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Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob a orientação da
Doutora Ana Paula Fernandes Monteiro Sampaio Carvalho
e da
Doutora Célia Sacramento Santos Pais

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Declaração

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Incorporation of *Candida albicans* Cell Wall Surface Proteins into Lipid Vesicles: Physicochemical Characterization and in vitro Cytotoxicity

Incorporação de Proteínas da Superfície da Parede Celular de *Candida albicans* em Vesículas Lipídicas: Caracterização Físico-química e Avaliação da Citotoxicidade in vitro

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Incorporation *of Candida albicans* Cell Wall Surface Proteins into Lipid Vesicles: Physicochemical Characterization and In vitro Cytotoxicity

Abstract

Over the past two decades, *Candida* species have become the fourth most common cause of bloodstream infections with mortality rates higher than 50%. A significant improvement has been made in chemotherapy for candidiasis with the availability of new azole derivatives and inhibitors of glucan synthase. However, the mortality rate for invasive candidiasis has remained stable. These observations underline the urgent need for the development of novel approaches to fight fungal infections. *Candida albicans* cell wall has in its composition cell wall surface proteins (CWSPs), which are important targets for the host immune system. Therefore, the present study was developed in order to compare two distinct lipid systems, one constituted by phosphatidylethanolamine (PE) and another composed by DODAB:MO, regarding their ability to incorporate antigenic CWSPs for a future development of a safe and effective vaccine against *C. albicans*.

After the successful incorporation of the CWSPs into lipid vesicles three characteristics were analyzed: average size of the lipid-CWSPs vesicles, their polydispersity index (PDI) and incorporation efficiency (IE). The PE-CWSPs vesicles presented average sizes between 268.9 nm and 492.4 nm and PDI values around 0.6. The MO, a neutral lipid, was included in the PE vesicles and the average size increased to 625.3 nm with PDI values of 0.5. DODAB:MO-CWSPs vesicles average size ranged between 250.9 nm to 732.5 nm and the PDI values were considerably lower, around 0.3 to 0.5. After optimization of the delipidation method IE was determined and PE-CWSPs presented values ranging from 16.5% to 42.3%, whether PE:MO-CWSPs vesicles presented lower values, around 25%. The DODAB:MO-CWSPs vesicles presented higher IE values, between 44.4% and 50,5%. Three formulations, PE (0.6mM)-CWSPs, DODAB:MO (0.1mM)-CWSPs and DODAB:MO (0.3mM)-CWSPs were chosen, from a total of eight, according to its size, PDI value and incorporation efficiency, to be tested for in vitro cytotoxicity and no significant cytotoxicity was observed.

With the information collected in this work both systems, PE-CWSPs and DODAB:MO-CWSPs, were considered to be suitable for the development of a vaccine against *C. albicans* infections.

Incorporação de Proteínas da Superfície da Parede Celular de *Candida albicans* em Vesículas Lipídicas: Caracterização Físico-química e Avaliação da Citotoxicidade in vitro

Resumo

Ao longo das últimas décadas, as espécies do género *Candida* tornaram-se a quarta causa mais frequente de infeções sistémicas com taxas de mortalidade superiores a 50%. A quimioterapia contra candidíase tem melhorado significativamente com a disponibilização de novos derivados azólicos e inibidores da sintase dos glucanos. Contudo, a taxa de mortalidade de candidíase invasiva tem-se mantido estável. Estes estudos apontam para a necessidade urgente de desenvolver novas estratégias contra as infeções fúngicas. A parede celular de *C. albicans* contem proteínas à sua superfície (CWSPs), que são alvos importantes para o sistema imune. Desta forma, este estudo foi desenvolvido no sentido de comparar dois sistemas lipídicos distintos, um constituído por fosfatidiletanolamina (PE) e outro composto por DODAB:MO, no que respeita à capacidade para incorporar as CWSPs, para o futuro desenvolvimento de uma vacina segura e eficiente contra *C. albicans.*

Após incorporação das CWSPs nas vesículas lipídicas foram analisadas três características: o tamanho médio das vesículas lipídicas, o seu índice de polidispersividade (PDI) e a sua eficiência de incorporação (IE). As vesículas PE-CWSPs apresentaram tamanhos médios entre 268,9 nm e 492,4 nm e valores de PDI entre 0,6. O lípido neutro MO foi incluído nas vesículas de PE e o tamanho destas aumentou para 625,3 nm com valores de PDI à volta de 0,5. As vesículas de DODAB:MO-CWSPs apresentaram tamanhos entre 250,9 nm e 732,5 nm e valores de PDI consideravelmente mais baixos, entre 0,3 e 0,5. Depois da otimização do método de remoção dos interferentes lipídicos foi determinada a IE e as vesículas de PE-CWSPs apresentaram valores entre 16,5% e 42,3% enquanto que as vesículas de PE:MO-CWSPs apresentaram valores mais baixos, 25%. As vesículas de DODAB:MO-CWSPs apresentaram valores de IE mais elevados, entre 44% e 50,5%. De um total de oito formulações foram escolhidas três de acordo com o seu tamanho, polidispersividade e eficiência de incorporação, PE (0,6)mM-CWSPs, DODAB:MO (0,1mM)-CWSPs e DODAB:MO (0,3mM)-CWSPs. A citotoxicidade in vitro das formulações selecionadas foi determinada não tendo sido observados valores significativos de citotoxicidade.

Com a informação obtida neste trabalho, ambos os sistemas PE-CWSPs e DODAB:MO-CWSPs foram considerados apropriados para o desenvolvimento de uma vacina contra as infeções provocadas por *C. albicans*.

Table of Contents

List of Abbreviations

AF – Autofluorescence

AIDS – Acquired Immunodeficiency Syndrome

ALS – Agglutinin like sequence

APCs – Antigen Presenting Cells

APCs – Antigen Presenting cells

ATP – Adenosine triphosphate

BCA – Bicinchoninic Acid

BSI – Bloodstream Infections

CBMA – Centre of Molecular and Environmental Biology

CLRs – C-type lectin receptors

CWSPs – Cell Wall Surface Proteins

DCs – Dendritic cells

DLS – Dynamic Light Scattering

DMEM - Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

DODAB - Dioctadecyldimethylammonium bromide

DTT – Dithiothreitol

ECMM – European Confederation of Medical Mycology

FTS – Freeze – Thaw – Sonicate

HFL – Film Hydration

HIV – Human Immunodeficiency Virus

HSCT – Hematopoietic Stem Cell Transplants

ICUs – Intensive Care units

IE – Incorporation Efficiency

IFN-γ – Interferon –gamma

IL-1 – Interleucin 1

IL-12 – Interleucin 12

IL-6 – Interleucin 6

LUVs – Large Unilamellar Vesicles

MBL – Mannose binding proteins

MHC – Major Histocompatibility Complex

MLVs – Multilamellar Vesicles

MO – Monoolein

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MVVs – Multivesicular Vesicles

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced form)

NK – Natural Killer

NNIS – National Nosocomial Infections Surveillance

NO – Nitric Oxide

PAMPs – Pathogen- associated molecular patterns

PBS – Phosphate Buffered Saline

PDI – Polidispersive Index

PE – Phosphatidylethanolamine

PRRs – Pattern Recognition Receptors

RNA – Ribonucleic acid

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

SDS – Sodium Dodecyl Sulfate

SDS PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SUVs – Small Unilamellar Vesicles

T_{EM} – Effector Memory T Cells

TCA – Trichloroacetic Acid

T_{CM}– Central Memory T Cells

TEMED – Tetramethylethylenediamine

Th – T helper cells

TLRs – Toll-like receptors

TNF α – Tumor Necrosis Factor alpha

USA – United States of America

YPD – Yeast Extract, Bactopeptone, Dextrose

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Chapter 1 - General Introduction

1.1 Fungal Infections

Over the last two decades, invasive fungal infections have assumed a great importance, essentially due to the increasing population at risk. This population includes people with human immunodeficiency virus (HIV) infection, recipients of solid organ or hematopoietic stem cell transplants (HSCT), patients with hematologic malignancies, burns or indwelling medical devices and low-birth weight infants. A retrospective study in the United States of America (USA) shows an increase of 207% of cases of sepsis caused by fungal infections: from 5231 cases in 1979 to 16042 cases in 2000 (Martin *et al.*, 2003). In a large pan-European Sepsis Ocurrence in Accutely Ill Patients study it was reported that fungal infection was observed in 17% of all septic patients in European Intensive Care Units (ICUs) (Vincent *et al.*, 2006). Medical progress has led to an expanding population of susceptible hosts with impaired immunological defenses and are at heightened risk for invasive fungal infections including, aspergillosis, cryptococcosis and candidiasis (Warnock, 2007).

Aspergillus fumigatus (*A. fumigatus*) is a saprophytic fungus that is important in recycling environmental carbon and nitrogen (Latge, 1999). Its natural ecological niche is the soil, where it survives and grows in organic debris. In the last years, *A. fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunocompromised hosts in developed countries (Andriole, 1993; Beck-Sague and Jarvis, 1993; Bodey *et al.*, 1993; Dixon *et al.*, 1996; Groll *et al.*, 1996; Denning, 1998; Latge, 1999).

Cryptococcus neoformans (*C. neoformans*) was first reported in Italy by a mycologist who cultured the fungus from peach juice (Sanfelice, 1984), it is also the etiologic agent of cryptococcosis, one of the most serious fungal infection encountered worldwide. Although *C. neoformans* can cause lesion in almost every organ, the most common clinical manifestation, as well as the most common cause of death is meningoencephalitis, an infection in the brain. Despite the most advanced medical treatment, the fatality rate for cryptococcosis is close to 25%. While cryptococcosis sporadically occurs in normal individuals without any underlying condition, this disease is frequently associated with patients with Acquired Immunodeficiency Syndrome (AIDS) (Kwon-Chung and Bennett, 2010).

1.2 *Candida* Infections and Candidemia

A significant cause of infection entails several species of *Candida.* Macroscopically, colonies of *Candida* spp. are cream-colored to yellowish. All species produces blastoconidia and many of them are dimorphic growing as budding yeast cells, pseudohyphae or true hyphae (Eggimann *et al.*, 2003). Figure 1 depicts macroscopic and microscopic features of *Candida albicans* (*C. albicans*) cells.

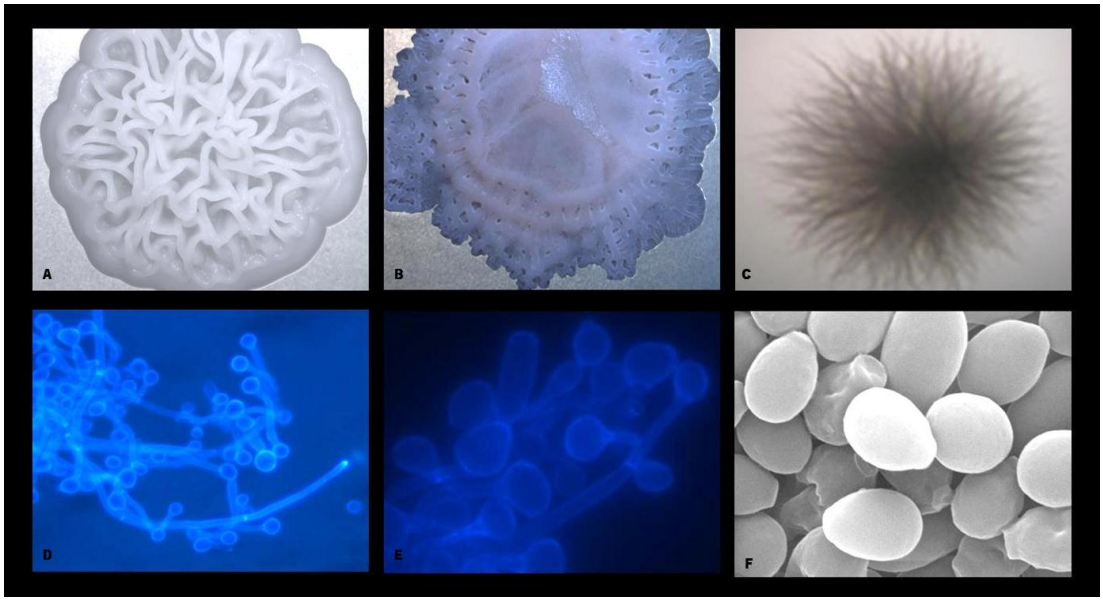

Figure 1- Macroscopic and microscopic features of *C. albicans* cells. Colonies of *C. albicans* cells in different filament inducing media (**A**, **B** and **C**). Fluorescence micrograph of calcofluor white stained *C. albicans* cells (**D** and **E**). Electronic Micrograph of *C. albicans cells* (**F**).

