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Review

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Nanoliposomes as Drug Delivery Systems for Antifungal Therapy

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Abstract

Nanoliposomes are lipid bilayer nanostructures recently gaining popularity as drug delivery vehicles. Their biocompatibility, safety, high entrapment efficiency and drug release profiles have made them suitable candidates for biomedical applications. The treatment of fungal infections is one of the most significantly researched fields in which nanoliposomes (NLPs) find a variety of uses. Although this topic has been referenced in several general reviews, it has never been thoroughly discussed. In our review, we give a detailed summary of the recent advancements made in this field, with a focus on encapsulated synthetic and natural antifungal agents for oral, parenteral, and transdermal applications, and the numerous advantages they have over conventional antifungal therapies, recent strategies at site-specific targeting using targeting moieties, as well as highlighting the safety concerns associated with NLP nanocarriers.

Keywords" Nanoliposomes, antifungal, targeted drug delivery

Purpose and Rationale

This review summarizes the state-of-the-art progress in using NLPs as drug delivery platforms for antifungal agents with a focus on recent advancements made in the last decade, and as such, will not undertake an in-depth analysis of the numerous data published in this field.

Introduction

Fungal infections have increased exponentially worldwide, with over 300 million cases accounting for approximately 1.7 million deaths annually [1]. The adaptability of these opportunistic pathogens to environmental conditions has made them difficult to treat especially in immunocompromised patients. Moreover, invasive treatment within intensive-care units, cancer treatments, increased use of prophylactic azole derivatives, immunosuppressive treatments after organ transplants, and the growing use of amphotericin B as empirical therapy, have all contributed significantly to the increasing rate of invasive fungal infections. Thus, resistance appeared for some strains such as *Candida spp., Aspergillus spp., Fusarium spp*., and zygomycetes among other fungal pathogens, which attracted researchers to find novel drug delivery systems to minimize drug resistance and toxicity [2-4].

One of the most prominent targeted drug delivery platforms that have garnered interest among researchers were liposomes (LPs) which are lipid structures that are composed of cylindrical-shaped molecules called phospholipids. Phospholipids are amphiphilic molecules with hydrophilic heads and hydrophobic tails that spontaneously form spherical bilayers when they come in contact with an aqueous phase, where the hydrophilic heads are aligned in the outer layer of the sphere whereas the hydrophobic tails are pointed towards each other. Liposomes are classified into categories that reflect their size and lamellarity: multivesicular vesicles (MVV, >1μm) that consist of hundreds of non-concentric aqueous chambers encapsulated in a single bilayer lipid membrane and have a honeycomb-like shape, multilamellar vesicles (MLV, >500 nm) are structures that exhibit an onion-like shape where several bilayers are organized as a stack. they form small unilamellar vesicles (SUV) that range in size from 30-100 nm and large unilamellar vesicles (LUV, >100

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nm) where their structure consists of a single phospholipid bilayer [3,4]. This arrangement allows the encapsulation of hydrophilic compounds inside the aqueous compartment of the LP, as well as incorporating hydrophobic compounds inside the membrane [5]. The size of the circulating LPs is an important parameter to determine its behavior in vivo, as larger LPs >1μm are more extensively taken up by the reticuloendothelial system (RES). Thus, the need to produce smaller sized LPs that have a longer circulating time is important. To achieve this, several techniques are employed to reduce the sizes of NLPs, for example, LUVs and SUVs are produced by extrusion and sonication, respectively.

Extrusion is an easy and relatively quick process that involves passing an LP suspension through a polycarbonate membrane filter of a determined pore size using an extruder, which is a machine fitted with a pump that pushes the suspension through a membrane to produce LPs with a relatively homogenous size distribution [6].

Sonication, is a process that involves utilizing acoustic energy from a bath or a probe tip sonicator to blend a lipid suspension to produce nanosized LPs, also called nanoliposomes (NLPs) that might be unilamellar or multilamellar [7]. NLPs have many advantages over conventional LPs depending on the intended use especially for the delivery of water-soluble drugs [7]. They offer larger surface-to-volume ratios, more surface area and thus a higher drug-loading capacity. Moreover, they have the potential to increase solubility, enhance the bioavailability, improve controlled release, and enable precision targeting of the encapsulated material to a greater extent. This is deemed beneficial and cost-effective because it allows using fewer concentrations of the drug, increasing therapeutic efficacy, and decreasing toxicity, especially with drugs that have a narrow therapeutic range.

However, it was noted that decreasing the size of the LPs is associated with a decrease in their physical stability, this is due to an increase in electrostatic interactions which results in aggregation and coalescence [8]. Thus, efforts have been made to preserve NLPs stability, such as employing a freeze-drying technique, addition of surfactants, modification with chitosan, and incorporation with a polymer gel [9]. Therefore, NLPs have become one of the most promising drug delivery tools owing to their biocompatibility, safety, versatility, high drug-loading capacity, and ability to carry hydrophobic and hydrophilic compounds within their structure [5-12]. Since their discovery in the 1960s, NLPs have been proposed in many applications. Therefore, notable progress has been made to investigate those compounds during the last two decades, especially for biomedical applications. Currently, many NLP-based formulations are either under clinical trials awaiting to be put on the market or already available for commercial use [12].

Among the first commercially available NLP-based antifungal products was AmBisome® (AmBi; Nexstar) which is a small unilamellar liposome (45-80 nm) that contains amphotericin B within its bilayer [13]. This formulation is known for its reduced renal toxicity, elevated bioavailability, improved safety, as well as its accurate targeting compared to conventional amphotericin B [13, 14].

In the last two decades, NLPs have been studied intensively as carriers to deliver antifungal agents by different routes of administration including parenteral, oral, and topical dosage forms, aiming at more precise targeting for increased efficacy and safety. Efforts have been made to further modify these compounds to increase their biocompatibility, such as modifying NLP's surface with polyethylene glycol (PEG) to produce non-toxic 'stealth' NLPs that were able to avoid being recognized by the RES. PEG is a bio-inert hydrophilic polymer that serves as a steric barrier, and grafting PEG on the surface of NLPs increases their hydrophilicity, thus hindering the interaction between them and serum protein opsonins that are involved with systemic clearance by the RES [15, 16]. As a result, extending the circulation half-lives of NLPs and allowing the drug to reach its target site more efficiently.

