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"Development of a *Toxoplasma gondii* membrane antigens delivery nanosystem for the achievement of an effective vaccine for Toxoplasmosis"

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Controlo de Qualidade. Trabalho realizado sob a orientação da Prof. Doutora Margarida Maria Coutinho Nogueira Marta Borges, e coorientação da Doutora Joana Fernandes da Silva Magalhães.

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Resumo

O *Toxoplasma gondii* é um dos parasitas zoonóticos mais bem-sucedidos do mundo, causando toxoplasmose. Esta doença tem importância clínica em indivíduos imuno-comprometidos em que a infeção pode levar a encefalite a até mesmo à morte e, em mulheres grávidas, em que a infeção primária durante a gravidez pode levar ao aborto ou à toxoplasmose congénita. A vacina é a estratégia ideal para controlar esta doença, uma vez que as terapias atuais diminuem o risco de transmissão congénita, mas não eliminam a forma parasitária latente. No momento atual não existe vacina disponível na clínica para a toxoplasmose. Estudos realizados demonstraram a necessidade da utilização de adjuvantes para desencadear uma resposta imune eficiente, induzindo somente uma proteção parcial contra a infeção. No entanto, os adjuvantes apresentam algumas desvantagens como o preço elevado e a necessidade de baixas temperaturas de armazenamento. É nesta área que a nanotecnologia tem proporcionado uma nova abordagem no desenvolvimento de vacinas superando as desvantagens associadas aos adjuvantes. Resultados anteriores demonstraram que a imunização intranasal de murganhos BALB\c com antigénios de membrana de *T. gondii* (TGMP) e o adjuvante consistindo em oligonucleótidos CpG (classe B; ODN; formulação original), induz uma proteção parcial contra a infeção. Este trabalho centra-se no desenvolvimento de um nanosistema de entrega de quitosano/carragenano contendo TGMP (NP-Chit:Carr-TGMP), que elimine a necessidade de utilização do adjuvante e que induza um efeito protetor efetivo. Dois métodos de cultura celular foram comparados para a obtenção de um número máximo de taquizoítos necessários para a extração de TGMP. Estudos *in vivo* utilizando o murganho foram realizados, para otimização das quantidades de TGMP e de ODN capazes de induzir um efeito protetor. A síntese de nanopartículas NP-Chit:Carr-TGMP foi otimizada e a eficiência de encapsulação de TGMP foi determinada. A citotoxicidade das NP-Chit:Carr-TGMP foi avaliada por ensaio de MTT utilizando uma linha celular de fibroblastos humanos. Os resultados obtidos permitiram determinar o processo de cultura celular ótimo permitindo obtenção de TGMP. Foi também possível aferir a quantidade ótima de TGMP e ODN (30 µg e 5 µg respetivamente) na formulação original capaz de induzir proteção parcial. Foi observado que a utilização de baixas quantidades de polímeros de Chit e Carr leva à obtenção de nanopartículas de ≈ 200 nm, e com uma eficiência de encapsulação de 60,5%. Os dados obtidos constituem uma base para prosseguir com a otimização das NP-Chit:Carr-TGMP, validação dos resultados de avaliação biológica *in vitro* e posteriormente à sua avaliação *in vivo*.

Toxoplasma gondii, Toxoplasmose, Nanopartículas, Quitosano, Carragenano

Abstract

Toxoplasma gondii is one of the world's most successful zoonotic parasites causing toxoplasmosis. This disease has clinical importance in immunocompromised individuals, where infection can lead to encephalitis and even death, and in pregnant women, where primary infection during pregnancy can lead to abortion or congenital toxoplasmosis. A vaccine is the ideal strategy to control this disease, as current therapies decrease the risk of congenital transmission, but do not eliminate the latent parasitic form. Currently, there is no available vaccine for toxoplasmosis. Studies to date have demonstrated the need for the use of adjuvants to trigger an efficient immune response, inducing only partial protection against infection. However, adjuvants have some disadvantages such as, high price and the need for low storage temperatures. Thus, nanotechnology has provided a new approach in vaccine development overcoming the disadvantages associated with adjuvants. Previous results by us have shown that intranasal immunization of BALB\c mice with *T. gondii* membrane antigens (TGMP) and the adjuvant consisting of CpG oligonucleotides (class B; ODN; original formulation), induces partial protection against infection. The goals of this work are the development of a chitosan/carrageenan delivery nanosystem containing TGMP (NP-Chit:Carr-TGMP), avoiding the need for adjuvant, and being able to induce an effective protective effect. Two cell culture methods were compared to obtain the maximum number of tachyzoites required for TGMP extraction. *In vivo* studies using the mice model were performed to optimise the amounts of TGMP and ODN able to induce a protective effect. The synthesis of NP-Chit:Carr-TGMP was optimized and the encapsulation efficiency (EE) of TGMP was determined. The cytotoxicity of NP-Chit:Carr-TGMP was evaluated by MTT assay using a human fibroblast cell line. The results obtained allowed the determination of the optimal cell culture conditions allowing the realization of this study. It was also possible to determine the optimum amount of TGMP and ODN (30 µg and 5 µg respectively) in the original formulation capable of inducing partial protection. It was observed that the use of low amounts of Chit and Carr polymers lead to nanoparticles with a size of ≈ 200 nm, with an EE of 60.5%. The results obtained constitute a basis to proceed with the optimization of NP-Chit:Carr-TGMP, validation of the *in vitro* biological data, and future *in vivo* experiments.

Toxoplama gondii, Toxoplasmosis, Nanoparticles, Chitosan, Carrageenan

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
Alum	Potassium aluminium sulphate
AMA1	Apical membrane antigen 1
APCs	Antigen-presenting cells
BSA	Bovine serum albumin
Carr	Carrageenan
Chit	Chitosan
CME	Clathrin-mediated andocytosis
CTLs	Cytotoxic T lymphocytes
CvME	Caveolin-mediated endocytosis
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EE	Encapsulation efficiency
FBS	Fetal bovine sérum
FDA	Food and drug administration
GRAs	Dense granule proteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
ICBAS	Institute of Biomedical Sciences Abel Salazar
Ig	Immunoglobulin
IL	Interleukin
IMC	Inner membrane complex
IN	Intranasal
INF-γ	Interferon-gamma
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MICs	Microneme proteins
MIP-1α	Macrophage inflammatory protein-1α

NALT	Nasal associated lymphoid tissue
NGAL	Neutrophil gelatinase-associated lipocalin
NKs	Natural killer cells
NLC	Nanostructured lipid carriers
NO	Nitric oxide
NP-Chit:Carr-TGMP	Chitosan/carrageenan delivery nanosystem containing TGMP
NPs	Nanoparticles
NTPase II	Nucleoside-triphosphatase II
PAMPs	Pathogen-associated patterns
PDI	Polydispersion index
PEC	Peritoneal exudate cells
PEG	Polyethylene glycol
PHPMA	N-(2-hydroxypropyl)-methacrylamide copolymer
PLA	Poly (lactic acid)
PLGA	Poly (lactic-co-glycolic acid)
PVP	Polyvinyl pyrrolidone
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROPs	Rhoptry-derived proteins
SAGs	Surface antigens
SD	Standard deviation
SEM	Standard error mean
SLN	Solid lipid nanoparticles
TE	Total extract
TGF-β	Transforming growth factor beta
TGMP	*Toxoplasma gondii* membrane proteins
T. gondii	*Toxoplasma gondii*
Th	T helper cells
TNF	Tumor necrosis factor

TNF-α	Tumor necrosis factor-alpha
VLPs	Virus-like particles

Chapter I – Introduction

1. *Toxoplasma gondii* overview

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan of the Apicomplexa phylum, discovered 100 years ago by scientists in North Africa and Brasil. Afterward, it's been found around the world infecting approximately one-third of the population able to infect all warm-blooded animals, including humans (1).

T. gondii life cycle comprises sexual reproduction in the definitive host (felines) and asexual reproduction in all the intermediate hosts, and it may present itself in various parasite forms: tachyzoite, bradyzoite, schizont, gametocyte and oocyst (2). However, only three of these forms are infective: tachyzoite, bradyzoite and the oocyst (with sporozoites). Once the definitive host ingests foods contaminated with one of these forms, the viable parasites invade the gut and differentiate into gametocytes allowing sexual reproduction and thereafter the development of oocysts. The immature oocysts are shed from the feline faeces sporulate in the environment becoming infective (3). All the warm-blooded intermediate hosts can be later infected by the ingestion of these oocysts, via contaminated water or meat, by congenital transmission, organ transplantation or blood transfusion, contaminated with tachyzoites (4, 5); (Figure 1).

The asexual replication begins in a process known as endodyogeny, when the parasites replicate their haploid genome and divide into two cells (tachyzoites), with a multiplication high rate during the acute phase of infection. Under host immune system pressure, tachyzoite converts to a slow replicating form within tissue cysts, the bradyzoite. This parasite form, seems to escape the immune response establishing a persistent infection (6). The brain is usually the main organ for encystment, but it can also occur in the lungs, liver and kidneys (7).

In immunocompetent individuals, *T. gondii* infection is usually asymptomatic, whereas in immunocompromised individuals, such as cancer patients, human immunodeficiency virus (HIV)-seropositive persons, or transplanted individuals under immunosuppressive treatment, the infection can cause serious health issues that can lead to encephalitis, pneumonia and even to dead (2, 8).

Although for healthy individuals this infection does not have great consequences, in pregnant women vertical transmission may occur, bringing complications such as abortion or congenital toxoplasmosis. The latter may lead to mental retardation, chorioretinitis, hydrocephalus or ocular disease of children who survived the prenatal infection (4). *T.*

gondii infection has been also associated with mental illnesses such as schizophrenia and bipolar disorder later in life (9, 10).

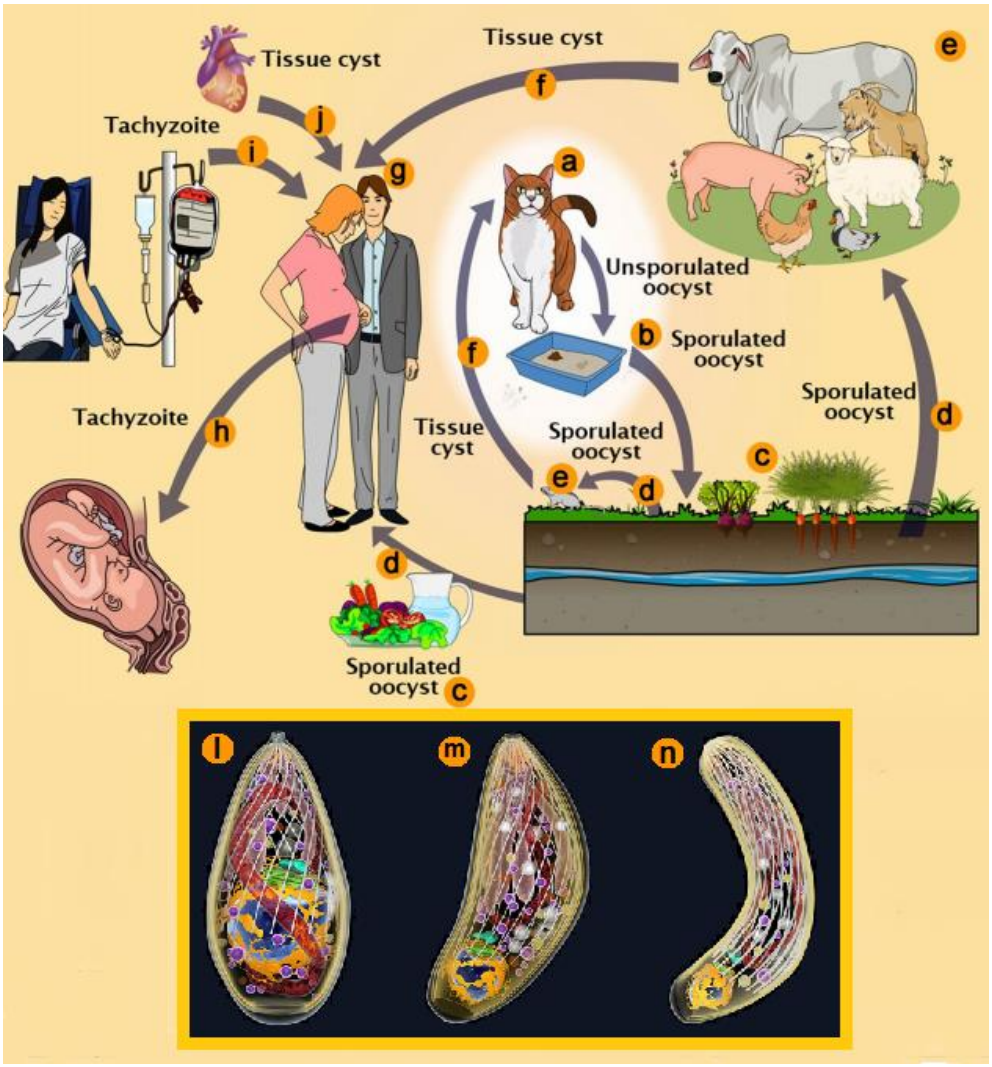


Figure 1. *T. gondii* infectious forms and transmission pathways. Adapted from (11).
(a) The definitive host (felines); (b) Unsporulated oocysts released in the faeces of the cat; (c) Sporulated oocysts present in vegetables; (d) Ingestion of oocysts present in contaminated water or raw vegetables by intermediate hosts; (e) Intermediate host (rodent); (f) Tissue cyst infection by ingestion of infected rodent; (g) Intermediate host (Human); (h) Human vertical transmission; (i) Blood transfusion transmission; (j) Organ transplant transmission; (l) Tachyzoite; (m) Bradyzoite and (n) Sporozoite.

1.1. Epidemiology

T. gondii infection is one of the most common parasites affecting about a third of the population worldwide and is the third most common cause of fatal foodborne illnesses (12, 13).

In Northern Europe, Southeast Asia, North America and Sahelian countries of Africa, low seroprevalences have been reported (10-30%). Central and Southern Europe have a moderate prevalence of 30-50% and high seroprevalences have been reported in Latin America and tropical African countries (14).

