	4	0.5	160.7
3	0.05	2	4	0.5	177
4	0.05	2	4	0.3	133.7
5	0.05	2	1	0.3	129.3
6	0.05	0.1	0.5	0.5	1627.8
7	0.025	2	4	1	133.4
8	0.025	1	2.5	1	93.4
9	0.025	1	2	1	98.6
10	0.025	1	1.5	1	210.7
11	0.025	1	1	1	119.7
12	0.025	1	2.5	-	2426.6
13	0.025	1	1.5	-	2149.1
14	0.025	1	1	-	1795.4
15	0.025	1	1	0.3	70.8
16	0.025	1	1	0.5	103.8
17	0.025	1	1	0.8	91.2
18	0.1	2	4	0.1	446.5

The curdlan is a neutral water-insoluble polysaccharide composed of β-1,3-glycosidic linkages (Novak, Vetvicka e Novak, 2008) (Zhang e Edgar, 2014) known for its gelling properties that are very convenient to food industry (Cai e Zhang, 2017). More recently, curdlan showed biomedical applications in the field of immunology. Some papers reported to use curdlan as a hydrogel like Min-Young Kim and co-workers (Kim *et al.*, 2012) or even introduced slightly modifications to improve its bioactivity as in the case of Dana Suflet and colleagues where

they added ammonium quaternary groups to curdlan to create a water-soluble derivative (Kim *et al.*, 2012) or Jing-Kun Yan and co-workers that produced carboxylic curdlan NPs to use as a carrier of doxorubicin (Yan *et al.*, 2015). Nanoparticle drug delivery system is then an application already described for curdlan. Besides the Jing-Kun Yan work, Byung-Do Kim and co-workers described the production of solid lipid nanoparticles (SLNs) using curdlan as the shell material and also tween 80 to prevent the gelling of curdlan and they obtained particles with similar size to ours (Kim, Na e Choi, 2005). Later, authors encapsulated the drug verapamil, producing a functional delivery system (Kim, Na e Choi, 2005). Another interesting characteristic of β-glucans (curdlan) is the fact that when it is formulated as a nanoparticle, it can interact with dectin-1 receptor and elicit an immune response in those cells like macrophages and because of that it was used like a target particle that can be driven to cells that expressed that receptor. Among studies, Hieu Vu-Quang reported iron oxide NPs coated with β-glucan to target immune cells (Vu-Quang *et al.*, 2012) or Matshawandile Tukulula and colleagues that described PLGA NPs with 1,3-β-glucan to target macrophages and then deliver higher quantities of rifampicin (Tukulula *et al.*, 2018). To the best of author's knowledge, it was never described curdlan NPs without any type of modification to encapsulate a drug. Curcumin, the drug chosen to be encapsulated, due to its low solubility in water, its poor bioavailability and the fact that it gets metabolized really fast makes it a perfect candidate to be encapsulated into NPs (Anand *et al.*, 2007). To the best of author's knowledge, it is the first time that curcumin is being encapsulated into curdlan NPs. However, in scientific literature it is possible to observe that an attempt to encapsulate curcumin has been tried with other polymer-based NPs. For example chitosan phosphate NPs was tried to encapsulate curcumin to improve the drug biological effect (Deka *et al.*, 2016).

Table 2.3 – Overall characterization of Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs. Size, polydispersity index (PDI) and zeta potential of glucan and glucan-curcumin nanoparticles were measured in the original medium (size; mean ± SEM, n = 8) or in water (size; zeta potential; mean ± SEM, n = 8) by dynamic light scattering and electrophoretic light scattering, respectively.

		Before sample concentration		After sample concentration		
	n	Size (nm) ± SEM	PDI ± SEM	Size (nm) ± SEM	PDI ± SEM	Zeta Potential (mV) ± SEM
Glu 100	8	129.7 ± 2.5	0.285 ± 0.009	139.2 ± 14.7	0.399 ± 0.039	-7.89 ± 0.76
GluCur 100	8	98.6 ± 3.8	0.289 ± 0.018	101.9 ± 9.1	0.415 ± 0.067	-4.41 ± 1.96
Glu 250	8	355.4 ± 41.0	0.249 ± 0.011	380.6 ± 38.1	0.359 ± 0.060	-1.18 ± 0.98
GluCur 350	8	518.7 ± 37.6	0.245 ± 0.011	378.9 ± 12.9	0.361 ± 0.070	-1.03 ± 0.08

2.2.2 Characterization of Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs

2.2.2.1 The methodology developed leads to particles with low PDI, however the process of concentration with Vivaspin increases the PDI

As we can see in table 2.3, it is summarized the size and PDI before and after sample concentration of all types of NPs. The encapsulation of the curcumin into both nanoparticles has modified their size. In fact, in the case of the Glu 100 and GluCur 100 NPs, the size decreased and for the Glu 250 and GluCur 350 NPs the size increased. After, the concentration of the particles it was observed the increase of the PDI that could be explained by the presence of a different suspension medium, the original medium was replaced by water. Additionally, the possibility to form agglomerates during the concentration process cannot be excluded as seen in figures 2.3 A-D and 2.4 A-D. Interestingly, with the exception of GluCur 350 NPs, the mean diameter of the particles did not change. In fact, GluCur 350 NPs decreased their size after centrifugation. This was observed because aggregates of smaller size were detected when analysing the results (Figure 2.4 C and D).

The zeta potential of the Glu 100, GluCur 100, Glu 250 and GluCur 350 was close to neutrality, which was what we expected since the curdlan polymer is neutral. The zeta potential was only measured after concentration because it was when NPs were dispersed in water and not in the original medium. If this last option was the case, depending on the chemical species present in the solvent we could have different zeta potential values, leading us to obtain results that could not be compared among them.

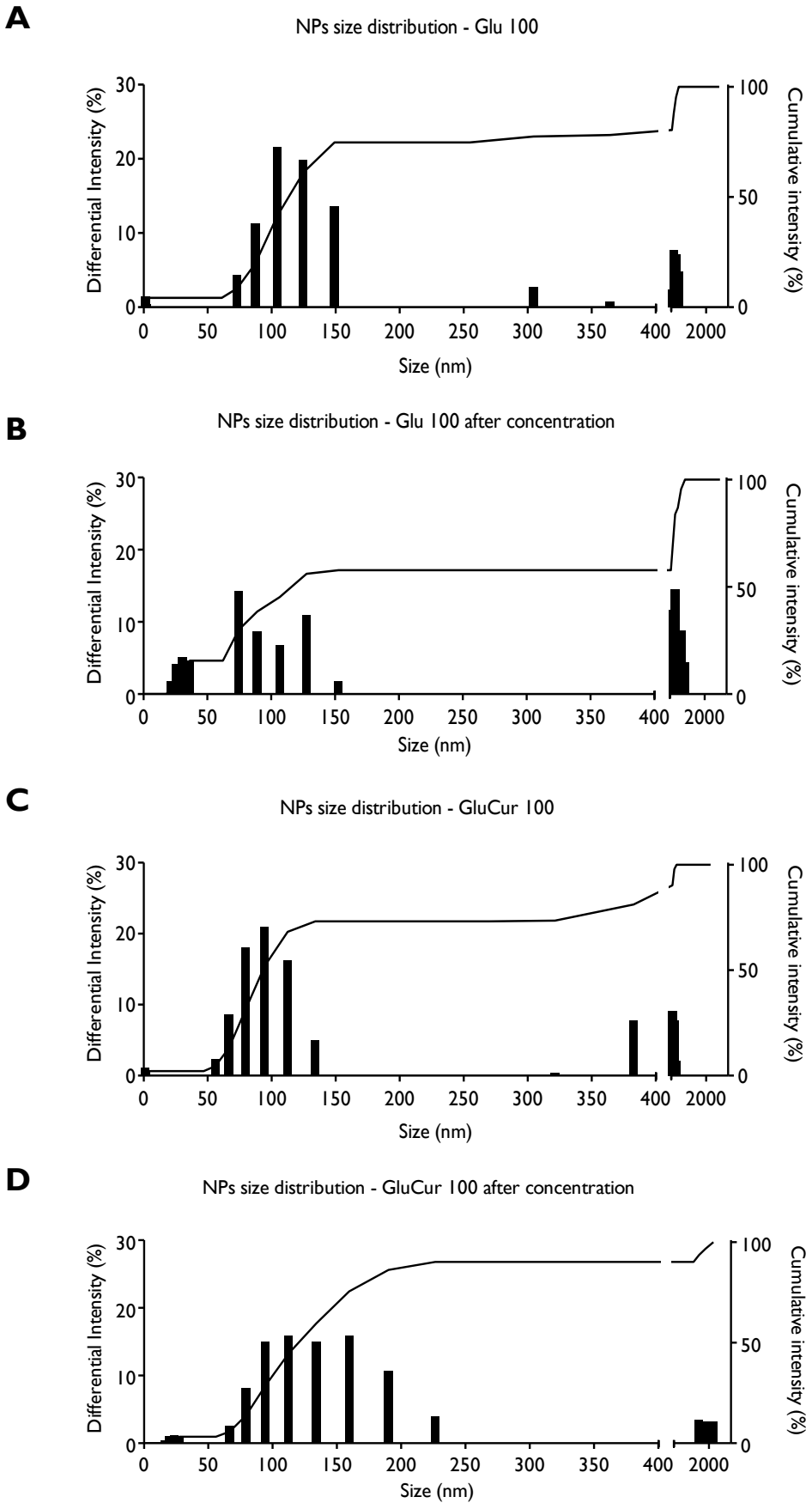

Figure 2.3 – Characterization of the size of NPs before and after concentration using Vivaspin: (A) and (B) for Glu 100, (C) and (D) for GluCur 100.

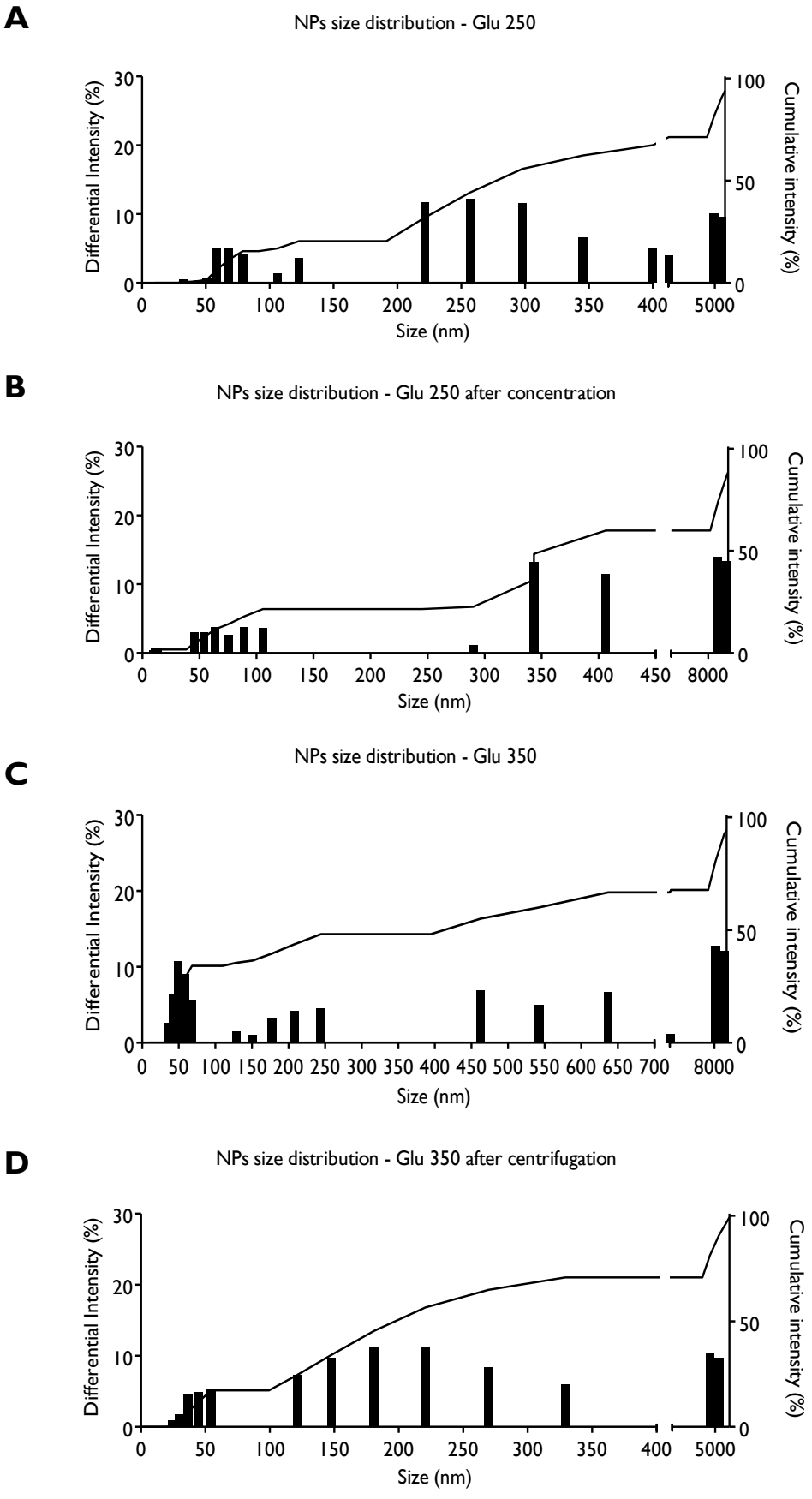

Figure 2.4 – Characterization of the size of NPs before and after concentration using Vivaspin: (A) and (B) for Glu 250, (C) and (D) for GluCur 350.

To summarize, in general, there were no important differences between NPs before and after concentration using Vivaspin. In Glu 100 we verified the increased presence of aggregates and an intensification of polydispersity populations after centrifugation (Figure 2.3 A and B). This was also noted by the results in table 2.3, where a slightly increased in size and PDI was observed. In GluCur 100 and in Glu 250, the aggregates presented a larger size and populations of GluCur 100 NPs were also more polydisperse after concentration (Figure 2.3 C, D; Figure 2.4 A and B). Finally, in GluCur 350 NPs, it was visualized aggregates of smaller dimension and also the disappearance of some populations with sizes ranging between 450 and 650 nm, leading to a decreased in the mean size of NPs (Table 2.3; Figure 2.4 C and D).

2.2.2.2 TEM analysis showed that curcumin loaded and unloaded Glu NPs with 100 nm had a similar morphology

To evaluate the morphology of the Glu 100 and GluCur 100 NPs, a TEM analysis was done. As seen in figure 2.5, both Glu 100 as GluCur 100 presented a rounded shape. In case of Glu 100 it was also detected particles with a rod shape and a size ranging between 50 and 110 nm, which was similar with the value of size obtained from DLS, indicating the polydispersity of populations. In case of GluCur 100 it seemed that the NPs had a tendency to aggregate, leading to an increase in the size of NPs compared with DLS.

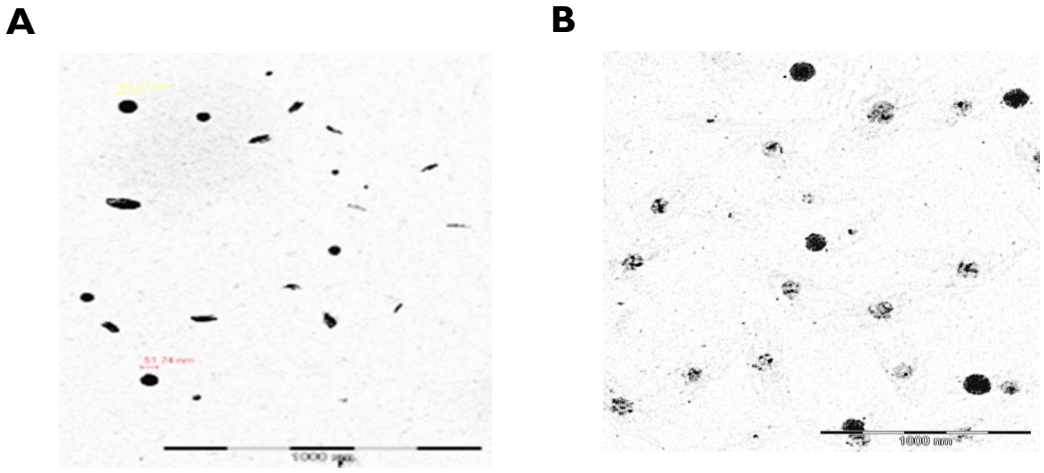

Figure 2.5 – TEM images of 100 nm Glu NPs after being concentrated and washed using Vivaspin: (A) Glu 100 NPs, (B) GluCur 100 NPs (scale bar: 1000 nm).

2.2.2.3 Cryo-SEM analysis showed that Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs presented a spherical shape

The morphology of Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs was also investigated through Cryo-SEM analysis. As seen in figure 2.6, Glu 100 and GluCur 100 NPs presented the same rounded shape as already observed by TEM analysis. The Glu 250 and GluCur 350 NPs presented the same morphology.