Summary of Recent Literature

1. Synthetic Antifungal Agents

1.1. Voriconazole (VCZ)-Loaded Nanoliposomes

Voriconazole (VCZ) is a second-generation triazole antifungal agent with a broad spectrum of action that has been used for many years as the first-line therapy against several systemic fungal infections, including those caused by Candida albicans. VCZ was approved by the

Food and Drug Administration in 2002 for the treatment of invasive aspergillosis and infections of *Scedosporium apiospermum* and *Fusarium spp.* which are refractory to other antifungal agents [17]. VCZ is known to have a highly variable, non-linear pharmacokinetic (PK) profile [18], partly because it's extensively metabolized in the liver into its primary metabolite Voriconazole-N-oxide (VNO) which has lower antifungal activity and is suspected to be causing adverse effects (AEs) [19, 20]. Furthermore, VCZ is a potent cytochrome (CYP) P450 inhibitor that exerts its effects on enzymes such as CYP2C19, CYP3A, and CYP2C9, which indicates several potential drug-drug interactions [20, 21].

VCZ is also associated with several AEs ranging from mild to severe, including hepatotoxic (mainly elevated liver function tests) and neurotoxic (hallucinations, confusion, or encephalopathy) AEs due to its narrow therapeutic window [22]. Moreover, VCZ has limited water solubility, which presents an issue for the development of intravenous (IV) formulations, this has prompted the use of sulfobutyl ether-beta-cyclodextrin (SBECD) in VCZ formulations to enhance the drug's solubility, SBECD has been shown to cause several AEs such as nephrotoxicity, hemolysis, blood vessel congestion, and its accumulation results in renal and hepatic toxicity, which further complicates the use of VCZ in clinical settings [22].

Veloso et al. (2018) have developed an IV formulation of nanosized liposomal VCZ composed of PC and cholesterol for the treatment of systemic fungal infections, by using the lipid-film hydration method followed by extrusion. This formulation was advantageous since it overcame the stability issues that were reported with previous intravenous VCZ products such as VFEND®. This stability improvement has decelerated VCZ rate of metabolism into VNO by 30%, and at the same time avoided SBECD's toxicity. The in vitro antifungal study comparing intravenous liposomal VCZ and VFEND® against *Candida spp.* and *Aspergillus spp.* yeasts have indicated that liposomal VCZ and VFEND® had similar potency against Candida spp. strains, yet liposomal VCZ triggered a higher fungicidal activity against the *C. albicans* 77 U strain, with the minimum fungicidal concentration (MFC) being 4-fold lower than that of VFEND®. Whereas among *Aspergillus spp.* strains, the minimum inhibitory concentrations (MIC) and MFC values were lower, with liposomal VCZ exhibiting higher inhibitory and fungicidal activities than with VFEND®. Furthermore, the histopathological findings of the in vivo mouse model study have suggested that the encapsulation of VCZ into NLPs has improved its PK properties and biodistribution. Also, the NLP's biocompatible nature was found to enhance cell tissue penetration properties, leading to higher VCZ quantities being released at the target site. In addition, VCZ's renal and hepatic accumulation was higher when the liposomal formulation was used, because the liver and kidneys are the target organs for fungal colonization. This finding further supports the mechanisms in which liposomal VCZ has higher antifungal activity and reduced toxicity to non-target tissues [22].

In a recent study by Hassanpour et al. (2020), the effects of VCZ-loaded nanoliposomes against VCZ-resistant *Aspergillus flavus* strains were investigated. Also, they examined the expression of CYP51A and multidrug resistance mutation 1 (MDR1), the genes involved in developing triazole resistance before and after VCZ and VCZ-loaded NLPs exert their effects. Liposomal VCZ was prepared using a modified thin-film hydration-sonication method. The prepared NLPs had a mean particle size smaller than 100 nm and a very high EE. Moreover, in vitro susceptibility test on resistant *A. flavus* was carried out using liposomal VCZ and pure VCZ, with liposomal VCZ exhibiting significantly lower MIC values than pure VCZ. In addition, the level of gene expression of CYP51A and MDR1 in VCZ-susceptible *A. flavus* and VCZ-resistant strains had more pronounced downregulation when exposed to LVCZ as opposed to pure VCZ [23].

In 2021, Hassanpour et al. conducted a study on LVCZ efficacy against fluconazole (FCZ)-resistant strains of *Candida albicans*. Using a quantitative reverse transcription PCR (qRT-PCR) instrument to measure mRNA levels of CDR1, and CDR2, the genes responsible for the development of resistance, and ERG11, the gene hindering the pathway in which FCZ exerts its therapeutic effects. LVCZ was produced using a slightly modified thin-film hydration-sonication technique. The in vitro antifungal susceptibility assay against *C. albicans* isolates was carried out using FCZ, VCZ, and LVCZ to assess the MBIC values for each formulation. Results showed that using VCZ-loaded NLPs

has remarkably lowered MBIC values in *C. albicans* isolates that were resistant to both FCZ and VCZ. Additionally, VCZ-loaded NLPs were able to reduce the expression of azole-resistant genes [24].

1.2. Fluconazole (FCZ)-Loaded Nanoliposomes

Fluconazole (FCZ) is a first-generation triazole compound with a broad spectrum of activity, it is the drug of choice for a wide variety of fungal infections most commonly those caused by *Candida sp.* FCZ is available as oral, intravenous dosage forms with both having similar PK profiles. Reports of fluconazole-resistant isolates from patients who have previously received FCZ therapy are increasing especially with immunocompromised patients. It was found that some *Candida* infections have started to develop from strains that are intrinsically resistant to FCZ such as *C. krusei*, or from strains that have reduced drug susceptibly and are developing resistance such as *C. glabrata*. FCZ is a potent inhibitor of the enzyme CYP2C9 and to some degree CYP3A4, and along with the drug's long half-life, this leads to a risk for multiple drug-drug interactions [25, 26].

Zandi et al. (2018) have conducted a study employing NLPs and nanoethosomes as potential drug delivery vehicles for FCZ therapy. Ethosomes are a modification of classical liposomes, with a high ethanol concentration of about (20-45%) and were investigated for their high drug entrapment and stability profiles [27]. The studied NLP formulation was prepared using the thin-film hydration method, whereas the ethanol injection method was used to prepare the nanoethosomal formulation. The particle size and zeta potential of NLPs and nanoethosomes were found to be 99.79 ± 11.1 nm, −7.25 ± 4.88 mV, and 114.37 ± 12.76 nm, 3.04 ± 5.47 mV respectively, both formulations exhibited similar EE and loading capacity (LC). Nevertheless, the NLP formulation was superior with higher EE and LC values, this appears to be attributed to the method used to prepare nanoethosomes since there was a higher chance of drug escaping with the ethanol injection method.

However, in vitro drug release study has shown that the NLP formulation might be better suited as a sustained drug release system, given that 40% of the drug was released after 48h, compared to 80% of that with the nanoethosomal formulation. This sustained release behavior might be attributed to the presence of cholesterol in the lipid bilayer which increased the latter rigidity. In nanoethosomes, the presence of ethanol decreased the rigidity, which ended up accelerating the drug's release from the vesicle [28].