The incidence of toxoplasmosis varies widely between countries and is affected by several factors, such as climatic conditions, which affect the survival of oocyst in the environment. This parasite form prefers warm and humid climates found in tropical countries, however, it can also survive in arid and cold countries. The low economic conditions have also a determinant role since the incidence of this infection is higher in developing countries compared to the developed ones (12, 14). However, the moderate prevalence's in European countries can be explained by dietary habits such as the preference for raw or undercooked meat (15).

Despite its worldwide distribution and based on data from Europe and the United States isolates, this parasite was described as having low genetic diversity and grouped in three major genotypes, types I, II and III (14). Type I genotype is associated with virulence in mice and humans, being reported in Acquired immunodeficiency syndrome (AIDS) patients, and in individuals exhibiting congenital toxoplasmosis or ocular toxoplasmosis. Type II is less virulent in mice, and is the most predominant in humans (mostly in Europe and North America), causing chronic infections and, reported in patients with congenital disease or AIDS. Lastly, although the type III genotype is not virulent for mice and is less virulent for humans is virulent and most frequent in other warm-blooded animals (16). However, nowadays a larger genetic diversity in isolates from South Africa, America and Asia that do not fit in the three major lineages has been found (except type III). These new genotypes lead to the description of 9 successful new haplogroups, making a total of 12 (14).

In Portugal, there is limited information on toxoplasmosis (17). However, a study carried out in 2003 reported that seroprevalence in the Portuguese population ranged from 35% in the south to 70% in the north, whereas for the woman of childbearing age it ranged from 30% to 60%. Another study indicated a seroprevalence of 21,9% in pregnant women. There were no data on the seroprevalence in newborns, although 11 cases per 10000 live births, with 124 children becoming congenitally infected each year has also been reported (17-19).

1.2. Host cell invasion mechanisms

T. gondii cell invasion occurs through a motility process called 'gliding', which consists of spiral movements of the parasite and makes it capable of invading any nucleated cell since this parasite does not need a specific host cell receptor for invasion (20).

The first contact of the parasite with the host cell it's through their surface antigens (SAGs) that recognise different receptors of different cell types such as proteoglycans, heparan sulphate and laminin (21). After the initial recognition, the parasite secretes the microneme proteins (MICs), such as MIC2, and then occurs the formation of the moving junction in cooperation with the rhoptry-derived proteins (ROPs), that allows the penetration through the host's plasma membrane (14, 22).

The parasite then drags the host membrane, being surrounded by the host membrane, establishing the parasitophorous vacuole (where replicates asexually). The ROPs and the dense granule proteins (GRAs) are both involved in the formation of a tubular network, allowing the connection between the parasite and the host cell cytoplasm (Figure 2); (20).

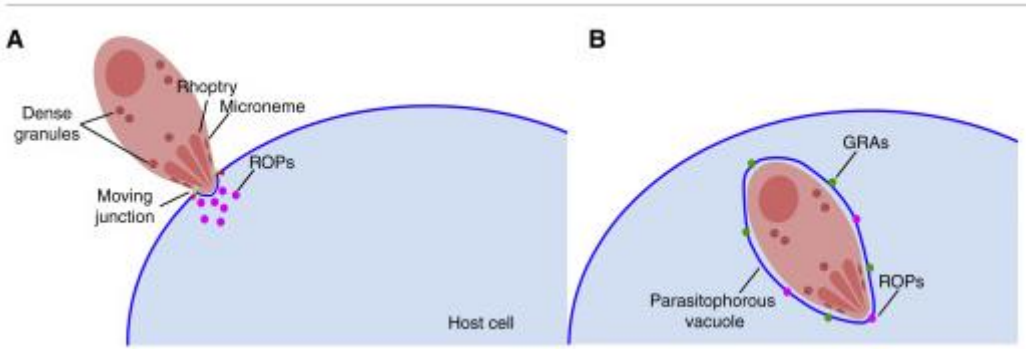


Figure 2. Host cell invasion by *T. gondii*. (A) Proteins secreted from the micronemes and rhoptries form the moving junction allowing the parasite to enter the cell creating the parasitophorous vacuole. (B) ROPs and GRAs allow the connection between the parasite and the host cell cytoplasm.

Studies carried out using *T. gondii* infected cells have shown that during parasite multiplication and the development of the parasitophorous vacuole there are changes in the distribution of cytoskeleton elements of the host cell. Moreover, interference in several mechanisms such as the regulation of glycolysis, lipids and cholesterol metabolism, cell cycle regulation and apoptosis to ensure their own survival has been reported (23).

1.3. *T. gondii* immune responses

T. gondii is most of the time, orally acquired. After the ingestion is released in the small intestine where the epithelial cells constitute the first line of defence against *T. gondii* (24). Nevertheless, the parasite can survive in various cells types and organs taking advantage of the dissemination of infected immune cells, such as neutrophils, dendritic cells (DCs), and natural killer cells (NKs), through blood circulation to target tissues (25).

T. gondii infection induces a strong Th1 immune response which is dependent on the interleukin (IL)-12 and interferon-γ (INF-γ) production. The early infected DCs, macrophages and neutrophils stimulate the synthesis of IL-12 and induce NKs and T lymphocytes to synthesize INF- γ, responsible for the control of the acute and chronic stages of infection (12).

T. gondii immune response comprises both innate and adaptive immune responses, whereas DCs play a key role in the starting of innate and adaptive immune responses (12). Regarding the innate immune response, neutrophils are the first phagocytic cells recruited to the local of infection, able to phagocyte and kill pathogens by the release of anti-microbial compounds, like calprotectin, lactoferrin, neutrophil gelatinase-associated lipocalin (NGAL), hCAP-18, and lysozymes (26), and the production of reactive oxygen intermediates (ROI) and nitric oxide (NO) (27, 28). Neutrophils secrete pro-inflammatory chemokines and cytokines acting on the Th1 cell surface receptors, such as IL-8, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and tumor necrosis factor-alpha (TNF-α), enabling neutrophils to support the immune response by attracting to the infection site and stimulating other Th1 immune cells (24, 27). Macrophage inflammatory proteins also attract to the site of infection monocytes, macrophages and dendritic cells (27).

The depletion of neutrophils, during early infection, results towards a Th2 response, since the decreased levels of IFN-γ, IL-12 and TNF-α lead to a weaker Th1 response, increasing susceptibility. However, the removal of neutrophils at later stages of infection has no effect on the susceptibility to infection, highlighting their important role in the immune initial response (29). The elimination of the parasites by neutrophils is dependent on IL-17 signalling, the main cytokine responsible for the development and recruitment of neutrophils (30).

DCs are the earliest antigen-presenting cell at the site of infection, and their maturation and activation in reaction to infection is very important for the initiation of innate immunity as well as the development of adaptive immunity. DCs maturation can be measured by up-regulation of the major histocompatibility complex (MHC) expression that leads to naïve T cells activation, proliferation and differentiation, and inflammatory cytokines production (31). During infection, DCs migrate to the site of infection in response to chemokines produced by neutrophils and macrophages. DCs are also able to phagocyte parasites and thereafter migrate to lymphoid organs, such as lymph nodes and spleen, where antigen-presentation occurs, driving the polarization of the Th response towards Th1, for instance, through the production of IL-12 (32, 33). A study has demonstrated that DCs depletion suppress the production of IL-12 and increment mice vulnerability to acute infection since the DCs are the major producers of this IL (34).

Macrophages are also essential for the prevention of the proliferation of microorganisms, exerting various functions, such as cytokines production, such as IL-12, antigen presentation to T cells, phagocytosis, phagolysosomal degradation, ROI and NO production (35, 36). IL-12 is very important for the control of *T. gondii* infection since activates natural killer and T cells to produce INF-γ, which induces the classical activation of macrophages, and later synergically acting with TNF-α. Activated macrophages present in the infection site promote parasite elimination and avoid intracellular replication by the release of lysosomal enzymes, ROI, NO and by inducing mechanisms of nutrient deprivation (35, 37). Immunopathology during infection may result from the overproduction of NO and inflammatory cytokines, which need to be closely regulated. Thus, signals from macrophages and the surrounding cells reduce macrophage classical activation, such as anti-inflammatory cytokines, like IL-10 and transforming growth factor-beta (TGF-β) (37).

NKs are also very important in early resistance to *T. gondii*, since they recognise and kill infected cells. They are the principal source of IFN-γ early in infection and therefore induce the classical activation of macrophages (38).

$CD4^+$ and $CD8^+$ T cells provide host defence against *T. gondii* through the production of IFN-γ, TNF-α, IL-6 and IL-1. $CD8^+$ T cells are effector lymphocytes against the parasite, while $CD4^+$ T cells regulate the immune responses (24).

$CD8^+$ T cells are an important mediator of the adaptive immune system, and the most important T lymphocyte population required in the control of chronic infection and prevention of *T. gondii* infection reactivation. The mechanisms of this cell population against parasitic infections include cytokines production, predominantly IFN-γ, which stimulated the IFN-γ dependent mechanisms in a wide variety of cells crucial in both acute and chronic stages of infection. $CD8^+$ T cells also produce IL-17 and IL-27, related to the down-regulation of the inflammatory response to *T. gondii* (12). *T. gondii* stimulates the production of $CD8^+$ cytotoxic T lymphocytes (CTLs) that have the ability to lyse infected cells by apoptosis (39).

With regard to humoral immune response, the first isotype class of antibodies produced after initial infection are immunoglobulin (Ig) M, activating the complement system, enabling the agglutination and cytotoxic activity against infected cells, whereas parasite's surface proteins are the main targets (40). The second class of antibodies to be produced is IgG. These immunoglobulins enable antibody-dependent cytotoxicity and play an important role in the protection of the foetus in congenital toxoplasmosis since they can cross the placenta. The IgA comprises two isotypes, IgA1 and IgA2 found predominantly in the serum and digestive tract, respectively (41, 42). There are few studies done regarding IgE since their appearance is random and associated with the beginning of complications like *T. gondii* reactivations in immune-depressed individuals, adenopathy and chorioretinitis (43).

These antibodies act on extracellular tachyzoites released by the lysis of infected cells and limit their replication by lysing them in the presence of the complement factors, helping opsonisation and macrophage phagocytosis. They are indispensable for the diagnosis of toxoplasmosis in humans (40).

1.4. Treatment

Immunocompetent individuals are normally asymptomatic and the disease remains unnoticed (44).

Currently, the treatment for toxoplasmosis targets only the tachyzoite form, not being able to eradicate the bradyzoite form inside the tissue cysts. A combination of pyrimethamine and sulfadiazine is used with leucovorin (folinic acid). The latter is used to prevent a reduction in platelet count induced by pyrimethamine, a folic acid antagonist, causing a dose-dependent suppression of the bone marrow (45). This combination treatment is used in patients exhibiting encephalitis and has a high rate of toxic effects (46).

If the patient is allergic to sulfadiazine, clindamycin is used, but the treatment is less effective and still induces high rates of toxicity. On the other hand, if the patient does not tolerate pyrimethamine, a combination of trimethoprim and sulfamethoxazole has shown to have similar efficacy results (47). All these drug treatments have similar rates of patient intolerance and some frequent side effects, that in some cases can be fatal, like agranulocytosis, toxic epidermal necrolysis, Stevens-Johnson syndrome and hepatic necrosis (45).

The treatment of pregnant women consists of two different strategies. If the infection is acquired before the 18 weeks of gestation, and there is no suspicion of foetus infection, spiramycin is used to prevent congenital transmission, since is non-toxic and does not cross the placenta. If the infection is acquired at or after the 18 weeks of gestation and there is suspicion of foetus infection, the usual treatment is an association of pyrimethamine-sulfadiazine and leucovorin (48).

Drugs that have isoprenoid pathway as a target, dihydrofolate reductase, *T. gondii* histone deacetylase, or type II fatty acid biosynthesis show some hope for future treatments against cysts in immunosuppressed individuals with chronic infection (49).

Recent studies focused on the use of chitosan nanoparticles (NPs) for the treatment of *T. gondii* infection, and considering their advantages, such as low toxicity and high inhibitory activity, these NPs have potential as an alternative treatment (50).

A vaccine strategy would be ideal for disease control, avoiding tissue cyst formation responsible for chronic infection (51).

2. Nanoparticles overview

NPs include materials of small length scale from nanometers (10^{-9} m) to microns (10^{-6} m) (52), that can be modified in order to overcome biological barriers and to be biocompatible (53). This feature has made scientists around the world begin to study NPs for medical and pharmaceutical applications. Some important treatment improvements have been achieved through the use of NPs, such as the significant reduction of the toxic effects of drugs used in cancer therapy (54), or the delivery optimization of anti-infective drugs (55), and nucleic acids (56-58), the entrance of drugs across the blood-brain barrier (59) and in HIV therapy (60).

Regarding their origin, there are three categories of NPs: the first category corresponding to the one's produced as a secondary product from industrial or natural processes; the second category are the one's specifically manufactured for human use, and the NPs naturally found in live organisms such as humans, animals and plants (61).

Different types of NPs are used for medical applications like drug delivery or vaccine production, including:

- Inorganic NPs (gold, carbon, silica, among others), almost all derived from unnatural sources, with a rigid structure, controlled and cheap synthesis, reproducibility and safety (62);
- Liposomes, spherical structures naturally found in human or animal bodies, formed by phospholipids around an aqueous nucleus, allowing the transportation of hydrophobic molecules as well as hydrophilic molecules (62);
- Other lipid NPs (triglycerides, partial glycerides, fatty acids, hard fats and waxes) developed as an alternative to liposomes to allow the control and target of drug release (63). There are two generations of lipid NPs, the solid lipid NPs (SLN) and the nanostructured lipid carriers (NLC) that allow the accommodation of a higher amount of substance (64);
- Virus-like particles (VLPs), protein complexes that mimic viruses in shape and structure but do not contain genetic viral material, capable of activating the immune system (65);
- Dendrimers, a three-dimensional, star-shaped macromolecule composed of a mixture of amines and amides that upon efficient uptake can induce a strong immune response (62, 66);
- Polymeric NPs, corresponding to the one used in most of the research in the last years, since they have an easy synthesis, are biodegradable, biocompatible and

have reduced cytotoxicity (62). Since this type of NP is the selected for this work, will be more detailed in the following subsection.

