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**LIPOSOMAL PHOTOSENSITIZER ENCAPSULATION AS A
STRATEGY TO ENHANCE ANTIMICROBIAL PHOTODYNAMIC
THERAPY AGAINST *Candida auris* BIOFILMS**

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THERAPY AGAINST *Candida auris* BIOFILMS**

Thesis submitted to the Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (UNESP), São José dos Campos, in partial fulfillment of the requirements for the degree of Doctor (PhD) in the Graduate Program in Applied Sciences on Oral Health.

Area: Microbiology and Immunology. Research line: Antimicrobial agents and control methods for infections of medical and dental interest.

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POTENTIAL IMPACT OF THIS RESEARCH

In this *in vitro* study, liposomal formulations were developed to enhance the efficacy of photodynamic therapy against *Candida auris* biofilms, an emerging fungal species that is difficult to treat due to its high level of resistance to antifungal agents. This research was carried out in cooperation between UNESP and Brown University, advancing the Sustainable Development Goals (SDGs) 3 and 9 by strengthening health innovation, capacity building, and international collaboration in microbiology and engineering.

IMPACTO POTENCIAL DA PESQUISA

Nesse trabalho *in vitro*, foram desenvolvidas formulações lipossomais capazes de aumentar a eficácia da terapia fotodinâmica contra biofilmes de *Candida auris*, uma espécie de fungo emergente de difícil tratamento pelo alto perfil de resistência aos antifúngicos. Essa pesquisa foi realizada em cooperação entre a Unesp e a Brown University, fortalecendo inovação em saúde, formação de recursos humanos e cooperação internacional nas áreas de microbiologia e engenharia.

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“Nothing in life is to be feared; it is only to be understood. Now is the time to understand more, so that we may fear less.” — *Marie Curie*

ABSTRACT

Lima PMN. Liposomal photosensitizer encapsulation as a strategy to enhance antimicrobial photodynamic therapy against *Candida auris* biofilms [doctorate thesis]. São José dos Campos (SP): Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (UNESP); 2026.

Candida auris poses a significant healthcare challenge, particularly within immunosuppressed patients. This pathogen can colonize the skin and develop biofilms associated with increased antifungal drug resistance that are difficult to treat. Some adjuvant treatments have been investigated, such as antimicrobial photodynamic therapy (aPDT), that employs a photosensitizer (PS) irradiated by light in the presence of oxygen. However, most PSs available suffer from poor biofilm penetration. This study aimed to analyze the effects of antimicrobial photodynamic therapy (aPDT) mediated by the photosensitizer methylene blue (MB), encapsulated in liposomal formulations, on *C. auris* biofilms. Initially, liposomes were fabricated by thin-film hydration followed by extrusion, yielding positively (MB-P) or negatively (MB-N) charged formulations. Liposomes were characterized by dynamic light scattering (hydrodynamic diameter, polydispersity index, and ζ -potential), encapsulation efficiency, and drug loading. After fabrication and characterization, the interaction of the developed formulations with *C. auris* (CBS 11220) in planktonic cultures and biofilms was evaluated by confocal microscopy. Subsequently, the effects of the formulations on aPDT against *C. auris* were studied using a 660-nm red LED and MB free or encapsulated in liposomes. Experimental conditions included NL and PL (empty liposomes), free MB, MB-P, and MB-N, each assessed with and without irradiation. The viability of fungal cells was evaluated by counting Colony Forming Units (CFU) in planktonic cultures and biofilms, as well as biomass by staining the biofilms with Crystal Violet. In addition, the production of reactive oxygen species (ROS) by *C. auris* cells was analyzed using H₂DCFDA fluorescence, and dark cytotoxicity toward mammalian cells was evaluated using the CCK-8 assay. The data were subjected to Analysis of Variance and Tukey's test, considering $p < 0.05$ as significant. The results confirmed that liposomal formulations with positive (MB-P) and negative (MB-N) charges were able to encapsulate methylene blue and provide efficient delivery to *C. auris* cells in planktonic cultures and biofilms. In the aPDT results, both MB-P or MB-N formulations resulted in the eradication of planktonic cells of *C. auris* at minimum fungicidal concentrations equivalent to free MB. MB-loaded liposomes showed enhanced penetration within biofilms and reduced *C. auris* biofilm burden up to 3× more compared to free MB. Additionally, biofilm biomass was reduced up to 37% with MB-loaded liposomes, while free MB only achieved ~3% reduction. Furthermore, aPDT mediated by MB-P or MB-N led to the production of ROS 2× higher than free MB, leading to greater oxidative damage towards *C. auris* biofilms. Finally, MB-loaded liposomes maintained ~80% of fibroblasts viability compared to ~58% viability for free MB. Promisingly, MB-P and MB-N liposomes were able to enhance the *in vitro* activity of PDT on *C. auris* biofilms, inciting the development of *in vivo* studies to validate its efficacy and safety.

Keywords: *Candida auris*; antimicrobial photodynamic therapy; methylene blue; liposomes; photosensitizer.

RESUMO

Lima PMN. Encapsulamento lipossomal de fotossensibilizador como estratégia para aprimorar a terapia fotodinâmica antimicrobiana contra biofilmes de *Candida auris* [tese]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2026.

Candida auris representa uma ameaça à saúde pública, principalmente aos indivíduos imunossuprimidos. Este patógeno é capaz de colonizar a pele e desenvolver biofilmes associados com o aumento da resistência antifúngica. Tratamentos adjuvantes tem sido investigado, como a terapia fotodinâmica antimicrobiana (TFDa), que emprega o uso de um fotossensibilizador e irradiação como fonte de luz na presença de oxigênio. Entretanto, a maioria dos fotossensibilizadores apresentam baixa penetração no interior dos biofilmes. Este trabalho teve como objetivo analisar os efeitos da TFDa mediada pelo azul de metileno (AM), encapsulado em formulações lipossomais, sobre biofilmes de *C. auris*. Lipossomas contendo AM foram produzidos por hidratação de filme fino seguida de extrusão, resultando em formulações carregadas positivamente (AM-P) ou negativamente (AM-N). Os lipossomas foram caracterizados por espalhamento dinâmico de luz (diâmetro hidrodinâmico, índice de polidispersão e potencial- ζ), eficiência de encapsulamento e carregamento do fármaco. Após, foi avaliada a interação das formulações com as células de *C. auris* (CBS 11220) em culturas planctônicas e biofilmes por microscopia confocal. A seguir, foram estudados os efeitos das formulações na TFDa contra *C. auris*, empregando-se LED vermelho (660 nm) e AM nas formas livre e lipossomal. As condições experimentais incluíram NL e PL (lipossomas vazios), AM livre, AM-P e AM-N, cada uma avaliada com e sem irradiação. A viabilidade das células fúngicas foi avaliada pela contagem de Unidades Formadoras de Colônias em culturas planctônicas e biofilmes, além da biomassa por coloração dos biofilmes com Cristal Violeta. Além disso, foram analisadas a produção de espécies reativas de oxigênio (EROs) pelas células de *C. auris* utilizando a sonda H₂DCFDA, e a citotoxicidade no escuro em fibroblastos (NIH 3T3) por meio do ensaio CCK-8. Os dados foram submetidos à análise de variância e teste de Tukey, considerando $p < 0,05$ como significativo. Os resultados confirmaram que as formulações lipossomais com carga positiva (AM-P) e negativa (AM-N) foram capazes de encapsular o AM e proporcionar uma entrega eficiente às células de *C. auris* em culturas planctônicas e em biofilmes. Nos resultados da TFDa, ambas as formulações levaram à erradicação das culturas planctônicas de *C. auris* com concentrações fungicidas mínimas equivalentes ao de AM livre. Os lipossomas AM-P e AM-N aumentaram a penetração do AM nos biofilmes e causaram redução da viabilidade celular 3 x maior do que a TFDa mediada por AM livre. Houve também, redução de até 37% da biomassa de biofilmes tratados com as formulações lipossomais, enquanto o AM livre reduziu apenas 3%. Além disso, a TFDa mediada pelas formulações de AM-P ou AM-N levou à produção de EROs 2 x maior do que o AM livre, resultando em maior dano oxidativo aos biofilmes de *C. auris*. Por fim, os lipossomas carregados com AM mantiveram cerca de 80% da viabilidade celular de fibroblastos, em comparação com cerca de 58% de viabilidade para o AM livre. Os lipossomas AM-P e AM-N foram capazes de aumentar a atividade *in vitro* da TFDa em biofilmes de *C. auris*, incentivando o desenvolvimento de estudos *in vivo* para validar

sua eficácia e segurança.

Palavras-chave: *Candida auris*; terapia fotodinâmica antimicrobiana; azul de metileno; lipossomas; fotossensibilizador.

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1 INTRODUCTION

Candidozyma auris (formerly known as *Candida auris*) is an opportunistic fungal pathogen that easily colonizes the skin of hospitalized patients and can enter the bloodstream through a wound or an inserted medical device, leading to invasive and fatal infections (Cristina et al., 2023). *C. auris* represents a global threat due to the rapid development of resistance towards antifungal agents and disinfectants (Halliday et al., 2023; Miramón et al., 2023; Proctor et al., 2021). Multidrug- and pan-resistant strains of *C. auris* have been identified globally, making treatment challenging (Center for Disease Control and Prevention, 2023; Chowdhary et al., 2017). Due to this concerning resistance pattern, *C. auris* was ranked in the critical priority group on the Fungal Priority Pathogen List released by the World Health Organization (WHO) (2022). Additionally, the Centers for Disease Control and Prevention (CDC) identified *C. auris* as an urgent threat in the 2019 Antibiotic Resistance Threats Report (centers for disease control and prevention, 2019).

The formation of *C. auris* biofilms, a community of fungal cells surrounded by secreted extracellular matrix (ECM), further complicates infection treatment. Biofilm structure limits the immune cell infiltration and antifungal drug penetration. Cells within the biofilm can also exhibit antifungal drug resistance due to increased metabolic activity and expression of drug efflux pumps (Cavalheiro, Teixeira, 2018; Wijaya et al., 2023; Ye et al., 2022). With resistance towards an already limited antifungal drug repertoire (~20 Food and Drug Administration approved) (Halliday et al., 2023; Torres et al., 2023), alternative treatments such as antimicrobial photodynamic therapy (aPDT) are of great interest to treat multidrug-resistant related biofilm infections.

aPDT is a therapeutic approach that involves light activation of a photosensitizer (PS), resulting in localized cell death through generation of cytotoxic reactive oxygen species (ROS) (Wu et al., 2018). Due to the rapid generation of ROS following light exposure and broad cell-targeting potential of aPDT, the development of fungal resistance to this treatment is highly unlikely (Zhu et al., 2022; Ziental et al., 2021), providing a promising strategy for the treatment of *C. auris* biofilm infections. However, the limited penetration of PSs within biofilm structures can impair the efficacy of aPDT (Lin et al., 2021; Xu et al., 2023).

Methylene blue (MB) is a highly attractive PS for PDT due to its strong biological performance with clear practical advantages. Its absorption peak around 660 nm closely matches widely available red light sources, enabling efficient photoactivation and great reactive oxygen species generation (ROS). In addition, MB have shown antimicrobial activity in PDT and has been associated with anti-inflammatory effects, which further support its therapeutic value in infectious and inflammatory conditions. Compared with many alternative PSs, it is inexpensive, broadly accessible, and has a favorable safety profile, with minimal tissue damage reported when appropriately applied. Moreover, MB is also an FDA-approved compound for clinical use for methemoglobinemia, which highlights its well-established pharmacological profile and supports its promise for applications in PDT. Altogether, these features makes MB one of the most versatile and clinically promising PSs for PDT (Alasqah, 2024; Boltes Cecatto et al., 2020; Food and Drug Administration, n.d.; Najm et al., 2024; Oliveira et al., 2025; Shen et al., 2020).

In this context, nanocarriers systems have been employed, such as liposomal formulations (Lin et al., 2021; Nsairat et al., 2022). Liposomes are lipid-based nanoparticles primarily composed of phospholipid bilayer with amphiphilic properties and can encapsulate both hydrophilic and hydrophobic drugs (Jiang et al., 2024; Taniguchi et al., 2022). The functionality of liposomes can be modulated through the optimization of lipid composition, the incorporation of stimuli-responsive compounds, or the use of ligand-specific receptors to stimulate targeted delivery. These modifications enhance their overall performance and functionality for pharmaceutical purposes (LaMastro et al., 2023; Nisini et al., 2018; Taniguchi et al., 2022), including improving drug stability and reducing drug toxicity (He et al., 2022). In addition, the use of liposomes as drug delivery system can lead to enhanced drug penetration into cell membranes through fusion, passive transport, or electrostatic interaction (Barba et al., 2019; Gbian, Omri, 2022). However, liposome interaction with fungal cells is little understood.

Recent studies have explored a range of liposomal formulations to encapsule different PSs. For instance, zinc phthalocyanine (ZnPC) in liposomes has demonstrated enhanced cytotoxicity of aPDT against oral and pharyngeal carcinoma cells. These liposomes led to effective delivery of ZnPC, improving its biodistribution and therapeutic impact (Cheung et al., 2020). Another PS, chlorine e6 (Ce6), was

encapsulated in cationic liposomes, which significantly enhanced the antifungal aPDT effect against both susceptible and drug-resistant clinical isolates of *Candida albicans* (Yang et al., 2013). Moreover, the encapsulation of Vertporphin (PS used in the treatment of macular degeneration) in liposomes improved its bioavailability by preventing self-aggregation and ensuring stability, leading to FDA approval of the photodynamic drug Visudyne® (Chang, Yeh, 2012; Nsairat et al., 2022).

However, while the advancements of aPDT using liposomes are significant, certain limitations persist. Many current liposomal formulations face challenges such as limited tissue penetration, suboptimal PSs release rates, and issues related to maintaining stability and effective light penetration (Saad, Hasan, 2022). Aiming to improve the treatment of *C. auris* biofilm-related infections, in this study we fabricated two different liposomal formulations containing MB. To investigate the MB delivery to *C. auris* through the liposomes obtained, we assessed their interaction with *C. auris* cells in both planktonic and biofilm states. We then examined the antifungal efficacy of aPDT using both MB-loaded liposomes against planktonic cells and biofilms of *C. auris*, as well as the production of ROS. Finally, we assessed the cytocompatibility of these liposome formulations in fibroblasts, a common cellular component of the skin.

This thesis presents an innovative strategy to improve aPDT against *C. auris* biofilms through the use of liposomal photosensitizer formulations, including cationic and anionic systems. To the best of our knowledge, based on the literature surveyed, no previous study has investigated aPDT against *C. auris* using liposome-encapsulated photosensitizers. Therefore, this work represents the first study to explore this approach, filling an important gap in the field and contributing to the development of new therapeutic strategies against biofilm-associated infections caused by this emerging multidrug-resistant pathogen.

2 LITERATURE REVIEW

2.1 Candidiasis and *C. auris*

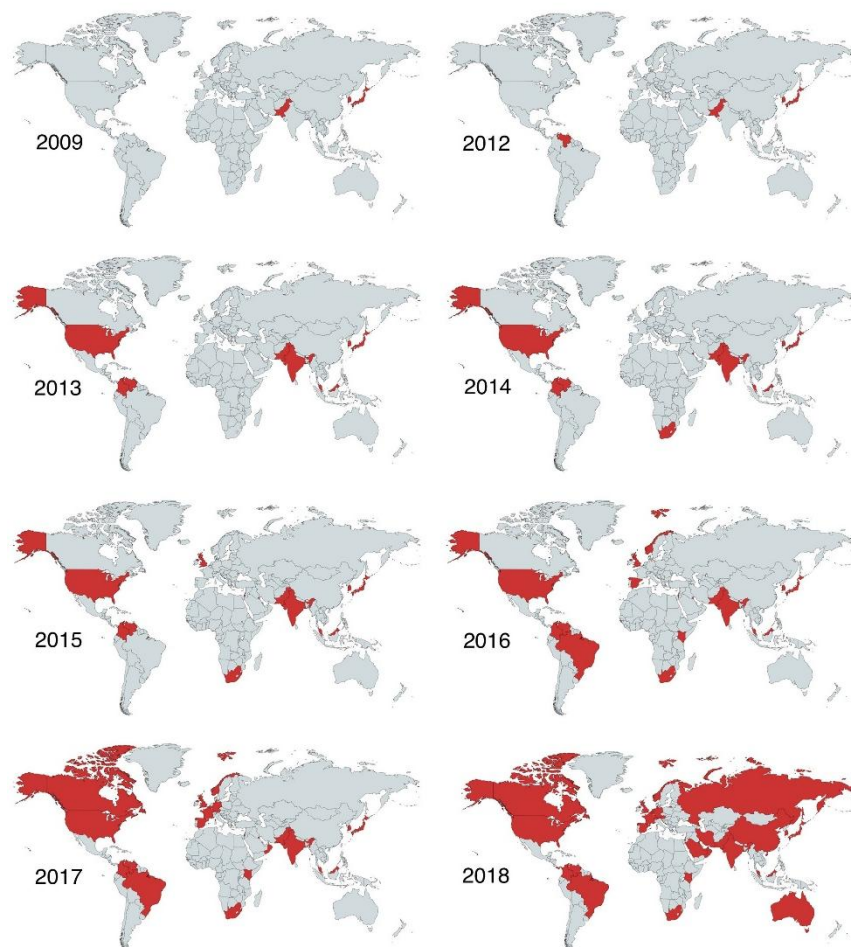
Fungal infections have become challenging to address due to the increase of antifungal resistant strains (Szymański et al., 2022). In 2013, 1.5 million deaths of HIV positive individuals were registered, from which approximately 47% was caused by fungal infections (Denning, 2016). Similarly, fungal infections are highly frequent in transplanted and oncologic patients. (Bongomin et al., 2017). The commensal fungus, *Candida* spp. are one of the most frequent pathogens to cause fungal infections in human beings (Lohse et al., 2018).