1.3. Itraconazole (ITZ)-Loaded Nanoliposomes

Itraconazole (ITZ) is a triazole antifungal agent with a broad spectrum of action, it is used against a variety of deep and superficial fungal infections caused by isolates of *Candida, Cryptococcus, Blastomyces, Histoplasma capsulatum*, and *Aspergillus* species and dermatophytes.

ITZ has been widely employed as an oral formulation for the treatment of histoplasmosis, blastomycosis, and refractory aspergillosis [29, 45] as well as a topical formulation for the treatment of vaginal candidiasis [46]. However, ITZ has very low water solubility and bioavailability which hinders its utilization in many clinical applications. Additionally, the conventional oral dosage form is associated with many AEs, most commonly gastrointestinal disturbances, and headaches [47,48]. Moreover, ITZ is contraindicated in pregnant and lactating women, as well as patients with hepatic and/or renal impairment. ITZ is a potent inducer of the enzyme CYP450, and as such interferes with the metabolism of drugs such as anticoagulants, sedatives, and hormonal products [33]. Consequently, researchers have been investigating the use of nanoparticles to utilize the full potential of ITZ by exploring different routes of administration to increase bioavailability and mitigate unwanted effects.

Ćurić et al (2013) have conducted a study to systematically characterize and optimize the encapsulation process of ITZ in PEGylated NLPs for parenteral administration using the thin-film hydration method combined with sonication. They analyzed the responses of the different interactions between multiple process parameters in the formulation process. The DoE methodology was advantageous in that responses such as particle size, polydispersity index (PDI), EE%, and optimal absolute loading were analyzed either together or separately using a relatively low number of experiments in a short amount of time leading to robust results.

Table 1. A summary of recent literature reports on antifungal drugs entrapped in nanoliposomes.

Anti-Fungal Drug	Route of Administration	Drug Loading Method	Size (nm)	Zeta Potential (mV)	Therapeutic Effect	Status	References
Voriconazole	Parenteral	lipid-film hydration followed by extrusion	95.3 ± 1.27	neutral	Increased antifungal activity and reduced toxicity	Preclinical *in vivo* study	[22]
Voriconazole	Not reported	Modified thin-film hydration-sonication	80.91 ± 2.78	−2.33 ± 0.54	Lower MIC values Downregulation of VCZ-resistant genes CYP51A and MDR1	Preclinical *in vitro* study	[23]
Voriconazole	Not reported	Modified thin-film hydration-sonication	110.0 ± 3.08	−9.30 ± 0.48	Lower MIC values Downregulation of FCZ-resistant genes CDR1, CDR2, and ERG11	Preclinical *in vitro* study	[24]
Fluconazole	Not reported	Thin-film hydration	99.79 ± 11.1	−7.25 ± 4.88	Prevention of fungal biofilm formation due to higher drug entrapment and sustained release	Preclinical *in vitro* study	[28]
Itraconazole	Parenteral	Thin-film hydration-sonication	<200	Not reported	Enhanced efficacy due to increased half-life	Not Reported	[29]
Itraconazole	Topical	Film hydration	~150	Variable	Increased antifungal activity and reduced toxicity	Preclinical *in vitro* study	[30]
Itraconazole	Topical	Lipid-film hydration-sonication	276.5	+32.7 ± 1.5	Increased antifungal activity	Preclinical *in vivo* study	[31]
Itraconazole	Oral	Thin-film dispersion	165.0 ± 2.5	−21.5 ± 1.3	Enhanced oral bioavailability	Preclinical *in vivo* study	[32]
Itraconazole	Topical	Thin-film hydration	358.2 ± 9.4	20.66 ± 0.74	Increased antifungal activity and reduced toxicity	Preclinical *in vivo* study	[33]
Amphotericin B	Parenteral	Lipid-film hydration-sonication	~100	~ −45	Increased antifungal activity and reduced toxicity	Preclinical *in vitro* study	[34]
Amphotericin B	Topical	Thin-film hydration	107 ± 8	−3 ± 0.2	Increased skin penetration and deposition	Preclinical *in vitro* study	[35]
Amphotericin B	Topical	Not Reported	100	Not Reported	Increased skin penetration.	Entering Phase 2 clinical trial	[37,38]
Nystatin	Parenteral	Thin-film hydration-extrusion	~ 100	Not reported	Increased antifungal activity and reduced toxicity	Preclinical *in vitro* study	[39]
Ciclopirox	Topical	Ethanol injection	196±1.73	−56.2±1.4	Sustained drug release	Preclinical *in vitro* study	[40]
Quercitin & Gallic Acid	Vaginal	Thin-film hydration	~200	~5.8	Enhanced deposition and therapeutic effect of active compounds	Preclinical *in vitro* study	[41]

This study has demonstrated that producing ITZ-loaded PEGylated NLPs have had a particle size smaller than 200 nm, with a high EE of about 90% [29]. In 2014, Alomrani et al. developed a novel ITZ-loaded deformable NLPs with hydroxypropyl-β-cyclodextrin (HPβCD) topical formulation for the treatment of skin infections caused by *Candida albicans* using the film hydration method. The employment of deformable (elastic) NLPs has increased the tissue