2.1. Polymeric nanoparticles

Polymeric NPs have between 10-1000 nm of diameter and are made of polymers and copolymers. They have a core-shell structure with a polymeric matrix in the interior and a hydrophilic polymer surface (for example polyethylene glycol (PEG) or polyvinyl pyrrolidone (PVP)) providing stearic stability. Hydrophobic drugs are usually housed in the polymeric matrix, while hydrophilic drugs prevail on the surface (67).

Polymeric NPs can exist as nanocapsules, with an oily core and a polymeric shell, or as nanospheres, with the core and the outer surface made of polymeric material (Figure 3). These NPs are biodegradable, non-toxic, non-inflammatory, non-immunogenic and don´t activate neutrophils (68).

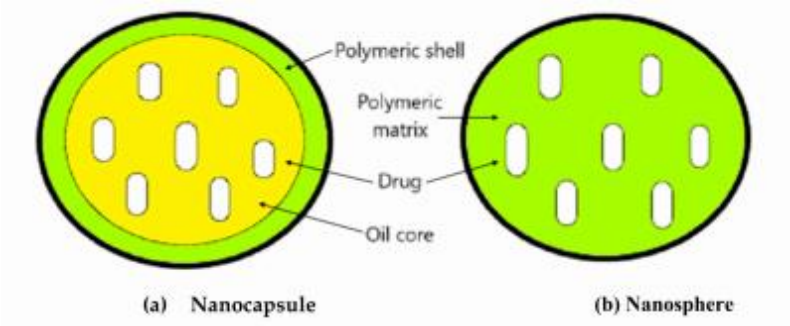


Figure 3. Ilustration of (a) nanocapsule and (b) nanosphere (67).

Polymeric NPs can be prepared with synthetic polymers like N-(2-hydroxypropyl)-methacrylamide copolymer (PHPMA), PEG, poly-L-glutamic acid, poly (lactic-co-glycolic acid); (PLGA), poly (sebacic acid), poly (acrylic acid), among others, or natural polymers like gelatin, albumin, inulin, heparin, chitosan (Chit), among others (Table 1); (67, 69). The food and drug administration (FDA) has approved PLGA and Poly (lactic acid) (PLA) for human use, and these are the most used for vaccine delivery (67, 70).

Table 1. Synthetic and natural polymers (67).

Synthetic Biodegradable Polymers		Natural Biodegradable Polymers	
Polyesters	Polyoxalates	Starch	Chitosan
Polyorthoesters	Polyiminocarbonates	Hyaluronic acid	Dextran
Polyanhydrides	Polyurethanes	Heparin	
Polydioxanones	Polyphosphazenes	Gelatin	
Poly(a-cyanoacrylates)		Albumin	

2.1.1. Chitosan

Chit is a deacetylated form of chitin, one of the most abundant polysaccharides in the world, present in the shells of crustaceans (71). Chitin is formed by units of D-glucosamine and N-acetyl-D-glucosamine, linked by β-1,4 glycosidic linkages (Figure 4). When the deacetylation of this compound reaches about a 50% level, it becomes soluble in aqueous acidic media (72). This copolymer is the only cationic marine polysaccharide since the amino groups of the chain protonate when Chit is dissolved in an acidic environment, allowing the interaction with other molecules (73).

The interaction of the positive charged Chit with the negatively charged cell membranes of microorganisms is thought to be the reason for its antimicrobial activity (74). Its cationic nature has been studied and explored for the development of drug delivery systems, since, can interact with negatively charged polymers and ability to form a gel, when in contact with specific polyanions (71). On the other hand, the Chit solubility makes it capable of being produced in many different forms like nanofibers, hydrogels, films, among others (75). Due to its biocompatibility, controlled biodegradability, cationic nature, non-toxic and antimicrobial properties, Chit has a wide diversity of applications in the medical fields such as wound dressing, tissue engineering, anticoagulant, antimicrobial agent and used in controlled drug delivery (72).

Recent studies have been focused on the use of Chit for the treatment of *T. gondii* infections as an alternative to the current treatment with pyrimethamine and sulfadiazine. These studies demonstrated that the NPs containing Chit have potential as an alternative treatment and in combination with other therapies could also provide a higher level of efficacy (50).

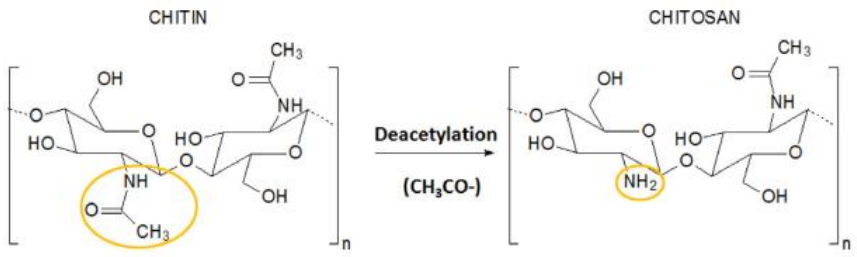


Figure 4. Chitin and Chitosan chemical structures (72).

2.1.2. Carrageenan

Carrageenan (Carr) is the name given to a family of high molecular weight sulphated polysaccharides extracted from red edible seaweeds and formed by alternate D-galactose and 3, 6-anhydro-galactose units, joined by α-1, 3 and β-1, 4-glycosidic linkage (76). Carr is also an anionic polysaccharide since is a sulphated polygalactan with 15-40% ester-sulphate content. Is used in the food industry, as a gelling, thickening and stabilizing agent as well as a substitute of fat, and in other industries, like cosmetic and pharmaceutical industries (76, 77).

There are three different classes of Carr based on their sulphate content: kappa-carrageenan with one sulphate group per disaccharide; iota-carrageenan with two sulphate groups per disaccharide; and lambda-carrageenan with three sulphate groups per disaccharide (76).

Carr possesses various pharmaceutical properties, like antitumor, anticoagulant and immunomodulatory activities (78, 79). In the last decades, Carr has been used as an antioxidant excipient in delivery systems of controlled drug release, in hydrogels and to increase the dissolution of poorly soluble substances (76). The mixture of Carr with natural polymers like Chit, cellulose, alginate, among others, is gaining more and more interest for new biodegradable materials development with excellent characteristics for biomedical applications, like drug delivery and tissue engineering (76, 80-82).

2.2. Synthesis of polymeric nanoparticles

There are different ways for the synthesis of polymeric nanoparticles (Figure 5), such as solvent evaporation, spontaneous emulsification, salting out, nanoprecipitation, polymerization, and ionic gelation.

The solvent evaporation method consists in the addition of the polymer in an organic solvent and then the dissolution of the drug in the polymeric solution, forming an oil in water solution with the presence of a surfactant. After, different physical methods like increasing pressure, temperature or continuous stirring can be done in order to evaporate the organic solvent (83).

The spontaneous emulsification method is a variation of the solvent evaporation method, where the oil phase comprises a water-soluble solvent and a water-insoluble organic solvent used as an oil phase. The spontaneous diffusion of the water-soluble solvent generates turbulence leading to the formation of nanoparticles (67).

The salting-out method is used to keep away from the use of organic solvents. The method starts with the dissolution of the drug in a water-soluble solvent that is later added to an aqueous solution with a stabilizer and salting-out agent, such as magnesium chloride for example. The mixture is then diluted to intensify the process of diffusion and create nanoparticles (84).

The nanoprecipitation method is usually used for lipophilic drugs. The method involves the addition of the polymer and the drug into an organic solvent, with or without a surfactant. After this, the solution is added to an aqueous solution with a stabilizer. Due to diffusion, the interfacial turbulence between the phases causes the precipitation of the polymer (67).

Polymerization methods involve nanoparticles synthesis by the polymerization of monomers. The monomer is added to a polymerization medium with a surfactant under vigorous stirring at an ambient temperature. The drug can be added before or after the polymerization reaction and ultracentrifugation is used to purify de NPs (83).

Nanoparticles development from hydrophilic polymers, like Chit and gelatin involving ionic gelation with the combination of two aqueous phases the first one containing the polymer, for example, the Chit and ethylene oxide, and the second one with polyanion sodium-tripolyphosphate (TTP); (85).

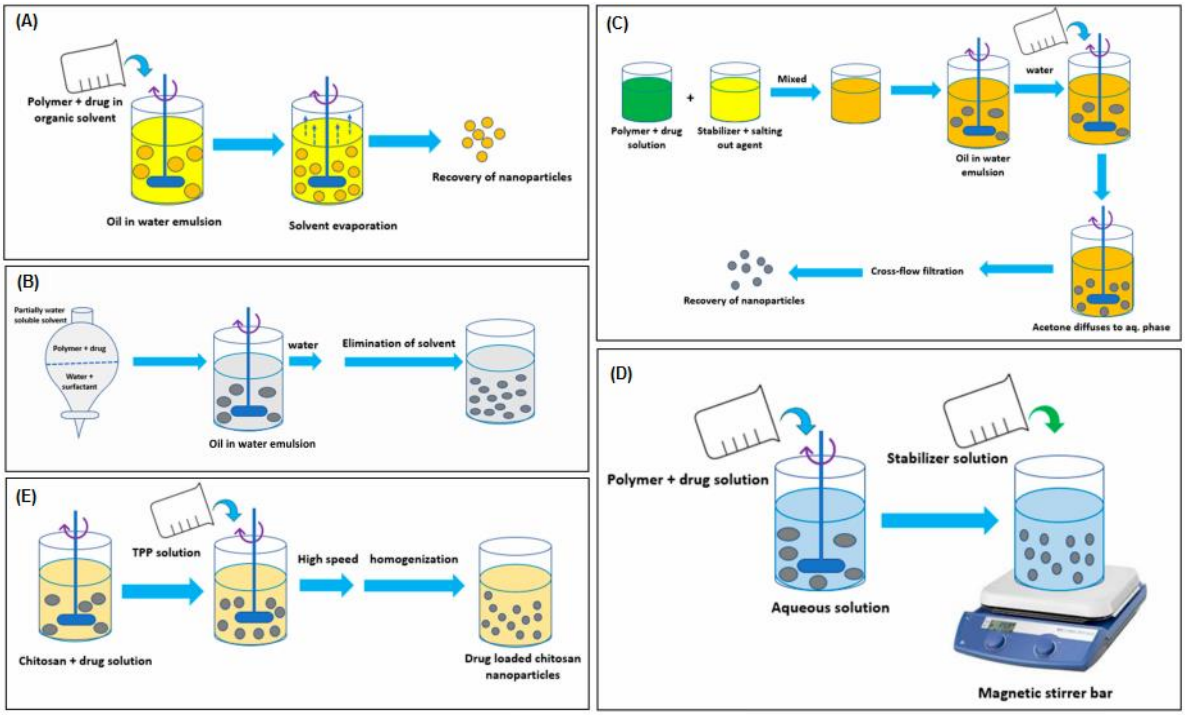


Figure 5. Synthesis of polymeric nanoparticles methods. Adapted from (67).

(A) Solvent evaporation method; (B) Spontaneous emulsification method; (C) Salting out method; (D) Nanoprecipitation method; and (E) Ion gelation method.

3. Nanoparticles and vaccination

Most fatal diseases like tetanus, smallpox or influenza in the past are controlled nowadays, and some are even almost eradicated. This fact has an explanation, such as vaccination, playing a determinant role in the control of these infectious diseases (86). However, some of the vaccines use live-attenuated or inactivated pathogens, and virulence reversion may occur and some unwanted side effects, like tissue damage (87). For diseases, such as AIDS, tuberculosis or toxoplasmosis, there is no effective vaccine available on the market (88). Afterward, there has been progress in this research subject, and the use of the whole microorganisms was replaced by subunit vaccines containing antigenic proteins, frequently less immunogenic than the whole microorganisms (89).

Recombinant protein and deoxyribonucleic acid (DNA)-based vaccines have shown some efficiency, but when co-administered with adjuvants, some of which expensive, shown to induce the establishment of both innate and adaptive immunity (88).

The use of NPs in the development of new vaccine formulations has advantages, since the incorporation of antigens into biomaterials, may achieve a desired immune response, able to replace the use of adjuvants. On the other hand, the physicochemical properties of the NPs can also be manipulated, allowing the controlled delivery and presentation of pathogen-associated molecular patterns (PAMPs) from microorganisms, targeting specific cells and triggering an adequate immune response (89).

3.1. Intranasal route

There are different vaccination methodologies, whereas the parenteral administration includes the subcutaneous, intradermal and intramuscular injection, while the mucosal immunization can be performed by intraocular, oral or intranasal (IN) administration (90). The vaccine efficacy and safety are quite determined by the administration route and the vaccination method since it influences the initiation of the immune response at the site of pathogen invasion. Normally mucosal pathogens such as *T. gondii,* require a mucosal vaccine, mimicking the natural route of infection, to induce adequate immune responses (91).

In fact, mucosal administration, more specifically the IN route is a promising strategy for vaccine delivery (92), since the nasal mucosa offers low metabolic activity and low concentration of proteolytic enzymes, absence of extreme pH values, as well as a wide, thin and high vascularized surface, allowing to bypass gastric degradation and hepatic first-pass metabolism (92, 93).

The primary site for induction of nasal immunity is the nasal associated lymphoid tissue (NALT) and is suggested that the NALT organogenesis is activated after birth and then matures as a consequence of the signals provided by environmental antigens (94). NALT is a rich area in DCs, B cells, T cells and macrophages, overlaid with epithelial cells and some M cells (95). Some studies have shown that after NALT stimulation by nasal antigen administration, strong humoral and cellular immune responses are induced at systemic and mucosal levels (96, 97).

Although the IN route has many advantages, it also has some limitations. Some of them are the low permeability to hydrophilic molecules, the small volumes that can be administered, the mucociliary clearance and the mucus barrier (98). However, different strategies have been developed to improve the vaccine formulation, such as the use of NPs as a delivery system (95).

The advances in the nanotechnology field have provided these systems the protection needed against biological degradation, allowing the passage of antigens encapsulated in NPs to pass across the nasal barriers and providing a prolonged time of antigen contact with the mucosal surface, leading to a better antigen presentation to the immune system (92, 99).