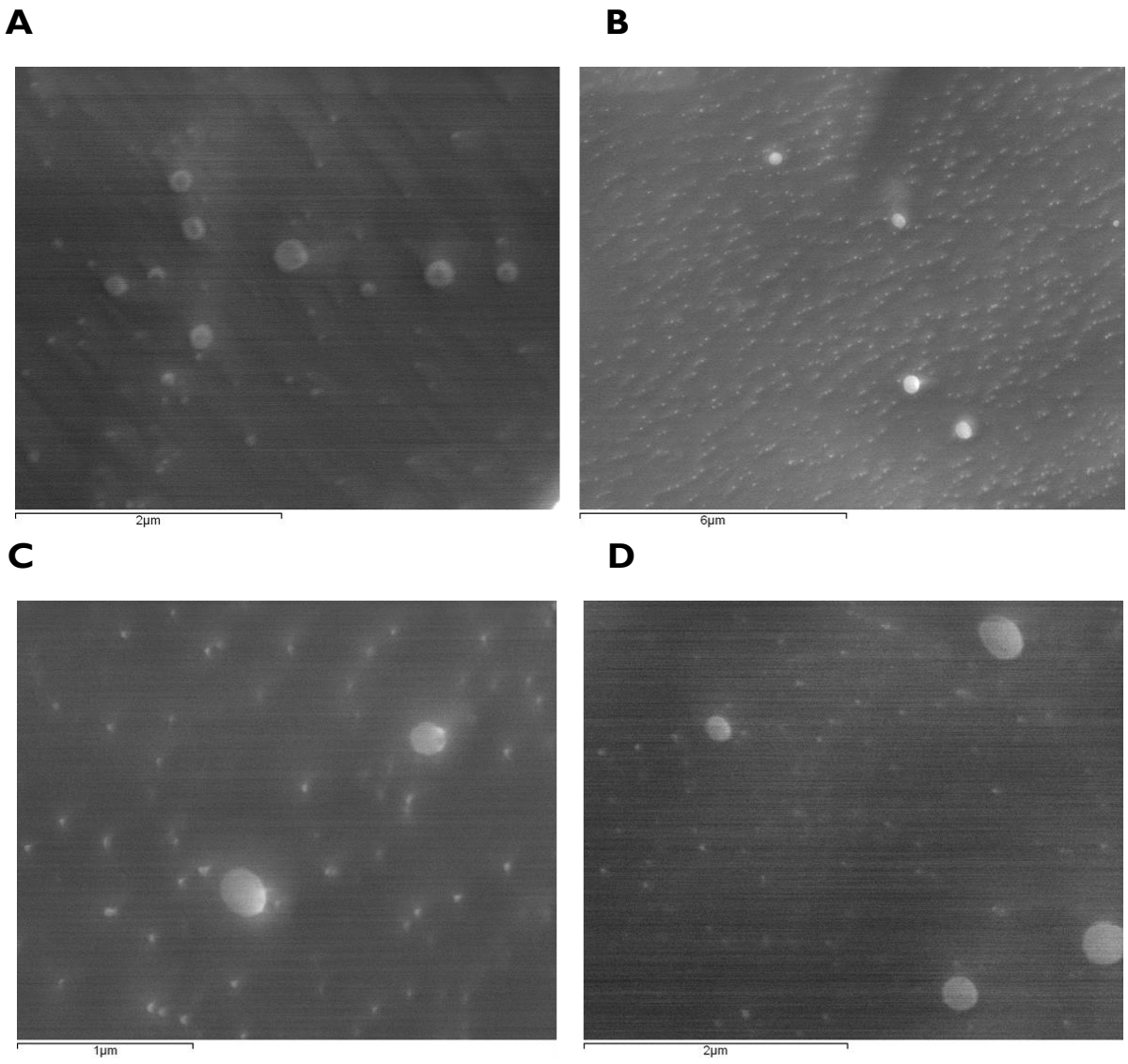

Figure 2.6 – Cryo - SEM images of 100 nm and 300 nm Glu NPs after being concentrated and washed using Vivaspin: (A) GluCur 100 NPs, (B) Glu 100 NPs, (C) GluCur 350 NPs and (D) Glu 250 NPs.

2.2.2.4 The precipitation method consumed almost all existing glucan to form NPs

Another important parameter to characterize the method to produce our NPs was to evaluate if all of the quantity of glucan that was dissolved into basic solution was, in fact, being used to produce the NPs. This is imperative for cellular studies because through the quantification of 1,3-β-glucan that was not used to form the NPs we could make an estimation of the concentration of NPs that we had. In literature, an analytical approach for quantifying 1,3-β-glucan by a fluoresce microassay was described (Ko e Lin, 2004). In this assay, aniline blue dye when in solution with 1,3-β-glucan forms a complex that exhibits fluorescence. That value will then be compared with the calibration curve constructed from the curdlan solutions.

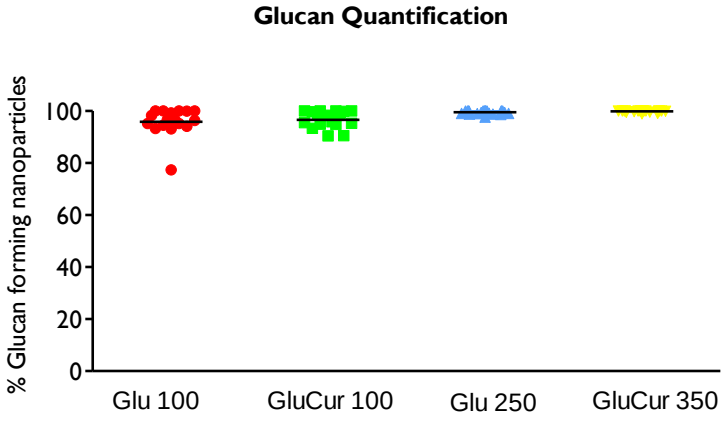

Figure 2.7 – Glucan quantification through a fluorescence dye-binding microassay: % of glucan that it is being used to produce the NPs.

This assay was done for all of the individuals batches of NPs (Glu 100, GluCur 100, Glu 250 and GluCur 350), being represented in figure 2.7 by a dot. We quantified glucan using the filtrate resulting from the centrifugation with Vivaspin. It could be realised that the great majority of glucan was being used to produce the NPs and that was not visualized any variance between different NPs. Having in account this result, we assumed that 100 % of glucan formed NPs, when quantifying its concentration for cellular studies.

2.2.2.5 The curcumin loading efficacy was close to 100 %

The curcumin encapsulated into glucan particles was measured by an indirect way, using the filtrate to quantify the free curcumin that was not inside of the particles. For that, a calibration curve was constructed with serial dilutions of known concentrations of curcumin in the solvent of NPs.

A **B**

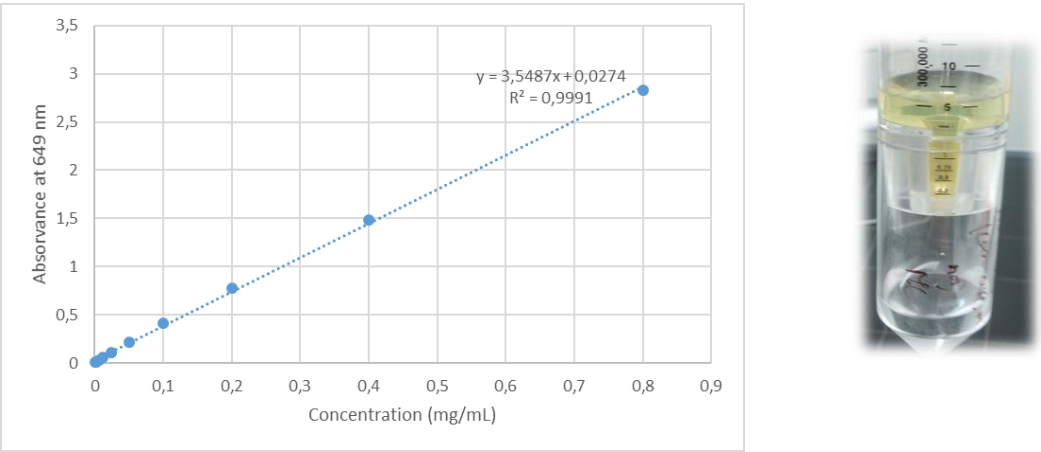

Figure 2.8 – (A) Quantification of curcumin using a calibration curve of known concentrations of the drug in the solvent of NPs. (B) Visualization of the filtrate obtained from the centrifugation of NPs with curcumin encapsulated using Vivaspin (lower part of the centrifugal tube).

One of the limitations of this method was that it was not possible to quantify the curcumin encapsulated in the NPs by a direct way, but only the curcumin that was actually present in the lower part of the tube in figure 2.8 B. The obtained absorbance values in both types of curcumin encapsulated NPs (GluCur 100 and GluCur 350) were always proximal to 0 and because of that we assumed that all curcumin added to the NPs was encapsulated.

2.2.2.6 All Glu NPs (Glu 100, GluCur 100, Glu 250 and GluCur 350) presented a great stability when stored in a refrigerator (4° C)

To evaluate the Glu NPs stability at long-term in laboratory, it was conducted a study for 6 weeks where individual batches of each NP type were placed at two different temperatures: 4 °C and 20 °C. This was relevant because it was desirable when doing the cellular studies to have the same batch for the different cellular assessments but for that to happen the characteristics of NPs have to remain unalterable.

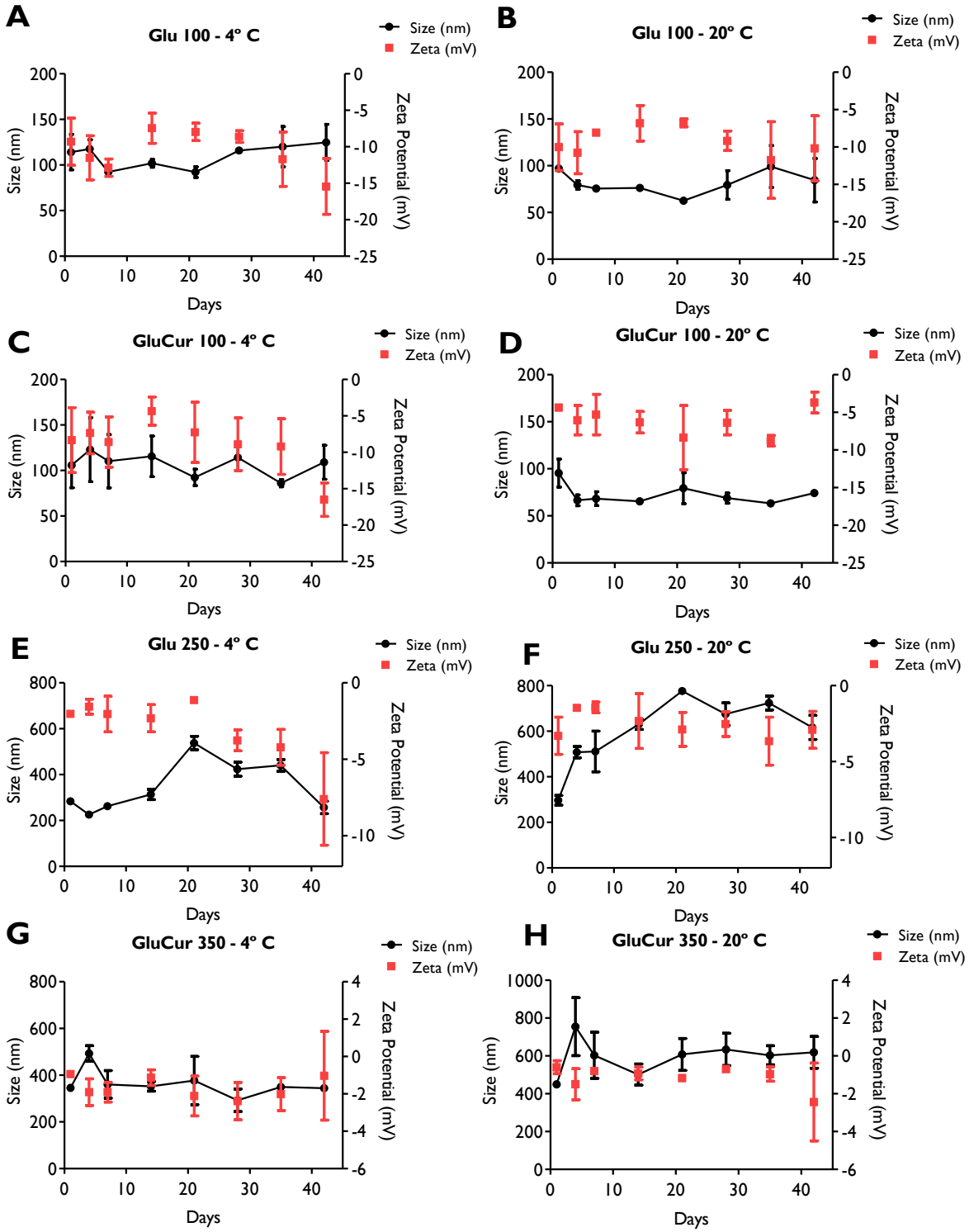

Figure 2.9 – Stability test of Glu 100, GluCur 100, Glu 250 and GluCur 350 performed at two temperatures: (A), (C), (E) and (G) at 4 °C and (B), (D), (F) and (H) at 20 ° C, presenting in each graph the mean size and the zeta potential over time. Results are the mean ± SEM, n = 3 (three independent experiments).

Table 2.4 – Measurement of PDI of the different types of NPs (Glu 100, GluCur 100, Glu 250 and GluCur 350) at two different temperatures (A) at 4 °C and (B) at 20 °C, over 42 days. Results are the mean ± SEM, n = 3 (three independent experiments).

A

4° C	PDI (Glu 100) ± SEM	PDI (GluCur 100) ± SEM	PDI (Glu 250) ± SEM	PDI (GluCur 350) ± SEM
1	0.379 ± 0.037	0.494 ± 0.137	0.416 ± 0.095	0.600 ± 0.071
4	0.479 ± 0.101	0.410 ± 0.118	0.510 ± 0.079	0.848 ± 0.048
7	0.315 ± 0.086	0.411 ± 0.143	0.421 ± 0.054	0.625 ± 0.091
14	0.440 ± 0.021	0.549 ± 0.145	0.493 ± 0.038	0.725 ± 0.077
21	0.407 ± 0.039	0.461 ± 0.098	0.547 ± 0.143	0.684 ± 0.087
28	0.452 ± 0.095	0.443 ± 0.098	0.413 ± 0.072	0.530 ± 0.067
35	0.492 ± 0.098	0.570 ± 0.084	0.465 ± 0.088	0.662 ± 0.089
42	0.450 ± 0.150	0.393 ± 0.060	0.447 ± 0.091	0.615 ± 0.105

B

20° C	PDI (Glu 100) ± SEM	PDI (GluCur 100) ± SEM	PDI (Glu 250) ± SEM	PDI (GluCur 350) ± SEM
1	0.463 ± 0.082	0.525 ± 0.104	0.411 ± 0.066	0.617 ± 0.039
4	0.327 ± 0.039	0.359 ± 0.030	0.456 ± 0.169	0.772 ± 0.046
7	0.331 ± 0.014	0.341 ± 0.039	0.493 ± 0.088	0.855 ± 0.013
14	0.357 ± 0.028	0.364 ± 0.033	0.545 ± 0.155	0.760 ± 0.072
21	0.376 ± 0.065	0.404 ± 0.058	0.493 ± 0.164	0.674 ± 0.130
28	0.372 ± 0.088	0.417 ± 0.058	0.663 ± 0.102	0.722 ± 0.104
35	0.470 ± 0.091	0.430 ± 0.055	0.552 ± 0.121	0.832 ± 0.021
42	0.373 ± 0.063	0.365 ± 0.038	0.395 ± 0.110	0.748 ± 0.034

In this stability test, Glu 100 and GluCur 100 showed at both temperatures not to suffer variations regarding their size and zeta potential during the 42 days (Figure 2.9 A, B, C and D). However, Glu 250 and GluCur 350 revealed a tendency to aggregate at 20 °C after only 1 week but remain stable for the 42 days at 4 °C (Figure 2.9 E, F, G and H). This tendency was not surprising since both types of NPs, Glu 250 and GluCur 350 after being produced already had a small population of aggregates and with the effect of temperature and the fact that the particles were not in the original medium but in water, the tendency to aggregate is larger. The zeta potential remained the same. As regards of the polydispersity index, for all types of NPs did not seem to exist significant differences during the 42 days and even between different temperatures (Table 2.4).

2.2.2.7 Glu 100 and GluCur 100 NPs over time showed a tendency to aggregate in cell culture media at 37 °C contrary to Glu 250 and GluCur 350 NPs where the population of aggregates disappeared

To simulate what happens when NPs were incubated with cells, it was done an experiment where all types of NPs (Glu 100, GluCur 100, Glu 250 and GluCur 350) were placed in contact with cell culture media, DMEM and RPMI, at 37 °C and different time points were chosen to evaluate the size and zeta potential of the formulations. Interestingly, Glu 100 and GluCur 100 NPs showed a tendency to aggregate over time but Glu 250 and GluCur 350 NPs on the contrary, did not present that tendency and it was even noticed that the population of aggregates that was present after the production of the particles disappeared, leading to a decrease on its mean size.

Table 2.5 – Measurement of zeta potential in mV of the different types of NPs (Glu 100, GluCur 100, Glu 250 and GluCur 350) after being incubated for 5 min and 72 h in two cell culture media (A) in DMEM and (B) in RPMI. Results are the mean ± SEM, n = 3 (three independent experiments).

A

DMEM	5 min	72 h
ZP Glu 100 ± SEM (mV)	-5.6 ± 2.1	-0.5 ± 1.6
ZP GluCur 100 ± SEM (mV)	0.4 ± 1.3	-2.8 ± 3.3
ZP Glu 250 ± SEM (mV)	-1.9 ± 1.0	0.8 ± 0.8
ZP GluCur 350 ± SEM (mV)	-3.6 ± 2.1	-4.2 ± 2.6

B

RPMI	5 min	72 h
ZP Glu 100 ± SEM (mV)	-4.1 ± 1.7	0.8 ± 0.4
ZP GluCur 100 ± SEM (mV)	-3.1 ± 3.1	-2.5 ± 2.8
ZP Glu 250 ± SEM (mV)	-0.2 ± 0.7	-1.3 ± 1.6
ZP GluCur 350 ± SEM (mV)	-0.3 ± 1.7	-1.8 ± 2.1

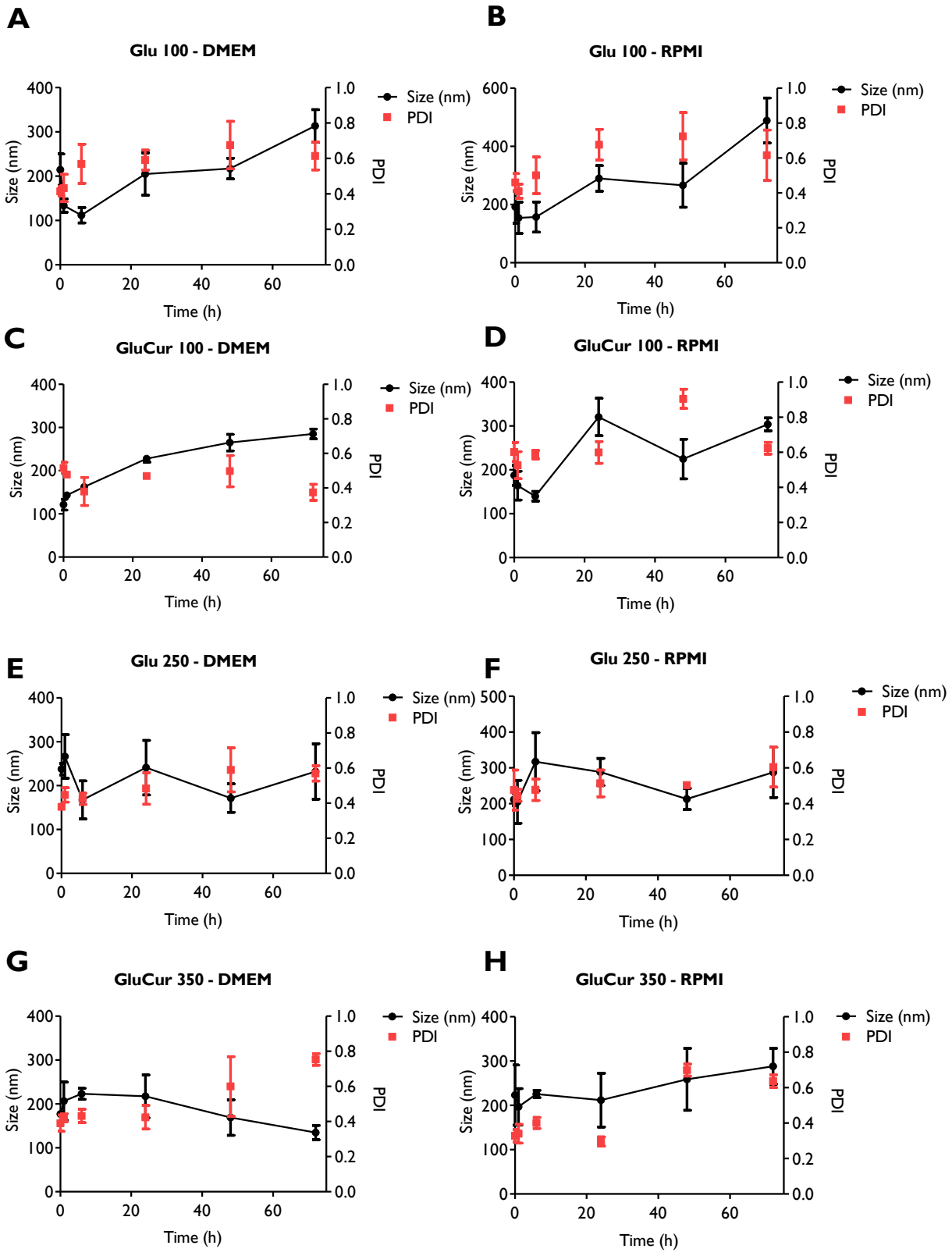

Figure 2.10 – Stability test of Glu 100, GluCur 100, Glu 250 and GluCur 350 performed in two cell culture media at 37 °C: (A), (C), (E) and (G) in DMEM and (B), (D), (F) and (H) in RPMI, presenting in each graph the mean size and PDI over time. Results are the mean ± SEM, n = 3 (three independent experiments).