penetration of ITZ beyond the stratum corneum, thus, enhancing antifungal efficacy. It was shown that the use of HPβCD has enhanced the solubility and permeation of ITZ compared to conventional LPs, by forming hydrophilic inclusion complexes with ITZ without hindering its intrinsic ability to permeate lipophilic membranes, consequently enhancing its stability. Moreover, adding HPβCD to the formulation has appeared to decrease the particle size down to 93 ± 3 nm, it's suggested that this size reduction is due to cyclodextrin's ability to reduce the surface tension of the medium, others suggested that this is due to its influence on the entropy of the lipid vesicle. Furthermore, the use of NLPs for the cutaneous delivery of ITZ-HPβCD for skin fungal infections has avoided a lot of issues that were reported with the oral formulation of ITZ-HPβCD such as Sporanox®, which was associated with several AEs [30]. Leal et al (2015) have investigated the antifungal activity of ITZ-loaded NLPs for the topical treatment of experimental keratitis with endophthlamitis caused by *Aspergillus flavus*. NLPs were prepared by the lipid film hydration method followed by sonication. They had a particle size distribution of around 276.5 nm and zeta potential of +32.7 ± 1.5 mV. In vivo study conducted on Wistrar rats has indicated that using NLPs as vehicles have enhanced the bioavailability and distribution of ITZ in the stroma and vitreous humor, increasing its activity against fungal infections in the eye, and thus it had favorable characteristics for the treatment of fungal keratitis with endophthalmitis compared to the free drug [31]. In 2016, Zhenbao Li et al. developed an NLP containing sodium deoxycholate (NaDC) to enhance the oral bioavailability of ITZ. The NLP consisted of egg yolk lecithin and NaDC and was prepared using the thin-film dispersion method. Maltose was later employed as a cryoprotectant during freeze drying to mitigate stability issues. Characterization of the resultant NLP has concluded that it had 165.0 ± 2.5 nm size and −25.1 ± 1.6 mV zeta potential, with an EE of about 88%. A bioavailability study in rats showed that the AUC was 1.67-fold higher in NaDC-NLP-ITZ than in the commercial product Sporanox® in terms of oral administration. Moreover, results have indicated that NaDC-NLP-ITZ had an improved dissolution efficiency and was able to increase transmembrane absorption, and as a result, enhance oral bioavailability of ITZ [32]. Kumar et al (2021) have conducted a study using a two-step DoE approach to develop, characterize and clinically evaluate ITZ-loaded NLPs for the topical treatment of dermatophytosis. This study aimed to produce statistically optimized ITZ-loaded NLPs using response surface methodology with full factorial DoE. The formulation was prepared as a hydrogel using the thin-film hydration method and was characterized using different techniques. The mean particle size was around 358.2 ± 9.4 nm, with a mean zeta potential of 20.66 ± 0.74 mV. The hydrogel had improved topical applicability, skin permeation, and residence time. Additionally, the in vivo antifungal activity was examined in Wistrar rats using standardized *Tinea pedis* as a model, and it suggested that the optimized NLP hydrogel had a faster alleviation of infection compared to conventional topical and antifungal treatments, making it a more advanced alternative for the treatment of dermatophyte infections [33].

1.4. Ciclopirox-Loaded Nanoliposomes

Ciclopirox Olamine (CPO) is a broad-spectrum synthetic antifungal agent commonly used to treat vaginal infections caused by *Candida albicans.* CPO acts by killing or inhibiting the growth of fungal cells through the chelation of metal ions resulting in the inhibition of iron-dependent enzymes responsible for degrading peroxides in fungal cells, leading to the intracellular depletion of essential substrates and/or ions [46,47]. However, the large dose and frequent application of CPO conventional cream have resulted in patient compliance issues. Karimunnisa et al. (2012) have developed an NLP-based mucoadhesive gel formulation for CPO, which enabled sustained release of the drug through NLPs. It also avoided the need for large dosing because of the mucoadhesive gel's ability to prolong contact with the vaginal cell wall, potentially leading to better patient compliance. The formulation was prepared using the ethanol injection method, with a mean particle size of around 196±1.73 nm and −56.2±1.4 mV zeta potential, with EE being about 44.89±3.2%. The in vitro antifungal activity against *Candida albicans* at pH 4.5 has shown that the NLP-based mucoadhesive formulation brought a significant reduction of fungal colonies after 3 hours and complete eradication after 6 hours, whereas the free drug has achieved complete

killing of *C. albicans* within 3 hours. These results suggest that the NLP-based formulation offered a sustained drug delivery with similar antifungal activity and is potentially a better and more convenient alternative for the treatment of vaginal infections [40].

2. Natural Antifungal Compounds

2.1. Amphotericin B (AmB)-Loaded Nanoliposomes

Amphotericin B is a highly potent macrocyclic polyene antibiotic agent isolated from *Streptomyces* species. AmB has strong antifungal and leishmanicidal properties due to its mechanism of action in which it binds to ergosterol, the main sterol in fungal and *Leishmania* cell membranes, disrupting the membrane and leading to cell death. AmB has a broad spectrum of activity against many fungal isolates, including *Candida spp.* and *Aspergillus spp.,* and filamentous fungi such as *Zygomycetes* [48]. However, AmB is associated with many severe side effects, including infusion-related events (fever, chills, nausea, and vomiting) and chronic nephrotoxicity, limiting its use in clinical settings. To mitigate those AEs and increase the therapeutic index of AmB, different lipid-based formulations have been approved for the effective treatment of invasive fungal infections, such as amphotericin B lipid complex (ABLC, Abelcet®), liposomal amphotericin B (LAmB, AmBisome®), and amphotericin B colloidal dispersion (ABCD, Amphotec®). All three formulations had an improved safety profile and better antifungal activity than conventional amphotericin B deoxycholate [14].

The components of the liposome in LAmB have significantly improved the mechanism of action in which AmB elicits its fungicidal properties. Firstly, LAmB includes hydrogenated soy phosphatidylcholine that has a gel-to-liquid crystalline phase transition temperature of approximately 55°C, this component ensures the stability of the formulation when it's injected intravenously with minimal release of the AmB until it reaches its target site. Moreover, Distearoyl phosphatidylglycerol (DSPG), an anionic bilayer component, can form an ion pair with the positively charged amino group of AmB, contributing to the strong integration of AmB inside the structure. Cholesterol, another major liposome component that contributes to the rigidity of the bilayer, binds with AmB through hydrophobic interaction, enabling AmB to remain firmly attached to the bilayer, and not readily released into the blood circulation [14, 34, 49]. Nevertheless, some liposomal formulations that contain free cholesterol and phospholipid molecules are prone to lose the free molecules to biomembranes and serum proteins when the liposome is in a biological environment, destabilizing the liposome and releasing its contents prematurely. As a result, Iman et al (2011) have designed and synthesized a family of chimeric sterol-modified glycerophospholipids (SMLs) to replace cholesterol to improve liposomal delivery and enhance its stability. They have synthesized 1,2-Distigmasterylhemisuccinoyl-sn-glycero3-phosphocholine (DSHemsPC), a new lipid in which two stigmasterol molecules are covalently linked to glycerophosphocholine. DSHemsPC showed excellent properties of stabilizing the NLP in biological fluids because stigmasterol in DSHemsPC does not transfer from the NLP into biological membranes. Additionally, stigmasterol, an unsaturated plant sterol, is similar yet less costly than cholesterol, making this a cost-effective, relatively easy method to produce AmB-loaded NLPs. Thirty-two different formulations with different molar ratios of components such as DSHemsPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1,2- dimyristoyl-sn-glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (DMPG), with different aliphatic chain lengths, were used to prepare the NLPs. They were prepared using thin-lipid film hydration followed by sonication, using different buffer solutions under different pH levels. They were examined for different characteristics and were tested for their in vitro antifungal and antileishmanial activities. The formulation had a mean particle size distribution of nearly 100 nm, negative zeta potential values, and low PDI. The in vitro antifungal activities of the prepared formulations were tested and compared to LAmB, with several formulations exhibiting similar or lower MIC values than LAmB. Moreover, the half maximal inhibitory concentration (IC50) of red blood cell potassium release (RBCPR) for formulations was tested some being comparable to or higher than LAmB IC50. Higher IC50 values indicate less potassium leakage, and as a result, lower toxicity. Among the prepared formulations, DSHemsPC/DMPC/DMPG/AmB with molar ratios of 1.25/5.0/1.5/1.0 prepared at pH