The pathogenicity of *Candida* spp. is associated with their high capacity to adapt and form biofilms. Biofilms are microbial communities involved in ECM and adhere to a biotic or abiotic surface. The biofilm formation ability holds a significant clinical importance in medical and dental practice, as biofilms provides increased microbial resistance to antimicrobial treatments (Pereira et al., 2021). Many *Candida* species can cause superficial or invasive infections, such as vulvovaginal candidiasis and ocular candidiasis, respectively. Among *Candida* spp., *Candida albicans* is one of the most virulent and frequently isolated species and have long been considered the primary responsible for candidiasis, that is, fungal infection caused by *Candida* spp., or candidemia, which is the bloodstream invasion by *Candida* spp. (Abe et al., 2024; Ajetunmobi et al., 2023; Lamothe et al., 2018). However, non-*albicans* *Candida* species, such as *Nakaseomyces glabratus* (*Candida glabrata*), *Candida tropicalis* and *Pichia kudriavzevii* (*Candida krusei*) may cause candidiasis and candidemia as well (Junqueira et al., 2012).

In 2009, *C. auris* an emerging fungal pathogen from the genus *Candida*, was identified. The first documented case of *C. auris* infection occurred from the ears of a 70-year-old woman hospitalized in Tokyo, Japan (Kim et al., 2024; Satoh et al., 2009). Since then, this yeast has rapidly spread across the five continents (Figure 1) (Rhodes, Fisher, 2019) and the number of published studies has been increasing exponentially all over the world, with special interest coming from Center of Disease and Control

(CDC) and the United States (Q. Wang et al., 2023).

Figure 1 - Timeline of identification of *C. auris* throughout the world from 2009 to 2018



Source: Adapted from Rhodes and Fisher, 2019.

According to Kim et al. (2024), the mortality rate for invasive or non-invasive *C. auris* infection can be up to 62%, and hospitalization may be necessary for up to 140 days. While (Chen et al., 2020) reported an overall mortality rate of 39% in non-invasive *C. auris* infection and 45% in candidemia caused by *C. auris*. The authors compiled numerous clinical reports of *C. auris* published between 2009 and 2019 and more than 4700 cases were reported in approximately 30 countries. Additionally, their findings demonstrated *C. auris* high resistance to fluconazole (91%) and moderate

resistance to amphotericin B and caspofungin (both approx. 12%). Similarly, Benedict et al. (2023) estimated a 34% death rate after analyzing 192 cases of hospitalization due to *C. auris* during 2017 to 2022 in the US, from which, 20% corresponded to candidemia. In the remaining cases, the axilla (38%) was the most common region for positive *C. auris* isolation. Moreover, 75% of these cases involved hospitalization in an intensive care unit (ICU) and 43% of cases needed mechanical ventilation. Over a 7 years period (from 2016 to 2023), the number of *C. auris* clinical cases reported in the US increased approximately 89-fold, from 51 to 4,514 (centers for disease control and prevention, 2021).

C. auris presents alarming data on antifungal resistance and is associated with high mortality rates, especially among hospitalized immunocompromised patients (Biswal et al., 2017; Scorzoni et al., 2021). *C. auris* is considered highly virulent due to their ability to form biofilms and to the expression of resistance genes involved in the escape from antifungal drugs and host immune cells (Chakrabarti, Singh, 2020; Chakrabarti, Sood, 2021). Polyene resistance is not fully understood; however, some mechanisms include the mutation in FLO8, a membrane transporter encoding gene, Cdr6, an efflux pump, and upregulation of ERG1, ERG2, ERG6 and ERG13, genes involved in ergosterol biosynthesis. Azole resistance is conferred by multiple mechanisms, including overexpression of ERG11 gene encoding lanosterol 14 α -demethylase, the fluconazole target and overexpression of CDR1, which is part of the ATP-binding cassette (ABC) transporter family, acting as an efflux pump of azoles. Finally, echinocandin resistance can be attributed to the reduced affinity of β -1,3-glucan synthase caused by mutations in genes like FKS1. Additionally, this effect can hinder immune recognition. Moreover, *C. auris* biofilms shows an increased resistance profile by sequestering the antifungal drug in the EPS, overexpressing efflux pumps like ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) and hosting persister cells, which can survive high levels of stress (Carolus et al., 2021; Chakrabarti, Sood, 2021). In addition, *C. auris* have an outer mannan layer on their cell wall, which protects the β -1,3-glucan, complicating immune cell recognition (Dakalbab et al., 2024).

C. auris shows close phylogenetic relationship to *Candida ruelliae*, which no isolation from humans or animals have been reported, and *Candida haemulonii* which have been associated with several invasive and non-invasive infections (Ciurea et al.,

2021; Jackson et al., 2019). Due to their genetic similarity, *C. auris* isolates are often misidentified as *C. haemulonii* (Escandón et al., 2018). The misidentification can lead to unsuccessful treatment and wrong surveillance (Dahiya et al., 2019).

Therefore, studies on *C. auris* aiming for the development of more effective antifungal therapies and infection prevention strategies are of critical importance due to its multidrug resistance, high mortality rates, and ability to persist in healthcare environments. Additionally, its misidentification in diagnostic methods further complicates infection control efforts.

2.2 Antimicrobial Photodynamic Therapy against *Candida* spp.

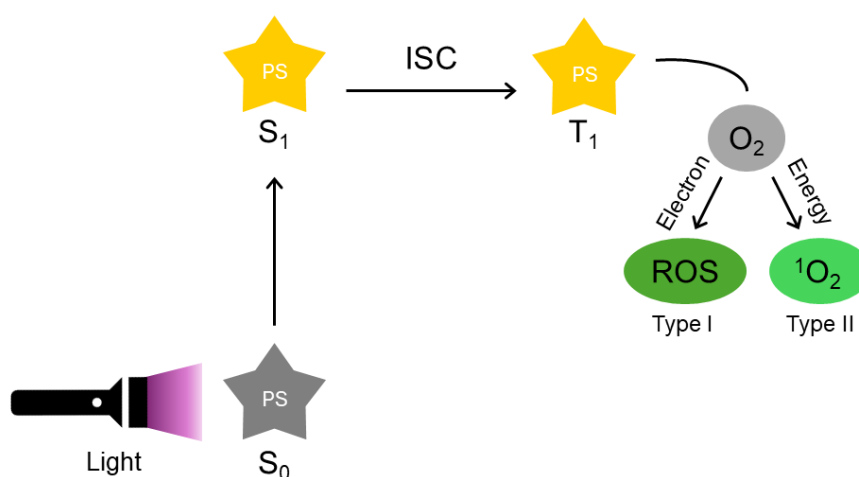
aPDT consists of the generation of ROS through irradiation of a PS. These ROS are highly toxic to cancer cells and microorganisms (Rodríguez-Cerdeira et al., 2021). aPDT can be used as both alternative and complementary treatment and shows promising potential for antimicrobial application-(Wu, Hu, 2022a).

The effectiveness of aPDT relies primarily on the PS. Ideally, a good PS should not be cytotoxic without light activation, present high quantum yield of ROS, absorption capacity at a wavelength that allows light penetration into the tissue, low cost and operational simplicity (Mfouo-Tynga et al., 2021; Wang et al., 2024; Zhang et al., 2017). In this scenario, different PSs have been studied, including classes of phenothiazines, chlorins (Cieplik et al., 2018; Quadrado et al., 2024; Silva et al., 2023) and porphyrins (Basso et al., 2019; Urquhart et al., 2023). Among phenothiazines, methylene blue (MB) is one of the most known and widely used due to their approved use in clinical settings (Bistas, Sanghavi, 2023; Da Collina et al., 2018).

A PS is typically found in their lowest energy state, called ground state (S_0) and when they absorb a photon coming from a light source, their state changes to an excited singlet state (S_1), which corresponds to a higher electronic energy state. Next, the PS can either return to the S_0 by releasing energy through fluorescence or undergo intersystem crossing (ISC) to a triplet state (T_1), that is, a state where electrons have the same spin orientation. In T_1 , the energy is transferred to molecular oxygen, which will absorb this energy and will generate ROS (Correia et al., 2021; Yu et al., 2022).

These ROS are highly reactive molecules of oxygen-centered radicals, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), and non-radical species such as singlet oxygen ($^1O_2$) (Arnaut et al., 2014; Edge, Truscott, 2021). In aPDT, ROS generation can result from two primary mechanisms. Type I mechanism involves electron transfer, producing O_2^- and OH . While type II mechanism involves energy transfer to molecular oxygen, forming $^1O_2$ (Figure 2) (Arnaut et al., 2014; Dąbrowski, 2017).

Figure 2 – Schematic representation of the mechanism of reactive oxygen species generation in aPDT based on the Jablonski diagram



Source: Author.

aPDT has emerged as a promising alternative for the treatment of fungal infections, particularly those caused by drug-resistant species due to their multiple-targeting mechanism making the development of resistance against aPDT very unlikely. The targeting site of aPDT depends on the location of the PS in the fungal cell (Cieplik et al., 2018). More specifically, the ROS production through Type I mechanism is described to induce protein degradation, DNA, lipids and cholesterol while type II is known for oxidation of aminoacids, proteins, DNA, lipids and cholesterol (Anas et al., 2021). aPDT membrane disruption and oxidative stress being the primary pathways leading to fungal cell death (Rosseti et al., 2014).

One of the most significant effects of aPDT is the disruption of fungal cell membranes because of PSs accumulation in the fungal membranes, particularly in regions rich in ergosterol, the primary sterol in fungal plasma membranes (Calzavara-Pinton et al., 2005). The ROS generated in aPDT initiate lipid peroxidation, damaging the structural integrity of the fungal plasma membrane (de Menezes et al., 2016). Due to membrane disruption and oxidative stress, fungal cells subjected to aPDT exhibit severe dysfunctions that lead to apoptotic and necrotic cell death (Zhang et al., 2017).

Several studies have shown significant antimicrobial activity of aPDT against *Candida* species (Dovigo et al., 2013; Figueiredo-Godoi et al., 2019; Valkov et al., 2021; Wu, Hu, 2022b). For instance, Figueiredo-Godoi et al. (2019) evaluated the *in vivo* effectiveness of aPDT using MB to treat *Candida* infections. To this end, *Galleria mellonella* larvae were systemically infected with *C. albicans* and treated with systemic application of MB with or without light activation. Larvae receiving only Phosphate Buffered Saline (PBS), with or without light activation were included as control group. aPDT using MB successfully prolonged larval survival. More specifically, infected larvae without aPDT treatment died within 24 (ATCC strain) or 72 (clinical strain) h while aPDT prolonged the survival of larvae infected with *C. albicans* ATCC or clinical strain by approximately 3 and 5 times, respectively, compared to the control group. Although aPDT treatment extended the lifespan of infected larvae, the total number of surviving *G. mellonella* remained low, indicating that despite beneficial, aPDT using MB was not sufficient to prevent high mortality.

Bapat and Nobile (2021) investigated the efficacy of aPDT against *C. auris* biofilms using various PSs (Toluidine blue O, New methylene blue and Rose Bengal) and light sources (blue, green and red). While their findings demonstrated that blue light alone exhibited antifungal effects against *C. auris*, this raises questions about its role in aPDT. On the other hand, red and green light combined with PS showed significant results against *C. auris* biofilm, suggesting that aPDT could be a promising alternative to combat *C. auris* infections. However, the long irradiation time (24 h) required to achieve these results is a major concern. Moreover, when the exposure time was reduced to 90 min, aPDT failed against *C. auris* biofilm. This extended treatment period poses challenges for clinical applications. Additionally, the study employed a high PS concentration (400 μ M), which may raise concerns regarding cytotoxicity. Despite these limitations, the findings by Bapat and Nobile (2021) provide

valuable insights into the potential of aPDT against *C. auris* biofilms, highlighting the need for further refinement before its clinical implementation.

2.3 Liposomes as photosensitizer nanocarrier

PSs are categorized into first, second and third generation, according to their historical development. For example, hematoporphyrins (i.e. porphyrins) are considered first-generation PSs. While PSs like toluidine blue, methylene blue, verteporfin and 5-Aminolevulinic acid are classified as second-generation. However, these PSs can present some limitations, such as poor aqueous solubility, stability and bioavailability. To overcome these and achieve better therapeutic outcomes, third generation PSs have been widely studied. To this end, second generation PSs have been conjugated with targeting moieties (e.g. antibodies and peptides) or nanoparticle formulations (Gupta et al., 2024; Mfouo-Tynga et al., 2021).

Liposomes are lipid-based nanoparticles composed of one or more lipid bilayers surrounding an aqueous core. They have been widely investigated as drug delivery systems to increase cellular uptake and ultimately enhance treatment effectiveness. Their drug delivery mechanism can be through membrane fusion, endocytosis, surface interaction between liposome and cellular membrane, lipid exchange and pinocytosis (Gbian, Omri, 2022; Mfouo-Tynga et al., 2021; Pivetta et al., 2022; Wu et al., 2018). Moreover, liposomes present versatility in their formulation, that is, you can control their sizes and modify their charge and add surface accessories to increase cellular interaction (Jiang et al., 2024; LaMastro et al., 2023). In recent years liposomes have been explored to enhance aPDT efficacy and have shown promising results against cancer cells and pathogenic microorganisms (Pivetta et al., 2022; Yang et al., 2013).

Currently there are several Food and Drug Administration (FDA) approved liposome formulations, for diverse therapeutic purposes, for example, Doxil (Doxorubicin) for chemotherapy, Ambisome (Amphotericin B) for antifungal therapy, Visudyne (verteporfin) for aPDT and Exparel (Bupivacaine) for anesthesia (Jiang et al., 2024).

A key challenge when treating microbial infections is biofilm formation, because of their enhanced antimicrobial resistance. Liposomes can overcome this issue by fusing into the microbial biofilm matrix and cell membrane, facilitating drug penetration within biofilms. This contributes to reduce therapeutic dosage and helps prevent drug resistance (Wang, 2021a).

For instance, phthalocyanines, a hydrophobic PS, have limited application due to their need for toxic organic solvents. However, studies have shown that liposomes encapsulating phthalocyanines can improve their solubility and consequently their biocompatibility. Moreover, liposomes encapsulating hydrophilic phthalocyanines formulations allowed for prolonged PS release and enhanced cellular uptake. Therefore, antimicrobial aPDT using liposomes encapsulating phthalocyanines have shown promising results (Siqueira et al., 2022).

Recent studies have shown the efficacy of aPDT against fungal keratitis; however, an effective trans-corneal drug delivery system is crucial. In this context, improved solubility, penetration and controlled release have been demonstrated using liposomes encapsulating rose bengal and riboflavin, suggesting that aPDT using liposomes to deliver PS across corneal epithelium can reduce the need for invasive pre-treatment procedures and enhance therapeutic outcomes (Hung et al., 2021). However, limited research has been conducted addressing the use of liposomes in aPDT against fungi, highlighting the need for further research.