The NPs used in this type of administration also need to have an adequate diameter to prevent their elimination through expiration and allow their deposition in the respiratory tract area, thus facilitating their cell internalization. They also need to have appropriate mucoadhesive properties, with the cationic NPs showing a better efficacy in bounding with the mucosa of the respiratory epithelium (100).

These evidences reinforce the need for knowledge towards the development of NPs to be administered by the mucosal route already shown to be most effective compared to the parental route for some particular purposes (92).

3.2. Internalization

The size of the NPs is important for the cellular uptake, affecting the efficiency, cellular distribution and internalization pathway (101).

When NPs come into contact with the cell membrane, they enter the cell mostly by endocytosis, an active form of transport consists on the NPs capture through the formation of vesicles and vacuoles into the cytoplasmatic membrane (102).

There are two different types of endocytosis, phagocytosis and pinocytosis (103). Phagocytosis is the election method for the internalization of large particles, occurring mainly by professional phagocytes, such as macrophages, neutrophils, monocytes and

dendritic cells (101, 104). The first step is the opsonisation, whereas immunoglobulins and complement proteins are responsible for the NPs recognition and their attachment to the phagocytes via Fc and complement receptors. This recognition starts a signalling cascade that culminates with the formation of cell extensions that capture the NPs (105).

On the other hand, the more common endocytosis type is pinocytosis, which can occur in many cell types and by four different mechanisms: clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis (CvME), clathrin/caveolae-independent endocytosis and micropinocytosis (103).

In the CME the internalization begins with the invagination of the region where the NPs interact with the membrane (or its receptor) due to the accumulation of clathrin, which later leads to the formation of the endosome that fuses with the pre-lysosomal vesicles to form the lysosome, where the NPs will be degraded (106). CvME consists on the formation of invaginations in the cell, surrounded by caveolin dimer protein, cholesterol and sphingolipids (107), resulting in the caveosome formation, a vesicle containing the internalized particle, but does not contain hydrolytic vesicles or acidic pH, which allows the particles to escape lysosomal degradation (108).

The internalization of NPs under 200 nm normally involves the CME mechanism, however, with the size increasing there is a shift towards the CvME mechanisms, which is the predominant pathway for particles of 500 nm (101).

The other internalization pathways, clathrin and caveolae-independent, are used by cells that do not have clathrin and caveolae and result in the escape of the particles into the lysosomes (109).

The micropinocytosis occurs for the internalization of larger NPs that would not be possible through the other mechanisms, and consists of a membrane extension, that creates a large vesicle capable of internalizing particles of 1 to 5 µm, that later will merge with lysosomes and suffer degradation (110).

The internalization pathways that lead to the lysosome degradation, are harmful when the NPs carry drugs since it can modify the concentration and the effectiveness of the active compound, however with regard to NPs carrying antigens, these pathways are determinant for the antigen presentation to the immune system cells and to start the desired host immune response (111).

3.3. Advantages

There are two main advantages to the use of NPs in vaccination. The first one is the fact that they can act as adjuvants enhancing the antigenicity of the antigens or act as an antigen

themselves, mimicking pathogens like viruses for example (88, 112, 113). The second one is the ability to induce innate responses as well as adaptive immune response, since they are used as antigen carriers enhancing the antigen processing and presenting (88), resulting in the efficient targeting of cells and the antigen controlled release (114).

Moreover, NPs also have a large ratio of volume to the surface area, which allows carrying large amounts of antigen if necessary and have an easily modifiable surface that can be covered with antibodies that detect cell-specific receptors, allowing a target delivery to a certain cell population, consequently preventing the damage of other cells or organs (115, 116). Furthermore, the use of NPs might lead to a reduction in the antigenic quantity to optimize therapeutic pharmacokinetics (117). In addition, they are also biodegradable and have low cytotoxicity, determinant characteristics for a delivery system (115).

In the past few years, with the appearance of new variants of pathogenic organisms, immunization against infectious diseases is increasingly a big challenge. The use of NPs in vaccine development has improved their efficacy to obtain the desired humoral and cell-mediated immunities, since nanocarriers can protect the precipitate degradation of antigens, facilitate their uptake and processing by antigen-presenting cells (APCs), and are safe for human use (62).

Interestingly, in the last year some of the vaccines developed against Covid-19 use NPs in their composition, for example, the mRNA-1273 SARS-CoV-2 Vaccine and the BNT162b2 mRNA Covid-19 Vaccine, which strengthens the inherent advantages of NPs as an asset for the development of new and upgraded vaccines (118, 119).

4. Recent advances in the use of Nanoparticles for *T. gondii* vaccination

In the last decade, several studies for the development of a vaccine against *T. gondii* have been carried out, however, an effective vaccine, capable of conferring sterile immunity against infection (elimination of tissue cysts) remains a challenge (120). Recently, new approaches have been made in vaccination strategies, like the use of NPs, which have been assessed mostly in rodents, showing promising results (121).

An ideal vaccine to control toxoplasmosis and prevent the development of chronic tissue cysts should induce a Th1- type immune response since it has been shown that the INF-γ-secreting $CD8^+$ T lymphocyte is the major cell involved in the long-term protective immunity against this disease (120).

The routes of vaccination can also induce different levels of protection since it has been shown that mucosal immunization routes can lead to better effective protection compared to systemic immunization routes (122).

The use of NPs has emerged as a novel vaccine platform. By using this strategy the antigens that otherwise were exposed to proteolytic degradation are preserved, guaranteeing a successful uptake by cells and inducing an immune response (121).

Regarding the last 10 years, different antigen strategies, like DNA vaccines, ribonucleic acid (RNA) vaccines, protein and recombinant subunit vaccines combined with NPs have been studied, in order to achieve a desired effective vaccine (121).

DNA vaccines are some of the most efficient platforms, and their low price and easy manufacture, and capability to stimulate humoral and cellular immune response are some of the features that make them one of the most studied nowadays (2). Some studies involving NPs encapsulated with DNA as antigens have shown some good results, for example, the calcium phosphate NPs with GRA14 DNA vaccine increased the *T. gondii* specific IgG1 and IgG2a antibody responses and lymphocyte proliferation (123). Mice immunized with a modified dendrimer vaccine with replicons encoding GRA6, ROP2A, ROP18, SAG1, SAG2 and apical membrane antigen 1 (AMA1), were protected against lethal challenge infection (124) and 20% of mice immunized with lipid nanoparticle encapsulated with nucleoside-triphosphatase II (NTPase II) survived post-challenge with *T. gondii* parasites (125).

Protein and recombinant subunit vaccines are also extremely safe and with a low probability of side effects, since the protein is highly purified, and there is a wide array of antigens that can be explored, ranging from antigenic epitopes to *T. gondii* total extract lysate antigens (121). Mice immunization with *T. gondii* total extract antigens delivered by porous NPs have shown to induce a Th1/Th17 immune response and all immunized mice survived the infection with significantly reduced brain cyst counts (126). Other polymer NPs loaded with *T. gondii* histone H2A1 showed mice protection against the parasite, prolonging their survival and the production of Th1 cytokines (127). A similar study using PLGA NPs loaded with T and B cell epitopes of AMA1, GRA4, ROP2 and SAG1 showed a strong Th1 immune response in mice immunized with these NPs compared to the same antigens adjuvanted with potassium aluminium sulphate (alum) (128). CS-based NPs with GRA10 epitope also induced a Th1-predominant immune response conferring partial protection against acute and chronic toxoplasmosis (129). Another study using maltodextrin-based NPs with *T. gondii* total antigen extract showed high protection against chronic and congenital toxoplasmosis in mice intranasally immunized, this vaccine was later used in sheep that also confirmed protection (130, 131).

Recent studies using only metallic NPs have also been done since inorganic metals such as gold, silver and platinum have an anti-parasitic effect achieved by a mechanism involving the alteration of the parasite redox status and mitochondrial membrane potential (132). A

study showed that the *in vitro* administration of silver NPs reduced the proliferation of *T. gondii* using a human trophoblast cell line (133).

On the other hand, studies using VLPs in vaccines are limited. However recent studies using VLPs have shown their value and, VLP-based vaccines are already commercially available for several human viral diseases, like Epaxal®, Gardasil®, GenHevac B®, among others (134). Some of the VLPs advantages are their safety; their size that allows rapid traffic into the lymph nodes and consequently a rapid immune response and the repetitive antigen presentation on the surface of the particle that promotes a potent immune response (135). A study showed that VLPs with inner membrane complex subcompartment protein 3 (ISP3) give protection to *T. gondii* ME49 infected mice (136). Thereafter multiple studies verified the efficacy of VLPs vaccines against ME49 strains using different antigens. Specifically, a study using VLPs expressing ROP4 or ROP13 conferred 100% survival against the challenge with ME49 strain and decreased cyst numbers (137).

A summary of the most recent studies carried out using NPs for the development of vaccines against *T. gondii* infection is given in Table 2.

The development of a vaccine against toxoplasmosis can be challenging and the proof of this is that a vaccine for clinical use is still lacking. The use of multi-antigenic vaccines seems to be more efficient compared to the single antigen ones (121). Therefore, the use of highly immunogenic antigens combined with NPs that might be used as adjuvants and delivery systems seems promising for the future achievement of an efficient vaccine.

Table 2. Recent studies carried out using NPs for the development of vaccines against *T. gondii* infection.

Type of NPs	Antigen	Doses	Administration route	Outcome	Reference
Dendrimers	mRNA replicons encoding GRA6, ROP2A, ROP18, SAG1, SAG2A and AMA1 proteins	1	IM	Induction of $CD8^+$ T cell responses	(66)
CaPNs and Alum	pcGRA14 and rGRA14	3	SB and IM	High levels of IgG, IgG2a and IFN-γ for CaPNs immunization and high levels of IgG1 and IL-4 for Aluminium hydroxide immunization	(138)
PLGA and Chit	rTgH2A1	1	SB	High levels of IgG2a	(127)
PLGA	Peptide sequence of SAG1	-	*In vitro*	Molecular docking of peptide and MHC molecules	(139)
DGNP	TE	3	IN	Induction of Th1/Th17 immune responses. Higher levels of total IgG detected.	(126)
PLGA and Alum	Recombinant fusion protein with epitopes of SAG1, AMA1, ROP2 and GRA4	3	IP	Higher levels of IgG2a for PLGA NPs and higher levels of IgG1 for Alum NPs	(128)

CaPNs	pcROM4 + pcGRA14	3	IM	Higher levels of INF-γ and IgG2a and prevalence of Th1 immune response	(140)
DGNP	TE	2	IN and ID	Specific Th1 cellular immune response (IFN-γ and IL-12). IL-10 regulated the Th1 response for ID but not IN vaccination. IN vaccination conferred protection against latent toxoplasmosis.	(131)
CaPNs	Plasmid encoding GRA14	3	IM	Increased levels of IgG1 and IgG2a. High levels of IFN-γ. Decrease of parasite load in mice tissues.	(123)
CaPNs	Multi epitope MIC3, ROP8 and SAG1	3	SB	Survival increase	(141)
VLPs	IMC proteins	2	IN	Reduced cyst load and size in the brain. IgA and IgG detection in faeces and intestines. Mixed Th1/Th2 cytokines and $CD4^+$/$CD8^+$ T cells.	(136)
Liposome	*T.gondii* ESA	3	IP	Increase in IgG antibody. Parasite load reduction.	(142)
PLGA	rROP18	3	IN	Elevated responses of specific IgA and IgG2a.	(143)
Lipid NPs	RNA vaccine encoding *T.gondii* NTPase-II protein	2	IM	High IgG antibody titters and IFN-γ production.	(125)
PLGA	rSAG1	3	IN	Elevated responses of IgA and IgG2a.	(144)

CaPNs: calcium phosphate nano-adjuvant; **Alum:** aluminium hydroxide nano-adjuvant; **Chit:** chitosan; **DGNP:** porous maltodextrin-based with lipid core nanoparticles; **pcGRA14:** GRA14 plasmid clones; **rGRA14:** GRA14 recombinant protein; **rTgH2A1:** recombinant *T. gondii* H2A1 histone; **TE:** total extract of *T. gondii* antigens; **pcROM4:** ROM4 plasmid clones; **IMC:** inner membrane complex; **ESA:** Excretory-secretory antigens; **rROP18:** ROP18 recombinant protein; **NTPase-II:** nucleoside thiophosphate hydrolase II; **rSAG1:** SAG1 recombinant protein; **IM:** intramuscular; **SB:** subcutaneous; **IN:** intranasal; **IP:** intraperitoneal; **ID:** intradermal.

Chapter II – Materials and Methods

1. Cell culture and *in vitro T. gondii* infection

Vero cells, a cell line isolated from renal epithelial cells from African green monkey, were provided by Prof. Manuel Vilanova from the Institute of Biomedical Sciences Abel Salazar (ICBAS) and grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate and 1% penicillin/streptomycin (purchased from Life Technologies). For their maintenance, when in confluent monolayer, Vero cells were subcultured using a 1:10 ratio and culture medium renewal was performed twice a week, being maintained in 25 cm^2 flasks at 37ºC, in a humidified atmosphere and with 5% CO_2. The cells detachment was done using 0.25% trypsin/1 mM EDTA solution for 5 minutes at 37ºC. A growth medium was added to inactivate the trypsin and the cell suspension was centrifuged at 2000 x g for 6 minutes at 4ºC. The supernatant was discarded, and cells were resuspended in a complete medium. Lastly, cells were divided into new flasks of 25 cm^2 for maintenance and 75 cm^2 for *in vitro* infection with *T. gondii* (in some experiments cells were placed at 175 cm^2 flasks for *in vitro* infection).

T. gondii strain ME49 was kindly provided by Dr. Marcus Meissner (Parasitology department of the Faculty of Medicine, Heidelberg University, Germany). Tachyzoites used for Vero infection came from a previously infected Vero cell culture reaching approximately 80% of lysis and with mobile extracellular tachyzoites.

Two different approaches were used for *T. gondii* tachyzoites production. In the first, Vero cells were sub-cultured in a 1:8 ratio and after 3 days (or when they reached confluent monolayer) were infected using a 10-fold dilution of viable tachyzoites. In the second, Vero cells were sub-cultured in a 1:25 ratio and were concomitantly infected using a 10-fold dilution of viable tachyzoites. In both approaches, tachyzoites were isolated after 5 days of infection.