5.5, had favorable characteristics, indicating that it has the potential of being a successful and economical alternative to LAmB [34]. Currently, topical AmB formulations are being investigated due to the many advantages the topical route of administration has to offer, such as avoidance of systemic cytotoxicity and ease of application. However, the cutaneous penetration of AmB is limited due to its high molecular weight as well as its amphoteric nature. Perez et al (2015) have developed ultradeformable AmB-loaded NLPs that were able to penetrate intact skin across the stratum corneum to the viable epidermis without the use of permeation enhancers. They developed highly deformable NLPs made of soy phosphatidylcholine (SPC), sodium cholate, or Tween 80 as edge activators (EA) and phospholipids. EAs are biocompatible single-chain surfactants with a high radius of curvature and mobility that destabilize the lipid bilayers and increase their deformability [50]. The key part of this formulation was the rearrangement of EA and phospholipids in the bilayer by relocating EA to higher curvature/stress zones and phospholipids to the smaller curvature zones, this allows the amount of AmB in the bilayer to be maximized while maintaining high deformability, thus ensuring that the formulation remains intact while it passes through the smallest pores. Ultradeformable NLPs were prepared using the thin film hydration method and had a particle size diameter of 107 ± 8 nm and −3 ± 0.2 mV zeta potential. They were tested in vitro against several fungal and leishmanial strains and had comparable efficacy to AmBisome. However, in vitro skin penetration studies showed that this formulation had provided higher AmB skin deposition compared to other liposomal preparations of AmB, as well as a significantly higher penetration to the deep epithelial layers without the aid of classical permeation enhancers, indicating that ultradeformable AmB-loaded NLPs are advantageous targeted delivery systems with clinical significance for the topical treatment of fungal and leishmanial infections [35]. Eskandari et al (2018) have studied the safety of AmB-loaded NLPs (SinaAmpholeish) with a particle size of 100 nm for the topical treatment of cutaneous leishmaniasis. They have conducted an irritancy Draize test in the eyes and skin of rabbits against Leishmania major, and an in vitro test against Leishmania tropica [37]. The formulation was deemed safe and has successfully passed a randomized, double-blind phase 1 clinical trial in human volunteers in 2019, and it is in the process to enter a phase 2 clinical trial to assess the efficacy of the formulation [38].

2.2. Nystatin-Loaded Nanoliposomes

Nystatin is a broad-spectrum polyene antibiotic isolated from *Streptomyces* species. It has antifungal activity against various fungal pathogens such as *Candida, Aspergillus*, and *Histoplasma spp*. It's available as a topical cream, oral suspension, and pastille for treating oral, vaginal, and cutaneous candidiasis and aspergillosis [51]. However, its clinical use against systemic fungal infections is limited because of its low water solubility and severe systemic toxicity. Saadat et al (2016) have encapsulated Nystatin inside NLPs to elucidate its solubility issues. They designed an efficient parenteral NLP-based formulation of nystatin with lower toxicity and higher antifungal activity. NLPs were developed using the thin-film hydration method followed by extrusion and contained nystatin, dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC), and had an average particle size of around 100 nm, sucrose was employed to prevent vesicle fusion during lyophilization, as well as playing a part alongside the NLP in enhancing EE of the drug. In vitro studies against *Candida albicans* have concluded that the drug release properties of NLPs were able to control and sustain the release rate of nystatin, as well as provide targeted delivery and enhanced penetration to fungal cell walls, compared to the free drug [39].

2.3. Natural Organic Compounds

A great deal of research into natural organic compounds such as flavonoids, has focused on their medicinal and pharmacological activities including their antifungal potentials. In 2019, Giordani et al demonstrated the utilization of liposomal quercitin (Q), a flavonoid polyphenol found in various vegetables and fruits [52], and gallic acid (GA), another polyphenol found in medicinal plants such as pomegranate rind [53], in the topical treatment of vulvovaginal candidiasis (VVC). They developed a liposomal formulation that was able to simultaneously deliver GA and Q that work synergistically to eradicate infection and alleviate symptoms of VVC. Generally, liposomal formulations are

suitable nanocarriers for the vaginal delivery of therapeutic compounds, as they do not interfere with vaginal microbiota, and they can protect cargo from enzymatic degradation that occurs in the vaginal cavity. Quercitin was proposed for this study due to its many advantages including its ability to scavenge reactive oxygen species (ROS) and chelate metal ions, as well as its anti-inflammatory effects as it inhibits cytokine production and histamine release. Previous studies have also suggested that quercetin may be an antinociceptive that is able to alleviate the pain associated with inflammation, as well as being lenitive with an anti-itch effect, making it a suitable candidate for the treatment of VVC. GA was reported to have antifungal effects against different clinical isolates of *Candida spp*., as it has demonstrated its ability to disrupt the biosynthesis of ergosterol, a key component of the fungal membrane. Although polyphenols possess numerous pharmacological properties, their full potential had not been fully realized due to their low solubility and bioavailability, as well as their high susceptibility to environmental conditions. This study has focused on incorporating these compounds into nanoliposomes to prevent degradation and promote their deposition into the target site to ensure enhanced synergistic therapeutic effect and the eradication of fungal infection. They have developed NLPs loaded with either Q or GA or both with an average size of around 200 nm, high EE, and average zeta potential of ~5.8 mV using the film hydration method. NLPs with both Q and GA (labeled LP-Q+GA) promoted the sustained release of both compounds and showed enhanced antioxidant activity compared to a single polyphenol alone. Moreover, the formulation was not cytotoxic, and it showed a greater anti-inflammatory effect compared to free polyphenols. Furthermore, incorporating GA into NLPs has significantly reduced *C. albicans* growth [41].

3. Selective Targeting Techniques

There have been several attempts to improve the targetability of NLPs to treat fungal infections. Meagher et al. (2021) have created a novel drug delivery system (DDS) with a particular affinity to fungal pathogens. They're based on Dectin-decorated NLPs (DectiSomes). This technology allows NLPs to be coated with proteins that bind to fungal cell walls and their exopolysaccharide matrices (Figure 1A) with high specificity, resulting in a higher concentration of antifungal drugs near fungal cells. In contrast, they will exist at a significantly lower concentration in host cells. This results in enhanced fungicidal activity, reduced toxicity, and off-target effects. They have designed 2 types of DectiSomes (Figure 1B) decorated with Dectin-1 and Dectin-2, the mammalian immunological receptors for oligoglucans and oligomannans, respectively.