In summary, although significant advances have been made in aPDT research, several limitations remain and poses substantial challenges. First, limitations arise from the PSs themselves: MB suffers from poor penetration into microbial biofilms, while highly lipophilic molecules such as hypericin display low solubility and poor bioavailability, often requiring complex pre-formulation steps before encapsulation. Second, technical challenges in liposome fabrication persist, including difficulties in maintaining consistent particle size and surface charge density, which are critical parameters that directly influence penetration, uptake, and aPDT efficacy. For lipophilic PSs, additional formulation complexity can be required, such as cyclodextrin inclusion complexes, a ring-shaped sugar molecules with a hydrophobic cavity that can trap lipophilic PSs, forming water-soluble complexes, highlighting that simple liposomal encapsulation is not universally suitable. Third, the performance of liposomal systems may be inconsistent; lipid composition can affect antimicrobial outcomes, and

in some cases, liposomal encapsulation may even reduce photodynamic activity relative to alternative delivery systems. Finally, clinical translation barriers persist, including scale-up challenges, manufacturing reproducibility, and ensuring stable, safe, and biologically effective formulations. These limitations demonstrate that while liposomes address key issues of PS delivery, they also introduce new physicochemical and challenges that must be overcome for optimal clinical application (Boccalini et al., 2017; Plenagl et al., 2019).

3 AIMS

3.1 General aims

The aim of this study was to investigate a liposomal nanocarrier system as a possible strategy to facilitate the methylene blue photosensitizer penetration into biofilm and improve aPDT action against *C. auris*.

3.2 Specific aims

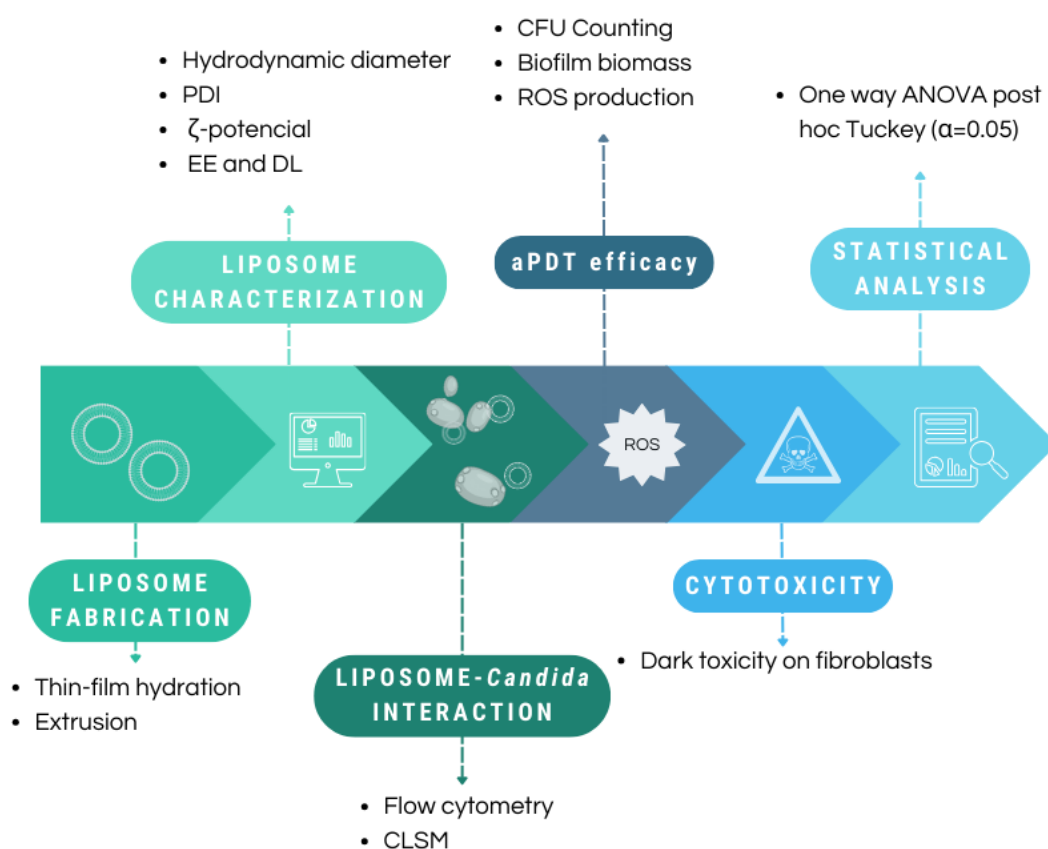
- a) to develop and characterize liposomal formulations containing MB;
- b) to study the interaction of liposomal formulations with *C. auris* in planktonic and biofilm stages;
- c) to evaluate the antifungal action of aPDT using MB-loaded liposomes on planktonic cultures and biofilms of *C. auris*;
- d) to evaluate the cytotoxic effects of MB-loaded liposomes on mammalian cells.

4 MATERIALS AND METHODS

4.1 Workflow overview

The methodological workflow used in this study is summarized in Figure 3. This schematic integrates all major stages of the study, from the preparation and characterization of MB-loaded liposomes to the investigation the aPDT efficacy against *C. auris*.

Figure 3 - Flowchart summarizing the experimental design



Caption: PDI: Polydispersity index; EE: Encapsulation efficiency; DL: Drug loading capacity; CFU: Colony Forming Unit; ROS: Reactive Oxygen Species; ANOVA: Analysis of variance; CLSM: Confocal Laser Scanning Microscopy.

Source: Author

4.2 Fabrication of MB-loaded liposomes

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (CHOL), and dimethyldioctadecylammonium bromide (DDAB) stocks were made in chloroform at a concentration of 25 mg/mL while dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt (DPPG) stocks were made in a chloroform: methanol solution (5:1 v/v) at a concentration of 2 mg/mL. All lipid stocks were stored at -20°C prior to use. A fresh aliquot of MB was prepared in 1× PBS (1 mg/mL) each time before use. MB-P and MB-N charged MB-loaded liposomes were fabricated using DDAB and DPPG, respectively.

To fabricate the MB-loaded liposomes, lipids were added into glass vials in the desired ratios (Chart 1); CHOL was added to achieve a final concentration of 20% (v/v) in all formulations. Chloroform and/or methanol were removed using a rotary evaporator in a 40°C water bath (Rotavapor R-300, Buchi, Sankt Gallen, Switzerland). The resulting dry lipid film was stored overnight in a desiccator. The dry lipid film was then rehydrated at 69°C in MB solution (MB at 1 mg/mL) to a final lipid concentration of 10 mg/mL. Rehydrated samples were then vortexed, sonicated at 69°C for 30 min, and extruded through 200 nm polycarbonate membrane for a total of 10 extrusions using a Liposofast LF-50 extruder (Avestin, Ottawa, Canada) at 70°C. Liposome formulations were purified to remove non-encapsulated MB by ultracentrifugation with Vivaspin® 20 centrifugal concentrator with 300 kDa pore size at 4000 × g for 1 h. Non-MB-loaded liposomes rehydrated with 1×PBS without MB and fabricated as described above. All liposomal formulations were stored at 4°C until further use (Figure 4). A schematic representation of the liposomes, illustrating MB encapsulation in MB-P and MB-N, is provided in Figure 5.

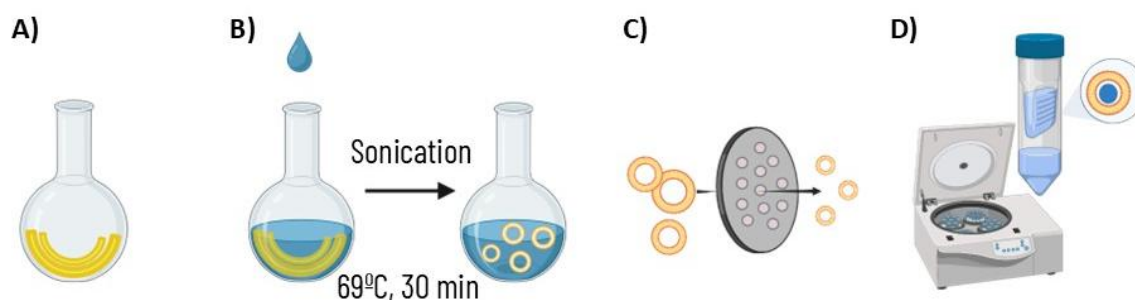
Chart 1 - Liposome composition of positively (MB-P) and negatively (MB-N) charged methylene blue (MB) loaded liposomes

Liposomes	Components (w/w)				MB in preparation solution (mg/mL)
	DPPC	DPPG	DDAB	CHOL	
MB-P	3	-	1	1	1
MB-N	3	1	-	1	1

Caption: MB-P: Positively charge MB-liposome; MB-N: Negatively charged MB-liposome; DPPC: (1,2-dipalmitoyl-sn-glycero-3-phosphocholine; CHOL: cholesterol; DPPG: 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt; DDAB: dimethyldioctadecylammonium bromide.

Source: Author.

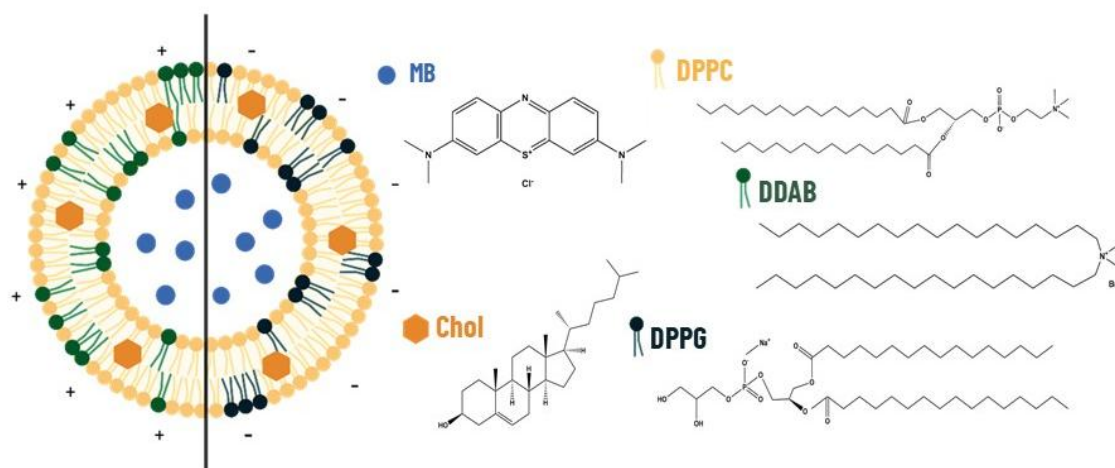
Figure 4 - Schematic overview of the fabrication process for methylene-blue-loaded liposomes



Caption: A) lipid thin-film formation using rotavapor R-300; B) Thin-film rehydration using 1mg/mL MB solution followed by sonication at 69°C for 30 min. C) Liposome extrusion through 200 nm polycarbonate membrane for a total of 10 extrusions using a Liposofast LF-50 extruder; D) Removal of non-encapsulated MB by ultracentrifugation with Vivaspin® 20 centrifugal concentrator with 300 kDa pore size at 4000 × g for 1 h.

Source: Author.

Figure 5 - Representative schematic of liposome design and chemical structures of the liposome components



Caption: MB: Methylene blue; Chol: cholesterol; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DDAB: dimethyldioctadecylammonium bromide; and DPPG: 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt.

Source: Author.

4.3 Characterization of liposomes according to physical properties, encapsulation efficiency, and drug loading capacity

To obtain the hydrodynamic diameter and polydispersity index (PDI) of each liposome formulation, dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS90 (Malvern Panalytical, Westborough, Massachusetts). An aliquot of 50 μ L of each sample was added to an UV-transparent disposable cuvette (Sarstedt, Nümbrecht, Germany). Three measurements were obtained per sample. The ζ -potential of each formulation was obtained by making a 10-fold dilution of each liposomal formulation in 1 $\times$ PBS; and measurements were collected using the Zetasizer Nano ZS90. The encapsulation efficiency (EE%) was assessed via absorbance measurements. Briefly, liposomes were lysed with 70% (v/v) methanol in a 1:1 liposome:methanol ratio (v/v). Lysed samples were transferred to a 96-well microtiter plate and the absorbance of each well was collected from 500 to 800 nm using a Cytation 3 plate reader (BioTek, Winooski, Vermont). Using a standard curve

of free MB prepared in 1× PBS and 70% (v/v) methanol, the EE% was calculated using Equation (1). To determine the drug loading (DL%) capacity of each formulation, liposomes were prepared in a solution of 1:1 (v/v) 1× PBS and lyophilized to obtain the liposome dry weight. The MB concentration was measured as described above and the DL% was calculated using Equation (2).

$$EE\% = \frac{\text{Total amount of MB in liposome (mg)}}{\text{Total amount of MB added initially (mg)}} \times 100 \quad (1)$$

$$DL\% = \frac{\text{Total amount of MB in liposome (mg)}}{\text{Liposome dry mass (mg)}} \times 100 \quad (2)$$

Moreover, a 60-day shelf-life stability study was performed, and DLS, EE, and DL were initially evaluated within the first 24 h after liposome fabrication.

4.4 *C. auris* strain

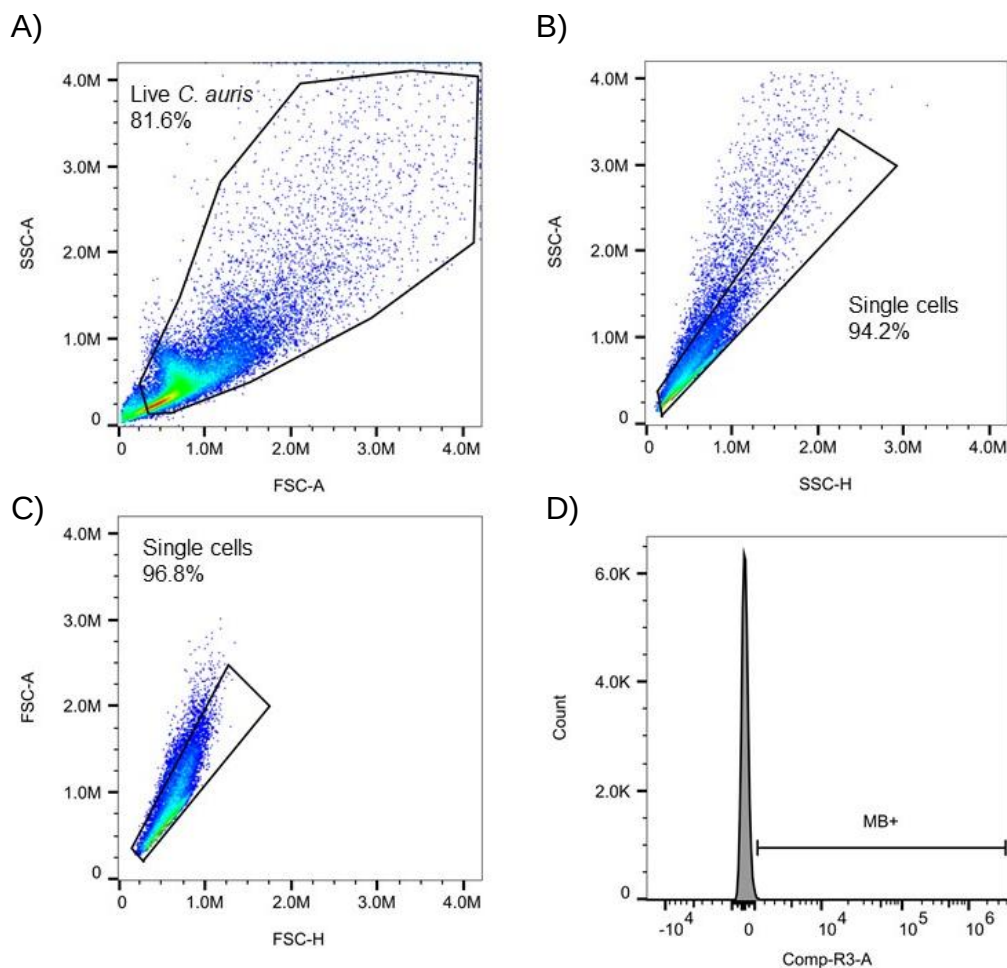
Standard strain of *C. auris* CBS11220, stored at -80°C, was used. For activation, *C. auris* was cultured on SD agar for 24 - 48 h at 37°C.