In healthy individuals, *Candida* species belong to the normal microbial flora of skin and mucosal surfaces namely oral cavity, gastrointestinal tract and the vagina (Mavor *et al.*, 2005). As commensals, *Candida* species are harmless, but if the balance of the normal flora is disrupted or the immune defenses are compromised, these fungi can outgrow and cause symptoms of disease, and consequently, two main types of infections can be observed: mucocutaneous or invasive candidosis.

There are several risk factors for *Candida* infections, such as prior colonization with *Candida* species; HIV infection; cancer chemotherapy; neutropenia; organ transplantation; indwelling catheters and devices; autoimmune diseases; burn; antimicrobial therapy; age, gender, abdominal surgery and perforation, polytrauma, heart disease, pulmonology,

radiotherapy, rheumatology and any therapy involving prolonged exposure to steroid drugs (Eggimann *et al.*, 2003; Mavor *et al.*, 2005; Sabino *et al.*, 2010)

In the USA, the National Nosocomial Infections Surveillance (NNIS) program showed that incidence of primary *Candida* bloodstream infections (BSI) has increased by as much as 487% over the decade of the 1980's in large teaching hospitals (Vincent *et al.*, 1998). The countries participating in the European Confederation of Medical Mycology (ECMM) survey reported candidemia rates (0.31 to 0.44 per 10 000 patients-days) lower than the rates reported in the USA (1,5 per 10 000 patients-days) (Tortorano *et al.*, 2006). Invasive candidiasis accounted for 17% of hospital-acquired infections reported during the "European Study on the Prevalence of Nosocomial Infections in Critically Ill Patients" and candidemia represented 10-20% of all candidiasis (Beck-Sague and Jarvis, 1993; Jarvis, 1995; Pfaller, 1996). This genus is now classified as fourth on the list of nosocomial agent of sepsis (Wisplinghoff *et al.*, 2004) and is associated with high morbidity and mortality. In patients hospitalized in ICUs and hospital wards, and those undergoing transplantation, the mortality attributed to candidemia ranged from 5 to 71% (Falagas *et al.*, 2006).

1.3 Cell Wall and Cell Wall Surface Proteins (CWSPs)

C. albicans is a dimorphic organism, capable of existing as yeast or in a filamentous hyphal state. Interest in the *C. albicans* cell wall and associated proteins derives from basic research into the dimorphic nature of the organism and the potential of using cell wall targets, in particular cell wall proteins, in drug development, since mammalian cells lack a cell wall (Ebanks *et al.*, 2006). The cell surface has two important roles: to maintain the integrity of the cell and to interact with the environment. Regarding its composition (Figure 2A and B) the cell wall has a flexible, three-dimensional network of β-1,3-glucan to which β-1,6-glucan and chitin are attached. The cell wall surface proteins (CWSPs) covalently attached to these two structural fibrillar polysaccharides can be divided in two classes. The first and most abundant class is linked to β-1,6-glucan through a glycophosphatidylinositol (GPI) remnant. The second class of proteins named Pir (proteins with internal repeats) are linked directly to β-1,3-glucan. Another class of proteins lacks the covalent attachment to the polysaccharide matrix and they may be heterogeneously distributed at the surface or secreted into the external milieu. "Atypical" proteins, such as succinate dehydrogenase (Sdh2) and pyruvate kinase (Cdc19), that were

previously identified in the cytosol and in other organelles have also been identified as belonging to the cell wall, however they lack the characteristics that are present in the "true" cell wall proteins: predicted signal peptide, potential O- and N- glycosylation site, GPI signature and series of internal repeats (Castillo *et al.*, 2008). Phospholipomannan is also present in the cell wall with α-1,2-mannose linkages (Chaffin, 2008).

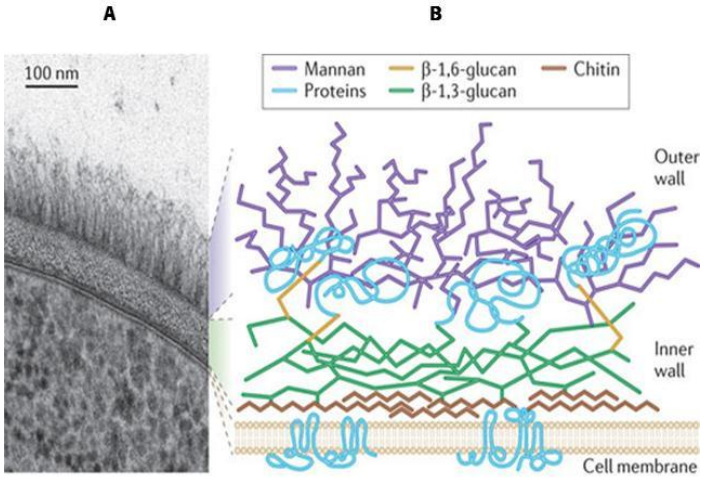

Figure 2- Structure of *C. albicans* cell wall. **A.** Electron Micrograph and **B**. Schematic representation of the cell wall of *C. albicans* (Gow *et al.*, 2012).

CWSPs are essential for the life of fungi and particularly in the dimorphic fungus *C. albicans.* They are important in several roles including: enzymatic, cell interaction, antigenicity, pathogenicity, wall structure and morphogenesis (Ruiz-Herrera *et al.*, 2006). Several proteins show enzymatic activities that are important in the degradation of complex molecules used as nutrients, while others are involved in the cleavage of the protective superficial structures of host cells and tissues enabling invasion. Proteins such as adhesins and lectins are important to allow the attachment of *C. albicans* cells to different host surfaces. Additionally, other enzymes are involved in the assembly of β-1,3 and β-1,6-glucans, and chitin. Specific cell wall components act as antigens or immunomodulators, whose effects can alter the balance between the fungus and its host (Castillo *et al.*, 2008). CWSPs are very immunogenic molecules and are overexpressed during pathogenesis in human disseminated candidiasis, which indicates that induced antibodies will recognize the yeast cells. They are very conservative and exposed on the surface of the yeast cell and present themselves as important targets for the host immune response (Xin *et al.*, 2008).

1.4 Host Immune Response

During the infection process the fungus has to face different infection stages such as entering the bloodstream, surviving in the blood and escaping from the bloodstream to deep-seated organs. There are two main routes of entry of *Candida* cells into the circulatory system: (i) the natural route which entails penetration through epithelial cells from mucosal surfaces into deeper tissues and blood capillaries or vessels and (ii) iatrogenic routes that include the use of medical devices and the ones caused by damaged barriers (Mavor *et al.*, 2005).

Effective host immune response against fungal organisms requires the coordinated contribution of both innate and humoral immunity (Antachopoulos and Roilides, 2005). The innate immune system confers rapid recognition of microbial agents and gives non-specific, broad spectrum responses. Physical barriers (skin, mucous membranes) are the first line of defense conferred by innate immunity and have antimicrobial substances on their surface. The skin and mucous membrane have a commensal microflora of saprophytic microorganisms that obstruct colonization by pathogenic microorganism. These barriers secret a group of peptides also called chemotactic factors that include leukotrienes and chemokines which can also be produced by leukocytes, endothelial cells, fibroblasts and smooth muscle cells after stimulation by cytokines (Blanco and Garcia, 2008). These chemotactic factors are critical factors in the defense once they enhance the effective recruitment of leucocytes to the site of infection (Blanco and Garcia, 2008).

When fungi overcome physical barriers they have to face cellular barriers. According to Heindenreich (Heindenreich, 2010) there are several types of effector cells that protect the body and provide immunity. Among them are neutrophils, natural killer cells (NK), dendritic cells (DCs) and macrophages that are essential for protection against fungal pathogens. Loss or defects on these cells in their fungal effector functions result in susceptibility of the host (Blanco and Garcia, 2008; Brown, 2011). Neutrophils are classically characterized by their ability to act as phagocytic cells, to release lytic enzymes from their granules and to produce reactive oxygen intermediates with antimicrobial potential (Mantovani *et al.*, 2011). NK cells are effector lymphocytes of the innate immune system that control microbial infections by limiting their spread and subsequent tissue damage and are regulatory cells engaged in reciprocal interactions with DCs, macrophages, T cells and endothelial cells (Vivier *et al.*, 2008). DCs are professional antigen-presenting cells (APCs) and are efficient stimulators of B

and T lymphocytes (Banchereau and Steinman, 1998). Forman and Torres (Forman and Torres, 2001) define macrophages as phagocytic cells that produce and release reactive oxygen species (ROS) in response to phagocytosis or stimulation with various agents. Macrophages play different roles in immunity once they are highly efficient in clearing pathogens and dying cells, and also in antigen presentation to primed T-cells. Macrophages can also secrete cytokines that affect migration and activation of other immune cells and reactive metabolites (Heinsbroek and Gordon, 2007). To eliminate pathogens, macrophages like other cells from the immune system, need to be able to distinguish self from non-self in either a direct or indirect way (Gordon and Taylor, 2005). The direct innate recognition of fungi by phagocytes is achieved mostly through the sensing of cell wall constituents and sometimes of internal compounds by Pattern Recognition Receptors (PRRs).

Since Medzhitov (Medzhitov and Janeway, 1997) proposed the concept of pattern recognition, several PRRs have been identified and they recognize the so called pathogen-associated molecular patterns (PAMPs). Figure 3 represents the PRR sensing *C. albicans* at the membrane level and the proinflammatory responses. The first PRRs discovered to recognize *C. albicans* were the Toll-like Receptors (TLRs) with TLR2 recognizing phospholipomannan, while TLR4 recognize O-linked mannan (Cheng *et al.*, 2012). The second major PRR family that recognizes *Candida* PAMPs is the C-type lectin receptors (CLRs). β-glucans are recognized by dectin-1 and the N-linked mannan is recognized by the macrophage mannose receptor. DC-SIGN is another important receptor cells that recognizes *Candida* mannan on the dendritic cells. Galectin-3 is important for the recognition of *C. albicans* β-mannosides and mannose-binding lectin (MBL) mediates *Candida* cells opsonization and uptake binding *Candida* cells to the receptor on the phagocyte (Netea and Marodi, 2010).

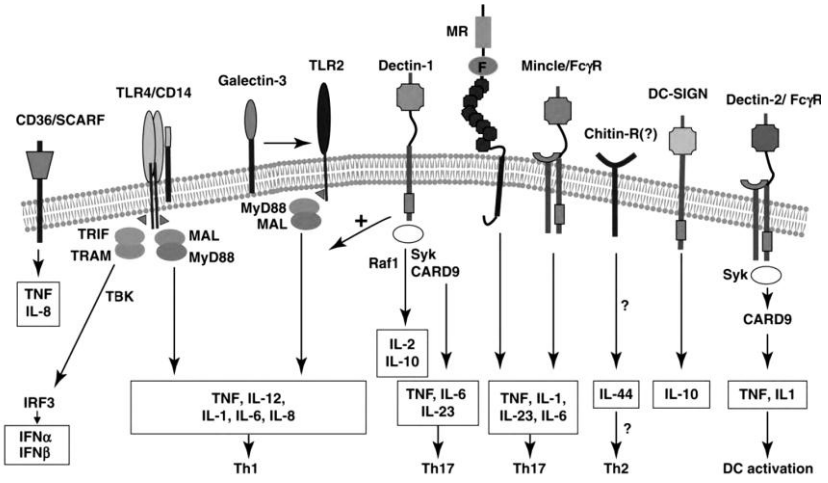

Figure 3 – PRR sensing *C. albicans* at the membrane level (Netea and Marodi, 2010).

The indirect recognition of the microorganism can be by coating and opsonization of the pathogen, allowing the recognition through membrane-bound opsonic receptors (Brown, 2011). In the case of *C. albicans,* MBL is an opsonin that binds mannans in the cell wall both in vivo and in vitro, leading to complement deposition via lectin pathway and subsequent phagocytosis (Lillegard *et al.*, 2006). Following internalization, the phagosomes matures through several sequential steps involving fusion and fission events, mostly with components of the endossomal network, leading to the formation of the phagolysossome, where the pH drops and antimicrobial molecules kill and digest the fungus (Brown, 2011). In addition to being important for intracellular killing, phagocytosis deliver signals for subsequent regulatory and microbicidal functions of the phagocytes (Vazquez-Torres and Balish, 1997). The process of phagocytosis is represented in Figure 4.