As it is shown in figure 2.10, 100 nm Glu NPs, both Glu 100 as GluCur 100 showed an increase in size with the longer permanence in culture media, DMEM and RPMI. The values of PDI for these particles were relatively high, demonstrating the polydispersity of populations. It is worth emphasizing the time 24 h because it was the time used to incubate NPs with cells in DMEM and also the 72 h because it was the time used to incubate NPs with cells in RPMI. At 24 h both Glu 100 as GluCur 100 presented a size of 200 nm in DMEM. However, at 72 h in RPMI, Glu 100 presented a size of 480 nm and GluCur 100 of 300 nm, being more visible the aggregation in this last cell culture media.

The Glu 250 and GluCur 350 NPs behaved in a similar way in both cell culture media, DMEM and RPMI, having revealed in both cases a size around 200 nm. The mean size in these particles was slightly lower than of that presented when the NPs were concentrated and dispersed in water because the populations of aggregates presented in water, disappeared in media. The values of PDI were also high due to the polydispersity of populations.

The zeta potential was measured in the beginning of the experiment and in the end with the objective of knowing if this parameter was affected over time. In all types of NPs, as it can be seen in table 2.5 the zeta potential was not altered. The fact that zeta potential is close to neutrality means that particles have a bigger disposition to form aggregates, since they are considered stable when values are lower than -25 mV and higher than 25 mV and all of our particles did not present values in that range (Tantra, Schulze e Quincey, 2010).

Chapter 3

Immunomodulatory effect of Glu NPs in different cell lines

3.1 Materials and Methods

3.1.1 Materials

Curdlan (P-Curdl) was purchased from Megazyme. Curcumin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and the murine cell line RAW 264.7 were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Vivaspin 20 centrifugal concentrator (MWCO 300KD) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Murine IL-1β and TNF-α standards for ELISA kits were acquired from PeproTech (Rocky Hill, NJ, USA). All the other chemicals were from analytical grade and purchased from normal suppliers.

3.1.2 Preparation of the nanoparticles

3.1.2.1 Glucan Nanoparticle (Glu 100)

The technique to produce glucan nanoparticles was based on a precipitation/coacervation method, where a solution of 8 % acetic acid was added, dropwise, under magnetic stirring, to a solution of 0.025 % glucan in 2 % sodium hydroxide and 1 % tween 80®. The addition of acetic acid was interrupted at different pH namely pH 11, 7, 6 and 5, where the mixture was kept under magnetic stirring during half an hour.

3.1.2.2 Curcumin encapsulated Glucan Nanoparticle (GluCur 100)

To encapsulate curcumin within the glucan nanoparticles with size of 100 nm, the same method of production of these last nanoparticles was done but instead of adding just a solution of 8 % acetic acid, between the pH of 11 to 5, it was introduced a solution of 8 % acetic acid containing 0.1 mg/mL curcumin and 1 % tween 80®.

3.1.2.3 Glucan Nanoparticle (Glu 250)

Glucan nanoparticles with size around 250 nm were prepared by a similar technique as described for glucan nanoparticles with size of 100 nm, which only differs in the concentrations of the solutions. Shortly, a solution of 4 % acetic acid was added, dropwise, under magnetic stirring to a solution of 0.1 % glucan in 2 % sodium hydroxide and 0.1 % tween 80®. This addition of acetic acid was done without interruption from the initial pH of

the solution until pH 5. The suspension of nanoparticles was left 1 h under magnetic stirring to achieve maturation.

3.1.2.4 Curcumin encapsulated Glucan Nanoparticle (GluCur 350)

To encapsulate curcumin within the 250 nm glucan nanoparticles, a similar method to its blank NPs was applied with slightly adjustments. The solution of 4 % of acetic acid was added until reaching pH 11 and then it was applied also dropwise 850 µL of a solution of 0.1 mg/mL curcumin in 8 % acetic acid and 1 % tween 80® to a solution of 0.1 % glucan in 2 % sodium hydroxide and 0.2 % tween 80® (final pH 5). The suspension of nanoparticles was left 1 h under magnetic stirring to achieve maturation.

3.1.3 Nanoparticle immunotoxicity assays

3.1.3.1 Macrophage cell line

3.1.3.1.1 Cell Culture

The murine macrophage cell line (RAW 264.7) was cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % inactivated FBS (Fetal Bovine Serum), 3.7 g/L sodium bicarbonate, 10 mM Hepes and 1 % penicillin/streptomycin. These cells semi-adherent were maintained in 75 cm^2 flasks with 20 mL of growth medium in a humidified atmosphere at 37 °C with 5 % CO_2. Subcultures were performed by detaching the cells mechanically after being grown at a confluence of approximately 70 % to 80 % and by diluting 1:2 to 1:6 every 2 to 3 days.

3.1.3.1.2 Cytotoxicity of the nanoparticles

To access cell viability, it was used the MTT tetrazolium reduction assay in which MTT is reduced to formazan, a purple coloured product in viable cells with active metabolism. The decrease of the purple colour means that cells are metabolic less active and do not mean necessarily that cells are death (Meerloo e Cloos, 2011). Briefly, the RAW 264.7 mouse macrophage cell line was seeded in a 96-well plate at a cell density of $2x10^5$ cell/mL in DMEM. After being left incubating for 24 h in a humidified atmosphere at 37 °C, 5 % CO_2, the medium was removed and was added 150 µL of fresh medium and 50 µL of the nanoparticles suspension for 24 h. Series of dilutions of the nanoparticles in water were prepared at a concentration range between 7.8 µg/mL and 494 µg/mL. To eliminate NPs

interferences, the medium was replaced by new medium and 20 µL of MTT solution (5 mg/mL in PBS pH=7.4) was added to each well followed by incubation during 1.5 h at 37 °C. Shortly, after, the supernatant was aspirated and 200 µL of DMSO was added to each well pipetted up and down to dissolve the formazan crystals. The absorbance values were then measured at 540 nm with 630 nm as wavelength reference using a microplate reader. The relative cell viability (%) was calculated in relation to the control, which was the cells in culture medium without the nanoparticles, using equation (1):

$$\text{Cell viability (\%)} = \frac{OD\ sample\ (540\ nm) - OD\ sample\ (630\ nm)}{\text{OD control (540 nm)} - OD\ control\ (630\ nm)}\ x\ 100 \qquad \text{(Eq. 1)}$$

3.1.3.1.3 Nitric Oxide Production Assay

Nitric Oxide Assay is based on the premise that NO is extremely unstable and degrades to nitrite (NO_2^-) and nitrate (NO_3^-), which are products that can be spectrophotometrically determined. The basis of this technique is that nitrites could be detected by reacting with sulphanilamide and N-(1-naphthyl) ethylenediamine forming a stable water soluble azodye, which can be quantified by measuring spectrophotometric absorption at 550 nm (Abcam, 2015). In brief, the RAW 264.7 mouse macrophage cell line was seeded in a 48-well plate at a cell density of $4.5x10^5$ cell/mL in DMEM and incubated for 24 h in a humidified atmosphere at 37 °C, 5 % CO_2. Then the medium was removed and it was added 375 µL of DMEM without phenol red and 125 µL of the nanoparticles suspensions to see if the formulations could induce the production of NO or it was added 250 µL of DMEM without phenol red and 125 µL of the nanoparticles suspensions and 125 µL of LPS (at a concentration of 1 µg/mL in each well) to see if the formulations inhibit the production of NO. LPS solution was diluted in DMEM without phenol red and it was used as a positive control to the NO production. Different concentrations of the nanoparticles were prepared in pyrogen-free water to give a final volume of 500 µL in each well of a microtiter plate and the formulations were left incubating during 24 h. For each formulation three different concentrations were chosen like it is presented in the following table:

Formulations	Concentrations (µg/mL)
Glu 100	15
	7.5
	3.75
GluCur 100	15
	7.5
	3.75
Glu 250	650
	325
	163
GluCur 350	650
	325
	163

After incubation, 100 µL of the supernatants were harvested and mixed with an equal volume of Griess reagent that had been previously prepared in a 96-well plate. The Griess reagent is a mixture of equal volumes of 1 % (w/v) sulphanilamide in 2.5 % (v/v) phosphoric acid and 0.1 % (w/v) naphthylethylenediamine dihydrochloride in 2.5 % (v/v) phosphoric acid and it has to be used immediately after being prepared. Subsequently to the addition of Griess reagent, the plate was incubated at room temperature for 10 min in the dark. The absorbance was measured at 550 nm using a microplate reader. DMEM without phenol red culture medium was used as the blank in all experiments. To determine the quantity of NO production, a calibration curve was done using dilutions of a stock solution of 1 mg/mL $NaNO_2$. The concentrations used were 0.625; 1; 1.25; 2.5; 5; 10; 20; 30; 50; 60; 70 and 80 µM of $NaNO_2$ diluted in DMEM without phenol red. After collecting the supernatants to make this assay, it was done the MTT reduction assay to evaluate the cytotoxicity of the used concentrations of the different formulations. For that, the remnant supernatant was removed and it was added 200 µL of DMEM and 20 µL of MTT solution (5 mg/mL in PBS pH=7.4) to each well followed by incubation during 1.5 h at 37 °C. The supernatant was then aspirated and 200 µL of DMSO was added to each well pipetted up and down to dissolve the formazan crystals. The absorbance values were then measured at 540 nm with 630 nm as wavelength reference using a microplate reader.

3.1.3.1.4 Quantification of cytokines

The RAW 264.7 mouse macrophage cell line was seeded in a 48-well plate at a cell density of $4.5x10^5$ cell/mL and incubated with formulations (Glu 100, GluCur 100, Glu 250 and GluCur 350) and controls (solvents of NPs, free curcumin, Glu Polymer, DMSO 0.4 % and LPS as a positive control) for 24 h in a humidified atmosphere at 37 °C, 5 % CO_2. The concentrations used were the same of the NO production assay. After 24 h, the supernatants were removed and stored at -80 °C until analysis of TNF-α and IL-1β. To measure cytokines release it was used ELISA technique according to kit manufacturer's instructions (Prepotech). Briefly, high binding 96 well-plates were coated with 100 µL of capture antibody and were left incubating overnight at room temperature. Plates were washed 5 times with wash buffer and blocked for 1 h. After washing, serial dilutions of the standard cytokines and 100 µL of each sample were added and incubated for 2 h at room temperature. Each sample represented a pool of one condition done in triplicate in one day. After extensive washing, it was applied 100 µL of the detection antibody, that was left incubating for 2 h. Plates were washed again and it was added 100 µL of avidin-HRP conjugate for 30 min. After the final washing, 100 µL of substrate solution was applied and left incubating at RT for colour development. The absorbance values were then measured at 405 nm with 630 nm as wavelength reference using a ELISA plate reader. Concentrations of the cytokines were extrapolated from absorbance values, using the calibration curves.

3.1.3.2 Whole Blood

3.1.3.2.1 Hemolysis Assay

This assay was adapted from an existing protocol and the principle of this method is that hemoglobin released by damage cells is oxidized to methemoglobin, which is then converted to cyanmethemoglobin. That amount of cyanmethemoglobin in the supernatant is measured spectrophotometrically at 540 nm. This value of absorbance is compared with a standard curve of hemoglobin to determine its concentration (Dobrovolskaia *et al.,* 2008). Briefly, blood was collected from volunteer healthy donors stabilized with Lithium heparin as anticoagulant. The fresh whole blood was diluted to adjust the total hemoglobin concentration to 10 mg/mL so that the values of absorbance of the samples were within the calibration range. A variety of concentrations of the different formulations (Glu 100, GluCur 100, Glu 250 and GluCur 350) were added to 100 µL of the diluted blood and 700 µL of PBS in a final volume of 900 µL. To determine the possible interferences of the nanoparticles, the

samples were incubated at the same concentrations with just PBS. To test the performance of hemolysis assay, PBS was used as a negative control (NC) and Triton-X-100 as a positive control (PC). The samples in the eppendorfs were mixed and incubated for 3 h at 37 °C, every 30 min the samples were shaken. After the incubation, the mixtures were centrifuged at 800 x g for 15 min. Apart from the eppendorfs with nanoparticles and the controls, 1 mL of the whole blood was also centrifuged to obtain plasma free hemoglobin (PFH). A hemoglobin standard was used to create a calibration curve, the dilutions were prepared from a stock solution and diluted with cyanmethemoglobin (CMH) reagent, the concentrations used in the curve ranged between 0.0125 mg/mL to 0.4 mg/mL. In a 96-well-plate, a volume of 200 µL of the standards was added and of the blank CMH reagent and of a solution of total blood hemoglobin sample (dTBH), which had been previously prepared by adding 400 µL of the diluted blood with 5 mL of CMH reagent. In the same plate, it was also added 100 µL of the positive and negative control, the samples with the nanoparticles and of PFH (samples were tested in duplicate). In these last samples 100 µL of CMH reagent were also added to each well. The 96-well-plate was left shaken during 2 to 3 min and the absorbance was measured at 540 nm using a microplate reader.

3.1.3.2.2 Coagulation

Through coagulation assay was possible to investigate if exists any effect caused by the prepared NPs on the blood coagulation cascade. The prothrombin time (PT) and the activated partial thromboplastin time (APTT) were measured to study the influence on the extrinsic and intrinsic pathways of blood coagulation, respectively. Briefly, blood was collected from volunteer healthy donors stabilized with sodium citrate as anticoagulant. The fresh whole blood was centrifuged for 10 min at 2500 x *g* and the resulting platelet-poor plasma (PPP) was collected to the same falcon (pool of plasma). A volume of 450 µL of plasma was treated with 50 µL of nanoparticles (final concentrations 2 and 200 µg/mL) and respective controls for 30 min at 37 °C. The samples were immediately evaluated using an hematology analyser (HmX Beckman Coulter Biomerieux option 4 plus). To assess the prothrombin time (PT), it was used Bio-TP LI, which determines the clotting time at 37 °C in the presence of tissular thromboplastin and calcium and to evaluate the activated partial thromboplastin time (APTT), it was used calcium chloride and Bio-CK which determines the clotting time at 37 °C in the presence of standardised amount of cephalin (platelet substitute) and a factor XII activator (Kaolin).

3.1.3.3 Human Peripheral Blood Mononuclear Cell Line (PBMCs)

3.1.3.3.1 Isolation of mononuclear cells from human peripheral blood by density gradient

To separate PBMCs from whole blood it was used a density gradient centrifugation using Lymphoprep. Briefly, in a 50 mL centrifuge tube the blood sample was diluted with saline solution (6 mL of blood: 30 mL of serum) and after 7.5 mL of the diluted sample was carefully layer by gently pipetting in a 15 mL tube conical, where 2.5 mL of lymphoprep had been previously added. Then, the tubes were centrifuged at 1190 x g for 20 min at 20 °C to form a ring of mononuclear cells between the plasma and the separation liquid (lymphoprep). The upper layer was aspirated leaving that mononuclear cell layer undisturbed at the interphase, so that it could be removed after to a new 15 mL tube conical (every two rings to a new tube). Afterwards, the PBMCs were washed with phosphate-buffered saline (PBS) previously heated at 37 °C by adding enough PBS to make up 12 mL and the samples were centrifuged at 487 x g for 10 min at 20 °C. The supernatant was removed, the pellet was loosen and two more washes were made with PBS (corresponds to the elimination of platelets). After the last wash, the cells (pellet) were resuspended in 6 mL of RPMI supplemented with 2 mM L-glutamine and 1 % penicillin/streptomycin previously heated at 37 °C. The cells were counted in the Neubauer Chamber and the cell viability was determined with trypan blue by mixing 180 µL of PBS with 20 µL of the cellular suspension with 50 µL of trypan blue in a 1.5 mL eppendorf tube.

3.1.3.3.2 Proliferation Assay

Cell proliferation assay can be used to evaluate cell activation to a stimulus like mitogens or even nanoparticles. The human peripheral blood mononuclear cells (PBMCs) were seeded in a 96-well plate at a cell density of $5x10^6$ cell/mL in RPMI 1640 medium supplemented with 1 % PenStrep, 2 mM L-glutamine and 10 % heat-inactivated FBS (100 µL in each well) and were left incubating in a humidified atmosphere at 37 °C, 5 % CO_2 for 2 h to stabilize. A serial of dilutions of the nanoparticles was prepared ranging from 0.3 µg/mL to 20 µg/mL to Glu 100 and GluCur 100 and from 3.85 µg/mL to 246 µg/mL to Glu 250, GluCur 350 and the polymer corresponding to non-cytotoxic concentrations of particles and polymer, respectively. After treating these cells with different doses of NPs during 72 h, the proliferation was measured by an indirect way using the MTT assay. A volume of 20 µL of MTT solution (5 mg/mL in PBS pH=7.4) was added to each well followed by incubation at 37

°C during 4 h. After that time, the 96-well plate was centrifuged for 25 min at 800 x g and the supernatant was carefully removed. 100 µL of DMSO was then added to each well pipette up and down to dissolve the formazan crystals. The absorbance values were then measured at 540 nm with 630 nm as wavelength reference using a microplate reader. The relative cell viability (%) was calculated in relation to the control, which was the cells in culture medium without the nanoparticles by the equation 1.