4.5 MB-loaded liposome interaction with planktonic cells of *C. auris* via flow cytometry

To verify interaction between liposomes to *C. auris* cells in planktonic cultures, flow cytometry was used as described by LaMastro et al. (2023), with modifications. An overnight culture of *C. auris* was prepared by selecting five colonies from a SD agar plate and incubating the culture in 5 mL of YPD media for 18 h at 37°C and shaking at 120 rpm. The overnight culture was adjusted to 10⁸ cell/mL in YNB media containing

100 μ M of glucose. Next, 10 μ L of adjusted *C. auris* culture was treated with either MB-P or MB-N liposomes in 1 $\times$ PBS (140 μ L/well) in a 96-well plate, achieving a 1.4:0.1:0.5 (v/v/v) ratio, and incubated for 15 min (pre-irradiation time) at 37°C, shaking at 120 rpm, and protected from light; 1 $\times$ PBS (50 μ L/well) was added instead of MB-loaded liposomes as a control. As MB is a fluorescent dye with λ_{ex} 665 nm and λ_{em} 686 nm (Selvam, Sarkar, 2017), no addition of other fluorescent dye was needed. Samples were then immediately analyzed via flow cytometry. At least 50,000 events per sample were collected. Data was analyzed with FlowJo Single Cell Analysis Software, v10 (Becton, Dickinson and Co. Biosciences, Franklin Lakes, New Jersey). Gating was performed utilizing samples containing cells only (no liposomes) and liposomes alone (no cells).

Flow cytometry samples were analyzed using FlowJo software (v 10). In each experiment, a single cell type was utilized, and forward scatter (FSC) and side scatter (SSC) profiles were employed to accurately gate each sample. A gate was established around *C. auris*, with exclusion of cell debris and/or dead cells. Subsequently, the population of interest was assessed using FSC Area (FSC-A) versus FSC Height (FSC-H) and SSC-A versus SSC-H. To eliminate doublets, only events falling along an approximately straight line to the densest event area (indicated by red/yellow/green hot spots) were selected for analysis. Lastly, the MB-positive gate was set using the negative cell control group (*C. auris* with no liposome treatment) in a single-parameter histogram. Initially, the main population of live *C. auris* cells was selected by FSC-A versus SSC-A to exclude debris and non-cellular events (A). This population was subsequently refined by sequential singlet discrimination using SSC-A versus SSC-H (B) and FSC-A versus FSC-H (C), thereby excluding doublets and aggregates. The resulting live single-cell population was then analyzed for methylene blue fluorescence, and the MB-positive (MB⁺) gate was established based on fluorescence intensity (D) (Figure 6).

Figure 6 - Flow cytometry gating strategy for *C. auris*-liposome interaction studies

Caption: Cellular debris/dead cells were gated out based on Forward Scatter Area (FSC-A)/Side Scatter Area (SSC-A). A single parameter histogram was used to set an appropriate MB-positive gate using the negative control containing no liposomes, where the frequency of non-specific MB-signal was set below 1%.

Source: Author.

4.6 MB-loaded liposome interaction with *C. auris* in planktonic culture and biofilms via Confocal Laser Scanning Microscopy

The interaction between the MB-loaded liposomes (MB-P and MB-N) and *C. auris* in planktonic and biofilm stages was examined using confocal laser scanning microscopy (CLSM). For the planktonic cultures, fungal cells were exposed to free MB, MB-P, MB-N, or 1× PBS for 15 min at 37°C and shaking at 120 rpm protected from

light. After incubation, *C. auris* were stained with 1 µg/mL of Calcofluor white (CFW) stain and 10 mM sodium hydroxide for 1 min protected from light. Next, 10 µL of the suspension was placed on a glass slide, covered with a glass coverslip, and imaged.

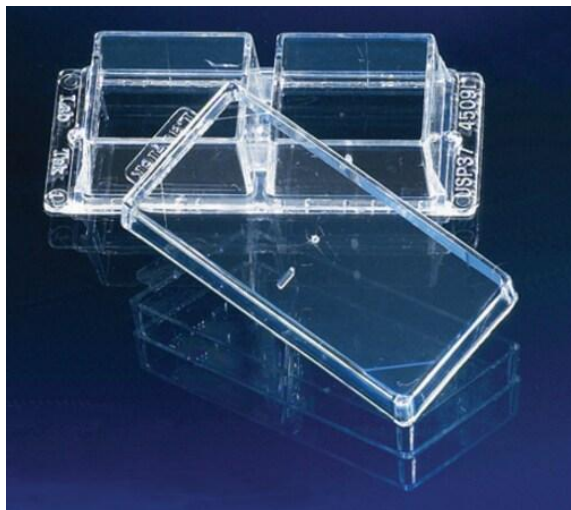
For the biofilm study, *C. auris* biofilms were first formed on glass chamber slides (Figure 7). For this, 200 µL of a fungal suspension adjusted to 10⁶ cell/mL in YNB medium was added to each chamber and incubated for 90 min at 37°C, 120 rpm to allow initial adhesion. The supernatant was aspirated, non-adherent cells were removed by washing twice with 1× PBS, 200 µL of YNB medium containing 2% glucose was added to each chamber, and the slides were incubated for 24 h at 37°C, 120 rpm to obtain mature biofilms. After 24 h, biofilms were washed twice with 1× PBS to remove unattached cells. MB-N, MB-P, free MB, or 1× PBS were added, and plates were incubated for 15 min in the dark at 37°C. After this, wells were washed gently with 1× PBS. CFW was added to each well and incubated for 2 min at room temperature protected from light. The supernatant from each well was removed; biofilm was washed with 1× PBS gently and imaged.

Planktonic cells and biofilms were imaged, and normalized MB fluorescence intensity was calculated using Equation (3).

$$\frac{\text{Sample (intensity/area)} - \text{Negative control (intensity/area)}}{\text{Positive control (intensity/area)} - \text{Negative control (intensity/area)}} \times 100 \quad (3)$$

All CLSM images were obtained using an Apo LWD 20×/1.10 water immersion objective and an A1R confocal laser microscope (Nikon Instruments, Inc., Melville, New York).

Figure 7 - Representative glass chamber slide used for growth of *C. auris* biofilms for confocal laser scanning microscopy (CLSM) analysis



Source: Sigma Aldrich, Nunc® Lab-Tek® II chambered coverglass.

4.7 Light source and experimental groups for the aPDT study

The light source used for aPDT was a red LED device (BlackBox Smart®, Biolambda, Brazil) emitting at a wavelength of 660 nm, with a power density of 40.11 mW/cm², an irradiation time of 712 s, and a total fluence of 28.56 J/cm². The experimental groups included: NL (negatively charged empty liposomes), PL (positively charged empty liposomes), free MB, MB-N (negatively charged liposomes encapsulating methylene blue), and MB-P (positively charged liposomes encapsulating methylene blue). All groups were assessed with and without irradiation, as summarized in Chart 2.

Chart 2 – Experimental groups

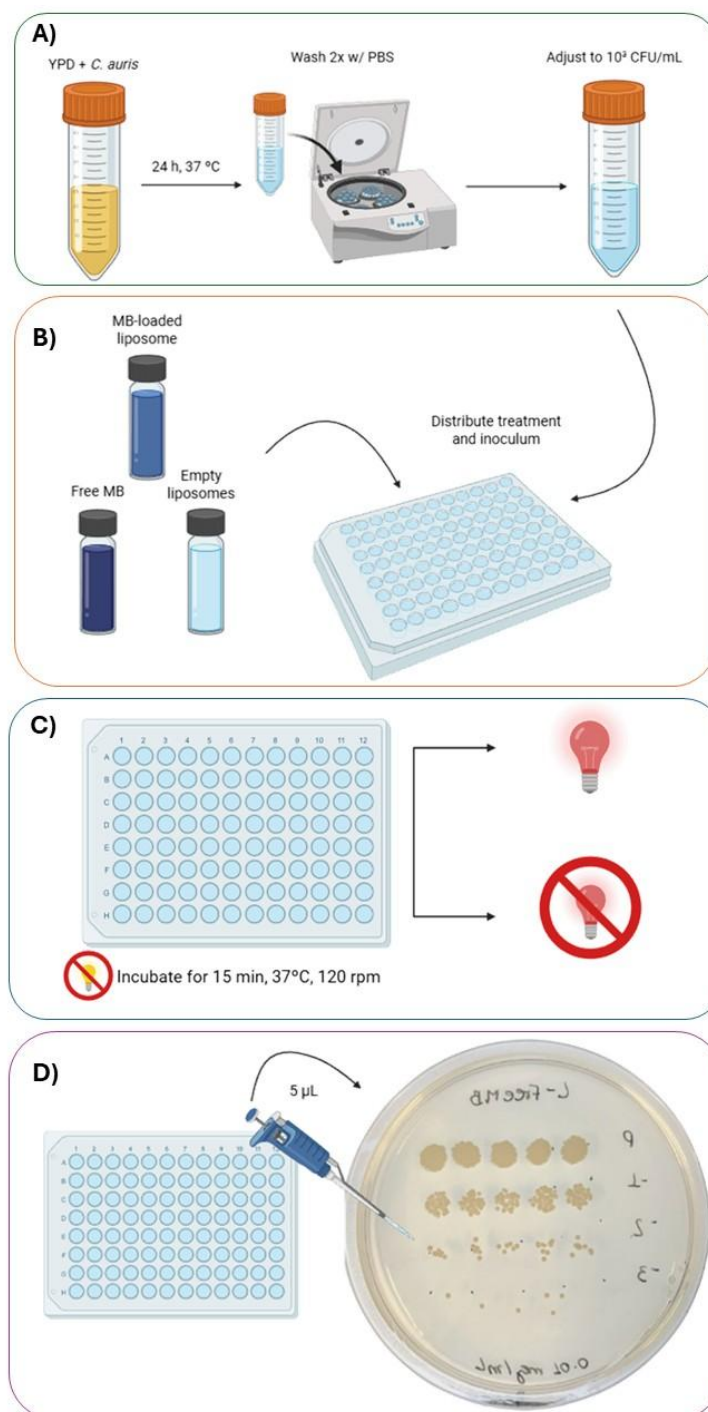
Name	Description
MB-P	Positively charged liposome encapsulating MB
MB-N	Positively charged liposome encapsulating MB
PL	Positively charged liposome encapsulating PBS
PN	Negatively charged liposome encapsulating PBS
MB	Free MB

Source: Author.

4.8 Activity of aPDT mediated by liposomal formulations against planktonic cells of *C. auris*

After 24 h growth of *C. auris* in YPD medium, *C. auris* was centrifuged and washed twice with 1×PBS. The fungal suspension was adjusted to 10^3 cell/mL in 1×PBS. Then, in a 96-well microtiter, 100 μ L/well of free MB, MB-P, MB-N, PL, NL or 1×PBS was added; free MB was freshly dissolved in sterile 1×PBS, filtered with a 0.22 μ m membrane, and protected from light. Next, 100 μ L of fungal suspension, adjusted to 10^3 cell/mL, was added to each well. Plates were then protected from light and incubated at 37°C, 120 rpm, for 15 min (pre-irradiation time). Then, samples were either irradiated, for 712 s (Mendes et al., 2025), or not subjected to irradiation as a control. Each well was serially diluted and 5 μ L of each experimental group was plated on SD agar and incubated at 37°C for 24 h. The fungal burden was then quantified via colony forming units (CFU) enumeration. *C. auris* cells treated with just 1×PBS or non-MB-containing liposomes were used as controls (Figure 8).

Figure 8 - Experimental workflow for evaluating MB-mediated aPDT against planktonic cells of *C. auris*



Caption: (A) Preparation of *Candida auris* CBS 11220 planktonic cells, including overnight growth, washing, and adjustment to 10³ cell/mL. (B) Distribution of treatments—free MB, MB-loaded liposomes (MB-P and MB-N), and empty liposomes (PL and NL) into 96-well plates along with the inoculum. (C) Incubation of samples for 15 min at 37°C followed by exposure or non-exposure to red LED light (660 nm). (D) Post-treatment viability assessment using spot-plating on agar for CFU quantification.

Source: Author.

4.9 Activity of aPDT mediated with MB-loaded liposomes on *C. auris* biofilms

Biofilm susceptibility was assessed against intermediate-stage (24 h) monotypic *C. auris* biofilms grown in 96-well plates. Initially, 200 μ L/well of fungal suspension, adjusted to 10^6 cell/mL in YNB medium, was added in a 96-well microtiter plate and incubated for 90 min at 37°C, 120 rpm to allow for initial adhesion. The supernatant was then aspirated, and the biofilm was washed twice with 1 $\times$ PBS to remove non-adherent cells. YNB medium with 2% glucose (200 μ L) was added in each well and the plates incubated at 37°C, 120 rpm. After 24 h, the supernatant was removed, and wells were washed twice with 1 $\times$ PBS. Biofilms were then treated with free MB, MB-loaded liposomes, or 1 $\times$ PBS at the desired concentration for 15 min (pre-irradiation time) at 37°C, 120 rpm, protected from light. After this, samples were irradiated (L+) as described above, while control samples (L-) were kept protected from light. Biofilms were then disrupted with vigorous pipetting, and CFU was quantified by plating 5 μ L of the dilutions of each well on SD agar. Biofilms treated with 1 $\times$ PBS and non-MB-loaded liposomes were included as controls.

To quantify the biofilm biomass after aPDT, monotypic *C. auris* biofilms were prepared, as described above. Following treatment and subsequent irradiation, biofilms were gently washed twice with 1 $\times$ PBS, air-dried for 45 minutes, and stained with a 0.1% (w/v) Crystal Violet (CV) solution (100 μ L/well) for 15 min. The stained biofilms were then washed twice with 1 $\times$ PBS. CV stain was eluted with 100 μ L/well of 100% methanol. The absorbance was measured at 540 nm and normalized biofilm biomass was calculated as follows (Equation 4):

$$\text{Biofilm biomass (\%)} = \frac{\text{Sample (Abs}_{540}) - \text{Negative control (Abs}_{540})}{\text{Positive control (Abs}_{540}) - \text{Negative control (Abs}_{540})} \times 100 \quad (4)$$

4.10 Reactive oxygen species production in biofilms treated with aPDT mediated by MB-loaded liposomes

Reactive oxygen species (ROS) production in biofilms after aPDT was monitored by the conversion of non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) to fluorescent 2',7'-dichlorofluorescein (DCF), as described by Dias et al. (2023). Biofilms were prepared as described previously and treated with 1× PBS, free MB, MB-P, MB-N, NL or PL. Hydrogen peroxide (H₂O₂) at a final concentration of 3 mM was included as a positive control. The plates were wrapped in aluminum foil and incubated for 15 min at 37°C and 120 rpm, then irradiated as previously described or kept in the dark. Then, wells were washed twice and 100 µL of the H₂DCFDA at a final concentration of 1 µM was added and maintained for 30 min at 37°C, and 120 rpm. All samples were transferred to a black 96-well plate without surface treatment. Fluorescence was measured in the spectral range of excitation of 485 nm and emission of 538 nm.

4.11 Analysis of cytotoxicity of MB-loaded liposome on mammalian cell

Cell viability of fibroblasts (NIH/3T3) after exposure to MB-P and MB-N was evaluated using a commercial Cell Counting Kit-8 (CCK-8; APEX BIO Technology, LLC, Houston, Texas), according to the manufacturer's recommendations and with minor adaptations to the experimental design. This assay is based on the reduction of the water-soluble tetrazolium salt WST-8 by mitochondrial and cytosolic dehydrogenases present in metabolically active cells, generating a water-soluble orange formazan product in the culture medium, whose amount is directly proportional to the number of viable cells (Bioscience - Bio-Techne Brand, n.d.). CCK-8 reagent was stored at 4°C, protected from light, and equilibrated to room temperature immediately before use to avoid condensation and to ensure homogeneous mixing.

For the assay, NIH/3T3 fibroblasts were seeded in sterile, flat-bottom 96-well tissue culture plates at a density of 10,000 cells/cm² in Dulbecco's Modified Eagle

Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), dispensing 200 μ L of cell suspension into each well using a micropipette. The plates were then incubated for 24 h in a humidified incubator at 37°C with 5% CO₂ to allow cell attachment and recovery prior to treatment.

After incubation period, fibroblasts were treated by adding 10% (v/v) of the test solutions directly to the culture medium in each well, resulting in final treatments containing 10% (v/v) of MB, MB-P, MB-N, PL, NL or 1× PBS, using the same MB concentrations previously established for the biofilm experiments (32 μ g/mL). A 10% (v/v) solution of dimethyl sulfoxide (DMSO) in culture medium was included as a positive cytotoxicity control under the same conditions. All treatment solutions were prepared immediately before use, gently mixed, and added gently along the wall of each well to avoid disturbance of the cell monolayer. The plates were incubated with the test solutions for 15 min at 37 °C and 5% CO₂ in the dark to avoid any unintended light activation of MB and to maintain stable culture conditions.