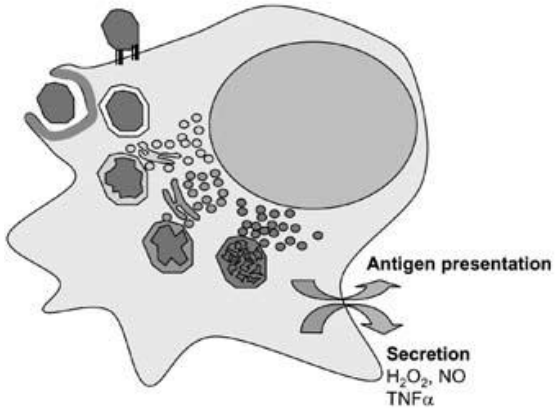

Figure 4– Schematic representation of phagocytosis (adapted from (Heinsbroek and Gordon, 2007).

Macrophages need to be activated to mobilize those potent microbicidal mechanisms that eliminate the intracellular pathogen. According to Gordon and Taylor (Gordon and Taylor, 2005) monocytes are recruited and differentiated into macrophages at the site of inflammatory lesion. Then they are activated, which is critical for stimulation of candidacidal activity. Of all cytokines that activate macrophages IFN-γ is considered an excellent stimulator of the capacity of macrophages to kill *C. albicans*. The enhanced killing of *C. albicans* coincides with the activation of multiple oxygen-dependent and independent mechanisms (Vazquez-Torres and Balish, 1997). ROS such as O_{2^-}, H_2O_2 are generated both enzymatically by oxidoreductases and non-enzymatically as side products of reactions that use electron transfer. Reactive Nitrogen Species (RNS) are also produced by macrophages to enhance killing in which the main

example is Nitric Oxide (NO) which is enzymatically produced by nitric oxide synthases through the oxidation of L-arginine in the presence of oxygen and NADPH (Forman and Torres, 2001). Figure 5 summarizes the reactions involved in the generation of ROS and RNS:

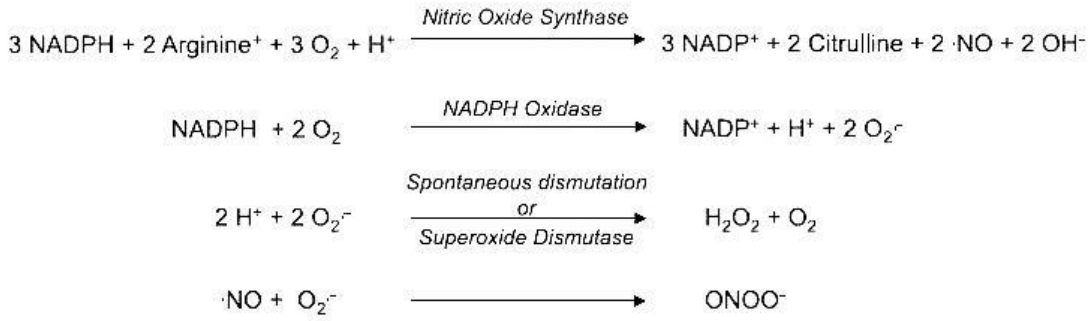

Figure 5 – Generation of ROS and RNS by macrophages (Forman and Torres, 2001).

C. albicans cells have been shown to be susceptible to some oxygen-dependent killing mechanisms of mononuclear phagocytic cells: $O_{2}^{\cdot-}$ anion, hydrogen peroxide-halide system, and RNS (Vazquez-Torres and Balish, 1997). Phagocytic cells also have nonoxidative mechanisms that help in the destruction of the fungus, namely the production of anti-microbial peptides such as α-defensins that bind to specific sites on the fungal membrane, inducing nonlytic permeabilization and the release of intracellular component such as ATP (Vylkova *et al.*, 2007). Hydrolases, lysozyme, collectin and lactoferrin also play an important role against pathogens. The antimicrobial activity of these proteins is related to lysis or opsonization of the pathogen (Jenssen and Hancock, 2009). According to Brown (Brown, 2011) the activation of these proteases by respiratory burst is a primary mechanism of microbial killing. Other antifungal mechanism is the limitation of nutrients. This is achieved by restraint of the ingested pathogen within the phagosomes and by active restriction of nutrients: limiting iron, sequestration of zinc among others.

As an APC the macrophage is able to load degraded material from the endocytic pathway, particularly from phagocytosis, onto Major Histocompatibility Complex Class II (MHC II) molecules for antigen presentation to primed T cells leading to T-cell activation (Brown and Netea, 2007). The activated T-cells produce IFN-γ which activates macrophages, increasing their antigen presentation (Brown and Netea, 2007). Once the antigen is eliminated, central memory (T_{CM}) and effector memory (T_{EM}) T cells persist in the memory pool to provide systemic

immune surveillance in lymphoid organs and in peripheral non-lymphoid tissues to react promptly in case of re-attack.

There is another barrier against fungi known as humoral barrier. It includes natural occurring antibodies and the complement system, the last refers to a group of proteins that interact in different pathways to assist immune cells in destroying foreign invaders (Heidenreich, 2010). According to Blanco and Garcia (Blanco and Garcia, 2008) humoral immunity can protect against fungal infections if certain types of protective antibodies are available in sufficient quantity. The main recognized functions of antibodies are antibody dependent cellular toxicity, prevention of adherence, antibody opsonization and toxin neutralization (Blanco and Garcia, 2008).

In general the innate immune system includes all aspects of the host's immune defense mechanism that are encoded in their mature functional forms by the germ-line genes of the host. In contrast to innate immunity, acquired immunity requires recognition of specific foreign molecules and develops memory T cells (Deepe, 1997). Fungal antigens are collected and processed by dendritic cells that transport them to draining lymph nodes where the antigens are presented to T helper and regulatory T cells. T helper cells give either Th1 (protective) cytokines such as TNF-α, IFN-γ, IL-1, IL-6 and IL-12 or Th2 (nonprotective) cytokines (IL-10, IL-4 and IL-5). TNF-α is an important cytokine against fungi and stimulates the antifungal activity of neutrophils and macrophages and also polarizes T helper cells towards a Th1 response (Ben-Ami *et al.*, 2008). More recently, Th17-type adaptive immunity was implicated in the control of fungal infections, particularly at the mucosa level, although this may be relevant only at specific mucosal sites and for specific pathogens (Brown, 2011).

Knowing the crucial role of the host's immune response in controlling invasive fungal infection the development of immunomodulation strategies is important to fight fungal infections (Ben-Ami *et al.*, 2008).

1.5 Antifungal Strategies

The advancements of medicine, surgery and transplantology in the last thirty years have caused dramatic increase in the number of immunocompromised individuals who are more susceptible to fungal infections. On the other hand, the pharmacologic treatment of invasive fungal infections has been mainly performed with only four antifungal drugs

(amphotericin B, flucytosine, fluconazole and caspofungin) which highlight the fact that the available antifungal agents are still very limited compared to antibiotics. The new azole derivatives and inhibitors of glucan synthase promised to improve the efficacy of antifungal prophylaxis and treatment in patients at risk and in refractory or resistant cases, but despite the introduction of these new agents the mortality rate for invasive candidiasis remained stable from 1997 to 2007 (Pfaller *et al.*, 2010).

Generally, the antifungal procedures are based on the differences between fungal and mammalian cells. The classes available include (Dan and Levitz, 2006):

- Amphotericin B (a polyene) which binds to ergosterol in the membrane, forming transmembrane channels through which intracellular molecules leak;
- Azoles, such as fluconazole, itraconazole, voriconazole, posaconazole, and allylamines (terbinafine), which target ergosterol enzymatic pathway, inhibiting its synthesis;
- Echinocandins, such as caspofungin and micafungin, which target and inhibits synthesis of the cell wall polysaccharide, β-1,3-glucan;
- Flucytosine that is an anti-metabolite of DNA and RNA synthesis.

Figure 6 summarizes the mechanisms of action of these antifungal drugs.

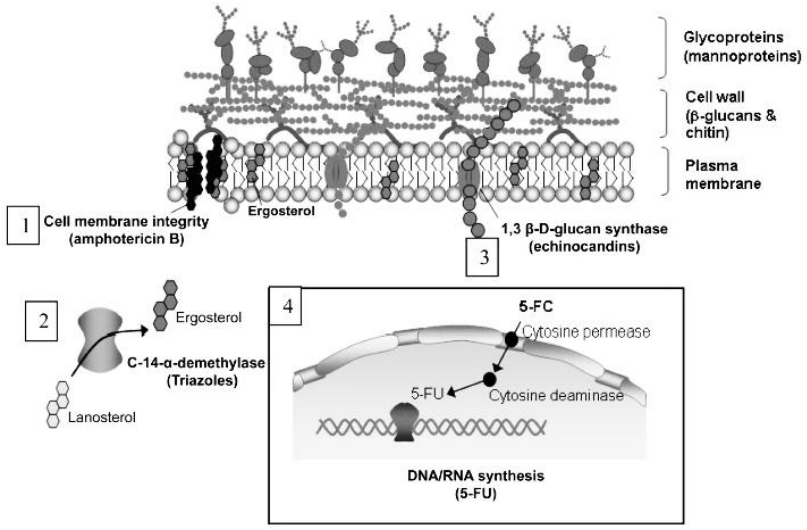

Figure 6– Mechanisms of action of antifungal drugs. (**1**) polyenes (amphotericin B) that disrupt membrane integrity; (**2**) triazoles that inhibit the key enzyme of ergosterol synthetic pathway; (**3**) echinochandins that inhibit the enzyme 1,3 β-D.glucan synthase; (**4**) 5-Flucytosine (5-FC) that is converted in 5-fluorouracil (5-FU) which is converted in metabolites that inhibit fungal DNA and RNA synthesis (Ben-Ami *et al.*, 2008).

However, according to the same author, success rates for many mycoses remain unacceptably low and drug therapy is often limited by toxicity, resistance and cost. Therefore,

this urges the need for the development of prophylactic and/or therapeutic vaccines effective against fungal infections (Torosantucci *et al.*, 2005).

Vaccination of high-risk groups is a particularly promising strategy to prevent invasive fungal infections because easily identifiable risk factors are clearly defined for these infections. These risk factors precede infections which afford a window of opportunity to vaccinate acutely at risk patients before the onset of the infection. One major concern about vaccinating such patients against invasive fungal infections has been the assumption that the immune system of patients at risk for these infections is too weak to respond to vaccination. However, only 10-20% of patients who develop candidemia are seriously immunocompromised (Spellberg, 2011).

Several studies have identified *Candida* molecules as antigen suitable for vaccine strategies such as ALS (agglutinin like sequence) proteins, or cell wall proteins such as Hyrp, or even other cell wall components such as β-glucan, using other proteins carriers or adjuvants to enhance activation (Spellberg *et al.*, 2006; Luo *et al.*, 2010; Pietrella *et al.*, 2010). According to Henrisken-Lacey and colleagues (Henriksen-Lacey *et al.*, 2011) vaccines constituting highly purified peptide or protein antigens are at the vanguard of vaccine research due to some important characteristics such as safety, relatively easy scale-up production and low production cost as compared with the live-attenuated or killed vaccines. However, they also possess some drawbacks that need to be taken into account: poor immunogenicity owing to the lack of PAMPs and rapid degradation which reduces the dose-effectiveness of such vaccines. These disadvantages raise the need for the use of new delivery systems. In the last ten years a significant amount of research has focused on using liposomes not only as antigen delivery system but also as a tool to increase immunogenicity of peptide and protein antigens (Henriksen-Lacey *et al.*, 2011).

1.6 Vesicular Systems

Vesicles are nano/microparticulate colloidal carriers which form spontaneously when certain lipids are hydrated in aqueous media (Elizondo *et al.*, 2011).

Almost 75 years ago Paul Elrich came up with the concept of “magic bullet” picturing a drug delivery mechanism that would target drugs to diseased cells and A.D. Bangham in the early 60´s established that a broad diversity of molecules could be encapsulated within

liposomes or inserted in their membranes. These notions were crucial for the development of vesicles as drug delivery systems. Examples of drugs that have been improved with the use of liposomes include AmBisome® that enhance the solubility and targeting of Amphotericin B, and Caelyx® or Myocet® that promote tumor targeting and minimize nonspecific drug toxicity of anticancer agents such as doxorubicin.

Liposomes have properties that make them valid for vaccine delivery: (i) entrap and protect antigen from degradation; (ii) deliver poor soluble antigens; (iii) present different pharmacokinetics depending on the vesicle composition, charge and size; (iv) reduce toxicity by shielding the antigen and (v) be avidly taken up by APCs (Henriksen-Lacey *et al.*, 2011).