3.1.4 Statistical analysis

Data were analysed using GraphPad Prim 5 (GraphPad Software, Inc., La Jolla, CA, USA), in which significant differences were obtained from one-way ANOVA Dunnett (compare all columns vs control column) and Bonferroni (compared selected pair of columns). P values were considered statistically different when $p < 0.05$. Statistical data was expressed as means ± standard error of the mean (SEM).

3.2 Results and Discussion

3.2.1 Smaller Glu NPs (size around 130 nm) are more toxic than the larger ones

In vitro toxicity could be very useful to predict the cytotoxic profile of the nanoparticles *in vivo*. To access cell viability of the nanoparticles it was used the MTT tetrazolium reduction assay, which determines cell viability in an indirect way because it measures the metabolic activity instead of the real number of viable cells. Through this method it was clearly observed that glucan nanoparticles with smaller size presented a completely different cytotoxic profile compared with nanoparticles around 300 nm. A mouse macrophage cell line (RAW 264.7) was used since it is a good representative of cells of the immune system and one of our main goals was to study how our particles could interact with the cells of the immune system.

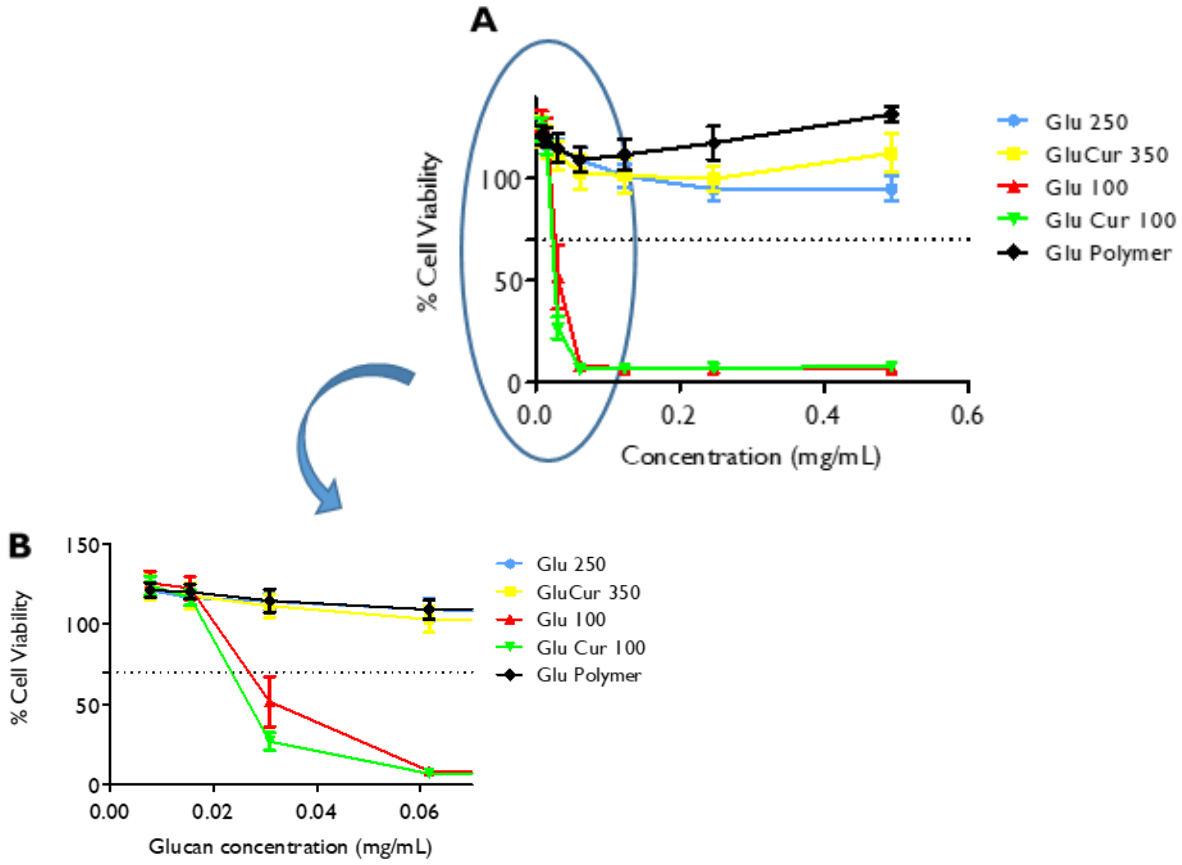

Figure 3.1 – Cytotoxicity evaluation through MTT tetrazolium reduction assay, after all of the NPs and polymer were in contact for 24 h with macrophages (mean ± SEM, n = 5, five independent experiments, each in triplicate): (A) Comparison of cytotoxicity of glucan nanoparticles with different sizes (Glu 100 with Glu 250 and GluCur 100 with GluCur 350) and with and without curcumin (Glu 100 with GluCur 100 and Glu 250 with GluCur 350) and also the polymer, (B) In more detail the cytotoxicity profile of the lower concentrations of all formulations and polymer.

It was observed that for concentrations below 0.02 mg/mL the cytotoxic profiles for nanoparticles and Glu polymer were the same (Figure 3.1 B). In fact, these viability values were around 120 %, making us consider the hypothesis that a stimulating effect may be occurring, leading to cell proliferation (increase of cell metabolic activity). However, for concentrations above 0.03 mg/mL, the viability of the cells in contact with the smaller NPs (Glu 100 and GluCur 100 nm), decreased drastically to values below 50 %, even reaching 7 % for concentrations above 0.06 mg/mL. It is important to mention that this highly cytotoxic profile of the smaller particles is not because of the solvent where NPs were done. That solvent was removed through centrifugation by washing the NPs with water and the supernatant obtained was used as control in the MTT assay and it did not present any cytotoxicity (supplemental information – Figure 3.1.1).

In contrast to the smaller particles, Glu 250 and GluCur 350 NPs did not show a decrease in cell viability in the several concentrations tested (until 0.5 mg/mL) and presented a very similar profile to the polymer (Figure 3.1 A). Interestingly, at the concentration of 0.5 mg/mL it seemed that exists again some cellular proliferation which was more visible in the case of the polymer and GluCur 350 NPs.

In the literature, a variety of studies demonstrated the biocompatibility of β-glucans (Vu-Quang *et al.*, 2012;) (Kim *et al.*, 2012) (Ganbold e Baigude, 2018) (Liu *et al.*, 2014). In one of those studies, Ji Lee and co-workers used laminarin, which is a type of 1,3-β-glucan and proved its effect on cell viability in RAW 264.7 mouse macrophages, having detected cell proliferation in concentrations similar to ours (100 to 500 μg/mL). That stimulation was also in a dose dependent manner (Lee *et al.*, 2012). Besides that, Wing Keung Chan and colleagues also had already proved the existence of proliferation in PBMCs caused by curdlan (Chan *et al.*, 2007). Surprisingly, despite the polymer used to produce both NPs did not cause toxicity, that result was not mirrored to Glu 100 and GluCur 100 NPs but it was for Glu 250 and GluCur 350 NPs. One of the reasons for that difference between the cytotoxicity profiles of Glu NPs could be because the variance of size can interfere with the activation of macrophages. A similar result was obtained by Margriet V.D.Z. Park and co-workers when they studied the effect of silver particles with different sizes (20, 80 and 113 nm) on the toxicity observed on macrophages (RAW 264.7). Despite the difference between sizes did not seem significantly, the results obtained for cytotoxicity were. Authors reported the smaller particles (20 nm) as the most potent in decreasing the cellular viability (Park *et al.*, 2011). This clearly showed that size is an important characteristic to have in mind, when developing a nanoparticle.

3.2.2 The cytotoxicity observed in smaller NPs containing curcumin is due to NPs and not to curcumin

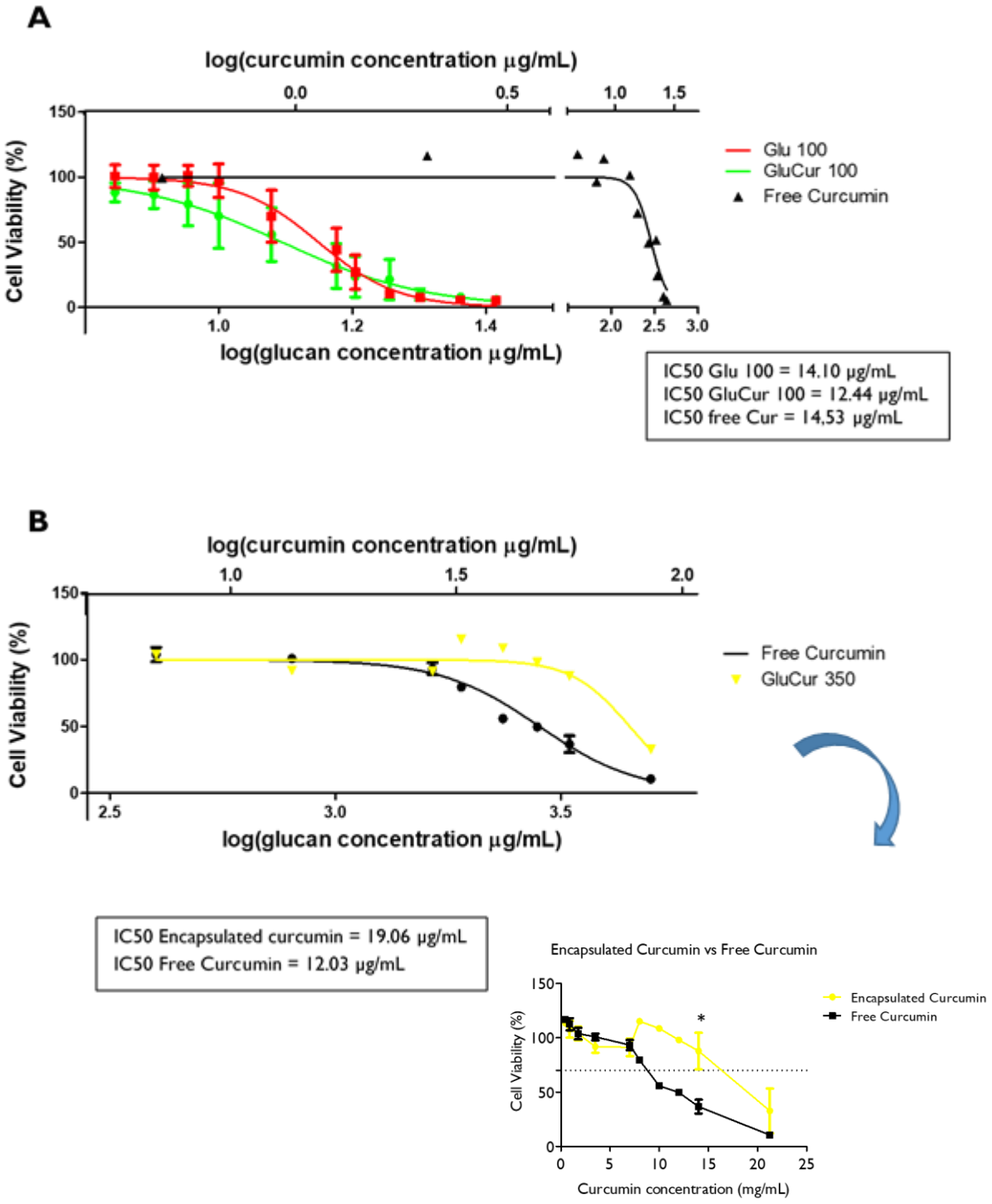

Figure 3.2 – Viability of macrophages in the presence of curcumin loaded nanoparticles and free curcumin using the MTT assay. Each concentration axis, above corresponds to curcumin concentration (µg/mL) and below corresponds to glucan concentration (µg/mL): (A) Concentration-response curve, after 24 h of incubation of the formulations with the mouse macrophages (RAW 264.7). The effect of curcumin free and curcumin loaded glucan nanoparticles (GluCur 100 nm), was evaluated. As a control, unloaded glucan nanoparticles (Glu 100 nm) was also tested. (B) Comparison of the cytotoxicity profile of curcumin free and curcumin loaded in glucan nanoparticles (GluCur 350 nm) on cell viability after 24 h of incubation with the macrophages. Comparison of IC50 for curcumin encapasulated in nanoparticles and free curcumin in RAW 264.7. Both results are the

mean ± SEM, n = 4 (four independent experiments, each in triplicate). * $p < 0.05$, significantly different from free curcumin.

Table 3.1 – Comparison of the viability through MTT tetrazolium reduction assay, of the correspondent curcumin encapsulated in glucan nanoparticles (GluCur 100 nm) to free curcumin showing that is not this compound that interferes in the decrease of the cell viability of the particles.

GluCur 100 nm concentration (µg/mL)	**NPs associated curcumin concentration (µg/mL)**	**% Viability NPs associated curcumin**	**% Viability free curcumin**
20	1	9.753	100
23	1.15	7.850	100
26	1.3	5.812	100

When compared, in more detail, Glu 100 and GluCur 100 particles with and without curcumin did not have a difference between them although the value of IC50 of GluCur 100 estimated was slightly lower (Figure 3.2 A) compared with the blank NPs, which meant it might present a higher toxicity.

The cytotoxicity profile of free curcumin was also determined including the value of IC 50 and it could be concluded indeed that it may not exist a difference between the smaller particles Glu 100 and GluCur 100 on the grounds that the concentrations of free curcumin that corresponded to the concentrations with curcumin encapsulated in the glucan nanoparticles did not present toxicity by themselves (Figure 3.2 A; Table 3.1).

Contrary to GluCur 100 NPs, in which it could not be seen the decrease of the cytotoxicity profile of curcumin for being encapsulated, in GluCur 350 NPs because the particle itself is less cytotoxic we could see that protective effect of the encapsulation of curcumin. Cytotoxicity results showed that for concentrations below 14 µg/mL the GluCur 350 NPs with curcumin encapsulated did not have any effect on cell viability, contrary to free curcumin that at the concentration of 14 µg/mL presented a decline in viability to 40 % (Figure 3.2 B).

These results are in agreement with the literature, regarding the effect of encapsulating curcumin in a nanoparticle on cell viability. Jiao Wang and co-workers studied the encapsulation of curcumin into SLNs and they concluded that it did not exist any difference in cell viability between just the nanocarrier, SLNs and curcumin encapsulated into SLNs in two cell lines, HEK293T and LO2 (Wang *et al.*, 2015) as it was our case. The cytotoxicity of free curcumin has been described by two groups in a mouse macrophage cell line (RAW

264.7), however the results showed a superior viability compared to what we obtained (Busari *et al.*, 2017) (Deka *et al.*, 2016). This difference between the reports is expected because despite using the same cell line, the cellular concentration, the cell passage or the time of incubation or even the solvent used to dissolve curcumin may be different and this lead to different outcomes. However, N. Sanoj Rejinold and co-workers obtained values of cellular viability to curcumin similar to ours using related concentrations in three cell lines, L929, PC3 and MCF7 (Sanoj Rejinold *et al.*, 2011).

In our results, depending on the cytotoxicity of the nanocarrier (Glu 100 or Glu 250) we could see or not, the protective effect of the encapsulation of curcumin. Mai Huong Le and colleagues demonstrated that a curcumin encapsulated glucan nanoparticle (glucan was isolated from a mushroom) was more toxic than free curcumin in both Hep-G2 and LU-1, which is similar to what happened with Glu 100 and GluCur 100 NPs (Le *et al.*, 2016). Authors argue that the increase in solubility of curcumin and compatibility between glucan and curcumin lead to an effectiveness in activity of this compound. Another study by Zulaikha Busari and co-workers revealed a similar result, curcumin loaded PLGA nanoparticle had a higher toxicity compared with free curcumin in RAW 264.7 cells (Busari *et al.*, 2017). They reported that this was due to the better absorption of the NPs influenced by its smaller size but they did not evaluate particles without curcumin with the same size. On the opposite side, a study with results similar with the Glu 250 and GluCur 350 NPs was reported by Jiao Wang and colleagues, where they showed that curcumin encapsulated in SLNs was less toxic than free curcumin in HEK293T and LO2 cells (Wang *et al.*, 2015). All in all, it could be concluded that depending on what we want to apply the curcumin, it is possible to choose a more cytotoxic nanocarrier or not.

3.2.3 Curcumin loaded Glu NPs inhibit NO production

Nitric oxide (NO) is an important molecule for host defense response against various pathogens such as bacteria, viruses, fungi, and parasites. However, overproduction of NO is known to be involved in the development of chronic inflammations (Joo *et al.*, 2014). To study the possible effects of our NPs on inflammation we performed a NO assay based on Griess reaction by incubating our particles with LPS-induced RAW 264.7 cells. For this assay, it was taken into account the previous results of viability in mouse macrophages, so that all concentrations chosen did not present any toxicity. It could be concluded that loaded

curcumin Glu NPs as well as curcumin in the higher concentrations tested inhibited NO production in LPS stimulated RAW 264.7 cells.

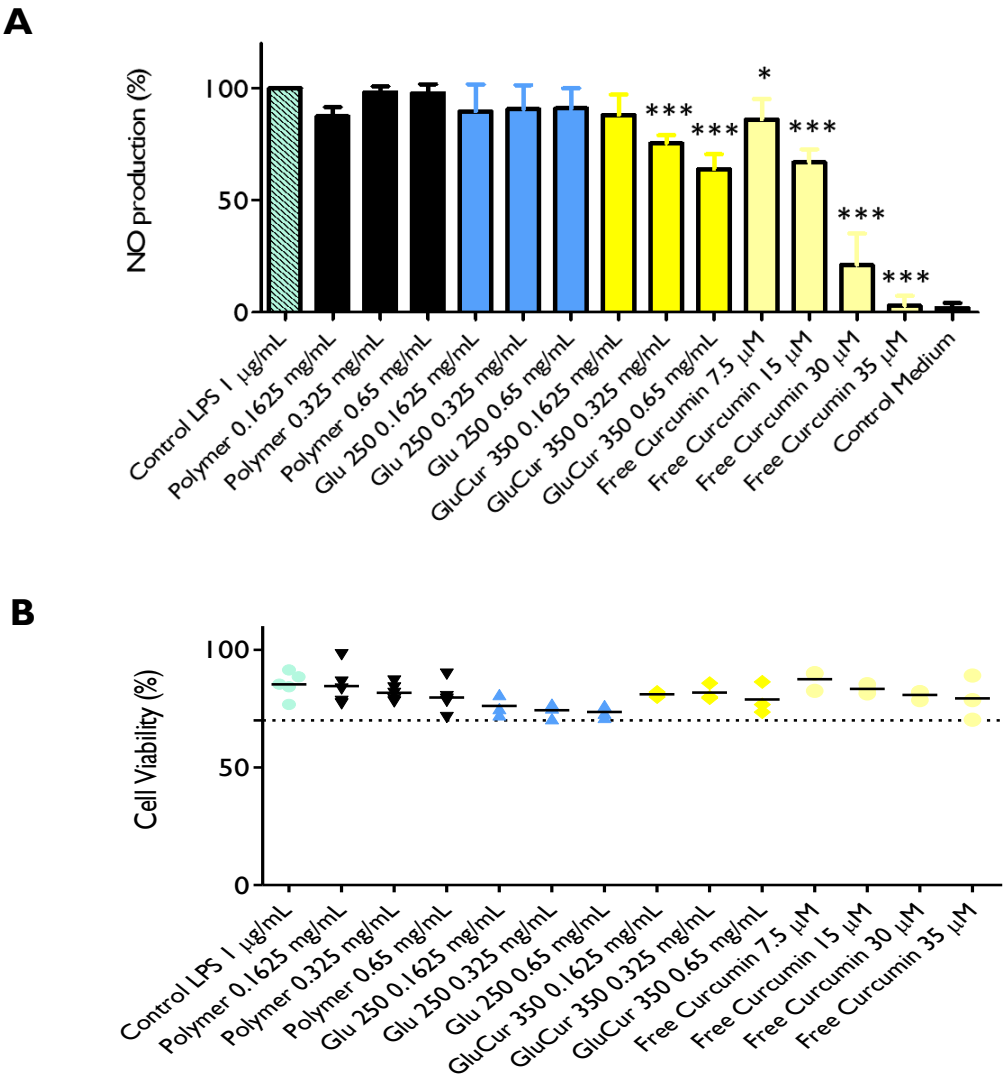

Figure 3.3 – Inhibitory effect of Glu NPs on NO production by LPS stimulated RAW 264.7 cells through Griess reaction after incubating the formulations for 24 h: (A) Comparison of NO production (%) between the Glu 250, GluCur 350, free curcumin in concentrations corresponding to encapsulated curcumin in GluCur 350 and Glu Polymer, (B) Effect on the viability after incubation with Glu 250, GluCur 350, free curcumin and Glu Polymer in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 µg/mL of LPS. Both results are the mean ± SEM, n = 3 (three independent experiments, each in triplicate). * $p < 0.05$, significantly different from the positive control (LPS).