At the end of the 15-min exposure period, the treatment media containing MB, liposomes, PBS, or DMSO were carefully aspirated from each well without touching the bottom of the wells in order to preserve the adherent cell monolayer, and immediately replaced with DMEM containing 10% (v/v) CCK-8 reagent freshly prepared by diluting the stock solution directly into DMEM. The plates were then returned to the incubator and maintained at 37 °C and 5% CO₂ for an additional 2 h to allow sufficient color development. During this incubation, the plates were kept protected from direct light and were handled gently to avoid agitation that could cause uneven distribution of the formazan product. After the 2-h incubation with CCK-8, the absorbance of each well was measured at 450 nm using a microplate reader. The raw absorbance values obtained were used to quantify cell viability after exposure to treatments, with higher absorbance indicating greater metabolic activity and, consequently, a higher number of viable fibroblasts under the tested conditions. Cell viability was calculated using Equation (5).

Normalized cell viability (%)

$$= \frac{\text{Sample (Abs}_{540}) - \text{Negative control (Abs}_{540})}{\text{Positive control (Abs}_{540}) - \text{Negative control (Abs}_{540})} \times 100$$

(5)

4.12 Statistical analysis

Statistical significance was analyzed using GraphPad Prism 10 with one-way analysis of variance (ANOVA) with post-hoc Tuckey's test where applicable ($\alpha = 0.05$; $p < .05$ was considered statistically significant; * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). All experiments were conducted in triplicate at minimum.

5 RESULTS

5.1 Characterization of liposomes according to physical properties, encapsulation efficiency and drug loading capacity

After fabrication, the liposomes obtained were characterized within 24 h based on their physicochemical properties related to hydrodynamic diameter, polydispersity index (PDI), ζ -potential and stability. The parameters of MB encapsulation efficiency (EE) and MB drug loading (DL) were also determined. These results are summarized in Table 2.

Table 2: Liposome characterization for the following parameters

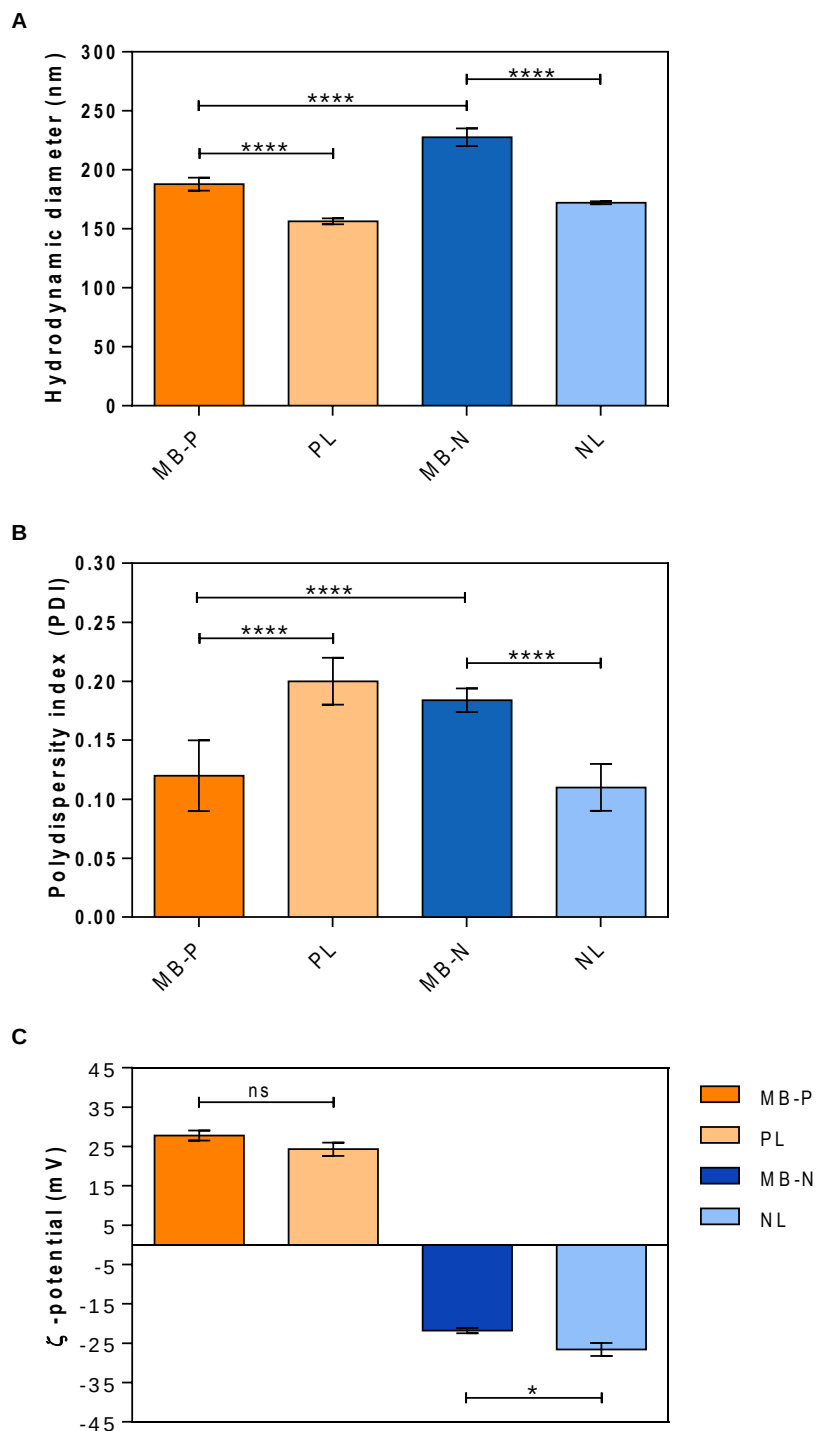
Sample	Hydrodynamic diameter (nm)	PDI	ζ -potential (mV)	EE (%)	DL (%)
MB-P	187.9 $\pm$ 5.5	0.12 $\pm$ 0.03	27.8 $\pm$ 1.3	10.75 $\pm$ 0.91	5.07 $\pm$ 0.52
MB-N	227.6 $\pm$ 7.4	0.18 $\pm$ 0.01	- 21.8 $\pm$ 0.7	14.41 $\pm$ 1.25	5.94 $\pm$ 0.98
PL	156.4 $\pm$ 2.5	0.2 $\pm$ 0.02	24.3 $\pm$ 1.7	--	--
NL	172.2 $\pm$ 1.2	0.11 $\pm$ 0.02	- 26.6 $\pm$ 1.7	--	--

Caption: Data are reported as mean $\pm$ standard deviations. MB-P: positively charged liposomes encapsulating methylene blue (MB); MB-N: negatively charged liposomes encapsulating MB; PL: positively charged liposomes; NL negatively charged liposomes; hydrodynamic diameter, PDI: polydispersity index; ζ -potential, EE: MB encapsulation efficiency; and DL: MB drug loading. Source: Author.

More specifically, we verified that NL and PL formulations exhibited hydrodynamic diameters of $\sim$ 156 nm and $\sim$ 172 nm, respectively. Upon the addition of MB, the diameter of each liposome formulation increased; NL containing MB (MB-N) exhibited hydrodynamic diameters $\sim$ 55 nm greater compared to blank NL formulations ($\sim$ 24% increase), and PL containing MB (MB-P) formulation increased $\sim$ 32 nm compared to their blank counterpart ($\sim$ 16% increase) (Figure 9A). Thus, this increase

in hydrodynamic diameter suggests successful encapsulation of MB. All liposome formulations exhibited PDI values of <0.2 , indicating minimal variation in the hydrodynamic diameters (Figure 9B). In relation to their electrostatic properties according to ζ -potential measurements, PL and NL formulations were positively and negatively charged, respectively, due to the charges of DDAB and DPPG. The addition of MB increased the ζ -potential from +24.3 mV to +27.8 mV for MB-P, while MB-N liposomes exhibited a decrease in magnitude (-21.8 mV) compared to NL formulations (-26.6 mV), most likely due to the cationic nature of MB (Figure 9C).

Figure 9 - Physicochemical properties of liposomal formulations analyzed using dynamic light scattering

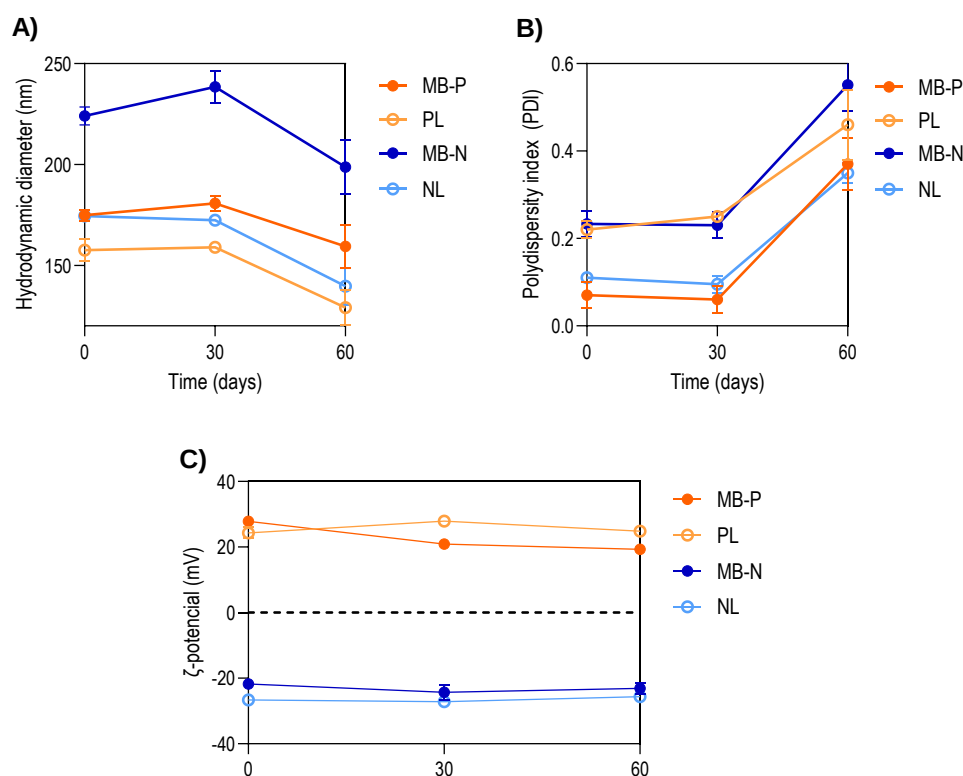


Caption: A) Hydrodynamic diameters; B) PDI: Polydispersity index; C) ζ -potential. MB-P: positively charged liposomes encapsulating methylene blue (MB); MB-N: negatively charged liposomes encapsulating methylene blue (MB); PL: positively charged liposomes; NL negatively charged liposomes. Results are reported as mean $\pm$ standard deviations (n=3).

Source: Author.

The stability of MB-P and MB-N, together with their corresponding control liposomes (PL and NL), was monitored for up to 60 days of storage at 4 °C by following the hydrodynamic diameter, PDI and ζ -potential (Figure 10). All formulations showed mean diameters in the nano-range and only minor fluctuations in size over the first 30 days, with values remaining close to their initial measurements (Figure 10A). Consistently, PDI values stayed below ~0.25 during this period for all formulations, indicating narrow size distributions and good stability (Figure 10B). The ζ -potential of cationic formulations (MB-P and PL) remained positive (around +20 to +30 mV), whereas MB-N and NL maintained negative values (around –20 to –30 mV) throughout the 30-day storage, with no marked changes over time (Figure 10C). After 60 days, however, all formulations exhibited a pronounced increase in PDI, particularly MB-N and MB-P, suggesting the onset of destabilization, although size and ζ -potential values were still within the same order of magnitude. Overall, these data indicate that MB-loaded liposomes and their respective controls remain physically stable for at least 30 days when stored at 4 °C.

Figure 10 - Stability of liposomal formulations evaluated at the day of fabrication (0 days), and after 30 and 60 days of storage at 4°C



Caption: Measurements were performed in 1× PBS at pH 7.4 using dynamic light scattering. A) Hydrodynamic diameter; B) Polydispersity index (PDI); C) ζ-potential. MB-P: positively charged liposomes encapsulating methylene blue (MB); MB-N: negatively charged liposomes encapsulating MB; PL: positively charged liposomes; NL: negatively charged liposomes. Results are reported as mean ± standard deviation (n = 3).

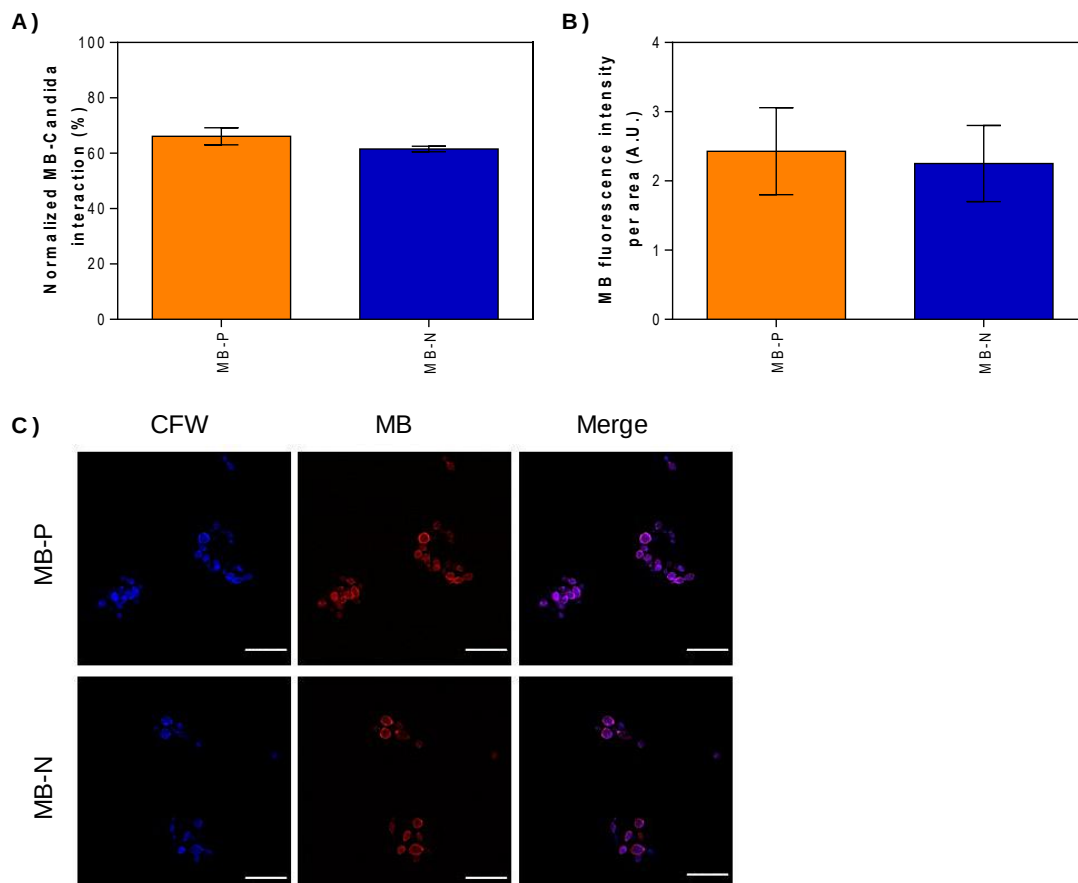
Source: Author.

5.2 MB-loaded liposome interaction with *C. auris* cells in planktonic stage

Initially, the liposome interaction with *C. auris* cells in planktonic state was assessed using flow cytometry, where the percentage of live fungal cells expressing MB fluorescence was quantified as an indication of fungal cell-liposome interaction. We verified that ~66% and ~61% of live *C. auris* cells expressed MB following incubation with MB-P and MB-N formulations, respectively (Figure 11A). Although MB-P showed slightly higher interaction with fungal cells, there was no statistical difference

between the two formulations. The liposomes and planktonic cells interaction was also confirmed by CLSM analysis. MB was found in *C. auris* cells treated with both MB-P and MB-N formulations, localized predominantly in the cell wall of the yeast cells. However, when quantifying the MB fluorescence intensity in CLSM images, no statistical difference was observed between cells treated with either MB-P or MB-N (Figure 11 B-C).