Among the different types of vesicular systems used for drug delivery the liposomes (constituted mainly by phospholipids) and vesicles containing nonionic (niosomes), cationic (cationic vesicles) or both cationic and anionic surfactants (catanionic vesicles) are the most investigated for this purpose (Elizondo *et al.*, 2011).

The good performance of vesicular systems is related to their structure at supramolecular, microscopic and nanoscopic level (Elizondo *et al.*, 2011). This highlights the importance of the lipid vesicular systems characterization mainly regarding the lamellarity, size and size distribution, zeta potential and incorporation efficiency.

According to Elizondo and coworkers (Elizondo *et al.*, 2011) regarding size and lamellarity different types of vesicles can be achieved from the self-assembly of a certain amphiphilic molecule: small unilamellar vesicles (SUVs, d≤200 nm), large unilamellar vesicles (LUVs, d≥200 nm), multilamellar vesicles (MLVs) and multivesicular vesicles (MVVs) all of them represented in Figure 7.

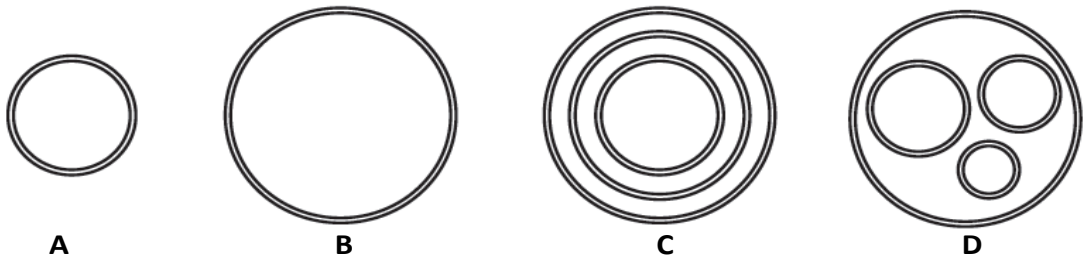

Figure 7 – Types of vesicles according to size and lamellarity: **A.** Small Unilamellar Vesicles (SUVs); **B.** Large Unilamellar Vesicles (LUVs); **C.** Multilamellar vesicles (MLVs); **D.** Multivesicular Vesicles (MVVs) (adapted from (Elizondo *et al.*, 2011)).

Several techniques are available for evaluating liposomes size and size distribution (Edwards and Baeumner, 2006): dynamic light scattering (DLS) (Ruf *et al.*, 1989; Ruozi *et al.*, 2005), microscopy techniques such as cryoelectron microscopy (McCracken and Sammons, 1987; Jass *et al.*, 2003; Frederik and Hubert, 2005), size exclusion chromatography (Grabielle-Madelmont *et al.*, 2003), field flow fractionation (Moon and Giddings, 1993; Korgel *et al.*, 1998; Arifin and Palmer, 2003) and analytical centrifugation (McCracken and Sammons, 1987). From all, the DLS technique is non-invasive, easier and faster than the other methods (Elizondo *et al.*, 2011). DLS measures the time-dependent fluctuations of light scattered from particles experiencing Brownian motion, which results from collisions between suspended particles and solvent molecules.

In order to achieve an optimal performance of the lipid vesicles it is also important to know the size distribution of the suspension and infer about the homogeneity of the lipid suspension. Figure 8 shows a schematic illustration of the response to an external stimulus presented by two hypothetical vesicular systems with homogenous and heterogeneous characteristics in terms of size and lamellarity. With the homogenous system the encapsulated drug would be released at the same time resulting in a sharp response while the heterogeneous system the drug release would take a longer period of time and the drug would not be liberated when required (Elizondo *et al.*, 2011).

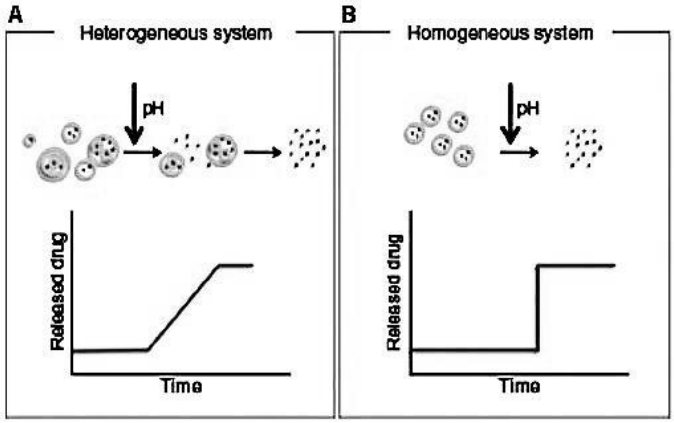

Figure 8- Schematic illustration of the response to an external stimulus presented by a heterogenous vesicular system **A.** and a homogenous vesicular systems **B.** (Elizondo *et al.*, 2011).

As well as size and size distribution, surface properties of vesicles are also important for the characterization of the vesicular systems. Zeta potential is a physical property that is exhibited by any particle in suspension and is explained as the overall charge that a particle

acquires in a particular medium. This measurement can be achieved by Laser Doppler Velocimetry technique, where a voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion (Clogston and Patri, 2011). Charged particles are attracted to the oppositely charged electrode and their velocity is measured in unit field strength as their electrophoretic mobility. It is described that colloids with low zeta potential are not stable and tend to coagulate or flocculate while colloids with high absolute zeta potentials (above 30mV) values are electrically stable (Clogston and Patri, 2011). So, Zeta Potential also gives important information about the stability of the vesicular systems.

Apart from the structural characterization the ability of a vesicular formulation to encapsulate the desired molecule is also very important. This ability of encapsulation can be referred as incorporation efficiency (IE) once it is more indicated for cases in which there is uncertainty whether the drug is encapsulated or integrated in the vesicular membrane.

The measurement of IE requires methods for the rapid physical separation of particles from their surrounding dispersion medium to enable real-time determination of the proportion of free molecules (Wallace *et al.*, 2012). Most methods for measurement of encapsulation separate the particles from the medium in which they are dispersed and there are numerous based on dialysis, filtration and centrifugation (Cui *et al.*, 2006; Ricci *et al.*, 2006; Zheng *et al.*, 2006; Wang *et al.*, 2009). After the separation it is important to quantify the encapsulated material. The methods used will depend on the type of molecule that is encapsulated.

Phospholipids are the most commonly used lipids as delivery systems, in particular the neutral phosphatidylcholine and the negatively charged phosphatidic acid, phosphatidylglycerol, phosphatidylserine and phosphatidylethanolamine, each of which has a different combination of fatty acid chains in the hydrophobic regions of the molecule. Besides the charge, the nature of the fatty acid residues in each lipid molecule, particularly the number of double bonds in the chain is responsible for bilayer properties such as phase behavior and elasticity are also important characteristics in the development of delivery systems (Jesorka and Orwar, 2008).

Augustyniak and co-workers (Augustyniak *et al.*, 2010) aimed to determine the effectiveness of phosphatidylcholine liposomes in potentiating a systemic immune response to *Moraxella catarrhalis* outer membrane proteins. For this study they used soya bean L-α-phosphatidylcholine to form liposomes and were able to show that the proteoliposomes prepared had immunogenic properties.

Geros and coworkers (Geros *et al.*, 1996) showed a successful reconstitution of a lactate-proton symport in a hybrid vesicle system obtained by fusing yeast plasma membrane with liposomes prepared from *Escherichia coli* (*E. coli*) phosphatidylethanolamine containing cytochrome c oxidase, a transmembrane protein. More, these vesicles were able to generate an electrical potential.

In the last years cationic lipids become commonly used as a transfection reagents for DNA, RNA or proteins and as a coadjuvant of antigens for vaccination trials (Lonez *et al.*, 2012). Cationic lipids are made of two domains: a positively charged headgroup and a hydrophobic domain. The assembly into liposomes can be dependent on the addition of neutral lipids, also called helper lipids, such as cholesterol. The surface of these liposomes is positively charged which enhance the adsorption to the negatively charged cell surface (Lonez *et al.*, 2012). Several studies have revealed that cationic vesicles induce stronger immune responses against co-administrated antigen when compared to neutral and anionic liposomes (Christensen *et al.*, 2012). Due to the fact that cationic liposomes are positively charged and cell membranes are negative Lincopan and coworkers (Lincopan *et al.*, 2009) believe that particles for vaccines should be positive and available in a range of sizes. Until now cationic particles of aluminum compounds, identified as having immunostimulatory properties, remain the only type of adjuvant licensed world-wide (Caputo *et al.*, 2008).

Neves Silva and coworkers (Neves Silva *et al.*, 2008) developed a Dioctadecyldimethylammonium Bromide (DODAB): Monoleein, 1-monooleoyol-*rac*-glycerol (MO) based lipoplexe. Lipoplexes are commonly used as non-viral delivery systems for transfection purposes. The long-chain cationic surfactant DODAB is a synthetic lipid, which in the presence of excess water and above the phase transition temperature tends to form LUVs. MO is an amphiphilic neutral lipid and it was reported (Neves Silva *et al.*, 2008; Oliveira *et al.*, 2010) that the inclusion of MO in the cationic liposomes formulations promotes the existence of inverted structures, different from the usual hexagonal morphology, due to MO tendency to form inverted cubic phases. The authors presented some important characteristics about this lipid system such as the non-cytotoxicity of the DODAB:MO lipoplexes and its transfection efficiency comparable to lipofectamine (Neves Silva *et al.*, 2008).

The understanding of the physicochemical characteristics of the lipid vesicles allied with the incorporation efficiency are important parameters in the study of lipid systems to guide in the selection for the most suitable system in the development of a vaccine against *C. albicans.*

Chapter 2 - Objectives and Strategy

This work is included in a laboratory project performed by our working group that proposes the development and characterization of a vesicular system composed by DODAB/MO as potential peptide antigen delivery systems for immunization trials.

The main objective of this study is to compare two lipid systems: one that is based on the phospholipid PE that has been successfully used in the incorporation of a lactate-proton symport in our Department with liposomes prepared from *E.coli* and another, the named DODAB:MO system that has been effectively used for genetic transfection, regarding their ability to encapsulate the antigenic *C. albicans* CWSPs in the development of a safe and effective vaccine against this pathogenic fungus.

Thus, this work enrolls several purposes:

1) Incorporate CWSPs in PE and DODAB:MO based lipid vesicles and characterize them according to vesicle size and size distribution;

2) Assess the incorporation efficiency of the formulation and select the most suitable formulations;

3) Evaluate the in vitro cytotoxicity of the selected formulations in the macrophage like J774 cell line.

The work presented was developed in the Centre of Molecular and Environmental Biology (CBMA) of Minho University.

Chapter 3 - Materials and Methods

3.1 Extraction of Cell Wall Surface Proteins

Proteins from the cell wall (CWSPs) of *C. albicans* (strain SC5314) were extracted after the optimization of the protocol described by Insenser and co-workers (Insenser *et al.*, 2010). Briefly, cells were grown in YPD medium (Yeast Extract 1%, Glucose 2%, Bactopeptone 1%) at 26 °C with agitation of 180 rpm until an OD of 5-6. Then, cells were harvested through centrifugation (8000 rpm, 5 minutes) and the pellet washed two times with 50 mM Tris-HCl, pH 7.5. After the washing step, dithiothreitol (DTT) was added to the buffer and incubated with the cell suspension for 2 hours at 4 °C with agitation. Then, the cell suspension was centrifuged (8000 rpm, 5 minutes), the supernatants collected and filtrated to remove the remnant cells. The supernatants were then centrifuged (25 minutes, 4000 g) in Amicon® filters with a membrane cut-off of 3kDa, for protein concentration. Protein was quantified using the Bradford Method and the concentration was adjusted to 50 μg/ml with Phosphate Buffered Saline (PBS) and stored in aliquots at -80 °C.

3.2 Evaluation of membrane integrity after CWSPs extraction

In order to extract only the CWSPs, a method based on DTT was used, as explained in 3.1. To ensure that this method of extraction did not disrupt the cell membrane and that cytoplasmic proteins were not extracted, membrane integrity, before and after cells incubation with DTT, was evaluated through flow cytometry. For this analysis, cells were incubated with a fluorescence probe Sytox Green, that intercalates in DNA when plasma membrane is compromised, according to a protocol described by Insenser and co-workers (Insenser *et al.*, 2010). Briefly, cells were incubated with Sytox Green (50 μM) after cell treatment with DTT for 5 minutes in the dark. An aliquot of the cell suspension before DTT treatment was also fluorescently stained for control. Cells boiled for 5 minutes were used as control of dead cells (plasma membrane totally compromised) and cells with no staining were used to determine autofluorescence (AF). The stained cells were analyzed with a Beckman Coulter Epics XI flow cytometer.