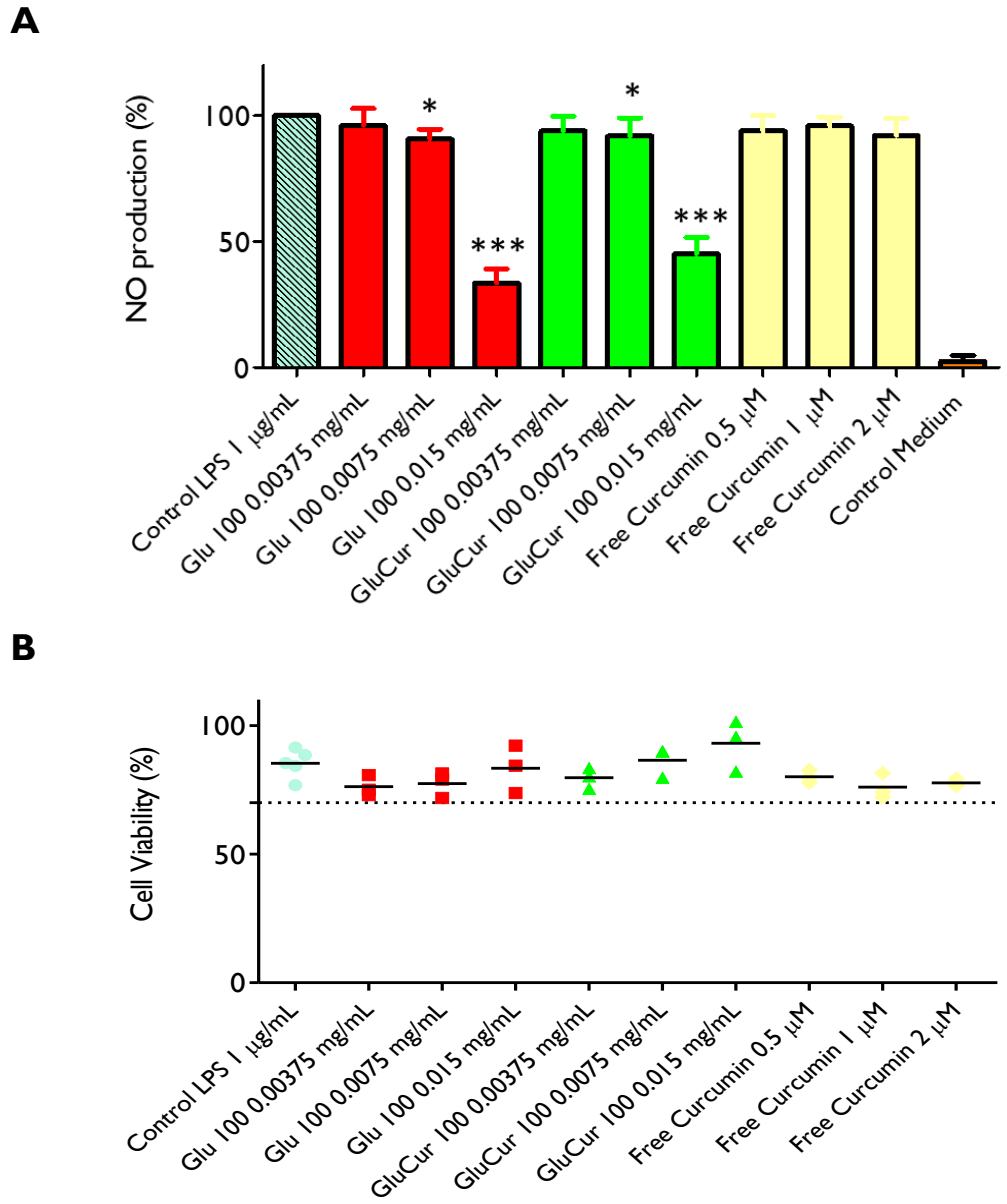

Figure 3.4 – Inhibitory effect of Glu NPs on NO production by LPS stimulated RAW 264.7 cells through Griess reaction after incubating the formulations for 24 h: (A) Comparison of NO production (%) between the Glu 100, GluCur 100 and free curcumin in concentrations corresponding to encapsulated curcumin in GluCur 100, (B) Effect on the viability after incubation with Glu 100, GluCur 100 and free curcumin in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 µg/mL of LPS. Both results are the mean ± SEM, n = 3 (three independent experiments, each in triplicate). * $p < 0.05$, significantly different from the positive control (LPS).

Curcumin possesses several pharmacological properties including antioxidant, anti-inflammatory, antiviral, antimicrobial and antifungal activities (Ciftci, Tanyildizi e Godekmerdan, 2010). If curcumin is known by its anti-inflammatory properties it is expected that this compound will have an inhibitory effect on NO production. It was no surprise when in our assay curcumin clearly inhibited NO production and this decrease was in a concentration-dependent manner (Figure 3.3 A). At concentration of 30 µM, it was observed

a NO production of 25 %, even reaching 5 % at 35 μM of free curcumin. One study was published by Bhaumik and colleagues, who also tested the inhibitory effect of NO production by curcumin (in comparable concentrations to what we used) in macrophages and their results were similar with ours (Bhaumik, Jyothi e Khar, 2000). The authors argue that this downregulation of NO production may prevent carcinogenesis. These effects were also proved in other types of cells lines (Onoda e Inano, 2000). Having established the effect of free curcumin in LPS stimulated RAW 264.7 cells, our next step was to test the encapsulated curcumin in glucan NPs (GluCur 350) that corresponded to the same concentrations of free curcumin. As it is shown in figure 3.3 A, the encapsulated curcumin also inhibited the NO production in a concentration-dependent manner but in this case the decrease of NO was not so strong. This fact was quite clear at the highest concentration tested where encapsulated curcumin presented a % of NO production of 62 %, contrasting with 25 % for free curcumin (both formulations were significantly different from the positive control LPS). It seems that because curcumin was encapsulated in the NPs, its effect was attenuated by the particle, most probably because the curcumin is not readily available and needs to be released from the particle which, on the other turn, is not a fast process considering the duration of the assay (24 h). It was also tested the blank nanoparticles (Glu 250) and Glu Polymer in the same concentrations of GluCur 350 and it was not observed any inhibition in NO production (Figure 3.3 A). Therefore, these Glu 250 NPs, at the concentrations tested, were not able to inhibit the NO production. This also proved that the effect of inhibition previously observed was due to curcumin and not to the glucan polymer or the particle itself.

In relation to 100 nm Glu NPs we wanted to know if this effect of encapsulated curcumin was reproducible, however Glu 100 and GluCur 100 presented a higher toxicity in this cell line and because of that the range of concentrations that were tested was significantly lower compared with Glu 250 and GluCur 350 NPs. Consequently, the amount of curcumin encapsulated into these particles and placed in contact with cells was much lower when compared with the GluCur 350 NPs. The same amount of free curcumin was also tested in the same conditions to have a direct comparison of the effect of the curcumin encapsulated (vs. free curcumin). In this particular case, the free curcumin did not inhibit the NO production explained by the small concentration used. So, it was not expected that the curcumin encapsulated would produce an effect on NO production. However, when we tested the curcumin loaded glucan NPs (GluCur 100), surprisingly, we observed that at NPs concentration of 0.015 mg/mL, corresponding to 2 μM of curcumin, it was seen a decrease

in NO production (Figure 3.4 A). The same effect was also observed in its corresponding blank nanoparticles (Glu 100) (same NPs concentration). In both cases that difference was significantly different from the positive control LPS. Therefore, in the smaller particles, the inhibitory effect of NO was due to the particles theirselves and not to the curcumin. There are a few studies that showed the inhibition/ production of NO by beta-glucans, however some results differ in their findings. Xiaojuan Xu and co-workers studied a beta-glucan isolated from a mushroom that at the concentrations that we used for the smaller particles (particularly 0.015 mg/mL) showed a similar inhibition of NO production (Joo *et al.*, 2014). They claimed that this inhibition was due to the selective suppression of JNK1/2 and ERK1/2 in the MAPK signaling pathways. Despite everything, it is intriguing and should be studied in the future the fact that Glu 250 NPs did not cause any inhibition of NO production but Glu 100 NPs did (despite being different concentrations between themselves). Even adding the fact that in literature curdlan is known to stimulate nitric oxide generation (Ljungman, Leanderson e Tagesson, 1998). This can be justified because the potency of immunomodulating effects (in this case inhibition of NO) differs among β-glucans and purified polysaccharides depending on their branching complexity and also depending on the polymer molecular weight (Kim *et al.*, 2011). Applying this information to our case, it can be excluded the differences between the branching complexity or the molecular weight since the polymer used for the production of both NPs was the same but the size of the particles was different, so that should be the main reason for the observed differences. During the tests, controls like the solvent of NPs and the solvent of curcumin (DMSO 0.4 %) were also tested and it was shown that they did not inhibit the production of NO. Besides we also tested the NPs with LPS, together with medium but without the cells, and that did not interfere with the NO assay. In each experiment, after removing the supernatant for NO assay, we also did MTT assay to confirm that the chosen concentrations did not cause any reduction on cell viability (Figure 3.3 B and Figure 3.4 B). This is important because if the viability of the cells was affected that could interfere with the quantity of NO that cells produce and this could lead us to false results.

3.2.4 The Glu 250 and GluCur 350 NPs can stimulate the production of NO in RAW 264.7 cells

Previously, we found that the Glu 250 NPs and the Glu Polymer did not inhibit the production of NO and that raised the question of whether these formulations would have

the capacity to produce NO on their own. Because of that we repeated the experience in the same concentrations where we already knew that the formulations did not affect the cellular viability. The only difference for this assay was that we did not apply the stimulus LPS so we could actually see the effect of NPs on NO production. It was also really important to produce the LPS-free NPs by making the procedure in aseptic conditions and using pyrogen-free water.

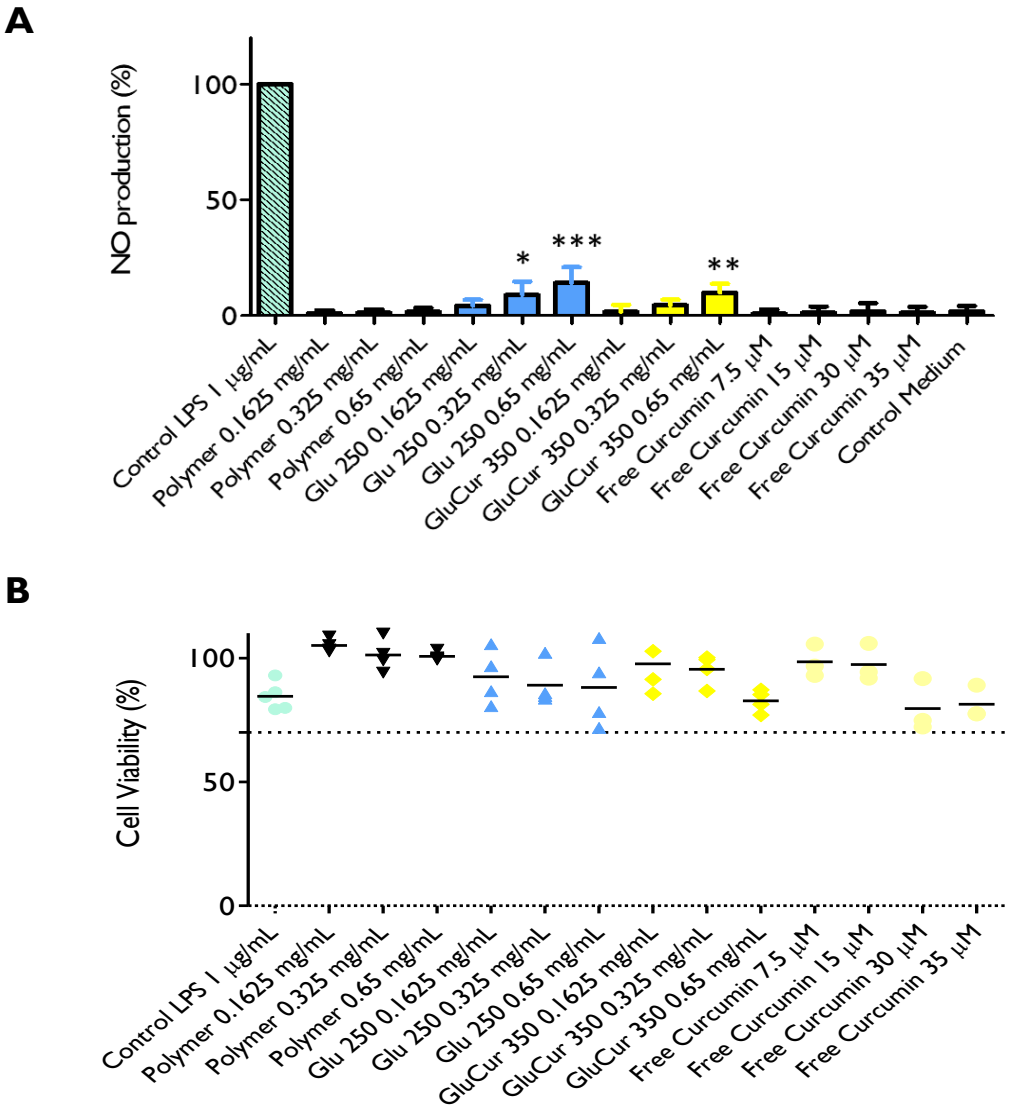

Figure 3.5 Stimulatory effect of Glu NPs on NO production by RAW 264.7 cells through Griess reaction after incubating the formulations LPS-free for 24 h: (A) Comparison of NO production (%) between the Glu 250, GluCur 350, free curcumin in concentrations corresponding to encapsulated curcumin in GluCur 350 and Glu Polymer, (B) Effect on the viability after incubation with Glu 250, GluCur 350, free curcumin and Glu Polymer in RAW 264.7 cells. All formulations were done in aseptic conditions and with pyrogen-free water. Both results are the mean ± SEM, n = 3 (three independent experiments, each in triplicate). * $p < 0.05$, significantly different from the control (DMEM without phenol red).

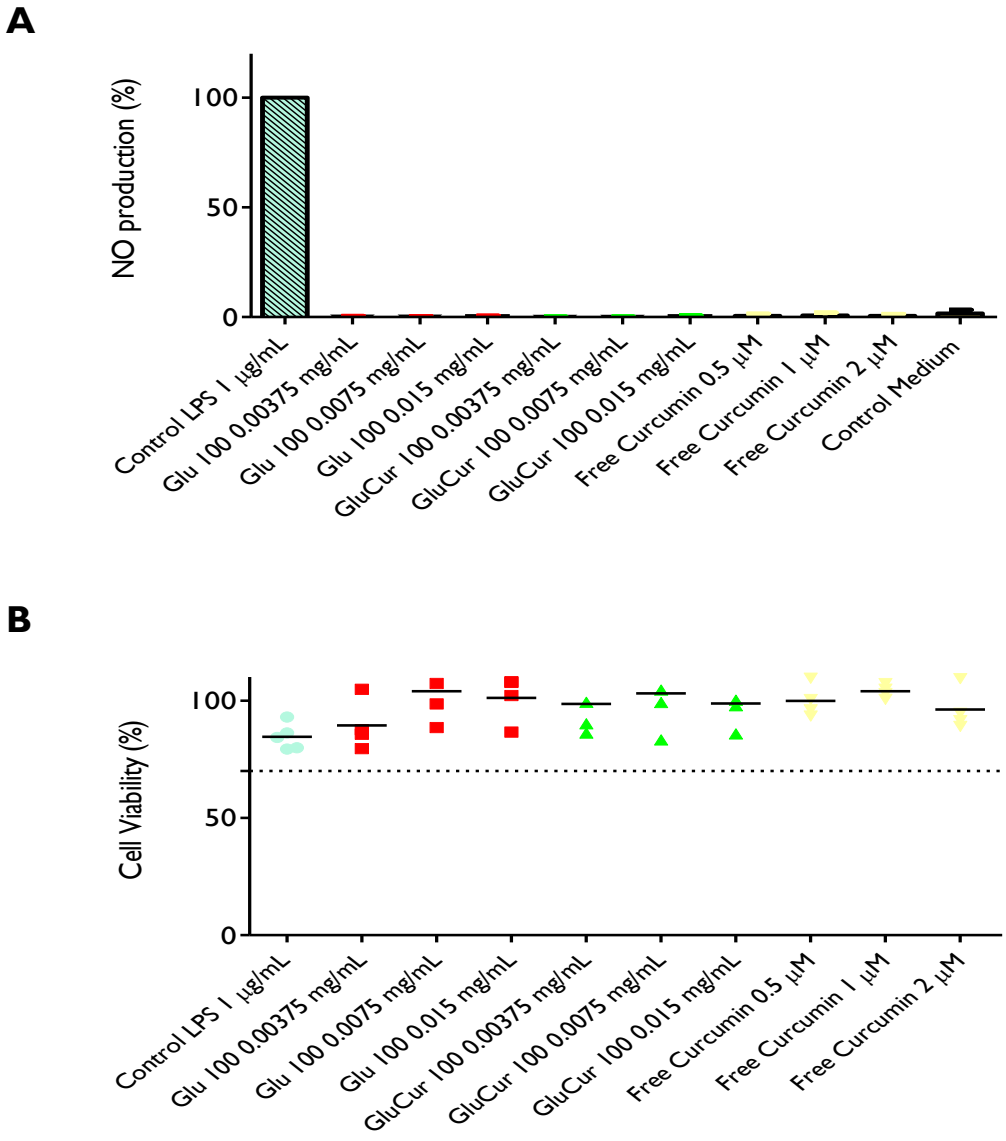

Figure 3.6 – Stimulatory effect of Glu NPs on NO production by RAW 264.7 cells through Griess reaction after incubating the formulations LPS-free for 24 h: (A) Comparison of NO production (%) between the Glu 100, GluCur 100 and free curcumin in concentrations corresponding to encapsulated curcumin in GluCur 100, (B) Effect on the viability after incubation with Glu 100, GluCur 100 and free curcumin in RAW 264.7 cells. All formulations were done in aseptic conditions and with pyrogen-free water. Both results are the mean ± SEM, n = 3 (three independent experiments, each in triplicate). * $p < 0.05$, significantly different from the control (DMEM without phenol red).