2. Tachyzoites isolation and extraction of *T. gondii* membrane proteins (TGMP)

Viable tachyzoites isolated from Vero cell cultures reaching approximately 80-90% of cell lysis were purified as follows: cells were detached using a cell scraper and transferred to a 50 mL falcon, followed by centrifugation at 200 x g for 15 minutes. The supernatant was discarded, and cells were resuspended in 5 mL of PBS. Vero cells were then disrupted by passaged at least 10 times through a 25G needle, followed by PBS addition until a total volume of 40 mL. Subsequently, the suspension was centrifuged at 1500 x g for 15 minutes

(this procedure was repeated 2 times) and the pellet was resuspended in 6 mL of PBS and passed through a PD-10 Desalting Column (GE, Healthcare, Freiburg, Germany), allowing the isolation of parasites from the cell debris. The parasite concentration was determined using a Neubauer chamber, afterwards, the suspension was centrifuged for another 15 minutes at 1500 x g. Pellets were stored at -80°C until future use.

For *in vivo* infection, the parasite inoculum was prepared in saline solution (0.9% NaCl) to a final concentration of 2.5 x 10^4 parasites/ml.

For TGMP extraction, the tachyzoite pellets previously stored were resuspended in a solution of 0.75% Triton X-114 (Sigma)/PBS (1 ml of solution per 10^8 parasites). The solution was incubated for 10 minutes on ice and centrifuged at 10000 x g for 30 minutes at 4ºC. The supernatant (with hydrophobic proteins) was recovered and placed in a hotplate at 30ºC for 3 minutes, followed by another centrifugation at 10000 x g for 30 minutes at 4ºC. The supernatant was collected and placed again in the hotplate at the same conditions followed by centrifugation at 1000 x g for 3 minutes at room temperature. After centrifugation, there were 2 phases: the upper phase with the hydrophilic proteins, and the lower phase with the hydrophobic proteins. The upper phase was discarded, and the TGMP was precipitated from the lower phase using absolute ethanol (4x the volume of the lower phase), vortexed for 15 seconds and incubated on ice for 1 hour. After centrifugation at 12000 x g for 20 minutes at 4ºC, the supernatant was discarded and the pellet dried for 30 minutes at room temperature. The pellet was resuspended in 50µl per 10^8 parasites of sterile PBS (TGMP/PBS) or in sterile 25mM HEPES (TGMP/HEPES) respectively and stored at -20ºC for future use.

Lowry protein assay was done for protein quantification. Bovine serum albumin (BSA) at 0.05% was used to create a standard curve with the desired concentrations (*i.e.,* 50,100 and 150 µg/ml). The samples were 50-fold diluted. A+B solution was freshly prepared, 50 ml of solution A (2g of $NaCO_3$ anhydrous, 0,02g of $KNaC_4H_4O_6$ up to 100 ml with NaOH 0,1M) plus 1 ml of solution B (0,5 g of $CuSO_4$ or 0,782 g of $CuSO_4.5H_2O$, one drop of H_2SO_4, and up to 100 ml with H_2O). Then, 750 µl of A+B solution was added to each tube, and the tubes were vortexed and incubated for 10 minutes in the dark. After incubation, 75 µl of Folin Reagent (1:1 Folin:H_2O) was added to each tube, vortexed and incubated for another 30 minutes in the dark. The samples were read at 750 nm by spectrophotometry and the protein concentration was calculated by the following formula:

$$f = \frac{\sum \ standards\ concentration}{\sum \ standards\ abs} \times Dilution\ factor$$

[Sample] = Sample Abs x f

3. SDS-PAGE analysis of protein profile

SDS-PAGE analysis was performed to determine the protein profile of the TGMP resuspended in PBS or HEPES. SDS-PAGE composed of 5% acrylamide stacking gel and 10% acrylamide running gel, was loaded with 8 µg of TGMP, previously boiled at 95ºC for 3 minutes. The protein migration profile was visualized with a silver nitrate staining protocol (145), and the relative molecular mass of the most noticeable bands was determined in comparison with the standard marker (sc-2361 Molecular weight Marker; Santa Cruz Biotechnology).

4. Immunization, *in vivo* infection and tissue sample collection

BALB\c mice obtained from Charles River (L'Arbresle, France) were maintained in ICBAS, University of Porto animal facilities.

A *in vivo* experiment comprised 6 groups (n=6) subjected to immunization with the following formulations: 10 µg CpG ODN 1826 (ODN, control group) purchased from InvivoGen; 10 µg ODN plus 10 µg TGMP/PBS; 10 µg ODN plus 30 µg TGMP; 5 µg ODN plus 30 µg TGMP/PBS; 10 µg ODN plus 10 µg TGMP/HEPES, and 10 µg plus 30 µg TGMP/HEPES. At day zero, under weak isoflurane anaesthesia, mice were immunized by IN route with 20 µl (10 µl in each nostril) of each formulation. After 3 weeks of the first immunization, a boost immunization was done, and 3 weeks after, mice were intraperitoneally infected with 5000 viable tachyzoites in a final volume of 200 µl.

The infected mice were monitored and weighed daily and euthanized with isoflurane when they reached the human endpoint (lost 10% of the initial weight) corresponding to 5 days after infection. After mice euthanasia, the heart, kidney, spleen, lung and liver, were collected and stored at -80ºC until use. The peritoneal exudate cells (PEC) were obtained after washing the peritoneal cavity with 3 ml of Hanks solution, and after centrifugation at 4000 x g, were stored at -80ºC until use.

All animal procedures were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes recommendations (ETC 123) and directive 2010/63/EU of the European Parliament and the council of 22 of September 2010 on the protection of animals used for scientific purposes, and Portuguese rules (DL 113/2013). The authorization to perform the experiments was issued by ORBEA Animal Ethics Committee from ICBAS (P315/2019/ORBEA) and by the responsible national board authority, Direcção-Geral de Alimentação e Veterinária.

5. Extraction of genomic DNA and quantification

Genomic DNA was extracted from the organs and PEC previously collected by the phenol-chloroform method and precipitation with ammonium acetate/ethanol (146). The tissues were first homogenized with 2 ml of lysis buffer (75 mM NaCl and 25 mM EDTA), and the sediment of the peritoneal exudate, was resuspended in 500 µl of lysis buffer and homogenised. Then, 500 µl of each homogenate was incubated overnight at 55ºC in a solution with 50 µl of 10% SDS and 10 µl of 25 mg/ml Proteinase K. After, 500 µl of phenol-chloroform (1:1, purchased from Sigma and Merck, respectively) were added and the samples were centrifuged at 6000 x g for 15 minutes at 4ºC. The supernatant was collected and transferred to another tube containing 500 µl of phenol-chloroform, then the sample was centrifuged again at the previous conditions. The resulting supernatant was transferred to another tube containing 7.5 M ammonium acetate/100% ethanol (the volume of ethanol was 2-fold the supernatant volume, and the volume of ammonium acetate added was 1/3 of the supernatant volume) for DNA precipitation.

The samples were then incubated for 2h at -20ºC followed by centrifugation for 15 minutes at 16000 x g at 4ºC. After centrifugation, the supernatant was discarded, the pellet was washed two times with 500 µl of ethanol 70% and centrifuged for 10 minutes at 16000 x g at 4ºC. After centrifugation, the supernatant was discarded and the pellet was incubated at 37ºC for 15 minutes, for ethanol evaporation. The pellet was then resuspended in 200 µl of ultra-pure water and stored at 4ºC for solubilisation.

For DNA quantification, a Thermo Scientific Nanodrop 100 spectrophotometer was used, and the samples were normalized for 200 ng/µl for the heart, kidney, spleen, lung and liver and 50 ng/µl for the PEC, for parasite load quantification by qPCR.

6. Quantitative real-time PCR (qPCR)

T. gondii DNA was detected using the genomic DNA extracted from kidney, heart, spleen, lung, liver and PEC, using primers and a probe designed for the SAG1 gene of *T. gondii*, giving rise to an amplification product of approximately 100 bp. qPCR was performed in Step-One Plus (Applied Biosystems by Life Technologies) device. Product amplification was performed with 1 µl of template DNA, corresponding to 200 ng (kidney, heart, spleen, lung and liver) and 50 ng (PEC) of DNA in a final volume of 10 µl containing 0.2 µM of each primer (SAG1 forward: CCAGAGCCTCATCGGTCGTC; SAG1 reverse: GGGTCCTTCCGCAGACAAC), 0.2 µM of probe (6FAM-CTGTyTGCACCGTAGGAGCACCT-BBQ; all designed by Till Molbiol) and KAPA PROBE

FAST qPCR Master Mix (Bio-Rad). The PCR programme run was as follows: 1) denaturation at 95°C, 3 minutes; 2) amplification in 40 cycles (denaturation at 95°C, 3 seconds; combined annealing/extension at 60°C, 30 seconds). DNA samples corresponding to $1.28x10^{2}$ to $1.28x10^{-3}$ µg of parasite DNA were included as external standards for the creation of a standard curve. Quantitative evaluation of fluorescence signals from PCR products was performed with the Step-One Plus programme (Applied Biosystems by Life Technologies).

7. Chitosan-Carrageenan nanoparticles synthesis and TGMP loading

The NP-Chit:Carr were prepared by ionic gelation method, through electrostatic interaction between the positively charged amino groups of the Chit, and the negatively charged sulphate groups of the Carr (147). Briefly, Chit was dissolved in 1% acetic acid (v/v) and the Carr in purified water previously heated to 60ºC to obtain a solution of 1 mg/ml (w/v) and 0.42 to 0.71 mg/ml (w/v), respectively, reaching the final ratios of 3.5/1, 4/1, 5/1 and 6/1 (w/w). NPs were spontaneously formed by the incorporation of 1.2 ml of each Carr solution into 3 ml of the Chit solution, under agitation at room temperature. To prepare the protein-loaded NPs, TGMP/HEPES was incorporated in the Carr solution. The concentration of TGMP/HEPES in the Carr solution was such that allowed the synthesis of NPs with 3 and 6% (w/w) of protein (TGMP/HEPES) respective to the Chit content.

Three syntheses were performed: in the 1st synthesis (NP#1), the method of NPs synthesis was as described above, without the loading of TGMP/HEPES; in the 2nd synthesis (NP#2) the agitation step was replaced by 30 seconds of sonication, without the loading of TGMP/HEPES; and in the 3rd synthesis (NP#3), the method was the same as the NP#1 but using half of the Chit and Carr solution volumes added (0,6 ml and 1,5 ml, respectively) and including the TGMP/HEPES.

8. Size and zeta potential measurements

For the measurements of size, polydispersion index (PDI) and surface charge (ζ-potential) of the NPs synthesized (#1, #2 and #3), dynamic and electrophoretic light scattering (ZetaPALS/ Zeta Potential Analyser, Brookhaven Instruments, USA) with a scattering angle of 90° at 20°C was used. The NPs were directly used (no dilution). For the measurement of size and PDI, six runs of 2 minutes were performed for each measurement for each synthesis. For the ζ-potential, ten runs of ten cycles were performed for each measurement

for each synthesis. Triplicates were done for all the measurements and the results were expressed as mean ± standard deviation (SD).

9. Quantification of NPs encapsulation efficiency

The quantification of the NPs EE of the NP#3 was determined upon the separation of the NP-Chit:Carr-TGMP from the preparation medium that contained the non-encapsulated TGMP by centrifugation at 11.500 x g for 30 minutes. The determination of free TGMP was done in the supernatant by the Lowry protein assay previously described. The medium used in the NPs preparation was used to do a calibration curve (to eliminate possible interferences). The percentage of free TGMP able to deposit at the same conditions was measured, using the solution employed in the NPs synthesis plus free TGMP in the same concentrations and experimental conditions.

The NPs EE was calculated from the equation indicated below:

$$\text{EE (\%)} = 100 - \left(\frac{\text{Free amount of protein x 100}}{\text{Total amount of protein}} \right) - \%\, Deposit$$

10. MTT cell viability analysis

HFF cells (from ATCC, USA) were plated in a 96-well plate at a density of 5 x 10^3 cells/well. After 72h, the medium was replaced with DMEM supplemented with 10% FBS, 1% sodium pyruvate and 1% penicillin/streptomycin solution in the presence of the 3.5/1 NP-Chit:Carr with or without TGMP/HEPES 3% and free TGMP3% in the following dilutions: 1/2, 1/10, 1/50, 1/100 and 1/500. DMEM medium was used in the control wells. Cells were then incubated for 24h at 37ºC. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was added to the wells (20 µl/ well; final concentration 500 µg/ml) and cell plates were left to rest for 3h at 37ºC. At the end of this period, the medium was removed, and 200 µl of a solution of dimethyl sulfoxide: isopropanol (3: 1) was added to each well. Cell plates were protected from light and agitated for 15 minutes. Absorbance was measured using a multi-mode microplate reader at 540 nm. One experiment was performed using 3 replicates for each condition.

11. Statistical analysis

The results were presented as mean ± standard error mean (SEM), except for the size and zeta potential measurements where the results were presented as mean ± standard deviation (SD). Statistical differences between two groups were determined by the unpaired

Student t-test. For multiple group comparisons, with one independent variable, the one-way ANOVA test with a Tukey's post-test was used. For group analysis with two independent variables, two-way ANOVA with a Bonferroni post-test was performed, using GraphPad Prism 5.0 software (San Diego, CA, USA). Statistically significance was labelled as * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.

Chapter III – Results and Discussion

1. Parasite and cell culture optimization

T. gondii tachyzoites are obligate intracellular parasites that can be maintained and cultured *in vitro* in the laboratory, with a duplication time of 6 to 9 hours *in vitro*, depending on the strain (148). When infected cells reach a concentration of 64 to 128 parasites/cell, cell rupture occurs and the free tachyzoites rapidly infect the neighbour cells (149).

The *in vitro* infection method was optimized to achieve the maximum number of parasites to obtain sufficient TGMP to be characterized and used in the NPs syntheses.