3.3 Analysis of CWSPs Electrophoretic Pattern

In order to analyze the electrophoretic pattern of the CWSPs extracted using the reducing agent DTT, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. For that, Resolution (12%) and Concentration gels (4%) were prepared as described in Table 1. CWSPs were prepared in denaturing conditions in which the sample buffer was constituted by Tris-HCl (63 mM, pH 6,8), Glycerol (10%), SDS (2%), Bromophenol Blue (0.0025%); DTT (7.5 M). Samples were then heated at 100 °C for 10 minutes, incubated on ice for 10 minutes and centrifuged (13400 g) for 2 minutes. The running buffer was prepared with 0.025 M Tris, 0.192 M glycin and 0.1% SDS. After the sample treatment, a volume of 15 μl of each sample was loaded on the gel and the electrophoresis was performed during 3 hours with a voltage of 60 V.

Table 1: Volumes used for Resolution and Concentration Gels for SDS-Page Electrophoresis of the CWSPs

	Resolution Gel (12%) (μl)	**Concentration Gel (4%)(μl)**
Polyacrilamide	8000	650
Tris-HCl (1.5M pH 8.8)	5000	0
Tris-HCl (0,5M pH 6,8)	0	1250
SDS 10%	2000	50
H_2O MiliQ	4890	3000
TEMED	10	5
Ammonium persulfate (APS) 10%	100	25

After the electrophoresis, proteins were silver stained, according to Cupo and co-workers (Cupo *et al.*, 1990). Briefly, gels were washed in deionized water for 5 minutes and soaked in fixative solution I (40% methanol, 10% acetic acid and 0.076% formalin in water) for 1 hour. Gels were then placed on fixative solution II (10% Ethanol and 5% acetic acid in water) for 2 hours or overnight. After the incubation time gels were washed in water, soaked in oxidizer solution (3.4 mM potassium dichromate and 3.2 mM nitric acid in water) for 30 minutes and washed again 3 times with water. Then gels were incubated in 12 mM silver nitrate for 30 minutes. After that, gels were washed 2 times in deionized water and developed by soaking them in developer solution (250 mM sodium carbonate and 0.05% of 37% w/v formalin water). When the bands appeared the development was stopped by placing the gels in stopping

solution (5% acetic acid in water). Gels were photographed and preserved in a solution of 30% ethanol, 10% acetic acid and 10% glycerol.

3.4 Lipid Vesicles Preparation

The step following the CWSPs extraction was the preparation of the lipid-CWSPs vesicles, using two lipid systems: phosphatidylethanolamine (PE) and DODAB:MO. In these two systems the lipid vesicles were prepared and the CWSPs incorporated, as explained below.

PE-CWSPs vesicles were prepared by two different methods: film hydration (HFL) and freeze-thaw-sonicate (FTS). PE-CWSPs liposomes were prepared through FTS according to Geros and co-workers (Geros *et al.*, 1996) with some alterations. Briefly, commercial *E. coli* PE (Sigma Aldrich P8068) was kept in aliquots of 10mg/ml in chloroform solution at – 80 °C. To prepare the liposomes, chloroform was removed under a stream of dry N_2. Lipids were then ressuspended in 50 mM-potassium phosphate (pH 6.2) and mixed with 1 ml of CWSPs (50 μg/ml). Then, the PE-CWSPs suspension was frozen in liquid nitrogen, thawed at room temperature and sonicated for 15 seconds and this procedure of FTS was repeated 5 times.

PE-CWSPs liposomes were also prepared through film hydration method. Defined volumes from the stock solution of PE were mixed with 1 ml of CWSPs (50 μg/ml) and placed in a rotary evaporator fitted with an aspirator and a bath at 150 rpm until the solvent was fully evaporated and the film formed. The transition temperature of the PE is 0 °C and DODAB is 50 °C so in order to standardize the bath conditions (in PE and in DODAB:MO) the bath was adjusted to approximately 55 °C.

After solvent evaporation, the lipid film was solubilized by adding the appropriate volume of Potassium Phosphate Buffer (50 mM, pH 6.2) and rotated until the lipid film was totally dispersed in the aqueous phase.

In order to confirm the formation of PE-CWSPs closed vesicles FM1-43 probe was incubated with the formulations. FM1-43, a membrane probe, was incorporated in the PE-CWSPs formulations after the formation of the lipid-CWSPs vesicles and allowed to incubate for 15 minutes. After the incubation time the fluorescence was analyzed by flow cytometry.

DODAB:MO-CWSPs formulations were prepared by film hydration method using a similar procedure of PE-CWSPs. Defined volumes from the stock solutions of DODAB and MO in ethanol were mixed. The mixture of DODAB, MO and 1 ml of CWSPs (50 μg/ml) was placed in

a rotary evaporator fitted with an aspirator and a bath with a temperature of 55 °C at 150 rpm until the solvent was fully evaporated and the film formed. After solvent evaporation, the lipid film was solubilized with the addition of appropriate volume of PBS and rotated until the lipid film was totally dispersed in the aqueous phase.

3.5 Lipid Vesicles Physicochemical Characterization

With the purpose to characterize the lipid-CWSPs vesicles two physicochemical parameters were assessed: size (z-average diameter) and Polydispersity Index (PDI). The size and PDI of all liposome formulations and preparations were determined by DLS using ZetaSizer (Malvern).

3.6 Incorporation Efficiency Determination

The incorporation efficiency is an important characteristic in the development of delivery systems. Thus, after lipid vesicles preparation with the CWSPs, ultracentrifugation was performed (100000 g, 15 minutes) in order to separate the supernatant with the non-incorporated CWSPs from the pellet with the lipid vesicles and the incorporated CWSPs.

Following this separation step it was important to quantify the amount of incorporated protein. However, lipids strongly affect protein quantification and to overcome this problem several procedures, that remove the lipid interference, were performed:

1. The N-Butanol Extraction, based on Zhu and co-workers (Zhu *et al.*, 2011) with some alterations. Briefly, each Lipid-CWSPs formulation was mixed with 2-butanol and vortexed for 10 seconds at maximum speed. Then, the mixture was allowed to incubate for 40 minutes at 55 °C. Afterwards, it was centrifuged at 13000 g for 1 minute and the aqueous phase transferred to new tubes and, this centrifugation was repeated. After that, a volume 100 µl of the aqueous phase was kept for protein quantification.

2. The methanol and chloroform delipidation method performed according to Wessel and Flugge (Wessel and Flugge, 1984). Firstly, 400 µl of methanol were added to 100 µl of the sample that was vortexed and centrifuged (10 seconds, 9000 g). Then, 100 µl or 200 µl of chloroform were added to the samples with lower and higher lipid content, respectively, vortexed and centrifuged again (10 seconds, 9000 g). Following, 300 µl of water were added,

and the suspension vortexed and centrifuged (1 minute, 9000 g). Here, the upper phase was removed and discarded. Then, 300 µl of methanol were added, vortexed and centrifuged (2 minutes, 9000 g). After this centrifugation the supernatant was removed and the protein pellet dried under a stream of air, for subsequent protein quantification.

3. The protocol based on sample treatment with Triton X-100 was performed according to Xu and co-workers (Xu *et al.*, 2012). Briefly, 100 µl of 6% (v/v) Triton X-100 were mixed with 500 µl of the sample and the sample maintained at 65 °C for 5 minutes to disrupt all vesicles. After this treatment the concentration of protein was quantified.

4. The Trichloroacetic Acid (TCA) protein precipitation method. This method was performed according to the Tech Tip #8 of Thermo Scientific (Pierce ®). Firstly, 450 µl of water were added to 50 µl of each sample. Then, 100 µl of a solution of 0.15 % (w/v) sodium deoxycholate were added followed by the addition of 100 µl of 72% (w/v) TCA. The mixture was allowed to incubate for 10 minutes at room temperature. After the incubation time, samples were vortexed and centrifuged (10 minutes, maximum speed). The supernatant was removed and the pellet solubilized in a solution of 5% Sodium dodecyl sulfate (SDS) (w/v) in 0.1 N sodium hydroxide (NaOH). Then, protein concentration was measured.

After delipidation optimization CWSPs were quantified in the supernatant and in the pellet through two methods: the Bradford method and the BCA method. The Bradford assay is based on the binding of the Coomassie Blue dye at acidic pH to arginine, histidine, phenylalanine, tryptophan and tyrosine residues and after binding to proteins there is a shift from 465 nm to 595 nm due to stabilization of the anionic form of the dye. For quantification 20 µl of each sample were plated in triplicate in a 96-well plate. A volume of 250 µl of Bradford solution (100 mg of Commassie Blue G-250; 50 ml of 95% ethanol; 100 ml of 85% phosphoric acid and 850 ml of ultrapure water) was added and incubated for 10 minutes. After the incubation time the absorbance was read at 595 nm. The BCA method is based on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions and the BCA reacts with Cu^{+} resulting in the development of an intense purple color with an absorbance maximum at 562 nm. For this assay the Pierce ® BCA Protein Assay Kit was used and quantification was performed according to the kit instructions.

3.7 Macrophage culture

The murine macrophage-like cell line J774A. 1 (American Type Culture Center number TIB 67 (Ralph and Nakoinz, 1975)), was routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FBS), 1% glutamine, 1% sodium pyruvate, and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in 5% CO_2 at 37 °C. Sub-cultures were performed into new cell-culture flasks every 2 days. After confluent growth, macrophage cells were recovered and washed. Viable cells were determined by Trypan blue exclusion counting with the hemocytometer, and re-suspended in DMEM to a final concentration of $4x10^5$ cells/ ml.

3.8 Determination of in vitro cytotoxicity of lipid vesicles – MTT Assay

In order to assess the in vitro cytotoxicity of the lipid-CWSPs formulations in the J774 cell line, the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983) was used. This is a rapid colorimetric assay that measures metabolic active cells once it is based on the reduction, by cellular reductases, of MTT in formazan (Mosmann, 1983).

Firstly, 1 ml of the J774 cell suspensions ($5x10^5$ cells/ ml) was placed in each well of 24 wells plates and macrophages allowed to incubate overnight for adherence. Then, defined volumes of the Lipid-CWSPs formulations were incubated with the cells for 24 hours. We used 2 controls: one of 100% of viability (cells without the lipid-CWSPs formulation) and another of 100% of toxicity (cells with toxic concentration of dimethyl sulfoxide (DMSO)). After the incubation time, 50 μl of MTT were added and incubated for 2 hours at 37 °C. Next, 500 μl DMSO/Ethanol (1:1 (v/v)) were added to allow crystals to dissolve. Absorbance was read at 570 nm. The blank used was DMSO/Ethanol (1:1 (v/v)).

Chapter 4 – Results and Discussion

4.1 Analysis of the CWSPs extracted

Candida species have become the fourth most common cause of bloodstream infections and despite the improvement in chemotherapy the mortality rate for invasive candidiasis has remained stable in these two past decades (Pfaller *et al.*, 2010). These observations underline the need for the development of novel approaches to tackle fungal infections, such as vaccines.

C. albicans cell wall surface proteins (CWSPs) are important targets for the host immune system (Castillo *et al.*, 2008). Thus, the main objective of this work was to prepare lipid-protein vesicles by the incorporation of CWSPs into two lipid systems and compare both their physical characteristics and in vitro cytotoxicity. The selected formulations could be used in a future immunomudulatory strategy against systemic candidiasis.

The first task of this work entailed the incorporation of CWSPs into two types of delivery systems, a phospholipid system based on phosphatidylethanolamine (PE) and a synthetic system based on DODAB:MO. In order to obtain the CWSPs, a method based on the reducing agent DTT was used to extract proteins that are non-covalently attached to *C. albicans* cell wall surface. To confirm that this method did not disrupt the cell membrane, because it was important to avoid cytoplasmic proteins, membrane integrity of *C. albicans* cells was evaluated, through flow cytometry, before and after the treatment with DTT (Figure 9).