As we can observe in figure 3.5A and figure 3.6A, the curcumin, in the concentrations tested, did not stimulate the production of NO. The Glu polymer also did not have the capacity to stimulate the production of nitrites. However, Glu 250 and GluCur 350 NPs, both, unloaded and loaded with curcumin, appeared to exert a slightly stimulation in NO production, which was concentration dependent. For GluCur 350 NPs the difference was significant at concentration of 0.65 mg/mL, having a % of NO production of 10 % while for Glu 250 NPs

the difference was significant at concentrations of 0.325 mg/mL and 0.65 mg/mL with a % of NO production of 8 % and 15 %, respectively (Figure 3.5 A). It was equally tested the Glu 100 and GluCur 100 NPs but again, because we were limited due to their toxicity profile, the concentrations we used were a lot lower than for Glu 250 and GluCur 350 NPs. With that in mind, it was no surprise when it was not detected any NO stimulation by these particles (Glu 100 and GluCur 100 – Figure 3.6 A). It was always confirmed the controls like the solvent of NPs or the solvent of curcumin in which it was shown that they did not stimulate the production of NO. Besides, as a control, we also tested the NPs together with medium without the cells and that did not interfere with the NO assay. Also in these experiments, after removing the supernatant for NO assay, we also did MTT assay to confirm that the chosen concentrations did not cause any reduction on cell viability (Figure 3.5 B and Figure 3.6 B).

These results were in agreement with the literature since it is said that β-glucan recognition by dectin-1, which is one of the receptors present in the macrophage cell line, triggers effective immune response, producing inflammatory mediators being NO, one of them (Kim *et al.*, 2011). It is also said that polysaccharides like glucan stimulate in a concentration-dependent manner, the increase of the NO by peritoneum macrophages (Akramien *et al.*, 2007). However, the curdlan polymer itself did not stimulate the production of NO, in contrast to what some papers reported like Ljungman and co-workers in 1998 and Córdova and colleagues in 2018, despite that in the last case the β-glucans were isolated not from a bacterium but from a marine yeast (Ljungman, Leanderson e Tagesson, 1998) (Medina-Córdova *et al.*, 2018). So, different results can be explained by the different sources of glucans or by different concentrations used during the assays.

3.2.5 The Glu NPs induce TNF – α production in RAW 264.7 cells

After cellular recognition, our NPs can be seen as invaders and induce the production of inflammatory mediators like TNF-α. TNF-α is a pro-inflammatory cytokine released from macrophages or activated T cells in response to microbes or other agents. This cytokine plays a crucial role in many immune and inflammatory processes, such as proliferation, apoptosis and cell survival (Young *et al.*, 2001) (Cai *et al.*, 2017). Because of that, the purpose of our experiment was to evaluate the ability of glucan particles to induce the macrophages to produce TNF- α. For that, a mouse macrophage cell line was stimulated with different concentrations of LPS-free NPs during 24 h and the supernatants of the cultures were

analysed by ELISA to quantify the TNF-α production. Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs induced TNF-α production in RAW 264.7 cells. The range of concentrations tested was the same used for NO production assay.

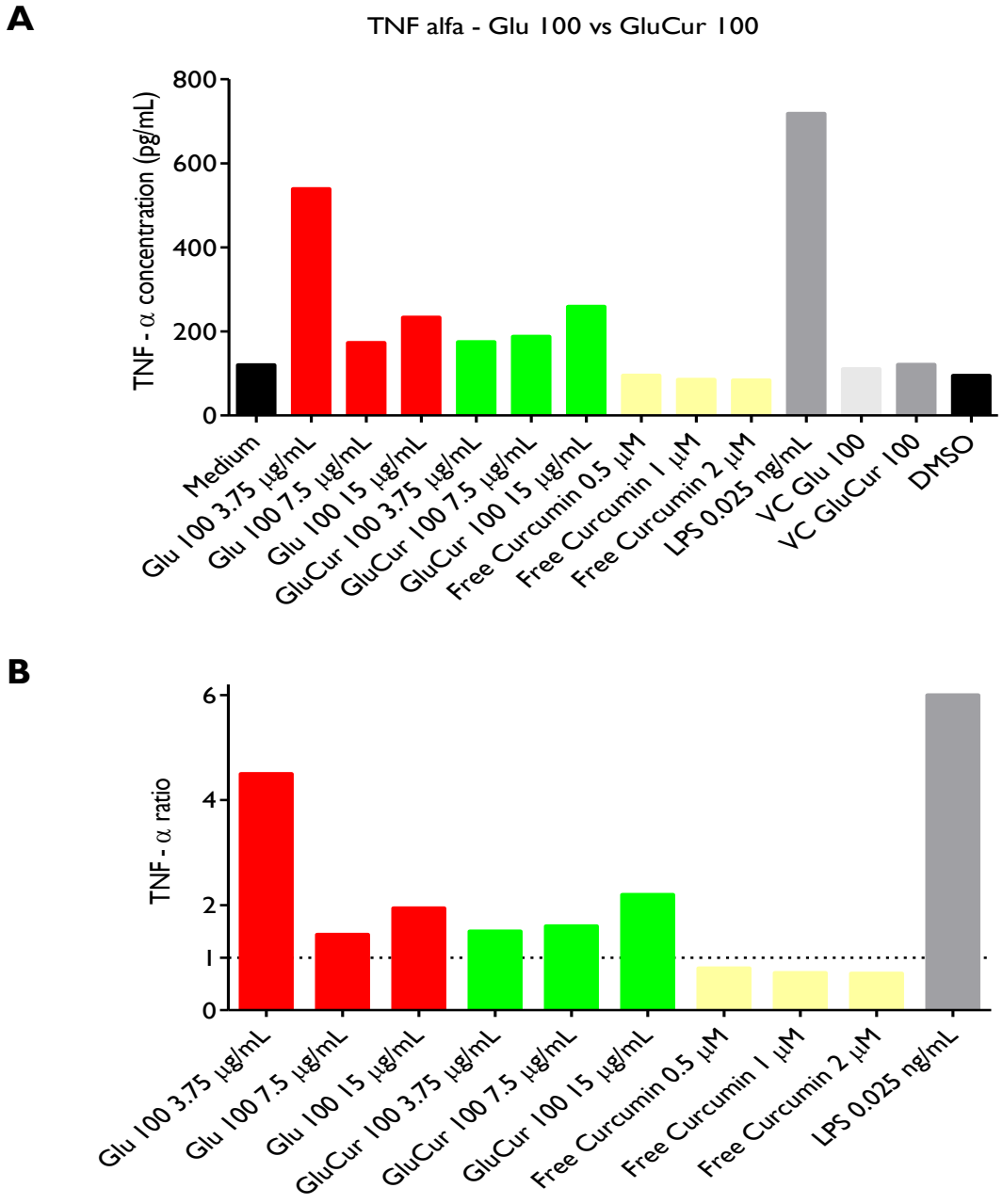

Figure 3.7 Effect of Glu 100 and GluCur 100 NPs on TNF-α production in mouse macrophage cell line (RAW 264.7): (A) The concentration of cytokine TNF-α represented in pg/mL, (B) Another representative way of showing the production of TNF-α: to obtain TNF-α ratio was needed to divide the concentration produced of TNF-α of each formulation by the concentration produced of TNF-α by the cells with just medium without any stimulus. This cytokine was measured using commercially available ELISA kits. LPS 0.025 ng/mL was used as a positive control and as expected induced elevated concentrations of this cytokine. Supernatants were harvested after 24 h of incubation with the formulations. Results represent the mean, n = 1 (one independent experiment).

As shown in figure 3.7, Glu 100 when used at the concentration of 3.75 μg/mL, caused a slightly (4 times more) increase in TNF-α production. However, that effect was not valid for GluCur 100 NPs or curcumin as showed in more detail in figure 3.7 B, where the ratio, particularly for curcumin was always below 1. Since it is described, that curcumin has an anti-inflammatory effect; we hypothesised and confirmed that, in particularly for the concentration of 3.75 μg/mL of GluCur 100 NPs, curcumin inhibited the TNF- α production observed for the same concentration of Glu 100 NPs. It is important to be investigated in future why was the lower concentration tested of the blank NPs (Glu 100) the one that showed the biggest increase in TNF-α production (ratio above 4), especially because this experiment was only done one time.

In this experiment, it was also observed the production of TNF-α by Glu 250 and GluCur 350 NPs and Glu Polymer. The production of the TNF- α for cells incubated with polymer was polymer concentration-dependent, a fact less obvious with the Glu 250 or GluCur 350 NPs. In fact, again, the particles with curcumin showed the lower TNF- α concentration that may be explained by the antagonist effect of curcumin. At the concentration of 650 μg/mL the Glu Polymer showed a TNF-α ratio of approximately 4, while the Glu 250 NPs at the same concentration had only a ratio of 2.7 (Figure 3.8 B). The GluCur 350 NPs had only a slightly production of TNF-α and the blank NPs (Glu 250) seem to stimulate more than the curcumin loaded glucan NPs. So, this could be the result of some inhibition of the production of TNF-α by curcumin. Finally, in the range of concentrations tested free curcumin (higher concentrations when compared with the curcumin tested in the experiment with the smaller particles) did not stimulate the production of TNF-α. It is important to refer that in both experiences, the controls were always done both the solvents of the NPs (VC NPs) and also DMSO 0.4 %, which was the solvent used for free curcumin and it was not detected production of TNF-α (Figure 3.7 A and 3.8 A).

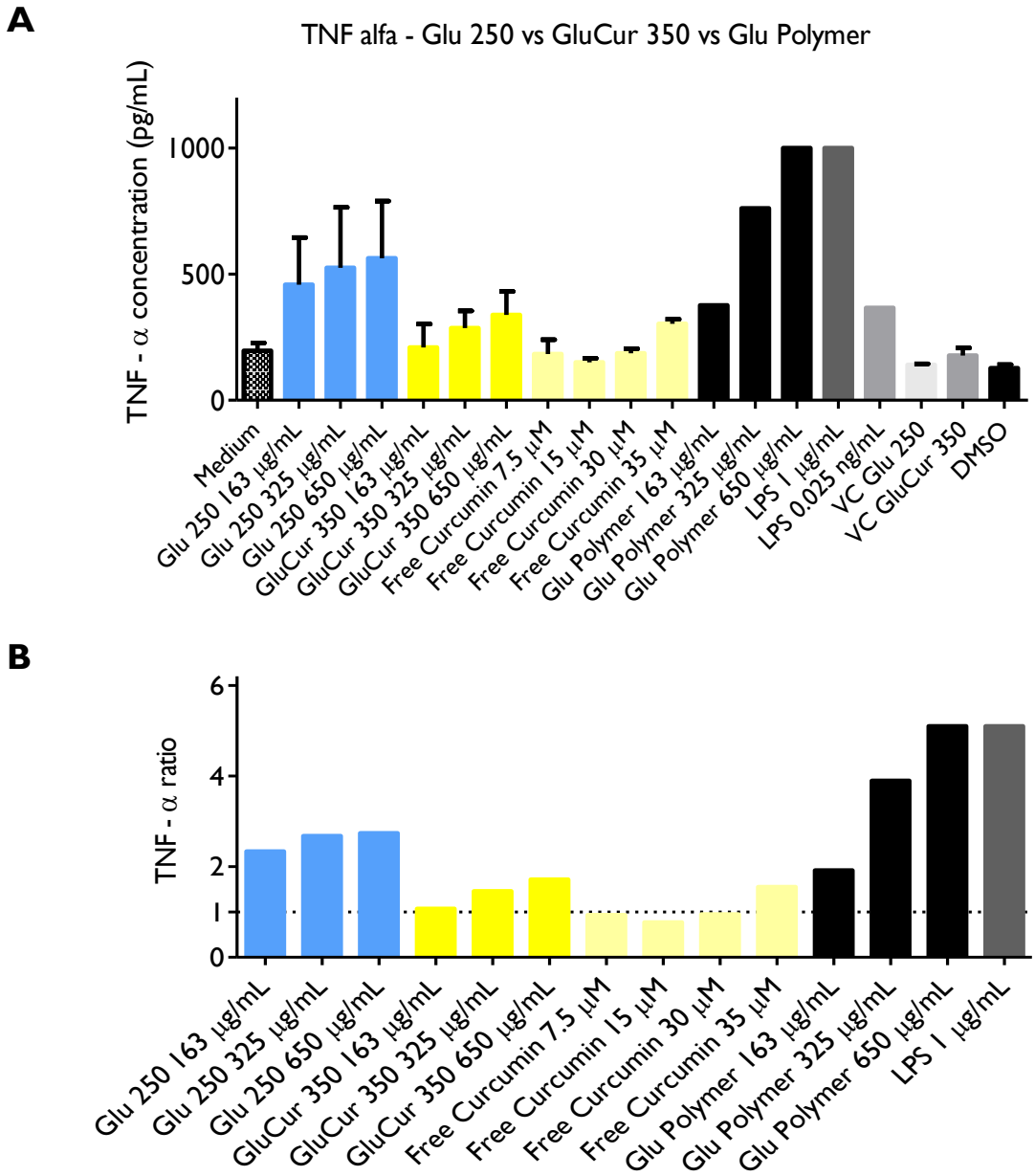

Figure 3.8 – Effect of Glu 250 and GluCur 350 NPs on TNF-α production in mouse macrophage cell line (RAW 264.7): (A) The concentration of cytokine TNF-α represented in pg/mL, (B) Another representative way of showing the production of TNF-α: to obtain TNF-α ratio was needed to divide the concentration produced of TNF-α of each formulation by the concentration produced of TNF-α by the cells with just medium without any stimulus. This cytokine was measured using commercially available ELISA kits. LPS 1 µg/mL was used as a positive control. Supernatants were harvested after 24 h of incubation with the formulations. Results represent the mean, n = 1 (one independent experiment).

All in considered, the results are in agreement with the literature, where studies like Hyung Kim showed the TNF-α production caused by β-glucans in this case in immature dendritic cells (Kim *et al.*, 2016). Moreover, another study was conducted by Sanja Stopinšek and co-workers, where they did a comparison between different kinds of β-glucans, being particulate curdlan one of them and they showed that, at the range of polymer concentrations tested, between 0 and 400 μg/mL, a concentration-dependent stimulation, with production of the TNF-α in human PBMCs, after 4 h of incubation, was observed (Stopinšek *et al.*, 2011). Eva Sonck and colleagues also revealed TNF-α production in PBMCs isolated from pigs after 24 h of incubation (Sonck *et al.*, 2010). It was also seen a dose dependent TNF-α production by curdlan on macrophages (Ljungman, Leanderson e Tagesson, 1998). In relation to curcumin, it is known to inhibit the synthesis of inflammatory molecules such as TNF-α, which is mainly responsible for the production of inflammatory products (Rai *et al.*, 2015). This inhibition was also showed by Jiao Wang and co-workers after incubating the curcumin for 24 h in RAW 264.7 cells (Wang *et al.*, 2015). Having that in mind, it was no surprise that curcumin did not stimulate the production of TNF-α.

3.2.6 The Glu 250 NPs induce IL –1β production in RAW 264.7 cells

Interleukin-1β (IL-1β) is also a potent pro-inflammatory cytokine that is crucial for host-defence responses to infection and injury (Lopez-Castejon e Brough, 2011). For that reason, the same study as for TNF-α was applied where a mouse macrophage cell line was stimulated with different concentrations of LPS-free NPs during 24 h. The Glu 100 and GluCur 100 NPs did not show the capacity to induce IL-1β production in RAW 264.7 cells, while the blank NPs, Glu 250 did. The range of concentrations tested was the same used for NO production assay (so that all concentrations were viable – above 70 %).

The 100 nm Glu NPs, both unloaded and loaded with curcumin (Glu 100 and GluCur 100) did not stimulate the IL-1β production (Figure 3.9). Actually, it seems that when added the NPs to the well it may exist some destabilization since the quantity of IL-1β detected was lower than in the control (medium). Free curcumin also did not induce the production of IL-1β, although it may appear at the concentration of 2 μM but that was not significantly different compared with the control.

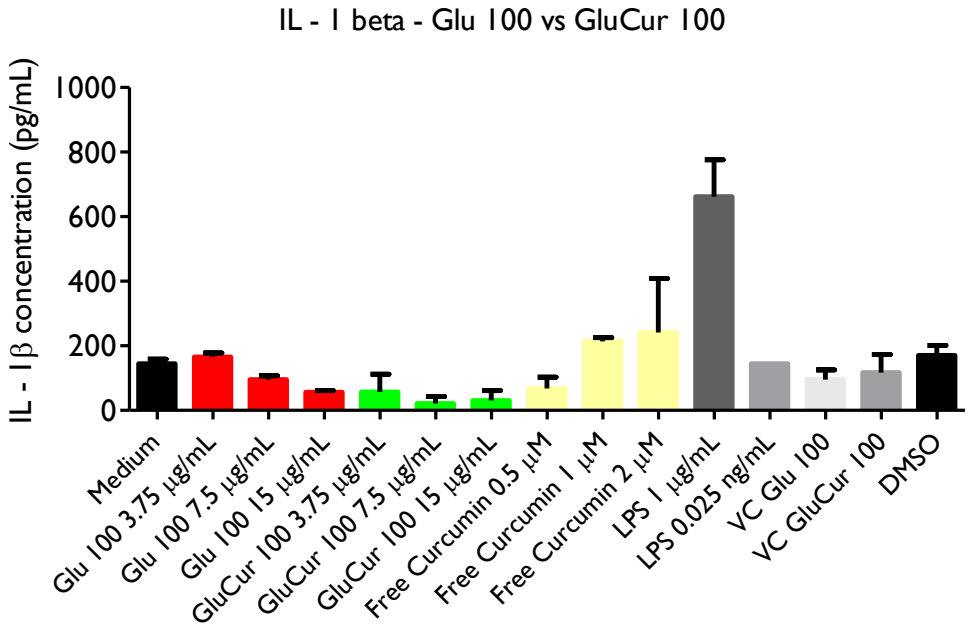

Figure 3.9 – Effect of Glu 100 and GluCur 100 NPs on IL-1β production in mouse macrophage cell line (RAW 264.7). The concentration of cytokine IL-1β represented in pg/mL. This cytokine was measured using commercially available ELISA kits. LPS 1 µg/mL was used as a positive control and as expected induced adequate concentrations of this cytokine. Supernatants were harvested after 24 h of incubation with the formulations. Results are the mean ± SEM, n = 3 (three independent experiments, each in triplicate).