They started by creating AmB-LLs which are PEGylated analogs of the popular pharmaceutical liposomal formulation AmBisome. Then, they coated the AmB-LLs with the carbohydrate recognition domains of Dectin-1 and Dectin-2, to produce DEC1-AmB-LLs and DEC2-AmB-LLs, respectively. The selective attachment occurs when two Dectin monomers float together and form dimers that bind specifically to the oligoglucans and oligomannans present in cell walls, exopolysaccharide matrices, and biofilms of fungal pathogens. When liposomes reach fungal ligands at multiple sites on fungal cells, the presence of a large number of Dectin molecules on each liposome enables efficient and high avidity binding. In vitro fungal cell binding and killing studies reveal that DEC1-AmB-LLs and DEC2-AmB-LLs have a 50- to 200-fold binding affinity to the cell walls and exopolysaccharide matrices of *Aspergillus fumigatus, Candida albicans,* and *Cryptococcus neoformans* as opposed to untargeted AmB-LLs.Moreover, the oligoglycan laminarin specifically inhibits the binding of DEC1-AmB-LL, whereas yeast oligomannan specifically inhibits the binding of DEC2-AmB-LL. In vitro assays against *A. fumigatus* cells have shown that the inhibitory and fungicidal effects of DEC1-AmB-LL are 5-to 50-fold larger than untargeted AmB-LLs delivering the same concentration of AmB as evidenced by a reduction in rates of conidial germination, a reduction in hyphal extension from germlings, and a loss of metabolic activity of growing hyphae. Similarly, DEC2-AmB-LL exhibited a 2- to 90-fold increase in efficiency against *A. fumigatus, C. albicans,* and *C. neoformans* [54].

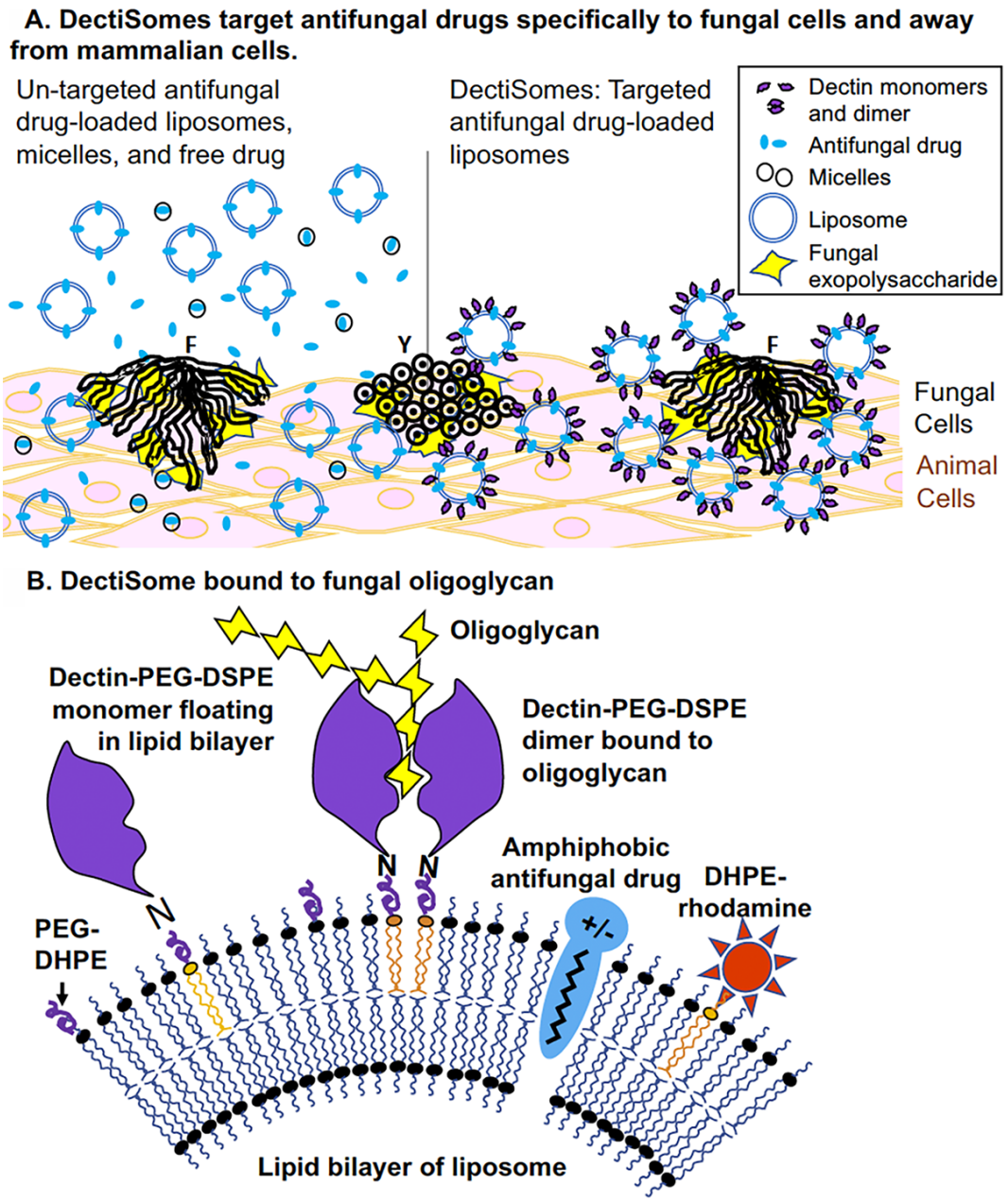

Figure 1. (A) Using glycan-binding proteins to coat antifungal drug-loaded liposomes concentrates drugs on fungal cells. Infection sites with fungal cells with Y or F morphologies are depicted. (B) DectiSomes structure, DEC-PEG-DSPE is formed by coupling its glycan-binding domain (purple globular structure) to a lipid carrier and is intercalated into the liposomal membrane via the DSPE moiety. Dimers (yellow sugar moieties). To allow fluorescence monitoring of liposome binding to fungal cells, rhodamine-B-DHPE is intercalated via its DHPE moiety. ""Liposomes' bilayer contains an antifungal agent (blue ovoid structure). DEC,dectin; DHPE,dihexadecanoyl-glycero-phospho-ethanolamine; DSPE,distearoyl-glycero-phospho-ethanolamine; F,filamentous; PEG,poly(ethylene glycol).Y, yeast. Reprinted from PLoS Pathog 17(7): e1009699. Meagher RB, Lewis ZA, Ambati S, Lin X. Aiming for a bull's-eye: Targeting antifungals to fungi with dectin-decorated liposomes. https://doi.org/10.1371/journal.ppat.1009699 Copyright (2021) with permission from PLoS.