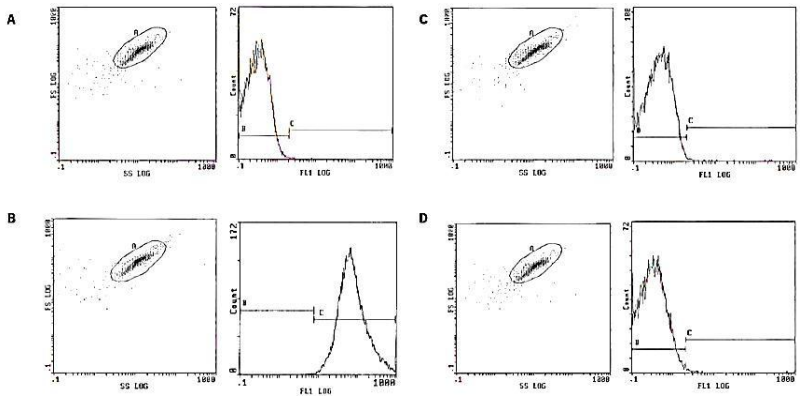

Figure 9 – Flow Cytometry results of yeast membrane integrity. **A.** Autofluorescence of *C. albicans* cells. **B**. Positive control with non-viable cells. **C**. Membrane integrity before treatment with DTT. **D**. Membrane integrity after treatment with DTT.

For autofluorescence determination a *C. albicans* cell suspension with no staining was analyzed and is represented in Figure 9 A. As positive control a *C. albicans* cell suspension was boiled and the majority of the cells are green stained, as shown in Figure 9 B. As it is possible to observe in Figure 9 C and D, there is no fluorescence either before or after yeast cells treatment with DTT. These results demonstrate that the majority of the cells were not altered with respect to their selective permeability. So, it was possible to confirm that the method of CWSPs extraction does not compromise the cell membrane and therefore, mainly proteins of the surface of the cell wall are extracted.

After the extraction procedure it was important to know the electrophoretic pattern of the extracted proteins. Therefore, CWSPs were prepared in denaturing conditions, separated through SDS-PAGE and silver stained. The CWSPs pattern obtained revealed one major band with a molecular weight of about 37kDa and other few less intense bands, as demonstrated in Figure 10.

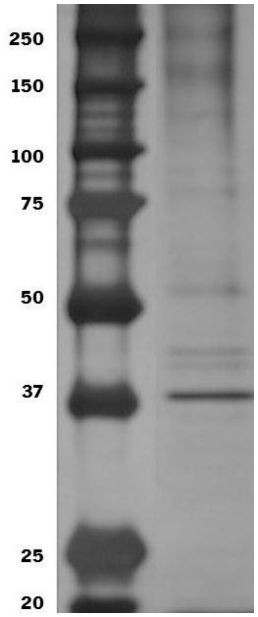

Figure 10- Electrophoretic pattern of the CWSPs extracted from *C. albicans* strain SC5314. Proteins were separated on a 12% SDS-PAGE gel and silver stained. The positions of the molecular mass standards (kDa) are indicated on the left.

The reduced number bands can be explained by two reasons. First, the extraction method, since DTT allows the extraction of proteins that are poorly attached to the surface of the cell wall of *C. albicans*. It is known that the majority of cell wall proteins are GPI anchor or covalently attached to the glucans' cell wall (Castillo *et al.*, 2008). On the other hand, the proteins from the yeast cell wall are highly glycosylated, which can unable proteins to pass

through the gel, due to its high molecular weight (Chaffin, 2008). Three distinct putative phosphatases described as being DTT-extractable and belonging to the PHO family (Pho112p, Ph113p and Pho100) presented molecular weights between 51.3kDa and 35KDa (www.candidagenome.org). So, the three most intense bands presented in gel could correspond to these proteins.

Although the number of bands is low the observed proteins are more than sufficient to be used as antigens and will be used for incorporation into the lipid vesicles purposed in this study.

4.2 Lipid Vesicles Preparation and Characterization

Once the extraction of the CWSPs was completed it was important to incorporate them into the lipid vesicles. For that, several formulations with PE were prepared according to two methods: lipid film hydration (HFL) and freeze thaw sonicate (FTS). The first requires evaporation of the PE-CWSPs mixture in a thin film and further hydration with a buffer. The last is simpler and is based on several cycles of freezing, thawing and sonication which enable the incorporation of CWSPs in the lipid vesicles.

The CWSPs concentration used in this study was 50 μg/ml. It is important to mention that these lipid vesicles will be used in an immunomudulatory strategy, in which mice will be immunized with a CWSPs concentration of around 10 μg/dose. For that reason and accepting that the incorporation efficiency of CWSPs into the lipid vesicles will be lower than 100%, we started with a higher concentration of protein (50 μg/ml).

Table 2 summarizes the variables used in the preparation of the different lipid-CWSPs formulations, namely: lipid concentration, preparation method (HFL or FTS), buffer (KPi, or PBS) used for lipid hydration, Lipid:MO ratio.

Table 2: Variables used in the lipid vesicles prepared.

	Lipid Concentration (mM)	**Preparation Method**	**Buffer**	**Lipid:Mo Ratio**
PE	0.3	HFL	KPi	-
	0.3	FTS		
	0.6	HFL		
	0.6	FTS		
	1.3	HFL		
	1.3	FTS		

PE:MO	0.3	HFL	KPi	1:2
	0.6	HFL		
	1.3	HFL		
DODAB:MO	0.1	HFL	PBS	1:2
	0.3	HFL		
	0.6	HFL		

- Not applicable

The first lipid-CWSPs vesicles prepared in this study were the PE-CWSPs using the two methods of preparation, the FTS and the HFL method. After the PE-CWSPs preparation two important physicochemical characteristics were evaluated: liposome size and polydispersity index (PDI) (Figure 11).

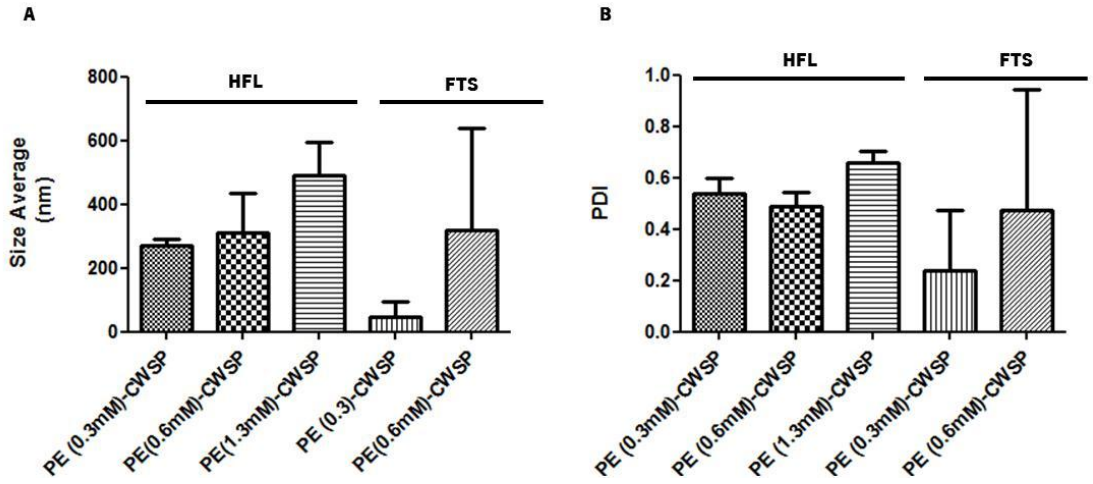

Figure 11- Physicochemical characteristics of PE-CWSPs lipid vesicles using two preparation methods: HFL and FTS. **A**. Size average of the lipid vesicles and **B**. Polydispersity Index (PDI) of the lipid vesicles.

The PE-CWSPs prepared through HFL presented size averages between 268.9 nm and 492.4 nm whether prepared through FTS presented sizes ranging from 93.5 nm to 339.7 nm.

Analyzing the variance of the PE-CWSPs prepared through HFL we observed that an increase in the PE concentration of the vesicles prepared through HFL was associated with an increase in the PE-CWSPs vesicles size average. The values of polydispersity index (PDI) in these vesicles were around 0.6.

Regarding PE-CWSPs vesicles prepared through FTS an increase in the PE concentration was also associated with an increase in the size. The PDI values vary from 0.48 to 0.94, however the deviation values were much higher. The formulation with a PE concentration of 1.3mM was prepared but, it was not possible to dissolve the lipid. Thus, no physicochemical characteristics could be determined. Since the preparation method that allowed the

characterization of the three formulations was HFL, this method was further used in the lipid vesicles preparation.

Monolein (MO) is an amphiphilic neutral lipid that has been used as a novel helper lipid with success in transfection experiments. It was demonstrated that the inclusion of helper lipids in the liposomal formulation facilitates the fusion of the complexes with the cell membrane (Oliveira *et al.*, 2010; Silva *et al.*, 2011). Thus, in this study MO was used to prepare the PE:MO-CWSPs formulations and these lipid formulation were compared with DODAB:MO-CWSPs previously tested by our group. DODAB:MO-CWSPs formulations have been prepared in our laboratory with different ratios, 2:1, 1:1 and 1:2, and it was observed that an increase in MO concentration resulted in DODAB:MO-CWSPs vesicles with lower average sizes and PDIs (data not shown). Thus, in this study the ratio that was selected was 1:2. Both PE:MO-CWSPs and DODAB:MO-CWSPs were prepared through HFL and the average sizes and PDI values are depicted on Figure 12 A and 12 B, respectively.

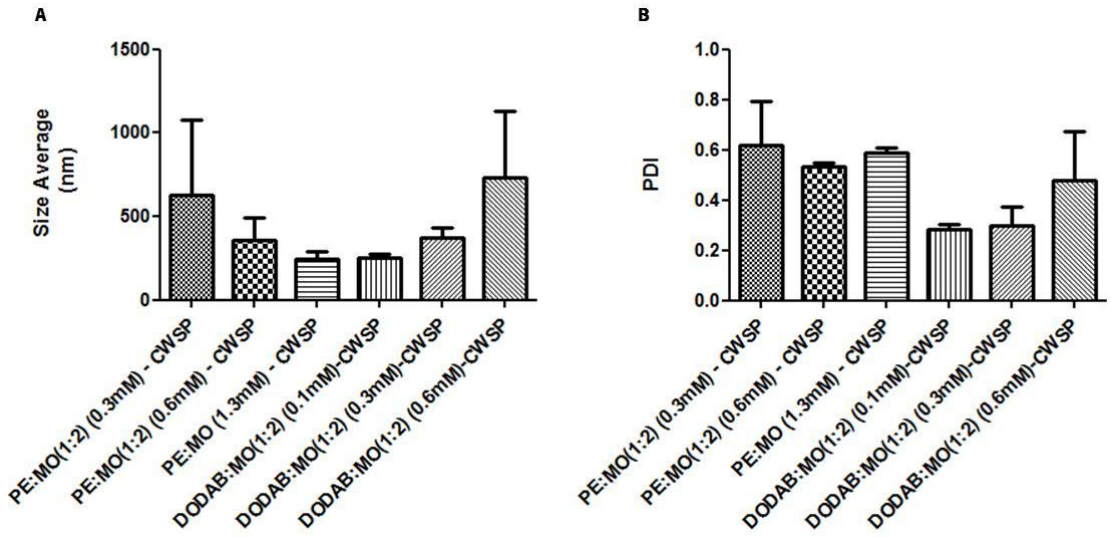

Figure 12- Physicochemical characteristics of PE:MO-CWSPs and DODAB:MO-CWSPs lipid vesicles using HFL method. **A**. Size average of the lipid vesicles and **B**. Polydispersity Index (PDI) of the lipid vesicles.

Regarding the PE:MO-CWSPs vesicles size, it ranged between 247.5 nm and 625.3 nm whether in DODAB:MO-CWSPs it varied between 250.8 nm and 732.5 nm. DODAB:MO-CWSPs presented lower PDI values (around 0.3 to 0.5) than the others lipid vesicles (around 0.6).

Curiously, in PE:MO-CWSPs vesicles an increase in the lipid concentration was associated with a decrease in the lipid vesicles size average contrary to the trend observed in DODAB:MO-CWSPs and PE-CWSPs vesicles.

In conclusion these results indicate that, in general, increasing the lipid content increases the size average of the lipid vesicles, except for PE:MO-CWSPs formulations. In order to understand these differences we reviewed the ultrastructures described for these lipid formulations.

PE vesicles constituted of phosphatidylethanolamine alone, an anionic lipid, form unilamellar closed vesicles resembling cell membranes (Shailesh *et al.*, 2009). In order to confirm the formation of closed vesicles the incorporation of a fluorescent probe in the PE-CWSP vesicles was performed. The probe used was FM1-43 that is described to be relatively non-fluorescent in aqueous solution but becomes highly fluorescent in lipid cell membrane environment (Nunez *et al.*, 2000). Thus, FM1-43 was incubated with the PE-CWSPs vesicles suspension and analyzed through flow cytometry. Results showed that the lipid vesicles emitted high fluorescence, indicating the incorporation of the probe (Figure 13). As this probe incorporates into the membrane it is possible to suggest a membranar closed structure for our vesicles.