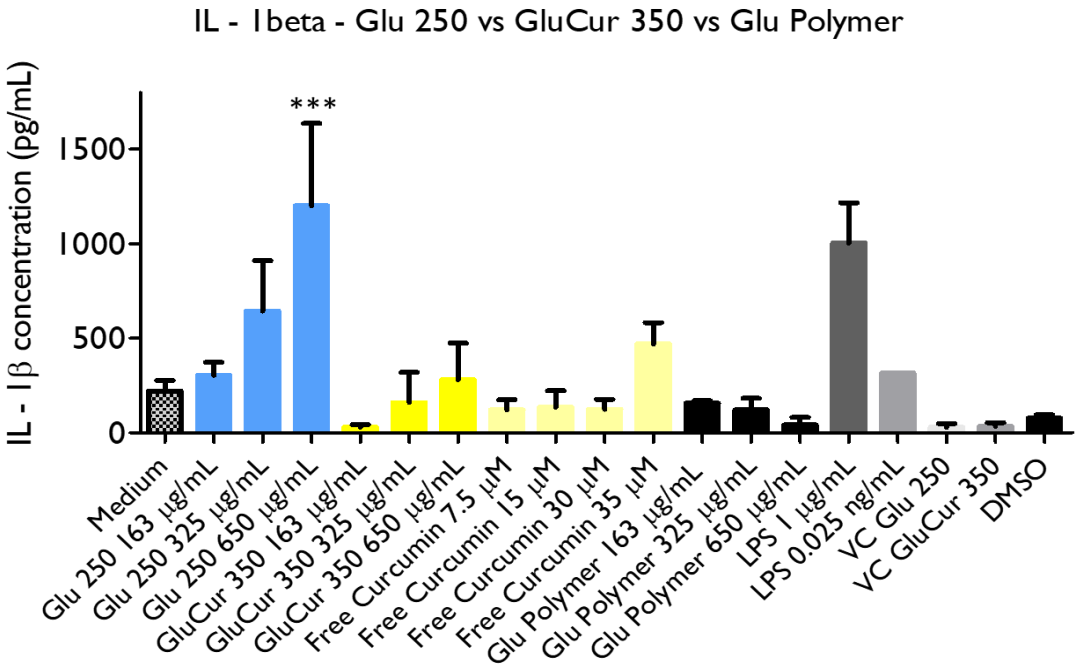

Figure 3.10 - Effect of Glu 250 and GluCur 350 NPs on IL-1β production in mouse macrophage cell line (RAW 264.7). The concentration of cytokine IL-1β represented in pg/mL. This cytokine was measured using commercially available ELISA kits. LPS 1 µg/mL was used as a positive control. Supernatants were harvested after 24 h of incubation with the formulations. Results are the mean ± SEM, n = 3 (three independent experiments, each in triplicate). * $p < 0.05$, significantly different from the control (DMEM without phenol red).

In this experiment, it was detected the production of IL-1β by Glu 250 NPs. A NPs concentration-dependent effect was observed, being the concentration of 650 μg/mL significantly different from the control (medium). Both, free curcumin and curcumin encapsulated Glu NPs (GluCur 350) did not show any production of IL-1β, when compared with the results observed for control (cells + medium). The fact that, blank NPs (Glu 250) showed induction of IL-1β and its corresponding with curcumin encapsulated did not, may be explained by some inhibition caused by curcumin as observed for the TNF- α. Glu Polymer (curdlan) in the range of concentrations tested also did not show IL-1β production. In both experiences, the controls of the solvent of the NPs (VC NPs) and DMSO 0.4 % were always done and did not present any production of IL-1β (Figure 3.9 and 3.10).

Series of studies were done proving the production of IL-1β by the polymer curdlan (Ljungman, Leanderson e Tagesson, 1998) (Sonck *et al.*, 2010) (Kankkunen *et al.*, 2018). Nevertheless, in our experiment that did not happen. One possible explanation could be as in Eva Sonck and co-workers where it was shown that lower concentrations of the particulate 1,3-β-glucan corresponded to a higher IL-1β production, while higher concentrations of the polymer (similar to concentrations that we used) corresponded to a lower stimulation of IL-1β production in human macrophages (Sonck *et al.*, 2010). As shown in figure 3.10, it can be actually seen some decrease in IL-1β production with the increase of quantity of the polymer. In literature, it is also possible to find reports were the inhibitory effect of curcumin on IL-1β production was observed (Jurenka, 2009) (Wang *et al.*, 2015) (Moon *et al.*, 2010). Some authors argue that the decrease expression of inflammatory cytokines like IL-1β and even TNF-α can result in growth inhibition of cancer cell lines by inhibition of NF-kB. This effect was confirmed by our results that showed the incapability of curcumin to induce cells to produce IL-1β and also the efficiency of this compound to inhibit the production of IL-1β in encapsulated curcumin Glu NPs (GluCur 350).

3.2.7 The Glu 100 and GluCur 100 NPs present a hemolytic activity higher than Glu 250 and GluCur 350 NPs

Hemolysis (destruction of red blood cells) *in vivo* can lead to anemia, jaundice and other pathological conditions, therefore the hemolytic potential of all intravenously administered pharmaceuticals must be evaluated (Dobrovolskaia *et al.*, 2009). In view of that, hemolysis is an immunotoxicological assay that should be performed to evaluate the biocompatibility of the nanoparticles with blood components. It is another way for us to assess the toxicity of

our particles. On the results showed in figures 3.11 and 3.12, it is possible to conclude that Glu 100 and GluCur 100 NPs had a superior hemolytic activity compared with Glu 250 and GluCur 350 NPs, where these last NPs turned out to be more biocompatible.

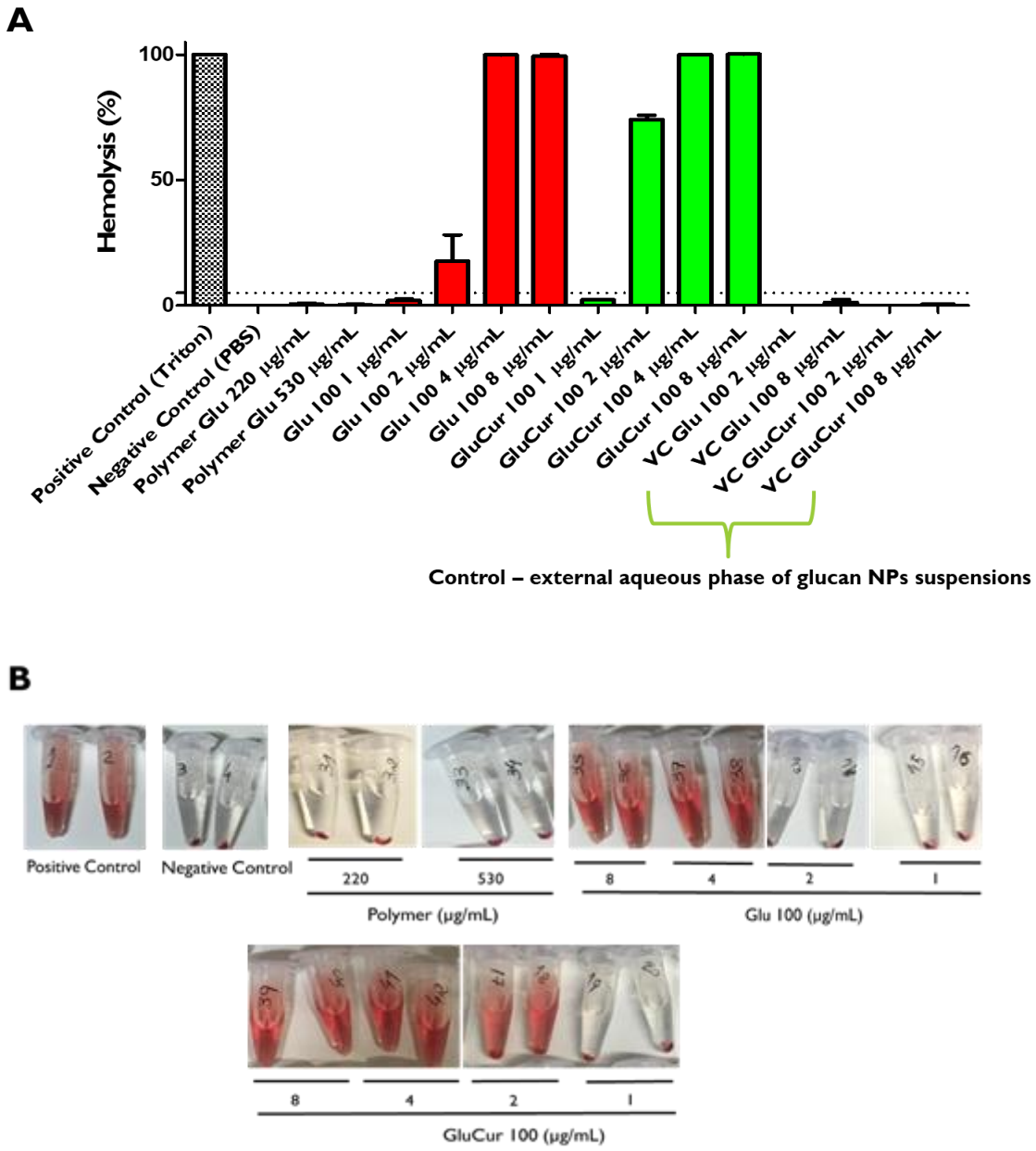

Figure 3.11 – (A) Hemolytic effect of different concentrations of glucan nanoparticles (Glu 100) and curcumin loaded glucan nanoparticles (GluCur 100) and just the glucan polymer in human whole blood (mean ± SEM, n = 3, three independent experiments, each in duplicate). Results are expressed in comparison with cells treated with Triton X-100 (positive control (PC) –100 % hemolysis) and with cells treated only with PBS (negative control (NC) – 0 % hemolysis). In vehicle control (VC), cells were incubated with the solvent of nanoparticles. (B) Visual evidence of the tubes containing diluted total blood after being incubated with different concentration of Glu NPs.

We started to evaluate the Glu 100 and GluCur 100 NPs and it was chosen concentrations taking into account the cytotoxicity assay in mouse macrophage cell line (RAW 264.7). It was observed that for concentrations above 4 μg/mL, the Glu 100 NPs caused almost 100 % hemolysis (Figure 3.11 A). Equally, the GluCur 100 NPs were also tested and in this case concentrations above 2 μg/mL already presented 75 % hemolysis, which lead us to conclude that particles with encapsulated curcumin are more toxic that its corresponding blank nanoparticles (Figure 3.11 A). Both, Glu 100 and GluCur 100 nm NPs showed concentration-dependent hemolysis and this can be supported by the visualisation of the eppendorfs containing the diluted total blood incubated with the different formulations (Figure 3.11 B). The control of the solvent of both NPs did not present hemolysis as expected (represented as VC followed by the type of nanoparticle in figure 3.11 A).

Through the cytotoxicity assay performed in RAW 264.7 cell line, we conclude that Glu 250 and GluCur 350 NPs presented a lower toxicity compared with the smaller nanoparticles and because of that we decided to test a broader range of concentrations for these specific NPs in this assay. In fact, at concentrations of 4 μg/mL and 8 μg/mL, both Glu 250 and GluCur 350 NPs did not show hemolytic activity in the human erythrocytes (Figure 3.12 A). The lysis of red blood cells was checked for concentrations above 100 μg/mL for curcumin loaded glucan NPs (GluCur 350) and for concentrations above 500 μg/mL for blank nanoparticles (Glu 250). Once again, in this case the NPs with encapsulated curcumin showed a higher toxicity. However, when free curcumin was tested in the same concentrations of the curcumin that was encapsulated in the particles, it did not cause by itself hemolysis (Figure 3.12 A). These results can be supported by the visualisation of the eppendorfs containing the diluted total blood incubated with the different formulations. It was also evaluated in this assay, the control of solvent of the NPs (represented as VC followed by the type of nanoparticle in figure 3.12 A) and DMSO 0.4 %, which was the solvent used for free curcumin and both did not present any hemolysis. In addition to these experiments, Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs were also incubated with just PBS (without blood) to assess the possible interference of the NPs with hemolysis assay and no interference was detected.

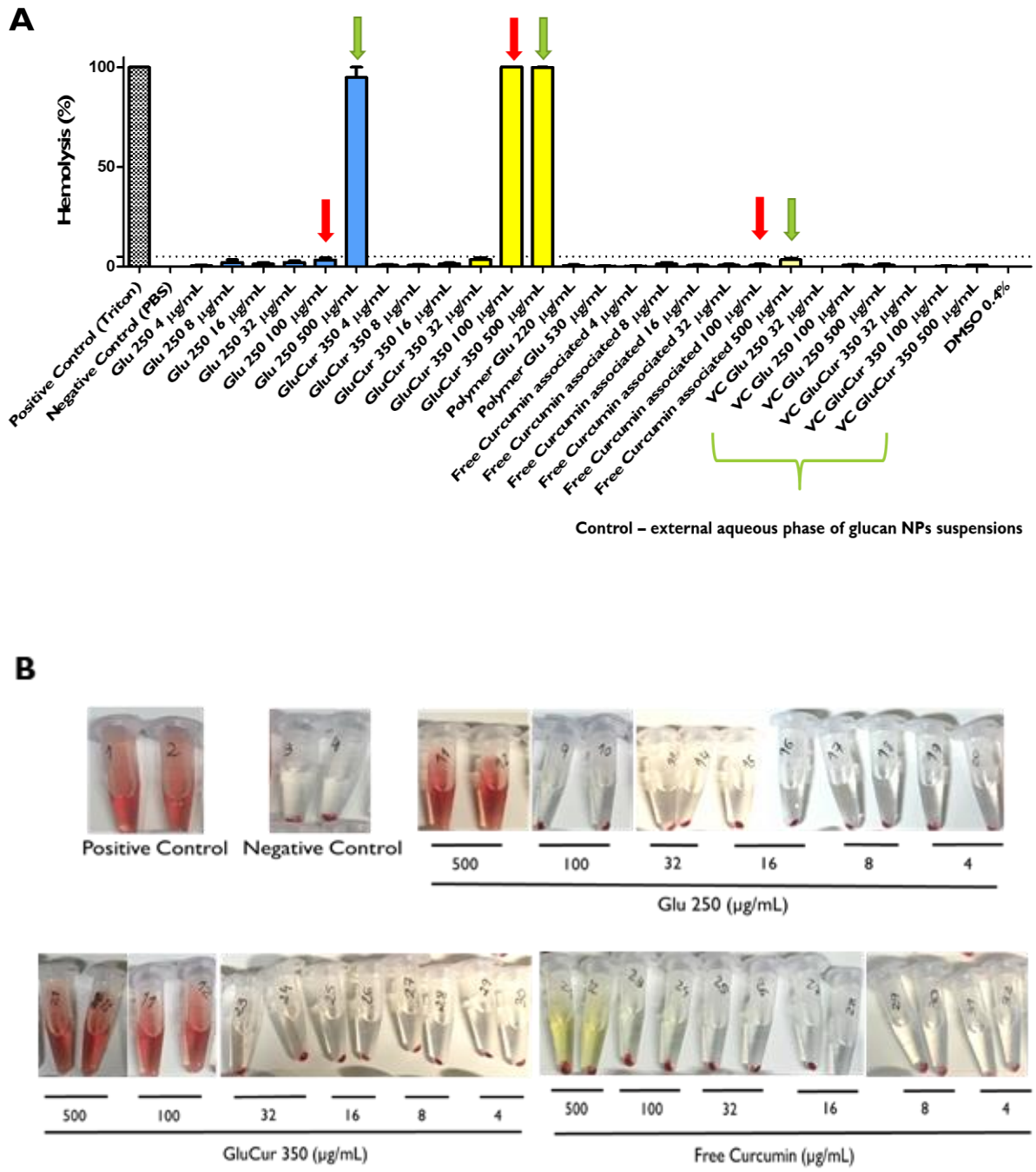

Figure 3.12 – (A) Hemolytic effect of different concentrations of glucan nanoparticles (Glu 250) and curcumin loaded glucan nanoparticles (GluCur 350) and its corresponding free curcumin in human whole blood (mean ± SEM, n = 3, three independent experiments, each in duplicate). Results were expressed in comparison with cells treated withTriton X-100 (positive control (PC) –100 % hemolysis) and with cells treated only with PBS (negative control (NC) –0 % hemolysis). In vehicle control (VC), cells were incubated with the solvent of nanoparticles. Another control tested was DMSO 0.4 %, which was the solvent used to dissolve curcumin. (B) Visual evidence of the tubes containing diluted total blood after being incubated with different concentration of Glu NPs.

Overall, there are not many studies about the cytotoxicity of Glu NPs in whole blood. Benjamin Lehtovaara and co-workers used curdlan as a shell to produce PLGA NPs and authors demonstrated that the use of a glucan polymer improved the biocompatibility of the

particles, by decreasing the hemolysis % in whole sheep blood erythrocytes (Lehtovaara, Verma e Gu, 2012). Our results showed this greater biocompatibility presented by the curdlan polymer. All concentrations tested for Glu Polymer were far below 5 % hemolysis, which is considered the critical safe hemolytic ratio for biomaterials accordingly to ASTM E2524-08 standard (Cit. por Choi *et al.*, 2011). Nevertheless, that biocompatibility was not shown in Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs. One of the possible reasons is that all of the particles were produced using a surfactant, tween 80 and this compound was reported to interact with the lipid bilayer of red blood cells, causing hemolysis (Pawar *et al.*, 2016). All particles were concentrated and washed using Vivaspin 20 centrifugal concentrator and because of that the residual % of tween should not be as significant. However, even as insignificant that % was it may still cause toxicity to red blood cells. Another fact that may add to this conclusion is that Glu 100 and GluCur 100 NPs presented a higher hemolytic activity than Glu 250 and GluCur 350 NPs and we know that one of the variables for the production of each one was that the smaller NPs were produced with 1 % of tween 80, while the others Glu NPs were produced with 0.1 % tween 80 (the GluCur 350 was 0.2 %). Therefore, if it was really the influence of tween 80 causing hemolysis in human whole blood erythrocytes, that effect would be more pronounced in Glu 100 and GluCur 100 NPs, as it was the case. Finally, curcumin is a natural compound that is known for being quite compatible with mammals and one study revealed this greater compatibility of curcumin with mammalian erythrocyte, where any hemolysis were caused as in our study (Deka *et al.*, 2016). Because of that, we can conclude that any toxicity did not come from curcumin.