To further assess DectiSomes, in vivo studies were conducted using a neutropenic/leukopenic mouse model of pulmonary aspergillosis as it mimics the chemo-immunosuppression observed in patients with solid tumors, hematopoietic malignancies, or immune disorders such as AIDS, as well as patients preconditioned for stem cell mobilization and hematopoietic cell transplantation. It was reported that DEC2-

AmB-LLs administered through oropharyngeal aspiration bind to exopolysaccharides associated with fungal infection sites in the lungs 30 times more effectively, as well as significantly reduce the fungal cell burden using a lower dose (0.2 mg/kg) compared with AmB-LL analog AmBisome (5 mg/kg). Overall, in vivo and in vitro studies show that Dectin targeting improves drug efficacy with a lower effective dose. However, there are concerns, notably with *C. neoformans*, encased with a thick capsule consisting of biochemically complicated exopolysaccharides that shed copiously upon infection. Furthermore, DectiSomes bound to soluble fungal glycans may exhibit less antifungal activity. Additionally, LPs are only moderately effective at crossing the blood-brain barrier, and the effects of coating them with dectins are yet to be addressed experimentally. DectiSomes can recognize pathogens such as *Aspergillus, Candida, Pneumocystis, Cryptococcus,* and *Mucor* species that are the main causes of global fungal infections, making them promising pan-antifungal agents [55].

In 2022, Choudhoury et. al studied the antifungal effects of DEC1-AmB-LLs against *Rhizopus delemar.* A fungal pathogen responsible for Mucormycosis (aka zygomycosis). Since Dectin-1 is an immune receptor for *R. delemar* infections, DEC1-AmB-LLs were found to have 100- to 1000-fold more binding capacity to the exopolysaccharide matrix of R. delemar germlings and mature hyphae than AmB-LLs. DEC1-AmB-LLs delivering submicromolar concentrations of AmB were an order of magnitude more effective than AmB-LLs at inhibiting and/or killing *R. delemar*, making DectiSomes a viable option to enhance mucormycosis therapy [56].

Similarly, Ambati et al. (2021) have developed another class of targeting proteins coated NLPs using the human DC-SIGN (a.k.a., CD-SIGN, ICAM-3) protein, which is a C-type lectin pathogen receptor that is encoded by the CD209 gene. Its carbohydrate recognition domain (CRD) binds crosslinked mannose-rich and fucosylated glycans (such as the LewisX trisaccharide) and lipomannans, which are commonly found in protist, bacterial, viral, helminth, and fungal pathogens, including *A. fumigatus, C. albicans,* and *C. neoformans.* Human DC-SIGN recognizes ligands via a CRD related to the membrane transit and signaling sequences. The orientation of the CRD may be altered by different combinations of the eight neck repeats (NR1 to NR8) expressed in different protein isoforms. They created two recombinant isoforms by combining the CRD with NR1 and NR2 in DCS12 and with NR7 and NR8 in DCS78 and coupling them to a lipid carrier, then loading them into the membrane of PEGylated AmB-LLs creating DCS12-AmB-LLs and DCS78-AmB-LLs. Compared to untargeted AmB-LLs and Bovine Serum Albumin coated BSA-AmB-LLs, DCS12-AmB-LL and DCS78-AmB-LL showed more effective binding to the exopolysaccharide matrices of *A. fumigatus, C. albicans,* and *C. neoformans*. Overall, DCS12-AmB-LLs outperformed DCS78-AmB-LLs in vitro. Furthermore, DCS12-AmB-LLs inhibited and/or killed all three species more effectively than AmB-LLs or BSA-AmB-LLs. In mice models of invasive candidiasis and pulmonary aspergillosis, a single low dose of DCS12-AmB-LLs decreased the fungal burden in the kidneys and lungs several-fold more than AmB-LLs (Figure 2) [57]

Santoso et al. (2022) have recently developed AmB-loaded hybrid NLPs decorated with engineered chitin-binding domain (LysM) of *P. ryukyuensis* chitinase. Because of its specificity toward chitin, which is absent in humans but abundant in fungal cell walls, the enzyme chitinase (EC. 3.2.1.14) is regarded as an effective, low-toxicity antifungal candidate. They have prepared several hybrid liposomal formulations including AmB and sodium deoxycholate. The prepared formulations had different surface charges (anionic, cationic, and neutral) with or without chitin-binding domains (LysM-Q or LysM-Pal). The design of this bioconjugate had a remarkable antifungal activity that was enhanced by adding a palmitoylated LysM domain (Lysm-Pal) and combined with conventional AmB formulation. This study suggests that functionalizing NLPs with fungal cell wall-binding proteins allows for specific targeting and enhances the therapeutic efficacy of antifungal agents. However, further studies are needed to validate this approach in practical applications [58].