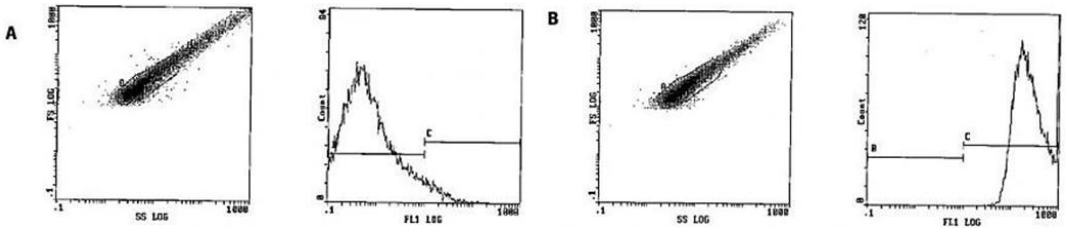

Figure 13 – Flow cytometry analysis of FM1-43 incorporated into PE vesicles. **A**. Autofluorescence of the PE vesicles. **B**. Fluorescence of PE vesicles after the incubation with FM1-43.

Thus, it is comprehensive that the increase in lipid concentration increases the size of the lipid vesicles.

Regarding the inclusion of MO in the PE-CWSPs vesicles it was not possible to evaluate them with the probe FM1-43. Considering the effect of MO on DODAB vesicles structures it has been shown that the inclusion of MO decreases the size of the lipid vesicles (Oliveira *et al.*, 2012). However, this pattern was not observed in this study from the PE-CWSPs to PE:MO-CWSPs vesicles, nevertheless it is important to refer that the deviation values are very high to confirm a trend. It is also possible that the incorporation of CWSPs could alter the structures of the lipid vesicles reverting the pattern observed with the DODAB:MO vesicles due to the interactions with PE, an anionic lipid, in contrast with the interactions with DODAB, a cationic lipid.

Regarding DODAB:MO-CWSPs vesicles, the increase in the lipid vesicles size related to an increase in the lipid concentration has already been shown for a wider range of DODAB:MO concentrations by our group (data not shown).

It is important to mention that the structural organization of these vesicles depends on the lipid concentration, the method of preparation, the solvent composition, the temperature and the presence of other substances (Neves Silva *et al.*, 2008). So, the real structures of PE-CWSPs and PE:MO-CWSPs need to be confirmed through a microscopic technique such as cryo-TEM.

It is also clear from the results of this study that PE-CWSPs vesicles present, in general, higher PDI values than DODAB:MO-CWSPs vesicles. This indicates that PE-CWSPs vesicles are more heterogeneous, once values close to 0 indicate a homogenous dispersion while those greater than 0.3 indicate heterogeneity (Varshosaz *et al.*, 2009).

These evaluation of the physicochemical characteristics together with the CWSPs incorporation efficiency are critical for the choice of which lipid vesicles will continue for further tests. Thus, next we calculated the incorporation efficiency of CWSPs into the prepared vesicles.

4.3 Determination of Incorporation Efficiency

More than the structural characterization, the ability of these vesicular systems to incorporate CWSPs is crucial (Mugabe *et al.*, 2006). This ability can be referred as incorporation efficiency (IE).

In this study, after the preparation of the lipid vesicles formulations ultracentrifugation was performed in order to separate the supernatant with the non-incorporated CWSPs from the pellet with the lipid vesicles and the incorporated CWSPs.

Thus, after the ultracentrifugation, the IE values were calculated by quantifying CWSPs through a protein assay quantification.

Firstly, the Bradford method was used to directly measure the non-encapsulated protein. As it is possible to observe in Figure 14 the protein concentration measured in a lipid-CWSPs formulation before centrifugation was higher than the total CWSPs used in the assay. Besides, the protein concentration in the pellet that has the majority of the lipid vesicles is also higher than in the initial CWSPs.

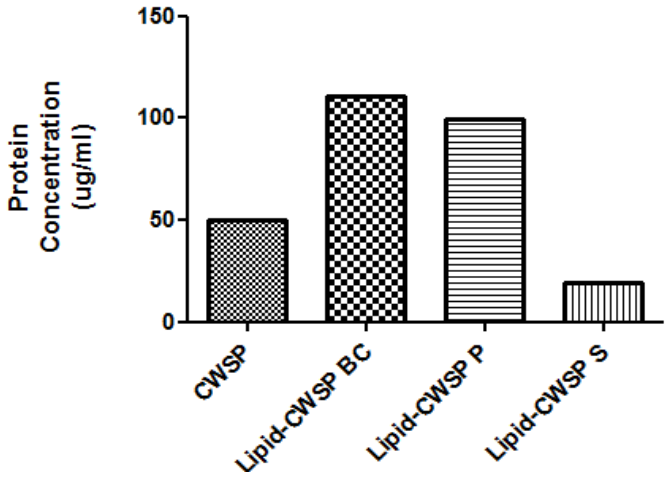

Figure 14 – Protein quantification using the Bradford Method. **P** refers to the pellet after centrifugation, whereas **S** refers to the supernatant. Lipid-CWSP **BC** refers to the Lipid-CWSP aliquot before centrifugation.

These results indicate that lipids interfere with the protein quantification through Bradford assay. Kruger (Kruger, 1994) had already stated the existence of several interferents in the Bradford assay, such as lipids and detergents, and to overcome this problem several methods were tested.

Zhu and coworkers (Zhu *et al.*, 2011) developed a vaccine anti-malaria (AMA1-C1/ISA720) using Montanide® ISA 720, a squalene-based water-in-oil adjuvant. They were able to quantify the total protein concentration in this vaccine after the removal of the oil phase through an n-butanol treatment of the formulation. Based on this study, we used the same protocol to try to remove the lipid interference from the samples. However, during the separation protocol the CWSPs instead of being solubilized in the aqueous phase precipitated between the aqueous and the organic phase. This problem turned this method inappropriate for these CWSPs quantification.

Thus, another separation protocol was assessed. In this second approach lipid vesicles were subjected to a delipidation method based on methanol and chloroform (Wessel and Flugge, 1984) and then CWSPs were quantified through the Bradford Method (Figure 15).

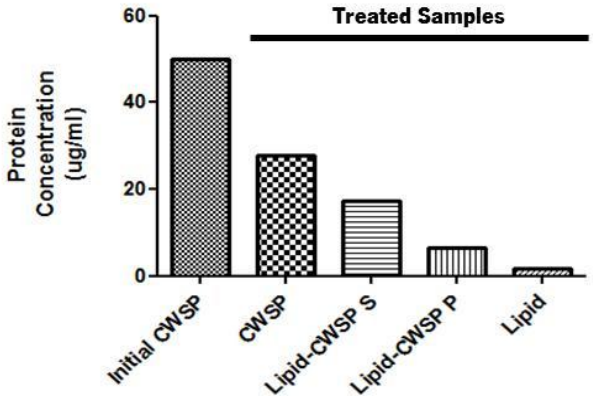

Figure 15 – Protein quantification through the Bradford Assay after samples treatment with methanol and chloroform (Wessel and Flugge, 1984). **P** refers to the pellet after centrifugation, whereas **S** refers to the supernatant.

Considering the amount of protein before and after methanol/chloroform treatment we can observe that before the treatment we have 50 μg/ml and after the treatment we recovered only 27.66 μg/ml. We also observed that from the 27.66 μg/ml of CWSPs 17.39 μg/ml were in the supernatant while 6.51 μg/ml were in the pellet, making up a total of 23.85 μg/ml. However, the yield of protein recovery with this method was just 45.47%. Since the incorporation efficiency of CWSPs in the lipid vesicles should be less than 100% and if we consider calculating the IE measuring the protein in the supernatant and in the pellet after ultracentrifugation with this procedure we would lose around 45% of the protein and consequently, could not measure the protein concentration accurately. As the yield of this procedure was low, another protocol was applied.

Xu and co-workers (Xu *et al.*, 2012) encapsulated the protein superoxide dismutase in liposomes and treated them with Triton X-100 before protein quantification. Thus, we tested the lipid-CWSPs vesicles following the same protocol. After the samples treatment with Triton X-100 we try to quantify the proteins with the Bradford assay. However, the interference of the Triton X-100 was clear since all the samples (CWSPs, Lipid-CWSPs supernatant, Lipid-CWSPs pellet) and PBS turned blue after the addition of the Bradford Reagent. As Triton X-100 interfered dramatically with the Bradford Reagent we tried another quantification method, the BCA method. As it is possible to observe in Figure 16 when comparing the initial CWSPs (50 μg/ml) with the CWSPs after the treatment with Triton X-100 the concentration of the protein was much higher, 111 μg/ml. It was also possible to see the same interference in the pellet

and in the supernatant of the lipid vesicles after ultracentrifugation. Regarding the lipid alone there is no measurement.

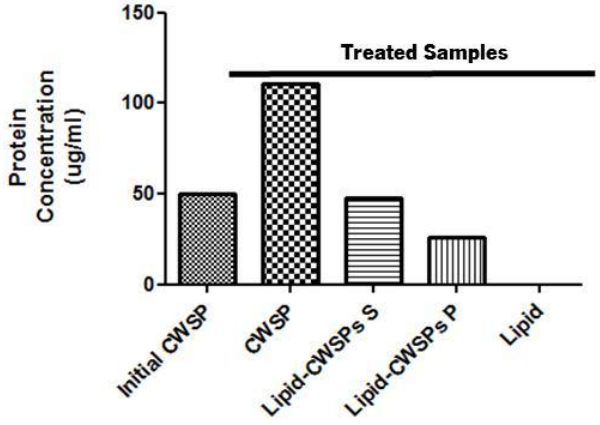

Figure 16 - Protein concentration measurement with BCA method, after samples treatment with Triton-X. **P** refers to the pellet after centrifugation, whereas **S** refers to the supernatant.

As the treatment with Triton–X seems to interfere with the protein in both the Bradford and the BCA measurements another method to remove lipid interference was used. For that, we followed a standard protocol of protein precipitation with TCA, according to Tech Tip #8 of Thermo Scientific. As it is possible to observe in Figure 17 A the concentration of CWSPs before (initial CWSPs) and after the treatment with TCA method is approximately the same. The sum of the protein concentration in the pellet and in the supernatant makes up 64.19 μg/ml and is higher than in the CWSPs after the TCA precipitation (45 μg/ml). So, this prompted us to measure the concentration of protein in a Lipid-CWSPs formulation before centrifugation and in fact the value (70.15 μg/ml) was similar to the obtained with the sum of the values obtained for the supernatant and the pellet. The lipid alone showed no measure at 562 nm.

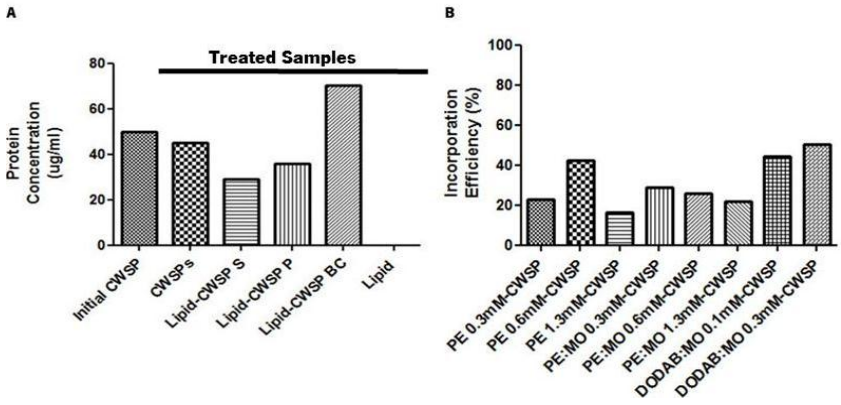

Figure 17– TCA precipitation and BCA quantification of CWSPs. **A**. Protein concentration measurements. **B**. Incorporation efficiency determined after protein concentration measurements.

We considered that with this protocol it was possible to successfully separate proteins from the lipid interferents, and with a residual loss of protein. This protocol was further used for the measurements of incorporated and non-incorporated proteins and the incorporation efficiency of the liposomes was calculated according to the following formula:

$$IE(\%)=100-\left(\frac{\text{Protein Concentration in Supernatant}}{\text{Protein Concentration in Supernatant+Protein Concentration in Pellet}}\times 100\right)$$

Results from the IE calculations of the lipid vesicles prepared in this study through the HFL are depicted on Figure 17 B. Regarding PE-CWSPs vesicles, the formulation with a lipid concentration of 0.6 mM presented the highest incorporation efficiency, 42.3%. The three PE:MO-CWSPs vesicles presented similar IE values (around 25%) with the formulation of PE:MO(0.3 mM)-CWSPs presenting a slight higher value (28.6%). Concerning DODAB:MO-CWSPs vesicles, the formulation with a lipid concentration of 0.3 mM presented the highest IE value of 50.6% and the DODAB:MO (0.1 mM)-CWSPs presented an IE value of 44.4%. The formulation DODAB:MO-CWSPs with the lipid concentration of 0.6 mM formed aggregates and thus, it was not possible to determine the IE value.