3.2.8 Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs do not have an effect on extrinsic and intrinsic pathways of blood coagulation

The assessment of the traditional clinical coagulation assays, PT and APTT is also present on the biocompatibility ISO because it evaluates the possible toxicity of the NPs with blood components (ISO 10993-5, 2008). Prothrombin time (PT) studies the influence on the extrinsic and common coagulation pathways and its value reflects the time in fibrin clot formation after the addition of tissue thromboplastin, while activated partial thromboplastin time (APTT) studies the influence on the intrinsic and common coagulation pathways and its value reflects the time in fibrin clot formation after the addition of a standardised amount of cephalin (platelet substitute) and a factor XII activator (Kaolin) and calcium chloride (Liu *et*

al., 2015). PT is strongly affected by the type of thromboplastin used and because of that it was created an international sensitivity index (ISI) to normalize all the results independently of the thromboplastin used. This index is presented as the international normalized ratio (INR) and it is obtained by the ratio of the PT of the plasma in study and of the PT of the reference plasma. Its reference value usually ranges from 0.9 to 1.2 (Tavares *et al.*, 2012). From the analysis of the results showed in figure 3.13, it could be concluded that Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs did not present any effect in the coagulation cascade by not affecting PT as APTT.

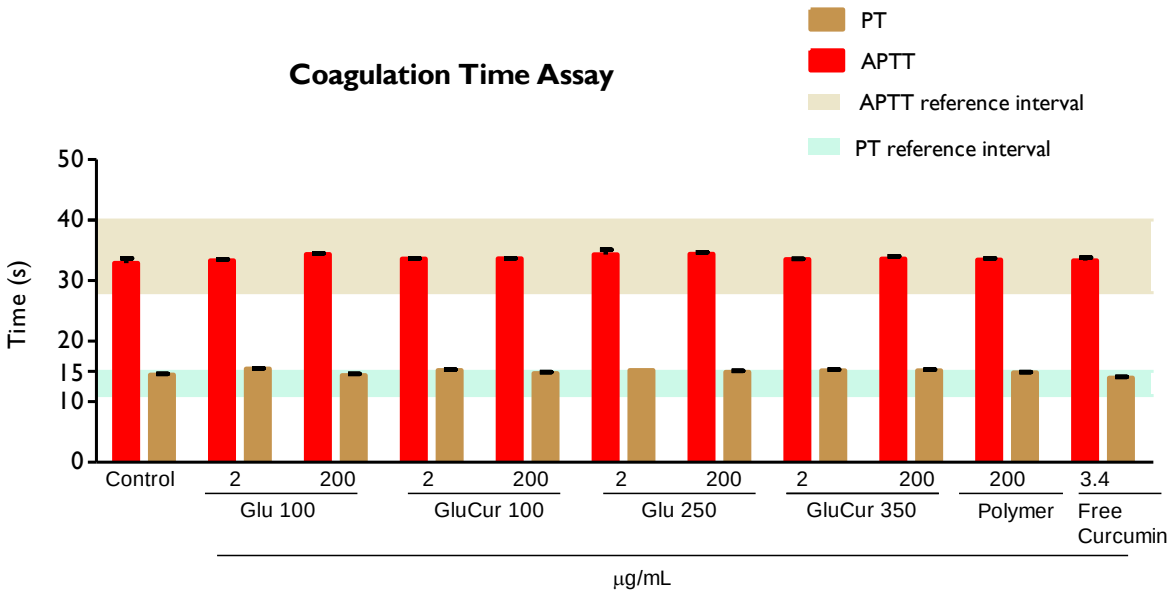

Figure 3.13 – Effect of Glu NPs on coagulation cascade. PT and APTT were measured by incubating platelet-poor plasma (PPP) with different concentrations of NPs (2 and 200 µg/mL) and also free curcumin for 30 min. The control used was PBS. The reference interval for PT and APTT is represented in the graph by light blue and pink respectively.

It was selected two concentrations for all NPs: 200 µg/mL because it was the maximum concentration that could be used and 2 µg/mL because it was where Glu 100 and GluCur 100 NPs did not cause hemolysis. Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs were in the normal range for PT and APTT, not having shown any distinctive effect between them and between control (Figure 3.13). The control used was PBS that showed a value of PT of 14.4 s and a value of APTT of 33 s. The INR was also within the normal range. These results may be explained because glucan more exactly curdlan is a neutral polymer and consequently it does not capture any coagulation factor of the cascade resulting in a normal mechanism. Besides, the control of the solvent of all types of NPs also did not interfere with

PT and APTT. It was concluded that in this study Glu NPs showed a greater biocompatibility with human blood cells.

In literature, a type of β-glucan isolated from the lichen *Parmotrema mantiqueirense* ranging between 0 to 1000 μg/mL, showed similar results, having no interference in both APTT and PT. Authors compared β-glucan that had been sulfated with its blank control β-glucan and they concluded that the presence of sulfated groups is an essential requirement for the anticoagulant activity, in other words to affect by increasing the coagulation times (Martinichen-Herrero *et al.*, 2005). Another study evaluated a mixture of β-glucan and poly(vinyl alcohol) (PVA), where the content of glucan varied from 10 % to 60 %. In the compound with the highest % of β-glucan, the APTT was within the normal range, showing a good blood compatibility (Huang e Yang, 2008). Nevertheless, our results showed that curcumin also did not affect the coagulation cascade, however these results are contradictory with the literature, where curcumin was shown to have a potent anticoagulant activity by prolonging APTT in a concentration dependent manner (Kim e Ku, 2011) (Pan *et al.*, 2007). Authors argue that it is the methoxy group in curcumin that positively regulates the anticoagulant function of curcumin (Kim e Ku, 2011). In our study at the concentration of 3.4 μg/mL (free curcumin), the APTT had a value of 33.3 s while in Dong-Chan Kim and colleagues APTT in a similar concentration had a value of 77.5 s.

3.2.9 At higher concentrations Glu 100 and GluCur 100 NPs as Glu 250 and GluCur 350 NPs induced the PBMCs proliferation

Cell proliferation is essential for research in many scientific fields, such as assessing cell health, determining genotoxicity and evaluating the impact of small molecules on cell cycle (Merckmillipore, 2015). In our case, we wanted to evaluate if Glu NPs could activate cells promoting their proliferation. It was chosen concentrations based on the studies done with macrophages and trying to choose those who did not present toxicity. Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs showed (Figure 3.14) proliferation in PBMCs at higher concentrations.

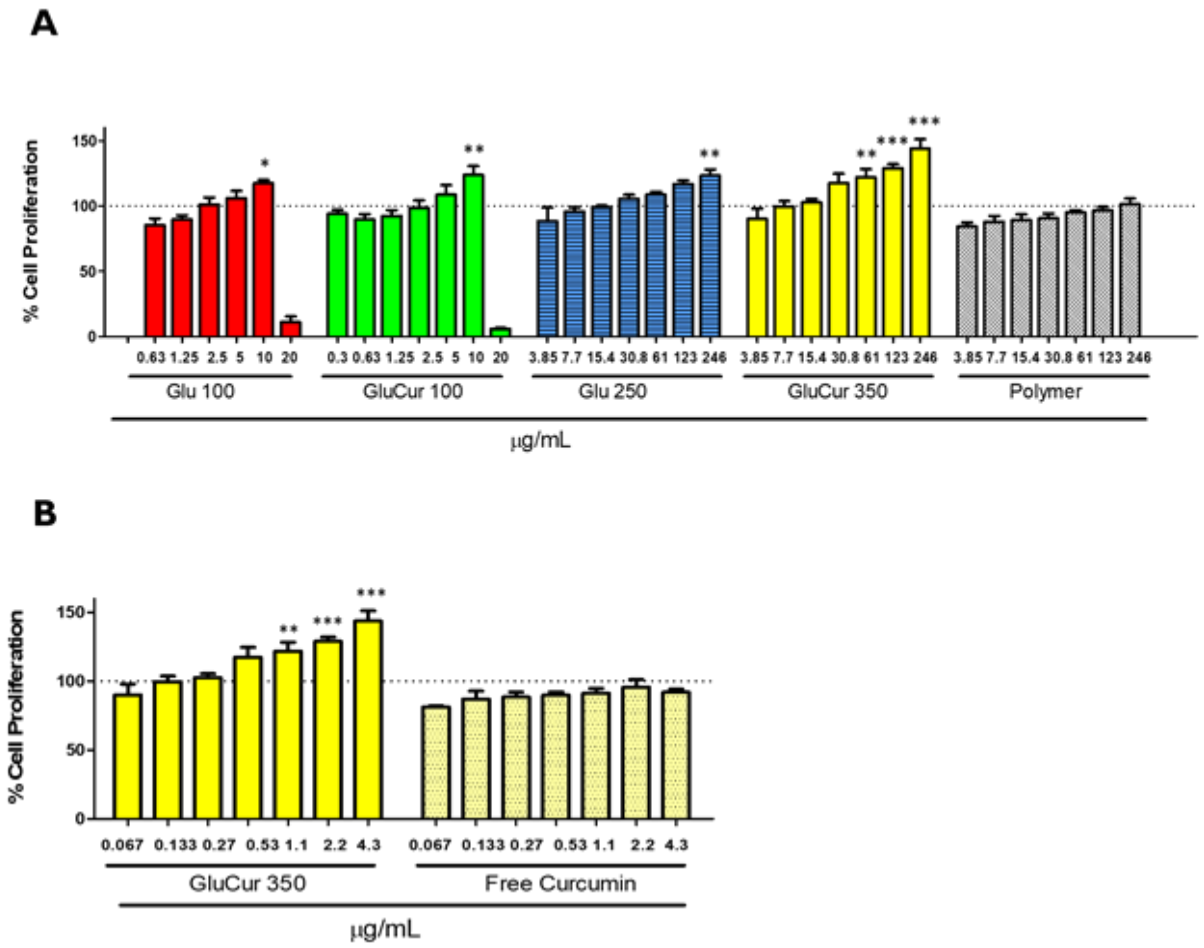

Figure 3.14 – Cell proliferation after treatment with Glu NPs and free curcumin: PBMCs were treated with different concentrations of (A) Glu 100, GluCur100, Glu 250, GluCur 350 NPs and Glu Polymer or (B) GluCur 350 and free curcumin in concentrations corresponding to the encapsulated curcumin for 72 h and then measured by MTT assay. RPMI 1640 was used as control (mean ± SEM, n = 4, four independent experiments, each in triplicate). * $p < 0.05$, significantly different from control.

Through MTT assay, we conclude that Glu Polymer did not induce proliferation (Figure 3.14 A). Contrary, in the Glu 250 NPs at the concentration of 246 μg/mL (the higher concentration tested), it was seen 120 % of cell viability, which was significantly different from the control (RPMI 1640). This was also observed for the curcumin loaded glucan NPs (GluCur 350) not only in the highest concentration (246 μg/mL) but also at concentrations of 61 and 123 μg/mL (all of these concentrations were significantly different from the control). In view of that, the GluCur 350 seem to have a greater capacity to stimulate proliferation in PBMCs than its corresponding blank NPs (Figure 3.14 A). With that in mind, our next step was to see if curcumin by itself also stimulate the proliferation of cells and for that it was tested free curcumin in the same concentrations of the corresponding

encapsulated curcumin in GluCur 350. In this interval of concentrations, free curcumin did not stimulate the cells to proliferate (Figure 3.14 B). In relation to Glu 100 and GluCur 100 NPs, the range of concentrations tested was inferior compared with Glu 250 and GluCur 350 NPs because it was discovered toxicity at the concentration of 20 μg/mL. That result was not surprising because when evaluated the toxicity in the macrophage cell line (RAW 264.4) the same differences between particles of different sizes was confirmed. At the concentration of 10 μg/mL, both Glu 100 and curcumin loaded glucan NPs GluCur 100 showed proliferation of PBMCs with a cell viability of 118 % and 124 %, respectively (Figure 3.14 A). In the smaller particles was not seen any difference between them in the proliferation profile. It was also evaluated the control of the solvent of NPs and DMSO 0.4 % and both controls did not present any cellular proliferation or cellular death (range of viability between 70 and 100 %). It is really important the use of controls in this assay, because by knowing that the solvent of the particles did not present cellular proliferation we can actually conclude that the particle itself was causing that effect (Figure 3.14 A).

These results are not in agreement with literature. Some studies demonstrated that β-glucan, curdlan was able to cause the proliferation of PBMCs and that proliferation was in a dose dependent manner (Chan *et al.*, 2007) (Sonck *et al.*, 2010). Despite that fact, we were not able to see that effect, induced by the polymer. However, the NPs could induce the proliferation of human PBMCs. Curcumin has been shown to have anti-proliferative properties (Hua *et al.*, 2010) and because of that our results of free curcumin to not induce the proliferation of PBMCs, was expected. Chie Amano and colleagues demonstrated the inhibitory activity of curcumin in a variety of cell lines as THP-1, mouse bone marrow-derived macrophages and L929 (Amano *et al.*, 2015). Michael Deters and co-workers showed the same inhibitory activity of proliferation in human PBMCs (Deters *et al.*, 2008).

Chapter 4

Concluding remarks and future perspectives

4. Concluding remarks and future perspectives

Nanotechnology is more and more a promising area that permits the study of structures to the nanoscale range and the development of nanomedicines which can be safer and more efficacious. The work presented here showed a production of a new polymeric delivery system as a nanocarrier for curcumin. The polymer chosen was glucan (curdlan) due to its immunomodulatory properties, being able to enhance both humoral and cellular immune responses and also because it has the ability to be vectorized to cells of the immune system, namely macrophages.

Curcumin, a natural product with medicinal properties presents a low bioavailability so the encapsulation of this compound into glucan nanoparticles may increase its therapeutic value.

One of our main goals was the development and characterization of curdlan delivery systems. For that, a simple method of nanoprecipitation was applied and it was obtained nanoparticles with different sizes (100 nm and around 300 nm). The next step was the incorporation of curcumin. It was possible to observe that all type of systems presented a good size, appropriate for drug delivery with a good polydispersity index. In addition, it was seen that the particles after suffering the process of concentration and washing, being dispersed in aqueous medium, maintained its size. In terms of stability, Glu 100, GluCur 100, Glu 250 and GluCur 350 NPs showed a great stability at the temperature of 4 °C for at least 42 days while in cell culture medium at 37 °C, Glu 100 and GluCur 100 NPs showed a tendency to aggregate over time but Glu 250 and GluCur 350 NPs continued stable.

Immunotoxicity tests were done in a macrophage cell line, RAW 264.7 and in human primary cells, PBMCs. Both type of cells represented cells of the immune system, being therefore suitable models. Besides it was also assessed the compatibility of these formulations with blood components through hemolysis and time coagulation assays. Surprisingly, it was observed that the cytotoxicity profile of the NPs was influenced by their different physicochemical characteristics, namely the size and composition (nanoparticles with or without curcumin). The smaller NPs presented a higher toxicity compared with Glu 250 and GluCur 350 NPs in both types of immune cells, being that toxicity associated with the particle and not with the incorporation of curcumin. The same cytotoxicity was also observed during the hemolysis assay. On the contrary, in the time coagulation assay all types of NPs showed a great compatibility by not altering the normal time of coagulation cascade.

As for the immunomodulation effects, it was performed 3 assays, the analysis of the inhibition or production of NO, the production or not of cytokines as TNF-α and IL-1β and

the proliferation of PBMCs when put in contact with the nanoparticle-based formulations. Overall bigger particles (without curcumin) showed a higher immunomodulatory effect since it produced NO, and the cytokines TNF-α and IL-1β and presented proliferation of PBMCs at the higher concentrations. Curcumin demonstrated a contrary effect of curdlan since this compound when encapsulated in the curdlan system altered their properties by inhibiting the production of NO and the excretion of the cytokines TNF-α and IL-1β. In relation to Glu 100 and GluCur 100 NPs, their effects were not so visible because these formulations were more toxic and to evaluate the immunomodulatory effects as the concentrations need to show viability above 70 %, the range of concentrations tested was a lot lower compared with Glu 250 and GluCur 350 NPs.

Overall, our results showed the production of curdlan NPs with great potential to encapsulate curcumin and to be applied as drug delivery systems in a near future. To design safer glucan-based nanomedicines it would be necessary to pay special attention to the size of the nanoparticles. Particles with a size around 100 nm would be very toxic. However, some aspects still remain to be considered as for example the mechanism of the internalization of the particles that would explain the effect of the size on the immunotoxicological parameters. It is known that the major receptor responsible for the uptake of 1,3-β-Glucans is dectin-1 but it would be interesting to confirm if this receptor is equally important for all the nanoparticles or if the size of glucan particles matters. In addition, a more extensive approach to immunomodulatory effects should be done *in vitro* as the determination of production of ROS or to evaluate the activation of complement, since it is essential to assess extensively the immunotoxicity of the formulations and their interaction with the immune system, prior to even considerer of going to *in vivo* studies.

Chapter 5

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Supplemental Information

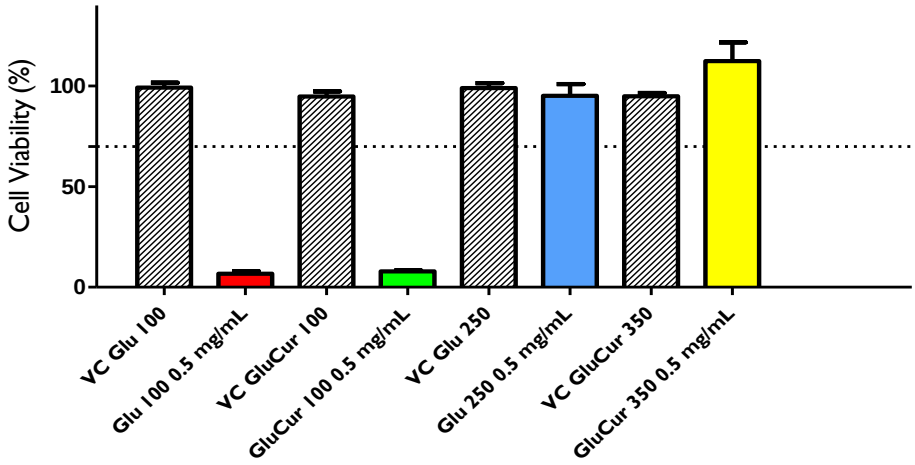

Figure 3.1.1 – Comparison of the cytotoxicity of the nanoparticles (Glu 100, GluCur 100, Glu 250 and GluCur 350 nm) at the concentration of 0.5 mg/mL and their corresponding vehicle controls through MTT assay after particles were in contact with macrophages for 24 h (mean ± SEM, n = 5, five independent experiments, each in triplicate).