Figure 11 - Interaction of MB-loaded liposome formulations with planktonic cells of *C. auris*



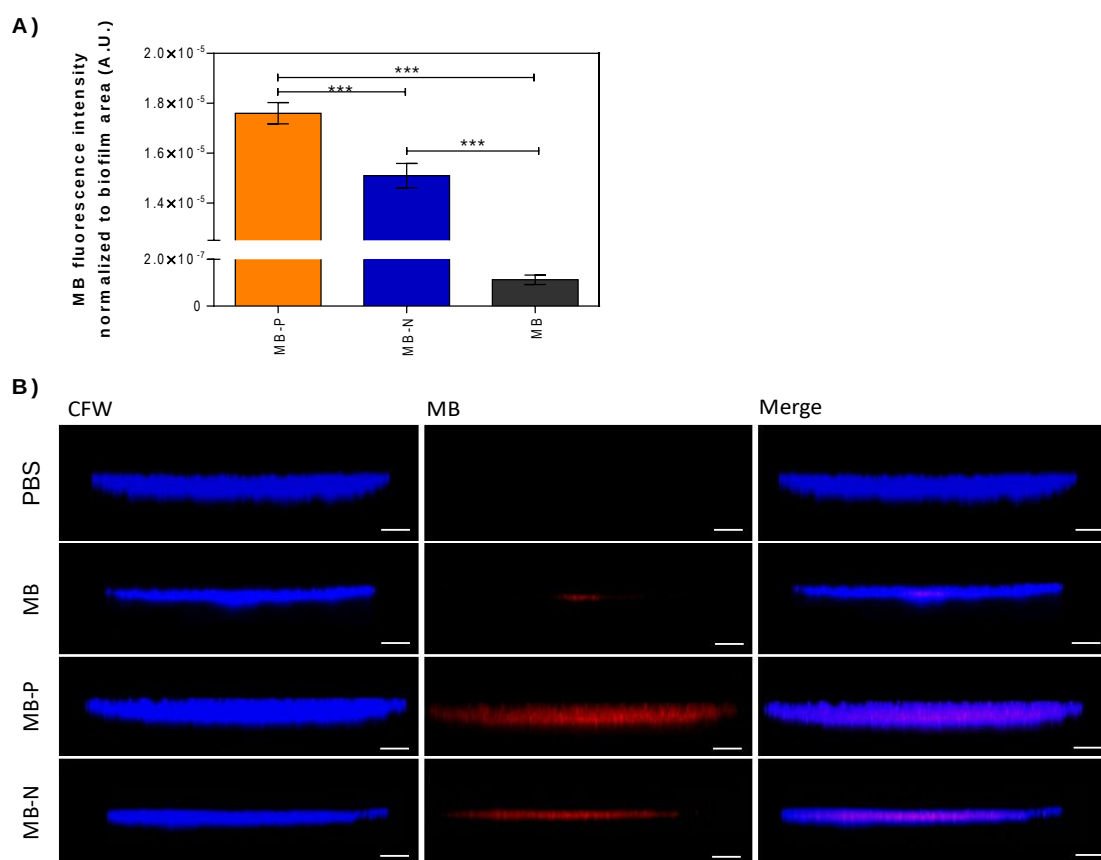
Caption: A) The percentage of live *C. auris* cells positive for MB fluorescence after 15 minutes of incubation with MB-loaded liposomes measured via flow cytometry. Data is reported as mean $\pm$ standard deviations (t-test two-tailed analysis, $n = 3$, $\alpha = 0.05$). B) MB fluorescence intensity per area after 15 minutes of incubation with MB-loaded liposomes measured from CLSM images. Data is reported as mean $\pm$ standard deviations (two-tailed t-test, $n = 12$, $\alpha = 0.05$). C) Representative confocal images of *C. auris* upon 15 min incubation with MB-P and MB-N formulations. *C. auris* cells were stained with CFW (blue), while MB is shown in red. Fungal cells displaying red fluorescence or purple hue suggest fungal cell-liposome interaction. Scale bar = 20 μ m. MB-P: positively charged liposomes encapsulating methylene blue (MB); MB-N: negatively charged liposomes encapsulating methylene blue (MB). Source: Author.

5.3 MB-loaded liposome interaction with *C. auris* cells in biofilm stage

To our study about liposome interaction with *C. auris* cells in biofilm state, we used CLSM analysis. The obtained images showed enhanced MB delivery for both liposome formulations when compared to free MB, with statistically significant

differences in the values of MB fluorescence intensity (Figure 12). Additionally, MB-P liposomes had a greater MB signal within *C. auris* biofilms compared to MB-N formulations, potentially due to enhanced electrostatic interaction between the *C. auris* biofilms and the positively charged liposomes.

Figure 12 - Interaction of MB-loaded liposome formulations with biofilms of *C. auris*



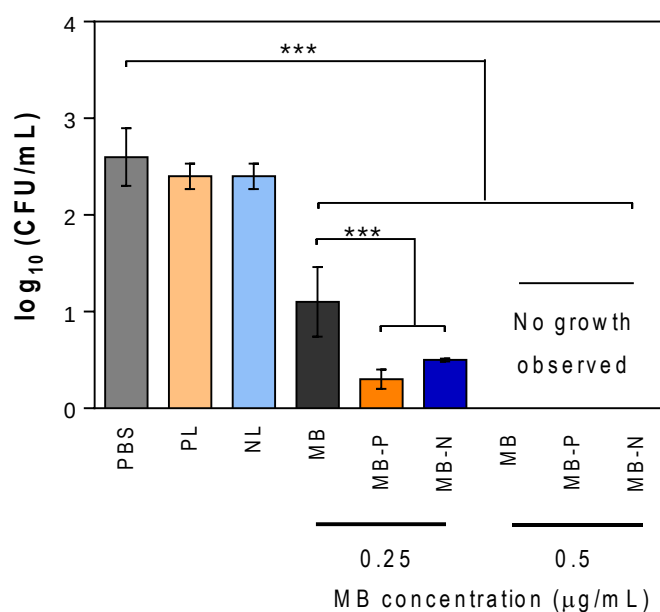
Caption: A) Corresponding MB fluorescence intensity in *C. auris* biofilms evaluated by CLSM, where higher fluorescence intensity indicated greater *C. auris* cell-liposome interaction and MB delivery within biofilms. Data are reported as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, *** $p \leq 0.001$, $n=3$, $\alpha = 0.05$). B) Representative confocal images of *C. auris* B11220 biofilm upon 15 min incubation with 1 $\times$ PBS only; free MB; MB-N and MB-P. *C. auris* cells were stained with CFW (blue), while MB is shown in red. Fungal cells displaying red fluorescence or purple hue suggest interaction with liposomes. Scale bar = 50 μ m. PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB.

Source: Author.

5.4 Activity of aPDT mediated by liposomal formulations against planktonic cells of *C. auris*

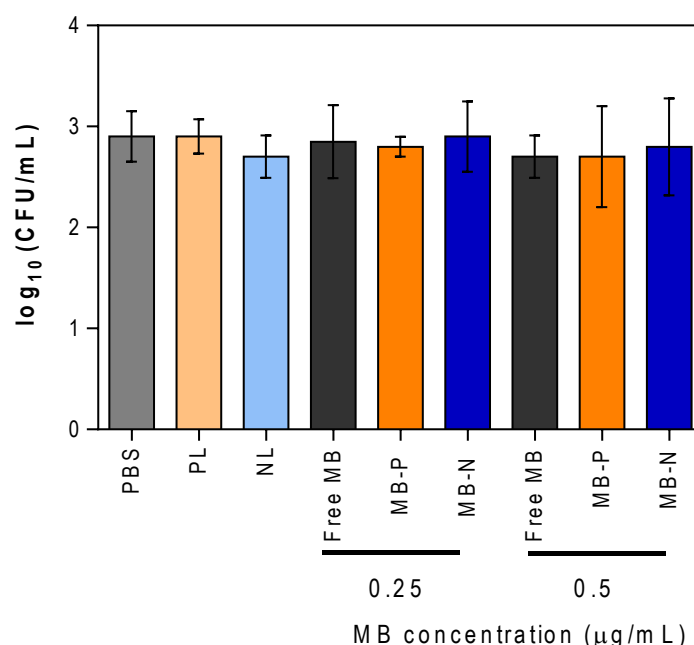
After confirming the interaction of liposomal formulations with *C. auris*, we moved on to assess the antifungal effect of aPDT using free MB, MB-P and MB-N formulations. Non-MB-liposome (PL and NL) formulations were also included as control groups. For this, planktonic cultures of *C. auris* were exposure to liposomes and irradiated by LED, then the antifungal activity of aPDT was determined by cell viability via CFU enumeration.

We verified that aPDT mediated by both MB-P and MB-N liposomes successfully eradicated *C. auris* at a Minimum Fungicidal Concentration (MFC) equivalent to free MB at 0.5 µg/mL, with a significant reduction in CFU values. Conversely, at lower MB concentrations (0.25 µg/mL), both MB-loaded liposome formulations caused greater reductions in *C. auris* burden compared to free MB. Non-MB-loaded liposomes (PL and NL) did not inhibit fungal growth, as expected (Figure 13). Furthermore, no significant reduction in fungal burden was observed for any treatment group without irradiation, confirming that light alone did not influence fungal viability, and light activation is required for antifungal activity of MB and MB-loaded liposomes (Figure 14).

Figure 13 - CFU counts of *C. auris* in planktonic cultures after aPDT

Caption: Number of CFU/mL of *C. auris* after 15 min incubation with liposome formulations (0.25 and 0.5 µg/mL of MB), followed by LED irradiation. PBS: phosphate buffered saline; PL: positively charged liposomes; NL: negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB. The data are represented as mean ± standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, n=5, α = 0.05, *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001). Source: Author.

Figure 14 - CFU counts of *C. auris* in planktonic cultures following a 15 min incubation with liposome formulations, without irradiation



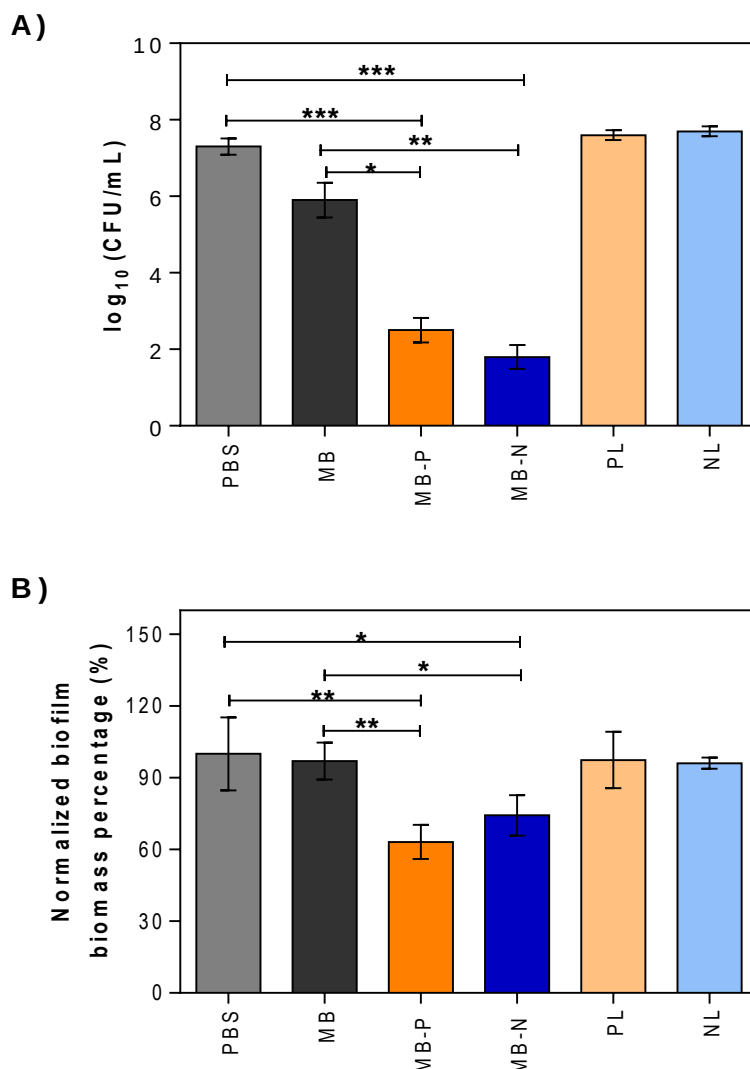
Caption: PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB. The data are represented as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, $n=5$, $\alpha = 0.05$). Source: Author.

5.5 Activity of aPDT mediated with MB-loaded liposomes on *C. auris* biofilms

Biofilms of *C. auris* were exposure to liposomes and irradiated by LED, then the antifungal activity of aPDT was determined by cell viability via CFU enumeration. Antibiofilm activity was also determined by biomass quantification using crystal violet (CV) staining assay.

When aPDT was applied on *C. auris* biofilms, both MB-P and MB-N liposome formulations significantly reduced fungal burden at concentrations equivalent to free MB (32 µg/mL). Specifically, MB-P and MB-N resulted in a ~ 4.8 and a ~ 5.5 log₁₀ (CFU/mL) reduction, respectively, compared to the control group treated with $1 \times$ PBS. In contrast, free MB only resulted in a 1.4 log (CFU/mL) reduction (Figure 15A).

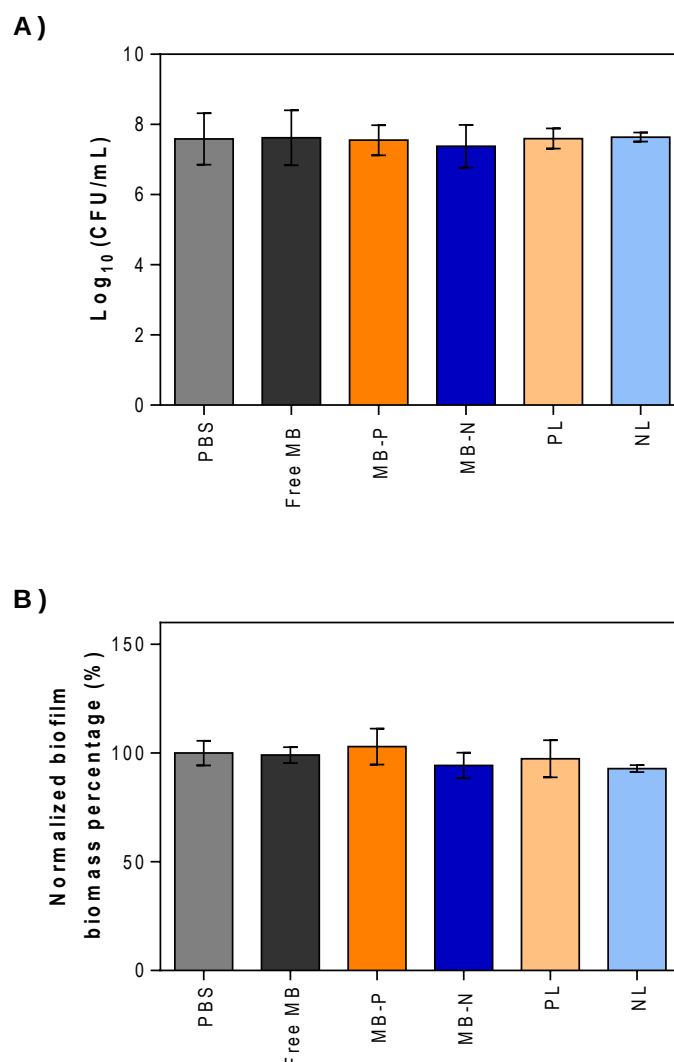
Additionally, a significant decrease in *C. auris* biofilm biomass was observed after aPDT mediated by MB-loaded liposomes compared to biofilms treated with free MB and untreated controls. aPDT mediated by MB-P and MB-N led to a ~37% and ~26% reduction, respectively, while free MB only achieved a ~3% reduction in biofilm biomass compared to the untreated control (Figure 15B). While not statistically significant, MB-P showed a slightly superior effectiveness in CFU and biomass reduction compared to MB-N. These observations are consistent with CLSM results, indicating that MB-P demonstrated superior penetration of MB within biofilm. Both non-MB-loaded liposomes (PL and NL), as well as treatments without irradiation (Figure 16), did not demonstrate any antibiofilm activity, as expected.

Figure 15 - Application of aPDT on *C. auris* biofilms

Caption: A) CFU counts of *C. auris* biofilms after a 15 min incubation with liposome formulations (32 $\mu\text{g/mL}$ of MB), followed by LED irradiation. MB-loaded liposomes significantly reduced fungal burden more effectively compared to free MB. The data are represented as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, $n=5$, $\alpha = 0.05$, $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$). B) Biofilm biomass percentage of *C. auris* post incubation with liposome formulations for 15 min followed by LED irradiation. MB-loaded liposomes significantly reduced biofilm biomass compared to free MB and untreated controls. Data are represented as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, $n=5$, $\alpha = 0.05$, $*p \leq 0.05$ and $**p \leq 0.01$). PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB.

Source: Author.