If we analyze trends, we observe that in PE-CWSPs vesicles increasing the lipid concentration from 0.3mM to 0.6mM an increase in the IE is observed, but in the formulation with 1.3mM the IE value decreased to 16.46%. In PE:MO-CWSPs an increase in lipid concentration seems to be related to a slight decrease in IE values from 28.6% to 21.8%, but the trend is not clear. In DODAB:MO-CWSPs an increase in lipid concentration is related to an increase in the IE values.

When comparing all the formulations, DODAB:MO-CWSPs vesicles presented the highest incorporation efficiencies. If we consider that the DODAB:MO-CWSPs vesicles presents similar structures as the described with DODAB:MO-DNA it is believed that the lamellarity of DODAB:MO vesicles, with cubic phases, promotes the entrapment of a higher number of molecules then the simpler structures with a phospholipid membrane.

When relating the IE values with the lipid vesicles size in all formulations an increase in the lipid vesicles is related to an increase with the IE. This tendency is only reverted from PE-CWSPs (0.6 mM) to PE-CWSPs (1.3mM) and we believed that this may be an error, and in the future more IE measurements will be made in order to confirm this. These results were

expectable because it is reasonable to infer that there are more CWSPs incorporated in larger vesicles.

4.4 In vitro assays

4.4.1 Cytotoxicity Assays

The following purpose of the study was the incubation of the lipid vesicles with cells from the J774 cell line, to assess the in vitro cytotoxicity. For that, we selected the lipid vesicles with the best features regarding the physicochemical characteristics (size and PDI) and the incorporation efficiency.

Liposomes size has a great influence on efficiency of liposomes as drug carriers, and on their targeting to specific cells. In this study, it is important that liposomes have a size average between 200 nm and 800 nm (Kelly *et al.*, 2011), because the main propose of the vesicles will as antigen carriers target to antigen presenting cells (Brewer *et al.*, 1998). In this view, all of the formulations respect this characteristic. The criterion used in regard to PDI was to choose the formulation with the lowest PDI in each group of the lipid vesicles. Regarding IE the criterion was to choose formulation with an IE higher than 40%.

In this view regarding PE-CWSPs vesicles the one selected to continue for the cytotoxicity assays was PE (0.6 mM)-CWSP that presented lower PDI and higher IE when comparing to the other PE-CWSPs vesicles. In PE:MO-CWSPs formulations, all presented IE values much lower than 40% and also high PDI values. So, all of these formulations were excluded for further tests. Concerning DODAB:MO-CWSPs vesicles, the lipid vesicles selected to be tested were both DODAB:MO (0.1mM)-CWSPs and DODAB:MO (0.3 mM)-CWSPs because they presented lower PDI then DODAB:MO (0.6mM)-CWSPs and IE higher than 40%.

In order to assess the in vitro cytotoxicity of the lipid-CWSP vesicles MTT assay was performed. For that, PE and DODAB:MO formulations with CWSPs were incubated with cells from J774 cell line for 24 hours then MTT was added and in viable cells it was converted into formazan. The viability results are presented in Figure 18.

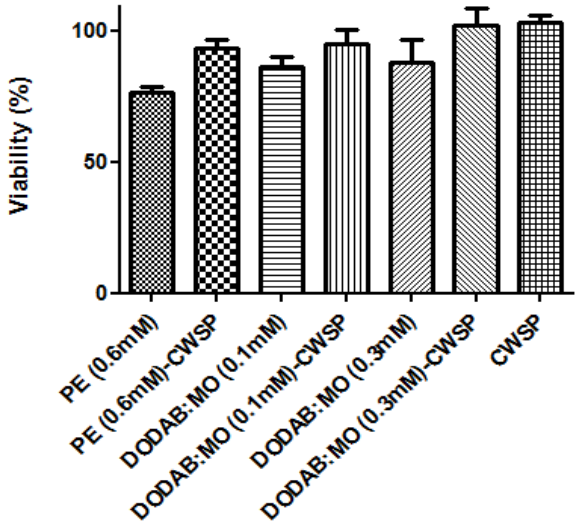

Figure 18- Viability percentage of J774 cells when incubated for 24 hours with the following formulations: PE (0.6mM), PE-CWSPs (0.6mM), DODAB:MO (0.1mM and 0.3mM), DODAB:MO-CWSPs (0.1mM and 0.3mM) and CWSPs.

Empty vesicles, i.e. without CWSPs, were also prepared in order to evaluate lipid cytotoxicity.

No effect on cells viability was observed after incubation with CWSPs alone, indicating that the protein was not cytotoxic. No cytotoxicity was observed when cells were incubated with the Lipid-CWSPs, indicating that regardless the lipid used, PE or DODAB:MO none is cytotoxic for these cells. It was also possible to observe that the incorporation of the CWSPs in the lipid vesicles resulted in an increase in cell viability, either in PE-CWSPs or in DODAB:MO-CWSPs formulations, indicating that the lipids alone are more cytotoxic than the formulation with protein. PE alone is more toxic, with a cell viability percentage of 76.5%, than DODAB:MO that presented a cell viability percentage of 85.9 % with a lipid concentration of 0.1mM and 87.75% in with a lipid concentration of 0.3mM.

According to Neves Silva and co-workers (Silva *et al.*, 2011) the lipoplexes, in which DNA was incorporated into DODAB:MO vesicles, did not demonstrate cytotoxicity effects in 293T cells, which is in accordance with our results.

The results obtained in this study are important since they show that neither PE-CWSP nor DODAB:MO–CWSP formulations are toxic for macrophages of J774 cell line. However, to further confirm these results another assay should be used, for example the LDH assay and another cell line should also be used to assess cytotoxicity.

In conclusion after the characterization of these two systems it is important to say that DODAB:MO delivery system is a more homogeneous system and with higher incorporation

efficiency (>44%) of the CWSPs when compared to PE system. Thus, after this work we would select DODAB:MO-CWSPs vesicles to continue the studies towards the development of a vaccine against *C. albicans*. However, other parameters must be considered.

Kelly and coworkers (Kelly *et al.*, 2011) reviewed the relation between liposomes size and liposome charge and its uptake by cells from the mononuclear phagocytic system (MPS).

They mention a study performed by Epstein-Barach and co-workers (Epstein-Barash *et al.*, 2010) where they conclude that small negatively charged liposomes were optimum for internalization by cells from MPS while large and positively charged liposomes induced cytokines and toxicity by these cells. Regarding the liposome charge it is described that, in general, cationic liposomes are associated with efficient cellular delivery of drugs but, can cause more cytotoxicity than neutral and anionic liposomes. This drawback can be mainly due to the charge mediated interactions between the cell (anionic) and the liposomes (cationic) causing high levels of uptake and subsequent lysis (Henriksen-Lacey *et al.*, 2011).

Thus, we opt to select the three formulations PE (0.6mM)-CWSPs, DODAB:MO (0.1mM)-CWSPs and DODAB:MO (0.3mM)-CWSPs prepared through HFL method to continue the studies towards the development of a vaccine against *C. albicans*.

Chapter 5 – Conclusions and Perspectives

Fungal pathogens represent the major eukaryotic agents of serious infection in European countries, in which infections due to *Candida albicans* and *Aspergillus fumigatus* are the most common and clinically important (Pfaller *et al.*, 2010).

The advancements of medicine, surgery and transplantology in the last thirty years have caused a dramatic increase in the number of immunocompromised individuals who are more susceptible to fungal infections. Cancer chemotherapy, neutropenia, organ transplantation, indwelling catheters and devices, autoimmune diseases, burns, antimicrobial therapy, abdominal surgery, radiotherapy or intensive care are among the main risk factors predisposing for *Candida* infection (Sabino *et al.*, 2010). It has been estimated that in the United States the healthcare cost associated with candidemia is around $2–4 billion/year. These observations underline the urgent need for novel approaches to combat fungal infections and, a vaccine that could prevent or ameliorate these infections would be of major benefit to global health, and national healthcare systems. The development of lipid vesicle delivery systems that can target monocytes/macrophages is crucial and could potentially open up new treatment strategies for a range of diseases in which the cell-mediated immune response is important, as it is for fungal infections(Christensen *et al.*, 2011).

The present study was developed in order to compare two distinct lipid systems, one constituted by phosphatidylethanolamine and another composed by DODAB:MO regarding their ability to incorporate antigenic CWSPs for a future development of a safe and effective vaccine against *C. albicans*.

With this work, it was possible to successfully extract proteins of the cell surface of *C. albicans* that are known to be highly immunogenic. The electrophoretic profile presented one major band at 37KDa with others less intense bands. The CWSPs were successfully incorporated into the lipid vesicles using two different methods: Lipid film hydration (HFL) and Freeze-Thaw-Sonicate (FTS). Three different types of vesicles were prepared, PE, PE:MO and DODAB:MO.

After the incorporation of the CWSPs in the lipid vesicles three characteristics were analyzed: size, PDI and incorporation efficiency. Regarding the vesicles size different behaviors were observed. In PE-CWSPs and DODAB:MO-CWSPs vesicles an increase in the lipid concentration was related with an increase in the lipid vesicles size, from 268.9 to 492.5nm

and from 250.9 nm to 732.5 nm respectively. However, in PE:MO-CWSPs formulations, increasing the lipid concentration led to a decrease in lipid vesicles size, from 247.5 nm to 625.3 nm. These different behaviors may be explained by the ultrastructures that these lipid vesicles form with the CWSPs that should be clarified by cryo-Tem tests.

According to previous results with DODAB, it was expected that the incorporation of the MO into the PE vesicles lead to a decrease in the vesicles size, however in this study this trend was not observed. This could be probably due to the different interactions between MO with the anionic lipid PE and with the cationic lipid DODAB.

In general, PE vesicles were more heterogeneous than DODAB:MO-CWSPs, presenting higher values of PDI.

The incorporation efficiency (IE) was determined after the optimization of the delipidation method. In PE-CWSP lipid vesicles the formulation with a lipid concentration of 0.6mM had the highest value of IE, 42.3%, while in PE:MO-CWSP lipid vesicles the IE values were similar and generally low, around 25%. Regarding the DODAB:MO-CWSPs vesicles the formulation with the highest IE value was DODAB:MO(0.3 mM)-CWSP with 50.6%.

It was also observed that in all formulations the IE values were correlated with the size of the lipid vesicles: an increase in the vesicle size was related with an increase in the IE values. These results were expected because it is reasonable to believe that there are more CWSPs incorporated in larger vesicles.

Three formulations (PE (0.6mM)-CWSP, DODAB:MO (0.1mM)-CWSP and DODAB:MO (0.3mM)–CWSP) were selected for the in vitro cytotoxicity assays according to three criteria: size average between 200 nm and 800 nm, low PDI values and IE higher than 40%. Cytotoxicity tests in the J774 cell line showed that all the lipid-vesicles are non-toxic when incubated for 24hours.

With the information collected in this work it is possible to conclude that the three formulations are suitable for the development of a vaccine against *C. albicans.* However, although with IE of around 40%, PE-CWSPs system showed high heterogeneity, PDI of 0.5. In order to overcome the heterogeneity of this system the lipid suspension could be subjected to extrusion. On the other hand, DODAB:MO delivery system was the most homogeneous system and the one with the highest IE values (>44%) of the Lipid-CWSPs formulations tested.

In order to complete this work zeta potential measurements are necessary as well as stability tests, two important characteristics of liposomes that were not approached during this work period. Regarding the in vitro tests it is important to perform several assays, including cytotoxicity tests with another cell line and using another method, quantification of ROS and NOS, ability of the lipid vesicles to enhance *C. albicans* phagocytosis and also evaluation of cellular uptake of the lipid-CWSPs vesicles.

In summary with this work we were able to incorporate the CWSPs extracted into two types of lipid vesicles and evaluate several important characteristics such as size, PDI, IE and in vitro cytotoxicity. Three lipid-CWSPs formulations were selected, PE-CWSP (0,6mM), DODAB:MO-CWSP (0.1mM) and DODAB:MO-CWSP (0.3mM), according to the evaluated parameters, and considered suitable for the development of a vaccine against *C. albicans*.

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