Two different methods of Vero cell culture and parasite infection were used, as described above. A significant increase in the number of parasites was observed in method 2 compared to method 1 (Figure 6). These results indicate that in our experimental conditions is more profitable to infect the cells when they are not yet in a confluent state. Similar results were obtained in a study where *T. gondii* infection was done when 100% confluent Vero cells was reached, resulting in a lower percentage of viable tachyzoites compared when using lower Vero cell confluences (150). These results are explained by the fact that lower confluences allow room for the multiplication of host cells concurrently with the tachyzoites intracellular replication, Vero cell lysis and parasite propagation in culture, preventing the accumulation of 'older' host cells (150).

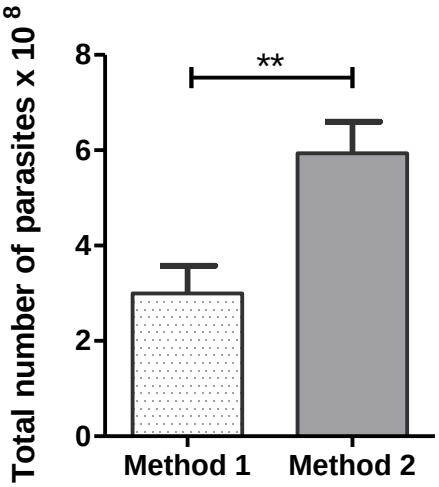


Figure 6. *T. gondii* cell culture optimization. Variable: timing of Vero *T. gondii* infection. Method 1: Vero cells were sub-cultured and after reaching confluence were infected. Method 2: Vero cells were sub-cultured and infected at the same day. Fifteen (n=15) cell culture of 75 cm^2 were used for each method. For statistical analysis an unpaired Student t-test was performed and statistical significance is indicated, P<0.01 (**).

Method 1 and 2 were carried out using 75 cm^2 culture flasks. However, to increase the numbers of parasites obtained, cell culture was carried out using 175 cm^2 culture flasks. Contrary to what was expected, the parasite numbers obtained in these flasks were significantly lower than the parasite number achieved using 75 cm^2 flasks (Figure 7).

As is known, *T. gondii* is an intracellular parasite, which to multiply it must enter the cell. A possible explanation for the reduced parasite number obtained from cell culture using larger flasks is that the Vero cells growth rate might be lower in flasks with larger areas.

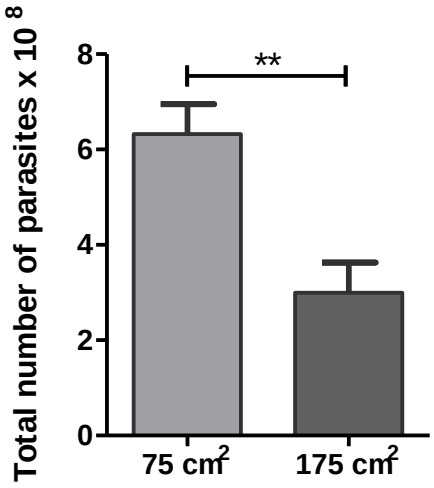


Figure 7. *T. gondii* cell culture optimization. Variable: culture flasks capacity. 15 flasks of 75 cm^2 and 7 flasks of 175 cm^2 were used in parallel using the method 2, in order to have approximately the same area of culture. For statistical analysis an unpaired Student t-test was performed and statistical significance is indicated, P<0.01 (**).

Another approach was carried out comparing the use of 8 or 15 culture flasks of 75 cm^2 (Figure 8), to achieve the maximum number of tachyzoites. There were no significant differences between the number of parasites obtained using 8 or 15 culture flasks of 75 cm^2.

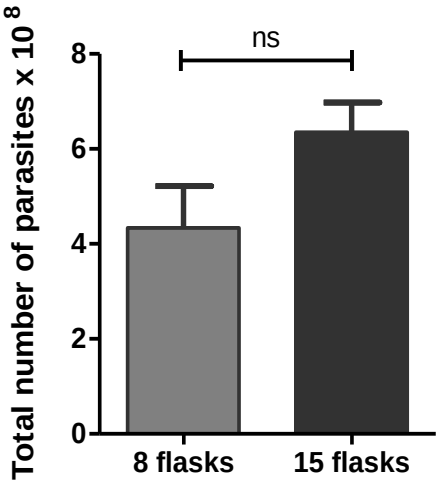


Figure 8. *T. gondii* cell culture optimization. Variable: number of cell culture flasks of 75 cm^2. 8 flasks of 75 cm^2 and 15 flasks of 75 cm^2 were used in parallel, using the method 2. For statistical analysis an unpaired Student t-test was performed. ns: not significant.

A PD-10 Desalting column that allows the separation of high molecular weight from low molecular weight molecules was used for the purification of the *T. gondii* tachyzoites from Vero cell debris.

The percentage of parasite retention in the column was determined by parasite counting before and after the passage of the cell suspension through the column. The results obtained indicated the retention of tachyzoites between 25 and 50%, taking into account 4 isolation procedures (Table 3). The large difference between experiments may be due to operator error during technical procedures. On average, it was observed 40% of parasite retention in the column employed.

Other types of columns or separation methods like elutriation (151), trypsin digestion, purification with a 3-µm filter, CF-11 cellulose purification or Percoll purification (152), should be tested to reduce this parasite retention.

Table 3. Percentage of parasite retention during purification.

Batch#	Total number of parasites before isolation	Total number of parasites after isolation	Total number of parasites retained in the column	Parasite retention (%)
1	1.77×10^8	8.79×10^7	8.91×10^7	50
2	7.65×10^8	3.96×10^8	3.69×10^8	48
3	5.43×10^8	3.48×10^8	1.95×10^8	36
4	1.02×10^9	7.62×10^8	2.58×10^8	25

A PD-10 Desalting Column was used.

2. Study of TGMP protein profile

Immunization studies, previously done by our group, using Balb\c mice demonstrated partial protection of mice immunized with TGMP resuspended in PBS plus ODN adjuvant (data not published).

However, due to the hydrophobic character of this set of proteins, TGMP was not completely soluble in PBS, becoming a particulate suspension. Due to its incomplete dissolution, some TGMP residues were retained in the extraction tubes, which were recovered with the addition of more PBS (TGMP/PBS residues).

For better solubilisation of TGMP, a 25 mM HEPES solution was used instead of PBS. To verify if HEPES did not interfere with the protein profile, an SDS-PAGE was performed followed by a silver nitrate staining (Figure 8). It was observed that the protein migration

profile is the same in the TGMP resuspended in PBS or in HEPES solution. The hydrophilic proteins profile, obtained in the process of protein extraction, were used as a control.

A high intense band was observed at 29 kDa in both formulations. According to the literature, in this range two proteins from *T. gondii* can be found: SAG1 with a molecular weight of 30 kDa, a membrane protein involved in the host cell invasion by the parasite (153); and dense granule protein 7 (GRA7) with a molecular weight of 29 kDa, which contributes to parasite's virulence, as it forms complexes with other proteins protecting the parasite from the host cell elimination mechanisms (154). Another protein with a molecular weight of 34 kDa can be found, the micronemal protein 6 (MIC6). It has been demonstrated by using a DNA vaccine coding for MIC6, the induction of both humoral and cellular Th1 immune responses able to increase the survival time of immunized and *T. gondii* infected mice (155).

The solubilisation of transmembrane proteins is one of the most critical stages during the extraction since the protein is extracted from the phospholipid bilayer of hydrophobic nature to an aqueous solution such as PBS. Efficient solubilisation of proteins must be obtained from a protein dissociation from lipid molecules or by varying the physical and chemical characteristics of the solutions used during their preparation. There is no standardised process that can be applied to all membrane proteins, so individual protocols must be established specifically for the protein of interest (156).

For future experiments, other solubilisation solutions like triton X-114, which showed efficacy in *T. gondii* membrane proteins solubilisation (157), should be tested.

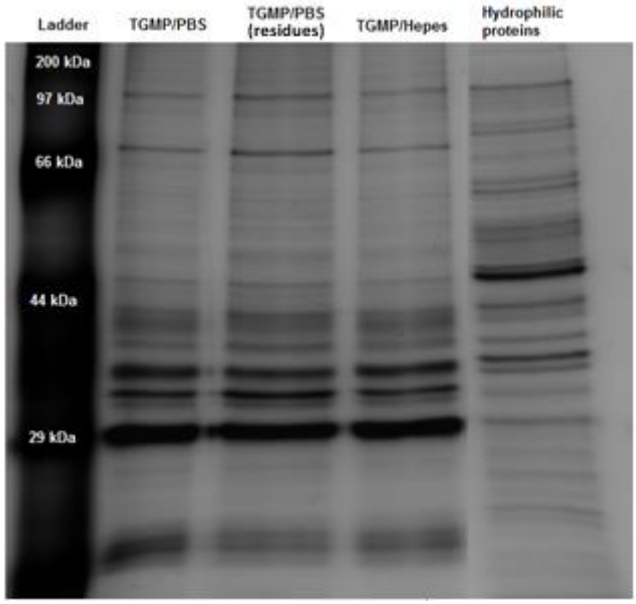


Figure 9. Protein profile of *T. gondii* hydrophobic (TGMP) and hydrophilic proteins. SDS-PAGE analyses (10% acrylamide gel, loaded with 8 µg of protein per lane), followed by silver nitrate staining. Ladder: molecular weight marker; TGMP/PBS: TGMP resuspended in PBS; TGMP/PBS (residues): residues of TGMP left in the extraction tube resuspended with PBS; and TGMP/HEPES: TGMP resuspended in HEPES.

3. Quantification of parasite load in different tissues from infected and immunized BALB\c mice

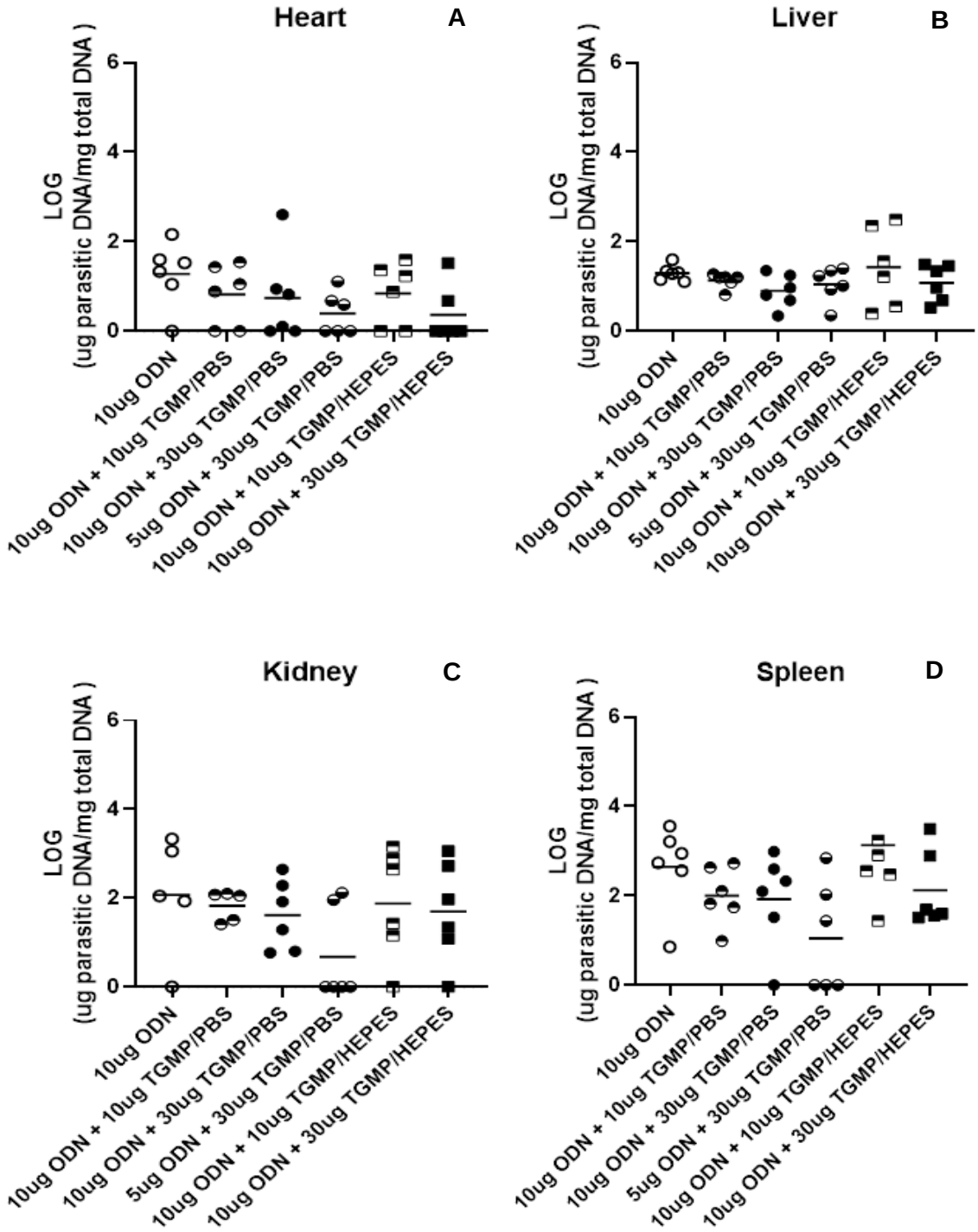
This experiment was conducted to determine the best combination of adjuvant and TGMP antigen amounts used in the immunization formulation, inducing the protective effect against *T. gondii* infection and, whether HEPES would induce any change in this protective effect. An experiment was performed using 6 groups of animals: mice immunized with adjuvant (10 µg ODN; control group); 10 µg ODN plus 10 µg TGMP/PBS; 10 µg ODN plus 30 µg TGMP/PBS; 5 µg ODN plus 30 µg TGMP/PBS; 10 µg ODN plus 10 µg TGMP/HEPES; and 10 µg ODN plus 30 µg TGMP/HEPES. The parasite load, in the different organs, is shown in Figure 10.

Regarding the heart, there were no significant differences between the groups of mice analysed indicating no protective effect in this organ. A trend towards a decrease in the parasite load was observed in the animals immunized with 5 µg ODN plus 30 µg TGMP/PBS or with 10 µg ODN +plus 30 µg TGMP/HEPES, compared with the control group. In some animals it was not possible to detect the parasite, being the heart one of the organs least affected by the parasite (Figure 10A).