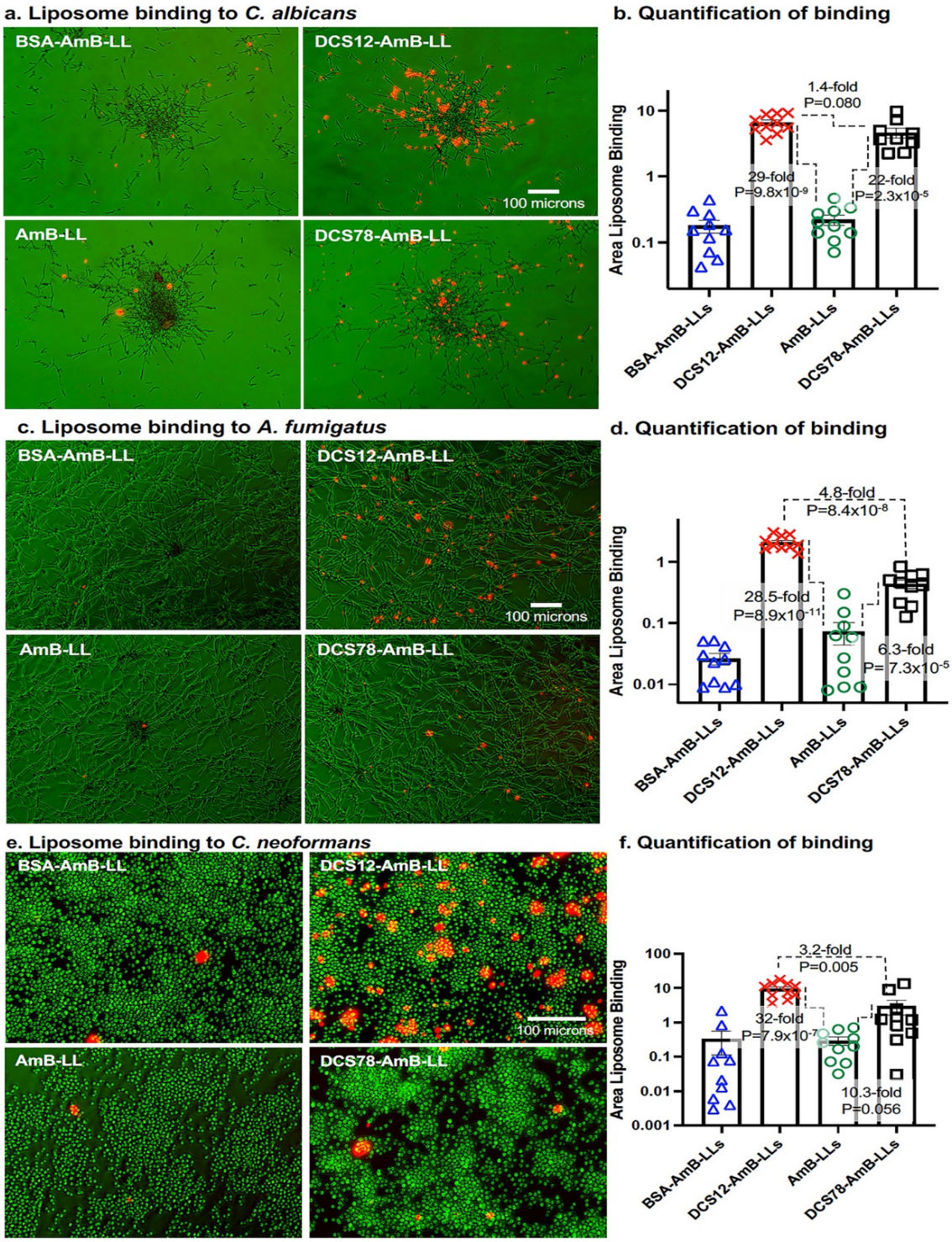

Figure 2. DC-SIGN targeted liposomes, DCS12-AmB-LLs and DCS78-AmB-LLs bind more efficiently to the exopolysaccharide matrices of three life-threatening fungal pathogens than AmB-LLs. a, c, e representative images of fluorescent liposomes binding to bright field images of fungal cells are shown. a C. albicans (×10 magnification). c A. fumigatus (×10 magnification). e C. neoformans (×20 magnification). b, d, f the relative area of red fluorescent liposome binding (log10) was measured as shown in scatter bar plots on the right. where N=10 for each bar. f the plot scale for C. neoformans had to be expanded from (3-5) logs to accommodate more widely distributed data. Standard errors and P values are indicated. Reprinted from Springer Fungal Biol Biotechnol 8, 22 (2021). Ambati, S., Pham, T., Lewis, Z.A. et al. DC-SIGN targets amphotericin B-loaded liposomes to diverse pathogenic fungi. https://doi.org/10.1186/s40694-021-00126-3 Copyright (2021) with permission.

Discussion

NLPs hold great promise as drug delivery systems. Their biocompatibility, biodegradability, controlled release, and the ability to modify their surface with targeting ligands to enhance their targetability makes them an attractive choice as dynamic and adaptable carriers for antifungal agents. However, the mechanism in which they aid with the fungicidal activity of their cargo is yet to be fully elucidated. For example, Walker et al. (2018) have conducted a study on the behavior of AmBisome®, a product that has been used for over 30 years, to try and understand the NLP's behavior. Amphotericin B has less affinity towards cholesterol than ergosterol, a main component in the fungal cell membrane, so when the AmB-loaded NLPs reach the target site, it's released from the NLP and bound with ergosterol where it exerts its antifungal effects. The study has shown that the AmB-loaded NLP remained intact while it travelled through the fungal cell wall of two fungal species (*Candida albicans* and *Candida neoformans),* both are species that differ in cell wall architecture and content. AmBisome® has managed to deliver its payload directly to the cell membrane despite cell wall pore size being smaller than that of the NLP vesicle. This means that the cell wall is deformable and viscoelastic, allowing transwall vesicular flow. It's worth noting that empty NLPs (without AmB) have failed to enter the inner cell wall as they stayed at the base of the fibrils of *C. albicans'* outer cell wall and did not enter the inner β-glucan-chitin layer. This means that the reason for AmBisome® transfection through the cell wall isn't necessarily related to the NLP structure. It was suggested that amphotericin B binds to ergosterol in the cell wall, and carrier NLPs are subsequently transported inwards by a pre-existing vesicular reshuffling motive mechanism such as cell wall melanin, as melanin is thought to be generated in fungal melanosomes and then transported to the cell wall. Since cell wall-associated melanin is continually modified throughout fungal cell morphogenesis and budding, mechanisms for transporting melanosomes must exist, which might be utilized by AmBisome® for cell wall transit [36]. Moreover, as with the case of other nano-sized carriers, NLPs loaded with antifungal agents will encounter natural defense systems (RES, opsonization, and immunogenicity) in the body to eliminate them as foreign substances, and the focus is being shifted to optimize liposomal formulations and bypass these obstacles, changing the lipid content and charge, as well as the addition of surface coatings and ligands, are examples of this. Recent efforts for improving conventional or stealth liposomal systems include active targeting with targeting ligands or triggering cargo release as a change of pH or temperature [59].

Conclusions

NLPs are one of the safest and most biocompatible nanocarriers for the delivery of therapeutic agents. Their applications for the treatment of fungal infections hold great promise as they offer drug targeting, and controlled release, and can be formulated for multiple routes of administration. However, it's imperative to assess their toxicity in clinical applications as it's influenced by parameters such as particle size and size distribution that directly impact the colloidal stability, drug release profile, bioavailability, mucoadhesive properties, cellular uptake, and clearance of the formulation. The influence of particle size on toxicity is more pronounced in nanocarriers as drug delivery systems than microcarriers, as the surface-to-volume ratio is significantly larger, leading to more drug molecules at the surface and higher drug release. Moreover, the particle size and the PDI heavily influence the biological fate of NLPs, and there have been numerous reports in the literature indicating that as the particle size decreases, its toxicity will increase. Due to their natural composition, small size, and high surface energy, NLPs tend to agglomerate. They have other stability issues like bilayer fusion and drug leakage, which will negatively impact their shelf life, so research that enhances their storage stability is currently being investigated. NLPs need to undergo extensive in vivo and in vitro testing to address the concerns associated with their safety by understanding the fate of these nanocarriers and their behavior during different physiological processes, establish a full safety profile, and ensure safe clinical translation. Lastly, the development of fabrication methods that are more scalable, reproducible, and economically viable as well as those that incorporate green practices while ensuring optimum particle properties must all be thoroughly investigated [60].

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose. For signed statements contact the journal office editor@precisionnanomedicine.com

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