Figure 16 - Antibiofilm activity of MB-loaded liposomes against *C. auris* biofilms following a 15 min incubation without irradiation

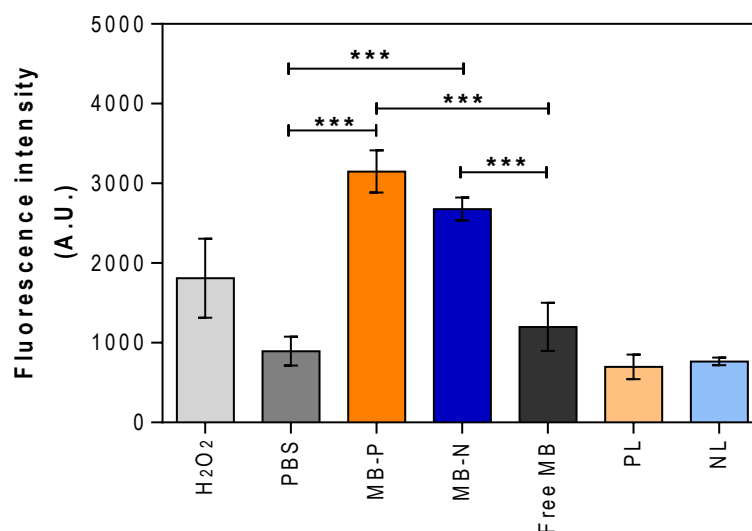


Caption: PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB. A) CFU counts of *C. auris* biofilms following a 15 min incubation with liposome formulations, without irradiation. The data are represented as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, $n=5$, $\alpha = 0.05$). B) Biofilm biomass percentage of *C. auris* B11220 post incubation with liposome formulations for 15 min, without irradiation. Data are represented as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, $n=5$, $\alpha = 0.05$). Source: Author.

5.6 Reactive oxygen species production in biofilms treated with aPDT mediated by MB-loaded liposomes

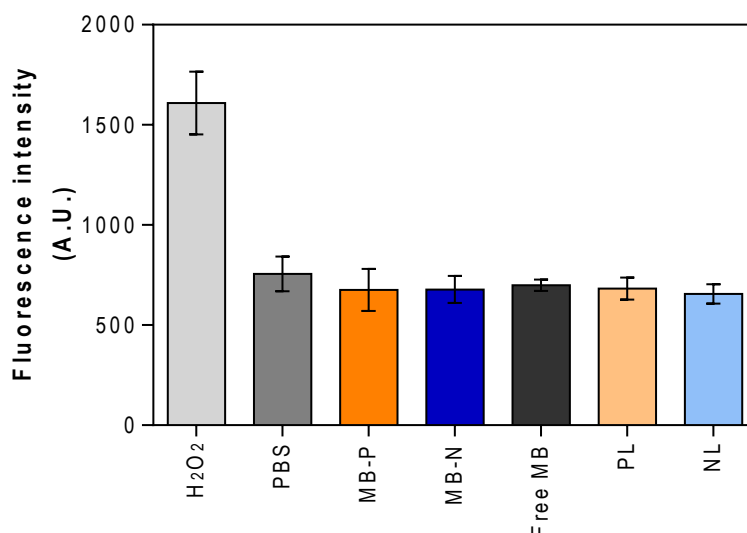
To confirm the aPDT effect of MB-P and MB-N formulations, we assessed the quantification of ROS production in *C. auris* biofilms after exposure to MB-loaded liposomes and LED irradiation. An increase in ROS generation was observed when aPDT was mediated by MB-loaded liposomes at a corresponding MB concentration of 32 µg/mL. MB-P increased ROS production by ~2.6× and MB-N by ~2.2×, contrasted with the significantly lower ROS generation observed with free MB at 32 µg/mL (Figure 17). The control groups, including PL, NL, 1× PBS with and without light activation, as well as MB-P, MB-N and free MB without light activation, did not exhibit increased ROS production (Figure 18).

Figure 17 - ROS production in *C. auris* biofilm post 15 min of incubation with liposome formulations or free MB (32 µg/mL) followed by aPDT



Caption: PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB. Data are presented as mean ± standard deviations. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis (n=5, α = 0.05). Significance levels are denoted as ***p ≤ 0.001. Source: Author.

Figure 18 - ROS production in *C. auris* biofilms after 15 min of incubation with liposome formulations, without irradiation

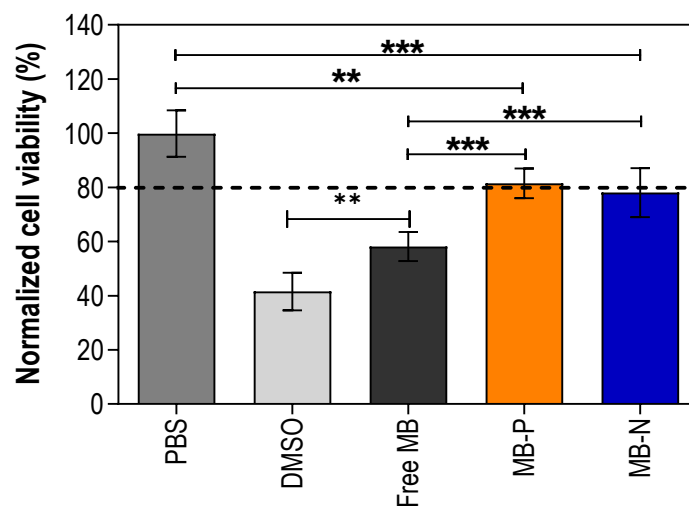


Caption: ROS production in *C. auris* biofilms after 15 min of incubation with liposome formulations, without irradiation. PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB. Data are presented as mean $\pm$ standard deviations. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis ($n=5$, $\alpha = 0.05$). Source: Author.

5.7 Analysis of cytotoxicity of MB-loaded liposome on mammalian cell

At a concentration of 32 $\mu\text{g/mL}$, free MB significantly reduced fibroblast metabolic activity to approximately 58% relative to untreated controls, whereas both liposomal formulations (MB-P and MB-N) maintained cell viability at around 80%. As expected, the lowest viability was observed in the 10% (v/v) DMSO control group (Figure 19). Overall, encapsulation of MB into liposomes reduced cytotoxicity, resulting in an increase of approximately 22 percentage points in fibroblast viability compared with free MB and indicating a safer profile under the tested conditions.

Figure 19 - Cell viability of NIH/3T3 fibroblasts following a 15 min incubation in dark with liposome formulations and free MB



Caption: PBS: phosphate buffered saline; PL: positively charged liposomes; NL negatively charged liposomes; MB: Methylene blue; MB-P: positively charged liposomes encapsulating MB; MB-N: negatively charged liposomes encapsulating MB. Data are presented as mean $\pm$ standard deviations (one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis, $n=5$, $\alpha = 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

Source: Author

6 DISCUSSION

In this study, we integrated principles from microbiology, nanotechnology, and phototherapy for designing liposome-based nanoparticles targeted to PDT. This interdisciplinary approach allowed to optimize the biocompatibility and customizable properties of liposomes (Almeida et al., 2020; LaMastro et al., 2023) to enhance the PS delivery within microbial biofilms. MB alone suffers from limited penetration into biofilms, as well as potential toxicity at high concentrations (Boccalini et al., 2017; Zhang et al., 2019). In general, liposomes can mitigate these issues by improving the solubility within biofilms and reducing the toxicity of MB, providing a more effective treatment (Guimarães et al., 2021; Large et al., 2021; Nsairat et al., 2022; Wang, 2021a).

Nanotechnology has recently gained attention as a strategy to boost PS efficacy by enabling more efficient, targeted delivery (De Leo et al., 2024). Studies have shown that liposomes can fuse with biofilm components of various types of bacterial and fungal biofilms, including those formed by *Candida* spp. (Foroozandeh, Aziz, 2018; LaMastro et al., 2023; Makhoul et al., 2023; Panthi et al., 2024; Wang, 2021b). However, little information is given about *C. auris*. In the context of PDT, while this is the first study to evaluate the PS-loaded liposomes on *C. auris* biofilms, related PS nanocarrier approaches have shown efficacy against *C. albicans* biofilms (Yang et al., 2013). The novelty of this study lies in combine the MB PS with liposomal formulations to control specifically the biofilms formed by *C. auris*.

6.1 MB-loaded liposome fabrication and characterization

Assessing the properties of liposomes is crucial for optimizing their efficacy as drug delivery vehicles. Key parameters such as size and surface charge play pivotal roles in determining their performance. Size affects biodistribution and cellular uptake, with smaller liposomes typically exhibiting better tissue penetration and clearance rates. Charge can influence stability and interactions with biological membranes,

affecting circulation time and targeting efficiency (Large et al., 2021; Nsairat et al., 2022).

In this study, two liposomal formulations were fabricated using a thin film hydration method followed by extrusion. DPPC, a saturated, zwitterionic lipid, was utilized as the main lipid in both formulations, as previous studies have shown good biocompatibility in therapeutic applications containing this lipid (Anderson, Omri, 2004) and previous liposome formulations containing DPPC have been used to encapsulate MB (Boccalini et al., 2017; Pivetta et al., 2022). Unsaturated phospholipids should be avoided for PDT use because they are highly susceptible to lipid peroxidation, making them well-known targets for ROS (Boccalini et al., 2017). The lipid DDAB was added to formulations to generate positively charged liposomes (PL) while DPPG was added to generate negatively charged liposomes (NL).

In this study, liposomal formulations were successfully fabricated. After adding MB, both formulations became larger: MB-N increased by ~55 nm (~24%) relative to blank NL, and MB-P increased by ~32 nm (~16%) relative to blank PL. Similarly, De Leo et al. (2024) observed an 18.9% increase in diameter of their liposomes after encapsulating MB. Thus, this increase in hydrodynamic diameter suggests successful encapsulation of MB. All liposomal formulations showed PDI values below 0.2. In drug-delivery applications, lipid-based carriers such as liposomes with $PDI < 0.2$ are generally considered highly monodisperse, reflecting good vesicle homogeneity and a narrow particle-size distribution (Haddad et al., 2023).

In relation to ζ -potential, the liposome formulations presented distinct electrostatic profiles due to their lipid composition. As expected, MB-P and PL liposomes were positively charged due to the presence of the cationic lipid DDAB (Khodaei et al., 2021), whereas MB-N and NL liposomes were negatively charged as a consequence of the anionic phospholipid DPPG (Sahin et al., 2023). These surface charges are important because they can strongly influence colloidal stability, interaction with biological interfaces, and affinity for negatively charged microbial cell envelopes and biofilm matrices (Barba et al., 2019; Gbian, Omri, 2022; LaMastro et al., 2023).

Incorporation of MB produced formulation-dependent shifts consistent with its cationic character. For MB-P, MB increased the ζ -potential from +24.3 mV to +27.8 mV, indicating an increase in the positive surface charge of the formulation. This

modest increase also indicates that MB loading did not disrupt the overall electrostatic identity of the cationic carrier, which may be advantageous for maintaining strong electrostatic interactions with negatively charged targets (Barba et al., 2019; Gbian, Omri, 2022).

In contrast, MB incorporation reduced the magnitude of the negative ζ -potential in MB-N (from -26.6 mV to -21.8 mV), indicating partial charge neutralization of the anionic surface. This behavior is consistent with adsorption of MB that counterbalances the negative contribution of DPPG. Notably, the ζ -potential remained negative after MB loading, implying that MB does not fully mask the anionic character of MB-N, but rather attenuates it. Such a shift could alter the balance between electrostatic repulsion, which supports dispersion stability, and biological interactions, potentially modifying adhesion to cell surfaces and penetration into biofilms (Barba et al., 2019; Gbian, Omri, 2022).

Overall, these results support successful MB association with both liposomal systems and highlight that lipid composition dictates how MB modulates surface charge. Even though both formulations retain ζ -potentials within ranges commonly associated with colloidal stability, the charge differences between MB-P and MB-N are likely to translate into distinct interaction patterns in biological environments, with cationic MB-P expected to favor stronger electrostatic attraction to negatively charged microbial surfaces, while MB-N may display comparatively reduced electrostatic driving forces after MB loading (Barba et al., 2019; Gbian, Omri, 2022; LaMastro et al., 2023).

Certainly, the physicochemical properties of liposomal formulations had influence in the parameters of EE and DL. The cationic nature of MB (Boccalini et al., 2017) suggests that the higher EE and DL values observed with MB-N liposomes may have resulted from electrostatic interactions between MB and the negatively charged liposome surfaces. Additionally, the increased hydrodynamic diameter of the MB-N formulations compared to blank negative liposomes (NL) supports the hypothesis that a greater quantity of MB was encapsulated within these liposomes. This finding underscores the potential of using negatively charged liposomal carriers for the effective delivery of cationic PSs.

MB-P which is composed of DPPC:DDAB:CHOL can be compared to those reported by Boccalini et al. (2017), who observed an EE of $\sim 8\%$ and a DL of $\sim 9\%$ in a

liposome composition DPPC:CHOL: Dioleyldimethylammonium chloride. Whereas Wu et al. (2018), observed a slightly lower EE (~5%) in a neutral charged liposome composed of DPPC:Poly(carboxybetaine) modified 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine. However, direct comparisons of these results with our formulation are limited by differences in liposome composition, initial MB concentration, and the lipid-to-drug ratio across studies.

Finally, stability was assessed by tracking hydrodynamic diameter, PDI, and ζ -potential on the day of preparation and after 30 and 60 days of storage at 4 °C in the dark. All formulations preserved their physicochemical profiles for up to 30 days; however, signs of instability became evident by 60 days. These results indicate that the current liposomes are suitable for use within a 30-day window, but further optimization is required to support longer-term storage.

Liposome shelf life is limited by both physical and chemical degradation pathways, as phospholipid bilayers are inherently fragile and susceptible to oxidation/peroxidation and hydrolysis. Consequently, stability (and ultimately product performance) is strongly affected by storage conditions, particularly temperature and light exposure, as well as lipid peroxidation. In general, liposomes tend to destabilize at elevated temperatures, and UV or direct sunlight can accelerate degradation. To extend shelf life, strategies that reduce oxidative stress are commonly recommended, including minimizing oxygen exposure (e.g., storage under nitrogen) or adopting lyophilization. Additional protective measures include using light-resistant containers, limiting metal contaminants introduced during processing, and, in some formulations, incorporating cholesterol to rigidify the membrane and reduce susceptibility to peroxidation (Pasarin et al., 2023).

Although various physicochemical properties of MB-loaded liposomes were evaluated in this study, a fully characterization is still required to consider them as an effective PS delivery system for PDT. For this, several analytical methods proposed by regulatory agencies, such as European Medicines Agency (EMA) and US Food and Drug Administration (FDA), must be employed in the next studies to determine the following properties: morphology by transmission electron microscopy (TEM), drug release in physiologically relevant media, liposome phase transition temperature, and liposome integrity in different salt concentration, pH and temperature (Wang, Grainger, 2022).

6.2 Interaction of liposomal formulations with *C. auris* cells in planktonic and biofilm stages

With the successful fabrication of MB-loaded liposomes, we then evaluated their interaction with *C. auris* cells in planktonic and biofilm stages. Given the negatively charged surface of fungal cells (Lenardon et al., 2020), we hypothesized that the cationic MB-P liposomes would exhibit greater interaction with *C. auris* compared to MB-N liposomes. This hypothesis is based on previous study demonstrating that positively charged nanoparticles interact more effectively with negatively charged fungal and mammalian cells due to electrostatic attraction (Large et al., 2021). Evaluating the interaction between liposomes and their target is crucial for optimizing drug delivery systems, as effective interaction ensures targeted delivery and minimizes off-target toxicity (Chavan et al., 2012; Jiang et al., 2024).

In our experiments with planktonic cultures using CLSM analysis, MB were found in *C. auris* cells treated with both MB-P and MB-N formulations, localized predominantly in the cell wall of the yeast cells. In a study conducted by Yang et al. (2013), cationic liposomes composed of dimyristoyl-sn-glycero-phosphatidylcholine (DMPC), cetyltrimethyl ammonium bromide (CTAB), and the PS chlorine e6 (Ce6) had a significantly increased binding to the planktonic form of *C. albicans* compared to negatively charged liposomes containing DMPC only. These results of Yang et al. (2013) can be attributed to the liposomes size, as their cationic liposomes were smaller (80 nm) compared to our cationic liposomes (187 nm). It is known that smaller liposomes have a higher surface area-to-volume ratio, potentially enhancing the interfacial interactions with the cell wall. In addition to liposome sizes, the differences in fungal species can also play a significant role. Yang et al. (2013) focused on *C. albicans*, whereas our study involved *C. auris*. Both species have cell walls composed of mannan, β -glucan, and chitin. However, *C. auris* has a thicker outer mannan layer, longer mannoprotein fibrils, a lower proportion of β -glucan, and lack of phosphomannan. *C. auris* is known for its unique cell wall properties and higher resistance to antifungal agents compared to *C. albicans*. These differences might influence the efficacy of liposomes in enhancing cellular uptake in *C. auris* and *C. albicans* species. This highlights the interplay between the type of PS, the

physicochemical properties of liposomal formulations, and the specific characteristics of the target fungal species as crucial factors determining the success of PSs delivery to *Candida* cells (Wang et al., 2022).