For the liver, no significant differences were observed and the parasite load was low, however, the parasite was detected in all the animals (Figure 10B, Table 4).

In the kidney and spleen, there were no significant differences in the TGMP immunized animals compared with the control group. However, the same downward trends in parasite load observed in the heart were observed in the kidney and spleen from the animals immunized with 5 µg ODN plus 30 µg TGMP/PBS compared with control, mainly because a large part of the animals in these groups were negative (Figure 10C-D, Table 4).

Considering the lung and PEC, there was a significant decrease in the parasite load of the animals immunized with 5 µg ODN plus 30 µg TGMP/PBS group compared with the control (Figure 10E-F). The parasite load was higher in PEC compared with the other organs, as expected, considering the route and time-point of infection.


Heart
A
Liver
B
Kidney
C
Spleen
D
LOG
(ug parasitic DNA/mg total DNA)
0
2
4
6
10ug ODN
10ug ODN + 10ug TGMP/PBS
10ug ODN + 30ug TGMP/PBS
5ug ODN + 30ug TGMP/PBS
10ug ODN + 10ug TGMP/HEPES
10ug ODN + 30ug TGMP/HEPES

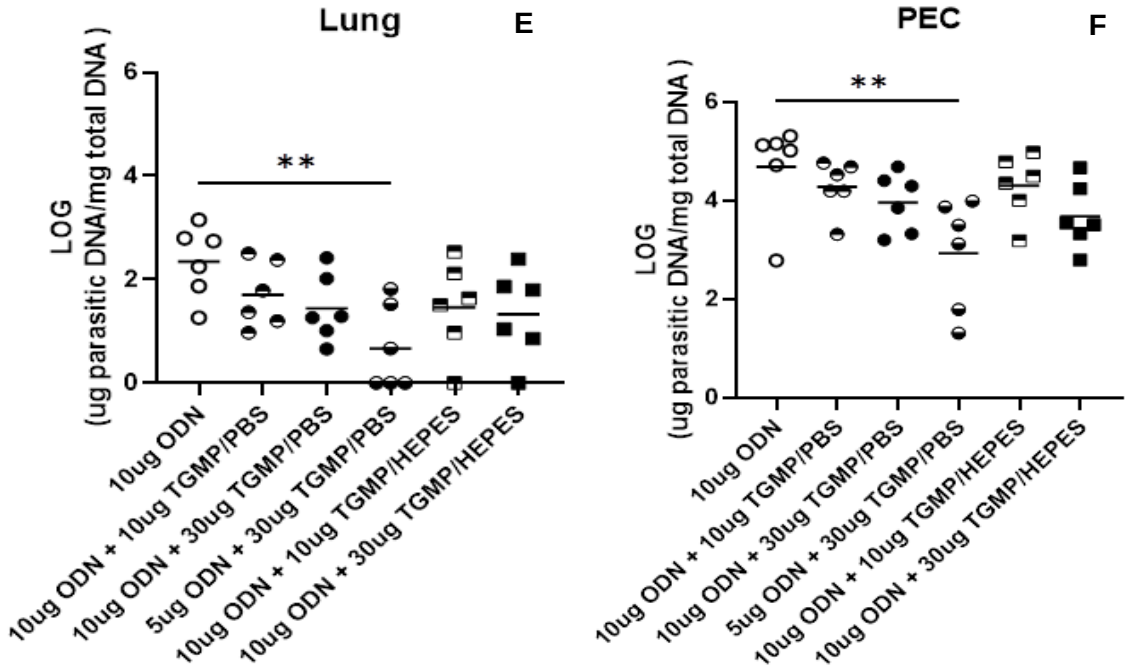


Figure 10. Parasite load quantification in the Heart (A), Liver (B) Kidney (C), Spleen (D), Lung (E) and PEC (F). *T. gondii* DNA was detected using primers and a probe designed for *SAG1*. PCR was performed using 200 ng of genomic DNA from Heart, Liver, Kidney, Spleen and Lung and 50 ng for PEC. DNA samples corresponding to $1.28x10^{2}$ to $1.28x10^{-3}$ ng/µl of parasite DNA were included as external standards for the creation of a standard curve. A control group with 10 µg of ODN and 5 other groups with different concentrations of ODN and TGMP/PBS or TGMP/HEPES were used (n=6 per group). For statistical analyses one-way ANOVA test with a Tukey's post-test was performed. Statistical significance is indicated, P<0.01 (**).

New studies will be carried out to better confirm the protective effect of immunization with TGMP and ODN, using a higher number of animals to obtain robust data and testing infection at different time points after immunization (long term).

The trend towards lower parasitic loads in organs and the significant reduction in lung and PEC observed in the 5 µg ODN plus 30 µg TGMP/PBS group might be a consequence of using a lower adjuvant concentration.

There are no statistically significant differences between the TGMP/PBS and TGMP/HEPES groups with the same amount of adjuvant, which indicates that the solution where the TGMP is dissolved has no influence on the data obtained.

The main goal of this work was the development of stable NPs able to efficiently encapsulate TGMP, and with adjuvant properties that allow the non-use of adjuvant (ODN) in inducing effective protection against infection. Therefore, the synthesis and optimization of NP-Chit:Carr-TGMP is described in the next sections.

Table 4 referred the infection rate in the tissues analysed from the animals immunized with the different formulations. Results revealed that in the heart, for all groups of mice, the

parasite was not detected, which indicates that immunization induced some degree of protection in the heart.

The chosen solution to increase TGMP solubilisation was the HEPES since has already been used for the dissolution of membrane proteins, for the development of a liposome delivery system in another infection model (158).

Table 4. *T. gondii* infection rate assessed by qPCR for *SAG1* in the tissues of infected animals.

	10µg ODN			10µg ODN + 10µg TGMP/PBS			10µg ODN + 30µg TGMP/PBS			5µg ODN + 30µg TGMP/PBS			10µg ODN + 10µg TGMP/HEPES			10µg ODN + 30µg TGMP/HEPES		
Tissue	**+**	**-**	**%**	**+**	**-**	**%**	**+**	**-**	**%**	**+**	**-**	**%**	**+**	**-**	**%**	**+**	**-**	**%**
Heart	5	1	83	4	2	67	4	2	67	3	3	50	4	2	67	2	4	33
Liver	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100
Kidney	4	1	80	5	0	100	6	0	100	2	4	33	5	1	83	5	1	83
Spleen	6	0	100	6	0	100	5	1	83	3	3	50	6	0	100	6	0	100
Lung	6	0	100	6	0	100	6	0	100	3	3	50	5	1	83	5	1	83
PEC	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100

+ represents the number of animals where the parasite load was above the detection limit; - represents the number of animals where the parasite load was below the detection limit; % represents the percentage of animals where it was possible to detect the parasitic load.

4. Nanoparticles optimization

The NP-Chit:Carr were successfully synthesized through electrostatic interaction between the positive charged Chit amino groups and the negative charged Carr sulphate groups as already described (147).

In the first (NP#1) and second (NP#2) syntheses (Figure 11) the NPs size ranged from 346 nm to 442 nm, and from 447 nm to 573 nm, respectively. There were no significant differences in the size between the different ratios of Chit:Carr used within each synthesis, which means that the different proportions between Chit and Carr used did not cause differences in the size of the NPs, contrary to what is described in the literature (147), where it is explained that the proportions with higher amounts of Carr lead to the formation of larger particles since the Carr is a large polymer. In fact, in the second synthesis, there is a tendency to size decrease when Carr proportion to Chit decreases, however, it's not statistically significant.

On the other hand, when the variable of agitation was replaced by sonication during the second synthesis procedure (NP#2), there was observed a significant increase of NPs size when using the 3.5/1 Chit:Carr ratio compared to NP#1.

Some articles claimed that NPs containing antigens greater than 225 nm tend to induce Th1 cytokines, such as INF-γ (71, 159), which indicates that the size obtained from both NP#1

and NP#2 is above the considered value and will probably exert influence in immune cells. This fact will be considered in future experiments.

Regarding the PDI (Figure 11B) there was a significant decrease in the NP#2 when using the Chit:Carr 3.5/1 ratio. The PDI ranges from 0 to 1 and demonstrates the size distribution of the NPs in the formulation. Briefly, in a monodisperse distribution the PDI is close to 0, and in a polydisperse distribution, the PDI is close to 1 (160). The PDI values obtained, ranged from 0.342 to 0.372 and from 0.294 to 0.330 in NP#1 and NP#2, respectively, which indicates that they are suitable for DLS analysis (161). However, in the NP#1, and in some ratios (4/1 and 5/1) of NP#2, PDI was higher than 0,3 that is the limit value usually used for nanocarriers used in IN administration (160).

Considering the zeta potential (Figure 11C) there was a significant decrease between the Chit:Carr 3.5/1 ratio or Chit:Carr 4/1 ratio compared to Chit:Carr 6/1 ratio in NP#1, and a significant increase in the Chit:Carr 5/1 ratio compared to the Chit:Carr 3.5/1 ratio in NP#2. There was a significant decrease in the zeta potential in the Chit:Carr 3,5/1 ratio comparing the NP#1 and NP#2.

NPs are considered approximately neutral when the zeta potential is between -10 to 10 mV and considered strongly cationic and anionic when the zeta potential is greater than +30 and less than -30 mV, respectively (162). A value of zeta potential lower than -30 mV and higher than + 30 mV is considered to have the sufficient repulsive force to obtain an optimal physical colloidal stability, since a zeta potential between these two values can result in the NPs aggregation and flocculation, becoming unstable particles (163). Interestingly, in the last decades, a high number of the developed NPs to target dendritic cells and induce cellular immune responses are of cationic nature, since they showed increased immunostimulatory properties compared to anionic NPs, and also showed to be potent inducers of antigen-specific T-cells (164). Even though significant differences have been observed, as mentioned above, the values obtained range from +50 mV to +62 mV and from +47 mV to 58 mV in NP#1 and NP#2 synthesis, respectively, which means they are all above the +30 mV. Therefore, is considered that the developed NPs have appropriate colloidal stability and electrical charge to stimulate the immune system as described by others.

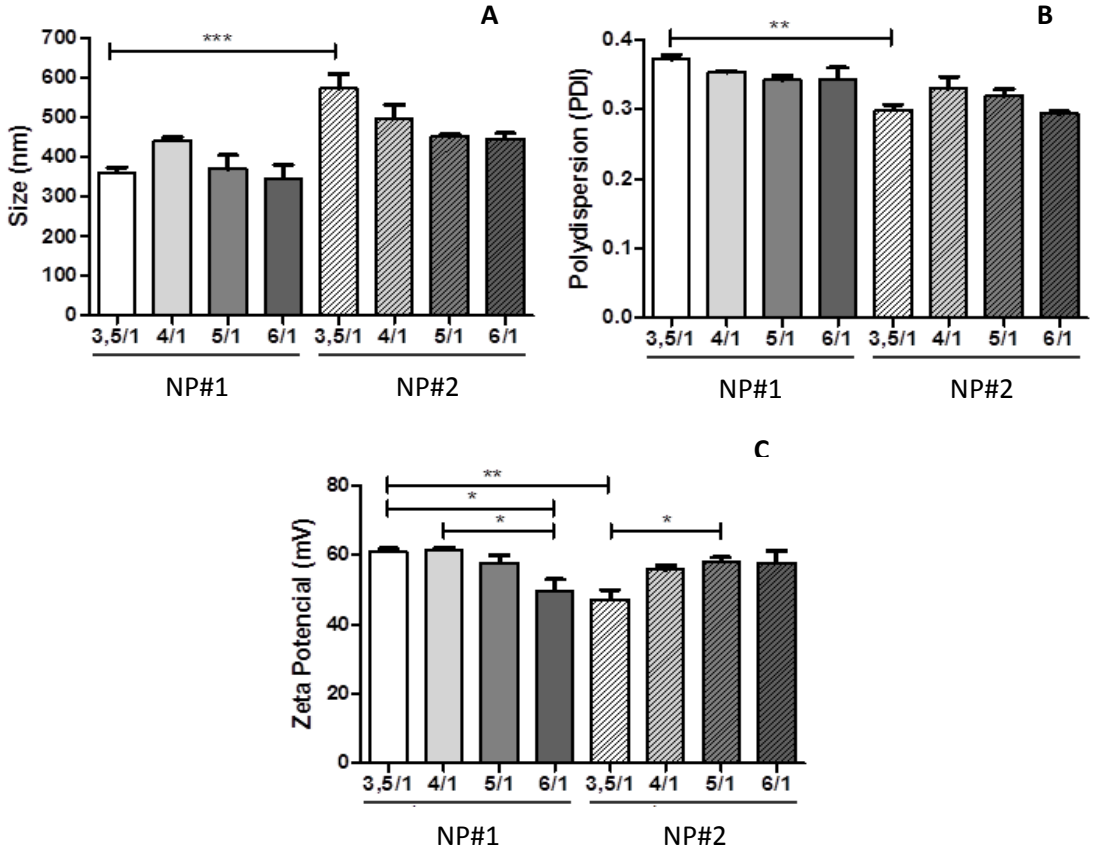


Figure 11. Physicochemical properties of the first (NP#1) and second (NP#2) nanoparticle synthesis. Size (A), polydispersion (B), zeta potential (C). Statistical significance is indicated, P<0.05 (*), P<0.01 (**) and P<0.001(***).

Based on these results, the Chit:Carr 3.5/1 and 5/1 ratios were chosen for the third synthesis (NP#3) including or not the encapsulation of TGMP/HEPES. This choice is justified because, when using the Chit:Carr 3.5/1 ratio, the NPs synthesized showed significant differences in size, PDI and zeta potential when comparing NP#1 and NP#2. Considering that the Chit:Carr 5/1 ratio led to a significant increase of zeta potential compared to the Chit:Carr 3.5/1 ratio in synthesis NP#2, it was also decided to include it in the NP#3 synthesis to better confirm the results achieved.

The agitation step was chosen for this synthesis (NP#3) procedure, since the range of NPs sizes were above the value described to stimulate an immune response, and we intend to keep the protocol as simple as possible. The variable introduced was the half volume of the Chit and Carr preparations used during synthesis.

Regarding the size of the NPs obtained in NP#3, there was a significant decrease from both ratios (3.5/1 and 5/1) compared to NP#1, which indicates that the reduced polymer volumes decreased the size of the NPs synthesized.

There were no significant differences between the non-encapsulated NPs (control) and the NPs encapsulated with TGMP/HEPES. This data was not expected since according to the literature when adding the protein to the NPs there is an effect of reticulation of the NPs induced by the protein presence (147) and the size tends to decrease.

In NP#3, is observed a significant increase in the NPs PDI when using the Chi:Carr 3.5/1 ratio plus TGMP 6% compared to the control. A significant increase of PDI is observed when using the Chit:Carr 5/1 ratio in NP#3 compared with the same ratio in NP#1. This might be explained by the lower volumes of Chit and Carr used in the NP#3.

Regarding the zeta potential, there were no significant differences between NP#1 and NP#3 syntheses, nor within each synthesis. Indeed, the developed NPs show physical colloidal stability since the zeta potential is far above the +30 mV, which is a good feature in NPs intended for vaccination.

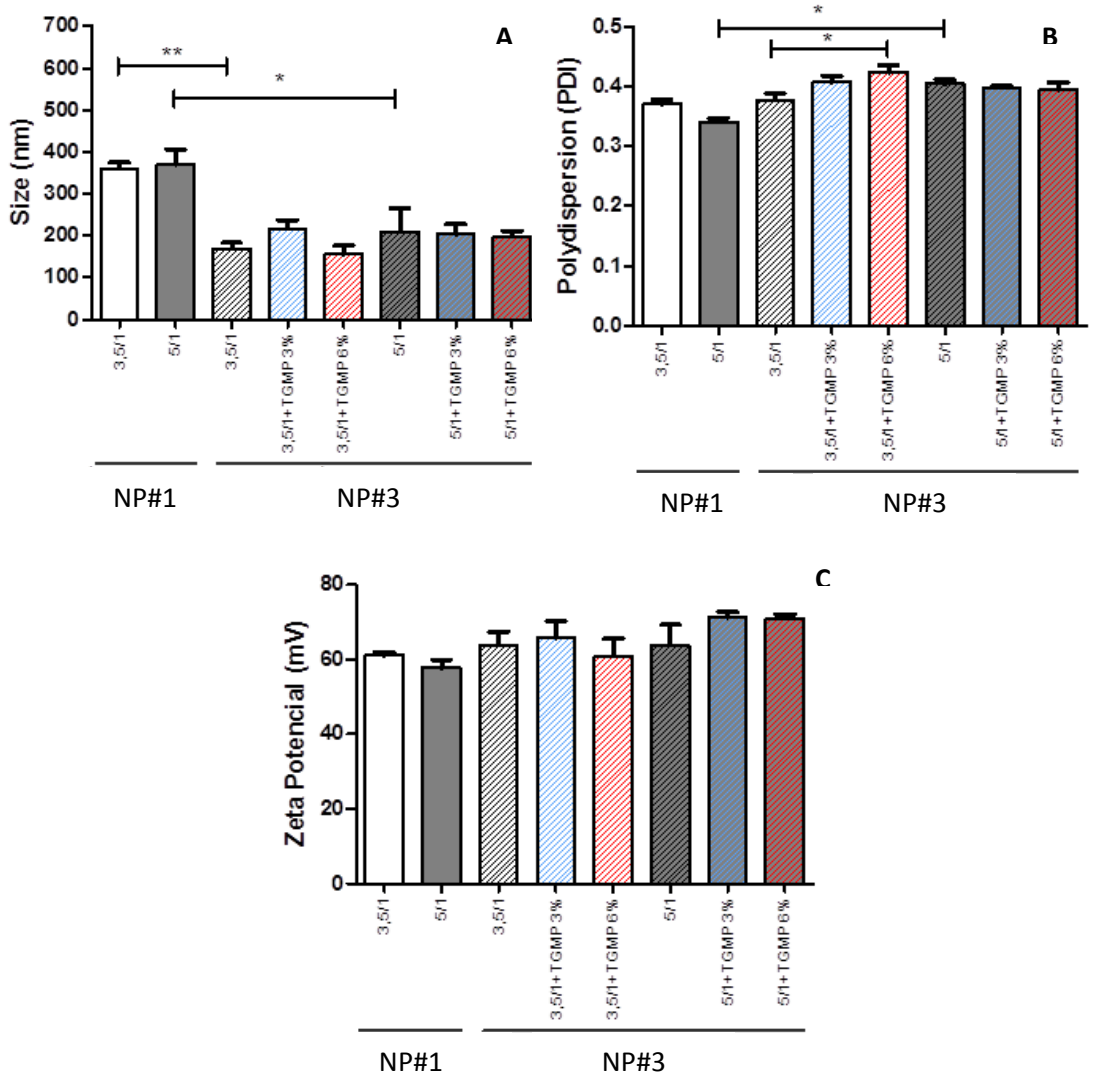


Figure 12. Physicochemical properties of the first (NP#1) and third (NP#3) nanoparticle synthesis. Size (A), polydispersion (B), zeta potential (C). Statistical significance is indicated, P<0.05 (*) and P<0.01 (**).

5. Encapsulation efficiency

To verify the EE of 3.5/1 and 5/1 Chit:Carr NPs ratios in NP#3, a centrifugation step was performed, and the amount of free (non-encapsulated) protein was quantified in the supernatant.

Since TGMP is in suspension (as it is not completed solubilized, even using the 25 mM HEPES solution), there is a strong possibility that some part of it will end up depositing under the used centrifugation conditions. To check the percentage of protein that ends up depositing, two solutions were prepared with the same amount of protein added to the NPs in NP#3: TGMP 3% and TGMP 6%. A percentage of deposition of 38.1% and 69.8% was verified, respectively (Table 6).

The EE was 61.9 and 23.2% for the 3.5/1 TGMP 3% and TGMP 6% ratio, respectively. While for the 5/1 TGMP 3% and TGMP 6%, the EE was 61.9 and 30.2%, respectively (Table 6).

Table 5. Deposit percentage of different concentrations of free TGMP after centrifugation and percentage of encapsulation efficiency (%EE) for the different chitosan/carrageenan ratios and different TGMP concentrations.

	[TGMP] before centrifugation (mg/ml)	[TGMP] after centrifugation (mg/ml)	% TGMP Deposition	% EE
TGMP 3%	0.021	0.013	38.1	-
TGMP 6%	0.043	0.013	69.8	-
3.5/1 + TGMP 3%	0.021	0.000	-	61.9
3.5/1 + TGMP 6%	0.043	0.003	-	23.2
5/1 + TGMP 3%	0.021	0.000	-	61.9
5/1 + TGMP 6%	0.043	0.000	-	30.2

[TGMP]: concentration of TGMP/HEPES determined by the Lowry method. Centrifugation at 11.500 x g during 30 minutes. The [TGMP] after centrifugation was quantified in the supernatant. % EE was determined by the formula: $100 - \left(\frac{\text{Free amount of protein x 100}}{\text{Total amount of protein}}\right) - \%\ Deposit.$

The results revealed that the % of EE for the Chit:Carr 3.5/1 ratio plus TGMP 6% concentration was already above what this ratio is capable of encapsulating, since some of the TGMP added to this formulation (0.003 mg/ml) was quantified in the supernatant after centrifugation, which means that it was non-encapsulated.

When incorporating 3% of TGMP the EE for both ratios is 61.9%. However, when incorporating 6% of TGMP the EE was much lower. Therefore, the concentration of 3% of TGMP will be selected for future synthesis.

Other methods can be used for better EE quantification, such as the ultrafiltration-based separation method (165) in order to efficiently separate the NPs from the free TGMP. This method will also be tested in our future studies.

6. MTT analysis

To determine the effect of the different formulations on cell viability, the cell line HFF was used. The cells were treated with non-encapsulated NPs, Chit:Carr 3.5/1 NPs encapsulated with TGMP 3% or TGMP 3%. Five dilutions of each formulation (1/2, 1/10, 1/50, 1/100 and 1/500) were tested. As is shown in Figure 13, a significant reduction in cell viability was observed for the treatment with the Chit:Carr 3.5/1 ratio NPs, the Chit:Carr 3.5/1 ratio NPs plus TGMP 3% and TGMP 3% at the 2-fold, 50-fold and 100-fold dilutions. A significant reduction in the cell viability was observed after treatment with a 10-fold dilution of Chit:Carr 3.5/1 ratio NPs but not with Chit:Carr 3.5/1 ratio NPs plus TGMP3% or TGMP 3%. The 500-fold dilution of the different formulations had no effect on cell viability.

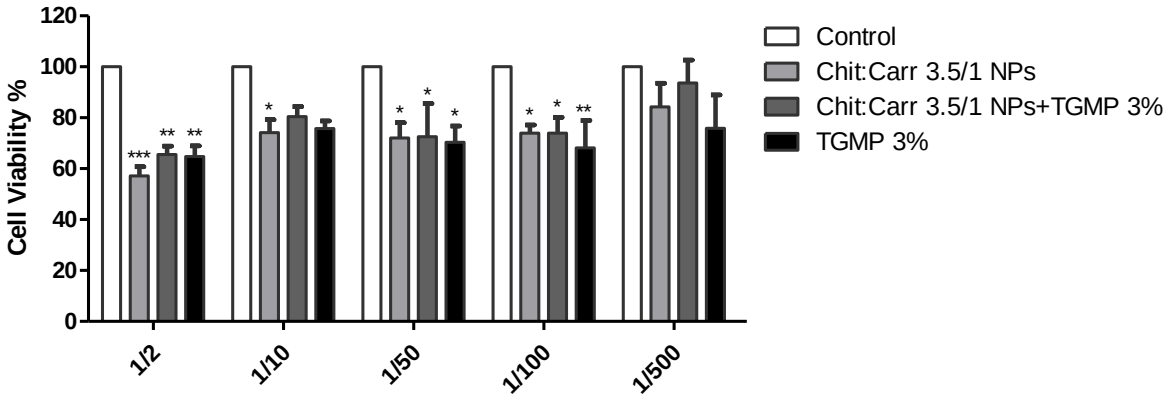


Figure 13. Evaluation of HFF cell viability by MTT assay. Cells were treated with Chit:Carr 3.5/1 NPs, Chit:Carr 3.5/1 NPs plus TGMP 3% or TGMP 3% at different dilutions. Cells untreated corresponding to the control group. Statistical significance is indicated, P<0.05 (*), P<0.01 (**) and P<0.001(***).

Since the cell viability values of the cells treated with the lowest dilution of the solutions (1/2) were still above 50%, it was not possible to calculate the IC_{50}. Future experiments will allow the IC_{50} determination by testing further dilutions.

Chapter IV – Conclusion

Regarding the cell culture and parasite *in vitro* infection, the second method was the most effective to obtain a better yield of parasites, and the 75 cm^2 cell culture flasks were also the most profitable.

The parasite load quantification showed partial protection after immunization with 5 µg ODN plus 30 µg TGMP/PBS compared to the control. A significant reduction was observed in PEC and lung. A tendency to decrease parasite load was observed in the other organs, although not significant due to the small number of animals used. Future experiments using n=8 will be done to verify whether this trend represents a significant reduction. When analysing the number of animals whereas was possible to detect the parasite it was observed a higher level of protection in the kidney, heart and in the lung of the mice immunized with TGMP/HEPES compared with the mice immunized with the TGMP/PBS.

Considering the level of parasite load, PEC and spleen were the ones with the higher levels, followed by the kidney, lung, liver and heart. The most affected organ after PEC was the spleen, followed by the kidney and the heart.

There was no effect on the parasite load when using TGMP in PBS or HEPES. Moreover, the protein profile of the TGMP in PBS and in HEPES was similar, both with intense bands at 29 kDa. In future studies, other reagents such as Triton X-114 or urea, or the employment of other TGMP solubilisation methods must be tested in order to obtain a homogeneous solution and increase the EE.

The ODN concentration seems to play an important role in the effectiveness of the immunization. Further experiments must be done to better confirm the results obtained.

The main goal of this work is to develop an effective nanoparticle formulation in the protection against *T. gondii* infection using a low amount of TGMP and ODN. The advantages will be important since ODN is an expensive adjuvant and the use of ODN plus TGMP only induces partial protection.

Regarding the optimization of Chi:Carr NPs synthesis, smaller amounts of polymers lead to the formation of smaller particles, and the optimized protocol allowed to obtain particles with physical colloidal stability and with a size suitable for vaccination use. However, further adjustments should be done to the protocol to reduce the PDI, since some of the obtained values were above the recommended, and a polydisperse solution has a greater tendency to aggregation compared to a monodisperse solution. Stability tests should be carried out as well, to assess the effect of time and temperature in NPs physical-chemical nature and to determine the best conditions to preserve it.

The incorporation of 3% of TGMP in the NPs led to the highest EE compared with TGMP 6%. However, the results were estimated considering the deposition of the free TGMP. To confirm these results, other methods to separate the NPs from the free TGMP, such as the use of a chromatographic column, should be tested to calculate with precision the EE of the NPs.

Cell viability results indicate that there is no significant toxicity for the 1/500 dilution of the Chi:Carr 3.5/1 NPs or Chit:Carr 3.5/1 NPs plus TGMP 3% and the TGMP 3% compared to untreated cells. The effect of the treatments is dependent on concentration considering the 2-fold and 500-fold dilutions.

Overall, this study was important as a preliminary analysis in the evaluation of the efficacy of TGMP/PBS and TGMP/HEPES against *T. gondii in vivo* infection. There appears to be no difference in parasite load when PBS or HEPES is used in the TGMP suspension. The data obtained regarding the optimisation of the synthesis of Chit and Carr polymeric NPs plus TGMP is certainly the beginning of very interesting work in the development of an effective vaccine against toxoplasmosis. Future *in vitro* and *in vivo* experiments will make it possible to study the immunogenic capacity of these polymeric NPs and their ability to lead to effective protection against the establishment of infection avoiding toxoplasmosis.

Chapter V. References

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