In our study about liposome interaction with *C. auris* cells in biofilm state, the CLSM images showed the enhanced MB delivery for both liposome formulations when compared to free MB. Additionally, MB-P liposomes had a greater MB signal within *C. auris* biofilms compared to MB-N formulations, potentially due to enhanced electrostatic interaction between the *C. auris* biofilms and the positively charged liposomes. Recent studies have also demonstrated the effectiveness of positively charged nano formulations against *C. auris* biofilms. Jaromin et al. (2024) studied a novel antifungal compound (PQA-Az-13) encapsulated in cationic liposomes. Their liposomal formulation exhibited strong activity against *C. auris* biofilms in both *in vitro* and *ex vivo* skin colonization models. In another study conducted by Lara et al. (2020), cationic silver nanoparticles (AgNPs) effectively inhibited *C. auris* biofilm, with IC₅₀ values of 0.06 ppm for biofilm in formation and 0.48 ppm for preformed biofilms. Moreover, AgNP-functionalized surfaces, such as silicone elastomers and bandage dressings, exhibited over 50% biofilm inhibition and retained efficacy after multiple washes, highlighting their potential in preventing *C. auris* infection.

Taken together, our results showed that both liposomal formulations were able to interact with planktonic cells and biofilms of *C. auris*. However, only in biofilm state, MB-P liposomes had a greater interaction with *C. auris* than MB-N liposomes. These results may be attributed to the distinct surface protein expression profiles of *Candida* cells in planktonic and biofilm stages. Cells of *C. auris* in biofilms overexpress several proteins, such as orthologs of *C. albicans* Spe3p, Tdh3p, Sod2p, Ywp1p, and Mdh1p, which are not as prominent in planktonic cells. Some of these proteins exhibit zinc ion binding activity, which typically involves negatively charged or neutral amino acid residues (Paudyal,Vediyappan, 2021). The presence of these proteins on the cell surface may contribute to the overall surface charge of biofilm cells, creating an environment more favorable for electrostatic interactions with MB-P. Further studies investigating the surface protein expression and the roles of these proteins under different growth conditions are required to provide deeper insights into the interaction dynamics with MB-loaded liposomes.

6.3 Antifungal action of PDT mediated by MB-loaded liposomes against planktonic cells and biofilms of *C. auris*

For aPDT study, *C. auris* in planktonic or biofilm stages were incubated with the formulations, irradiated with LED light, and antifungal efficacy was measured by CFU counts and biofilm biomass. In planktonic assays, MB-P and MB-N eradicated *C. auris* at the same MFC as free MB (0.5 µg/mL) and outperformed free MB at a lower MB dose (0.25 µg/mL). In biofilms, MB-P and MB-N produced markedly greater killing and biomass reduction than free MB at equivalent MB concentrations.

Thus, our results support the central premise that liposomal delivery can potentiate MB-mediated PDT against *C. auris*, particularly in the biofilm context where conventional PS delivery is limited. While free MB can be effective in planktonic settings, biofilms impose well-known barriers—dense extracellular matrix, altered metabolic states, and restricted diffusion—that collectively reduce PS penetration and limit the spatial overlap between generated ROS and critical cellular targets (Cavalheiro, Teixeira, 2018; Wijaya et al., 2023; Ye et al., 2022). In this study, incorporating MB into liposomes appears to address these barriers by enhancing MB localization and retention within the biofilm structure, thereby improving PDT outcomes under irradiation.

A key interpretation is that the improved performance of MB-loaded liposomes likely reflects delivery and distribution effects rather than changes in MB photochemistry. PDT efficacy depends heavily on (I) how much PS reaches the target microenvironment, (II) how close the PS is to vulnerable structures at the moment of light activation, and (III) local oxygen availability. Liposomes can improve each of these factors by acting as carriers that concentrate MB near fungal cells, protect MB from unfavorable interactions in solution (e.g., sequestration by matrix components), and facilitate penetration into deeper biofilm regions. Because ROS have short lifetimes and diffusion distances, improving MB positioning can lead to a high leverage effect, especially in biofilms (Gunaydin et al., 2021; Sun et al., 2024).

The comparison between MB-P and MB-N suggests that surface charge and lipid composition influence delivery within biofilm. The trend toward stronger activity with the positively charged formulation is mechanistically plausible: cationic particles generally show stronger electrostatic interactions with negatively charged microbial cell

envelopes and anionic components of the biofilm matrix (Gow and Lenardon, 2023; Lenardon et al., 2020). This can increase adhesion, reduce washout, and promote deeper permeation or more homogeneous distribution—consistent with the CLSM observation of improved MB penetration for MB-P. Even when statistical differences are modest, these charge-driven effects remain biologically meaningful because PDT killing is highly sensitive to local PS concentration and spatial distribution.

It is also important to note in our results that blank liposomes lacked antifungal activity and that treatments without irradiation did not reduce viability. Together, these controls indicate that the observed effects arise from true photodynamic action rather than dark toxicity or nonspecific membrane disruption. This is directly relevant to the feasibility of clinical translation: a formulation that is inert in the absence of light can offer a more controlled safety profile and potentially reduce off-target effects.

Beyond viability, the reduction in biofilm biomass after liposomal MB-PDT is consistent with a broader impact on the biofilm community, potentially involving changes in architecture and/or matrix-associated components, although dedicated structural and matrix assays are required to confirm this (Haval et al., 2025). This is clinically significant because *C. auris* biofilms are associated with surface persistence and device colonization, and matrix disruption can facilitate clearance and improve susceptibility to adjunctive therapies (Horton, Nett, 2020; Nett, Andes, 2020). In practice, a modality that both reduces viable burden and weakens the biofilm scaffold could be advantageous for decontamination strategies and localized treatment of colonized surfaces. However, because biofilm physiology varies with surface type and environmental conditions, future work should validate efficacy on clinically relevant materials (e.g., catheter polymers, stainless steel) and under more complex conditions, including polymicrobial communities.

Overall, these findings reinforce that PS delivery is a key determinant of PDT efficacy in *C. auris* biofilms and that liposomal encapsulation—particularly with a cationic surface—can meaningfully improve the likelihood that MB reaches and remains within biofilm regions where light-activated ROS can exert lethal damage. This positions MB-loaded liposomes as a promising platform for enhancing PDT against *C. auris*.

Comparing with other studies in literature, we can suggest that *C. auris* presents susceptibility to liposome-based PDT with results comparable to *C. albicans* and

bacteria. Romio et al. (2017) explored the effects of PDT mediated by liposomes loaded with triclosan and acridine orange against *C. albicans*, verifying 40% inhibition of planktonic cells. In relation to bacteria, Miretti et al. (2022) studied the effects of PDT, using Zn-phthalocyanine (ZnPc) liposomes, on non-tuberculous mycobacteria. PDT with ZnPc-liposomes achieved reductions varying of 2.9 to 3.6 log₁₀ (CFU/mL) for *Mycobacterium fortuitum* and of 2.0 to 2.6 log₁₀ (CFU/mL) for *Mycobacterium chelonae*. Free ZnPc showed minor reductions in bacterial viability (~0.9 log₁₀ CFU/mL), emphasizing the importance of liposome encapsulation in enhancing efficacy. With focus on a Gram-negative bacterium, Afrasiabi et al. (2023) developed a doxycycline-loaded liposomes doped with curcumin (NL-Cur+Dox) to combat *Aggregatibacter actinomycetemcomitans*, a key pathogen in periodontitis. NL-Cur+Dox irradiated with LED light reduced bacterial biofilms by 82%, while light irradiation of non-formulated drugs showed reductions of approximately 40%. These authors suggested that liposomes improved the solubility, stability, and delivery of curcumin and doxycycline, facilitating better drug-cell interactions, potentially enhancing penetration and accumulation at the bacterial site.

These results underline the importance of optimizing PS using liposomal formulations for antimicrobial PDT applications. Liposomal formulations designed here were able to increase the delivery of MB in biofilms formed *in vitro* and to improve PDT effectiveness on *C. auris*. However, additional studies in animal models are required to validate their *in vivo* efficacy and then translate for clinical studies. As the clinical application of liposome-based PDT is mainly directed at the treatment of cutaneous and mucosal infections, where the light can be easily applied, future studies using wound mouse infection models should be incited.

6.4 Effects of PDT mediated by MB-loaded liposomes on reactive oxygen species production in *C. auris* biofilm

ROS generation is crucial for effective PDT approaches, as ROS are responsible for causing oxidative damage to microbial cells, leading to cell death (Wu,Hu, 2022b). ROS target cellular components, particularly the cell membrane,

causing lipid peroxidation, protein oxidation, and nucleic acid damage (Dantas et al., 2015; Gonzalez-Jimenez et al., 2023). In PDT process, the PS dictates the efficiency of ROS production, then ROS quantification is a prime target for optimization of drug delivery systems as liposomes (Zhao et al., 2025a). To confirm the PDT effect of MB-P and MB-N formulations, we assessed the quantification of ROS production in *C. auris* biofilms after exposure to MB-loaded liposomes and LED irradiation.

All experiments included the following groups: empty liposomes (PL and NL), MB-loaded liposomes (MB-P and MB-N), PBS only, and hydrogen peroxide (positive control). Each condition was tested both with and without light irradiation. No increased ROS generation was detected in empty liposomes or PBS under either condition, confirming that the nanocarriers themselves do not produce ROS or interfere with the assay. In contrast, hydrogen peroxide and MB-loaded liposomes under irradiation showed significant ROS production. More specifically, under irradiation, MB-P increased ROS production by $\sim 2.6\times$ and MB-N by $\sim 2.2\times$, contrasted with the significantly lower ROS generation observed with free MB at 32 $\mu\text{g/mL}$.

Therefore, greater oxidative damage was generated using MB-loaded liposomes on *C. auris* biofilms compared to free MB, aligning with the CFU count and biomass results. These findings further underscore the relationship between the enhanced MB delivery and increased ROS production within biofilms. Wu et al. (2018) also developed liposomes using a zwitterionic polymer-lipid (poly(12-(methacryloyloxy)dodecyl phosphorylcholine)) to encapsulate MB, however their study was focused on antitumoral photodynamic therapy. These authors employed the DCFH-DA assay to quantify the ROS production in breast cancer cells treated with MB-loaded liposomes and compared to free MB. A strong green fluorescence was observed in cancer cells treated with MB-liposomes after light exposure. The fluorescence intensity in MB-liposome-treated cells was significantly higher than in cells treated with free MB under the same conditions. According to the authors, the improved intracellular ROS generation was attributed to the enhanced cellular uptake of MB when encapsulated in liposomes. The liposomal delivery system facilitated better membrane penetration and higher intracellular accumulation of MB.

For antimicrobial PDT, besides targeting cellular sites, ROS generated should also target biofilm components. It has been demonstrated that ROS produced after PDT can attack proteins, lipids, and nucleic acids present within the biofilm matrix,

contributing to disrupt the extracellular matrix and disaggregate the biofilms (Melo et al., 2021). However, biofilms are more resistant to the damage by ROS than planktonic cells. Naturally, biofilm environment suffers with accumulation of oxidant agents produced endogenously by the aerobic metabolism of microorganisms and by the mammalian immune system. To defend from ROS damage, biofilms develop protective mechanisms, such as an increased production of specific detoxifying enzymes like catalases and superoxide dismutases (SODs). It is known that there is an intricate interplay between the response to oxidative stress and biofilm formation stages, and many other resistance mechanisms can exist behind the action of detoxifying enzymes (Nizer et al., 2024). There is evidence that common mechanism for oxidative stress resistance and multicellular behavior exist in *C. albicans* biofilms, since cell adhesion, biofilm formation, and oxidative stress resistance are influenced by a common factor, the cell wall protein Hwp2p (Čáp et al., 2012).

Therefore, for the effectiveness of PDT on biofilms, PSs must be designed to generate high levels of intra- and extracellular reactive oxygen species (iROS and eROS), and MB-loaded liposomes developed in our study reinforce the notion that optimized PS delivery can improve PDT outcomes against biofilms. Seeking to increase the ROS production, recent studies have also focused on synergistic approaches, integrating liposome-based PDT with other ROS generation strategies, such as sonodynamic therapy (SDT) and photothermal therapy (PTT) (X. Wang et al., 2023; Yan et al., 2022; Zhao et al., 2025b).

6.5 Evaluation of possible cytotoxic effects of MB-loaded liposomes on mammalian cells

For a safe clinical application, liposomal formulations must combine antimicrobial efficacy without toxicity on host cells (Frigaard et al., 2023; Liu et al., 2022). Thus, in this study, we measured the cell viability of NIH/3T3 fibroblasts after contact with MB-loaded liposomes for a period of 15 min in dark. Fibroblasts were chosen as a model cell line, as they are one of the main cell types found in the skin and in localized *C. auris* infections (e.g., burn wounds, skin infections) (Centers for disease control and prevention, 2021; J. Wang et al., 2023).

At a concentration of 32 $\mu\text{g/mL}$, free MB significantly reduced the metabolic activity of fibroblasts (~58%) compared to untreated controls, while both liposome formulations (MB-P and MB-N) maintained fibroblast viability at ~80%.

It has been seen that MB can influence cell proliferation, cell signaling, and induce apoptosis in a dose and time dependent manner. Moreover, systemic administration of MB has been associated with adverse effects such as hemolysis, methemoglobinemia, and nausea. (Zhang et al., 2019) Our findings indicate that MB-loaded liposome formulations can be used to reduce MB toxicity towards fibroblasts at therapeutic concentrations (e.g., 32 $\mu\text{g/mL}$). This observed phenomenon may be attributed to the liposome drug delivery mechanism, which probably caused a gradual release of MB, decreasing its direct contact with fibroblasts compared to free MB. In addition, in the context of PDT, it has been proposed that when liposomes are exposed to light, their thermosensitive lipid composition, such as DPPC, induces degradation and subsequent release of their inner content. (Abu Dayyih et al., 2021) Consequently, a certain proportion of liposomes may only release their content in response to light exposure. In the absence of light, these liposomes may exhibit a protective role. Overall, this suggests a promising avenue for the development of safe and effective antifungal PDT using MB-loaded liposomes.

Similar results were reported by Afrasiabi et al. (2023), who designed doxycycline-loaded liposomes doped with curcumin (NL-Cur+Dox) against *A. actinomycetemcomitans*, testing their possible toxic effects on Human Gingival Fibroblasts (HGFs) and red blood cells. The cytotoxicity and hemocompatibility of NL-Cur+Dox was compared to free doxycycline (Dox) and curcumin (Cur). NL-Cur+Dox and NL-Cur maintained cell viability up to 90%, while treatment with free Dox reduced cell viability significantly, indicating that liposomal encapsulation protected HGFs from the cytotoxic effects of free Dox. NL-Cur+Dox exhibited low hemolysis (<5%), comparable to the negative control (PBS). Red blood cells (RBCs) treated with NL-Cur+Dox showed no morphological changes, further confirming its safety for systemic use.

By combining our results with data from the literature, we can confirm that the encapsulation of PSs in liposomes not only improves therapeutic efficacy but also minimizes cytotoxic effects on mammalian cells, increasing the selectivity of PDT.

7 CONCLUSION

In this *in vitro* study, the designed liposomes using saturated lipids were able to encapsulate MB PS and provide an efficient delivery on *C. auris* cells and biofilms. Among the formulations developed, MB-Positive liposomes had a greater interaction with *C. auris* biofilms than MB-Negative liposomes, which provided a higher penetration of MB within biofilms. When PDT was applied, both MB-loaded liposomes caused an eradication of *C. auris* planktonic cells and an inhibition of biofilms compared to free MB. The decrease of biofilms after PDT mediated by MB-loaded liposomes were accompanied by an increase in the ROS production in biofilms. Moreover, MB-loaded liposomes demonstrated biocompatibility with mammalian fibroblasts. Based on the literature reviewed, this study is the first study to demonstrate the *in vitro* effectiveness of aPDT using PS-loaded liposomes to control *C. auris* biofilms, providing support for further *in vivo* studies that can validate its efficacy and safety for the treatment of *C. auris* infections.

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